only. For patients with this risk factor, treatment modalities with potential of more curative intent, such as RFA combined with TACE[13] or hepatic resection might have to be selected if possible. A randomized controlled trial might be necessary to solve this issue.

To prevent late recurrence, therapeutic approaches effective at suppressing multicentric occurrence such as polyprenoic acid and interferon (IFN) therapy may be indicated in patients with cirrhotic liver.[29–31] Of 88 patients who underwent RFA, 79 were hepatitis C virus-positive, 21 of whom received IFN therapy. Of these, a sustained virological response was achieved in five. Because the number of cases is small, the effect of IFN therapy could not be analyzed. Our policy is to evaluate for the complete ablation after RFA and to implement rigorous CT and US surveillance. On this basis, effective treatment modalities (hepatic resection, repeated RFA, or TACE) can be considered as early as possible before recurrent tumor progression.

A total of five complications (5.7% per treatment, 3.9% per session) were observed during the follow-up period, but none of these was major or required the cessation of therapy.

In conclusion, under our RFA protocol percutaneous RFA is considered a reliable treatment for small HCC in terms of therapeutic efficacy and safety. Although the present study has some limitations, such as the small number of patients and retrospective design, our results demonstrate that percutaneous RFA can be used successfully as first-line treatment for small HCC. In addition, we also demonstrated that early and late intrahepatic recurrence after RFA of HCC were associated with prognosis. These findings may suggest a need for different strategies in the prevention and management of early and late intrahepatic recurrence.

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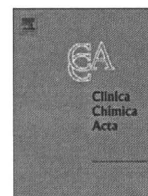
# Efficient detection of hepatocellular carcinoma by a hybrid blood test of epigenetic and classical protein markers

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## ARTICLE INFO

## 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].

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 pro-thrombin-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



**Training phase**

1) Measurement of AFP, PIVKA-II, and 7 methylated genes (*APC*, *RASSF1A*, *SRD5A2*, *BASP1*, *SPINT2*, *CCND2*, and *CFTR*).

2) Determination of an optimal combination of serum biomarkers (n=2∼9).

3) Construction of an optimal hybrid detector.

★ **164 serum samples obtained between May 1998 and April 2006.** All samples were obtained at Yamaguchi University Hospital.

**Validation phase**

1) Measurement of AFP, PIVKA-II, and 2 methylated genes (*SRD5A2* and *SPINT2*).

2) Assessment of diagnostic performance of the optimal hybrid detector determined.

**Total 258 serum samples obtained between May 2006 and April 2008.**

★ 77 serum samples obtained at Yamaguchi University Hospital.

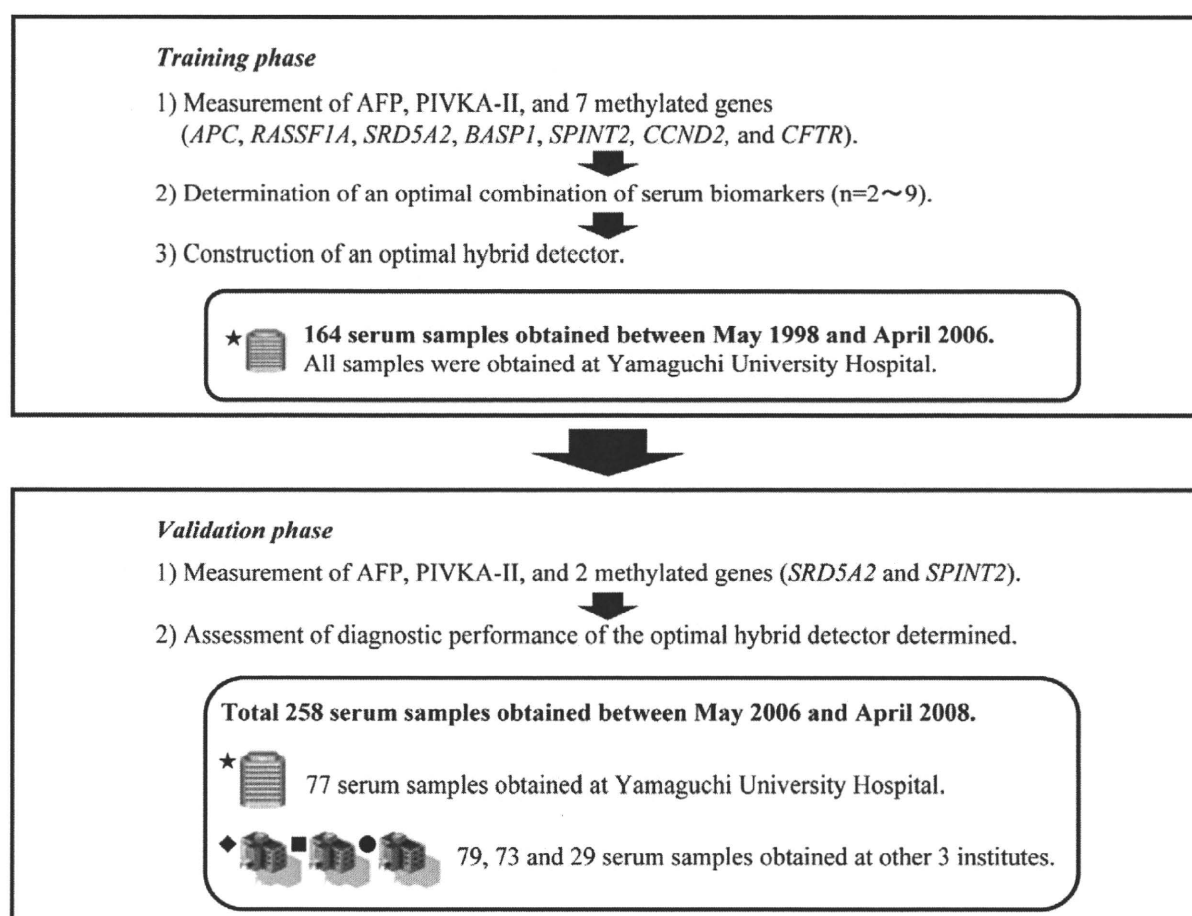◆ 79, 73 and 29 serum samples obtained at other 3 institutes.

**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/mm³) (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 amino-transferase (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).
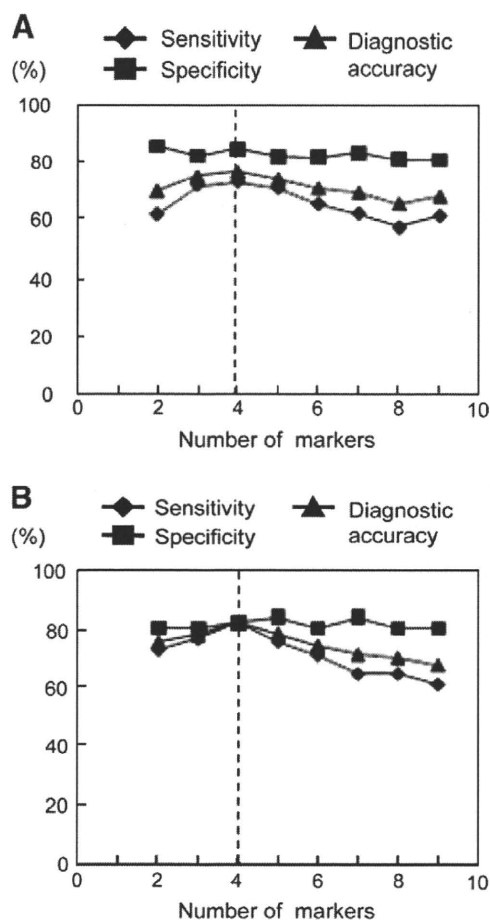


Fig. 2. Performances of markers in the training cohort. We input data for $n$ markers ($n = 2$-9) of the 164 training samples into Fisher linear classifier (FLC). This procedure was repeated for all combinations (from 9 C2 to 9 C9) of $n$ markers and performances of the constructed individual FLCs were 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

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

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 $\chi^2$ 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].
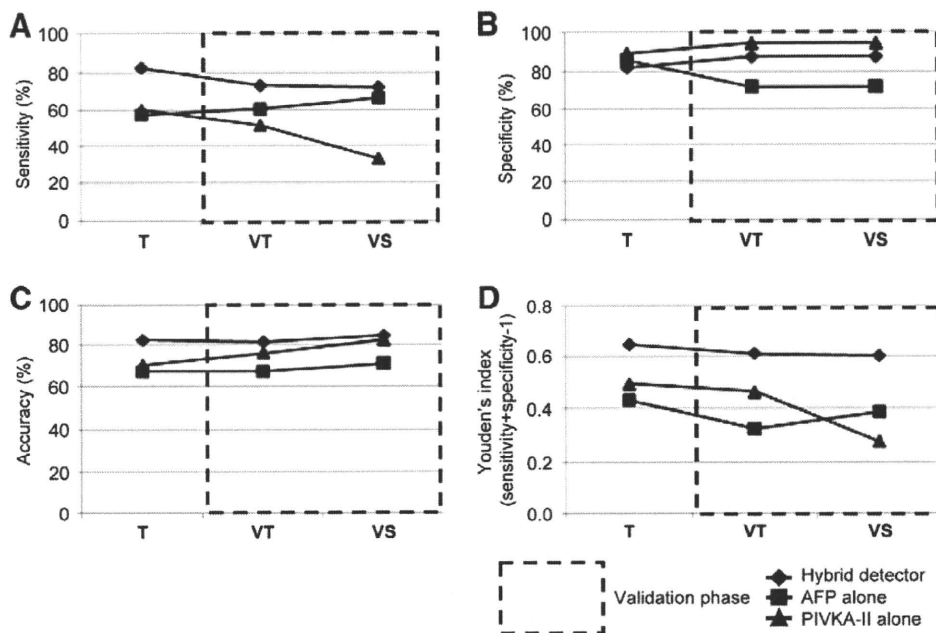


**Fig. 3.** Performances of the Optimal Hybrid detector (diamond), AFP (square), and PIVKA-II (triangle) in the validation cohort. The optimal hybrid detector showed the most robust performances for detection of HCC (A-D). T, training cohort of 108 HCC patients and 56 HCV carriers without HCC used for 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.
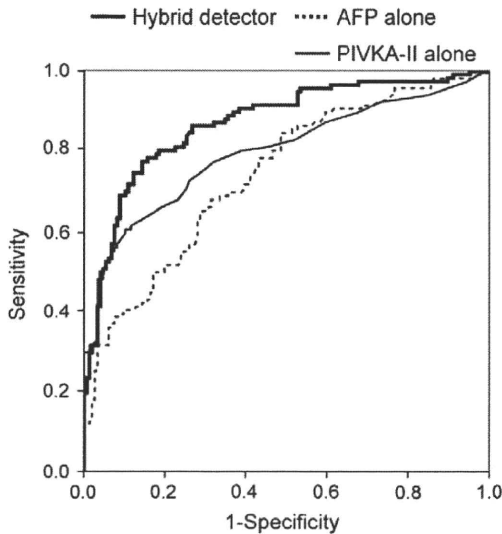
**Fig. 4.** Receiver operating characteristic curve analysis of the optimal hybrid detector, AFP, and PIVKA-II for the validation cohort.

## 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.

## Acknowledgements

## 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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# A novel transcatheter arterial infusion chemotherapy using iodized oil and degradable starch microspheres for hepatocellular carcinoma: a prospective randomized trial

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Koichi Uchida · Shuji Terai · Isao Sakaida

## Abstract

*Background* We designed a novel transcatheter arterial infusion chemotherapy (TAI) using iodized oil (lipiodol) and degradable starch microspheres (DSM) for hepatocellular carcinoma (HCC) patients. In this study, we investigated the efficacy of TAI using lipiodol and DSM in a prospective randomized trial.

*Methods* We randomly divided 45 patients with HCC into 3 groups: TAI using lipiodol (lipiodol group, $n = 15$), TAI using DSM (DSM group, $n = 15$), and TAI using lipiodol and DSM (lipiodol + DSM group, $n = 15$). In the lipiodol group, a mixture of cisplatin and lipiodol was administered. In the DSM group, a mixture of cisplatin and DSM was administered. In the lipiodol + DSM group, a mixture of cisplatin and lipiodol was administered, followed by DSM.

*Results* The response rates were 40% in the lipiodol group, 53.4% in the DSM group, and 80% in the lipiodol + DSM group, respectively. The response rate tended to improve in the lipiodol + DSM group (lipiodol group vs. lipiodol + DSM group, $P = 0.07$). The median progression-free survival time was 177 days in the lipiodol group, 287 days in the DSM group, and 377 days in the lipiodol + DSM group. The progression-free survival in the lipiodol + DSM group was significantly better than those in the DSM group ($P = 0.020$) and the lipiodol group ($P = 0.035$). There were no serious adverse effects among the 3 groups.

*Conclusions* TAI using lipiodol and DSM was superior to TAI using lipiodol only and TAI using DSM only because of improvements in therapeutic effects and progression-free survival.

**Keywords** Hepatocellular carcinoma ·
Transcatheter arterial infusion chemotherapy ·
Iodized oil · Degradable starch microspheres ·
Randomized trial

## Introduction

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer in the world [1]. Deaths due to HCC are increasing in almost all countries worldwide, including Japan [2–4]. Recent advancements in several therapeutic techniques such as hepatic resection, percutaneous ethanol injection, radiofrequency ablation (RFA), transcatheter arterial chemoembolization (TACE), sorafenib, and transplantation have improved the prognosis of HCC patients [5–10].

Of these treatments, TACE has become one of the most popular for HCC patients. TACE in Japan has generally used several anticancer agents, iodized oil (lipiodol) and gelatin sponge particles [11]. On the other hand, polyvinyl alcohol (PVA), drug-eluting beads (DEB), and embospheres have been used as embolizing agents in Europe and the United States [12]. Studies prior to 2000 failed to prove a survival benefit of TACE in the treatment of HCC [13, 14]. However, the survival benefit of TACE was proven by meta-analysis in recent reports [15, 16]. In addition, with the development of the microcatheter, the catheter can be inserted in the segmental or subsegmental hepatic artery, and segmental or subsegmental TACE has been reported to be a useful treatment [7, 17]. On the other hand, transcatheter arterial infusion chemotherapy (TAI) using an emulsion of lipiodol and

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an anticancer agent (without gelatin sponge particles) has usually been performed for HCC patients in whom the catheter could not be inserted in the targeted segment or a feeding artery was not detected in the tumor. In addition, TAI without gelatin sponge particles has been also used for HCC in high-risk patients (for example, main portal vein occlusion, Child–Pugh B or C) [18]. We have also experienced that repeated TACE therapy is not possible due to obstruction of the hepatic artery in HCC patients. Therefore, we have been performing segmental or subsegmental TACE for selected HCC patients. However, it has been reported that the effect of TAI using lipiodol was lower than that of TACE in local tumor control [19]. Many interventional radiologists desire a novel therapy that is both more effective than TAI using lipiodol in local tumor control and is less damaging to the hepatic artery than TACE.

Degradable starch microspheres (DSM) were developed to provide transient occlusion of small arteries [20, 21]. The duration of occlusion in the hepatic arteries by DSM is limited to 80 min [22]. Several studies of metastatic liver tumors indicate that intra-arterial therapy with DSM and an anticancer agent improves the therapeutic effects compared with therapy using an anticancer agent alone [22–24]. However, few studies have evaluated TAI using DSM in HCC patients [25–27].

Given this background, we designed a novel TAI using lipiodol and DSM for use in HCC patients [28]. After a mixture of an anticancer agent and lipiodol is injected, DSM is administered until stasis or reflux of the arterial flow. We postulate that TAI using two occlusion materials may be beneficial because of the tight interruption of blood supply for HCC. In this study, we investigated the efficacy of a novel TAI using lipiodol and DSM in a prospective randomized trial.

## Materials and methods

### Patients

The eligibility criteria for inclusion in this study were as follows: (1) age 20–80 years; (2) Child–Pugh score of A or B; leukocyte count $\geq 3000/mm^3$; (3) hemoglobin level $\geq 9.5$ g/dL; (4) platelet count $\geq 50000/mm^3$; (5) serum creatinine level <1.2 mg/dL; (6) total bilirubin <3.0 mg/dL; (7) locally nodular disease without extrahepatic metastasis and/or vascular tumor thrombosis (portal vein, hepatic vein, and bile duct); (8) no indication for surgical resection and local ablation, or patients rejected surgical resection; and (9) Eastern Cooperative Oncology Group (EOGG) performance status of 0–1 [29].

We studied 45 patients with HCC who had been admitted to the Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, between February 2006 and May 2008. We randomly divided the patients into 3 groups before the angiography: TAI using lipiodol (lipiodol group, $n = 15$), TAI using DSM (DSM group, $n = 15$), and TAI using lipiodol and DSM (lipiodol + DSM group, $n = 15$). The primary outcome measure was tumor response. Secondary outcome measures included progression-free survival and toxicity (Fig. 1). HCC was diagnosed on the basis of imaging results (hyperattenuation in the arterial phase and hypoattenuation in the portal-venous phase) and elevated serum levels of α-fetoprotein (AFP) and/or des-γ-carboxyprothrombin (DCP).

Patients provided their written informed consent before participating in the study, which was approved by the Institutional Review Board of Yamaguchi University Hospital.

**Fig. 1** Study design. We randomly divided patients into 3 groups: transcatheter arterial infusion chemotherapy (TAI) using lipiodol (lipiodol group, $n = 15$), TAI using degradable starch microspheres (DSM) (DSM group, $n = 15$), and TAI using lipiodol and DSM (lipiodol + DSM group, $n = 15$)



HCC (February, 2006-May, 2008)
Locally nodular disease that did not have extrahepatic metastasis and/or vascular tumor thrombosis

Informed consent

Randomized

lipiodol group
15 patients

DSM group
15 patients

lipiodol+DSM group
15 patients

Primary endpoint: Tumor response
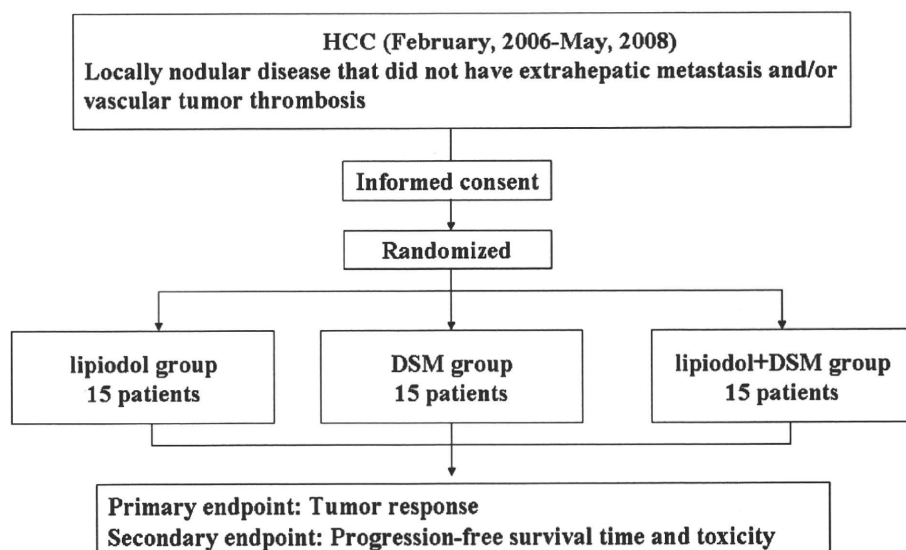Secondary endpoint: Progression-free survival time and toxicity

**Table 1** Clinical profiles of the 45 patients with hepatocellular carcinoma

| Clinical characteristics | Lipiodol group ($n = 15$) | DSM group ($n = 15$) | Lipiodol + DSM group ($n = 15$) | P value |
|---|---|---|---|---|
| Age | $69.5 \pm 4.4$ | $68.0 \pm 7.9$ | $69.3 \pm 9.1$ | NS |
| Gender (male/female) | 12/3 | 12/3 | 10/5 | NS |
| HCV Ab(+)/HBs Ag(+)/others | 12/2/1 | 12/2/1 | 11/3/1 | NS |
| Child–Pugh A/B | 11/4 | 8/7 | 12/3 | NS |
| Maximum tumor size (mm) | $27.1 \pm 16.2$ | $33.6 \pm 12.8$ | $27.7 \pm 15.1$ | NS |
| Tumor stage I/II/III[a] | 2/4/9 | 0/2/13 | 0/7/8 | NS |
| Number of tumors 1/2/3/4/5$\leqq$ | 2/2/3/1/7 | 1/2/2/2/8 | 1/3/3/3/5 | NS |
| Previous treatment (yes/no) | 15/0 | 15/0 | 15/0 | NS |

*DSM* degradable microspheres, *NS* not significant

[a] According to the criteria of the Liver Cancer Study Group of Japan

Table 1 summarizes the clinical profiles of the patients in the 3 groups. There were no significant differences between the 3 groups with regard to age, gender ratio, proportion of patients with hepatitis B virus and hepatitis C virus infections, Child–Pugh score, maximum tumor size, tumor stage, number of tumors, or previous treatment. Tumor stage was determined according to the criteria of the Liver Cancer Study Group of Japan [30, 31]. Tumor staging was based on the following 3 parameters (T factor): solitary tumor, <2 cm in diameter and no vessel invasion. Stage I was defined as one fulfilling all of the above 3 criteria (T1); stage II as one fulfilling 2 of the above 3 criteria (T2); stage III as one fulfilling 1 of the above 3 criteria (T3); stage IV A as one fulfilling none of the above 3 criteria (T4) with no distant metastasis or as one with any T factor with lymph node metastasis; and stage IV B as one with any T factor with distant metastasis.

Embolization technique

Hepatic angiography was performed with a 4-French (4-Fr) or 5-Fr angiographic catheter. After digital subtraction angiography (DSA), angiography combined with a computed tomography (angio-CT) [32] system using a Somatom plus 4 (Siemens, Erlagen, Germany) was performed to carefully evaluate HCC tumors. In this study, a fine-powder formulation of cisplatin (IA-call; Nippon Kayaku Co., Tokyo, Japan) was used as the anticancer agent. The dose of cisplatin was limited to 80 mg. According to the tumor vascularization and distribution, TAI was performed by selectively introducing a catheter into the right or left hepatic artery or a segmental branch of the hepatic artery. Gelatin sponge particles were not used in this study.

In the lipiodol group, a mixture of cisplatin and lipiodol (Lipiodol Ultra Fluid; Andre Guerbet, Paris, France) was administered through the tumor-supplying vessels. In the DSM group, a mixture of cisplatin and emulsion obtained by mixing DSM (Spherex; Yakult Honsha Co., Tokyo, Japan) and contrast agent was administered. If this procedure was insufficient, lipiodol or DSM alone was injected until stasis and reflux were achieved.

A mixture of cisplatin and lipiodol was administered in the lipiodol + DSM group. After that point, emulsion obtained by mixing DSM and contrast agent was injected until stasis and reflux were achieved.

The serotonin antagonist ondansetron hydrochloride was administered intravenously as an antiemetic prior to treatment in all 3 groups. To prevent kidney damage, adequate hydration was ensured before and after the treatment by an intravenous drip infusion of 1000–2000 mL of an infusion solution.

After the treatment, a follow-up examination including CT, tumor marker measurement, and serum biochemistry, was performed, first at 1 month after treatment completion and subsequently every 3–4 months. In principle, the same transcatheter arterial treatments were repeated unless the tumors progressed, when a follow-up CT examination showed new lesions in the liver or regrowth of previously treated tumors.

Response and toxicity evaluation

The antitumor effect was assessed by dynamic CT 1 month or more after treatment. The response was classified according to the Liver Cancer Study Group of Japan criteria [30]. In the response evaluation criteria, lipiodol accumulation in the tumors is regarded as an indication of necrosis because significant positive correlations have been reported between lipiodol accumulation observed on CT images and the necrotic regions in the resected tumors examined pathologically after TACE and TAI [33–35]. Therapeutic effect IV (TE IV) is defined as the disappearance or 100% necrosis of all tumors, and TE III as a greater than 50% reduction in tumor size and/or greater than 50% necrosis. TE I is defined as a greater than 25% increase in tumor size. TE II is defined as disease that does not qualify for classification as TE IV, III, or I.

When repeated TAI was performed, the greatest antitumor effect was assessed as the final response.

The severity of adverse reactions was evaluated during the first treatment cycle according to the Common Terminology Criteria for Adverse Events v.4.0 (CTCAE v.4.0) [36].

## Statistical analysis

The data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using the unpaired $t$ test and the Mann–Whitney $U$ test as appropriate. Progression-free survival and cumulative survival were calculated by the Kaplan–Meier method [37] and significance was determined by the log-rank test. Progression-free survival time was defined as the interval between the first TAI after randomization and death or the progression of the last follow-up period. Survival time was defined as the interval between the first TAI after randomization and death or the last follow-up period. The follow-up period ended on April 30, 2010. Statistical significance was defined as a $P < 0.05$.

## Results

### Information on the anticancer agent and embolizing agents

The median doses of cisplatin at first TAI in the lipiodol group, the DSM group, and the lipiodol + DSM group were 64.3 ± 22.0 mg (20–80 mg), 59.4 ± 20.0 mg (20–80 mg), and 60.5 ± 20.1 mg (10–80 mg), respectively. There was no significant difference in cisplatin dose among the 3 groups. In the lipiodol group, the dose of lipiodol at first TAI was 4.8 ± 2.0 mL (1–8 mL). In the DSM group, the dose of DSM at first TAI was 1164.6 ± 1013.1 mg (120–3000 mg). In the lipiodol + DSM group, the doses of lipiodol and DSM at first TAI were 4.1 ± 2.0 mL (0.5–8 mL) and 426.6 ± 404.8 mg (60–1500 mg), respectively.

### Response to therapy

The total number of treatment courses was 23 with a mean of 1.5 courses per patient (range 1–5 courses) in the lipiodol group, 29 with a mean of 1.9 courses per patient (range 1–6 courses) in the DSM group, and 29 with a mean of 1.9 courses per patient (range 1–6 courses) in the lipiodol + DSM group.

Table 2 shows the final response to therapy. In the lipiodol group ($n = 15$), 4 (26.7%), 2 (13.3%), 4 (26.7%), and 5 (33.3%) patients exhibited TE VI, III, II, and I, respectively [response rate (patients with TE VI and III/all patients) = 40%; complete response (CR) rate (patients with TE VI/all patients) = 26.7%]. In the DSM group ($n = 15$), 4 (26.7%), 4 (26.7%), 7 (46.6%), and 0 (0%)

**Table 2** Response to therapy

| Group | TE[a] | | | | Response rate[b] (CR rate[c]) |
|---|---|---|---|---|---|
| | IV | III | II | I | |
| Lipiodol group (n = 15) | 4 | 2 | 4 | 5 | 40% (26.7%) |
| DSM group (n = 15) | 4 | 4 | 7 | 0 | 53.4% (26.7%) |
| Lipiodol + DSM group (n = 15) | 6 | 6 | 2 | 1 | 80% (40%) |

*TE* therapeutic effect, *CR* complete response, *DSM* degradable microspheres

[a] According to the criteria of the Liver Cancer Study Group of Japan

[b] Response rate, patients with TE IV and III/all patients

[c] CR rate, patients with TE IV/all patients

# Lipiodol group versus DSM group, $P = 0.21$

## DSM group versus lipiodol + DSM group, $P = 0.25$

### Lipiodol group versus lipiodol + DSM group, $P = 0.07$

patients exhibited TE IV, III, II, and I, respectively (response rate = 53.4%; CR rate = 26.7%). In the lipiodol + DSM group ($n = 15$), 6 (40%), 6 (40%), 2 (13.3%), and 1 (6.7%) patient exhibited TE IV, III, II, and I, respectively (response rate = 80%; CR rate = 40%). The response rate tended to improve in the lipiodol + DSM group (lipiodol group vs. lipiodol + DSM group, $P = 0.07$; Mann–Whitney $U$ test). However, no significant differences were seen between the 3 groups (lipiodol group vs. DSM group, $P = 0.21$; DSM group vs. lipiodol + DSM group, $P = 0.25$; Mann–Whitney $U$ test).

### Progression-free survival

Figure 2 shows the progression-free survival rates for the 3 groups. The 1- and 2-year progression-free survival rates in the lipiodol group were 13 and 13%, respectively. The 1-year progression-free survival rate was 27% in the DSM group. The 1-, 2-, and 3-year progression-free survival rates in the lipiodol + DSM group were 53, 13, and 7%, respectively. The median progression-free survival times were 177 days in the lipiodol group, 287 days in the DSM group, and 377 days in the lipiodol + DSM group. No significant difference in progression-free survival was seen between the lipiodol group and the DSM group ($P = 0.515$). On the other hand, progression-free survival in the lipiodol + DSM group was significantly better than that in the DSM group ($P = 0.020$) and the lipiodol group ($P = 0.035$).

### Survival

In the lipiodol group, the 1- and 2-year cumulative survival rates were 80 and 60%, respectively. In the DSM group, they were 87 and 40%, respectively. In the lipiodol +DSM

group, they were 87 and 67%, respectively. No significant differences between the 3 groups were seen in survival (lipiodol group vs. DSM group, $P = 0.377$; lipiodol group vs. lipiodol + DSM group, $P = 0.560$; DSM group vs. lipiodol + DSM group, $P = 0.212$).

By the final follow-up, 21 patients remained alive (lipiodol group, $n = 8$; DSM group, $n = 6$; lipiodol + DSM group, $n = 7$), while the other 24 patients had died (lipiodol group, $n = 7$; DSM group, $n = 9$; lipiodol + DSM group, $n = 8$). In the lipiodol group, the cause of death was cancer



**Fig. 2** Progression-free survival rates for the 3 groups. The 1- and 2-year progression-free survival rates in the lipiodol group were 13 and 13%, respectively. The 1-year progression-free survival rate was 27% in the DSM group. The 1-, 2-, and 3-year progression-free survival rates in the lipiodol + DSM group were 53, 13, and 7%, respectively. No significant difference in progression-free survival was seen between the lipiodol group and the DSM group ($P = 0.515$). On the other hand, progression-free survival in the lipiodol + DSM group was significantly better than that in the DSM group ($P = 0.020$) and the lipiodol group ($P = 0.035$)

progression in 6 patients and hepatic failure in 1 patient. In the DSM group, the cause of death was cancer progression in 7 patients, hepatic failure in 1 patient, and another disease in 1 patient. In the lipiodol + DSM group, the cause of death was cancer progression in 3 patients, hepatic failure in 2 patients, another disease in 2 patients, and rupture of esophageal varices in 1 patient.

Adverse effects of therapy

Table 3 shows the adverse effects of therapy. There was no significant difference in thrombocytopenia between the 3 groups, although grade 3 thrombocytopenia occurred in 4 patients of the lipiodol group (26.7%) and grade 3 or 4 thrombocytopenia occurred in 5 patients of the lipiodol + DSM group (33.3%). However, only 1 patient in the lipiodol + DSM group required a blood transfusion. The grade of elevated alanine aminotransferase (ALT) levels was significantly higher in the lipiodol + DSM group than in the lipiodol group ($P = 0.043$), although there were no significant differences in any other adverse effects between the 3 groups. No treatment-related deaths were observed in the 3 groups.

Figure 3 shows the changes in serum ALT or platelets before and after treatment in the lipiodol + DSM group. Transient increases in serum ALT concentration were observed in almost all patients; however, 2 weeks after treatment, concentrations decreased almost to pretreatment levels. Transient decreases in platelets were observed in almost all patients, and platelet counts at 3 days after treatment were the lowest before and after treatment; 2 weeks after treatment, the count increased almost to pretreatment levels.
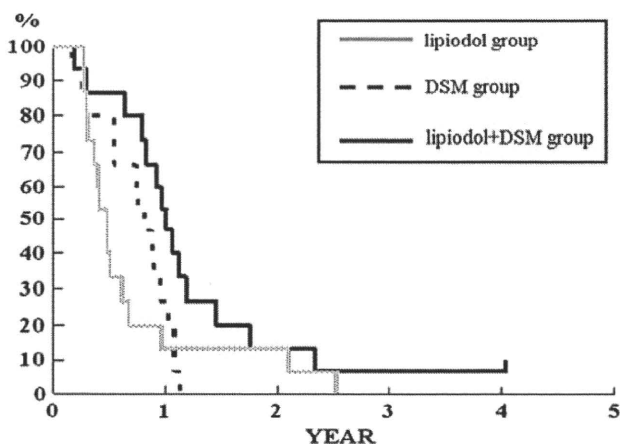
| Adverse effect | Lipiodol group ($n = 15$)/DSM group ($n = 15$)/lipiodol + DSM group ($n = 15$) | | | | P value |
|---|---|---|---|---|---|
| | Grade 1 | Grade 2 | Grade 3 | Grade 4 | |
| Fever | 12/5/8 | 0/1/0 | 0/0/0 | 0/0/0 | NS |
| Nausea | 0/2/1 | 0/0/1 | 0/0/0 | 0/0/0 | NS |
| Appetite loss | 2/5/2 | 0/0/0 | 0/0/0 | 0/0/0 | NS |
| General fatigue | 3/5/3 | 0/0/0 | 0/0/0 | 0/0/0 | NS |
| Thrombocytopenia | 3/0/0 | 4/5/5 | 4/1/3 | 0/0/2 | NS |
| Creatinine | 2/2/1 | 0/0/0 | 0/0/0 | 0/0/0 | NS |
| ALT | 12/5/5 | 3/5/6 | 0/3/3 | 0/0/0 | 0.043[#] |
| Diarrhea | 0/0/1 | 0/0/0 | 0/0/0 | 0/0/0 | NS |
| Ulcer | 0/0/0 | 0/0/1 | 0/0/0 | 0/0/0 | NS |
| Pleural effusion | 0/0/0 | 0/0/1 | 0/0/0 | 0/0/0 | NS |
| Pulmonary embolism | 0/0/0 | 0/0/0 | 1/0/0 | 0/0/0 | NS |
| Ascites | 0/1/0 | 0/0/0 | 0/0/0 | 0/0/0 | NS |
| Biloma | 0/0/1 | 0/0/0 | 0/0/0 | 0/0/0 | NS |

**Table 3** Adverse effects of therapy

According to Common Terminology Criteria for Adverse Events v. 4.0

*DSM* degradable microspheres, *ALT* alanine aminotransferase, *NS* not significant

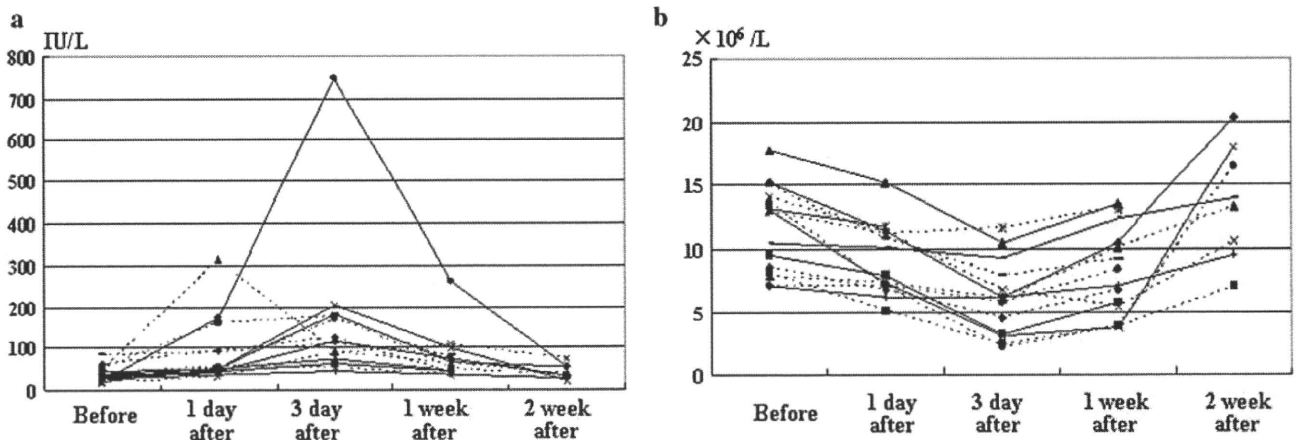[#] Lipiodol group versus lipiodol + DSM group

**a**



**b**



**Fig. 3** Changes in serum alanine aminotransferase (ALT) (**a**) or platelet (**b**) levels before and after treatment in the lipiodol + DSM group. Transient increases in serum ALT concentration were observed in almost all patients; however, 2 weeks after treatment, the concentration decreased almost to pretreatment levels. Transient decreases in platelet levels were observed in almost all patients, and platelet counts at 3 days after treatment were lower than before and after treatment; 2 weeks after treatment, the count increased almost to pretreatment levels

## Discussion

We designed a novel TAI using lipiodol and DSM for use in HCC patients, and reported the usefulness of this procedure [28]. In this study, we investigated the efficacy of this novel TAI using lipiodol and DSM in a prospective randomized trial (lipiodol vs. DSM vs. lipiodol + DSM).

We used a fine-powder formulation of cisplatin (IA-call; Nippon Kayaku Co., Tokyo, Japan) as the anticancer agent. The most common single-agent anticancer drug was doxorubicin, followed by cisplatin [12]. Although there is no evidence of the superiority of any chemotherapeutic agents [12], only a nonrandomized trial by Ono et al. [38] showed that cisplatin was better than doxorubicin. A Phase II study of hepatic arterial infusion of a fine-powder formulation of cisplatin reported that the response rate was 33.8% [39]. Therefore, we selected IA-call as the anticancer agent.

In our study, the response rates (patients with TE VI and III/all patients) in the lipiodol group, DSM group, and lipiodol + DSM group were 40, 53.4, and 80%, respectively. The CR rate (patients with TE IV/all patients) in particular was 40% in the lipiodol + DSM group. Although no significant differences between the 3 groups were seen due to the small population, the response rate tended to improve in the lipiodol + DSM group (lipiodol group vs. lipiodol + DSM group, $P = 0.07$; Mann–Whitney $U$ test). Because of the response rate results, progression-free survival in the lipiodol + DSM group was significantly better than that in the DSM group ($P = 0.020$) and the lipiodol group ($P = 0.035$). On the other hand, no significant difference in progression-free survival was seen between the lipiodol group and the DSM group ($P = 0.515$).

Previous reports associated with our study are shown in Table 4. The response rate was 51% (CR rate 29%) in TAI using cisplatin and lipiodol [19]. On the other hand, the response rates were 73% (CR rate 32%) [19] and 45% (CR rate 0%) [38] in TACE using cisplatin and lipiodol. Although there is a difference in anticancer drugs, the response rates were 52.9% (CR rate 11.8%) [26] and 26% (CR rate 0%) [27] in TAI using DSM. The present findings showed that the response rates in the lipiodol group and in the DSM group were 40% (CR rate 26.7%) and 53.4% (CR rate 26.7%), respectively. Although it is difficult to compare the response rates of our data with those of previous reports, the response rates of the lipiodol group and the DSM group were similar to those of previous reports. On the other hand, only two clinical studies have evaluated TAI using lipiodol and DSM in HCC patients [40, 41]. However, there were some differences in embolization technique. Although the procedure of Vogl et al. [40] was similar to ours, the DSM dose was low (2–10 mg) compared with our procedure (60–1500 mg; mean, 426.6 ± 404.8 mg). Kirchhoff et al. [41] reported administering a mixture of anticancer drugs, DSM, and lipiodol and seeing a response rate of 36% (CR rate 0%). The particles of the emulsion using anticancer agents and lipiodol (lipiodol emulsion) are <30 μm [42] and those of DSM are 45 ± 7 μm in diameter [43]. Because the DSM particles are larger in diameter than those of the lipiodol emulsion, DSM may cause the occlusion of feeding tumor vessels before the accumulation of lipiodol emulsion by means of a mixture of DSM and lipiodol emulsion. In our study, the response rate was 80% (CR rate 40%). Our response rate is better than that reported by Kirchhoff et al. [41], and is similar to that of TACE reported by Ikeda et al. [19].

**Table 4** Previous reports associated with our study

| Author and reference | Embolizing agents | Anticancer drugs | Case no. | Response rate (CR rate) | Survival (%) |
|---|---|---|---|---|---|
| Ikeda [19] | Lipiodol | Cisplatin | 94 | 51% (29%) | 81.6/39.8 (1/3 year) |
| | Lipiodol, gelform | Cisplatin | 74 | 73% (32%) | 87.8/52.2 (1/3 year) |
| Fruse [26] | DSM | Epirubicin | 17 | 52.9% (11.8%) | 64.7/45.3 (1/2 year) |
| Kirchoff [27] | DSM | Cisplatin, doxorubicin | 35 | 26% (0%) | 57/31 (1/2 year) |
| Kirchoff [41] | Lipiodol, DSM | Cisplatin, doxorubicin | 47 | 36% (0%) | 75/59 (1/2 year) |
| | Lipiodol | Cisplatin | 15 | 40% (26.7%) | 80/60 (1/2 year) |
| Our study | DSM | Cisplatin | 15 | 53.4% (26.7%) | 87/40 (1/2 year) |
| | Lipiodol, DSM | Cisplatin | 15 | 80% (40%) | 87/67 (1/2 year) |

*DSM* degradable microspheres

Both animal and clinical studies have reported that lipiodol injected into the hepatic artery occasionally appears in the portal veins through multiple arterioportal communications [44, 45], and that lipiodol can be used to temporarily embolize both the hepatic arteries and the portal veins. We speculate that lipiodol emulsion may be pushed out in the portal vein, the drainage vein of HCC, by DSM. Consequently, we may achieve as tight an interruption of blood supply as TACE for HCC.

There were no significant differences between the 3 groups in adverse effects other than the grade of elevated ALT levels. However, we consider that the high level of ALT in the lipiodol + DSM group reflects the effect of embolization. Transient increases in serum ALT concentration decreased almost to pretreatment levels 2 weeks after TAI using lipiodol and DSM. Because no serious adverse effects were seen in the lipiodol + DSM group, we consider TAI using lipiodol and DSM to be a safe treatment.

In conclusion, our developed TAI using lipiodol and DSM was superior to TAI using lipiodol only and TAI using DSM only because of improvements in therapeutic effects and progression-free survival. This procedure is both a safe and an effective therapy for HCC patients. TAI using lipiodol and DSM may be expected to serve as an alternative to TACE. Since our study examined only a small population, further investigations are necessary.

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## Original Article

# Effect of a late evening snack using branched-chain amino acid-enriched nutrients in patients undergoing hepatic arterial infusion chemotherapy for advanced hepatocellular carcinoma

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*Aim:* A late evening snack (LES) is recommended for protein-energy malnutrition in patients with liver cirrhosis. This study investigated energy metabolism in cirrhotic patients with hepatocellular carcinoma (HCC) and the effects of LES using a branched-chain amino acid (BCAA)-enriched nutrient in cirrhotic patients with advanced HCC undergoing hepatic arterial infusion chemotherapy (HAIC).

*Methods:* Energy metabolism was measured using indirect calorimetry for 10 cirrhotic patients without HCC and 36 patients with various stages of HCC. Next, in 23 cirrhotic patients with advanced HCC undergoing HAIC, 13 patients received LES (LES group), and 10 patients received ordinary food (control group). Changes in energy metabolism and glucose tolerance were examined using indirect calorimetry and 75-g oral glucose tolerance test (OGTT) before and after 1 cycle of treatment.

*Results:* Non-protein respiratory quotient (npRQ) was significantly lower in patients with advanced HCC than in cirrhotic patients without HCC, or in patients with early-stage HCC. In cirrhotic patients with advanced HCC undergoing HAIC, npRQ, BCAA/tyrosine ratio (BTR), and prealbumin and ALT levels were significantly improved in the LES group, but not in controls. In addition, area under the concentration curve for glucose (AUC glucose) tended to be improved in the LES group.

*Conclusions:* LES using BCAA-enriched nutrients appears to improve energy metabolism and glucose tolerance in cirrhotic patients with advanced HCC undergoing HAIC.

**Key words:** advanced hepatocellular carcinoma, branched-chain amino acid, hepatic arterial infusion chemotherapy, late evening snack, nutritional therapy

## INTRODUCTION

THE LIVER PLAYS an important role in energy metabolism, and liver diseases lead to abnormalities in nutrient metabolism and subsequent malnutrition.[1] Protein-energy malnutrition (PEM) is a common finding in cirrhotic patients.[2,3] Owen *et al.* reported that patients with cirrhosis show marked decreases in

glucose oxidation after an overnight fast, with enhanced fat and protein catabolism similar to that observed in healthy controls after 2–3 days of starvation.[4] PEM is a significant factor in establishing the vital prognosis of liver cirrhosis.[3]

In an attempt to improve the state of energy malnutrition, a late evening snack (LES) has been developed for use by patients with liver cirrhosis, resulting in improved energy substrate metabolism.[5–8] A LES is recommended in the present guidelines of the American Society for Parenteral and Enteral Nutrition[9] and the European Society for Clinical Nutrition and Metabolism.[10] We have also reported that a LES using branched-chain amino acid (BCAA)-enriched nutrients improves energy malnutrition, imbalances in amino acids, and

glucose intolerance in patients with liver cirrhosis.[11-13] However, those studies focused on the effects of LES in patients with liver cirrhosis.

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer in the world.[14] Deaths due to HCC are increasing in almost all countries around the world, including Japan.[15-17] In particular, the prognosis for patients with advanced HCC showing portal vein tumor thrombosis (PVTT) remains poor.[18] Such patients are thus generally treated with hepatic arterial infusion chemotherapy (HAIC).[19-21] Our previous study identified Child-Pugh score[22] as an independent prognostic factor in cirrhotic patients with advanced HCC treated using HAIC.[20,21] In addition, energy expenditure in cirrhotic patients with HCC is reportedly increased compared with that in cirrhotic patient without HCC,[23] and a hypermetabolic rate in patients with gastrointestinal malignancy has been associated with the most advanced stage of the disease.[24]

Given this background, we consider that nutritional support is required for cirrhotic patients with advanced HCC undergoing HAIC. Few reports have examined nutritional support in cirrhotic patients with HCC.[25,26] Furthermore, no clinical studies have evaluated energy metabolism using indirect calorimetry in patients with HCC. We therefore investigated energy metabolism in patients with HCC and the efficacy of nutritional support using LES in patients with advanced HCC undergoing HAIC.

## MATERIALS AND METHODS

### Energy metabolism in patients with HCC

#### Patients

WE INVESTIGATED ENERGY metabolism using indirect calorimetry in cirrhotic patients without HCC and with various stages of HCC under the same conditions of liver capacity. Subjects comprised 10 cirrhotic patients without HCC and 36 patients with HCC before treatment ($n = 46$). No patients had received BCAA-enriched nutrients, and all were classified as Child-Pugh A.[22] Table 1 summarizes the clinical profiles of the 46 patients in this study. Tumor stage was determined according to the criteria of the Liver Cancer Study Group of Japan.[27,28] Liver cirrhosis was present in 10 patients, stage I/II HCC in 13 patients, stage III HCC in 13 patients, and stage IV HCC in 10 patients. The 4 groups showed no significant differences in clinical characteristics other than age (HCC stage III group vs. HCC stage IV group, $P = 0.017$). In addition, no significant differences in laboratory parameters, including BCAA/tyrosine ratio (BTR),[29] were identified among the 4 groups.

Energy metabolism was analyzed using indirect calorimetry (Deltatrac II; Detex Ohmeda, Helsinki, Finland). Indirect calorimetry was performed for 30 min after overnight bed rest and fasting. We measured oxygen consumption per minute ($VO_2$), carbon dioxide

**Table 1** Clinical profiles of the 46 patients with and without hepatocellular carcinoma

| Clinical characteristics | LC (n = 10) | HCC (n = 36) | | |
| --- | --- | --- | --- | --- |
| | | Stage I/II† (n = 13) | Stage III† (n = 13) | Stage IV† (n = 10) |
| Age | 66.9 ± 9.2 | 69.1 ± 8.0 | 73.8 ± 9.4* | 62.2 ± 11.2* |
| Sex (male/female) | 4/6 | 9/4 | 8/5 | 8/2 |
| HCV Ab(+)/HBs Ag(+)/others | 5/2/3 | 11/1/1 | 8/4/1 | 5/3/2 |
| Child-Pugh A(5)/A(6) | 6/4 | 8/5 | 10/3 | 7/3 |
| Total Protein (g/dL) | 7.20 ± 0.58 | 7.25 ± 0.76 | 7.45 ± 0.57 | 7.45 ± 0.58 |
| Albumin (g/dL) | 3.62 ± 0.46 | 3.78 ± 0.51 | 3.88 ± 0.25 | 3.66 ± 0.28 |
| BTR | 4.46 ± 1.16 | 4.52 ± 1.45 | 5.19 ± 0.96 | 4.86 ± 1.06 |
| NH3 | 46.6 ± 11.7 | 45.6 ± 25.0 | 40.9 ± 16.9 | 59.6 ± 30.2 |
| Total cholesterol | 160.3 ± 30.1 | 166.5 ± 30.1 | 176.9 ± 35.1 | 159.8 ± 13.7 |
| ChE | 193.0 ± 73.6 | 214.9 ± 89.6 | 255.0 ± 81.4 | 204.8 ± 60.1 |
| CHI | 79.7 ± 16.7 | 66.2 ± 17.6 | 68.0 ± 13.4 | 78.6 ± 18.5 |
| BMI | 23.1 ± 1.8 | 21.4 ± 2.6 | 22.9 ± 4.8 | 23.7 ± 3.5 |

*$P = 0.017$.
†According to the criteria of the Liver Cancer Study Group of Japan.
BMI, body mass index; BTR, branched-chain amino acid/tyrosine ratio; ChE, cholinesterase; CHI, creatinine height index; HCC, hepatocellular carciunoma; HCV, hepatitis C virus; LC, liver cirrhosis; NH3, ammonia.
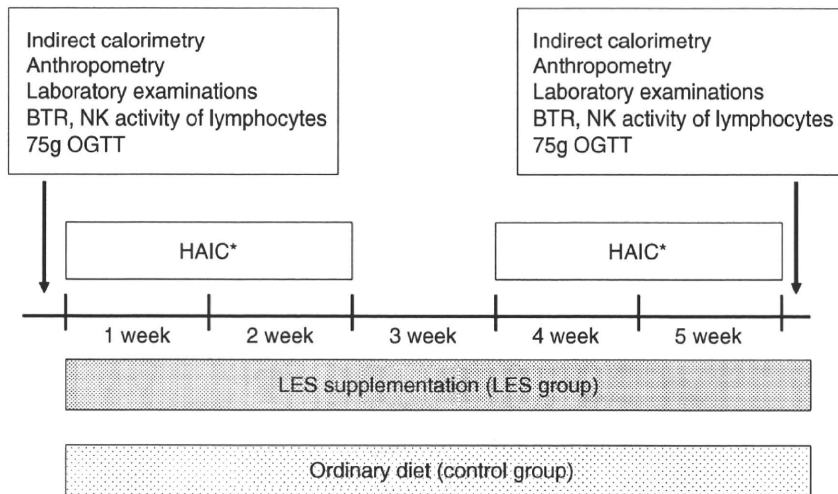
**Figure 1** Study protocol. In the late evening snack (LES) group, patients received a LES supplement comprising branched-chain amino acid-enriched nutrients. In the control group, patients received ordinary food to the same amount of calories as the LES group. Before and after 1 cycle of hepatic arterial infusion chemotherapy (HAIC), nutritional evaluation using indirect calorimetry and InBody, laboratory examinations, and glucose tolerance using the 75-g oral glucose tolerance test were measured.

production per minute ($VCO_2$) and total urine nitrogen (TUN) on the day prior to examination, and the non-protein respiratory quotient (npRQ) was calculated as a measure of energy metabolism for the 4 groups.

## Effect of LES for advanced HCC during HAIC

### Patients

This study was performed on cirrhotic patients with unresectable HCC undergoing HAIC who had been admitted to the Department of Gastroenterology and Hepatology at Yamaguchi University Graduate School of Medicine. Eligibility criteria for this study were as follows: age, 20–80 years; Child-Pugh score A or B;[22] leukocyte count, ≥3000/$mm^3$; platelet count, ≥50 000/$mm^3$; serum creatinine, <1.2 mg/dL; unresectable HCC due to extensive, locally advanced disease that did not permit resection, bilobar disease, extrahepatic metasta-

sis, or PVTT; and Eastern Cooperative Oncology Group (ECOG) performance status 0–2.[30]

Between December 2007 and February 2009, 26 patients were enrolled in this study. After randomization using a random number table, 13 patients received LES using a BCAA-enriched nutrient (LES group), and 13 received ordinary food (control group). However, 3 patients dropped out of the control group after withdrawing from the study during treatment. Thus, 13 patients in the LES group and 10 patients in the control group were subjected to analysis.

All patients provided written informed consent prior to enrolment into the study, and all protocols were approved by the Institutional Review Board of Yamaguchi University Hospital.

Table 2 summarizes the clinical profiles of patients in the 2 groups. No significant differences between groups were seen in clinical characteristics.

**Table 2** Clinical profiles of the 23 patients with hepatocellular carcinoma

| Clinical characteristics | LES group (n = 13) | Control group (n = 10) | *P*-value |
|---|---|---|---|
| Age | 64.5 ± 9.5 | 66.4 ± 12.8 | 0.69 |
| Sex (male/female) | 11/2 | 8/2 | 0.78 |
| HCV Ab(+)/HBs Ag(+)/others | 7/5/1 | 8/1/1 | 0.35 |
| Child-Pugh A/B | 6/7 | 6/4 | 0.58 |
| Maximum tumor size (mm) | 77.7 ± 50.5 | 88.0 ± 39.7 | 0.60 |
| Tumor stage II/III/IV A/IV B† | 1/3/3/6 | 1/2/6/1 | 0.31 |
| CHI | 66.7 ± 16.3 | 72.0 ± 22.7 | 0.65 |
| BMI | 23.3 ± 3.9 | 21.4 ± 2.8 | 0.22 |

†According to the criteria of the Liver Cancer Study Group of Japan.
BMI, body mass index; CHI, creatinine height index; LES, late evening snack.