malignant potential of HCC with subsequent unfavorable prognosis after treatment [12–16]. However, there have been few reports of the relationship between AFP-L3 status and prognosis in subgroups of HCC patients receiving different therapeutic modalities, such as hepatectomy and percutaneous ablative therapy.

The aim of this collaborative retrospective and prospective study was to evaluate the clinical usefulness of measuring AFP-L3 for prognostic predictor in patients with HCC after curative treatment.

Patients and Methods

Study Design

A total of 336 HCC patients underwent curative treatment at four participating hospitals (Niigata University Hospital, Ehime University Hospital, Shinsyu University Hospital, and Gunma University Hospital) from January 1998 to March 2005 and were investigated retrospectively. Of these patients, 232 underwent percutaneous ablative therapy and 104 underwent hepatectomy. Percutaneous ablative therapy comprised PEI in 90 patients, MCT in four patients, and RFA in 138 patients. Long-term survival data on these patients were confirmed as of the end of March 2005.

To evaluate the prognostic influence of AFP-L3 in two subgroups comparable for tumor extension, we prospectively investigated 189 patients diagnosed with early stage HCC initially at four hospitals from April 2005 to October 2007. We considered patients who had multiple (up to three) tumors measuring 3 cm or less in diameter as having early stage HCC. Forty-eight of 189 patients were excluded in this study, as they were received transcatheter treatment. As a result, 141 HCC patients, 99 who underwent percutaneous ablative therapy and 42 who underwent hepatectomy, were enrolled in the prospective study. Percutaneous ablative therapy comprised PEI in ten patients, MCT in two patients, and RFA in 87 patients. In these 141 patients, HCC recurrence was assessed by imaging modalities every 3 or 4 months after treatment and recurrence free survival was evaluated as of the end of December 2007. Informed consent was obtained from each patient, and the study protocol conformed with the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in the a priori approval by our institution's human research committee.

Diagnosis of HCC and Laboratory Examination

In our study, the diagnosis was based essentially on imaging findings together with increments of tumor marker levels. We employed methods such as computed tomography (CT), magnetic resonance imaging, and CT during hepatic arteriography, considering hyperattenuation in the arterial phase with washout in the late phase to be a typical feature of HCC. In nine cases that showed atypical features on imaging, ultrasound-guided biopsies were performed.

Hepatic functional reserve was ranked by the criteria of the Child-Pugh scoring system. Serum alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) were determined at each hospital by using commercially available kits. AFP-L3 percentage was measured at each hospital by liquid-binding assay (Wako Pure Chemical Industries Ltd, Osaka, Japan) [17]. AFP, AFP-L3, and DCP were measured in the same serum before treatment. Cut-off values for positivity for AFP, AFP-L3, and DCP were set at 20 ng/ml, 15%, and 40 mAU/ml, respectively, based on previous studies [18–20].

Treatment

Therapeutic modalities for individual patients were chosen according to hepatic functional reserve, tumor multiplicity, and tumor size. Percutaneous local ablative therapies were performed under a US-guided procedure, and its efficacy was evaluated with dynamic CT within a few days after treatment. Complete ablation of HCC was defined as non-enhancement of the lesion with surrounding liver parenchyma. Patients received additional sessions of an ablative therapy until the treatment was judged as complete. During the study, a Cool-tip RF System attached to a 200-W power generator (Radionics, Burlington, Massachusetts, USA) was the main device used for RFA treatment and Microtaze OT-110M (Alfresa-Pharma Co., Inc., Osaka, Japan) was used for MCT.

Statistical Analysis

Differences in the proportions of the independent binary variables were determined by Fisher's exact test. Continuous variables were compared by Student's *t*-test. Univariate survival and recurrence-free survival were determined by the Kaplan–Meier method. Log-rank test was used to test for equality of long-term survival and recurrence-free survival between the groups. Multivariate analyses of prognostic factors in the clinical features were performed by using Cox's stepwise proportional hazard model. The factors included for multivariate analyses were patient age, gender (female/male), HBsAg (negative/positive), Anti-HCV (negative/positive), Child-Pugh class (A/B, C), AFP (ng/ml) (<20/≥20), DCP (mAU/ml) (<40/≥40), AFP-L3 (%) (<15/≥15), tumor size (cm) (<3/≥3 or ≤2/>2), and number of tumors (single/multiple). Statistical analyses were performed with SPSS 15.0 software (SPSS Japan Inc. Tokyo, Japan). A *P*-value of less than 0.05 was considered as statistically significant.

Results

Retrospective Study

Clinical Features of Patients Classified by Therapeutic Modality

A total of 336 HCC patients who underwent hepatectomy and percutaneous ablative therapy were investigated retrospectively. Patients who underwent percutaneous ablative therapy were characterized by older age ($P < 0.05$), positivity for antibody to hepatitis C virus (anti-HCV) ($P < 0.05$), and advanced Child-Pugh classification ($P < 0.05$). In contrast, patients who underwent hepatectomy were characterized by positivity for hepatitis B surface antigen (HBsAg) ($P < 0.05$), AFP-L3 ($P < 0.05$), and DCP ($P < 0.05$) elevation, as well as large tumor size ($P < 0.05$). No significant differences were observed between the two groups in terms of gender, AFP level, or number of tumors (Table 1A).

Univariate and Multivariate Analyses of the Factors Predicting Long-Term Patient Survival

The median observation time after treatment was 38.3 months (range, 1.0–146.2 months). Of the 232 patients who underwent percutaneous ablative therapy, 172 were alive and 60 had died from HCC, hepatic failure, and/or complications of cirrhosis. Of the 104 HCC patients who underwent hepatectomy, 68 were alive and 36 had died. The median survival time was 69.0 months in patients who had undergone percutaneous ablative therapy and 114.9 months in those who had undergone hepatectomy.

In the univariate analysis, anti-HCV status ($P = 0.034$), AFP status ($P = 0.007$), AFP-L3 status ($P = 0.001$), tumor size ($P = 0.001$), and number of tumors ($P = 0.045$) were significant prognostic factors of long-term survival in patients who underwent percutaneous ablative therapy. AFP status ($P = 0.011$), tumor size ($P = 0.006$), and number of tumors ($P < 0.001$) were significant prognostic factors in patients who underwent hepatectomy (Table 2).

Multivariate analysis by Cox's stepwise proportional hazard model revealed that tumor size ($P = 0.018$) and AFP-L3 status ($P = 0.013$) were significant independent prognostic factors for long-term survival in patients who underwent percutaneous ablative therapy. Tumor size ($P = 0.013$) and number of tumors ($P = 0.004$) were significant independent prognostic factors in patients who underwent hepatectomy (Table 3). We showed the long-term survival curves of two groups (with or without AFP-L3 elevation) in patients who underwent percutaneous ablative therapy and in those who underwent hepatectomy (Fig. 1). No significant difference in survival was observed

Table 1 Clinical features of patients with HCC classified by therapeutic modality in the retrospective and prospective studies

Variables	Percutaneous ablation ($n = 232$)	Hepatectomy ($n = 104$)
(A) Retrospective study		
Age (median, range)	68 (39–89)	65 (35–81)*
Gender		
Male	145 (62.5%)	66 (63.5%)
Female	87 (37.5%)	38 (36.5%)
HBsAg		
Negative	209 (90.1%)	73 (70.2%)
Positive	23 (9.9%)	31 (29.8%)*
Anti-HCV		
Negative	28 (12.1%)	45 (43.3%)
Positive	204 (87.9%)	59 (56.7%)*
Child-Pugh class		
A	177 (76.3%)	95 (91.3%)
B and C	55 (23.7%)	9 (8.7%)*
AFP (ng/ml)		
<20	65 (28.0%)	22 (21.2%)
≥20	167 (72.0%)	82 (78.8%)
DCP (mAU/ml)		
<40	149 (67.4%)	48 (51.1%)
≥40	72 (32.6%)	46 (48.9%)*
AFP-L3 (%)		
<15	181 (78.0%)	61 (58.7%)
≥15	51 (22.0%)	43 (41.3%)*
Tumor size (cm)		
<3	185 (79.7%)	33 (31.7%)
≥3	47 (20.3%)	71 (68.3%)*
Tumor number		
Single	148 (63.8%)	75 (72.1%)
Multiple	84 (36.2%)	29 (27.9%)
Variables	Percutaneous ablation ($n = 99$)	Hepatectomy ($n = 42$)
(B) Prospective study		
Age (median, range)	69 (36–85)	65 (40–80)
Gender		
Male	66 (66.7%)	24 (57.1%)
Female	33 (33.3%)	18 (42.9%)
HBsAg		
Negative	85 (85.9%)	29 (69.0%)
Positive	14 (14.1%)	13 (31.0%)*
Anti-HCV		
Negative	27 (27.3%)	15 (35.7%)
Positive	72 (72.7%)	27 (64.3%)
Child-Pugh class		
A	79 (79.8%)	39 (92.9%)
B and C	20 (20.2%)	3 (7.1%)

Table 1 continued

Variables	Percutaneous ablation ($n = 99$)	Hepatectomy ($n = 42$)
AFP (ng/ml)		
<20	64 (64.6%)	22 (52.40%)
≥20	35 (35.4%)	20 (47.6%)
DCP (mAU/ml)		
<40	63 (63.6%)	27 (64.3%)
≥40	35 (35.4%)	15 (35.7%)
AFP-L3 (%)		
<15	85 (85.9%)	33 (78.6%)
≥15	14 (14.1%)	9 (21.4%)
Tumor size (cm)		
≤2	63 (63.6%)	27 (64.3%)
>2	36 (36.4%)	15 (35.7%)
Tumor number		
Single	78 (78.8%)	34 (81.0%)
Multiple	21 (21.2%)	8 (19.0%)

HBsAg hepatitis B surface antigen, *HCV* hepatitis C virus, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin. Percentages are shown in parentheses

* $P < 0.05$ between groups by Fisher's exact test and Student's *t*-test

between the two AFP-L3 groups in patients who underwent hepatectomy ($P = 0.308$). In contrast, patients in the ablative therapy group whose AFP-L3 levels were below 15% lived significantly longer than those whose values were more than 15% ($P = 0.001$).

Prospective Study

Clinical Features of Patients with Early Stage HCC Classified by Therapeutic Modality

A total of 141 patients with early stage HCC were evaluated prospectively. Patients who underwent hepatectomy were characterized by positive for hepatitis B surface antigen (HBsAg) ($P < 0.05$). No significant differences were observed in age, gender, anti-HCV positivity, AFP status, AFP-L3 status, DCP status tumor size, and number of tumors between the two groups. Patients who underwent percutaneous ablative therapies tended to have an advanced Child-Pugh classification ($P = 0.055$) (Table 1B).

Univariate and Multivariate Analysis of the Factors Predicting Recurrence-Free Survival in Patients with Early Stage HCC

The median follow-up time after treatment was 12.0 months (range, 1.0–30.5 months). Among the 99 patients who underwent percutaneous ablation, recurrences were observed in 36 (36.4%). Among the 42 patients who underwent hepatectomy, recurrences were observed in six (14.3%).

In the univariate analysis, we found no significant difference in recurrence-free survival rates by pretreatment variables in patients who underwent percutaneous ablation, although AFP-L3 elevation ($P = 0.054$) tended to decrease recurrence-free survival. In contrast, tumor size ($P = 0.038$) and number of tumors ($P = 0.034$) were significant prognostic factors in patients who underwent hepatectomy (Table 2).

Although this prospective study was conducted over a short period of time, multivariate analysis of prognostic factors among the clinical features was performed and Cox's stepwise proportional hazard model revealed that HBsAg status ($P = 0.033$), DCP status ($P = 0.011$), and AFP-L3 status ($P = 0.006$) were significant independent prognostic factors of recurrence-free survival in patients who underwent percutaneous ablative therapies. On the other hand, we found no significant independent prognostic factors in patients who underwent hepatectomy (Table 3).

We showed recurrence-free survival rates between two groups—with or without AFP-L3 elevation—among

Table 2 Univariate analysis of the factors predicting long-term survival in the retrospective study and recurrence-free survival in the prospective study for patients who underwent percutaneous ablation and in those who underwent hepatectomy

Variables	Long-term survival		Recurrence-free survival	
	Percutaneous ablation *P*-value	Hepatectomy *P*-value	Percutaneous ablation *P*-value	Hepatectomy *P*-value
Gender (female/male)	0.907	0.525	0.225	0.194
HBsAg (negative/positive)	0.139	0.801	0.151	0.314
Anti-HCV (negative/positive)	0.034	0.963	0.194	0.171
Child-Pugh class (A/B,C)	0.083	0.235	0.293	0.487
AFP (ng/ml) (<20/≥20)	0.007	0.011	0.117	0.994
DCP (mAU/ml) (<40/≥40)	0.328	0.153	0.075	0.059
AFP-L3 (%) (<15/≥15)	0.001	0.308	0.054	0.530
Tumor size (cm) (<3/≥3)	0.001	0.006	0.063	0.038
Tumor number (single/multiple)	0.045	<0.001	0.667	0.034

HBsAg hepatitis B surface antigen, *HCV* hepatitis C virus, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin. *P*-value was calculated using Log-rank test

Table 3 Multivariate analysis of factors predicting long-term survival in the retrospective study and recurrence-free survival in the prospective study for patients who underwent percutaneous ablation and in those who underwent hepatectomy

Long-term survival			Recurrence-free survival		
Variables	Hazard ratio (95% CI)	*P*-value	Variables	Hazard ratio (95% CI)	*P*-value
Percutaneous ablation			Percutaneous ablation		
AFP-L3 (%)			HBsAg		
<15	1		Negative	1	
≥15	2.098 (1.169–3.765)	0.013	Positive	2.823 (1.090–7.310)	0.033
Tumor size (cm)			DCP		
<3	1		<40 (mAU/ml)	1	
≥3	1.998 (1.123–3.553)	0.018	≥40 (mAU/ml)	2.767 (1.267–6.046)	0.011
			AFP-L3		
			<15 (%)	1	
			≥15 (%)	3.463 (1.437–8.347)	0.006
Hepatectomy			Hepatectomy		
Tumor size (cm)			Tumor number		
<3	1		Single	1	
≥3	6.162 (1.457–26.064)	0.013	Multiple	4.654 (0.936–23.149)	0.060
Tumor number					
Single	1				
Multiple	3.170 (1.442–6.921)	0.004			

Hazard ratio and *P*-value were calculated using Cox's stepwise proportional hazard model

CI confidence interval, *AFP* alpha-fetoprotein, *HBsAg* hepatitis B surface antigen, *DCP* des-gamma-carboxy prothrombin

patients with early stage HCC who underwent percutaneous ablation and patients who underwent hepatectomy (Fig. 1). No significant difference was observed between groups with or without AFP-L3 elevation ($P = 0.53$) in patients who underwent hepatectomy. In contrast, a close-to-significant ($P = 0.054$) difference was observed between the groups of patients with and without AFP-L3 elevation who underwent percutaneous ablative therapy.

In summary, the results of the retrospective and prospective studies demonstrated that AFP-L3 status was a statistically significant prognostic factor of long-term survival and recurrence-free survival in patients who underwent percutaneous ablative therapy, but did not affect prognosis in patients who underwent hepatectomy.

Discussion

AFP-L3, a fucosylated species of AFP, is the product of alpha 1-6 fucosyltransferase (FUT8) in the presence of GDP-fucose. Our previous result revealed that FUT8 levels in HCC tissue were higher than those in the surrounding non-cancerous tissues and that FUT8 levels of HCC tissue increased in accordance with tumor dedifferentiation [21]. Several reports have shown the relationship between AFP-L3 status and histologic grade in HCC. Miyaaki et al. [16] showed that the frequency of poorly differentiated HCC was significantly higher in AFP-L3-positive patients than in AFP-L3-negative patients. Oka et al. [14] reported that AFP-L3-positive HCC was characterized by portal vein invasion and poorer differentiation, and that tumors in AFP-L3-positive HCC were advanced, even if they were small and the patient had a low serum AFP concentration. These results indicate the relationship between increased AFP-L3 level and increased degree of malignant behavior of HCC tissue.

Recurrence after treatment is an important factor affecting prognosis. Vascular invasion is an established adverse prognostic indicator of recurrence of HCC [22, 23]. Yamashita et al. [24] suggested that portal vein invasion is associated with AFP-L3 positivity, and that there is a strong possibility of intrahepatic invasion when there is positive conversion of this marker. Hayashi et al. [13] reported the relationship between AFP-L3 status and pattern of recurrence in patients with HCC. In their report, intrahepatic metastasis was significantly more common in AFP-L3-positive patients than in negative patients, although the recurrence rate of multicentric tumors did not differ significantly between the two groups with or without AFP-L3 elevation. From this point of view, hepatectomy—especially anatomical resection, which can remove venous tumor thrombi together with the primary lesion—is more suitable than local ablative therapies for the treatment of AFP-L3-positive patients.

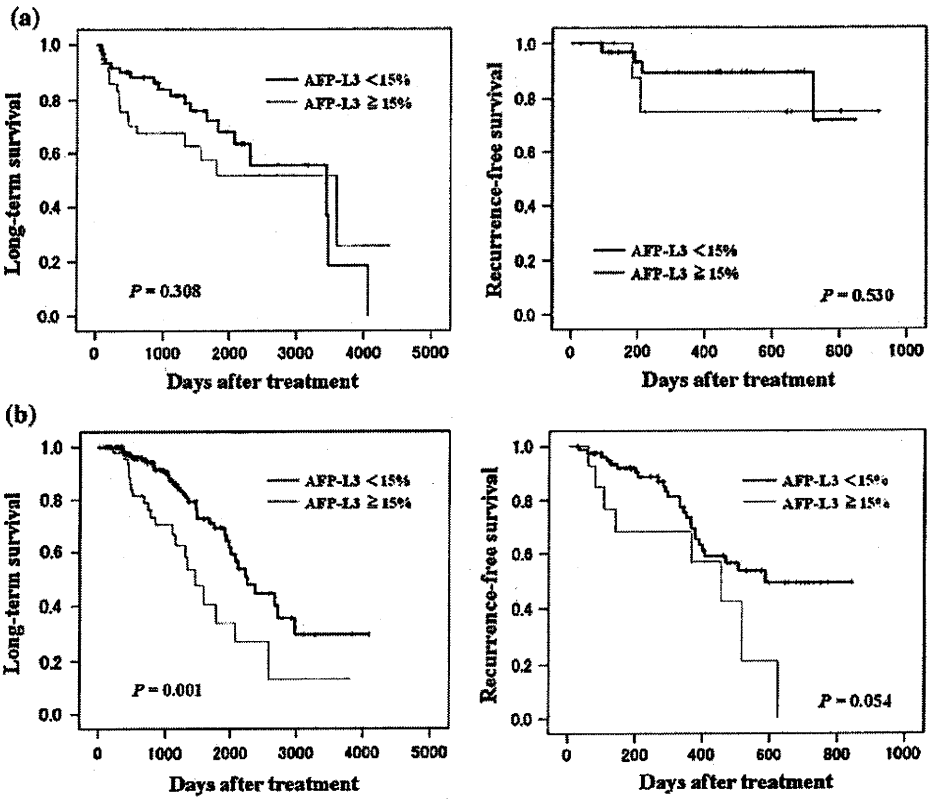

Fig. 1 Comparison of long-term survival rates and recurrence-free survival rates between patients with and without AFP-L3 elevation who underwent hepatectomy (a) and who underwent percutaneous ablation (b)

In our study, the pathological diagnosis was made by individual pathologists at each hospital. At Niigata University Hospital, 58 HCC patients underwent hepatectomy, of whom 23 had an elevated serum AFP-L3 level (≥15%) and the remaining 35 were negative for AFP-L3 (<15%). Among the 23 patients with AFP-L3 elevation, only two (8.7%) were diagnosed as having well-differentiated HCC on the basis of the resected specimens, 14 (60.9%) had moderately differentiated HCC, and seven (30.4%) had poorly differentiated HCC. In contrast, among the 35 patients who were negative for AFP-L3, 7 (20.0%) were diagnosed as having well-differentiated HCC, 24 (68.6%) had moderately differentiated HCC, and only four (11.4%) had poorly differentiated HCC. Although no statistically significant differences were observed by Fisher's exact test, the group showing AFP-L3 elevation tended to have a poorer histopathological grading ($P = 0.141$). Only eight out of 331 patients who underwent percutaneous ablative therapy were diagnosed as having HCC on the basis of histological findings in four hospitals. Therefore, we were unable to investigate whether poorly differentiated tumors were more frequent in the groups who underwent percutaneous ablative therapy and hepatectomy. Portal vein invasion was investigated similarly in 58 patients, and was found to be present in six of 23 AFP-L3-positive patients and six of 35 AFP-L3-negative patients. No significant difference was observed between AFP-L3 and portal vein invasion in this limited investigation.

We demonstrated here in a multicenter retrospective study that AFP-L3 status was a significant prognostic factor affecting the long-term survival of patients who underwent percutaneous ablative therapy. In addition, to evaluate the prognostic influence of AFP-L3 in two subgroups comparable for tumor extension, we performed a multicenter prospective study to identify the prognostic factors for recurrence-free survival in patients with early stage HCC. Although this evaluation was conducted over a short period of time, we confirmed that AFP-L3 status was a significant prognostic predictor of recurrence-free survival in patients who underwent percutaneous ablative therapy, but it did not affect the prognosis of patients who underwent hepatectomy.

A number of studies have shown that AFP-L3 status is an independent prognostic factor in patients with HCC [12, 13, 15]. We previously reported that AFP-L3-positive (>15%) patients had a lower survival rate than negative (<15%) patients in subgroups with a low serum AFP concentration. Moreover, the statistically significant differences were more distinct in the subgroups with lower AFP concentrations [20]. However, the patients in these studies had received various treatments such as hepatectomy, RFA, and transcatheter arterial embolization, and

there have been few reports of the relationship between AFP-L3 status and prognosis in subgroups of HCC patients receiving different therapeutic modalities. Tateishi et al. [15] demonstrated that pre-treatment AFP-L3 positivity (>15%) was a significant predictor of HCC recurrence in patients who underwent curative ablation, and that AFP-L3 positivity after ablation was the strongest predictor of HCC recurrence by multivariate analysis. Although their study was performed in only one center and did not evaluate long-term survival, their results are compatible with ours.

Treatment of HCC patients with cirrhosis faces a dilemma in that minimization of damage to noncancerous liver tissue improves long-term survival, but incomplete treatment of subsequent HCC recurrences results in a poor prognosis. Accordingly, if a useful indicator of choice of therapeutic modality were to be available before the initial therapy, there would be several advantages in not only the treatment, but also the follow-up, of patients with HCC.

In conclusion, present results revealed that AFP-L3 had different impacts on prognosis in patients with HCC who underwent percutaneous ablative therapy and hepatectomy. Although this study was not a randomized control trial, AFP-L3 might be a promising scale to improve the prognostic estimate and appraisal of therapeutic outcome in patients with HCC.

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Multidrug resistance-associated protein 2 determines the efficacy of cisplatin in patients with hepatocellular carcinoma

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Abstract. We hypothesized that expression of multidrug resistance-associated protein 2 (MRP2), a major cisplatin transporter, may determine the efficacy of cisplatin as a treatment for patients with hepatocellular carcinoma (HCC). A prospective analysis was conducted of 49 consecutive patients who underwent resection for HCC (16 patients treated with cisplatin-based neoadjuvant chemotherapy and 33 patients treated without neoadjuvant chemotherapy). Expression of MRP2 in resected specimens was assessed by immunohistochemical and Western blot analyses. The extent of tumor necrosis was assessed histologically in the greatest dimension of the tumor specimen from each patient. The median percentage of tumor necrosis was 81% (range: 0-100%) and complete tumor necrosis was found in 3 patients. Overexpression of MRP2 was detected in 24/46 (52%) tumor specimens. In 16 patients treated with cisplatin, tumor size and dose of cisplatin did not correlate with tumor necrosis of the resected specimens (P=0.706 and P=0.555, respectively). Of 13 tumor specimens containing vivid tumor from 16 patients treated with cisplatin, 8 had overexpression of MRP2. Tumor specimens with overexpression of MRP2 showed a lower percentage of tumor necrosis than those with non-overexpression (median percentage of tumor necrosis, 19% vs. 99%, P=0.003). In conclusion, overexpression of MRP2 correlates with a lower percentage of tumor necrosis in patients treated with cisplatin-based neoadjuvant chemotherapy for HCC, whereas either tumor size or dose of cisplatin does not. Expression of MRP2 determines the efficacy of cisplatin-based chemotherapy in patients with HCC.

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Key words: hepatocellular carcinoma, drug resistance, multidrug resistance-associated protein 2, chemotherapy, cisplatin

Introduction

Multidrug resistance-associated protein 2 (MRP2; ABCC2), formally known as ATP-binding cassette (ABC), sub-family C, member 2, is a member of the superfamily of ABC transporters (1). MRP2 is localized to the canalicular (apical) membrane of hepatocytes (2-4), where it functions as a major exporter of organic anions, drugs, conjugated bilirubin, and bile salts to bile canaliculi (2-6).

MRP2 is one of the major transporters of cisplatin (7-9). *In vitro* experiments have shown that elevated expression of MRP2 decreases cisplatin accumulation in HCC cells and contributes to cisplatin resistance (9). Transfection of MRP2 antisense cDNA into a human hepatoma cell line decreased the MRP2 protein level and increased sensitivity to cisplatin (10). MRP2 expression in resected tumor specimens of patients with HCC, as detected by immunohistochemical analysis, ranges from 63 to 90% (11-13). In addition, MRP2 expression in tumor specimens is increased compared to non-neoplastic liver tissues using quantitative RT-PCR and Western blot analyses (13,14). The effect of tumor expression of MRP2 on the efficacy of cisplatin administration for patients with HCC has not been investigated previously. The aim of the current study was to test the hypothesis that expression of MRP2 may determine the efficacy of cisplatin-based neoadjuvant chemotherapy for patients with HCC.

Materials and methods

Patient population. From March 2007 through December 2008, a total of 59 consecutive Japanese patients with resectable HCC were referred to the Division of Digestive and General Surgery, Niigata University Medical and Dental Hospital (Niigata, Japan). Ten patients who received ablation therapy prior to surgical resection were excluded. The remaining 49 patients formed the basis of this prospective pilot study and included 39 men and 10 women with a median age of 70 years (range: 40-81 years). Signed informed consent

to participate in the current study was obtained from all patients. The current study was approved by the Institutional Review Board of Niigata University Medical and Dental Hospital.

Neoadjuvant chemotherapy. During the study period, neoadjuvant chemotherapy was applied to prevent tumor progression when a patient was on the waiting list for definitive operation for HCC for more than one month. The decision to use neoadjuvant chemotherapy was made by the Institutional Cancer Committee of Niigata University Medical and Dental Hospital. Indications for neoadjuvant chemotherapy included multiple hepatic tumors or a solitary tumor >3 cm in diameter, because these preoperative factors are closely associated with vascular invasion or poor post-resection survival (15-17). In the current series, 16 patients received neoadjuvant chemotherapy, which consisted of hepatic arterial infusion of a fine-powder formulation of cisplatin (IA-call®, Nippon Kayaku, Co., Ltd., Tokyo, Japan; recommended dose of 65 mg/m^2) under the guidance of hepatic angiography. The remaining 33 patients did not undergo neoadjuvant chemotherapy for HCC. The size of the largest hepatic tumor ranged from 1.5 to 12.1 cm (median tumor size, 3.5 cm) on contrast-enhanced spiral computed tomography (CT) images before neoadjuvant chemotherapy.

The response to neoadjuvant chemotherapy was assessed by contrast-enhanced spiral CT and was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines (18). Treatment-related toxicity was evaluated according to the Common Terminology Criteria of Adverse Events (CTCAE version 4.0; National Cancer Institute, Bethesda, MD, USA) (19).

Hepatectomy procedures. A hepatectomy procedure was selected for each patient, taking the primary tumor status (size, number, location), the hepatic functional reserve, and the patient's general condition into account (16). In the current study, the term 'major hepatectomy' indicated formal hemihepatectomy or more extensive resection, whereas less extensive hemihepatectomy was designated 'minor hepatectomy'. Postoperative morbidity was defined as any postoperative complication that lengthened the hospital stay (16).

Pathologic evaluation. Resected specimens were submitted to the Department of Surgical Pathology of Niigata University Medical and Dental Hospital. Each specimen was examined to determine the presence of cirrhosis, the number of hepatic tumors, the size of the largest hepatic tumor, the histologic grade, and gross or microscopic vascular invasion. The pathologic findings were described according to the TNM Classification of Malignant Tumours by the International Union Against Cancer (6th edition, 2002) (20).

A total of 90 hepatic tumors were resected in the current series. Twenty-eight patients had a solitary tumor and 21 had multiple tumors. The number of hepatic tumors was determined by gross examination of multiple slices from each resected specimen, but did not include satellite nodules. The definition of satellite nodules followed that of Taylor *et al* (21), regarding colorectal carcinoma liver metastasis. In patients with multiple tumors, the largest tumor was chosen as representative of all tumors.

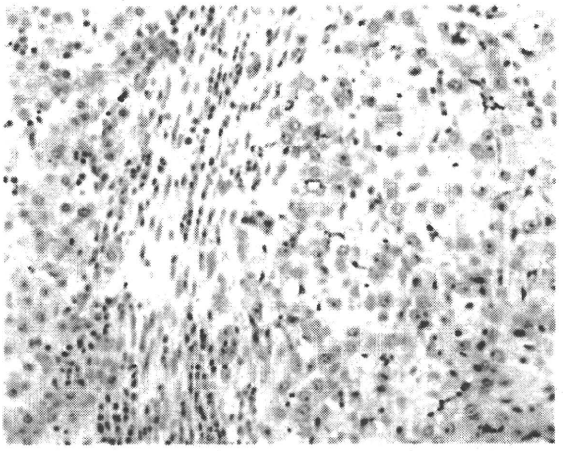

Figure 1. Hepatocellular carcinoma with overexpression of multidrug resistance-associated protein 2 (MRP2). Non-neoplastic hepatocytes in the left half show faint MRP2 expression in the canalicular membranes of hepatocytes, whereas cancerous tissue in the right half has overexpression of MRP2. Immunohistochemical staining: original magnification, x400.

The microscopic diagnosis of cirrhosis in the adjacent non-neoplastic liver was defined as the presence of regenerative nodules surrounded by fibrous septa. Using these criteria 33 patients had liver cirrhosis histologically verified. The median tumor size was 2.8 cm (range: 1.1-10 cm) in the resected specimens and the histologic grade was determined according to the Edmondson-Steiner classification (22), which is based on the areas of the tumor with the highest grade. Vascular invasion was defined as gross or microscopic involvement of the vessels (portal vein or hepatic vein) within the peritumoral liver tissue (17).

The extent of tumor necrosis was assessed histologically in the greatest dimension of the tumor specimen from each patient. The percentage of tumor necrosis was defined as the ratio between total necrotic area and the whole area of the tumor, multiplied by 100. In the greatest dimension of the tumor specimens from 16 patients who received neoadjuvant chemotherapy, the median necrotic area was 113.4 mm^2 (range: 0-2862 mm^2), whereas the median whole tumor area was 336.5 mm^2 (range: 57-5498 mm^2).

Immunohistochemistry. From each resected specimen, 1 to 3 paraffin-embedded block(s) (median, 2 blocks) were used for immunohistochemistry. Three serial 3-μm sections were re-cut and prepared from each block; 1 for hematoxylin and eosin staining, 1 for MRP2-immunohistochemical staining, and 1 as a negative control. Two independent surgical pathologists blinded to the clinical details assessed each section.

The streptavidin-biotin immunoperoxidase method was performed using the Histofine SAB-PO (M) kit (Nichirei Biosciences Inc., Tokyo, Japan). The sections were deparaffinized and rehydrated, then microwaved at 500 W for 7 cycles of 3 min in 10 mmol/l sodium citrate buffer (pH 6.0) to retrieve antigenic activity. Endogenous peroxidase activity was inhibited by incubation with 0.3% hydrogen peroxidase in methanol for 20 min. Sections were blocked against non-specific reactions

Table I. Tumor response in 16 patients treated with cisplatin-based neoadjuvant chemotherapy.

	No. of patients					
	CR	PR	SD	PD	Response rate (%)	P-value
Total	0	4	12	0	25	
No. of tumors						0.569
Solitary	0	3	5	0	37.5	
Multiple	0	1	7	0	12.5	
Tumor size (cm)						>0.999
≤3	0	2	6	0	25	
>3	0	2	6	0	25	
Stage						0.435
I	0	3	4	0	43	
II	0	1	6	0	14	
III	0	0	2	0	0	

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

with 10% normal rabbit serum, and were then incubated overnight at 4˚C with mouse anti-MRP2 monoclonal antibody (clone M2III-6; Monosan, Uden, The Netherlands; dilution at 1:20). Sections were then incubated with biotinylated rabbit anti-mouse immunoglobulin for 30 min followed by incubation with the streptavidin-peroxidase complex for 10 min. Diaminobenzidine was used as the chromogen, and the sections were counterstained with hematoxylin. Normal mouse immunoglobulin was substituted for the primary antibody as a negative control, whereas the immunoreactivity of adjacent non-neoplastic liver tissue was used as an internal positive control.

Pattern of MRP2 immunohistochemical expression in HCC. MRP2 expression was defined as the immunoreactivity of canalicular (apical) membranes of hepatocytes according to the description of Paulusma *et al* (4). Non-neoplastic hepatocytes showed weak to moderate intensity of MRP2 expression in the canalicular membrane. Immunoreactivity of MRP2 in tumor specimens was evaluated by comparison with adjacent non-neoplastic hepatocytes and classified into 3 categories: unchanged expression, when immunoreactivity of the tumor specimen was similar to that of non-neoplastic hepatocytes; loss-of-expression, characterized by totally negative immunoreactivity throughout the tumor specimen; and diffuse expression, characterized by strong positive immunoreactivity throughout the tumor specimen (Fig. 1). In the current study, overexpression of MRP2 was defined as diffuse expression, whereas non-overexpression was defined as unchanged or loss-of-expression.

Detection of MRP2 expression by Western blot analysis. Tissue samples were prepared for Western blotting by first snap-freezing and then stored at -80˚C until used for analysis. Tissue samples from 3 normal livers obtained at surgery for other conditions were processed for analysis as normal controls. Lysate from tissue samples were obtained by homogenization in the lysis buffer [20 mM Tris-buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 8.0, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 M sodium chloride, 0.5% sodium deoxycholate, 0.5% Triton X-100, 0.25 M sucrose, 50 mM 2-mercapto-ethanol, 250 μM phenylmethylsulfonyl fluoride and 1 μM pepstatin]. The lysate samples were kept on ice for 30 min, filtered through gauze and precleared by centrifugation at 15,000 rpm for 15 min at 4˚C. Following protein quantification using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA), 50 μg aliquots of samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon membranes (Millipore, Bedford, MA, USA). Nonspecific sites binding sites on the membranes were blocked in 5% skim milk, whereupon filters were incubated with anti-MRP2 antibody (clone M2III-6; Monosan, Uden; dilution at 1:1000) and then the appropriate horseradish peroxidase-conjugated secondary antibodies. After the detection was performed using enhanced chemiluminescence reagent (GE Healthcare, Buckingham, UK), the blots were stripped, washed, and reprobed for ß-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution at 1:5000).

Statistical analysis. Medical records were obtained from all 49 patients. Categorical variables were compared by the Fisher exact test or the Pearson χ^2 test; continuous variables were compared by the Mann-Whitney U test. Correlation between 2 continuous variables was evaluated by the Spearman rank correlation. All statistical evaluations were performed using the SPSS 16.0J software package (SPSS Japan, Tokyo, Japan). All tests were two-sided and $P<0.05$ was considered statistically significant.

Results

Tumor response in 16 patients treated with cisplatin-based neoadjuvant chemotherapy. In 16 patients who received

Table II. Toxicity in 16 patients treated with cisplatin-based neoadjuvant chemotherapy.

Characteristics	No. of patients			
	Grade 1	Grade 2	Grade 3	Grade 4
Hematological toxicity				
Leukocytopenia	2	1	0	0
Anemia	0	1	0	0
Thrombocytopenia	2	2	0	0
Non-hematological toxicity				
Fever	2	0	0	0
Diarrhea	1	0	0	0
Decreased albumin level	2	7	0	0
Elevated total bilirubin level	4	1	0	0
Elevated AST level	2	8	2	0
Elevated ALT level	3	7	1	0
Elevated creatinine level	6	0	0	0

AST, aspartate aminotransferase; ALT, alanine aminotransferase.

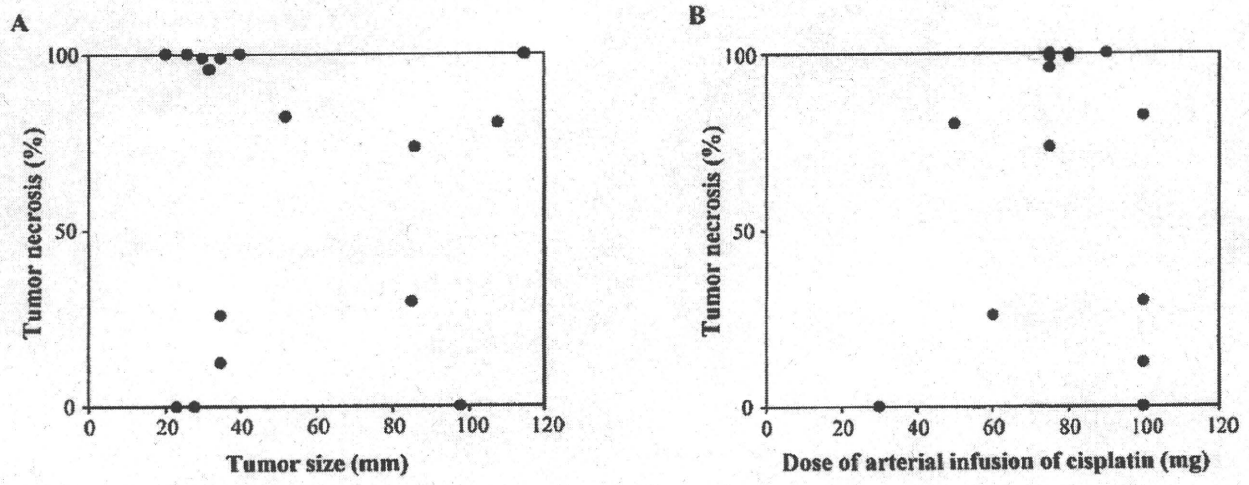

Figure 2. Correlation of tumor necrosis with tumor size and dose of cisplatin. (A) Tumor size prior to neoadjuvant chemotherapy does not correlate with tumor necrosis (correlation coefficient = -0.102; P=0.706). (B) The dose of hepatic arterial infusion of cisplatin has no correlation with tumor necrosis (correlation coefficient = -0.160; P=0.555).

cisplatin-based neoadjuvant chemotherapy, the median dose of cisplatin was 77.5 mg per body (range: 30-100 mg per body). The overall response rate of these patients was 25%; the therapeutic efficacy according to the RECIST guidelines was not associated with tested tumor-related factors (Table I).

Toxicity in 16 patients treated with cisplatin-based neoadjuvant chemotherapy. All 16 patients were assessed for toxicity and no toxic deaths occurred. The incidences of the main treatment-related toxicity according to the CTCAE version 4.0 are listed in Table II as the maximum grade seen per patient. No patients with grade 4 toxicities were identified. Three grade 3 non-hematological toxicities were observed. All hematological toxicities were grade 2.

Surgical resection. Hepatectomy procedures that were planned before neoadjuvant chemotherapy was administered were performed in all 16 patients who received the neoadjuvant chemotherapy. The interval between neoadjuvant chemotherapy and delayed surgery ranged from 30 to 114 days (median: 53 days). Operative procedures included major hepatectomy in 13 patients and minor hepatectomy in 36 patients. Complications during the postresection hospital stay occurred in 10 (20%) patients. Intra-abdominal sepsis (n=5) was the most common complication, followed by wound infection (n=4), biliary fistula (n=2), and pneumonia (n=1). The incidence of postoperative morbidity was 13% (2 of 16 patients) in patients treated with neoadjuvant chemotherapy compared with 24% (8 of 33 patients) in patients treated

Table III. Factors associated with MRP2 expression in 46 tumor specimens.

Variable	No. of patients		P-value
	Non-overexpression of MRP2	Overexpression of MRP2	
Age			0.568
≤70	13	12	
>70	9	12	
Sex			>0.999
Male	18	20	
Female	4	4	
Neoadjuvant chemotherapy			0.521
Absent	17	16	
Present	5	8	
Liver cirrhosis			0.763
Absent	7	9	
Present	15	15	
Tumor size (cm)			0.388
≤3	14	12	
>3	8	12	
Number of hepatic tumors			0.080
Solitary	16	11	
Multiple	6	13	
Edmondson-Steiner grade			0.507
I	4	4	
II	17	16	
III	1	4	
pT classification			0.625
pT1	13	11	
pT2	8	10	
pT3	1	3	
Vascular invasion			>0.999
Absent	18	20	
Present	4	4	

MRP2, multidrug resistance-associated protein 2; pT, pathologic T classification.

without neoadjuvant chemotherapy (P=0.464). There were no in-hospital deaths in the current series.

Tumor necrosis in resected specimens of 16 patients treated with cisplatin. The median percentage of histologically verified tumor necrosis was 81% (range: 0-100%). Complete tumor necrosis (no evidence of vivid tumor) was found in 3 patients [1 patient with PR (partial response) and 2 patients with SD (stable disease)], whereas no evidence of tumor necrosis was observed in 3 patients. Tumor size on CT images prior to neoadjuvant chemotherapy did not correlate with tumor necrosis of the resected specimens (P=0.706; Fig. 2A). The dose of hepatic arterial infusion of cisplatin did not correlate with tumor necrosis of the resected specimens (P=0.555; Fig. 2B). The median percentage of tumor necrosis was 99.4% (range: 73.6-100%) in 4 patients with PR, whereas it was 54.9% (range: 0-100%) in 12 patients with SD. There were no apparent differences in the percentage of tumor necrosis between 2 groups (PR vs. SD) according to the RECIST criteria by Mann-Whitney U test (P=0.138).

Immunohistochemical analysis of MRP2 expression. Three tissue samples with complete tumor necrosis were excluded from immunohistochemical analysis for MRP2. In the remaining 46 tissue samples, overexpression of MRP2 was detected in 24/46 (52%) of tumor specimens (Table III).

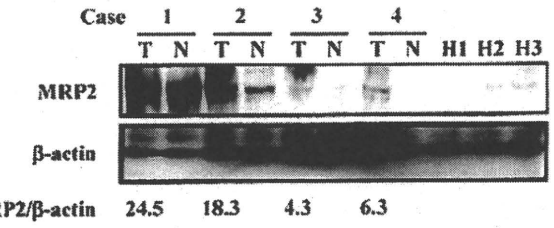

Figure 3. Western blot analysis for multidrug resistance-associated protein 2 (MRP2) levels. Based on immunohistochemical analysis, case 1 and 2 show overexpression of MRP2, whereas case 3 and 4 show non-overexpression of MRP2. The band intensities of tumor samples tested by Western blot analysis show a close correlation with the results of immunohistochemical analysis. MRP2 expression is faint in all healthy liver tissues. T; tumor, N; non-tumor, H1-3; healthy liver.

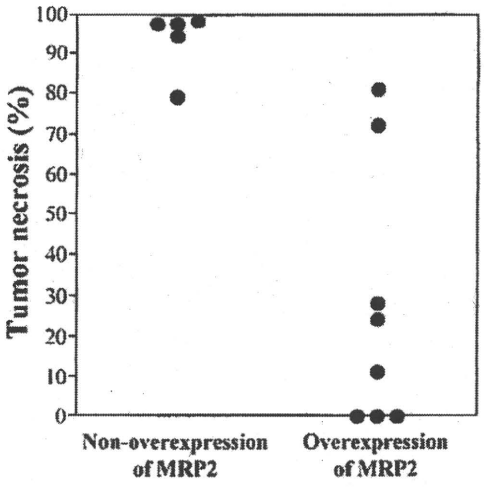

Figure 4. The extent of tumor necrosis in relation to the level of multidrug resistance-associated protein 2 (MRP2) expression. The percentage of tumor necrosis is significantly lower in tumor specimens with overexpression of MRP2 (median percentage of necrosis, 19%) than in tumor specimens with non-overexpression of MRP2 (median percentage of necrosis, 99%; P=0.003).

Loss-of-expression of MRP2 was found in 2/46 (4%) of tumor specimens; unchanged expression of MRP2 was observed in 20/46 (44%) of tumor specimens; and thus 22 tumor specimens were categorized as specimens with non-overexpression of MRP2.

Western blot analyses of MRP2 expression levels. To confirm the results of the immunohistochemical analysis for MRP2, we performed Western blot analysis on 5 randomly selected tumor samples with overexpression of MRP2, 5 tumor samples with non-overexpression of MRP2, and 3 tissue samples from healthy liver. Fig. 3 shows the representative results of Western blotting for MRP2 of selected tissue samples. MRP2 expression was faint in all healthy liver tissues tested by Western blot analysis, whereas the band intensities of tumor samples showed a close correlation with the results of immunohistochemical analysis.

Factors associated with MRP2 expression in 46 tumor specimens. Overexpression of MRP2 in tumor specimens was not associated with tested clinical-pathologic factors including neoadjuvant chemotherapy (Table III). Overexpression of MRP2 was detected in 16/33 (48%) of tumor specimens from patients treated without neoadjuvant chemotherapy, suggesting that nearly half of the patients with HCC have intrinsic overexpression of MRP2.

MRP2 expression and tumor necrosis in 13 tumor specimens containing vivid tumor from patients treated with cisplatin. We identified 8 tumor specimens with overexpression of MRP2 out of 13 tumor specimens containing vivid tumor from 16 patients treated with cisplatin-based neoadjuvant chemotherapy (Table III). Tumor specimens with overexpression of MRP2 showed a lower percentage of tumor necrosis (median percentage of necrosis, 19%) compared to tumor specimens with non-overexpression of MRP2 (median percentage of necrosis, 99%; P=0.003; Fig. 4).

Discussion

The activity of ABC transporters is one of the major causes of resistance to chemotherapy in patients with HCC (23). Of several identified ABC transporters, MRP2 is the principal cisplatin transporter (7-9). There is a paucity of clinical data regarding any relationship between MRP2 expression and tumor necrosis in patients treated with cisplatin-based neoadjuvant chemotherapy prior to hepatectomy for HCC. This prompted us to conduct the current study. This is the first study to demonstrate that MRP2 overexpression correlates with a lower percentage of tumor necrosis in tumors from patients treated with cisplatin-based neoadjuvant chemotherapy for HCC and thus expression of MRP2 determines the efficacy of cisplatin-based chemotherapy in patients with HCC.

Cisplatin is a widely used chemotherapeutic agent in the treatment of HCC (24). The fine-powder formulation of cisplatin has 3 times higher concentration compared to conventional formulations of cisplatin (25). Yoshikawa *et al* (25) reported that hepatic arterial infusion of a fine-powder formulation of cisplatin had a high therapeutic efficacy with a response rate of 33.8%. In the current study, we confirmed the results of Yoshikawa *et al* (25). Severe adverse effects (grade 3, liver dysfunction) were observed in 3 patients but they were manageable and transient. Planned hepatectomy procedures were performed in all 16 patients treated with neoadjuvant chemotherapy without any increase in postoperative complications. Thus, neoadjuvant chemotherapy with a fine-powder formulation of cisplatin is well tolerated and does not impair planned hepatectomy procedures.

We observed high tumor necrosis in the tumor specimens of patients who were treated with cisplatin-based neoadjuvant chemotherapy for HCC. In contrast, there were no apparent differences in the percentage of tumor necrosis between patients with PR and patients with SD according to RECIST criteria. In fact, complete tumor necrosis (no evidence of vivid tumor) was found in 2 patients with SD. Forner *et al* (26) have the questioned the reliability of RECIST criteria, which are based on the evaluation of unidimensional tumor measurements and disregard the extent of necrosis in solid liver tumors. Forner *et al* (26) recommended the evaluation

of tumor response based on measurements of the reduction in viable tumor burden as determined by dynamic imaging studies. Michaelis and Ratain (27) also suggested that RECIST criteria have limited value in the assessment of tumor response after chemotherapy because some anti-cancer agents have cytostatic, rather than cytotoxic properties, so that shrinkage of the tumor after cytostatic chemotherapy is not expected. Further investigation is required to develop effective criteria for the assessment of tumor response after chemotherapy.

MRP2 is a major transporter of conjugated bilirubin and bile salts into the bile canaliculi (5,6). In the current study, half of the tumor samples showed MRP2 overexpression, irrespective of administration of cisplatin (Table III). Since HCCs often produce bile, the intrinsic expression of MRP2 in HCC may partly explain their low sensitivity to some MRP2-dependent anti-cancer agents including cisplatin, doxorubicin, etoposide, and vincristine (7,28). Bonin *et al* documented that MRP2 mRNA expression level significantly increases in HCC compared to non-neoplastic liver tissue (14). In addition, Zollner *et al* using Western blot analysis found that MRP2 protein expression was elevated in all 4 HCC samples examined (13). In the current study, overexpression of MRP2 correlated with a lower percentage of tumor necrosis in patients treated with cisplatin-based neoadjuvant chemotherapy for HCC, whereas tumor size or dose of cisplatin did not. Collectively, these above findings suggest that expression of MRP2 determines the efficacy of cisplatin-based chemotherapy in patients with HCC.

Various classic compounds that inhibit MRP2 activity *in vitro* such as MK-571 or cyclosporin A have been proposed to be used to increase antitumor therapeutic effectiveness (29,30). The intrinsic toxicity of MRP2 inhibitors at doses necessary for their activity and their poor specificity are the major obstacles in applying them *in vivo* (8). In attempt to develop alternative, less toxic, and more efficient therapy, Meterna *et al* specifically inhibited MRP2 protein expression by 2 anti-MRP2 hammerhead ribozymes. This gene therapeutic approach may be applicable to overcome cisplatin resistance in tumor cells (8). Folmer *et al* demonstrated that tumors resulting from MRP2-overexpressing subcutaneously grown hepatoma cells, regressed in size upon antisense MRP2 expression in combination with vincristine (28). Wakamatsu *et al* reported that co-treatment with cisplatin and both glycyrrhizin and lamivudine inhibited the cisplatin efflux from Huh7 HCC cell line and concluded this was because glycyrrhizin is a competitive substrate for MRP2 (9). Therefore, it appears that inhibition of MRP2 may be an approach to develop an effective therapy to overcome cisplatin resistance in patients with HCC.

In interpreting the current study, we have considered the limitations of the analysis of a small number of patients and incomplete follow-up assessment of performed chemotherapeutic treatment using RECIST criteria. In fact, we believe that these limitations do not greatly influence the results of the study because the differences among groups were too marked to have resulted from these procedural biases.

In conclusion, overexpression of MRP2 correlates with a lower percentage of tumor necrosis in patients treated with cisplatin-based neoadjuvant chemotherapy for HCC, whereas either tumor size or dose of cisplatin does not. Expression of MRP2 determines the efficacy of cisplatin-based chemotherapy in patients with HCC.

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ORIGINAL ARTICLE

Clinical Advantage of Highly Sensitive On-Chip Immunoassay for Fucosylated Fraction of Alpha-Fetoprotein in Patients with Hepatocellular Carcinoma

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Abstract

Background Alpha-fetoprotein (AFP) has been widely used as a diagnostic master for hepatocellular carcinoma (HCC), and the fucosylated fraction of AFP (AFP-L3) has been reported to be a specific marker for HCC. However, AFP-L3 has not always been reliable in cases with low serum AFP concentrations. Recently, a novel automated immunoassay for AFP-L3, the micro-total analysis system (μ-TAS), has been developed.

Aim The aim of this study is to evaluate the clinical usefulness of μ-TAS AFP-L3.

Methods Serum AFP-L3 was measured in 295 patients with HCC and in 350 with benign liver diseases. The diagnostic accuracy of μ-TAS AFP-L3 was compared with that of the conventional assay (liquid-phase binding assay; LiBASys). The relationship between μ-TAS AFP-L3 and clinical features was investigated.

Results When the cutoff value was set at 7%, the sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of μ-TAS AFP-L3 were 60.0%, 90.3%, 76.4%, 83.9%, and 72.8%, respectively. Its sensitivity was particularly good (41.1%) in HCC subgroups with lower AFP concentrations (<20 ng/ml). The positivity rates for μ-TAS AFP-L3 were higher at each tumor stage than those of LiBASys AFP-L3 (μ-TAS/LiBASys: stage I, 44.2%/16.3%; stage II, 52.9%/37.5%; stage III, 66.4%/44.5%; stage IV, 82.8%/65.5%).

Conclusions μ-TAS AFP-L3 is more sensitive for discriminating HCC than the conventional LiBASys AFP-L3, particularly in subgroups with lower AFP concentrations and early-stage HCC.

Keywords Alpha-fetoprotein · AFP-L3 · Diagnosis · Hepatocellular carcinoma

Introduction

Alpha-fetoprotein (AFP) has been widely used as a diagnostic marker for hepatocellular carcinoma (HCC) [1, 2]. However, AFP levels are sometimes elevated in patients with chronic hepatitis and cirrhosis who have no evidence of HCC [3–5]. Therefore, the usefulness of AFP as a screening marker of HCC has been limited by its impaired specificity.

The fucosylated fraction of AFP (AFP-L3) has been reported to be a specific marker for HCC [6–8]. Moreover, its level predicts the malignant potential of HCC with subsequent unfavorable prognosis after treatment [9–13]. However, measurement of AFP-L3 has not always been reliable for serum samples with low total AFP concentration determined by conventional lectin affinity electrophoresis or using a liquid-phase binding assay system (LiBASys) [14].

Recently, a novel automated immunoassay for AFP-L3 using on-chip electrokinetic reaction and separation by affinity electrophoresis (micro-total analysis system; μ-TAS) has been developed [15, 16]. This system involves microchip capillary electrophoresis and a liquid-phase

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binding assay system, and the assay can be run in less than 10 min.

The aim of this study is to evaluate the clinical usefulness of measuring μ-TAS AFP-L3 as a diagnostic and prognostic marker in comparison with the conventional LiBASys AFP-L3 assay.

Methods

Patients

Frozen serum samples were obtained from 295 patients with HCC and from 350 patients with benign liver diseases (BLD) who had chronic hepatitis or liver cirrhosis between April 1999 and September 2009. Among the BLD patients, 80 were positive for hepatitis B surface antigen (HBsAg), 167 were positive for anti-hepatitis C virus (HCV), 19 had autoimmune hepatitis, 51 had primary biliary cirrhosis, 17 had alcoholic liver disease, 11 had nonalcoholic steatohepatitis, and 5 had other conditions. HCC patients were diagnosed using imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), and CT during hepatic arteriography, considering hyperattenuation in the arterial phase with washout in the late phase. Vascular invasion was evaluated by imaging modalities. In some cases that showed atypical features on imaging, ultrasound-guided biopsies were performed. Tumor stage on imaging findings was ranked using the tumor–node–metastasis (TNM) staging system of the Liver Cancer Study Group of Japan [17, 18]: T1 (fulfilling the following three conditions: solitary, ≤2 cm, no vessel invasion), T2 (fulfilling two of the three conditions), T3 (fulfilling one of the three conditions), T4 (fulfilling none of the three conditions or showing presence of distant metastasis), N0 (no lymph nodes metastasis), N1 (metastasis to lymph nodes), M0 (no distant metastasis), M1 (distant metastasis), and stage I (T1N0M0), stage II (T2N0M0), stage III (T3N0M0), and stage IV (T4N0M0, or any TN1M0, or any TN0-1M1).

Therapeutic modalities for HCC patients were chosen on the basis of hepatic functional reserve, tumor multiplicity, and tumor size. Among the 295 HCC patients, 94 underwent hepatic resection, 60 underwent radiofrequency ablation (RFA) or microwave coagulation therapy (MCT), 32 underwent percutaneous ethanol injection (PEI), 48 underwent transcatheter arterial chemoembolization (TACE), 40 underwent transcatheter arterial infusion chemotherapy (TAI), 10 underwent systemic chemotherapy, and 11 received best supportive care. Long-term survival data for these HCC patients were confirmed at the end of October 2009.

Informed consent was obtained from each patient, and the study protocol conformed with the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in the a priori approval by our institution's human research committee.

Laboratory Examination

Simultaneous quantitative measurement of AFP-L1 (ng/ml) and AFP-L3 (ng/ml) was achieved using the μ-TAS assay (Wako Pure Chemical Industries Ltd., Osaka, Japan) [15, 16], and then the percentage of AFP-L3 was calculated. As a reference method, AFP-L1 and AFP-L3 were also measured using a liquid-phase binding assay system (LiBASys) at the same time (Wako Pure Chemical Industries Ltd., Osaka, Japan) [14]. Total AFP concentration (ng/ml) in the serum sample was determined by addition of AFP-L3 to AFP-L1. Serum des-gamma-carboxy prothrombin (DCP) was measured using a μ-TAS assay for DCP (Wako Pure Chemical Industries Ltd, Osaka, Japan). The lower limits of quantitation for μ-TAS assay AFP (L1 and L3), LiBASys assay AFP (L1 and L3), and μ-TAS assay DCP are 0.3 ng/ml AFP (L1 and L3), 0.8 ng/ml AFP (L1 and L3), and 5 mAU/ml DCP, respectively [14–16]. When AFP-L3 is not detectable, the percentage of AFP-L3 is defined as 0.5%. AFP, AFP-L3, and DCP were measured in the same serum before treatment of HCC. Hepatic functional reserve was ranked using the criteria of the Child–Pugh scoring system.

Statistical Analysis

The correlations of the μ-TAS assay with the LiBASys assay were evaluated using the Pearson product-moment correlation coefficient. Receiver operating characteristic (ROC) analysis was used to evaluate the diagnostic value of the μ-TAS AFP-L3 assay and the LiBASys assay. Sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of the μ-TAS assay AFP, AFP-L3, DCP, and LiBASys assay AFP-L3 were calculated.

Long-term survival of patients with HCC was determined by Kaplan–Meier method. Log-rank test was used to test for equality of long-term survival between groups. Multivariate analyses of prognostic factors in the clinical features were performed using the Cox proportional-hazards model. The factors included in multivariate analysis were patient age, gender (female/male), HBsAg (negative/positive), anti-HCV (negative/positive), alcohol intake (not frequent/frequent), Child–Pugh class (A/B, C), log (AFP ng/mL), log (DCP mAU/mL), μ-TAS assay AFP-L3 (%) (<7/≥7), tumor size (mm), number of tumors (single/multiple), vascular invasion (negative/positive), and

treatment method (hepatic resection/RFA, PEI, TACE, TAI). Statistical analyses were performed by using the SPSS 15.0 software package (SPSS Japan Inc., Tokyo, Japan). Differences at $P < 0.05$ were considered to be statistically significant.

Results

Clinical Features of Patients

Demographics, etiology of liver disease, hepatic functional reserve ranked by Child–Pugh classification, and tumor stage of the study patients are summarized in Table 1. Two hundred ninety-five patients with HCC comprised 43 patients with stage I, 104 with stage II, 119 with stage III, and 29 with stage IV.

Serum total AFP concentration and AFP-L3% in the HCC group and the BLD group are shown in Table 2.

Table 1 Clinical features of patients with HCC and BLD

	HCC ($n = 295$)	BLD ($n = 350$)
Age, median (range), years	70 (38–89)	60 (16–86)
Gender (female/male)	95/200	196/154
Etiology		
HBsAg (+)	69	80
Anti-HCV (+)	172	167
AIH	4	19
PBC	3	51
Alcoholic	27	17
NASH	7	11
Others	13	5
Child–Pugh class		
A/B/C	193/85/13	246/22/12
Tumor stage		
I/II/III/IV	43/104/119/29	
Therapeutic modality		
Hepatic resection	94	
RFA or MCT	60	
PEI	32	
TACE	48	
TAI	40	
Systemic chemotherapy	10	
Best supportive care	11	

HCC hepatocellular carcinoma, *BLD* benign liver disease, *HBsAg* hepatitis B surface antigen, *HCV* hepatitis C virus, *AIH* autoimmune hepatitis, *PBC* primary biliary cirrhosis, *NASH* nonalcoholic steatohepatitis, *RFA* radiofrequency ablation, *PEI* percutaneous ethanol injection, *TACE* transcatheter arterial chemoembolization, *TAI* transcatheter arterial infusion chemotherapy

Table 2 Serum total AFP concentration and AFP-L3% in patients with HCC and BLD

	HCC ($n = 295$)	BLD ($n = 350$)
LiBASys assay		
AFP ng/ml, median (range)	43.2 (0.8–1054374.1)	1.2 (0.8–1,767)
AFP-L3%, median (range)	1.3 (0.5–94.5)	0.5 (0.5–16.5)
μ-TAS assay		
AFP ng/ml, median (range)	51.1 (1.1–921613.9)	2.8 (0.3–1648.8)
AFP-L3%, median (range)	9.2 (0.5–97.0)	0.5 (0.5–17.3)

HCC hepatocellular carcinoma, *BLD* benign liver disease, *AFP* alpha-fetoprotein, *AFP-L3* fucosylated fraction of AFP, *LiBASys* liquid-phase binding assay system, *μ-TAS* micro-total analysis system

Correlations of the μ-TAS Assay with the LiBASys Assay for Total AFP Concentration and AFP-L3%

The correlation of the μ-TAS assay with the LiBASys assay for total AFP was evaluated. A correlation coefficient of 0.997 and a slope of 0.926 indicated good correlation between the two methods in 586 patients whose serum total AFP concentration was less than 1,000 ng/ml (Fig. 1a). AFP-L3 was also evaluated, and this revealed that the serum AFP-L3 level determined by using the μ-TAS assay correlated well ($r = 0.965$) with that determined by the LiBASys assay (Fig. 1b). However, the correlation became weaker for serum in which AFP was less than 20 ng/ml ($r = 0.387$) (Fig. 1c).

Comparison of Diagnostic Efficacy Between μ-TAS AFP-L3 and LiBASys AFP-L3

ROC curves for serum AFP-L3 examined by using the μ-TAS assay and the LiBASys assay are shown in Fig. 2. The area under the curve of μ-TAS AFP-L3 (0.858) was larger than that of LiBASys AFP-L3 (0.744), indicating that the diagnostic accuracy of AFP-L3 examined using the μ-TAS assay was superior to that obtained using the LiBASys assay.

We compared the diagnostic efficacy between μ-TAS AFP-L3 and LiBASys AFP-L3 at several cutoff values. The sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of μ-TAS AFP-L3, LiBASys AFP-L3, μ-TAS AFP (cutoff 200 ng/ml), and μ-TAS DCP (cutoff 40 mAU/ml) are shown in Table 3a.

A cutoff value of 1% yielded the maximum value of Youden index (specificity/100 + sensitivity/100 − 1) of 0.637, when examined using the μ-TAS AFP-L3 assay. A cutoff value of 10% has been used for diagnosis of HCC in previous reports [8, 10–13, 19]. Additionally, we have

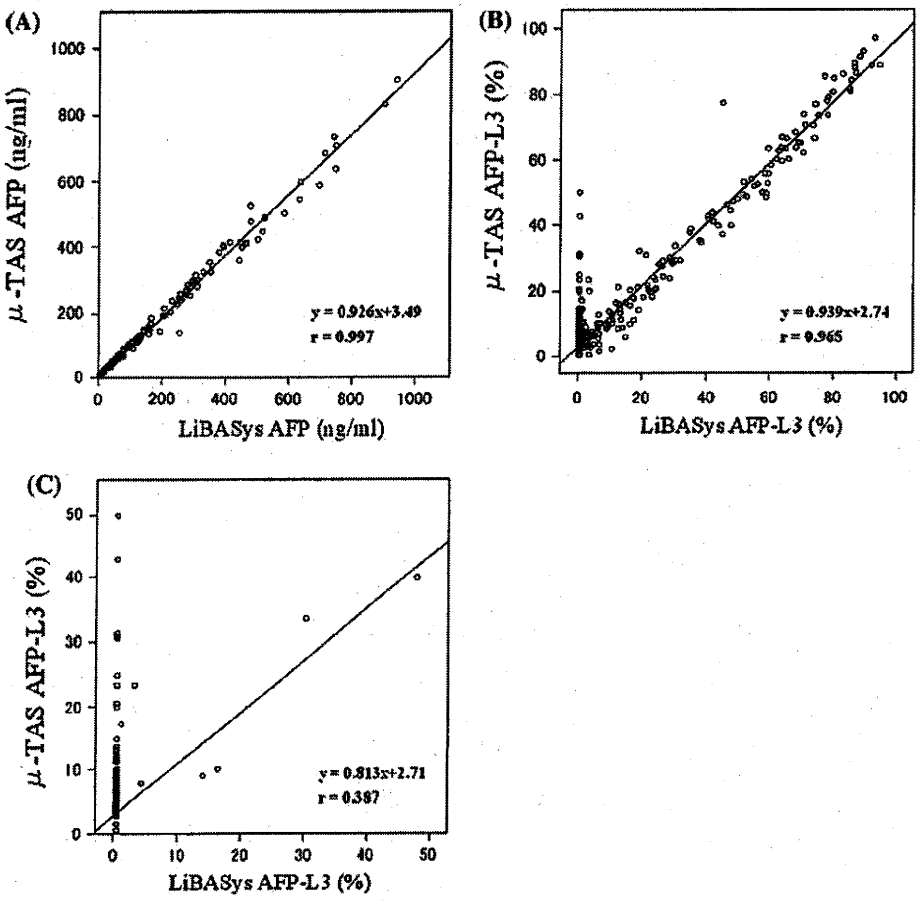

Fig. 1 Correlations of the μ-TAS assay with the LiBASys assay in terms of total AFP concentration (a), AFP-L3% (b), and AFP-L3% in subgroup with lower serum AFP concentration (<20 ng/ml) (c). Good correlation for AFP (correlation coefficient $r = 0.997$; slope 0.926) was observed between the μ-TAS assay and the LiBASys assay (a). The serum AFP-L3 level determined by the μ-TAS assay was well correlated ($r = 0.965$) with that determined by the LiBASys assay (b). The correlation became weaker for serum in which AFP was less than 20 ng/ml ($r = 0.387$) (c)

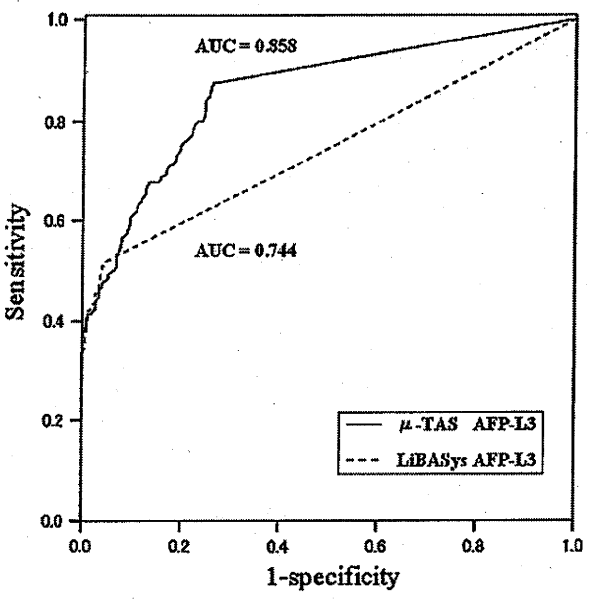

Fig. 2 Receiver operating characteristic curve for serum AFP-L3 examined using the μ-TAS and LiBASys assays. The area under the curve of μ-TAS AFP-L3 (0.858) was larger than that of LiBASys AFP-L3 (0.744)

chosen a cutoff value of 7%, which had been demonstrated to improve the sensitivity of the μ-TAS AFP-L3 assay greatly, and to maintain the specificity at 90% or more. The μ-TAS AFP-L3 was more sensitive for discriminating HCC than the LiBASys AFP-L3 at three cutoff levels with a small decrease of specificity compared with the LiBASys AFP-L3. In addition, μ-TAS AFP-L3 (cutoff 7%, 60.0%) was more sensitive for discriminating HCC than the total AFP level (cutoff 200 ng/ml, 33.6%) and μ-TAS DCP (cutoff 40 mAU/ml, 55.8%), which has been used as a standard marker for HCC. The diagnostic accuracies of μ-TAS AFP-L3 were superior to those of LiBASys AFP-L3 and μ-TAS AFP.

This diagnostic advantage of μ-TAS AFP-L3 was also observed in 432 patients (HCC, 112; BLD, 320) who had lower serum total AFP concentrations (less than 20 ng/ml). Serum AFP-L3% measured using the μ-TAS assay and the LiBASys assay in the lower serum AFP group is shown in Fig. 3a, b. The sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of serum AFP-L3% calculated using three cutoff values (1%, 7%, and 10%) to determine HCC in the lower serum AFP group are shown in Table 3b. The sensitivity of μ-TAS AFP-L3 was especially good (μ-TAS/LiBASys: cutoff 1%,

Table 3 Sensitivity, specificity, accuracy, positive predictive values, and negative predictive values of μ-TAS AFP-L3, LiBASys AFP-L3, μ-TAS AFP, and μ-TAS DCP

	Cutoff	Sensitivity	Specificity	Accuracy	PPV	NPV
(A)						
LiBASys AFP-L3	1%	50.8%	95.7	75.2	90.9	69.8
	7%	40.0	99.1	72.1	97.5	66.2
	10%	38.3	99.4	71.5	98.3	65.7
μ-TAS AFP-L3	1%	87.5	73.7	80.0	73.7	87.5
	7%	60.0	90.3	76.4	83.9	72.8
	10%	47.5	96.0	73.8	90.9	68.4
μ-TAS AFP	200 ng/ml	33.6	98.0	68.5	93.4	63.6
μ-TAS DCP	40 mAU/ml	55.8	95.3	76.9	91.1	71.5
(B)						
LiBASys AFP-L3	1%	4.5	99.4	74.8	71.4	74.8
	7%	3.6	100	75.0	100	74.8
	10%	3.6	100	75.0	100	74.8
μ-TAS AFP-L3	1%	67.9	80.6	77.3	55.1	87.8
	7%	41.1	91.9	78.7	63.9	81.7
	10%	21.4	96.9	77.3	70.6	77.9

PPV positive predictive value, *NPV* negative predictive value, *AFP* alpha-fetoprotein, *AFP-L3* fucosylated fraction of AFP, *LiBASys* liquid-phase binding assay system, *μ-TAS* micro-total analysis system, *DCP* des-gamma-carboxy prothrombin

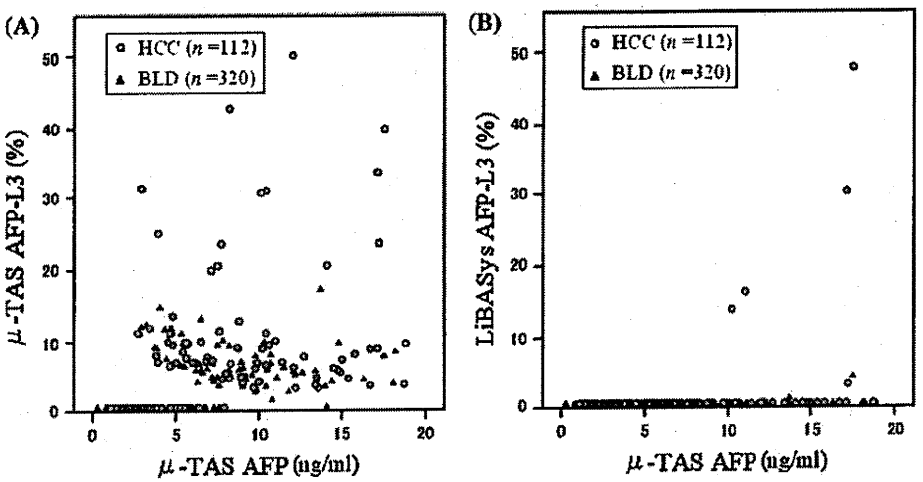

Fig. 3 Serum AFP-L3% measured using the μ-TAS and LiBASys assays in the lower serum AFP group (less than 20 ng/ml). μ-TAS assay (**a**) and LiBASys assay (**b**)

67.9%/4.5%; cutoff 7%, 41.1%/3.6%; cutoff 10%, 21.4%/3.6%) in the subgroups with lower AFP concentrations (<20 ng/ml). The diagnostic accuracy of μ-TAS AFP-L3 was superior to that of LiBASys AFP-L3, and the cutoff value of 7% for μ-TAS AFP-L3 had the most accurate diagnostic power (accuracy, 78.7%).

AFP-L3 Positivity Rate in Relation to Tumor Characteristics

When the cutoff value of AFP-L3 was set at 7%, the positivity rates for μ-TAS AFP-L3 were higher for each tumor stage (44.2% for stage I, 52.9% for stage II, 66.4% for stage III, 82.8% for stage IV) than those for LiBASys AFP-L3 (16.3% for stage I, 37.5% for stage II, 44.5% for stage III, 65.5% for stage IV). High positivity rates for μ-TAS AFP-L3 were also found in patients classified by tumor size and tumor number (μ-TAS/LiBASys: tumor size ≤2 cm, 43.5%/22.9%; 2 cm < tumor size ≤ 3 cm, 57.8%/37.1%; 3 cm < tumor size, 76.4%/57.5%; single tumor, 53.5%/32.7%; multiple tumors, 63.4%/43.8%) (Table 4).

Impact of μ-TAS AFP-L3 on Long-Term Survival of Patients with HCC

We evaluated the impact of μ-TAS AFP-L3 on the long-term survival of patients with HCC. The mean observation time after treatment was 23.5 months (range 0–106 months). The survival rate of patients with elevated μ-TAS

Table 4 Positivity rate of AFP-L3 at a 7% cutoff value according to tumor characteristics

	μ-TAS AFP-L3 (%)	LiBASys AFP-L3 (%)
Stage		
I	44.2	16.3
II	52.9	37.5
III	66.4	44.5
IV	82.8	65.5
Size (cm)		
≤2.0	43.5	22.9
2.1–3.0	57.8	37.1
3.0<	76.4	57.5
Number		
Single	53.5	32.7
Multiple	63.4	43.8

AFP-L3 fucosylated fraction of AFP, *μ-TAS* micro-total analysis system, *LiBASys* liquid-phase binding assay system

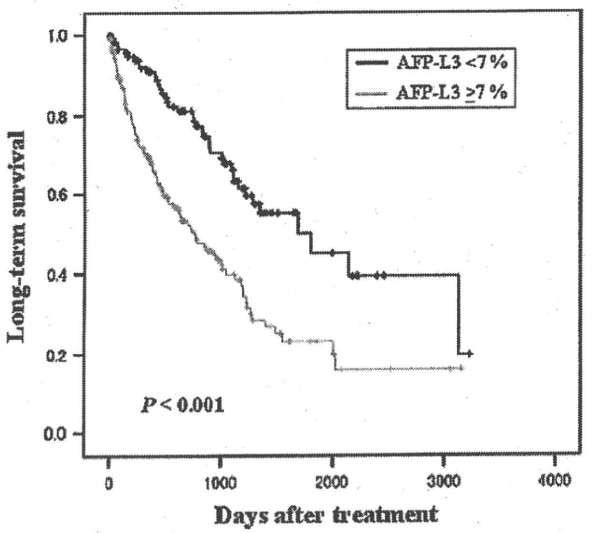

Fig. 4 Comparison of long-term survival rates between HCC patients with and without μ-TAS AFP-L3 elevation. *P* value was calculated using log-rank test

AFP-L3 level (≥7%) was significantly lower than that of patients without such elevation ($P < 0.001$) (Fig. 4).

Multivariate analysis using a Cox proportional-hazard model revealed that μ-TAS AFP-L3 status ($P = 0.021$) was a significant independent factor predictive of long-term survival in patients with HCC (Table 5). Child–Pugh class ($P < 0.001$), log (AFP ng/mL) ($P = 0.002$), number of tumors ($P = 0.001$), vascular invasion ($P = 0.010$), and treatment method ($P = 0.019$) were also significant independent prognostic factors.

Discussion

Many studies have shown that AFP-L3 status is a specific marker for HCC. However, measurement of AFP-L3 has not always been reliable in cases with low serum total AFP concentration. Furthermore, several studies have reported that the sensitivity of AFP-L3 is relatively low (22.2–38.0%) in early-stage HCCs less than 2 cm in diameter [8, 19].

In the present study we demonstrated that μ-TAS AFP-L3 was more sensitive and accurate for discriminating HCC from BLD than the conventional LiBASys AFP-L3 and total AFP level (cutoff 200 ng/ml), which have been used as a standard marker for HCC.

Diagnostic sensitivity was especially good in subgroups with lower AFP concentrations (<20 ng/ml). A cutoff level of 10% or 15% for AFP-L3 has been used for diagnosis of HCC in many previous studies [8, 10–13, 19]. In the present study, we compared the sensitivity, specificity, accuracy, positive predictive value, and negative predictive value for AFP-L3 between the μ-TAS assay and the conventional LiBASys assay at several cutoff values. We had suggested that a cutoff value of 7% was appropriate for discriminating HCC from BLD in the μ-TAS AFP-L3 assay. This cutoff value of 7% has been demonstrated to improve sensitivity greatly with a slight decrease of specificity as compared with the conventional LiBASys assay. The 7% cutoff value in the μ-TAS AFP-L3 had the highest diagnostic accuracy in the subgroups with lower AFP concentrations.

The difficulty in the treatment of HCC is related to the underlying impairment of hepatic functional reserve and late detection, thus limiting the possibility of curative therapy such as hepatic resection and RFA. Therefore, early detection of HCC followed by adequate curative treatment is an important issue for HCC surveillance in high-risk populations such as patients with cirrhosis due to any cause and chronic infection with hepatitis B virus or hepatitis C virus. From this viewpoint, μ-TAS AFP-L3 is an extremely powerful tool for detection of HCC at an early stage. The present study revealed that μ-TAS AFP-L3 (cutoff 7%) was able to detect HCC in 41.1% of patients with low AFP concentration (<20 ng/ml), compared with only 3.6% in the same subgroup using LiBASys AFP-L3. Theoretically, the μ-TAS AFP-L3% cutoff level could be measured for samples containing as little as 3 ng/ml total AFP [16]. Indeed, the μ-TAS AFP-L3 at a 7% cutoff value was able to detect 28 out of 80 (35.0%) HCC patients whose serum total AFP concentrations were less than 10 ng/ml. In contrast, AFP-L3% was not detected by the LiBASys assay at such low total AFP concentrations. In addition, the AFP-L3 positivity rate for the μ-TAS assay at