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Clinical utility of highly sensitive *Lens culinaris* agglutinin-reactive alpha-fetoprotein in hepatocellular carcinoma patients with alpha-fetoprotein <20 ng/mL

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The *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3) has been used as a diagnostic and prognostic marker of hepatocellular carcinoma (HCC). The analytical sensitivity of a conventional method for AFP-L3% is not sufficient in patients with a low AFP level. This study was performed to determine the clinical utility of a newly developed highly sensitive AFP-L3% (hs-AFP-L3%) assay in patients with an AFP level <20 ng/mL. In the cohort study, serum samples obtained from 270 patients with newly diagnosed HCC before treatment and 396 patients with chronic liver disease at Ogaki Municipal Hospital, in both of which the AFP level was <20 ng/mL, were measured for conventional AFP-L3% (c-AFP-L3%), hs-AFP-L3% and des-gamma-carboxy prothrombin (DCP). Diagnostic sensitivity and specificity of hs-AFP-L3% at a cut-off level of 5% were 41.5% and 85.1%, respectively, significantly increasing the sensitivity from 7.0% for c-AFP-L3%. Multivariate analysis identified hs-AFP-L3% as an independent factor associated with reduced long-term survival. The survival rate of patients with high hs-AFP-L3% (≥5%) before treatment was significantly poorer than that of patients with low hs-AFP-L3% (<5%) ($P < 0.001$). In patients with AFP <20 ng/mL, measurements of AFP-L3% by the highly sensitive method before treatment were more useful for diagnosis and prognosis of HCC than by the conventional method. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2011.01875.x, 2011)

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world, and the third most common cause of cancer-related death.[1] A diagnosis of HCC is typically marked with a poor prognosis, largely because early HCC is difficult to diagnose. Three tumor markers, alpha-fetoprotein (AFP),[2–5] *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3)[6–8] and des-gamma-carboxy prothrombin (DCP),[9–11] are currently available as serological markers of HCC for surveillance, diagnosis and patient outcome prediction. Alpha-fetoprotein has been widely used as an *in vitro* diagnostic, and different cut-off values have been proposed by previous studies.[12–14] However, total AFP is not always specific for HCC, especially when HCC is in the early stages. In contrast, a fucosylated fraction of AFP (AFP-L3) is highly specific for HCC. A combination assay of AFP-L3% and DCP results in an improvement in diagnostic sensitivity compared with AFP-L3% or DCP alone.[15] Furthermore, higher levels of AFP-L3% prior to treatment are also associated with poorer prognosis.[8,16,17]

The percentage of AFP-L3 is determined by AFP-L3 concentration divided by the total AFP concentration in serum. Therefore, the analytical sensitivity for AFP-L3% can be affected inversely by the total AFP concentration. In addition, the clinical usefulness of AFP-L3% has been hampered by insufficient analytical sensitivity of the conventional assay system in patients with a low AFP level, which is a liquid-phase binding assay on an auto-analyzer (LiBASys).[18,19]

Recent technical improvements to the highly sensitive analytical methods, by using novel and advanced microfluidics-based separation science, have improved the analytical sensitivity of this assay.[20] This new generation of assays [micro-total analysis system (μTAS)] has enabled the accurate measurement of AFP-L3% at very low AFP concentrations. In patients with an AFP level of ≥20 ng/mL, μTAS AFP-L3% correlated well with LiBASys AFP-L3%.[20] Therefore, we conducted a retrospective cohort study to determine the clinical utility of the new highly sensitive AFP-L3% (hs-AFP-L3%) assay for diagnosis of HCC of our patient population under surveillance who were at risk of HCC and with AFP <20 ng/mL. In addition, we evaluated the clinical usefulness of this new AFP-L3% as a prognostic factor in patients with HCC with AFP <20 ng/mL.

Methods

Patients. Between January 1995 and December 2007 there were 1004 patients diagnosed with primary HCC at Ogaki Municipal Hospital. The AFP levels of all patients had been measured, and 461 patients (46%) had AFP <20 ng/mL. Of these 461 patients, 270 met the eligibility criteria (availability of stored serum samples, patient informed consent and Child–Pugh class A or B). Patients with Child–Pugh class C were not enrolled in the present study, because the influence of poor liver function on patients' survival was too dominant to accurately evaluate HCC-related death in the prognosis part of this study.

Hepatocellular carcinoma was diagnosed by histological examination or the appropriate imaging characteristics using criteria similar to the guidelines accepted by the American Association for the Study of Liver Diseases.[21] Tumor stage on imaging findings was assessed on the basis of the TNM classification of the Liver Cancer Study Group of Japan.[22]

Control samples were obtained from 396 patients with chronic liver disease without HCC, which were collected during routine HCC surveillance in the same period. These patients had AFP <20 ng/mL and met the eligibility criteria of stored samples availability and patient informed consent. There were 98 patients classified by histological confirmation of chronic liver disease, and 298 patients were diagnosed by imaging findings and biochemical tests. To ensure that controls did not have

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HCC, these patients were followed for 3 years after serum sampling by ultrasonography, computed tomography or magnetic resonance imaging to ensure that none had developed HCC.

Individual decisions regarding treatment were made principally on the basis of the treatment guidelines for HCC in Japan.[23] Patients were initially assessed for eligibility for surgical treatment. When patients declined or were deemed ineligible for surgical treatment, they underwent non-surgical treatment. Patients were first offered locoregional ablative therapy (LAT) with percutaneous ethanol injection or, more recently, radiofrequency ablation. Patients who were not eligible for either resection or locoregional ablative therapy were offered transcatheter arterial chemoembolization (TACE). No patient underwent liver transplantation as a treatment.

Each HCC patient's follow-up period began between 1995 and 2007 and continued until death or December 2008, whichever came first. The follow-up period ranged from 0.3 to 101.6 months (a median of 28.2 months).

The study protocol was approved by the Institutional Ethics Review Board and was in compliance with the Declaration of Helsinki.

Assays of AFP, AFP-L3% and DCP. AFP, AFP-L3% and DCP were measured in the same serum sample obtained at the time of HCC diagnosis before any treatment (for HCC group) or from a sample obtained at least 3 years before the examination that confirmed the absence of HCC (for the control group). The measurements of hs-AFP-L3% and DCP were achieved by using a microchip capillary electrophoresis and liquid-phase binding assay on a μTASWako i30 auto analyzer (Wako Pure Chemical Industries, Ltd, Osaka, Japan).[20] The measurements of conventional AFP-L3% (c-AFP-L3%) were performed using a column chromatography and liquid-phase binding assay on a LiBASys auto analyzer (Wako Pure Chemical Industries, Ltd).[18,19] Analytical sensitivity of μTAS is 0.3 ng/mL AFP, and the percentage of AFP-L3 can be measured when AFP-L3 is over 0.3 ng/mL. Analytical sensitivity of LiBASys is 0.8 ng/mL AFP, but AFP-L3% can not be calculated at AFP <10 ng/mL. Therefore the correlation between μTAS and LiBASys was poor at AFP <20 ng/mL.

Statistical analyses. To evaluate the diagnostic value of hs-AFP-L3% and DCP, sensitivity and specificity were calculated. For the evaluation of prognosis, the long-term survival of

Table 1. Demographics of the study population

Characteristics	Patients with HCC (n = 270)	Patients without HCC (n = 396)
Gender (%)		
Male/Female	209 (77)/61 (23)	207 (52)/189 (48)
Age	67.9 ± 8.8	63.5 ± 12.2
Mean ± SD		
Infection of hepatitis virus (%)	38 (14)/193 (71)/7 (3)/32 (12)	82 (21)/217 (55)/12 (3)/85 (21)
HBV/HCV/HBV + HCV/none		
Child–Pugh class (%)	215 (80)/55 (20)/0	323 (82)/73 (18)/0
A/B/C		
Platelet count ($\times 10^3/mm^3$)	12.4 (7.6, 17.1)	11.3 (8.8, 17.6)
Median (25%, 75% quartile)		
ALT (IU/L)	41.5 (27.0, 64.8)	31.0 (20.0, 57.3)
Median (25%, 75% quartile)		
AST (IU/L)	48.0 (34.0, 68.5)	36.0 (26.0, 61.0)
Median (25%, 75% quartile)		
Total bilirubin (mg/dL)	0.8 (0.5, 1.0)	0.7 (0.5, 1.0)
Median (25%, 75% quartile)		
Albumin (g/dL)	3.7 (3.3, 4.1)	3.9 (3.4, 4.2)
Median (25%, 75% quartile)		
AFP (ng/mL)	6.7 (3.6, 10.3)	2.8 (1.8, 4.9)
Median (25%, 75% quartile)		
c-AFP-L3% (%)	0.5 (0.5, 0.5)	0.5 (0.5, 0.5)
Median (25%, 75% quartile)		
hs-AFP-L3% (%)	4.2 (0.5, 7.1)	0.5 (0.5, 0.5)
Median (25%, 75% quartile)		
DCP (mAU/mL)	33 (18, 182)	19 (15, 27)
Median (25%, 75% quartile)		
Tumor stage†		
I	89	NA
II	127	NA
III	47	NA
IV	7	NA
Tumor size		
≤2 cm	123	NA
>2 and ≤3 cm	63	NA
>3 and ≤5 cm	52	NA
>5 cm	32	NA
Tumor number		
Single	189	NA
Multiple	81	NA

†According to TNM staging by the Liver Cancer Study Group of Japan. AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-gamma-carboxy prothrombin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

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Table 2. Sensitivity and specificity. (a) Patients with AFP <20 ng/mL (HCC, n = 270; control, n = 396), and (b) patients with AFP <10 ng/mL (HCC, n = 199; control, n = 357)

	Cut-off (%)	Sensitivity (%)	Specificity (%)
(a) AFP <20 ng/mL			
c-AFP-L3%	5	7.0	98.5
	7	5.2	98.7
	10	4.1	99.0
hs-AFP-L3%	5	41.5	85.1
	7	26.7	92.4
	10	14.8	98.2
(b) AFP <10 ng/mL			
c-AFP-L3%	5	0.0	100.0
	7	0.0	100.0
	10	0.0	100.0
hs-AFP-L3%	5	36.2	88.5
	7	23.6	93.8
	10	11.6	98.3

AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma.

patients with HCC was determined by the Kaplan–Meier method, and the log-rank test was used to compare the survival rates. The Cox proportional hazards model was used for multivariate analysis for factors that influenced patient survival. The values were considered significant when the P value was <0.05. All analyses were performed using JMP6 statistical software (SAS Institute Japan, Japan).

Results

Clinical features of patients. The demographics, etiology of liver disease, hepatic functional reserve ranked by Child–Pugh classification, tumor stage, tumor size and tumor number of the study patients are summarized in Table 1. Overall, there were 270 patients with HCC, including 89 in stage I, 127 in stage II, 47 in stage III and seven in stage IV. The majority of HCC (80.0%) were diagnosed stages I and II. The levels of median and quartile for AFP concentration, c-AFP-L3%, hs-AFP-L3%, DCP and other laboratory tests are shown in Table 1. The numbers of HCC patients and non-HCC patients with AFP <10 ng/mL were 199 out of 270 (73.7%) and 357 out of 396 (90.2%), respectively. In addition to evaluating AFP-L3% in patients with AFP <20 ng/mL, we further evaluated the patient group with AFP <10 ng/mL.

Sensitivity and specificity. The receiver-operating characteristic (ROC) curve was constructed to evaluate the area under the curve (AUC) for AFP-L3%. In all patients (HCC, n = 270; control, n = 396), the AUC of μTAS hs-AFP-L3% and prior-generation methodology LiBASys c-AFP-L3% were 0.707 and 0.537 respectively ($P < 0.05$) (data not shown). In patients with AFP <10 ng/mL (HCC, n = 199; control, n = 357), the AUC of μTAS hs-AFP-L3% was 0.668, but that of LiBASys c-AFP-L3% was not measured because AFP-L3% was out of the reportable range (data not shown). These indicated that the diagnostic accuracy of μTAS hs-AFP-L3% was superior to that of LiBASys c-AFP-L3%.

Overall sensitivity and specificity calculated for patients with AFP <20 ng/mL and for those with AFP <10 ng/mL using three cut-off values (5%, 7% and 10%) are shown in Table 2. In

Table 3. Sensitivity by tumor characteristics. (a) Patients with AFP <20 ng/mL (n = 270), and (b) <10 ng/mL (n = 199)

	Analyte	c-AFP-L3%	hs-AFP-L3%	DCP (%)	hs-AFP-L3% and DCP (%)
	Cut-off, n	10%	5%	40 mAU/mL	5% + 40mAU/mL
(a)					
Stage†					
I	89	4.5	34.8	20.2	44.9
II	127	2.4	42.5	57.5	71.7
III	47	6.4	53.2	53.2	74.5
IV	7	14.3	28.6	71.4	85.7
Size					
≤2 cm	123	4.1	36.6	24.4	48.8
>2 and ≤3 cm	63	3.2	46.0	52.4	65.1
>3 and ≤5 cm	52	3.8	44.2	63.5	80.8
>5 cm	32	6.3	46.9	78.1	90.6
Number					
Single	189	3.2	39.2	43.9	60.8
Multiple	81	6.2	46.9	46.9	70.4
(b)					
Stage†					
I	66	0.0	28.8	18.2	40.9
II	97	0.0	37.1	58.8	70.1
III	30	0.0	53.3	56.7	73.3
IV	6	0.0	16.7	83.3	83.3
Size					
≤2 cm	90	0.0	31.1	23.3	45.6
>2 and ≤3 cm	43	0.0	37.2	46.5	55.8
>3 and ≤5 cm	42	0.0	40.5	69.0	81.0
>5 cm	24	0.0	45.8	87.5	95.8
Number					
Single	146	0.0	33.6	43.8	58.2
Multiple	53	0.0	43.4	50.9	69.8

†According to TNM staging by the Liver Cancer Study Group of Japan. AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin.

patients with AFP <20 ng/mL, when the cut-off value for AFP-L3% was set at 5%, the sensitivity and specificity of c-AFP-L3% were 7.0% and 98.5%, respectively. Those of hs-AFP-L3% were 41.5% and 85.1%, respectively. Sensitivity of hs-AFP-L3% was significantly higher than that of c-AFP-L3% ($P < 0.05$). Focusing on patients with AFP <10 ng/mL, the sensitivity of hs-AFP-L3% was 36.2%, which was still much higher than that for c-AFP-L3%. A cut-off value of 10% has been reported for diagnosis of HCC using the earlier generation methodology. For this study, to maintain the specificity at 85% or more, we chose a cut-off value of 5% for hs-AFP-L3% and 10% for c-AFP-L3%.

Sensitivity with respect to tumor characteristics. Patients were classified by tumor stage (I, II, III and IV), tumor size (<2, 2–3, 3–5 and >5 cm) and tumor number (single tumor and multiple tumors). In patients with AFP <20 ng/mL, sensitivities by tumor characteristics are shown for c-AFP-L3% (cut-off 10%), hs-AFP-L3% (cut-off 5%), DCP (cut-off 40 mAU/mL) and hs-AFP-L3%-DCP combined in Table 3. Sensitivities of hs-AFP-L3% in stages I and II were 34.8% and 42.5%, respectively, whereas those of c-AFP-L3% were only 4.5% and 2.4%, respectively. Those of DCP in stage I and II were 20.2% and 57.5%, respectively. Combination of hs-AFP-L3% and DCP resulted in an improvement in sensitivity compared with hs-AFP-L3% or DCP alone. Those of the combination in stage I and II were 44.9% and 71.7%, respectively. Focusing on patients with AFP <10 ng/mL, sensitivity using the combination in stages I and II were 40.9% and 70.1%, respectively.

In patients treated by hepatectomy, 13 patients had well-differentiated HCC by postoperative pathological examination. Hs-AFP-L3% was elevated (≥5%) in four patients (30.8%). Hypervascularity of the tumor was not detected with computed tomography during hepatic arteriography, the most sensitive imaging modality to detect hypervascularity, in five patients. Hs-AFP-L3% was elevated in one of these hypovascular HCC (20.0%).

Survival rates of patients with HCC. We evaluated the significance of hs-AFP-L3% on the survival rate of HCC patients (Fig. 1). Statistical significance were not observed between the patients with high c-AFP-L3% (≥10%) and the patients with low c-AFP-L3% (<10%) ($P = 0.175$). The survival rate of patients with high hs-AFP-L3% (≥5%) was significantly lower than that of patients with low hs-AFP-L3% (<5%) by the log-rank test ($P < 0.001$). Statistical significance was not observed between the patients with high DCP (≥40 mAU/mL) and the patients with low DCP (<40 mAU/mL) ($P = 0.197$). Focusing on patients with AFP <10 ng/mL, statistical significance was still observed between the patients with high hs-AFP-L3% (≥5%) and the patients with low hs-AFP-L3% (<5%) ($P = 0.035$).

Univariate and multivariate analyses for prognostic factors for HCC. Table 4 shows the results of univariate and multivariate analysis of prognostic factors evaluated by Cox proportional hazards model in patients with AFP <20 ng/mL. The factors in the analysis were c-AFP-L3%, hs-AFP-L3%, DCP, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, albumin, type of treatment, tumor stage, age and gender. In the univariate analysis, the hazard ratios of hs-AFP-L3%, total bilirubin, albumin, type of treatment and tumor stage were statistically significantly high ($P = 0.001$, <0.001, 0.001, 0.001 and 0.006, respectively). Those of c-AFP-L3% and DCP were not statistically significant ($P = 0.218$ and 0.202, respectively). In the multivariate analysis, hs-AFP-L3% and non-resection were independent prognostic factors with significantly high hazard ratios ($P = 0.026$ and <0.001. respectively). For patients with AFP <10 ng/mL, hs-AFP-L3% was identified as a prognostic factor by univariate analysis ($P = 0.045$) but not by multivariate analysis ($P = 0.457$) (data not shown).

Survival rates of patients stratified by the type of treatment. In patients with AFP <20 ng/mL and classified into stages I and II, survival rates evaluated by treatment and by hs-AFP-L3% status are shown in Figure 2. All patients with any treatments ($n = 216$) are shown in Figure 2a, patients with

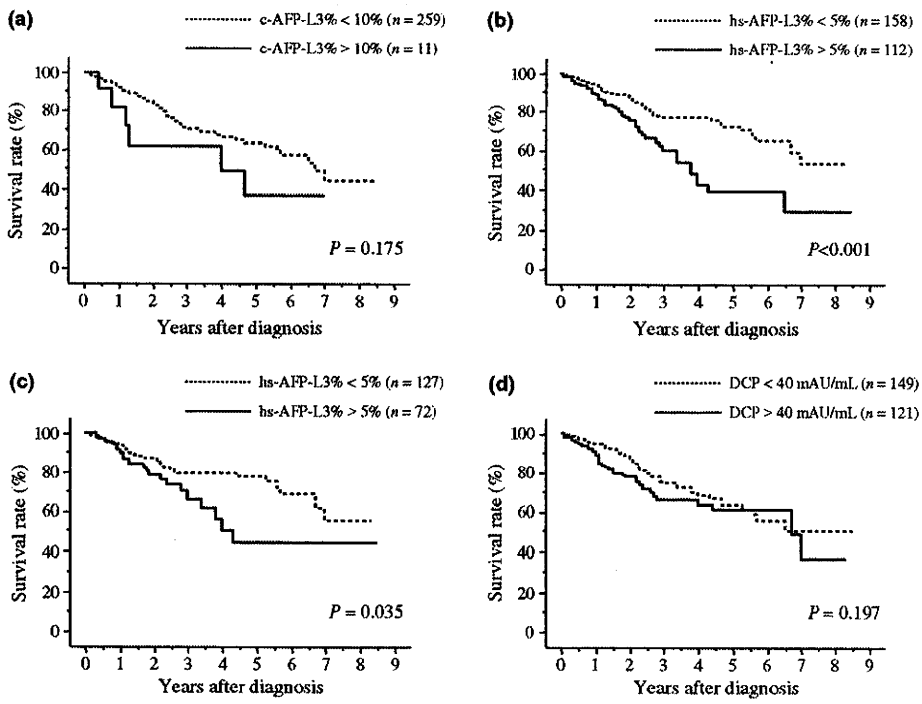

Fig. 1. Survival rates. (a) c-AFP-L3% in patients with AFP <20 ng/mL ($n = 270$), (b) hs-AFP-L3% in patients with AFP <20 ng/mL ($n = 270$), (c) hs-AFP-L3% in patients with AFP <10 ng/mL ($n = 199$), and (d) Des-gamma-carboxy prothrombin (DCP) in patients with AFP <20 ng/mL ($n = 270$).

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Table 4. (a) Univariate and (b) multivariate analyses for prognostic factors of hepatocellular carcinoma in patients with alpha-fetoprotein <20 ng/mL

	Variables	Hazard ratio (95% confidence interval)	*P* value
(a) Univariate analyses			
c-AFP-L3%	≥10%	1.765 (0.683–3.739)	0.218
hs-AFP-L3%	≥5%	2.195 (1.401–3.450)	0.001
DCP	≥40 mAU/mL	1.335 (0.855–2.080)	0.202
ALT	≥40 IU/L	1.132 (0.725–1.792)	0.587
AST	≥40 IU/L	1.370 (0.845–2.310)	0.207
Total bilirubin	≥1 mg/dL	2.466 (1.543–3.901)	<0.001
Albumin	<3 g/dL	2.868 (1.567–4.923)	0.001
Treatment	(LAT + TACE/ resection)	4.893 (2.876–8.832)	<0.001
Stage†	III + IV/I + II	2.111 (1.247–3.440)	0.006
Age		1.009 (0.983–1.037)	0.504
Gender	Male/Female	1.185 (0.902–1.616)	0.232
(b) Multivariate analysis			
hs-AFP-L3%	≥5%	1.697 (1.066–2.709)	0.026
Total bilirubin	≥1 mg/dL	1.575 (0.961–2.558)	0.071
Albumin	<3 g/dL	1.650 (0.878–2.930)	0.116
Treatment	(LAT + TACE/ resection)	3.627 (2.066–6.708)	<0.001
Stage‡	III + IV/I + II	1.675 (0.982–2.753)	0.058

†According to TNM staging by the Liver Cancer Study Group of Japan. AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-gamma-carboxy prothrombin; LAT, locoregional ablative therapies; TACE, transcatheter arterial chemoembolization.

resection (n = 103) in Figure 2b, patients with LAT (n = 56) in Figure 2c and patients with TACE (n = 57) in Figure 2d. The difference in the survival rate of patients with resection was not statistically significant between patients with high hs-AFP-L3% and with low hs-AFP-L3% (P = 0.813). In the case of LAT, the survival rate of patients with high hs-AFP-L3% was significantly lower than that of patients with low hs-AFP-L3% (P = 0.037). The survival rate of patients with high hs-AFP-L3% tended to be lower than that of patients with low hs-AFP-L3%, but the difference was not significant in the case of TACE. The survival rate of patients with resection was significantly higher than that of patients with ablation and TACE regardless of the hs-AFP-L3% level (P = 0.002) (data not shown).

Discussion

Alpha-fetoprotein, AFP-L3% and DCP are used as markers for HCC, and their utility in the diagnosis of HCC and the evaluation of tumor progression and prognosis has been reported. Alpha-fetoprotein is the most widely used marker for monitoring HCC development. However, elevated AFP is not typically observed in patients with a small tumor or early stage HCC. Recent advances in diagnostic imaging techniques have allowed for the detection of small tumors and early stage HCC,[24–28] and the establishment of surveillance programs for HCC in the high-risk group have also contributed to diagnosis of early stage HCC.[29,30] These trends have resulted in an increase in the number of HCC patients diagnosed by imaging without elevation of AFP. Thus, HCC patients with low AFP represent the appropriate study population in a successful HCC surveillance program. Among the tumor markers, AFP-L3% is highly specific for HCC, and elevated AFP-L3% correlates with tumor progression, poor tumor differentiation and unfavorable prognosis.[8,11,30–32]

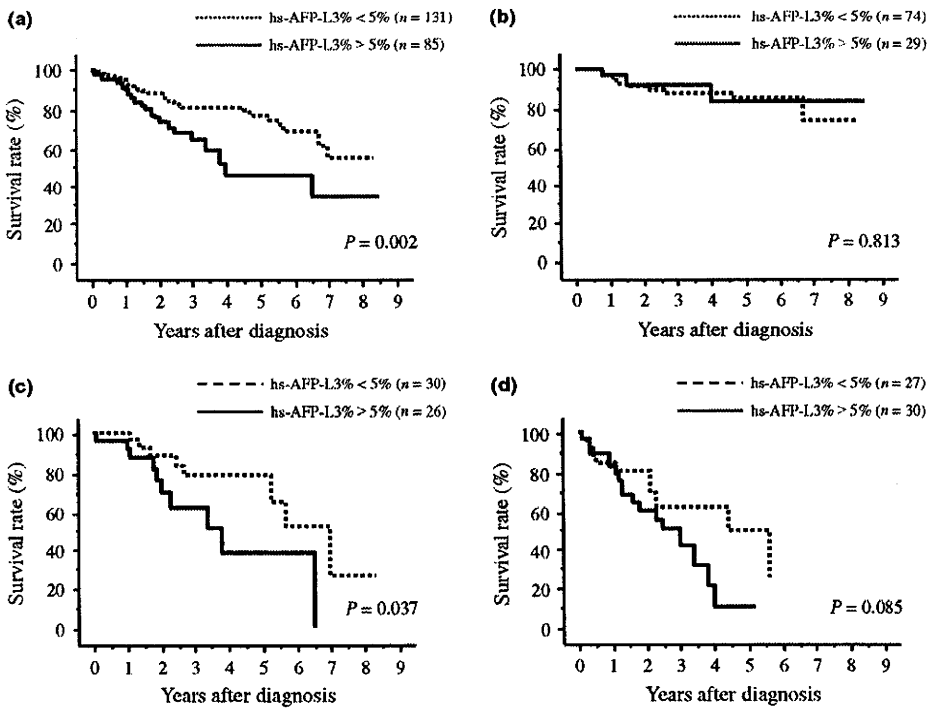

Fig. 2. Survival rates of patients stratified by the type of treatment in patients with alpha-fetoprotein (AFP) <20 ng/mL and classified into stages I and II. (a) All patients with any treatments (n = 216), (b) patients treated by surgical resection (n = 103), (c) patients treated by locoregional ablative therapies (n = 56), and (d) patients treated by transcatheter arterial chemoembolization (n = 57).

However, measurement of AFP-L3% by the conventional assay system has not always been reliable in patients with AFP <20 ng/mL due to low analytical sensitivity. Therefore, the clinical utility of conventional AFP-L3% has limited use in the diagnosis and prediction of outcome of this subpopulation. The present study focused on patients with AFP <20 ng/mL, and further, the subgroup with AFP <10 ng/mL, and revealed that the hs-AFP-L3% assay could diagnose earlier stage HCC than the c-AFP-L3 assay (cut-off 5%). The combination assay with DCP resulted in a significant improvement in diagnostic sensitivity. Parallel measurement of hs-AFP-L3% and DCP will identify additional HCC patients in the early stage because the markers are complementary for different subgroups of HCC.

Regarding prognosis, in patients with AFP <20 ng/mL, the survival rate of patients with elevated hs-AFP-L3% (≥5%) was significantly lower than that of patients with low hs-AFP-L3% (<5%). Univariate and multivariate analysis identified hs-AFP-L3% as an independent factor associated with long-term survival. Furthermore, high hs-AFP-L3% (≥5%) in the present study suggested an unfavorable prognosis, even when focusing on patients with stages I and II. In patients with stages I and II HCC treated by surgical resection, there was no statistically significant difference in survival between patients with high hs-AFP-L3% (≥5%) and those with low hs-AFP-L3% (<5%). The survival rate of patients treated by hepatic resection was much higher than that of patients with LAT or TACE. Thus, hepatic resection demonstrated favorable effects on survival compared with the other treatments, which might confound the clinical utilities of hs-AFP-L3%. In patients with AFP <10 ng/mL, hs-AFP-L3% was not identified as a prognostic factor by multivariate analysis, probably for the same reason. Although in our previous study using conventional AFP-L3% there was no difference in survival between patients with high AFP-L3% and those with low AFP-L3% in the patients treated surgically,[34] postoperative AFP-L3% has been reported as a predictive marker for recurrence and long-term survival.[35] To evaluate the prognosis of patients with resection, measurements of hs-AFP-L3% using samples after treatment should be performed.

The lower survival rate of patients with elevated hs-AFP-L3% and high rate of elevation in early stage HCC indicated that hs-AFP-L3 will be useful in identifying early stage HCC but with poorer prognosis, for which early diagnosis and treatment would be important. It may be advisable that hs-AFP-L3% should be included as a routine screening tool for HCC in the surveillance of patients at high risk of the development of HCC, together with imaging modalities.

In conclusion, the present study shows that hs-AFP-L3% was a useful marker for the diagnosis of early stage HCC in patients with AFP <20 ng/mL, and parallel measurement with DCP improved sensitivity. In addition, measurement of hs-AFP-L3% before treatment could help predict patient prognosis.

Disclosure Statement

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Evolution of hypointense hepatocellular nodules observed only in the hepatobiliary phase using Gd-EOB-DTPA enhanced magnetic resonance imaging

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Short running title: Evolution of hypointense hepatocellular nodules.

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Abstract

Objective: We sought to determine whether hypointense hepatocellular nodules observed at the hepatobiliary phase using gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTP)–enhanced magnetic resonance imaging (MRI) progress to hypervascular hepatocellular carcinoma (HCC).

Materials and Methods: Repeated Gd-EOB-DTPA–enhanced MRI was performed in 30 patients with 49 nodules that were determined to be hypointense in the hepatobiliary phase but be not enhanced in the arterial phase of dynamic MRI. The correlation between characteristics of hypointense nodules which showed slightly or markedly low signal intensity relative to surrounding liver parenchyma and their progression to hypervascular HCC were analyzed in cirrhotic liver. Angiography-assisted CT was performed in all patients at the time of start. The rate of progression to classic HCC was calculated using the Kaplan-Meier method.

Results: The overall 6-month and 12-month cumulative incidence of vascularization was 27.6% and 43.5%, respectively. The 6-month and 12-month cumulative incidence of vascularized nodules with maximum diameters $\geq$ 15 mm were 43.3% and 77.3%, respectively. In contrast, the 6-month and 12-month cumulative incidence of vascularized nodules with maximum diameters <15 mm were 16.9% and 16.9%,

respectively. The difference between these incidences was significant ($P = 0.0147$).

Conclusion: Hypointense nodules with maximum diameters of at least 15mm often become hypervascular. Therefore, patients with hypointense nodules characterized by a maximum diameter $\geq$ 15 mm should be followed carefully because of the high incidence of vascularizartion.

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent types of cancer, particularly in southern and eastern Asia. It is the sixth most common cancer in the world, and the third most common cause of cancer-related death[1]. In Japan, HCC is the third most common cause of cancer-related death in men, and the fifth most common among women[2]. Periodic follow-up of chronic liver disease, in particular cirrhosis, using ultrasound (US), multidetector computed tomography (MDCT)[3,4], magnetic resonance imaging (MRI)[5], and/or tumor markers[6] allows detection of small early-stage HCC. Additionally, follow-up of high-risk patients frequently reveals various types of hepatocellular nodules, many of which are difficult to differentially diagnosis[7-11].

Despite the importance of a differential diagnosis, it is nearly impossible to characterize these hepatocellular nodules specifically using US, MDCT, and/or MRI. Therefore, a histological diagnosis is usually obtained with percutaneous liver biopsy under US guidance. This procedure is not always possible, however, due to the need for multiple samples and its invasive nature. Matsui et al.[7-11] reported the utility of evaluating the intranodular blood supply with CT during arterial portography (CTAP) and CT during hepatic arteriography (CTHA) to establish a differential diagnosis. This

procedure, however, is also invasive and hard to repeat in the absence of hepatic tumor embolization.

Recently, the liver-specific contrast agent gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA), which is taken up by hepatocytes, has been used for dynamic MRI studies. Gd-EOB-DTPA results in both dynamic and liver-specific hepatobiliary MR images[12-14]. In the hepatobiliary phase, hepatic lesions that lack normally functioning hepatocytes are imaged as an absence of hepatocyte-selective enhancement compared with normal parenchyma[15]. Furthermore, evaluation of vascularity and hepatocyte-specific uptake enables accurate detection and characterization of focal liver lesions. Differential diagnosis of dysplastic nodules and early HCC can be made based on findings with or without Gd-EOB-DTPA uptake[16]. Differentiation of early HCC from dysplastic nodule using hepatobiliary images, however, is controversial.

The purpose of this study was to analyze the correlation between characteristics of the hypointense nodules, as observed at the hepatobiliary phase using Gd-EOB-DTPA-enhanced MRI, and their progression to hypervascular HCC in cirrhotic liver.

Materials and Methods

Patient selection

Between February 1, 2008 and July 31, 2009, 430 consecutive patients underwent Gd-EOB-DTPA–enhanced MRI at our institution. The patients were suspected of having HCC on the basis of sonographic findings or elevated levels of tumor markers (alpha-fetoprotein [AFP] and des-γ-carboxy prothrombin [DCP]). Among these subjects, repeated Gd-EOB-DTPA–enhanced MRI was performed in 30 patients with 49 nodules that were hypointense in the hepatobiliary phase with a maximum diameter of 0.8-4.0 cm (median, 1.4 cm) that were not enhanced in the arterial phase of dynamic MRI. In this series, hypointense nodules meant slightly or markedly low signal intensity relative to surrounding liver parenchyma in the hepatobiliary phase obtained 20 minutes after contrast materials injection. The current study included the following criteria for enrollment: (1) CTAP and CTHA were carried out within at least 1 month after Gd-EOB-DTPA-enhanced MRI by which hypointense nodules were detected initially, (2) hypointense nodules were not visualized by CTAP and did not show hyperattenuating area by CTHA, (3) hypointense nodules showed oval or round shape with well defined margin. The patients included 19 males and 11 females with a median age of 75.0 years (58-81 years). The cause of cirrhosis was

related to hepatitis C in 25patients, both hepatitis B and C in 1 patient, and neither hepatitis B nor C in 4 patients. All patients belonged to Child-Pugh class A. AFP and DCP values showed form 0.8 ng/mL to 287 ng/mL (median, 7.4ng/mL) and from 10 mAU/L to 791 mAU/L (median, 29 mAU/L). The presence of hypervascular HCC was observed in 10 patients on another site of the liver at the start of follow-up. Observation periods ranged from 3.0 months to 15.0 months (median, 6.0 months). Four patients had 3-month follow-up and 12 patients had more than 6-month follow-up. Hypointense nodules followed with Gd-EOB-DTPA-enhanced MRI and ultrasound in turn at intervals 3 months as a general rule. Gd-EOB-DTPA-enhanced MRI was performed sequentially if the increase of the nodule diameter was confirmed by ultrasound examination or the elevation of tumor markers was observed. The number of MRI examinations ranged from 2 to 9 (median, 3 examinations) per each patient. The interval of MRI examination ranged from 3 to 9 months (median, 3months).

Imaging methods

MRI was performed using a 1.5-T whole-body MRI system (Intera Achieva 1.5T NOVA; Philips Medical Systems) with a phased-array body coil as the receiver coil. T1-weighted sequences were acquired with the following parameters: T1-weighted turbo field echo (TFE) in-phase and opposed-phase transverse (TE, opposed-phase 2.3,

in-phase 4.6; flip angle, 12°; matrix size, 256 × 512; scan percentage,70) with a 3.5-mm section thickness, a 0-mm intersection gap, and a field of view of 38 cm. After intravenous injection of Gd-EOB-DTPA (Primovist; Bayer Schering Pharma, Osaka, Japan), T1-weighted transverse gradient-echo sequences (high-resolution isotropic volume examination [THRIVE] with spectral presaturation with inversion recovery [SPIR], 4/1.8; flip angle, 12°; matrix size, 256 × 512; scan percentage, 78.54) with a 3.5-mm section thickness, a 0-mm intersection gap, and a field of view of 38 cm were obtained. Gd-EOB-DTPA was administered intravenously as a bolus at a rate of 2 mL/sec (0.1mL/kg, maximal dose of 10mL) through an intravenous cubital line (20–22 gauge), which was flushed with 20 mL of saline using a power injector (Sonic Shot; Nemoto Kyourindo, Tokyo, Japan). The time for the dynamic arterial phase imaging was determined using the MR fluoroscopic bolus detection of descending aorta (Bolus Trak: Philips Medical Systems). The mean delay times (the time interval between the start of bolus administration and the start of image acquisition) for the arterial, portal, and delayed phases were 20, 60, and 180 seconds, respectively. Immediately after the dynamic study, a respiration-triggered single-shot T2-weighted sequence with a reduction factor of 4 (1,200/100; flip angle, 90°; matrix size, 400 × 512) with a 7-mm section thickness, a 1-mm intersection gap, and a field of view of 38 cm was obtained

with SPIR. The 20-min delayed hepatobiliary phase[17] was obtained with a T1-weighted TFE sequence (TR/TE, 4/1.8; flip angle, 12°; matrix size, 256 × 512) with a 3.5-mm section thickness, a 0-mm intersection gap, and a field of view of 38 cm. All the sequences were obtained with parallel imaging (SENSE).

CTAP and CTHA were performed with an IVR-CT/Angio system (CT: X Vision Real; DSA: DPF 2000A; Toshiba Medical Systems, Tokyo, Japan). CTAP was performed after an infusion of 70 ml of contrast medium (Iopamiron 150; Japan Schering, Osaka, Japan) at a rate of 2.0 mL/sec in the superior mesenteric artery using a power injector (Autoenhance A50; Nemoto Kyourindo, Tokyo, Japan). CTAP data acquisition began 30 sec after initiation of a transcatheter injection of contrast medium. CTHA was performed after an infusion of 20-25 mL of contrast medium at a rate of 1.0 mL/sec in the common or proper hepatic artery using a power injector. CTHA data acquisition began 10 sec after initiation of a transcatheter injection of contrast medium. Helical CT scanning was performed with a section thickness of 7 mm and a table feed speed of 7-mm increments.

Image Analysis

A diagnosis of hepatic nodules was rendered when the hepatobiliary images depicted a round or oval distinct nodule larger than or equal 8 mm[11] in widest diameter that was

distinguishable from cyst and cavernous hemangioma in cirrhotic livers. The hepatic nodules which showed irregular shape with small size (maximum diameter less than 8 mm) or ill defined margin were excluded. The findings of the dynamic phase of MRI were categorized into three patterns: hypointensity, isointensity, and hyperintensity relative to the surrounding cirrhotic liver tissue. The CTHA findings were categorized into three patterns: hypoattenuating, isoattenuating, and hyperattenuating relative to the surrounding cirrhotic liver tissue. The CTAP findings were classified into three groups: isoattenuating, slightly hypoattenuating, hypoattenuating relative to the findings of the surrounding cirrhotic liver tissue. Two blinded observers with at least 5 years of experience interpreted independently and randomly reviewed the MR and CT images. Nodules that showed enhancement on contrast-enhanced arterial phase, a washout pattern as depicted on contrast portal/venous phase, and hypointensity relative to the surrounding liver parenchyma on hepatobiliary phase were regarded as HCCs at follow-up Gd-EOB-DTPA enhanced MRI. In addition, Vascularization of hypointense nodules in these cases were confirmed as the typical findings of hypervascular HCC which showed hyperattenuating areas in CTHA and hypoattenuating areas in CTAP by angiography assisted-CT in all patients. If the hypervascularization of hypointense nodules was not detected by the dynamic phase of Gd-EOB-DTPA enhanced MRI at

follow-up examination, angiography assisted-CT was not performed.

Statistical analysis

Statistical analysis was performed with the Statistical Program for Social Science (SPSS ver.17.0 for Windows; SPSS Japan, Tokyo, Japan). Continuous variables are shown as medians (range). Continuous variables such as tumor size, frequency of MRI examination, and observation periods were compared by using a Mann-Whitney U test and categorical variables such as T1-weighted and T2-weighted image, the findings of CTHA, the grade of hypo intensity in hepatobiliary phase were compared using Fisher's exact test or the chi-square test. Actuarial analysis of the cumulative incidence of vascularization was performed using the Kaplan-Meier method and differences were tested by log-rank test. The cutoff point of tumor size was determined using Cox proportional hazards model. Statistical significance was set at $P < 0.05$.

Our institution did not require institutional approval or informed consent when reviewing patient records and images for this retrospective study.