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Efficient detection of hepatocellular carcinoma by a hybrid blood test of epigenetic and classical protein markers

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ABSTRACT

Background: There are few blood tests for an efficient detection of hepatocellular carcinoma (HCC) associated with hepatitis C virus (HCV) infection.
Methods: The abilities of quantitative analyses of 7 genes hypermethylation in serum DNA, α-fetoprotein (AFP) and prothrombin-induced vitamin K absence II (PIVKA-II), and various combinations to detect HCC were evaluated in a training cohort of 164 HCV-infected patients (108 HCCs; 56 non-HCCs). An optimal hybrid detector, built using data for 2 methylated genes (*SPINT2* and *SRD5A2*), AFP, and PIVKA-II, achieved the most satisfactory ability to detect HCC in the training cohort. We evaluated the ability of the optimal hybrid detector to detect HCC in an independent validation cohort of 258 consecutive HCV-infected patients (112 HCCs; 146 non-HCCs) who were newly enrolled in 4 distinct institutes.
Results: In the validation cohort of 258 patients, accuracy, sensitivity, and specificity of the hybrid detector for detection of HCC were 81.4%, 73.2%, and 87.7%, respectively. Notably, even when detecting HCC ≤ 2 cm in diameter, the hybrid detector maintained markedly high abilities (84.6% accuracy, 72.2% sensitivity, 87.7% specificity). Youden's index (sensitivity + specificity − 1) for HCC ≤ 2 cm was 0.60, vastly much superior to the 0.39 for AFP at a cut-off value of 20 ng/ml and the 0.28 for PIVKA-II at a cut-off value of 40 mAU/ml.
Conclusions: These results show that the optimal hybrid blood detector can detect HCV-related HCC more accurately.

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1. Introduction

For the last decade, evidence has been accumulating in various countries that hepatocellular carcinoma (HCC) is increasing [1–4] . This phenomenon can be explained partly by endemic infection with hepatitis C virus (HCV), one of the major etiological agents for development of HCC [5,6]. Despite the recent advent of treatment, HCC detected after the onset of symptoms shows a dismal prognosis (5-year survival, <10%) [5], indicating an urgent need for efficient detection systems to identify small, asymptomatic HCV-related HCC.

Current methods for diagnosis and screening of HCC include physical examination, various imaging techniques including ultrasonography (US), and measurements of serum α-fetoprotein (AFP) in certain risky populations, such as HCV-infected patients with liver cirrhosis (LC) [4,7]. AFP measurement for the detection of small HCCs (diameter ≤ 2 cm) has been questioned due to the low sensitivity and unstable cut-off values among studies or institutes [8]. The detection ability of US depends on examiner expertise, degree of patient obesity, presence of LC, and size of the liver tumor [9].

Epigenetic inactivation of transcription by aberrant methylation of CpG islands is a fundamental contributor to carcinogenesis [10].

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Several genes reportedly undergo hypermethylation in the process of hepatocarcinogenesis [11–13]. Some studies have revealed the presence of circulating methylated genes in the bloodstream of HCC patients, but none has been applied to daily clinical use as a diagnostic tool [14,15].

In a genome-wide search using DNA array data, our recent study used a quantitative methylation-specific PCR (qMSP) technique to identify 2 unique genes (*BASP1* and *SRD5A2*) for which promoter methylation is specific for small HCC associated with HCV infection [16]. Moreover, we found that 5 known genes (*APC*, *RASSF1A*, *SPINT2*, *CCND2* and *CFTR*) were exclusively methylated in early HCC tissues [17].

Taken together, these prompted us to develop a serological parameter for the efficient detection of HCC associated with HCV. The present study therefore quantified levels of the 7 methylated marker genes [16,17], and classical tumor markers AFP and prothrombin-induced vitamin K absence II (PIVKA-II) in the blood of HCV-infected patients.

2. Materials and methods

2.1. Patients of the training cohort

In the present study, we utilized a training-validation approach [18,19] in which a hybrid detector was built *in silico* on the basis of information from only a training cohort, then the ability of this detector to identify HCC was evaluated in an independent validation cohort at multiple institutions (Fig. 1). Written informed consent was obtained from all patients. The study protocol was undertaken according to the REMARK criteria (http://www.cancerdiagnosis.nci.nih.gov/assessment/progress/remark.htm), and was approved by the Institutional Review Board for the Use of Human Subjects at Yamaguchi University School of Medicine and Review Boards for the Use of Human Subjects at another 3 institutes defined below.

Our training cohort (Table 1) included 164 patients positive for HCV antibody, all of whom were treated at Yamaguchi University Hospital between May 1998 and April 2006, and were subjected to analyses of AFP and PIVKA-II, routine radiography, US, computed tomography (CT), magnetic resonance imaging (MRI), and, if necessary, hepatic angiography, dynamic CT, or dynamic MRI before and after treatment. On the basis of those imaging techniques, 108 of the 164 patients were diagnosed with HCC. Subsequently, 95 of these 108 patients (88.0%) bearing HCC underwent hepatic surgery or biopsy; and all tumors from the 95 patients were pathologically confirmed as HCC. Moreover, none of the 108 HCC patients showed any other malignancies at enrollment. We confirmed that none of the remaining 56 patients developed HCC during the follow-up period of >2 years. On the basis of these findings, we classified the 108 patients with HCC and the remaining 56 patients into HCC and non-HCC groups, respectively (Table 1). Using the results of imaging techniques and pathological examinations, we judged that 79 of the 164 patients (48.2%) had liver cirrhosis (LC). As summarized in Table 1, we used the tumor-node-metastasis (TNM) staging system as revised by the Liver Cancer Study Group of Japan (LCSGJ) [20]. The present study defined HCC≤2 cm in diameter as "small HCC".

2.2. Patients of the validation cohort

Our validation cohort comprised 262 consecutive HCV-infected patients (Table 1) who were enrolled in 4 distinct institutes between

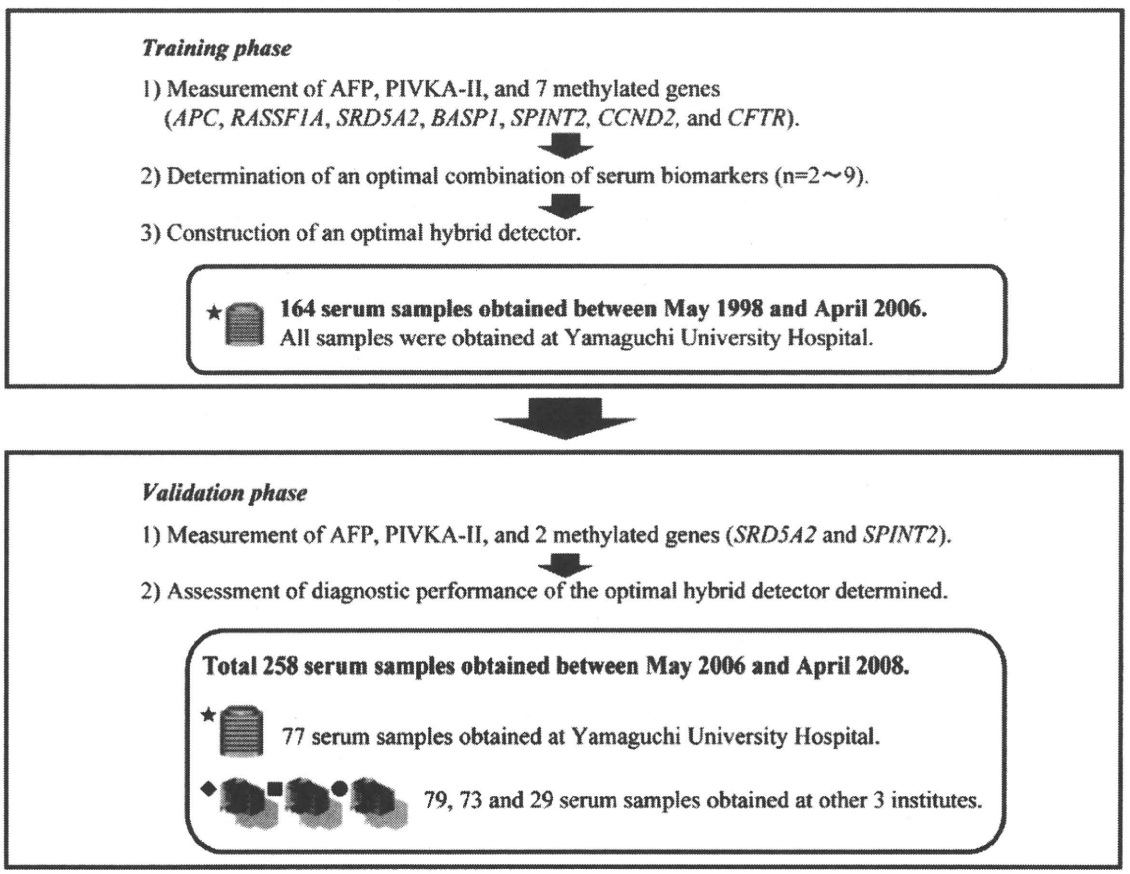

Fig. 1. Overview of the Training-Validation approach used for construction and evaluation of the hybrid detector for hepatocellular carcinoma.

Table 1
Patient characteristics in training and validation cohorts.

	HCC patients			Non-HCC patients		
	Training cohort (n = 108) (%)	Validation cohort (n = 112) (%)		Training cohort (n = 56) (%)	Validation cohort (n = 146) (%)	
Sex			$P = 0.004^a$			$P = 0.062^a$
Male	83 (76.8)	66 (58.9)		30 (53.6)	57 (39.0)	
Female	25 (23.2)	46 (41.1)		26 (46.4)	89 (61.0)	
Age (years) (mean ± SD)	66.6 ± 7.9	70.4 ± 8.0	$P < 0.0001^b$	64.6 ± 7.8	64.6 ± 10.3	$P = 0.985^b$
Serum ALT (U/L) (mean ± SD)	62.2 ± 65.4	55.9 ± 36.9	$P = 0.376^b$	49.1 ± 34.0	51.0 ± 39.3	$P = 0.749^b$
Platelet (10,000/mm^3) (mean ± SD)	12.3 ± 5.8	10.3 ± 5.4	$P = 0.008^b$	14.5 ± 7.7	11.9 ± 6.1	$P = 0.012^b$
Non-cancerous liver			$P = 0.028^a$			$P < 0.0001^a$
Chronic hepatitis	43 (39.8)	29 (25.9)		42 (75.0)	68 (46.6)	
Cirrhosis	65 (60.2)	83 (74.1)		14 (25.0)	78 (53.4)	
a feto-protein			$P = 0.618^a$			$P = 0.041^a$
<20 ng/ml	46 (42.6)	44 (39.3)		48 (85.7)	105 (71.9)	
>20 ng/ml	62 (57.4)	68 (60.7)		8 (14.3)	41 (28.1)	
PIVKA-II			$P = 0.207^a$			$P = 0.088^a$
<40 mAU/ml)	42 (38.9)	59 (52.7)		49 (87.5)	138 (94.5)	
>40 mAU/ml	66 (61.1)	53 (47.3)		7 (12.5)	8 (5.5)	
Tumor size			$P = 0.006^a$			
<2.0 cm	22 (20.4)	36 (32.1)				
2.1–5.0 cm	62 (57.5)	67 (59.8)				
>5.0 cm	24 (22.1)	9 (8.1)				
Primary lesion			$P = 0.992^a$			
Single	52 (48.1)	54 (48.2)				
Multiple	56 (51.9)	58 (51.8)				
Histological grading			$P = 0.900^c$			
G1	21 (22.1)	12 (23.5)				
G2	63 (66.3)	32 (62.7)				
G3–G4	11 (11.6)	7 (13.8)				
Stage			$P = 0.077^a$			
I	12 (11.1)	21 (18.7)				
II	42 (38.9)	32 (28.6)				
III	36 (33.3)	30 (26.8)				
IVA + IVB	18 (16.7)	29 (25.9)				

PIVKA-II, Prothrombin Induced Vitamin K Absence II.
[a] Chi-square test.
[b] Student's *t* test.
[c] Fisher exact test.

May 2006 and April 2008. Out of the 262 patients, 1 was excluded due to daily intake of warfarin, which may affect serum levels of PIVKA-II, and 3 were excluded because of small amounts of extracted cell-free DNA (cfDNA). Among the remaining 258 patients, 77 were treated at Yamaguchi University hospital, 73 at Shimonoseki Kohsei Hospital, 79 at Sapporo-Kosei General Hospital, and 29 at Kurume University Hospital. The detection program for HCC in individual institutes was performed according to the nationwide follow-up survey conducted by the LCSGJ [20] and/or the guidelines of the American Association for the Study of Liver Diseases (AASLD) [4]. On the basis of findings from multiple imaging modalities (US, CT, MRI, hepatic angiography, dynamic CT, and dynamic MRI), hepatologists from the individual institutes diagnosed 112 of the 258 patients (43.4%) as HCC. Among the 112 HCC patients, 52 were diagnosed at Yamaguchi University Hospital, 23 at Shimonoseki Kohsei Hospital, 24 at Sapporo-Kosei General Hospital, and 13 at Kurume University Hospital. Hepatic surgery or biopsy was subsequently performed for 51 of the 112 HCC patients (45.5%). All tumors, including 15 tumors ≤ 2 cm in diameter, from the 51 patients were pathologically confirmed as HCC, indicating the justification of our detection programs for HCC. Our follow-up program did not detect HCCs in any of the 146 patients initially defined as without HCC for 6 months after enrollment. Collectively, we categorized the 112 patients with HCC and the remaining 146 patients as HCC and non-HCC groups, respectively, in the validation cohort (Table 1).

2.3. Extraction and quantification of DNA in sera

Blood samples were collected from patients before treatment to measure methylated marker genes, AFP, PIVKA-II, alanine aminotransferase (ALT) and platelet count. We set a cut-off value of 20 ng/ml for AFP and a cut-off value of 40 mAU/ml for PIVKA-II for the discrimination of HCC, as these values have been shown to offer the highest diagnostic ability for HCV-related HCC and have been used most frequently in clinical practice [8,21]. As a source for methylation analysis, cfDNA was extracted from 1 ml of sera using a DNA Extractor SP Kit for Serum and Plasma (Wako Pure Chemical Industries, Osaka, Japan) according to the instructions from the manufacturer, and was quantified as described previously [22].

2.4. Measurement of methylated gene fragments circulating in sera

We performed qMSP assays for 2 novel methylated genes (*SRD5A2* and *BASP1*) and 5 other genes (*APC*, *RASSF1A*, *SPINT2*, *CCND2*, and *CFTR*), as described previously [16,17] (For gene selection, see supplementary material). In the training phase (Fig. 1), methylated forms of the 7 genes in patient sera were measured and calculated as methylated DNA amount in serum (picograms per 1 ml of serum). In the validation phase (Fig. 1), methylated forms of only *SRD5A2* and *SPINT2* in sera of patients were measured and calculated.

2.5. Development and evaluation of the hybrid detector

We used the Fisher linear classifier (FLC) [19] to construct a hybrid detector *in silico* where "HCC" and "non-HCC" are defined as groups A and B, respectively.

In FLC, the score is defined by

$$T(x) = f_A(x) - f_B(x)$$

where

$$f_A(x) = \frac{1}{2}(x-\hat{\mu}_A)^T\left[P(A)\hat{\Sigma}_A + P(B)\hat{\Sigma}_B\right]^{-1}(x-\hat{\mu}_A) + C(A).$$

$\hat{\mu}_A$ and $\hat{\Sigma}_A$ in $f_A(x)$ are the sample mean vector and sample covariance matrix for Group A, respectively, and $P(A)$ is a prior probability for Group A. $C(A)$–$C(B)$ in $T(x)$ is called Cut off. The value of Cut off can be optimized by minimizing the error rate estimated on the training samples. Then, FLC assigns a given x to be classified to Group A (i.e., HCC) if $T(x)<0$. FLC assigns a given x to be classified to Group B (i.e., non-HCC) if $T(x)>0$.

We input data for n markers ($n=2$–9) from the 164 training samples into FLC and evaluated the ability of constructed individual FLCs to detect HCC in the 164 training samples. Mean detection ability (i.e., sensitivity and accuracy) of top-10 combinations was maximal when the FLC was built using 4 markers (Fig. 2A). We next plotted specificity, sensitivity and diagnostic accuracy of each top-ranked combination of n markers ($n=2$–9). Likewise, a 4-marker combination (*SRD5A2*, *SPINT2*, AFP and PIVKA-II) achieved the highest sensitivity and accuracy among combinations of n markers (Fig. 2B).

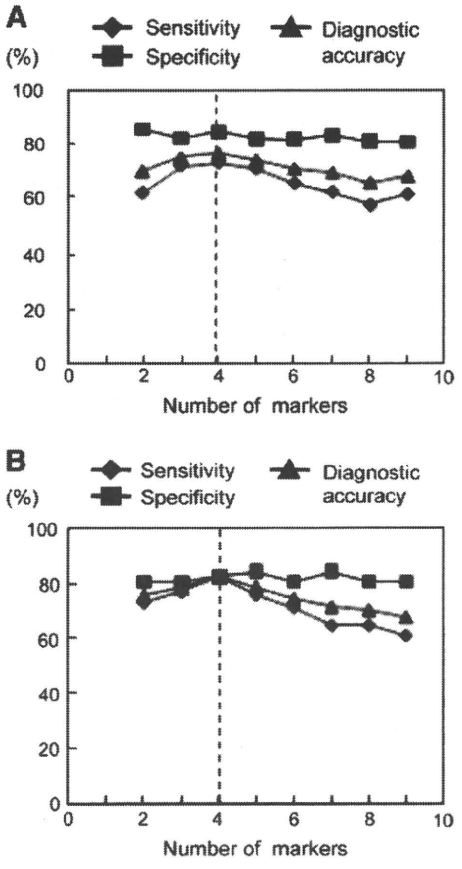

Fig. 2. Performances of markers in the training cohort. We input data for n markers ($n=2$–9) of the 164 training samples into Fisher linear classifier (FLC). This procedure was repeated for all combinations (from 9 C2 to 9 C9) of n markers and performances of the constructed individual FLCs were computed. Mean specificity, sensitivity and accuracy of the top 10 combinations of 2–7 markers and 9 combinations of 8 markers, and specificity, sensitivity and diagnostic accuracy for all 9 markers were plotted (A). Sensitivity and diagnostic accuracy were greater as the number of markers increased to 4; however, sensitivity and accuracy obtained using more than 4 markers were rather inferior to those obtained with 4 markers. We next plotted specificity, sensitivity and accuracy of each top-ranked combination of n markers ($n=2$–9) (B). A 4-marker combination of *SRD5A2*, *SPINT2*, AFP and PIVKA-II achieved the highest sensitivity and accuracy among combinations of n markers.

Collectively, using the optimal combination of 4 markers (*SRD5A2*, *SPINT2*, AFP and PIVKA-II), score was defined by

$$\begin{aligned} T(x) &= f_A(x) - f_B(x) \\ &= -136.28 \times (SRD5A2) - 1.78 \times (SPINT2) - 1.07 \times (\text{AFP}) - 1.99 \\ &\quad \times (\text{PIVKA-II}) + 131. \end{aligned}$$

where sample mean vectors and sample covariance matrices were estimated using the 164 training samples. Our hybrid detector classified samples as HCC or non-HCC for values of $T(x)<0$ and $T(x)>0$, respectively.

2.6. Perfectly blinded assessment of the validation cohort

To evaluate detection ability of the optimal hybrid detector established in the training cohort, we recruited another 258 patients with chronic HCV infection as the validation cohort. These patients were consecutively enrolled at each institute to maintain the independence of patient selection. In the present study, information regarding sample characteristics in the validation cohort was perfectly blinded for analysts of serum markers (TMo, TM, and NK) and bioinformaticians (YH and YF), who constructed a hybrid detector *in silico*.

2.7. Statistical analysis

The χ^2 test, Student's t test and Mann–Whitney U test were used to evaluate differences in tumor and patient characteristics between training and validation cohorts. Receiver operating characteristic (ROC) curve analysis was performed using SPSS for Windows version 11.0 J software (SPSS, Chicago, IL). Values of $P<0.05$ were considered significant.

3. Results

3.1. Patient characteristics

Significant differences in age and sex of HCC patients were seen between the training and validation cohorts ($P=0.004$ and $P<0.0001$, respectively; Table 1). HCC patients in the validation cohort showed significantly fewer platelets, higher frequency of coexisting LC, and smaller tumors compared to the training cohort ($P=0.008$, $P=0.028$ and $P=0.006$, respectively; Table 1). Non-HCC patients in the validation cohort showed significantly fewer platelets in peripheral blood, higher frequency of coexisting LC, and higher AFP levels than patients in the training cohort ($P=0.012$, $P<0.0001$, and $P=0.041$, respectively; Table 1).

3.2. Training phase

Among the 9 markers tested (Table 2), *SPINT2* and *SRD5A2* displayed high specificities (98.2% and 92.9%) but low sensitivities (35.2% and 8.3%) for HCC detection. *RASSF1A* for HCC detection had the highest sensitivity (83.3%), but showed a low specificity of 58.9%. No markers showed a Youden's index (sensitivity + specificity − 1) >0.6 for HCC detection in our training cohort, suggesting limitations to the single use of each marker. To improve this low detection ability, we attempted to build a hybrid detector system by combining data from several markers. We calculated all combinations of markers *in silico* and found that an optimal hybrid detector built using a 4-marker combination (*SRD5A2*, *SPINT2*, AFP and PIVKA-II) achieved the highest sensitivity, specificity and accuracy (82.4%, 82.1% and 82.3%, respectively) in the training cohort among all combinations of markers (Fig. 2B). This optimal hybrid detector showed a higher Youden's index (0.65) than any of the 9 markers tested (Table 2). We also

Table 2
Sensitivity, specificity, and accuracy of 9 biomarkers and the hybrid system for diagnosis of HCC or small HCC in the training cohort.

	Sensitivity (%)	Specificity (%)	Accuracy (%)	Youden's index
Methylation markers (cut-off value)				
BASP1 (0.2 pg per 1-ml serum)	62.0	78.6	71.2	0.41
CCND2 (0.2 pg per 1-ml serum)	64.8	42.9	60.3	0.08
APC (0.2 pg per 1-ml serum)	17.6	78.6	40.4	< 0
SPINT2[a] (0.2 pg per 1-ml serum)	35.2	98.2	59.6	0.33
SRD5A2[a] (0.2 pg per 1-ml serum)	8.3	92.9	39.1	0.01
CFTR (0.2 pg per 1-ml serum)	56.5	83.9	69.2	0.40
RASSF1A (0.2 pg per 1-ml serum)	83.3	58.9	72.4	0.42
Classical protein markers				
AFP[a] (20 ng/ml)	57.4	85.7	67.1	0.43
PIVKA-II[a] (40 mAU/ml)	60.2	89.3	70.1	0.50
Four-marker combination (cut-off value)				
Optimal hybrid system (0)	82.4	82.1	82.3	0.65

[a] Four markers used in the optimal hybrid system.

examined the methylation levels of the 7 methylated genes in the three groups consisting of patients who underwent previously or undergo currently therapies of interferon (IFN) combined with ribavirin, and patients who had no therapies of IFN combined with ribavirin. No significant differences in the methylation levels were found between ribavirin and non-rivavirin therapies (data not shown).

3.3. Validation phase

The ability of the optimal hybrid detector to detect HCC was evaluated using 258 sera from 258 HCV-infected patients in the validation cohort. Notably, sensitivity of PIVKA-II for HCC detection decreased from 60.2% in the training cohort to 51.8% in the validation cohort (Fig. 3A). The specificity of AFP for HCC detection decreased from 85.7% in the training cohort to 71.9% in the validation cohort (Fig. 3B). By contrast, the optimal hybrid detector maintained high sensitivity (73.2%), specificity (87.7%), and accuracy (81.4%) for HCC detection in the validation cohort (Fig. 3A–C). The positive predictive value and negative predictive value for HCC detection were 82.2% and 80.8%, respectively. Even for the detection of small HCC in the validation cohort, the optimal hybrid detector showed high sensitivity (72.2%), specificity (87.7%), and accuracy (84.6%) (Fig. 3A–C). As a result, the optimal hybrid detector for detection of HCC and/or small HCC maintained a Youden's index ≥ 0.6 throughout both training and validation cohorts (Fig. 3D). The optimal hybrid detector also judged all of 4 healthy peoples as non-HCC (data not shown).

The present study arbitrarily determined cut-off values of AFP and PIVKA-II, and directly applied these values to the validation cohort. We therefore had to compare the ability of the optimal hybrid detector with the maximal abilities of AFP and PIVKA-II alone in the validation samples. For this purpose, ROC curve analysis for the detection of HCC was performed for the validation cohort. AFP and PIVKA-II alone had areas under the ROC curve of 0.739 (95% confidence interval (CI), 0.678-0.799) and 0.794 (95% CI, 0.736-0.853), respectively, for HCC detection (Fig. 4). The optimal hybrid detector had a more global area under the ROC curve of 0.868 (95% CI, 0.822-0.913) compared to AFP and PIVKA-II, indicating that ability of the optimal hybrid detector was superior to the maximal abilities of AFP and PIVKA-II alone for detecting HCC in the validation cohort.

As summarized in Table 3, *SPINT2* and *SRD5A2* showed the highest accuracy in detecting non-HCC patients with chronic hepatitis or cirrhosis. AFP was most robust in detecting small HCC and PIVKA-II was most robust in detecting HCC > 2 cm in diameter. Apparently, the optimal hybrid detector possessed all of individual merits of the 2 methylated markers, AFP and PIVKA-II.

In the present study, the cost per each test of AFP, PIVKA-2, SRD5A2 and SPINT2 was $4, $17.6, $11.7 and $10.6, respectively. In the validation group, the specificity and diagnostic accuracy of AFP alone and the hybrid detector were 71.9% and 67.0%, and 87.7% and 81.4%, respectively (Fig. 3). Thus, AFP test plus $40 resulted in an increase of 15.8% and 14.4% of specificity and diagnostic accuracy, respectively. The areas under ROC curves of AFP alone and the hybrid detector were 0.739 and 0.868, respectively (Fig. 4). AFP test plus $40 resulted in an increase of 0.129 of the area.

In diagnosing HCC, the performance of the combined blood test of *SPINT2*, *SRD5A2*, AFP and PIVKA-2 was superior to that of the methylation test of 3 genes (*RASSF1*, *CCND2* and *SPINT2*) in HCC tissue developed in our previous study [17].

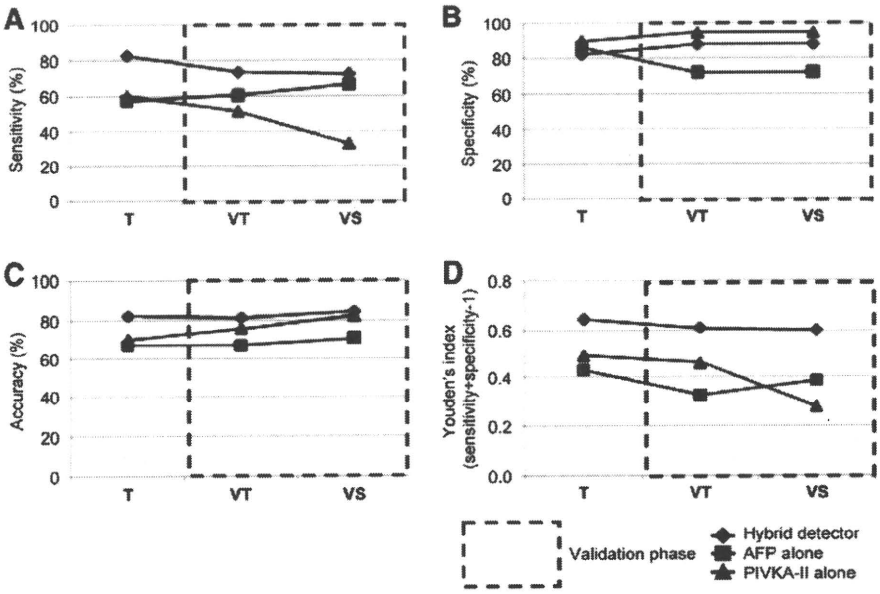

Fig. 3. Performances of the Optimal Hybrid detector (diamond), AFP (square), and PIVKA-II (triangle) in the validation cohort. The optimal hybrid detector showed the most robust performances for detection of HCC (A-D). T, training cohort of 108 HCC patients and 56 HCV carriers without HCC used for comparison with data from the validation cohort; VT, validation cohort of all 112 HCC patients and 146 HCV carriers without HCC; VS, validation cohort of 36 small HCC patients and 146 HCV carriers without HCC.

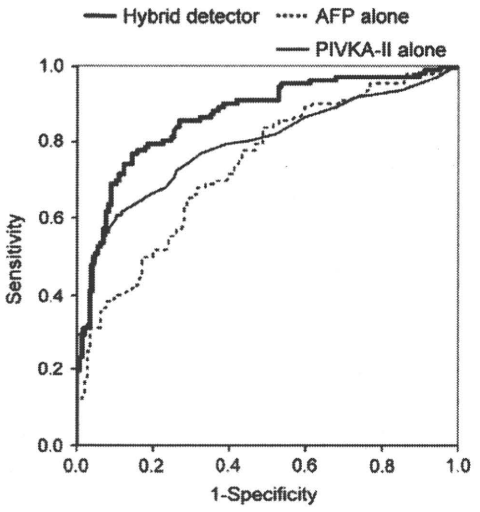

Fig. 4. Receiver operating characteristic curve analysis of the optimal hybrid detector, AFP, and PIVKA-II for the validation cohort.

4. Discussion

Many studies have evaluated AFP and PIVKA-II as detection tools for HCC, particularly small HCC. To the best of our knowledge, among studies using >100 samples, one study [23] showed a maximum sensitivity of 54.8%, but a specificity of only 49.1%, while another study [24] showed a maximum specificity of 71.0%, but a sensitivity of 25.0% in the ability of AFP to detect small HCC at a cut-off value of 20 ng/ml. A recent work by Marrero and colleagues showed that the optimal AFP cut-off value for diagnosis of HCC was 10.9 ng/ml leading to a sensitivity of 70% and a specificity of 82% [25]. However, the performance decreased to a sensitivity of 66% in diagnosing early HCC [25]. Another study showed that an AFP elevation (optimal cut-off value of 16 ng/ml) was indicative of HCC in non-infected patients, but not in HCV-infected patients [26]. For PIVKA-II, most studies with more than 100 samples showed sensitivities <40% for the detection of small HCC, with one study [27] reaching 53.5% sensitivity. Thus, reliance on the classical tumor markers AFP and PIVKA-II for the detection of HCC thus remains unsatisfactory, particularly given the low diagnostic powers and unstable cut-off values used between institutes [4,5,28]. To address these issues, we carefully conducted a multi-institutional study with multiple parameters, designed to develop a hybrid detector with more stable performance by searching for all combinations of marker candidates including methylated markers, as demonstrated previously by our laboratory [19]. The present study was also intended to minimize selection bias by using data collected consecutively only from HCV-infected patients [18,29]. We thus successfully developed a hybrid detector that accurately detected HCV-related HCC, particularly HCC≤2 cm in diameter, in a perfectly blinded manner in a multi-institutional large cohort.

Since the disclosure of epigenetic regulation in key genes, many studies [30–32] have shown the clinical efficacy of measuring promoter hypermethylation in various specimens such as tumor tissue, feces, and urine for determining the diagnosis and prognosis of cancer patients. Most studies measuring methylated DNA in the bloodstream of HCC patients have reported positive results, but almost all have been far from the setting of daily clinical use because of the insufficient performance due to the single use of a methylated marker gene [13–15,33]. We have provided herein the first evidence that a hybrid of methylation and classical protein markers has high potential for detecting HCV-related HCC in a blinded setting, opening new avenues toward the daily clinical application of methylated genes as tumor markers.

SPINT2 encodes hepatocyte growth factor (HGF) activator inhibitor type 2 (HAI-2) (http/www.ncbi.nlm.nih.gov/gene/10653), which regulates HGF activity. Epigenetic inactivation of *SPINT2* reportedly causes loss of tumor suppressor activity in renal cancer cells [34] and this gene is frequently hypermethylated in human HCC [12]. Consistent with those findings, our recent study [17] showed that *SPINT2* was frequently methylated in small HCC tissues, but unmethylated in non-HCC liver tissues, promising a high specificity for methylation patterns of *SPINT2* circulating in the bloodstream. *SRD5A2* encodes an enzyme that converts testosterone to the more active androgen dihydrotestosterone. Several polymorphisms in *SRD5A2* gene have been implicated as risk factors for prostate cancer [35]; however, how these polymorphisms act in the pathogenesis of HCC remains unclear.

We found that *RASSF1A*, *BASP1*, and *CCND2* offered more robust diagnostic performances than *SPINT2* and *SRD5A2* in the training phase. However, our *in silico* procedure predominantly selected the latter 2 genes for the optimal hybrid detector (Table 2). This result was consistent with our previous work [19,36], in which the diagnostic power of a detector built using several markers was independent of the ranking for diagnostic power of individual markers when combination was considered. In the validation phase, *SPINT2* and *SRD5A2* were very robust in detecting non-HCC patients, expectedly complementing the low detection ability of AFP and PIVKA-II (Table 3). Methylated *SPINT2* was also detectable in sera from 2 HCC cases negative for both AFP and PIVKA-II. This complementary effect is attributable to the absence of correlations between serum concentrations of AFP and PIVKA-II and those of methylated *SPINT2* and *SRD5A2* (data not shown). In addition to these independent expression patterns, our successful results might be partly attributable to a harmony of genetic features of *SPINT2* and *SRD5A2* and proteomic features of AFP and PIVKA-II. These features might maximize the synergistic power of the 4 markers.

The diagnostic accuracy of any test is related to the frequency of the underlying disease in the population being studied [4]. In the present study, many differences were seen between patient characteristics in the training and validation cohorts. In particular, the validation cohort included a significantly larger number of small HCCs than the training cohort ($P=0.006$; 36/112 vs. 22/108). This sample heterogeneity indeed resulted in decreased sensitivity of PIVKA-II alone and decreased specificity of AFP alone (Fig. 3A, B) for detecting small HCC in the validation cohort. The sensitivity and specificity of any test are inversely related. As a result, most studies have reported a Youden's index <0.5 for the diagnosis of small HCC. In contrast, our

Table 3
Diagnostic accuracy of markers and disease progression in the validation cohort.

Markers (cut-off value)	CH (%)	LC (%)	HCC≤2 cm (%)	HCC (2.1–5 cm) (%)	HCC>5 cm (%)	Total accuracy (%)
Optimal hybrid system (0)	65/68 (95.5)	63/78 (80.8)	26/36 (72.2)	48/67 (71.6)	8/9 (88.9)	210/258 (81.4)
SPINT2 (0.2 pg per 1-ml serum)	68/68 (100)	78/78 (100)	1/36 (2.78)	15/67 (22.4)	2/9 (22.2)	164/258 (63.6)
SRD5A2 (0.2 pg per 1-ml serum)	68/68 (100)	76/78 (97.4)	2/36 (5.56)	1/67 (1.50)	1/9 (11.1)	148/258 (57.4)
AFP (20 ng/ml)	60/68 (88.2)	45/78 (57.7)	24/36 (66.7)	37/67 (55.2)	7/9 (77.8)	173/258 (67.0)
PIVKA-II (40 mAU/ml)	67/68 (98.5)	71/78 (91.0)	12/36 (33.3)	40/67 (59.7)	7/9 (77.8)	197/258 (76.3)

CH, chronic hepatitis; LC, liver cirrhosis without HCC.
AFP, α-feto protein; PIVKA-II, prothrombin induced vitamin K Absence II.

hybrid detector showed markedly high performance (72.2% sensitivity, 87.7% specificity, 84.6% accuracy) and a Youden's index of approximately 0.6 for the detection of small HCC. The high accuracy of our hybrid detector in the present blinded, multi-institutional setting is thus fascinating from the perspective of screening for heterogeneous samples within or among various institutes.

We found that AFP test plus $40 resulted in increases of 15.8% and 14.4% of specificity and diagnostic accuracy, respectively. However, the cost-effectiveness of the hybrid detector in surveillance setting remains unclear; further studies are needed to clarify whether the hybrid detector we built could serve as a non-invasive and easy-to-use tool in surveillance programs for HCV-related HCC in the near future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.cca.2010.09.028.

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INTERNAL MEDICINE

CASE REPORT

Hepatocellular Carcinoma with a "nodule-in-nodule" Appearance Reflecting an Unusual Dilated Pseudoglandular Structure

Yusuke Ishida[1], Hiroaki Nagamatsu[2], Hironori Koga[1], Hiroshi Yoshida[2], Masamichi Kojiro[3] and Michio Sata[1]

Abstract

We present a case of hepatocellular carcinoma (HCC), showing an atypical "nodule-in-nodule" appearance on ultrasonogram (US). In general, a "nodule-in-nodule" appearance is found as a hyperechoic tumor containing a hypoechoic nodule. In the present case, however, there was a hyperechoic subnodule in the center of the tumor, which was surrounded by a hypoechoic tumor area. Histologically, the subnodule consisted of moderately differentiated HCC with a markedly dilated pseudoglandular structure, and the outer tumor consisted of well-differentiated HCC with a thin-trabecular pattern. It should be noted that there is a rare HCC with dilated pseudoglandular structure showing the inversed "nodule-in-nodule" appearance.

Key words: liver cancer, nodule-in-nodule, dedifferentiation, pseudogland, ultrasonography

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Introduction

Small hepatocellular carcinoma (HCC) is sometimes detected as a hyperechoic nodule by ultrasonography (1, 2). Most of these HCCs consist of well-differentiated cancerous tissues with fatty change (3, 4). During the dedifferentiation process, the well-differentiated HCC tissue with fatty change is often recognized as the outer rim of moderately differentiated HCC tissue, showing a hypo-in-hyper-echoic US finding, which is called the "nodule-in-nodule" appearance (4, 5). Here, we report a case with a hyper-in-hypo-echoic HCC, demonstrating an atypical "nodule-in-nodule" appearance caused by moderately differentiated cancerous tissue with markedly dilated pseudoglandular structure in the center of the tumor.

Case Report

A 51-year-old man was referred to our hospital for a further examination of a liver tumor detected at periodic follow-up of hepatitis C virus antibody-positive chronic hepatitis. Laboratory examination on admission revealed elevated values of PIVKA-II, serum alkaline phosphatase, and gamma glutamyl transpeptidase. Serum levels of α-fetoprotein, aspartate aminotransferase, and alanine aminotransferase, and total bilirubin were normal (Table 1). Ultrasonography (US) demonstrated a hypoechoic tumor, 24×34 mm in size, containing a hyperechoic subnodule (Fig. 1). T2-weighted magnetic resonance (MR) images showed a markedly hyperintense area in the center of the tumor; a finding consistent with the hyperechoic nodule seen on US (Fig. 2). On dynamic MR images, the inner part of the tumor was gradually and weakly enhanced by contrast media, followed by a slow washout. On the other hand, the outer part of the tumor was slightly enhanced only at the delayed phase (Fig. 3). He underwent a right hepatic lobectomy with a biopsy diagnosis of moderately differentiated HCC (Fig. 4). Grossly, the resected tumor, 27×30 mm in size, showed a "nodule-in-nodule" appearance (Fig. 5). Histologically, the inner subnodule consisted of moderately differentiated HCC with a markedly dilated pseudoglandular

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Table 1. Laboratory Findings on Admission

RBC	484 × 10⁴/mm³	PT	104.5%
Hb	15.5 g/dL	PT-INR	0.97
Ht	45.6%	Hyaluronic acid	84 ng/mL
WBC	2400 /mm³	$ICGR_{15}$	8.6%
Plt	33.0 × 10⁴/mm³	BUN	11.7 mg/dL
		Cr	0.56 mg/dL
T.B	0.4 mg/dL	Na	145 mEq/L
AST	36 U/L	K	4.2 mEq/L
ALT	41 U/L	Cl	102 mEq/L
LDH	245 U/L		
ALP	333 U/L	AFP	6.3 ng/mL
γGTP	198 U/L	PIVKA-II	97 mAU/mL
T.P	8.3 g/dL	CEA	2.9 ng/mL
Alb	4.4 g/dL	CA19-9	51.6 U/mL
T.cho	159 mg/dL	HBs Ag	(-)
		Anti-HCV Ab	(+)

structure. In contrast, the outer tumor consisted of well-differentiated HCC with a thin-trabecular pattern (Figs. 6-8).

Discussion

It is known that a "nodule-in-nodule" appearance in relatively small HCC reflects a dedifferentiation process from well-differentiated to moderately differentiated HCC, and it is frequently depicted on US imaging.

The "nodule-in-nodule" appearance is generally found as a hyperechoic tumor containing a hypoechoic nodule, and it reflects the development of moderately differentiated HCC without fatty change within a well-differentiated HCC showing diffuse fatty change. In the present case, however, moderately differentiated HCC was depicted as a hyperechoic

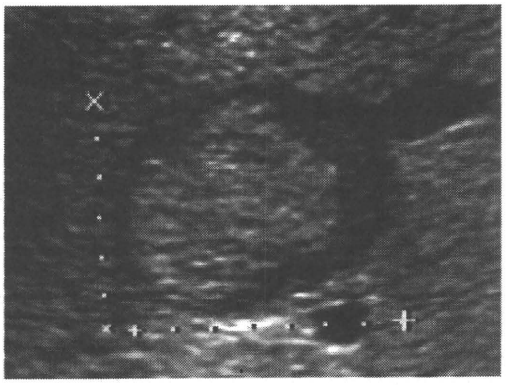

Figure 1. US image of the tumor.

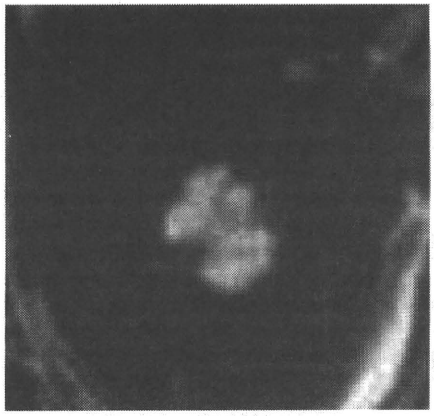

Figure 2. T2-weighted MR images for the tumor.

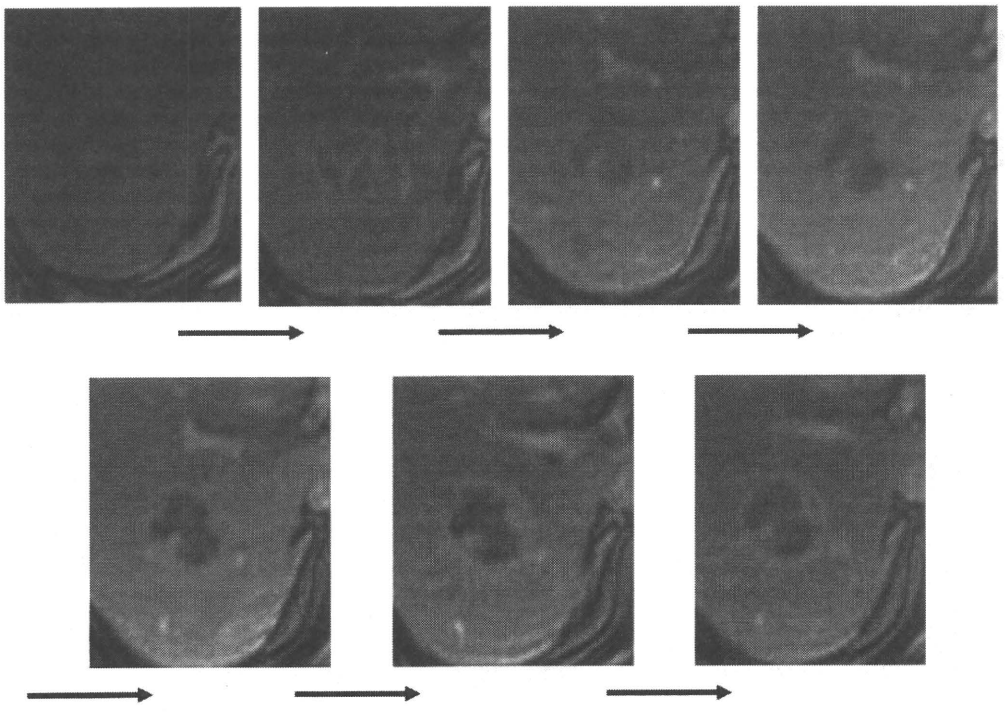

Figure 3. Dynamic MR study on the tumor.

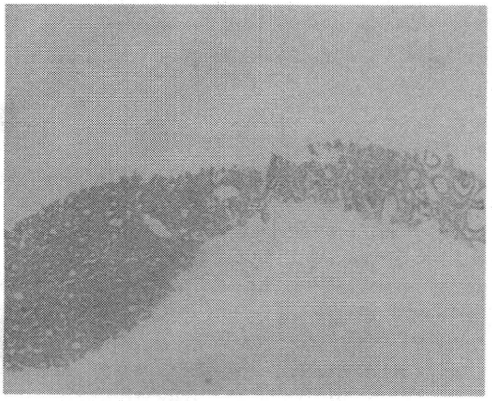

Figure 4. HCC tissue obtained by aspiration biopsy, showing the boundary between the two different structures of the tumor.

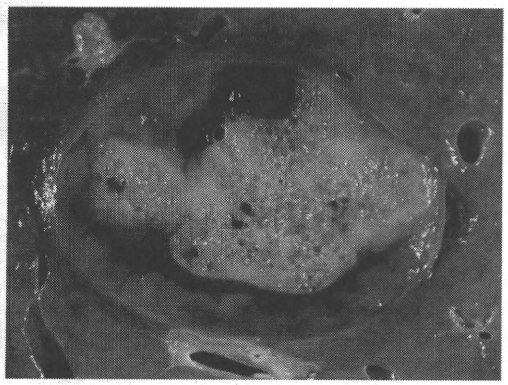

Figure 5. Gross appearance of the resected HCC nodule.

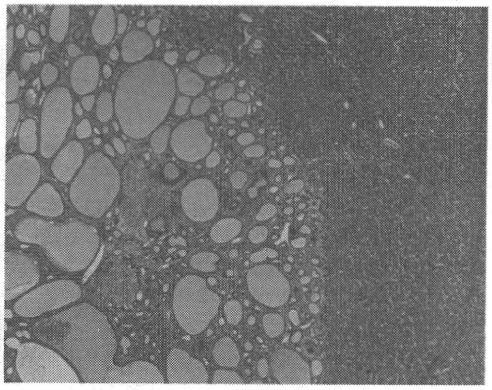

Figure 6. The boundary of the different histological grades of HCC (magnification: ×40).

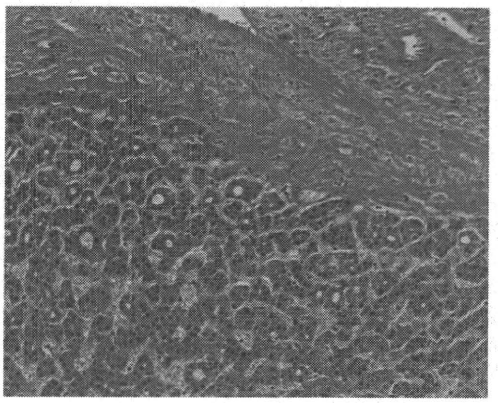

Figure 7. Well-differentiated HCC tissue (magnification: ×100).

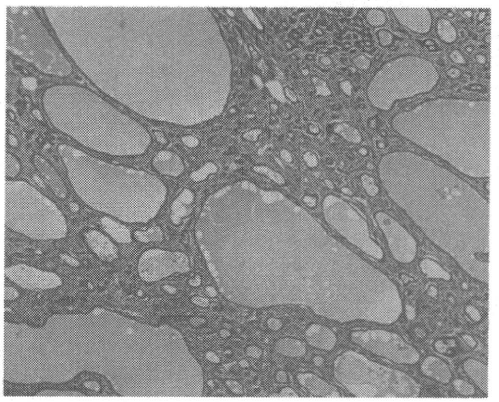

Figure 8. Moderately differentiated HCC tissue, showing the markedly dilated pseudoglands (magnification: ×100).

and hyperintense subnodule in the center of the tumor, and surrounded by a hypoechoic tumor area consisted of well-differentiated HCC without any fatty change. Possible causes for such atypical or inversed "nodule-in-nodule" appearance have not yet been fully discussed. A previous report demonstrated that the HCC hyperintensity on T2-weighted images is correlated with expansive growth, peliotic change, and hypervascularity (6), suggesting that the inner subnodule in the present case had different biological features. Indeed, the subnodule in our case showed a marked hyperintensity on T2-weighted images, but it was not clearly enhanced by contrast media. The unidentified substance within peliotic change-like dilated pseudoglands might contribute to the MR imaging findings seen in our case.

Although the precise mechanism of hyperechogenicity for the inner nodule in this case remained unclear, it is noteworthy that hyaline cast deposition within the collecting tubules in the kidney of the newborns accounts for the US finding of hyperechoic renal pyramids (7). Thus, it was possible that the hyperechogenicity of the inner nodule reflected the pinkish substance reminiscent of an amorphous hyaline in the dilated pseudoglands. The inner substance was also described as colloid-like material (8). Indeed, the large pseudoglandular structure resembles thyroid follicles, and the thyroid adenoma with colloid type follicles is often demonstrated as hyperechoic (9). From these points of view, it is speculated that the degree of hyperintensity and hyperechogenicity may, at least in part, be dependent on not only the size of the peliotic change-like dilated pseudoglands but also the nature of substances within the pseudoglands.

When we encounter HCC, featuring an atypical or inversed "nodule-in-nodule" appearance, we should pay care-

ful attention to its unique pathological architecture in addition to the commonly-seen fatty change.

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Microvascular Invasion in Patients with Hepatocellular Carcinoma and Its Predictable Clinicopathological Factors

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Background: Macroscopic vascular invasion is known to be a poor prognostic factor in hepatocellular carcinoma (HCC). The aim of this study was to determine the outcomes and predictive factors after hepatic resection for HCC with microvascular invasion (MVI).

Methods: One hundred ten patients who underwent curative resection for HCC without macroscopic vascular invasion were included in this retrospective study. The risk factors of these patients for recurrence-free and disease-specific survival were investigated, and the clinicopathological factors predicting the presence of MVI were also determined.

Results: Of the 110 resected specimens, 49 (45%) had evidence of MVI. By univariate analysis, MVI was found to be statistically significantly associated with greater tumor size, gross classification, histological grade, and intrahepatic micrometastasis. Gross classification proved to be the only independent predictive factor for MVI by multiple logistic regression analysis. By multivariate analysis, cirrhosis and MVI were identified as independent risk factors for recurrence-free survival. The 5-year recurrence-free survival rates for patients with and without MVI were 20.8% and 52.6%, respectively. By multivariate analysis, the number of tumors, presence of MVI, and intrahepatic micrometastasis were identified as independent predictors of disease-specific survival. The 5-year disease-specific survival rates for patients with and without MVI were 59.3% and 92.0%, respectively.

Conclusions: The presence of MVI was the most important risk factor affecting recurrence and survival in HCC patients after curative resection. Furthermore, this study showed that gross classification of HCC can be very helpful in predicting the presence of MVI.

Key Words: Hepatocellular carcinoma—Microvascular invasion—Gross classification—Hepatic resection—Recurrence—Survival.

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. Recent advances in imaging procedures have led to increased detection of early-stage HCC and improved survival because of the greater number of patients identified in which curative hepatic resection is possible.[1,2] However, the long-term survival rate is still unsatisfactory as a result of the high rate of intra- and extrahepatic recurrences.[3,4] Tumor invasion in the portal and/or hepatic vein is associated with increased risk leading to early recurrences of HCC.[5–9] Moreover, the fatal recurrence of HCC with vascular invasion limits additional attempts at

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various curative therapies, such as hepatic resection, percutaneous ethanol injection, microwave coagulation therapy, and radiofrequency ablation, thereby contributing to poor survival.[10] Macroscopic vascular invasion, such as a tumor thrombus in the major portal vein, is known to be the crucial survival risk factor after resection or liver transplantation for HCC.[11–14] A previous study of natural history of HCC with macroscopic vascular invasion has shown a patient median survival time of 2.7 months.[15]

Microvascular invasion (MVI) is difficult to detect before treatment of HCC is undertaken, even if recent superior imaging procedures are used during patient evaluation. Although several studies have suggested that the presence of MVI is an independent factor predictive of poor survival after resection of HCC,[16–18] the significance of MVI is still unclear. The aim of this study was to evaluate whether the MVI of HCC is associated with tumor recurrence and affects patient long-term survival after curative resection, and to identify preoperative predictors of MVI.

PATIENTS AND METHODS

Patients

Between January 1995 and December 2005, 142 patients were diagnosed and underwent hepatic resection for HCC at the Kurume University School of Medicine. Patient inclusion criteria for this study were as follows: (1) patients with a single tumor up to 5 cm or three or fewer tumors each up to 3 cm, (2) patients without radiological evidence of macroscopic portal and hepatic vein tumor invasion, (3) patients without extrahepatic metastasis, and (4) patients who underwent curative hepatic resection defined as the removal of all macroscopic residual tumors. Of these 142 patients, 110 patients met these criteria and were retrospectively included in this study. There were 90 male (82%) and 20 female patients, with a mean age of 62.6 ± 10.5 years. With respect to viral markers, 74 patients (67%) were positive for hepatitis C virus (HCV) antibody, 27 patients (25%) were positive for hepatitis B surface antigen, and 9 patients were negative for both markers. Liver cirrhosis was present in 53 patients (48%). The mean maximum tumor size was 28.1 ± 9.6 mm, and 85 patients (77%) had a solitary tumor. In the surgical procedures, lobectomy, segmentectomy, and subsegmentectomy are anatomic resections of Couinaud's segment classification and were performed in 13, 50, and 22 patients, respectively. Partial hepatectomy is a nonanatomic resection consisting of less than subsegmentectomy and was performed in 25 patients.

Preoperative Evaluation

In 21 of 110 patients, preoperative diagnosis of HCC was histologically confirmed by needle biopsy under ultrasonographic guidance. In the remaining 89 patients, HCC was diagnosed on the basis of the findings of typical radiological features on ultrasonography, contrast-enhanced dynamic computed tomography (CT), and magnetic resonance imaging (MRI), along with high alfa-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) levels. The preoperative hepatic functional reserve was evaluated by the Child-Pugh scoring system[19] and the 15-minute retention rate for indocyanine green. Tumor size was determined on the basis of the largest dimension observed on ultrasonography and CT.

Histological Criteria

MVI was defined as microscopic tumor invasion identified in portal or hepatic vein of the surrounding liver tissue, which was contiguous to the tumor. Intrahepatic micrometastasis (IM) was defined as satellite micronodule in the surrounding liver tissue, which was isolated from the main tumor. Tumor differentiation was histologically graded according to the classification of the Liver Cancer Study Group of Japan.[20] Gross classification of nodular type HCC was based primarily on the definition of Kanai et al.[21] and the Liver Cancer Study Group of Japan[20]: vaguely nodular type (VN type, nodule has distinct margins and contains portal tracts), single nodular type (SN type, round nodule with clear demarcation), single nodular with extranodular growth type (SNEG type, similar to SN type but showing extranodular growth), and confluent multinodular type (CMN type, a nodule formed by a cluster of small and confluent nodules). The tumors of the 110 patients were classified grossly as VN type (3 tumors, 2.7%), SN type (59 tumors, 53.6%), SNEG type (29 tumors, 26.4%), and CMN type (19 tumors, 17.3%).

Patient Follow-up

After surgical resection, each patient was followed carefully. Serum biochemistries, AFP levels,

TABLE 1. *Comparison of patients' characteristics based on microvascular invasion (MVI)*

Characteristic	MVI		*P* value
	Present (n = 49)	Absent (n = 61)	
Sex (M/F)	40/9	50/11	.964
Age (y)	61.5 ± 11.3	63.4 ± 9.87	.336
Cause (HCV/HBV/both negative)	26/23	31/30	.579
Background liver (normal or CH/cirrhosis)	26/23	31/30	.815
Bilirubin (mg/dL)	.8 ± .4	.9 ± .4	.302
Albumin (g/dL)	4.0 ± .4	3.9 ± .4	.286
Prothrombin time (%)	91.5 ± 11.8	88.0 ± 12.7	.149
Prothrombin time (%)	17.6 ± 10.9	19.5 ± 10.9	.333
AFP (ng/mL) (≤100/>100)	28/21	43/18	.210
DCP (AU/mL) (≤100/>100)	29/20	45/16	.156
Maximum tumor size (mm)	30.8 ± 9.56	26.0 ± 9.2	.009
No. tumors (single/≥2)	36/13	49/12	.394
Gross classification (VN + SN/SNEG + CMN)	12/37	50/11	<.0001
Histological grade (well/moderate/poor)	15/34	2/59	.033
Intrahepatic micrometastasis (present/absent)	15/34	2/59	<.0001
Cell encapsulation (present/absent)	35/14	42/19	.770
Type of surgical resection (lobectomy + segmentectomy/subsegmentectomy + partial hepatectomy)	27/22	36/35	.637

HCV, hepatitis C virus; HBV, hepatitis B virus; CH, chronic hepatitis; $ICGR_{15}$, indocyanine green retention at 15 minutes; AFP, alfa-fetoprotein; DCP, des-gamma-carboxy prothrombin; VN, vaguely nodular type; SN, single nodular type; SNEG, single nodular with extranodular growth type; CMN, confluent multinodular type.

DCP levels, and Child-Pugh scores were measured, and ultrasonography was performed monthly. Contrast-enhanced dynamic CT was performed every 3 months until 6 months after treatment and every 6 months thereafter. MRI was performed as a supplemental examination. The closing date of this study was December 2006 or the date of the patient's death. Follow-up ranged from 8 to 130 months (median, 47 months). Of the 110 patients, 27 patients died during follow-up. Of these 27 patients, 23 patients died from HCC-related causes.

Statistical Analysis

All data are expressed as mean ± standard deviation. Comparisons between the two groups were performed by the Mann-Whitney *U*-test for continuous variables, and the χ^2 test or the Fisher exact test for discrete variables. The multiple logistic regression model was used to identify factors related to MVI. Survival curves were constructed by the Kaplan-Meier method, and curves were compared by the log rank test. A Cox proportional hazard stepwise model was used for univariate and multivariate analysis to identify any independent variables that were related to survival. All *P* values were two-tailed, and a level of <.05 was considered to be statistically significant. Statistical analysis was performed by SPSS software (SPSS, Chicago, IL).

TABLE 2. *Independent predictor of microvascular invasion*

Predictor	HR (95% CI)	*P* value
AFP (ng/mL) (>100)	1.25 (.44–3.57)	.679
DCP (AU/mL) (>100)	1.31 (.45–3.86)	.621
Maximum tumor size (mm) (>30)	1.77 (.57–5.43)	.321
No. of tumors (≥2)	2.19 (.67–7.18)	.195
Gross classification (SNEG + CMN)	11.81 (3.93–37.80)	<.0001
Histological grade (poor)	2.31 (.38–14.14)	.364
Intrahepatic micrometastasis (present)	3.32 (.58–18.97)	.178
Cell encapsulation (absent)	1.73 (.57–5.26)	.337

HR, hazard ratio; 95% CI, 95% confidence interval; AFP, alfa-fetoprotein; DCP, des-gamma-carboxy prothrombin; SNEG, single nodular with extranodular growth type; CMN, confluent multinodular type.

RESULTS

Clinicopathological Characteristics Predictive of MVI

Of the 110 resected specimens, 49 (45%) had evidence of MVI. Comparisons of patient clinicopathological characteristics according to presence or absence of MVI are shown in Table 1. Baseline variables, including age, sex, etiology, background liver function, and serum AFP and DCP levels, were similar in both groups. In addition, there was no difference in surgical procedures between the two groups.

Of the pathological features, the present of cell encapsulation and the number of tumors were not

TABLE 3. *Univariate and multivariate analyses of recurrence-free survival for hepatocellular carcinoma*

Characteristic	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Sex (M)	.90 (.46–1.79)	.770		
Age (>65 years)	1.17 (.70–1.96)	.540		
HCV (positive)	1.15 (.66–1.98)	.622		
HBV (positive)	.96 (.54–1.73)	.902		
Background liver (cirrhosis)	1.65 (.99–2.76)	.056	1.79 (1.05–3.06)	.032
$ICGR_{15}$ (%)	1.26 (.75–2.13)	.380		
AFP (ng/mL) (>100)	1.17 (.69–2.21)	.556		
DCP (AU/mL) (>100)	1.28 (.75–2.19)	.359		
Maximum tumor size (mm) (>30)	1.28 (.76–2.15)	.362		
No. of tumors (≥2)	1.79 (1.02–3.16)	.044		
Gross classification (SNEG + CMN)	1.98 (1.19–3.32)	.009		
Histological grade (poor)	1.23 (.56–2.72)	.601		
Microvascular invasion (present)	2.75 (1.62–4.67)	.0002	2.97 (1.69–5.24)	.0002
Intrahepatic micrometastasis (present)	2.14 (1.13–4.04)	.020		
Cell encapsulation (absent)	1.02 (.59–1.74)	.958		
Type of surgical resection (subsegmentectomy + partial hepatectomy)	1.59 (.95–2.65)	.076		

HR, Hazard ratio; 95% CI, 95% confidence interval; HCV, hepatitis C virus; HBV, hepatitis B virus; CH, chronic hepatitis; $ICGR_{15}$, indocyanine green retention at 15 minutes; AFP, alfa-fetoprotein; DCP, des-gamma-carboxy prothrombin; SNEG, single nodular with extranodular growth type; CMN, confluent multinodular type.

significantly associated with MVI. The maximum tumor size of patients with MVI was larger than those without MVI ($P = .009$). None of the patients with well-differentiated HCC had MVI, in contrast to the 41 (43%) and 8 (73%) patients with moderately and poorly differentiated HCC, respectively ($P = .033$). IM was evident in 17 (15%) of the 110 patients enrolled, and the presence of IM was significantly associated with MVI ($P < .0001$). In addition, the gross classifications of SNEG type (21 tumors, 72%) and CMN type (16 tumors, 84%) showed MVI more frequently than those of VN type (0%) and SN type (12 tumors, 20%) ($P < .0001$).

Multiple logistic regression analysis was performed to identify which of eight variables was independently associated with MVI (Table 2). The gross classifications of SNEG and CMN types were independent predictors of MVI (hazard ratio [HR], 11.81; 95% confidence interval [95% CI], 3.93–37.80; $P < .0001$).

Predictors of Recurrence-Free Survival After Hepatic Resection

Cox proportional hazard regression analysis was performed to identify independent predictors of recurrence-free survival (Table 3). The results of univariate analysis show that number of tumors (two or more; $P = .044$), gross classification (SNEG + CMN type; $P = .009$), presence of MVI ($P = .0002$), and presence of IM ($P = .02$) were found to be significant risk factors affecting recurrence-free survival. By multivariate analysis, liver

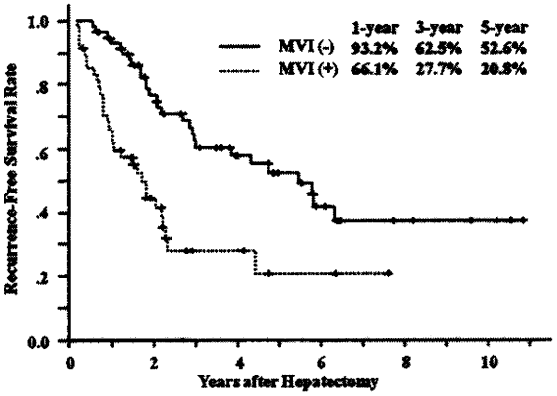

FIG. 1. Comparison of recurrence-free survival rates in patients with and without microvascular invasion (MVI). The 1-, 3-, and 5-year recurrence-free survival rates are shown. The recurrence-free survival of patients with MVI was significantly shorter compared with survival of patients without MVI ($P = .0001$).

cirrhosis (HR, 1.79; 95% CI, 1.05–3.06; $P = .032$) and presence of MVI (HR, 2.97; 95% CI, 1.69–5.24; $P = .0002$) were identified as independent predictors of recurrence-free survival. Recurrence-free survival curves of patients with and without MVI are shown in Fig. 1. The recurrence-free survival of patients with MVI was significantly shorter compared with survival of patients without MVI ($P = .0001$). During the follow-up period, tumor recurrence developed in 59 patients (54%), consisting of 31 patients (63%) with MVI and 28 patients (46%) without MVI. As the pattern of recurrence in 31 patients with MVI, 24 patients (77%) showed intrahepatic recurrence near

TABLE 4. *Univariate and multivariate analyses of disease-specific survival for hepatocellular carcinoma*

Characteristic	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Sex (M)	1.08 (.39–2.95)	.885		
Age (>65 years)	.95 (.42–2.16)	.902		
HCV (positive)	1.07 (.45–2.52)	.881		
HBV (positive)	1.53 (.62–3.77)	.356		
Background liver (cirrhosis)	1.97 (.83–4.67)	.126		
$ICGR_{15}$ (%)	1.35 (.57–3.20)	.495		
AFP (ng/mL) (>100)	2.19 (.96–4.97)	.061		
DCP (AU/mL) (>100)	1.10 (.46–2.60)	.835		
Maximum tumor size (mm) (>30)	1.24 (.54–2.83)	.613	2.93 (1.03–8.29)	
No. of tumors (≥2)	2.19 (.89–5.40)	.088		.043
Gross classification (SNEG + CMN)	2.99 (1.23–7.26)	.016		
Histological grade (poor)	2.09 (.61–7.22)	.243		
Microvascular invasion (present)	3.88 (1.65–9.13)	.002	3.51 (1.27–9.74)	.016
Intrahepatic micrometastasis (present)	3.53 (1.36–9.16)	.009	3.28 (1.04–10.34)	.043
Cell encapsulation (absent)	1.00 (.41–2.47)	.997		
Type of surgical resection (subsegmentectomy + partial hepatectomy)	1.31 (.57–3.03)	.531		

HR, hazard ratio; 95% CI, 95% confidence interval; HCV, hepatitis C virus; HBV, hepatitis B virus; CH, chronic hepatitis; $ICGR_{15}$, indocyanine green retention at 15 minutes; AFP, alfa-fetoprotein; DCP, des-gamma-carboxy prothrombin; SNEG, single nodular with extranodular growth type; CMN, confluent multinodular type.

to the resected tumor or in the same lobe of the resected tumor, and four patients (13%) had extrahepatic recurrence (two peritoneum, one lung, and one bone).

Predictors of Disease-Specific Survival After Hepatic Resection

Cox proportional hazard regression analysis was performed to identify independent predictors of recurrence-free survival (Table 4). The results of univariate analysis show that gross classifications (SNEG + CMN types; $P = .016$), presence of MVI ($P = .002$), and presence of IM ($P = .009$) were found to be significant risk factors affecting disease-specific survival. By multivariate analysis, number of tumors (two or more; HR, 2.93; 95% CI, 1.03–8.29; $P = .043$), presence of MVI (HR, 3.51; 95% CI, 1.27–9.74; $P = .016$), and presence of IM (HR, 3.28; 95% CI, 1.04–10.34; $P = .043$) were identified as independent predictors of disease-specific survival. Disease-specific survival curves of patients with and without MVI are shown in Fig. 2. The short-term survival of patients with MVI was significantly shorter compared with those of patients without MVI ($P = .001$).

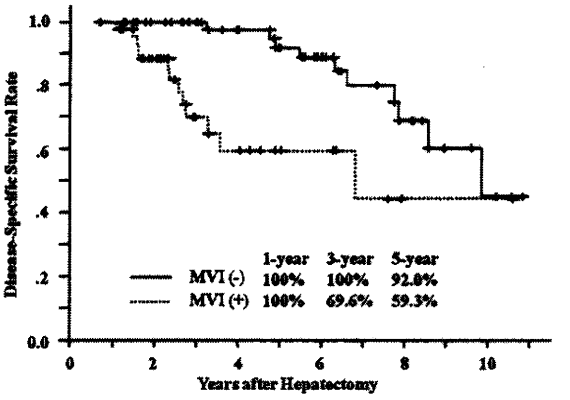

FIG. 2. Comparison of disease-specific survival rates in patients with and without microvascular invasion (MVI). The 1-, 3-, and 5-year disease-specific survival rates are shown. The short-term survival of patients with MVI was significantly shorter compared with those of patients without MVI ($P = .001$).

DISCUSSION

In this study, we show that the presence of MVI is the most statistically significant independent risk factor affecting recurrence-free and disease-specific survival in patients with HCC after curative resection. Therefore, our results suggest that when determining a treatment plan for a patient with HCC, it is important to detect the presence of MVI as soon as possible before hepatic resection. However, it is difficult to diagnose MVI even with the most recent diagnostic imaging procedures, such as ultrasonography, CT, and MRI because it is a histopathological finding.

Several studies of HCC patients who underwent orthotopic liver transplantation have shown that MVI was strongly associated with tumor size and histological grade.[22,23] Adachi et al.[24] reported that tumors >3 cm and high histological grades were

strong predictors of microscopic portal venous invasion by histological analysis of resected HCC. Similarly, in our study, univariate analysis showed that tumor size and histological grade were associated with MVI. However, in the small HCCs (<3 cm) that are frequently detected by recent advanced imaging procedures, it is difficult to predict the presence of MVI by imaging findings before hepatic resection.

We showed by univariate and multivariate analyses that gross classification of HCC was a strongly independent predictor of MVI. Kanai et al.[21] proposed a new gross classification that divides the Eggel's nodular type HCC into the three groups on the basis of patterns of tumor growth and extension, as follows: single nodular type (type 1), single nodular with extranodular growth type (type 2), and confluent multinodular type (type 3). This classification of nodular type HCC has been accepted by the Liver Cancer Study Group of Japan[20] and is widely used in Japan. As to the incidence of portal vein invasion and intrahepatic metastasis in HCC, several authors have reported that these frequencies were lower in type 1 tumors than in type 2 and 3 tumors. Kanai et al.[21] reported on histopathological findings that type 2 and type 3 tumors exhibit cancer cells invading peripheral nontumoral hepatocytes in a replacement growth pattern, whereas type 1 tumors show a clear boundary between tumor and nontumoral parenchyma in an expanding growth pattern. Moreover, single nodular type HCC has a subcategory defined as vaguely nodular type by the Liver Cancer Study Group of Japan,[20] which is characterized as early HCC. Nakashima et al.[25] reported that vaguely nodular type tumors had small diameters, most of them consisted solely of well-differentiated HCC tissues, and did not have portal vein invasion or intrahepatic metastasis. Similarly, in this study, all three cases of VN type HCC were histologically well-differentiated HCC and did not have MVI. Thus, our results supported the correlation between MVI and gross classification of HCC. Gross classification can be mostly determined by combining preoperative imaging modalities such as contrast-enhanced ultrasonography and dynamic or angio-CT. Therefore, gross classification may be helpful in predicting MVI even if the tumor is small.

Although surgical resection has been one of the mainstays in the curative treatment for HCC, the incidence of fatal recurrence is high.[3,9] After curative resection for HCC, two patterns of recurrence are primarily observed: multicentric occurrence and intrahepatic metastasis. Poon et al.[5] reported that early recurrences seem to arise mainly from intrahepatic metastasis, whereas late recurrences are more likely to be multicentric in origin. In addition, they showed by multivariate analysis that vascular invasion is an independent risk factor for early recurrence, and cirrhosis is an important risk factor for late recurrence. Similarly, in this study, cirrhosis and MVI were independent risk factors associated with disease-free survival after curative resection of HCC.

The presence of vascular invasion is consistently reported as strongly predictive of intrahepatic metastasis,[26,27] as also shown in this study. Moreover, our results showed that intrahepatic micrometastases occurred in approximately 31% of HCC patients with MVI. These findings suggest that cancer cell spreading via the portal vein is main mechanism for such intrahepatic metastasis. Several other authors have also demonstrated that early recurrence after curative resection is observed with high frequency in HCC with MVI.[6–8,18,28] Therefore, before treating a patient with newly diagnosed HCC, it is important to predict whether MVI and IM are present because of their highly malignant potential.

Irrespective of factors such as MVI in the resected primary tumor, multicentric occurrence of HCC is also caused by the background condition of the liver. Cirrhosis, in which premalignant lesions such as adenomatous hyperplasia frequently occur, is known to have high carcinogenic potential.[29,30] In addition, previous studies have shown that the incidence of multicentric HCC is higher in liver cirrhosis patients with HCV infection than in cirrhosis patients with other factors, including hepatitis B virus infection.[31,32] Our study included 74 patients (67%) with HCV infection.

In this study, patients with MVI experienced disease recurrence far more frequently after hepatic resection for HCC than patients without MVI. However, it has been suggested that our results may include detection bias: it is possible that both the factors of multicentric occurrence and intrahepatic metastasis caused the recurrence of HCC.

It has been generally known that recurrence of HCC is the major risk factor affecting survival after hepatic resection.[33,34] Especially, it is thought that the survival in the HCC patients with early recurrence is influenced by primary tumor factors that affect intrahepatic metastasis.[5,35] In this study, factors with high malignant potential, such as number of tumors, IM, and MVI, were independent risk factors affecting survival. Moreover, among these factors, the presence of MVI was found to be the most strongly prognostic factor. Several authors have reported an association between MVI and survival,[16,17] and

Kondo et al.[18] demonstrated that MVI was a statistically significant predictor for early recurrence and early death within 2 years after hepatectomy in patients with a solitary HCC. Similarly, in this study, the 3-year recurrence-free and disease-specific survivals of patients with MVI were far shorter compared with those of patients without MVI. Thus, the presence of MVI may play an important role in short-term survival after hepatic resection.

In conclusion, our study found that the presence of MVI was the strongest predictor for recurrence and survival in HCC patients after curative resection. This result suggests that it is important to predict the presence of MVI before hepatic resection for help in determining treatment strategies. Furthermore, gross classification of HCC can be mostly helpful in predicting the presence of MVI.

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ORIGINAL ARTICLE

Fucosylated Fraction of Alpha-Fetoprotein as a Predictor of Prognosis in Patients with Hepatocellular Carcinoma After Curative Treatment

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Abstract
Aim The aim of this study was to evaluate the clinical usefulness of measuring the *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3) for prognostic predictor in patients with hepatocellular carcinoma (HCC).
Methods A total of 477 HCC patients who underwent percutaneous ablative therapy or hepatectomy were enrolled. Overall survival and recurrence-free survival were respectively evaluated retrospectively and prospectively. Multivariate analyses of clinical prognostic factors were performed by Cox's stepwise proportional hazard model.
Results AFP-L3 status was a statistically significant independent prognostic factor of long-term survival ($P = 0.013$) and recurrence-free survival ($P = 0.006$) in patients who underwent percutaneous ablative therapy. In contrast, AFP-L3 did not affect prognosis in patients who underwent hepatectomy.
Conclusions AFP-L3 had different impacts on prognosis in patients with HCC who underwent percutaneous ablative therapy and hepatectomy. Our results suggest that AFP-L3 positivity (≥15%) might be a promising indicator for choosing therapeutic modalities in HCC patients.

Keywords Alpha-fetoprotein · AFP-L3 · DCP (des-γ-carboxy prothrombin) · Hepatocellular carcinoma · Prognostic factor

Introduction

Hepatectomy is a generally accepted method that improves the long-term outcome in patients with hepatocellular carcinoma (HCC) [1]. However, patients with HCC frequently have coexisting liver cirrhosis with impaired hepatic functional reserve, and this may prevent surgical intervention. On the other hand, percutaneous ablative therapies, including percutaneous ethanol injection (PEI), microwave coagulation therapy (MCT), and percutaneous radiofrequency ablation (RFA), have been developed and applied as alternative therapeutic options in cases of small HCC [2–8]. Recently, RFA has been performed as a first-line therapeutic option for early stage HCC; its survival outcomes are similar to those of hepatectomy [6–8]. However, a method for making the correct choice among therapeutic modalities to suit individual patients with early stage HCC remains to be determined.

The *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3) has been reported to be a specific marker for HCC [9–11]. Moreover, its level predicts the

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