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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* 2011; 102: 1025–1031)**

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, Cary, NC, USA).

## 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 (*n*) | c-AFP-L3% (%) | hs-AFP-L3% (%) | DCP (%) | hs-AFP-L3% and DCP (%) |
|---|---|---|---|---|---|
| | Cut-off | 10% | 5% | 40 mAU/mL | 5% + 40 mAU/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.[8] 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 analyses 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

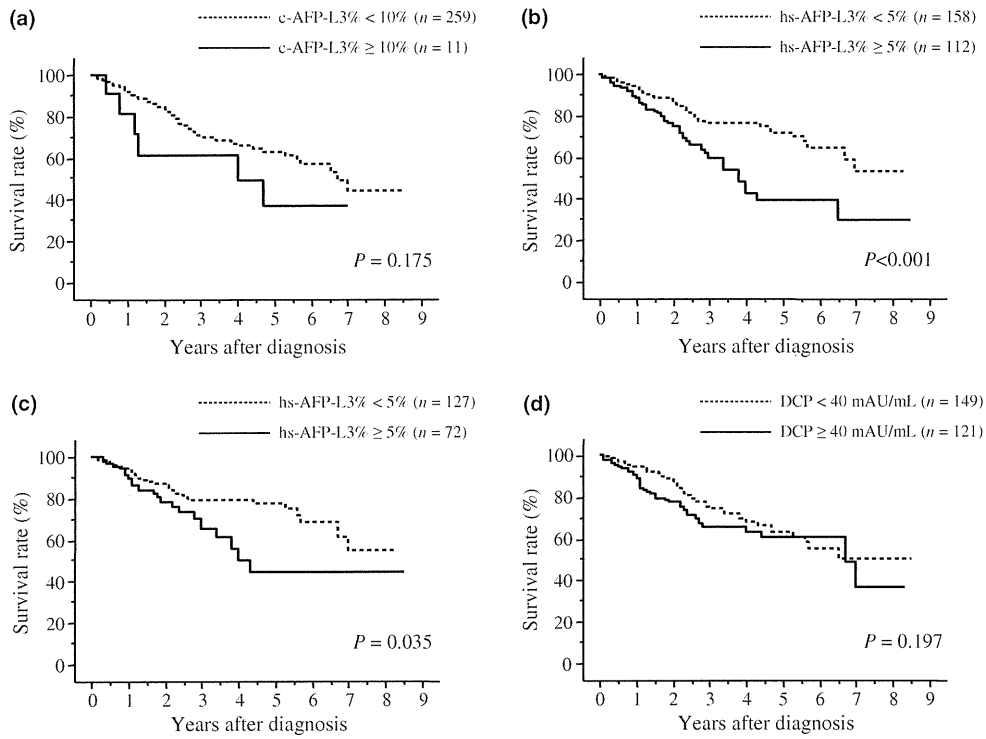


**Fig. 1.** Survival rates. (a) c-AFP-L3% in patients with AFP <20 ng/mL ($n = 270$), (b) hs-AFP-L3% in patients with AFP <20 ng/mL ($n = 270$), (c) hs-AFP-L3% in patients with AFP <10 ng/mL ($n = 199$), and (d) Des-gamma-carboxy prothrombin (DCP) in patients with AFP <20 ng/mL ($n = 270$). c-AFP-L3%,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



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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 found 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 statistically 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.[8,11,31–33]

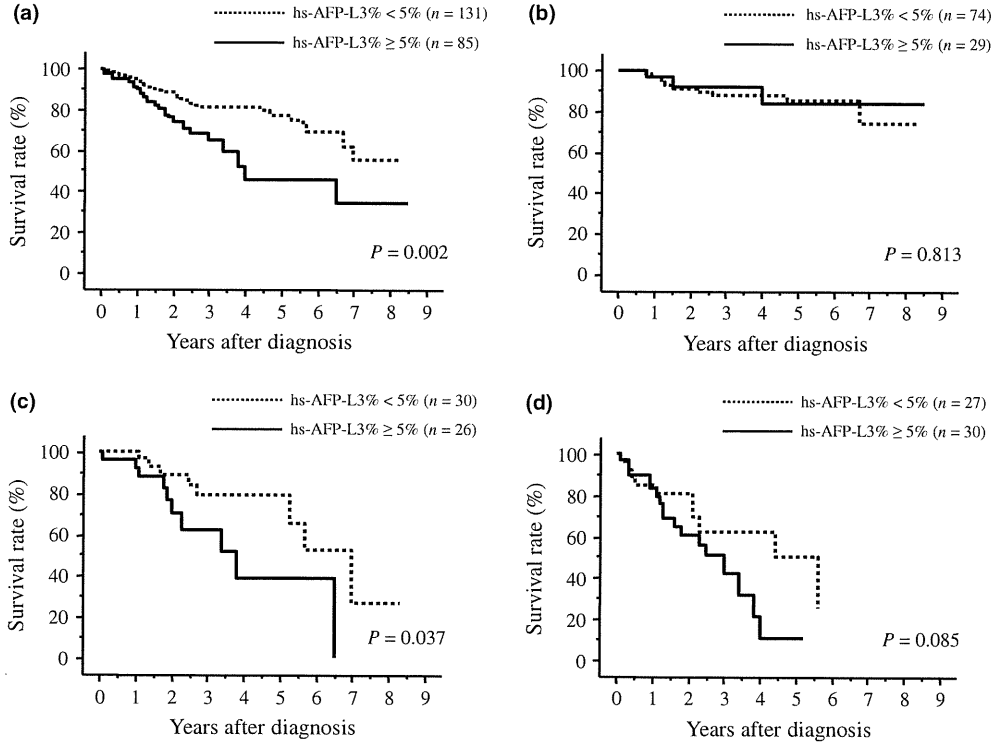


**Fig. 2.** Survival rates of patients stratified by the type of treatment in patients with alpha-fetoprotein (AFP) <20 ng/mL and classified into stages I and II. (a) All patients with any treatments ($n$ = 216), (b) patients treated by surgical resection ($n$ = 103), (c) patients treated by locoregional 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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# Amino Acid Substitutions in the Hepatitis C Virus Core Region Are Associated With Postoperative Recurrence and Survival of Patients With HCV Genotype 1b-Associated Hepatocellular Carcinoma

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**Objective:** We researched the molecular marker for prognosis of postoperative patients with hepatocellular carcinoma (HCC).

**Background:** The association of amino acid substitutions in the hepatitis C virus (HCV) core region and hepatocarcinogenesis has recently been explored. We investigated if these amino acid substitutions are associated with recurrence or survival in patients with HCC after attempted curative treatment by hepatectomy.

**Methods:** A total of 163 patients infected with HCV genotype 1b who previously underwent hepatectomy for primary, not recurrent HCC were analyzed. Amino acid substitutions in the HCV core region were measured by direct sequencing. Postoperative recurrence or survival rates were compared according to tumor characteristics, tumor markers, and amino acid substitutions in the core region.

**Results:** Recurrence rates after hepatectomy were higher in patients bearing a methionine at residue 91 of the HCV core region than in patients with leucine ($P = 0.0002$). Survival was also decreased in patients with methionine at this residue from that seen in patients with leucine at this position ($P = 0.0061$). The associations between amino acid substitutions at residue 91 of the HCV core region and either recurrence or survival rates were independent of liver function, progression of HCC, or tumor marker levels.

**Conclusions:** Amino acid substitutions at residue 91 of the HCV core region are associated with postoperative recurrence or survival in patients infected with HCV genotype 1b who developed HCC and treated by hepatectomy. This factor should be taken into consideration for the postoperative management of patients with HCC.

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Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third most common cause of cancer-related death.[1,2] In Japan, HCC is the third and fifth most common causes of death from cancer in men and women, respectively.[3] One of the most important risk factors for the development of HCC[4,5] is chronic viral hepatitis. Hepatitis C virus (HCV) infection, one of main causes of chronic viral hepatitis, can result in liver cirrhosis and HCC.[6] The majority of patients in Japan with HCC exhibit chronic HCV infection.[7]

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Amino acid substitutions in the HCV core region, especially at residues 70 and/or 91 have been associated with poor responses to antiviral therapy with peginterferon and ribavirin as curative therapy for HCV (eradication of HCV) in patients infected with HCV genotype 1b.[8,9] More recently, an association between substitutions in this region and aspects of hepatocarcinogenesis, including the incidence of HCC and patient prognosis, has been reported.[10–13] In previous reports, researchers demonstrated a difference in the incidence of HCC according to amino acid differences in residues 70 and/or 91 of the HCV core region.[10–12] In addition, Ogura et al[13] reported that the mortality of patients with HCC was affected by amino acid substitutions at residue 91 in the HCV core region as well as the type of initial treatment or preservation of liver function. This study, however, included patients who underwent a variety of treatments, both surgical and nonsurgical, and only survival rate was analyzed. It remains unclear if amino acid substitutions in the HCV core region affect the prognosis of patients with HCC who have been treated with curative intent.

In this study, we evaluated the impact of amino acid substitutions in the HCV core region (residues 70 and 91) on the survival and recurrence rates in patients with HCC after hepatectomy with curative intent.

## METHODS

### Patients

A total of 969 patients were diagnosed with primary, not recurrent HCC between January 1999 and December 2008 at Ogaki Municipal Hospital. Of these patients, 331 patients were treated with hepatectomy. Decisions regarding individual treatments were made based on the treatment guidelines for HCC in Japan.[14] In all patients, HCC tumors were resected with ample margins; enucleation of tumors without margins was not performed. HCV infection was confirmed in 229 of the 331 patients by positive serum HCV RNA at the time of HCC diagnosis using PCR-based detection. HCV genotype was assessed in 209 patients with PCR amplifying the core gene sequences using genotype-specific primers,[15] determining that 166 patients had infections with HCV genotype 1b. We excluded 3 patients who had coinfection with hepatitis B virus to avoid a possible impact of HBV on outcomes, resulting in a final study population of 163 patients (Supplemental Digital Content 1, available at: http://links.lww.com/SLA/A144). A diagnosis of HCC was confirmed by pathologic diagnosis of resected specimens.

After hepatectomy, all patients were followed for 1.65 to 128.9 months (median follow-up period, 48.3 months) at our institution with US and CT or MRI performed every 3 to 6 months. Regular monitoring of serum tumor markers (alpha-fetoprotein [AFP], *lens culinaris* agglutinin-reactive AFP [AFP-L3], and des-gamma-carboxy prothrombin [DCP]) was performed every 3 months. When an elevation of tumor markers was detected, additional imaging examination (usually by CT or MRI) was performed to check for a

recurrence of HCC. If the presence of a recurrence was confirmed, patients underwent treatment for recurrent HCC based on the treatment guidlines. Recurrent HCC was categorized into intrahepatic metastasis and multicentric recurrence according to the previous study.[16] Intrahepatic metastasis was defined as recurrent tumors consisting of moderately or poorly differentiated HCC with the same or lower degree of differentiation compared with the differentiation of the primary tumors. Multicentric recurrence was defined according to previously reported criteria with some modifications[17] as follows: (1) The recurrent tumors consisted of well-differentiated HCC occurring in a different hepatic segment, than even moderately or poorly differentiated preexisting HCCs; (2) Both the primary and recurrent tumors were well-differentiated HCCs; and (3) The recurrent tumor contained regions of dysplastic nodule in peripheral areas.

The entire protocol was approved by the hospital institutional review board and carried out in compliance with the Helsinki Declaration.

## Measurements of Amino Acid Substitutions of HCV Core Region

Amino acid substitutions in the core region of HCV were analyzed by direct sequencing of amino acids 1–191 of genotype 1b[8] using stored serum samples. HCV RNA was extracted from serum samples and were PCR amplified using the following primer pairs:

5'-GCCATAGTGGTCTGCGGAAC-3' (CC11: outer, sense primer)

5'-GGAGCAGTCCTTCGTGACATG-3' (e14: outer, antisense primer),

5'-GCTAGCCGAGTAGTGTT-3' (CC9: inner, sense primer), and

5'-GGAGCAGTCCTTCGTGACATG-3' (e14: inner, antisense primer).

Amplified PCR products were purified and used for direct sequencing. Sequencing results were used to detect substitutions of arginine or glutamine at amino acid 70 and leucine or methionine at amino acid 91.

## Measurement of the Tumor Markers for HCC

Three tumor markers for HCC, AFP, AFP-L3, and DCP, were measured in serum samples taken at the time of HCC diagnosis. Serum AFP levels were determined by enzyme-linked immunosorbent assay using a commercially available kit (ELISA-AFP, International Reagents, Kobe, Japan). A cut-off value of 20 ng/mL AFP was used to define AFP positivity, as proposed by Oka *et al.*[18] Serum AFP-L3, expressed as the percentage of total AFP (AFP-L3 level/total AFP level × 100), was measured by lectin-affinity electrophoresis followed by antibody-affinity blotting (AFP Differentiation Kit L, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cut-off value used to establish AFP-L3 positivity was 10%, as proposed by Shimizu *et al.*[19] The serum DCP level was determined by a specific enzyme immunoassay (Eitest PIVKA-II kit, Eisai Laboratory, Tokyo, Japan) according to the manufacturer's instructions. The cut-off value used to establish DCP positivity was 40 mAU/mL, as proposed by Okuda *et al.*[20]

## Statistical Analyses

Differences in percentages between groups were analyzed by the $\chi^2$ test. Differences in mean quantitative values were analyzed by the Mann–Whitney *U* test. The date of treatment (hepatectomy) was defined as time zero for calculations of patient recurrence and survival rates. In the analysis of recurrence rate, patients in whom HCC recurred were noncensored, and those in whom HCC did not recur were censored. In the analysis of cancer specific survival rates, patients who died from HCC-related cause were noncensored and the other patients were censored. In the analysis of overall survival rate, all patients who died were not censored and surviving patients were censored. The Kaplan–Meier method[21] was used to calculate survival rates, whereas the log-rank test[22] was used to analyze differences in survival.

The Cox proportional hazards model[23] was used for univariate and multivariate analyses of factors related to recurrence and survival. Variables analyzed included patient age and gender, Child-Pugh class (A/B), tumor size (≤2 cm / >2 cm and ≤5 cm / >5 cm), number of tumors (single/multiple), macroscopic portal vein invasion (absent/present), amino acid substitutions of the HCV core region (at residue 70: arginine/glutamine, at residue 91: leucine/methionine), pretreatment serum AFP level (<20 ng/mL/≥20 ng/mL), pretreatment AFP-L3 proportion (<10%/≥10%), and pretreatment serum DCP level (<40 mAU/mL/≥40 mAU/mL). Data analyses were performed using the JMP statistical software, version 6.0 (Macintosh version; SAS Institute, Cary, NC, USA). All *P* values were derived from 2-tailed tests, with a $P < 0.05$ accepted as statistically significant.

# RESULTS

## Patients Characteristics

Table 1 summarizes the pretreatment characteristics of the study patients. This population was composed of 121 males and

**TABLE 1.** Characteristics of Study Patients (n = 163)

| | |
|---|---|
| Age (mean ± SD, years) (range) | 67.4 ± 7.1 (47–83) |
| Sex ratio (female/male) | 42 (25.8)/121 (74.2) |
| Surveillance state at diagnosis (our institution/ others/ none)* | 114 (69.9)/ 45 (27.6)/ 4 (2.4) |
| HCV infection | 163 (100) |
| Child-Pugh class (A/B)† | 152 (93.3)/ 11 (6.7) |
| Albumin (mean ± SD, g/dL) | 3.85 ± 0.43 |
| Total bilirubin (mean ± SD, mg/dL) | 0.74 ± 0.35 |
| 15-minute retention rate of ICG (%) | 15.5 ± 7.3 |
| Prothrombin (%) | 90.4 ± 14.3 |
| Platelet (×1000/μL) | 124 ± 52 |
| Tumor size (mean ± SD, cm) (range) | 2.93 ± 2.02 (0.6-7.6) |
| ≤ 2 cm / > 2 cm and ≤ 5 cm / > 5 cm | 63 (38.6)/ 80 (49.1)/ 20 (12.3) |
| Tumor number (single/multiple) | 137 (84.0)/ 26 (16.0) |
| Macroscopic-portal vein invasion (absent/present) | 143 (87.7)/ 20 (12.3) |
| Microscopic-portal vein invasion (absent/present) | 133 (81.6)/ 30 (18.4) |
| AFP (median, ng/mL) (range)‡ | 18.0 (0.8–5280) |
| ≥20 ng/mL / <20 ng/mL | 84 (52.2)/ 77 (47.8) |
| AFP-L3 (median, %) (range)‡ | 0.5 (0–87.2) |
| ≥ 10% / <10% | 125 (82.8) /26 (17.2) |
| DCP (median, mAU/mL) (range)‡ | 42.0 (10–36,164) |
| ≥ 40 mAU/mL / <40 mAU/mL | 78 (49.4)/ 80 (50.6) |
| Interferon therapy after hepatectomy (no/yes) | 139 (85.3)/ 24 (14.7) |

Percentages were in parentheses.

HCV indicates hepatitis C virus; ICG, indocyanine green test; AFP, alpha-fetoprotein;AFP-L3, *lens culinaris* agglutinin-reactive AFP; DCP, des-gamma-carboxy prothrombin.

*Our institution, patients had been under surveillance at our institution before the detection of HCC; others, patients had been under surveillance at family physician before the detection of HCC; none, patients had not beem under surveillance and had admitted to our institution with symptoms.

†Category of Child-Pugh class A includes patients without cirrhosis.

‡AFP, AFP-L3, and DCP were not measured in 2, 12, and 5 patients, respectively.

42 females with a mean age of 67.4 ± 7.1 years. HCC was detected under surveillance at our institution in 69.9% of patients, and 93.3% of patients exhibited Child-Pugh class[24] A liver function. Multiple tumors were present in 16.0% of patients. Macroscopic portal vein invasion was observed in 11.7% of patients, whereas microscopic portal vein invasion was observed in 18.4% of patients. Pretreatment AFP, AFP-L3, and DCP were above the specified cut-off levels in 47.8%, 17.2%, and 50.6% of patients, respectively. Antiviral therapy with interferon was performed in 24 (14.7%) patients after hepatectomy.

## Recurrence Rate after Hepatectomy Based on Amino Acid Substitutions of the HCV Core Region and Pretreatment Serum Tumor Markers

Sequencing of the HCV core region failed in 6 patients, preventing detection of amino acid substitutions at residues 70 and 91. At residue 70, 87 of 157 patients (55.4%) possessed arginine, whereas 70 patients (44.6%) had glutamine at that position. At residue 91, 97 of 157 patients (61.8%) had leucine and 60 patients (38.2%) had methionine.

We determined the rates of recurrence for patients after attentive curative treatment with hepatectomy based on the amino acid residue at position 91 of the HCV core region (Fig. 1). The recurrence rate for patients with methionine at this position was significantly higher than that of patients bearing a leucine ($P = 0.0002$). In contrast, we found no difference in recurrence according to amino acid substitutions at residue 70 (Supplemental Digital Content 2, available at: http://links.lww.com/SLA/A145). When we analyzed recurrence rates according to pretreatment tumor markers, there was no difference in recurrence rate according to pretreatment serum AFP, AFP-L3, or DCP levels (Supplemental Digital Content 3, available at: http://links.lww.com/SLA/A146). Univariate analysis identified tumor size (>5 cm), macroscopic portal vein invasion, and amino acid substitution at residue 91 of the HCV core region as factors that significantly associated with recurrence rate after hepatectomy. By multivariate analysis, these 3 factors were also selected as independent factors associated with increased recurrence rates (Table 2). Recurrent HCC was categorized into multicentric recurrence in 25 of 55 patients (45.5%) with recurrence bearing a leucine and in 33 of 48 patients (68.8%) with methionine, the prevalence being higher in patients with methionin ($P = 0.0292$).

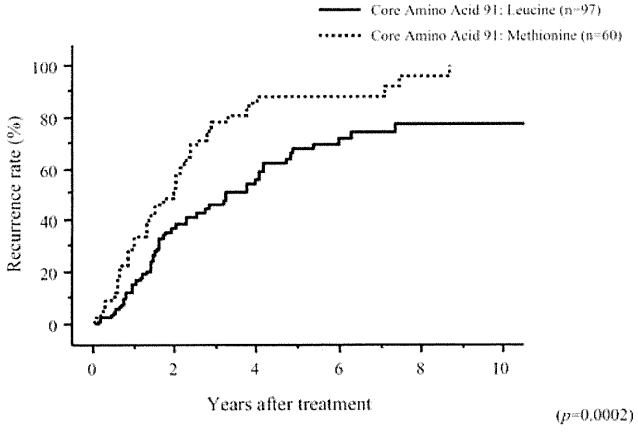


**FIGURE 1.** Recurrence rates after hepatectomy in patients bearing leucine (solid line) or methionine (dotted line) at residue 91 of the HCV core region. Recurrence rates were significantly higher in patients with methionine than in patients with leucine at residue 91 of the HCV core region ($P = 0.0002$).

Comparison of the characteristics of patients according to amino acid substitution at residue 91 of the HCV core region did not reveal differences in patient age, gender, liver function, the progression of HCC, or pretreatment AFP, AFP-L3, and DCP levels between patients with leucine and methionine at residue 91 (Table 3). The rate of patients who underwent postoperative interferon therapy and the rate of patients who achieved the eradication of HCV by interferon therapy were not different between these 2 groups.

## Cancer Specific and Overall Survival Rates after Hepatectomy According to Amino Acid Substitutions of the HCV Core Region and Pretreatment Serum Tumor Markers

We determined the cancer specific and overall survival rates of patients after hepatectomy as a function of amino acid substitution at residue 91 of the HCV core region. The cancer specific survival of patients bearing methionine at residue 91 was significantly lower than that of patients with leucine at residue 91 ($P = 0.0010$, Supplemental Digital Content 4, available at: http://links.lww.com/SLA/A147) and consequently, overall survival of patients with methionine was significantly lower than that of patients with leucine ($P = 0.0061$, Fig. 2). In contrast, we did not find a difference in survival correlating with the amino acid substitution at residue 70 (Supplemental Digital Content 5, available at: http://links.lww.com/SLA/A148). When we analyzed patient survival rates according to pretreatment tumor markers, there was no difference in patient survival according to pretreatment serum AFP or DCP levels. We did identify a significant difference in survival associated with pretreatment AFP-L3 proportions ($P = 0.0473$) (Supplemental Digital Content 6, available at: http://links.lww.com/SLA/A150). We found significantly higher survival rate of patients who underwent postoperative antiviral therapy with interferon than that of patients who did not ($P = 0.0065$, Supplemental Digital Content 7, available at: http://links.lww.com/SLA/A151). Univariate analysis identified patient age, tumor number, macroscopic portal vein invasion, amino acid substitution at residue 91 of the HCV core region, and postoperative interferon therapy as factors significantly associated with survival after hepatectomy. By multivariate analysis, tumor number, portal vein invasion, amino acid substitution at residue 91 of the HCV core region, and postoperative interferon therapy were selected as independent factors associated with patient survival (Table 4).

## DISCUSSION

The results of this study demonstrated that amino acid substitutions at residue 91 in the HCV core region were associated with recurrence and survival rates for patients with HCC bearing HCV genotype 1b infection who underwent hepatectomy with curative intent. Comparison of patient background characteristics, including liver function at diagnosis and HCC tumor progression (size, number, and portal vein invasion), did not reveal any difference between HCV genotype 1b-positive patients with leucine at residue 91 and those with methionine at that position, indicating that the differences in recurrence and survival seen for different amino acid substitutions at residue 91 were not due to the differences in liver function or tumor progression before treatment. Multivariate analyses identified that methionine substitution at this position was an independent factor associated with increased recurrence and decreased survival after hepatectomy. In previous reports, Akuta et al[10] and Nakamoto et al[12] studied the association between the incidence of primary, not recurrent HCC and amino acid substitutions in the HCV core region, combining substitutions at residues 70 and 91. They

**TABLE 2.** Univariate and Multivariate Analyses for Factors Associated with Postoperative Recurrence in HCC Patients Infected with HCV Genotype 1b (n = 157)

| Factor | | Univariate analysis | Multivariate analysis | Risk ratio (95% confidence interval) |
|---|---|---|---|---|
| Age | | 0.0731 | – | |
| Sex | Male | | | |
| | Female | 0.0892 | – | |
| Child-Pugh class | A | | | |
| | B | 0.7315 | – | |
| Tumor size | ≤2 cm | | | 1 |
| | >2 cm and ≤ 5 cm | 0.1061 | – | |
| | >5 cm | 0.0018 | 0.0165 | 1.5254 (1.0869–2.0542) |
| Tumor number | Single | | | |
| | Multiple | 0.5379 | – | |
| Macroscopic-PV invasion | Absent | | | 1 |
| | Present | 0.0011 | 0.0011 | 1.8240 (1.2972–2.4585) |
| Core-70 amino acid | Arginine | | | |
| | Glutamine | 0.1130 | – | |
| Core-91 amino acid | Leucine | | | 1 |
| | Methionine | 0.0023 | 0.0207 | 1.2878 (1.0399–1.5895) |
| Pretreatment AFP | <20 ng/mL | | | |
| | ≥20 ng/mL | 0.6394 | – | |
| Pretreatment AFP-L3 | <10% | | | |
| | ≥10% | 0.0763 | – | |
| Pretreatment DCP | <40 mAU/mL | | | |
| | ≥40 mAU/mL | 0.0643 | – | |
| IFN therapy after hepatectomy | No | | | |
| | Yes | 0.1859 | – | |

PV indicates portal vein; IFN, interferon; AFP, alpha-fetoprotein; AFP-L3, *lens culinaris* agglutinin-reactive AFP; DCP, des-gamma-carboxy prothrombin.
*Category of Child-Pugh class A includes patients without cirrhosis.

**TABLE 3.** Comparison of Clinical Characteristics of Study Patients Based on the Amino Acid Substitutions at Residue 91 of the HCV Core Region (n = 157)

| | Core Amino Acid 91: Leucine (n = 97) | Core Amino Acid 91: Methionine (n = 60) | *P* value |
|---|---|---|---|
| Age (mean ± SD, years) (range) | 66.8 ± 6.9 (47-79) | 68.7 ± 7.3 (49-83) | 0.0737 |
| Sex ratio (female/male) | 28 (28.9)/ 69 (71.1) | 14 (23.3)/ 46 (76.7) | 0.5642 |
| Child-Pugh class (A/B)* | 93 (95.9)/ 4 (4.1) | 54 (90.0)/ 6 (10.0) | 0.2581 |
| Albumin (mean ± SD, g/dL) | 3.85 ± 0.42 | 3.83 ± 0.45 | 0.5523 |
| Total bilirubin (mean ± SD, mg/dL) | 0.73 ± 0.33 | 0.76 ± 0.37 | 0.5546 |
| 15-minute retention rate of ICG (%) | 15.7 ± 6.7 | 15.4 ± 8.4 | 0.8114 |
| Prothrombin (%) | 90.4 ± 14.2 | 90.1 ± 14.6 | 0.6752 |
| Platelet ( × 1000/μL) | 127 ± 55 | 117 ± 46 | 0.1679 |
| Tumor size (mean ± SD, cm) (range) | 2.86 ± 2.25 (0.6-17.6) | 2.93 ± 1.64 (0.7-11.0) | 0.2020 |
| ≤2 cm/>2 cm and ≤5 cm/>5 cm | 37 (38.2)/ 46 (47.4)/ 14 (14.4) | 25 (41.7)/ 30 (50.0)/ 5 (8.3) | 0.5047 |
| Tumor number (single/multiple) | 82 (84.5)/ 15 (15.5) | 50 (83.3)/ 10 (16.7) | 0.8414 |
| Macroscopic-portal vein invasion (absent/present) | 84 (86.6)/ 13 (13.4) | 54 (90.0)/ 6 (10.0) | 0.7003 |
| Microscopic-portal vein invasion (absent/present) | 77 (79.4)/ 20 (20.6) | 51 (85.0)/ 9 (15.0) | 0.5021 |
| AFP (median, ng/mL) (range)† | 16.0 (0.8-5280) | 22.6 (0.8-3480) | 0.1780 |
| ≥ 20 ng/mL / <20 ng/mL | 53 (55.2)/ 43 (44.8) | 27 (45.8)/ 32 (54.2) | 0.3280 |
| AFP-L3 (median, %) (range)† | 0.5 (0-87.2) | 0.5 (0-65.7) | 0.0893 |
| ≥ 10% / <10% | 77 (87.5)/11 (12.5) | 43 (75.4)/ 14 (24.6) | 0.0980 |
| DCP (median, mAU/mL) (range)† | 45.5 (10-36164) | 40.0 (10-11638) | 0.7514 |
| ≥ 40 mAU/mL / <40 mAU/mL | 45 (47.9)/ 49 (52.1) | 30 (51.7)/ 28 (48.3) | 0.7684 |
| Interferon therapy after hepatectomy (no/yes) | 85 (87.6)/ 12 (12.4) | 53 (88.3)/ 7 (11.7) | 0.8954 |
| Eradication of HCV by interferon therapy (no/yes) | 94 (96.9)/ 3 (3.1) | 57 (95.0)/ 3 (5.0) | 0.8571 |

Percentages were in parentheses.
ICG indicates indocyanine green test; AFP, alpha-fetoprotein; AFP-L3, *lens culinaris* agglutinin-reactive AFP; DCP, des-gamma-carboxy prothrombin; HCV, hepatitis C virus.
*Category of Child-Pugh class A includes patients without cirrhosis.
†AFP, AFP-L3, and DCP were not measured in 2, 12, and 5 patients, respectively.

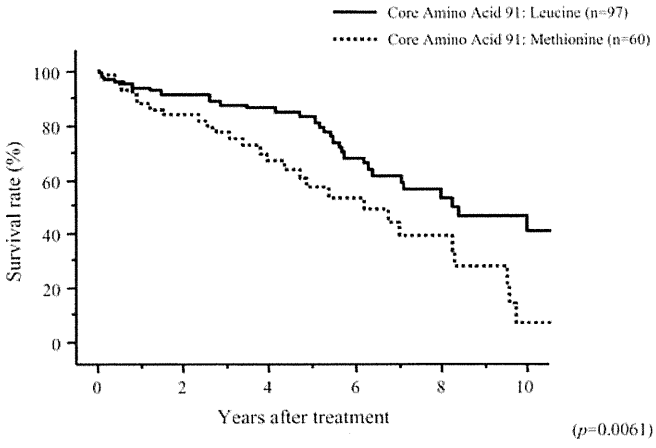


**FIGURE 2.** Overall survival rates after hepatectomy in patients bearing leucine (solid line) or methionine (dotted line) at residue 91 of the HCV core region. Survival rates were significantly lower in patients with methionine at residue 91 of the HCV core region than those with leucine at residue 91 ($P = 0.0061$).

**TABLE 4.** Univariate and Multivariate Analyses for Factors Associated with Postoperative Survival in HCC Patients Infected with HCV Genotype 1b (n = 157)

| Factor | | Univariate analysis | Multivariate analysis | Risk ratio (95% confidence interval) |
|---|---|---|---|---|
| Age | | 0.0198 | 0.1698 | |
| Sex | Male | | | |
| | Female | 0.0934 | – | |
| Child-Pugh class | A | | | |
| | B | 0.3398 | – | |
| Tumor size | ≤2 cm | | | |
| | >2 cm and ≤5 cm | 0.3474 | – | |
| | >5 cm | 0.0898 | – | |
| Tumor number | Single | | | 1 |
| | Multiple | 0.0190 | 0.0434 | 1.3841 (1.0102–1.8466) |
| Macroscopic-PV invasion | Absent | | | 1 |
| | Present | 0.0022 | 0.0031 | 1.8280 (1.2460–2.5621) |
| Core-70 amino acid | Arginine | | | |
| | Glutamine | 0.1483 | – | |
| Core-91 amino acid | Leucine | | | 1 |
| | Methionine | 0.0063 | 0.0076 | 1.4517 (1.1063–1.8994) |
| Pretreatment AFP | <20 ng/mL | | | |
| | ≥20 ng/mL | 0.3632 | – | |
| Pretreatment AFP-L3 | <10% | | | |
| | ≥10% | 0.0617 | – | |
| Pretreatment DCP | <40 mAU/mL | | | |
| | ≥40 mAU/mL | 0.5713 | – | |
| IFN therapy after hepatectomy | No | | | 1 |
| | Yes | 0.0013 | 0.0203 | 0.5052 (0.2036–0.9141) |

PV indicates portal vein; IFN, interferon; AFP, alpha-fetoprotein; AFP-L3, *lens culinaris* agglutinin-reactive AFP; DCP, des-gamma-carboxy prothrombin.
*Category of Child-Pugh class A includes patients without cirrhosis.

reported that lower incidence of the development of HCC in patients bearing an arginine at residue 70 and a leucine at residue 91 (double-wild type) than in patients with other substitutions at these positions (nondouble-wild type). We also found significant difference in both recurrence and survival rates after hepatectomy between these 2 groups ($P = 0.0219$ and 0.0384, Supplemental Digital Content 8 and 9, available at: http://links.lww.com/SLA/A152 and http://links.lww.com/SLA/A153). However, the differences in recurrence and survival rates after hepatectomy were more marked when stratifying patients according to amino acid substitutions at residue 91 in the HCV core region, indicating different effects of the amino acid substitutions of the HCV core region between de novo HCC cases and recurrences after curative resection.

Pretreatment elevations of tumor markers for HCC, especially AFP-L3 and DCP, have been reported to indicate malignant potential of HCC tumor and be associated with higher recurrence rates and lower survival rates.[25,26] In this study, however, we were unable to identify differences in recurrence or survival according to pretreatment elevations of tumor markers, except for a mild association of AFP-L3 elevation and decreased survival rates. This may be due to a focus in our study on patients who underwent hepatectomy as a curative radical treatment and our exclusion of patients who underwent nonsurgical treatment or no treatment. Hepatectomy may overcome the malignant potential of HCC tumor associated with a pretreatment elevation of tumor markers.

Postoperative interferon therapy has been reported to decrease recurrence rate[27] and increase survival rate[28] after hepatectomy, especially in patients who achieved the eradication of HCV by the therapy. We found higher survival rates in patients who underwent postoperative interferon therapy than in those who did not. The effect

of interferon therapy to improve liver function might have contributed to the increased survival[28] in our study patients. In contrast, we failed to find the effect of interferon on the suppression of recurrence of HCC after hepatectomy. Recent studies revealed that the efficacy of interferon therapy is strongly associated with amino acid substitutions in the HCV core region,[8] mainly with amino acid substitutions at residue 70.[9,29] In contrast, our results showed the association between amino acid substitutions at residue 91 and postoperative recurrence and survival rates of patients with HCC after hepatectomy. Together with previous reports and our results, it seems that postoperative interferon therapy did not play a role in the association between postoperative recurrence and survival of patients with HCC and amino acid substitutions in the HCV core region observed in this study.

Previous studies examining the patterns of HCC recurrence in patients with HCV-related HCC reported that intrahepatic metastases are predominant within 2 to 3 years of treatment; multicentric recurrence of HCC becomes predominant after that period.[16] A comparison of the recurrence curve for patients with leucine at residue 91 of the HCV core region with that for patients bearing methionine at that position (Fig. 1) revealed that the difference in recurrence rates became marked 2 years after hepatectomy and suggests that amino acid substitution at residue 91 is associated with multicentric recurrence of HCC in patients with HCV genotype 1b infection. Indeed, the prevalence of multicentric recurrence was significantly higher in patients bearing a methionine at residue 91 in the HCV core region than in patients with leucine.

There are several limitations to this study. The study population included only patients infected with HCV genotype 1b; our study did not examine the association between amino acid substitutions at residue 91 and recurrence or survival rates in patients with other HCV genotypes. All patients were Mongoloid Japanese; these results, therefore, should be evaluated in other ethnicities to demonstrate the generalization of our findings. In addition, the percentage of macroscopic and microscopic portal vein invasion in the study patients was lower in comparison to HCC patients from Japanese general population.[30] This will be because of the high rate of patients in whom HCC was detected under surveillance at our liver center in this study; HCC was diagnosed in early stage in most of these patients.[31] Furthermore, as none of our patients were treated with liver transplantation, we have no data on the effect of amino acid substitutions at residue 91 on recurrence or survival in this subpopulation. Finally, the mechanism underlying the effect of this amino acid substitution on patient recurrence and survival remains unknown, which we hope will be investigated in the future to shed light on potential treatment approaches.

In conclusion, our examination of 163 patients treated by hepatectomy with curative intent revealed that amino acid substitution at residue 91 of the HCV core region influenced recurrence and survival after hepatectomy; patients bearing methionine at this position demonstrated higher recurrence and lower survival rates. Further studies will be needed to confirm this association in other population and elucidate the mechanism underlying this effect.

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AJR FOCUS ON:

# Evolution of Hypointense Hepatocellular Nodules Observed Only in the Hepatobiliary Phase of Gadoxetate Disodium–Enhanced MRI

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**OBJECTIVE.** We sought to determine whether hypointense hepatocellular nodules observed in the hepatobiliary phase of MRI enhanced with gadolinium–ethoxybenzyl–diethylenetriamine pentaacetic acid (gadoxetate disodium) progress to hypervascular hepatocellular carcinoma.

**MATERIALS AND METHODS.** Gadoxetate disodium–enhanced MRI was repeated for 30 patients with 49 nodules determined to be hypointense in the hepatobiliary phase but nonenhancing in the arterial phase of dynamic MRI. The correlation between characteristics of hypointense nodules with slightly or markedly low signal intensity relative to surrounding liver parenchyma and their progression to hypervascular hepatocellular carcinoma was analyzed in cirrhotic livers. All patients underwent angiography-assisted CT before MRI. The rate of progression to classic hepatocellular carcinoma was calculated by the Kaplan-Meier method.

**RESULTS.** The overall 6- and 12-month cumulative incidences of vascularization were 27.6% and 43.5%. The 6- and 12-month cumulative incidences of vascularized nodules with a maximum diameter 15 mm or greater were 43.3% and 77.3% and a maximum diameter less than 15 mm were 16.9% and 16.9%. The difference between these incidences was significant ($p = 0.0147$).

**CONCLUSION.** Hypointense nodules with a maximum diameter of at least 15 mm often become hypervascular. Therefore, patients with hypointense nodules characterized by a maximum diameter of 15 mm or greater should be observed carefully because of the high incidence of vascularization.

**Keywords:** gadoxetate disodium, hepatocellular carcinoma, MRI, vascularization

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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 among men and the fifth most common among women [2]. Periodic follow-up of chronic liver disease, particularly cirrhosis, with ultrasound, MDCT [3, 4], MRI [5], or measurement of tumor markers [6] aids detection of small, early-stage HCC. In addition, follow-up of patients at high risk frequently reveals various types of hepatocellular nodules, many of which are difficult to differentiate [7–11].

Despite the importance of differential diagnosis, it is nearly impossible to characterize these hepatocellular nodules specifically with ultrasound, MDCT, and MRI. Therefore, a histologic diagnosis is usually obtained with percutaneous liver biopsy under ultrasound guidance. This procedure is not always possible, however, because of the need for multiple samples and its invasive nature. Nakanuma et al. [7], Matsui et al. [8, 9], Takayasu et al. [10], and Hayashi et al. [11] reported on the utility of evaluating the intranodular blood supply with CT with arterioportography (CTAP) and CT hepatic angiography (CTHA) to establish a differential diagnosis. This procedure, however, also is invasive and difficult to repeat in the absence of hepatic tumor embolization.

The liver-specific contrast agent gadolinium–ethoxybenzyl–diethylenetriamine pentaacetic acid (gadoxetate disodium), which is taken up by hepatocytes, has been used for dynamic MRI studies. Gadoxetate disodium is used to acquire both dynamic and liver-specific hepatobiliary MR images [12–14]. In the hepatobiliary phase, imaging evidence of hepatic lesions that lack normally

functioning hepatocytes is absence of hepatocyte-selective enhancement compared with normal parenchyma [15]. Evaluation of vascularity and hepatocyte-specific uptake facilitates accurate detection and characterization of focal liver lesions. The differential diagnosis of dysplastic nodules and early HCC can be made on the basis of evidence obtained with or without gadoxetate disodium uptake [16]. Differentiation of early HCC from a dysplastic nodule on hepatobiliary phase images, however, is controversial. The purpose of this study was to analyze the correlation between the characteristics of hypointense nodules in the hepatobiliary phase of gadoxetate disodium–enhanced MRI and the progression of these nodules to hypervascular HCC in cirrhotic liver.

## Materials and Methods

### *Patient Selection*

Our institution did not require institutional approval or informed consent for review of patient records and images in this retrospective study. Between February 1, 2008, and July 31, 2009, 430 consecutively registered patients underwent gadoxetate disodium–enhanced MRI at our institution. The presence of HCC was suspected on the basis of the sonographic findings or elevated levels of tumor markers (α-fetoprotein and des-γ-carboxy prothrombin). Gadoxetate disodium–enhanced MRI was repeated for 30 of these patients (19 men, 11 women; median age, 75.0 years; range, 58–81 years) with 49 nodules that were hypointense in the hepatobiliary phase, had a maximum diameter of 0.8–4.0 cm (median, 1.4 cm), and were nonenhancing in the arterial phase of dynamic MRI. In this series, hypointense was defined as slightly or markedly low signal intensity relative to surrounding liver parenchyma in the hepatobiliary phase 20 minutes after contrast injection.

The following criteria were used for enrollment in this study: CTAP and CTHA within at least 1 month after gadoxetate disodium–enhanced MRI at which hypointense nodules were detected initially; hypointense nodules not visualized at CTAP and not having a hyperattenuating area at CTHA; and oval or round hypointense nodules with well-defined margins. The cause of cirrhosis was related to hepatitis C in 25 patients, both hepatitis B and C in one patient, and neither hepatitis B nor C in four patients. All patients had Child-Pugh class A disease. The median α-fetoprotein concentration was 7.4 ng/mL (range, 0.8–287 ng/mL), and the median des-γ-carboxy prothrombin concentration was 29 mAU/L (range, 10–791 mAU/L). Hypervascular HCC was found in 10 patients in another site in the liver at the start of follow-up.

The median observation period was 6.0 months (range, 3.0–15.0 months). Four patients underwent 3 months of follow-up, and 12 patients underwent more than 6 months of follow-up. In general, hypointense nodules were followed up with gadoxetate disodium–enhanced MRI and ultrasound in turn at 3-month intervals. Gadoxetate disodium–enhanced MRI was performed sequentially if the increase in nodule diameter was confirmed at ultrasound examination or elevation in tumor marker concentration was observed. A median of three MRI examinations (range, 2–9 examinations) were performed per patient. The mean interval between MRI examinations was 3 months (range, 3–9 months).

### *Imaging Methods*

MRI was performed with a 1.5-T whole-body MRI system (Intera Achieva 1.5 T Nova, Philips Healthcare) with a phased-array body coil as the receiver coil. T1-weighted sequences were performed with a T1-weighted turbo field-echo in-phase and opposed-phase transverse sequence (opposed phase TE, 2.3; in-phase TE, 4.6; flip angle, 12°; matrix size, 256 × 512; scan percentage, 70; section thickness, 3.5 mm; intersection gap, 0 mm; field of view, 38 $cm^2$). After IV injection of gadoxetate disodium (Primovist, Bayer Schering Pharma), a T1-weighted transverse gradient-echo sequence was performed (high-resolution isotropic volume examination with spectral presaturation with inversion recovery; TR/TE, 4/1.8; flip angle, 12°; matrix size, 256 × 512; scan percentage, 78.54; section thickness, 3.5 mm; intersection gap, 0 mm; field of view, 38 $cm^2$). Gadoxetate disodium was administered IV as a bolus at a rate of 2 mL/s (0.1 mL/kg; maximal dose, 10 mL) through a cubital IV line (20–22 gauge), which was flushed with 20 mL of saline solution through a power injector (Sonic Shot, Nemoto Kyourindo).

The timing of dynamic arterial phase imaging was determined with MRI fluoroscopic bolus detection (Bolus Trak, Philips Healthcare) of the descending aorta. The mean delay times (interval between start of bolus administration and start of image acquisition) for the arterial, portal, and delayed phases were 20, 60, and 180 seconds. Immediately after the dynamic study, a respiratory-triggered single-shot T2-weighted sequence was performed with a reduction factor of 4 (TR/TE, 1200/100; flip angle, 90°; matrix size, 400 × 512; section thickness, 7 mm; intersection gap, 1 mm; field of view, 38 $cm^2$; spectral presaturation with inversion recovery). The 20-minute delayed hepatobiliary phase acquisition [17] was performed with a T1-weighted turbo field-echo sequence (TR/TE, 4/1.8; flip angle, 12°; matrix size, 256 × 512; section thickness, 3.5 mm; intersection gap, 0-mm; field of view, 38 $cm^2$). All sequences were performed with parallel imaging (sensitivity encoding).

CTAP and CTHA were performed with an interventional radiology CT angiography system (CT, X Vision Real; digital subtraction angiography, DFP 2000A; Toshiba Medical Systems). CTAP was performed after infusion of 70 mL of contrast medium (iopamidol, Iopamiron 150, Bayer Schering Pharma) at a rate of 2.0 mL/s into the superior mesenteric artery through a power injector (Autoenhance A50, Nemoto Kyourindo). CTAP data acquisition was begun 30 seconds after initiation of a transcatheter injection of contrast medium. CTHA was performed after infusion of 20–25 mL of contrast medium at a rate of 1.0 mL/s into the common or proper hepatic artery through a power injector. CTHA data acquisition was begun 10 seconds after initiation of a transcatheter injection of contrast medium. Helical CT was performed with a section thickness of 7 mm and a table feed speed of 7-mm increments.

### *Image Analysis*

A diagnosis of hepatic nodule was rendered when the hepatobiliary images depicted a round or oval distinct nodule 8 mm or more in widest diameter [11] that was distinguishable from a cyst or cavernous hemangioma in cirrhotic liver. Hepatic nodules with an irregular shape, maximum diameter less than 8 mm, or ill-defined margin were excluded. The dynamic MRI findings 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, and hypoattenuating relative to the findings in 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 enhancing in the arterial phase, having a washout pattern in the portal venous phase, and hypointense relative to the surrounding liver parenchyma in the hepatobiliary phase were regarded as HCC at follow-up gadoxetate disodium–enhanced MRI. In addition, vascularization of hypointense nodules in these cases was confirmed as the typical finding of hypervascular HCC showing hyperattenuating areas during CTHA and hypoattenuating areas during CTAP in all patients. If hypervascularization of hypointense nodules was not detected in the dynamic phase of follow-up gadoxetate disodium–enhanced MRI, angiography-assisted CT was not performed.

*Statistical Analysis*

Statistical analysis was performed with SPSS software (version 17.0 for Microsoft Windows, SPSS Japan). Continuous variables were presented as median and range. Continuous variables such as tumor size, frequency of MRI examinations, and observation period were compared by Mann-Whitney *U* test, and categoric variables such as T1-weighted and T2-weighted image, findings at CTHA, and grade of hypointensity in the hepatobiliary phase were compared by Fisher exact test or chi-square test. Actuarial analysis of the cumulative incidence of vascularization was performed with the Kaplan-Meier method, and differences were tested by log-rank test. The cutoff point of tumor size was determined with the Cox proportional hazards model. Statistical significance was set at $p < 0.05$.

## Results

*Nodule Characteristics*

Of the 49 nodules imaged with repeated gadoxetate disodium–enhanced MRI, 13 (26.5%) became hyperintense in the arterial phase of dynamic MRI during the follow-up period. Table 1 shows the characteristics of the nodules that were vascularized and those that were not. At the start of follow-up, there were no differences between nodules with and those without vascularization with respect to findings on T1-weighted, T2-weighted, and hepatobiliary phase MR images and CTHA images; presence of typical HCC at other sites; frequency of MRI examinations; or observation period. The nodules with vascularization, however, were significantly larger than those without vascularization ($p = 0.0260$), and the change in nodule size with vascularization was significantly greater than that without vascularization during the follow-up period ($p = 0.0153$). At the end of follow-up period, two of the vascularized nodules were hypointense on T1-weighted images, 10 were isointense, and one was hyperintense. On T2-weighted images, none of the vascularized nodules was hypointense, 10 were isointense, and three were hyperintense. The corresponding characteristics of nonvascularized nodules were three hypointense, 23 isointense, and 10 hyperintense on T1-weighted images and one hypointense, 33 isointense, and two hyperintense on T2-weighted images.

*Cumulative Incidence of Vascularization*

The overall 6- and 12-month cumulative incidences of vascularization were 27.6% and 43.5%. The optimal cutoff point of tumor size determined with the Cox proportional hazards model was 15 mm (Table 2). The 6- and 12-month cumulative incidences of vascularization of nodules with a maximum diameter of 15 mm or greater were 43.3% and 77.3%. In contrast, the 6- and 12-month cumulative incidences of vascularization of nodules with a maximum diameter less than 15 mm were 16.9% and 16.9%. The difference between these incidences was significant ($p = 0.0147$, log-rank test) (Fig. 1). Figures 2 and 3 show two cases of progression to hypervascular HCC.

**TABLE 1: Nodule Characteristics**

| Characteristic | Vascularization | | *p* |
|---|---|---|---|
| | Present | Absent | |
| No. of nodules | 13 | 36 | |
| Size of nodules (mm) | 20 (12–40) | 14 (8–40) | 0.0260 |
| Change in size (mm) | 4 (0–8) | 1 (–8 to 13) | 0.0153 |
| T1-weighted imaging finding | | | 0.6592 |
| Hypointensity | 5 | 3 | |
| Isointensity | 7 | 24 | |
| Hyperintensity | 2 | 9 | |
| T2-weighted imaging finding | | | 0.1033 |
| Hypointensity | 1 | 4 | |
| Isointensity | 9 | 31 | |
| Hyperintensity | 3 | 1 | |
| Hepatobiliary phase imaging finding | | | 0.1834 |
| Hypointensity | 9 | 32 | |
| Slight hypointensity | 4 | 4 | |
| Hyperintensity | | | |
| CT hepatic angiographic finding | | | 0.3629 |
| Isoattenuating | 3 | 4 | |
| Hypoattenuating | 10 | 32 | |
| Typical hepatocellular carcinoma at other site | | | 0.5018 |
| Present | 3 | 13 | |
| Absent | 10 | 23 | |
| No. of MRI examinations | 4 (2–8) | 4 (2–9) | 0.6608 |
| Observation period (mo) | 5 (3–11) | 6 (3–13) | 0.0971 |

Note—Continuous variables are expressed as median with range in parentheses.

## Discussion

A multistep process is thought to be the main mechanism of hepatocarcinogenesis. Correlations between imaging results and the development of hypervascular HCC, however, have not been sufficiently elucidated. In this study, 13 of 49 nodules (26.5%) exhibited hypervascular spots during the follow-up period, and the overall 1-year cumulative incidence of vascularization was 43.5%. Hypointense hepatocellular nodules found in the hepatobiliary phase may be precancerous lesions,

**TABLE 2: Hazard Ratios for Tumor Size Cutoff Points**

| Cutoff Point (mm) | Hazard Ratio | *p* |
|---|---|---|
| 10 | 26.748 (0.048–14895.852) | 0.308 |
| 13 | 2.228 (0.610–8.138) | 0.225 |
| 15 | 3.117 (1.171–12.446) | 0.026 |
| 18 | 3.135 (1.022–9.612) | 0.046 |
| 20 | 1.768 (0.576–5.425) | 0.319 |

Note—Value in parentheses are ranges.

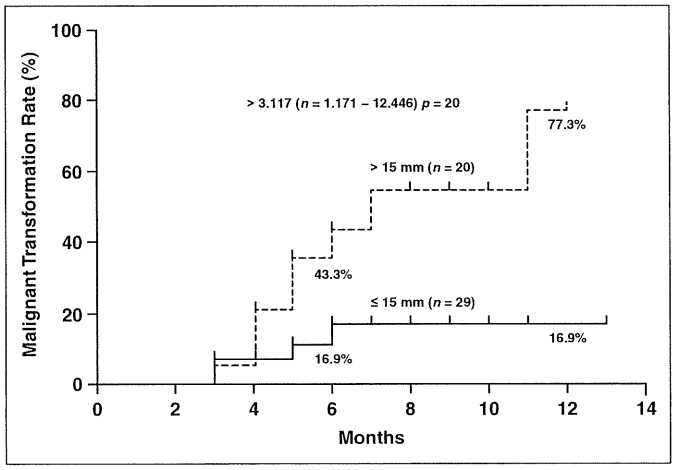


**Fig. 1**—Graph shows cumulative incidence of vascularization of nodules based on maximum diameter. Six- and 12-month cumulative incidence of vascularized nodules with maximum diameter of 15 mm or greater was significantly higher than that of vascularized nodules with maximum diameter less than 15 mm. Dotted line represents development of arterial vascularity for tumors ≥ 15 mm; solid line represents similar development for tumors < 15 mm.

referred to as dysplastic nodules or early HCC in the multistep carcinogenesis process. It is thought that the frequency of vascularization among these nodules is high.

We also evaluated signal intensity at MRI at the start of the follow-up period. According to previous reports [9, 18], high-grade dysplastic nodules and early HCC occasionally are hypointense relative to surrounding cirrhotic liver on T2-weighted MR images and are hyperintense on T1-weighted MR images, and almost all moderately differentiated HCCs are hyperintense and some early HCCs are isointense on T2-weighted MR images. In our study, however, T1- and T2-weighted images showed no differences in signal intensity between nodules that progressed to classic HCC and those that did not. It is still unclear why dysplastic nodules and early HCC are hypointense on T2-weighted MR images and hyperintense on T1-weighted images. Further investigation is necessary. Nodule size at the start of follow-up was significantly associated with progression of hypointense hepatocellular nodules to hypervascular HCC. Kojiro [19] wrote that tumors that grow to a diameter of approximately 1.5 cm begin to proliferate more actively owing to dedifferentiation. In addition, the development of unpaired arteries is insufficient until tumors reach this size [20]. A tumor

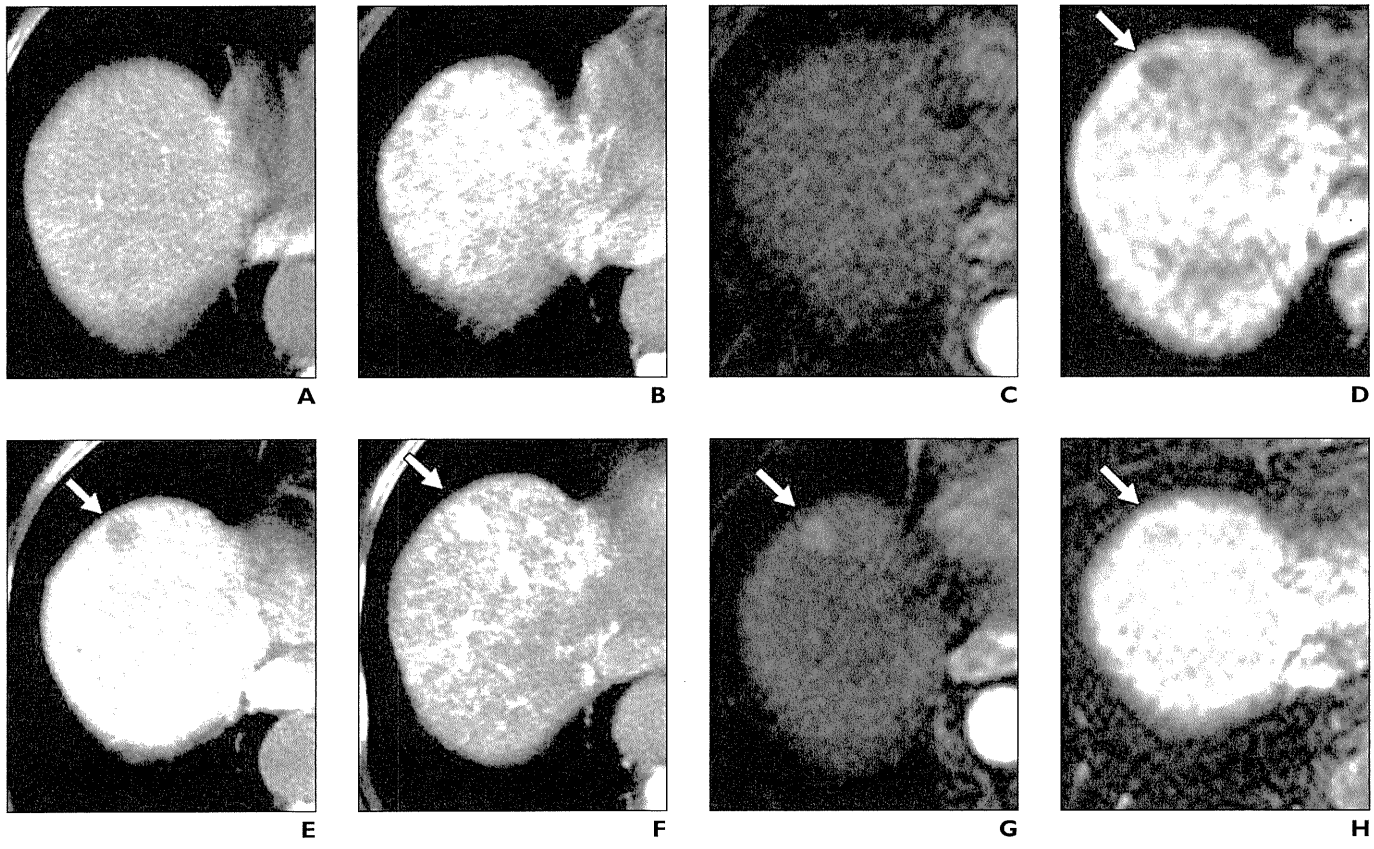


**Fig. 2**—75-year-old woman with hepatitis C–related cirrhosis.
**A,** CT arterial portogram at beginning of follow-up shows no nodules.
**B,** CT hepatic arteriogram at beginning of follow-up shows no nodules.
**C,** Arterial phase of gadoxetate disodium–enhanced MR images at beginning of follow-up shows no nodules.
**D,** Hepatobiliary phase gadoxetate disodium–enhanced MR image at beginning of follow-up shows markedly hypointense nodule (*arrow*).
**E,** CT arterial portogram image 9 months after **A–D** shows hypoattenuating nodule (*arrow*).
**F,** CT hepatic arteriogram 9 months after **A–D** shows hyperattenuating foci in nodule (*arrow*).
**G,** Arterial phase gadoxetate disodium–enhanced MR image 9 months after **A–D** shows hyperattenuating nodule (*arrow*).
**H,** Hepatobiliary phase gadoxetate disodium–enhanced MR image 9 months after **A–D** shows slightly hypointense nodule (*arrow*).

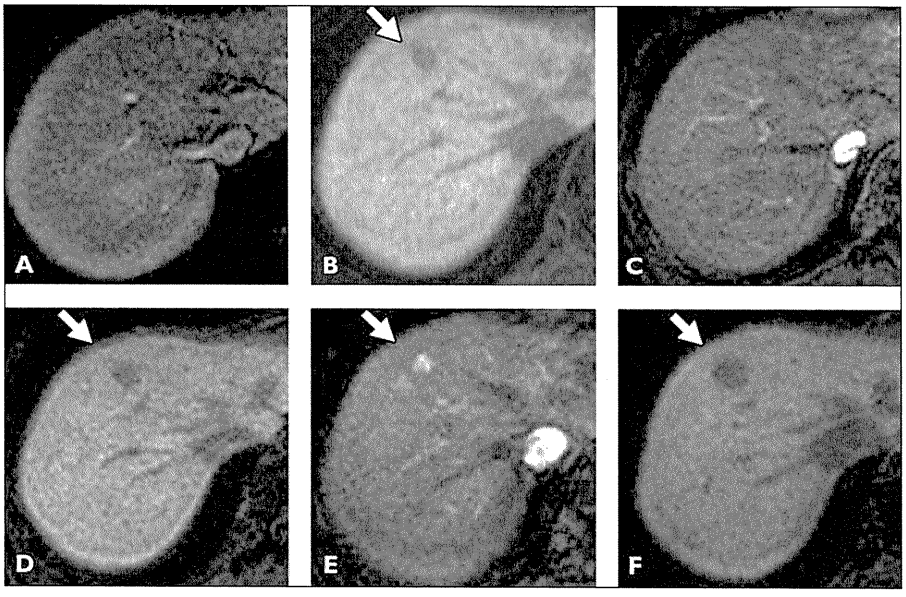


**Fig. 3**—75-year-old man with hepatitis C–related cirrhosis.
**A,** Arterial phase gadoxetate disodium–enhanced MR image at start of follow-up shows no nodules.
**B,** Hepatobiliary phase gadoxetate disodium–enhanced MR image at start of follow-up shows slightly hypointense nodule (*arrow*).
**C,** Arterial phase dynamic gadoxetate disodium–enhanced MR image 9 months after start of follow-up shows no nodules.
**D,** Hepatobiliary phase gadoxetate disodium–enhanced MR image 9 months after start of follow-up shows markedly hypointense nodule (*arrow*).
**E,** Arterial phase gadoxetate disodium–enhanced MR image 15 months after start of follow-up shows hyperattenuating foci in nodule (*arrow*).
**F,** Hepatobiliary phase gadoxetate disodium–enhanced MR image 15 months after start of follow-up shows markedly hypointense nodule (*arrow*).

diameter of 1.5 cm is thought to be the critical threshold for vascularization of hypointense hepatocellular nodules.

Gadoxetate disodium is transported into hepatocytes via organic anion transporters (OATPs) and excreted into bile canaliculi via a multidrug resistance-associated protein [21]. OAT P1B3, a sodium-independent organic anion transporter expressed in the basolateral membrane of hepatocytes, is critical for the transport of agents such as gadoxetate disodium and uptake of endogenous substances and xenobiotics into hepatocytes [12, 22–24]. Narita et al. [25] did not observe OAT P1B3 expression in most HCCs that also did not take up gadoxetate disodium. Those authors observed that gadoxetate disodium was taken up during the hepatobiliary phase only in moderately differentiated HCCs and that three well-differentiated HCCs did not accumulate gadoxetate disodium. Using a rat model of hyperplastic nodules, which corresponded to human low- or high-grade dysplastic nodules and well-differentiated HCC, Tsuda and Matsui [26] found that organic anion transporter P1 activity decreased in comparison with that observed in control samples. In this study, we found that hepatocellular nodules that were hypointense only during the hepatobiliary phase but were nonenhancing during the arterial phase of dynamic MRI were thought to represent dysplastic nodules or early (well-differentiated) HCC.

This study had limitations. First, the hepatocellular nodules evaluated were not identified pathologically. One of the major purposes of this study, however, was to define standard criteria for predicting the nature of borderline lesions—low- and high-grade dysplastic nodules and early HCC—with imaging rather than biopsy. Second, the interval between follow-up examinations varied among and within patients. Prospective studies of gadoxetate disodium–enhanced MRI performed at constant intervals are required to resolve this limitation. Most of the MRI examinations in this study, however, were performed at intervals of 3 and 6 months, which is consistent with usual practice for follow-up of patients at high risk of HCC. Third, there was no control group without hypointense nodules in the hepatobiliary phase. Comparison of groups with and without hypointense nodules in the hepatobiliary phase is recommended to calculate the precise incidence of vascularization.

## Conclusion

Hypointense nodules with a maximum diameter of at least 15 mm in the hepatobiliary phase but nonenhancing in the arterial phase of dynamic MRI often progress to hypervascular HCC. Therefore, patients with hypointense nodules that have a maximum diameter of 15 mm or more need careful follow-up because of the high incidence of vascularization.

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