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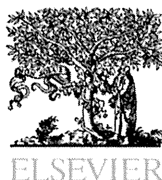

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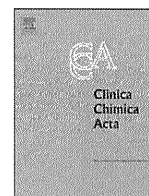

Efficient detection of hepatocellular carcinoma by a hybrid blood test of epigenetic and classical protein markers

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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

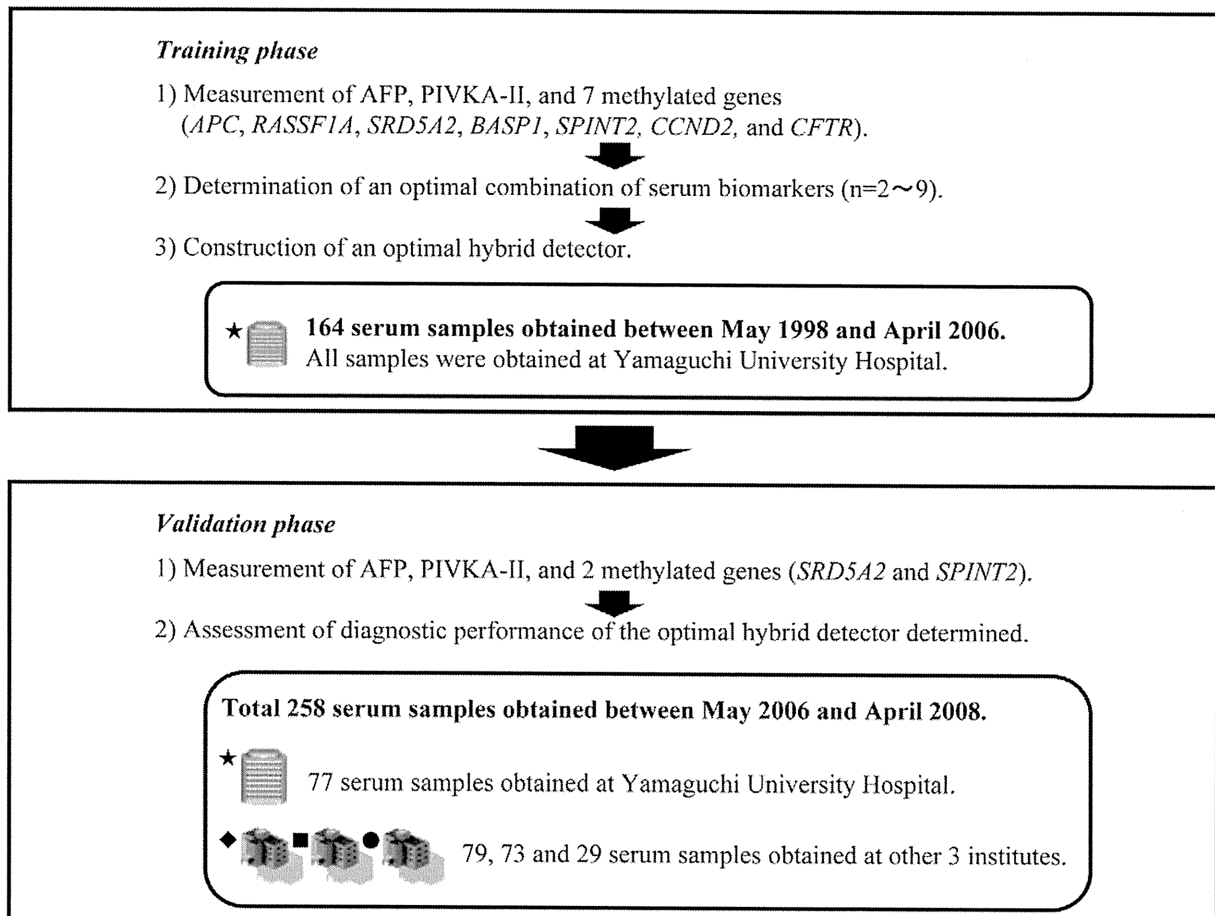

Fig. 1. Overview of the Training-Validation approach used for construction and evaluation of the hybrid detector for hepatocellular carcinoma.

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).

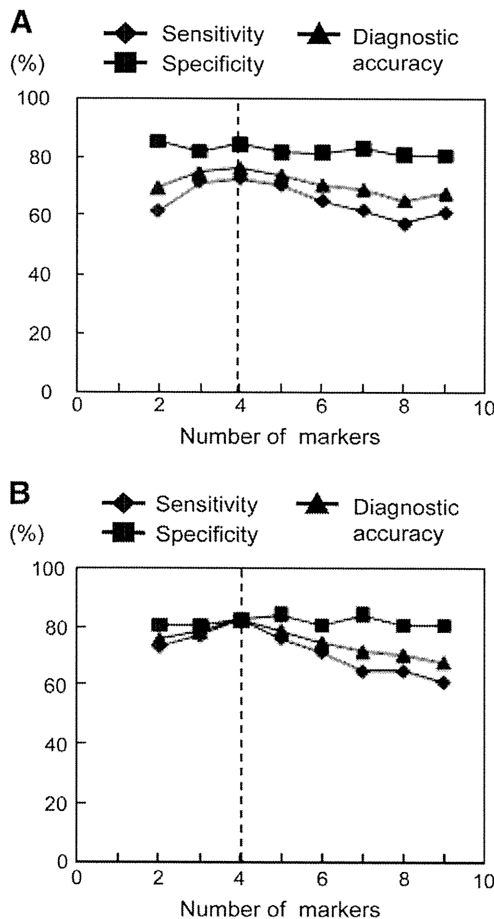

Fig. 2. Performances of markers in the training cohort. We input data for n markers ($n=2$–9) of the 164 training samples into Fisher linear classifier (FLC). This procedure was repeated for all combinations (from 9 C2 to 9 C9) of n markers and performances of the constructed individual FLCs were computed.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

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

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].

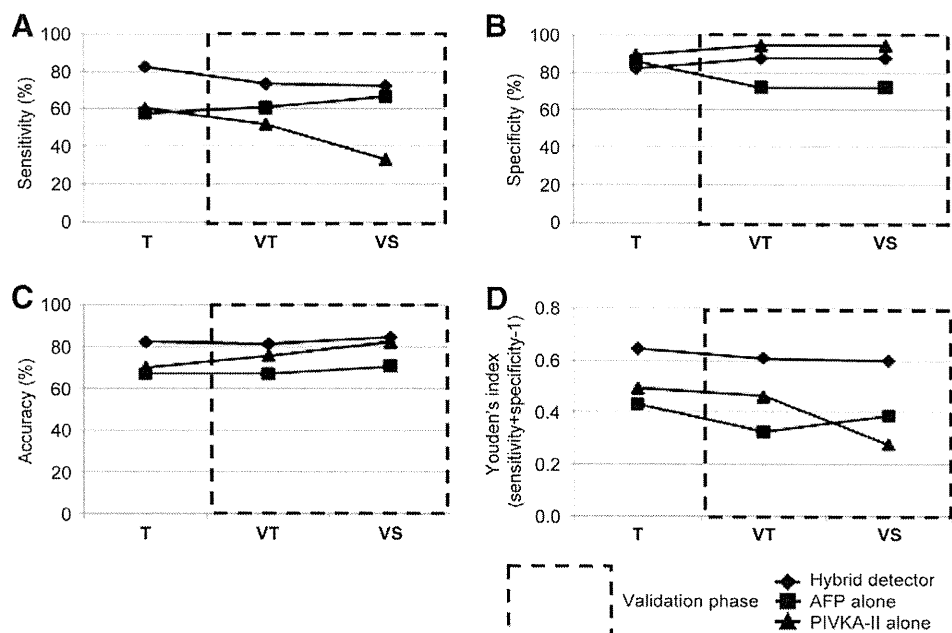

Fig. 3. Performances of the Optimal Hybrid detector (diamond), AFP (square), and PIVKA-II (triangle) in the validation cohort. The optimal hybrid detector showed the most robust performances for detection of HCC (A-D). T, training cohort of 108 HCC patients and 56 HCV carriers without HCC used for comparison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

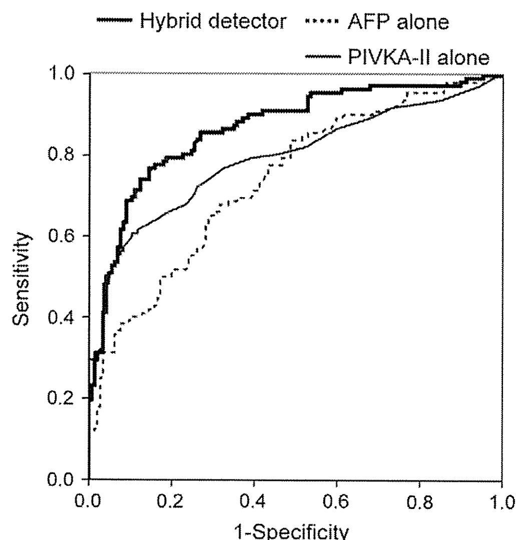

Fig. 4. Receiver operating characteristic curve analysis of the optimal hybrid detector, AFP, and PIVKA-II for the validation cohort.

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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Original Article

Data mining reveals complex interactions of risk factors and clinical feature profiling associated with the staging of non-hepatitis B virus/non-hepatitis C virus-related hepatocellular carcinoma

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Aim: Non-hepatitis B virus/non-hepatitis C virus-related hepatocellular carcinoma (NBNC-HCC) is often detected at an advanced stage, and the pathology associated with the staging of NBNC-HCC remains unclear. Data mining is a set of statistical techniques which uncovers interactions and meaningful patterns of factors from a large data collection. The aims of this study were to reveal complex interactions of the risk factors and clinical feature profiling associated with the staging of NBNC-HCC using data mining techniques.

Methods: A database was created from 663 patients with NBNC-HCC at 20 institutions. The Milan criteria were used as staging of HCC. Complex associations of variables and clinical feature profiling with the Milan criteria were analyzed by graphical modeling and decision tree algorithm methods, respectively.

Results: Graphical modeling identified six factors independently associated with the Milan criteria: diagnostic year of HCC; diagnosis of liver cirrhosis; serum aspartate aminotransferase (AST); alanine aminotransferase (ALT); α-fetoprotein (AFP); and des-γ-carboxy prothrombin (DCP) levels. The decision trees were created with five variables to classify six groups of patients. Sixty-nine percent of the patients were

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within the Milan criteria, when patients showed an AFP level of 200 ng/mL or less, diagnosis of liver cirrhosis and an AST level of less than 93 IU/mL. On the other hand, 18% of the patients were within the Milan criteria, when patients showed an AFP level of more than 200 ng/mL and ALT level of 20 IU/mL or more.

Conclusion: Data mining disclosed complex interactions of the risk factors and clinical feature profiling associated with the staging of NBNC-HCC.

Key words: data mining, disease progression, hepatoma, non-viral hepatitis, tumor marker

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the fifth most common cancer and the third most common cause of cancer-related deaths worldwide.[1–3] Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) is a risk factor for HCC. Recent developments in the management of patients with viral hepatitis have resulted in early detection of HCC and improvement of prognosis.[4–8]

The number of patients with non-HBV/non-HCV-related HCC (NBNC-HCC) has been increasing, and NBNC-HCC now accounts for 12–16% of all the HCC cases in Japan.[8,9] A variety of factors are involved in the development and progression of this cancer including age, sex, alcoholic liver disease and diabetes mellitus.[10–12] Therefore, neither early detection nor improved prognosis has been achieved in NBNC-HCC.[6] Radical treatment is applicable to patients with NBNC-HCC who meet the Milan criteria;[13] however, this cancer is often detected at an advanced stage. For earlier detection, it is important to understand the complex interactions of the risk factors and clinical feature profiling associated with the Milan criteria, a staging system for NBNC-HCC.

Data mining, a set of statistical techniques, uncovers meaningful patterns and interactions of variables from a large data collection even when there is no a priori hypothesis imposed.[13] Graphical modeling is an exploratory multivariate analysis of data mining that reveals complex associations between variables.[14] This analysis assumes that the response variable is influenced by multiple factors.[15] Therefore, different from results of univariate analysis, an association between a risk factor and an outcome variable may disappear or appear because of the effects of another set of variables known as "confounding factors".[16,17] Furthermore, its findings are visualized as a graph, which provides an idea of how variables interact and denotes the conditional independence structure between random variables.[15] Therefore, graphical modeling is now identified as a new approach to model clinical data.[18]

Decision tree making is another exploratory technique of data mining that represents a series of rules for classification by identifying priorities.[19–21] It is an explicit, quantitative and systematic approach to decision-making under conditions of uncertainty and allows clinicians to choose an option that maximizes the net benefit to the patient.[22] Recently, decision trees were used to reveal the clinical feature profiling for staging of pancreatic cancer[23] and ovarian cancer.[24] However, decision trees have never been applied to identify the clinical feature profiling associated with the staging of NBNC-HCC.

The aims of this study were to reveal complex interactions of the risk factors and clinical feature profiling associated with the staging of NBNC-HCC using data mining techniques.

METHODS

Patient database

BETWEEN 1995 AND 2006, a total of 10 133 patients were diagnosed with HCC at 23 institutions located in Kyushu, a high morbidity area of HCC in Japan. Among them, 1363 patients were diagnosed with NBNC-HCC according to the negative results of both serum hepatitis B surface antigen and serum anti-HCV antibody or HCV RNA.

In order to examine the clinical variables associated with the staging of NBNC-HCC, a database of 663 patients with NBNC-HCC at 20 institutions was created on the basis of the following variables: diagnostic year of HCC; age; sex; family history of liver disease; past history of blood transfusion; alcohol intake; diagnosis of liver cirrhosis; diagnosis of liver disease; diagnosis of diabetes mellitus; serum aspartate aminotransferase (AST) level; serum alanine aminotransferase (ALT) level; serum α-fetoprotein (AFP) level; serum des-γ-carboxy prothrombin (DCP) level; size of HCC; and number of HCC.

For practical use, alcohol intake, serum AFP level and serum DCP level were categorized as follows. Alcohol intake: none; 60 g/day or less; 60–100 g/day; or more than 100 g/day. AFP level: 20 ng/mL or less; 20–200 ng/mL; or more than 200 ng/mL. DCP level: 40 mAU/mL or less; 40–100 mAU/mL; or more than 100 mAU/mL.

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The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected by the approval of the Ethics Committee of the Kurume University School of Medicine.

Diagnosis and staging of HCC

The diagnosis of HCC was based on the clinical practice manual proposed by the Japan Society of Hepatology,[25] by using serum AFP and DCP levels and imaging techniques including ultrasonography, computerized tomography, magnetic resonance imaging, hepatic angiography and/or tumor biopsy. The Milan criteria (single nodule ≤5 cm or three nodules <3 cm) were used for the staging of HCC.[26]

Data mining

An association between the Milan criteria and each risk factor was examined by Student's t-test and χ^2-test. Because of the insufficient scientific evidence for testing specific clinical hypotheses, graphical modeling and decision trees were employed to explore complex associations between the Milan criteria and a set of risk factors.

MIM software (http://www.hypergraph.dk/) was used for graphical modeling. R package rpart (recursive partitioning and regression trees by Terry Therneau and Beth Atkinson; http://www.mayo.edu/biostatistics) was used to construct a decision tree algorithm. In order to evaluate the prediction error, the original data ($n = 663$) were randomly divided into a training dataset ($n = 442$) and a test dataset ($n = 221$). Ten-hold cross-validation was conducted to construct the initial tree on the basis of the training dataset; then, the optimal-size tree was constructed by examining a set of cost–complexity parameters. The overall prediction error rate as well as the sensitivity and specificity were calculated by applying the results of the decision tree algorithm to the test dataset.

Table 1 Characteristics of all patients

Variable	
n	663
Diagnostic year of HCC (years)	2002 ± 3
Age (years)	68.1 ± 9.9
Male/female	480/183
Family history of liver disease (yes/no/unclear)	79/547/37
History of blood transfusion (no/before 1989/after 1989/unclear)	584/29/22/28
Daily alcohol intake (none/<60 g/60–100 g/>100 g)	254/183/141/85
Etiology of chronic liver disease (none/alcohol/others)	296/188/179
Diagnosis of liver cirrhosis (yes/no)	260/403
Diagnosis of diabetes mellitus (no/yes without medication/yes with medication)	396/109/158
Serum AST level (U/L)	53.3 ± 51.3
Serum ALT level (U/L)	51.8 ± 49.9
Serum AFP level (ng/mL)	9397 ± 71066
Serum DCP level (mAU/mL)	8003 ± 37377
Size of HCC (cm)	5.0 ± 3.4
Number of HCC	2.8 ± 2.9

Data are expressed as the mean ± standard deviation or the number of patients.
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma.

RESULTS

Characteristics of patients with NBNC-HCC

THE PATIENTS' CHARACTERISTICS are summarized in Table 1. Family history of liver disease and history of blood transfusion were not noted in more than 80% of the patients. Approximately 40% of the patients did not have any etiology of chronic liver disease.

Univariate analysis of variables associated with the Milan criteria

Univariate analysis showed that diagnosis of liver cirrhosis, serum AST level, serum ALT level, serum AFP level and serum DCP level were significantly associated with the Milan criteria (Table 2).

Graphical modeling

Complex interactions of the risk factors associated with the Milan criteria were visualized graphically (Fig. 1). Graphical modeling identified six independent factors directly associated with the Milan criteria: diagnostic year of HCC; diagnosis of liver cirrhosis; serum AST level; serum ALT level; serum AFP level; and serum DCP level (Fig. 1). Although alcohol intake, diagnosis of liver disease and diagnosis of diabetes mellitus were not directly associated with the Milan criteria, they were associated with the Milan criteria through diagnosis of liver cirrhosis (Fig. 1).

Decision tree algorithm

With the training dataset ($n = 442$), a decision tree algorithm was created by using five variables to classify six groups of patients (Fig. 2). A serum AFP level of 200 ng/mL or less was the cut-off value for the initial

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Table 2 Univariate analysis of the variables associated with the Milan criteria

Variable	Statistical method	Test statistics	Degree of freedom (df)	*P*
Diagnostic year of HCC (years)	χ^2	13.4013	11	0.2679
Age (years)	Pooled	−1.07	661	0.2843
Sex	χ^2	0.2975	1	0.5854
Family history of liver disease	χ^2	1.7412	1	0.187
History of blood transfusion	χ^2	4.9527	2	0.084
Daily alcohol intake	χ^2	2.4158	3	0.4907
Liver cirrhosis	χ^2	28.9521	1	<0.0001
Diabetes mellitus	χ^2	0.926	2	0.6294
AST level (U/L)	Satterthwaite	3.06	387.51	0.0023
ALT level (U/L)	Satterthwaite	4.79	546.95	<0.0001
AFP level (ng/mL)	χ^2	63.1357	2	<0.0001
DCP level (mAU/mL)	χ^2	47.7161	2	<0.0001

Associations between the variables and the Milan criteria were analyzed by the indicated statistical methods. $P < 0.05$ was considered significant.
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma.

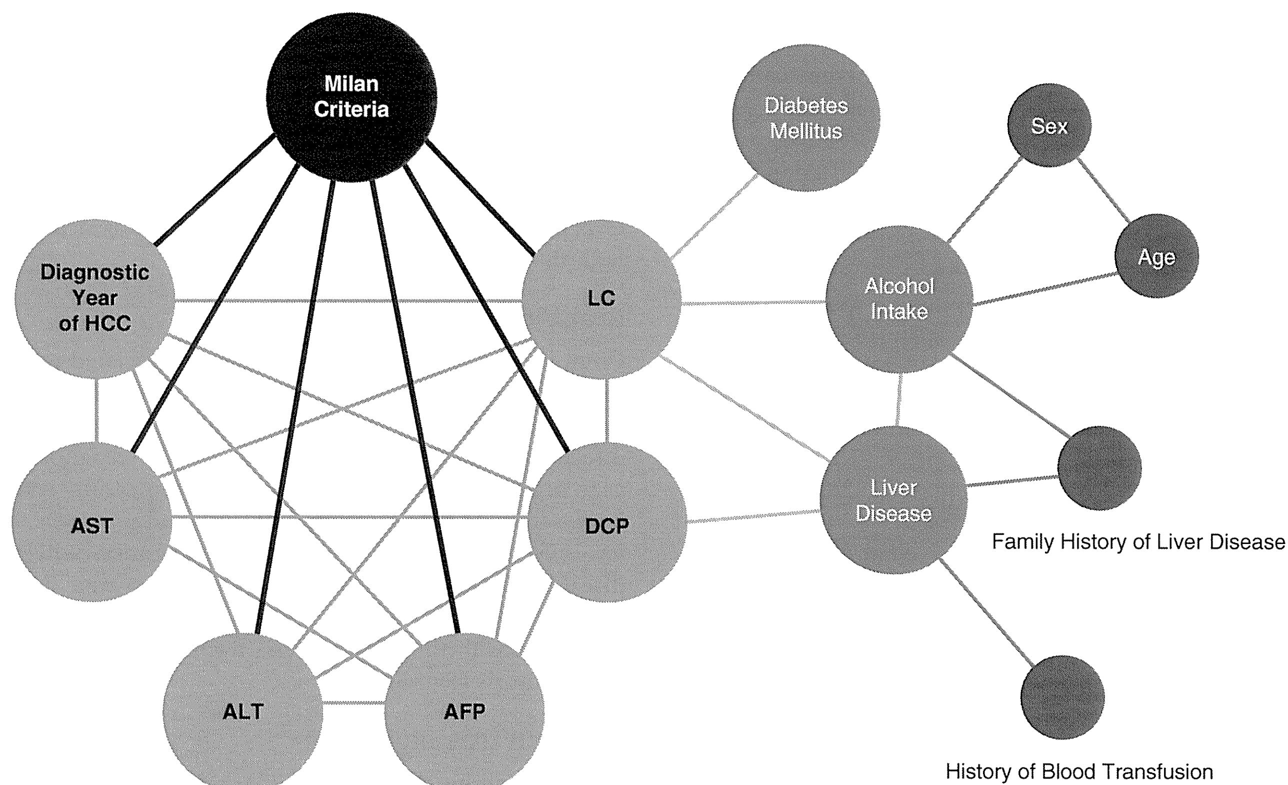

Figure 1 Graphical modeling of the interactions of the risk factors associated with the Milan criteria. AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; LC, liver cirrhosis.

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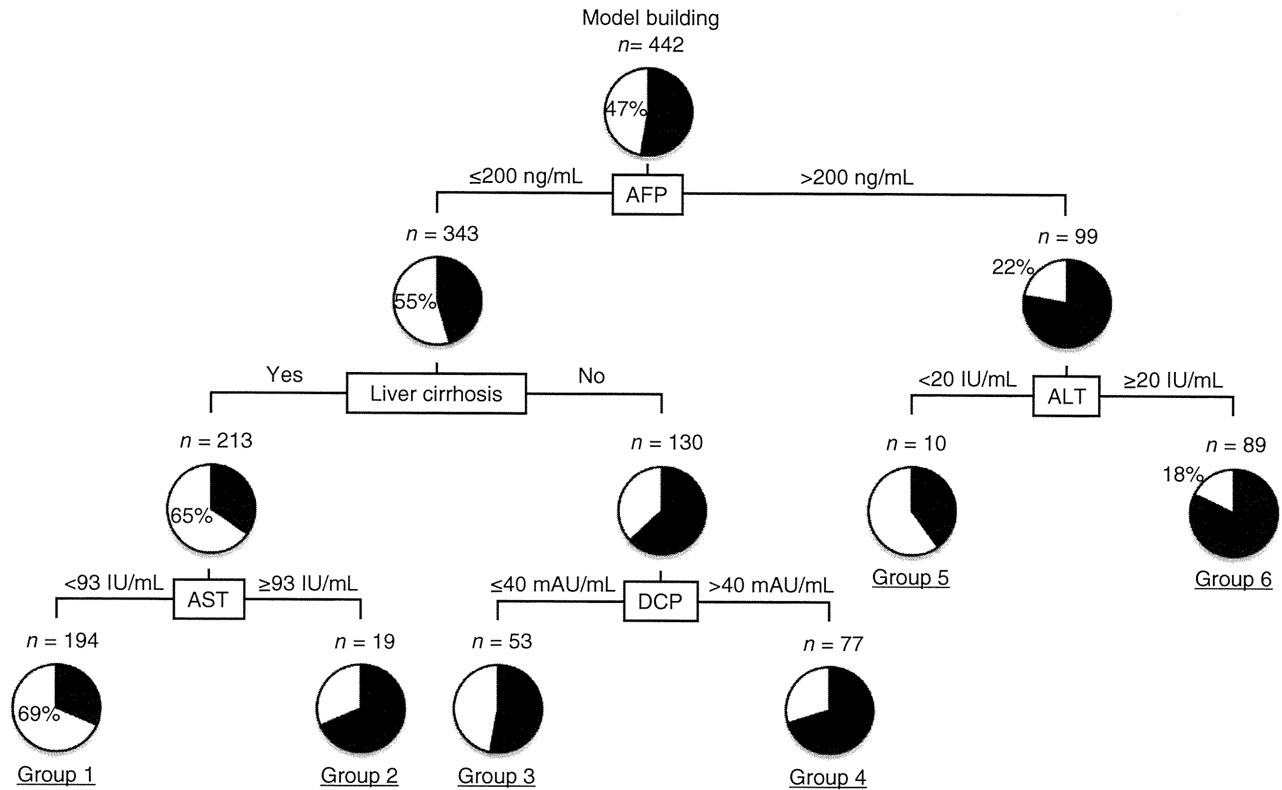

Figure 2 Decision tree algorithm of the variables associated with the Milan criteria. The patients were classified according to the indicated cut-off values of the variables. The pie graphs indicate the percentage of patients with HCC within (white)/beyond the Milan criteria in each group. AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma.

classification. Among the patients with an AFP level of 200 ng/mL or less, diagnosis of liver cirrhosis was used as the variable for the second division. Among the patients with liver cirrhosis, a serum AST level of less than 93 IU/mL was the cut-off value for the third division. Thus, 69% of the patients were within the Milan criteria, when the patients met all of the following conditions: AFP of 200 ng/mL or less; diagnosis of liver cirrhosis; and AST of less than 93 IU/mL (group 1; Fig. 2). On the other hand, only 18% of the patients were within the Milan criteria, when patients showed an AFP level of more than 200 ng/mL and an ALT level of 20 IU/mL or more (group 6; Fig. 2).

There were no significant differences in the patients' characteristics between the training dataset and the test dataset. Prediction error was obtained by applying the results of the decision tree algorithm to the test dataset. The sensitivity (proportion of patients with HCC correctly classified as beyond the Milan criteria) and specificity (proportion of patients with HCC correctly classified as within the Milan criteria) were 72.1% (75/104) and 68.4% (80/117), respectively; the overall prediction error rate was 29.8% (66/221).

DISCUSSION

IN THIS STUDY, we revealed the complex interactions of the risk factors associated with staging of NBNC-HCC using graphical modeling. In addition, we presented a decision tree algorithm to identify clinical feature profiling associated with the staging of NBNC-HCC.

Various factors seem to be intricately related to the progression of NBNC-HCC. In this study, by graphical modeling, we identified six variables directly associated with the Milan criteria: serum AST level; serum ALT level; serum AFP level; serum DCP level; diagnosis of liver cirrhosis; and diagnostic year of HCC. Chronic hepatic inflammation modulates many of the signaling cascades involved in cell proliferation, survival and invasion of

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HCC.[27,28] Further, AFP and DCP are directly associated with HCC progression through the induction of cancer cell proliferation and angiogenesis, respectively.[29,30] Thus, our results are in good accordance with previous basic investigations and suggest that hepatic inflammation as well as elevated AFP and DCP levels independently accelerate the progression of NBNC-HCC.

Diagnostic year of HCC was also directly associated with the Milan criteria in this study. Although the reason for this association is unclear, a progress in serum tumor markers is a possible explanation. Because sensitivities of AFP and DCP were improved during this study period (1995–2006),[31–33] one would think that serum AFP and DCP levels are confounding factors for an association between diagnostic year of HCC and the Milan criteria.

Recently, lifestyle-related factors including alcohol intake and diabetes mellitus have been noted as risk factors for the development of NBNC-HCC.[2,10–12,34–38] Previous *in vitro* studies showed that ethanol and glucose stimulate the proliferation and migration of HCC,[39,40] indicating the direct association of alcohol intake and diabetes mellitus with NBNC-HCC progression. However, in this study, these factors were not directly associated with the Milan criteria. Although the reason for this discrepancy remains unclear, alcohol intake and diabetes mellitus were associated with the Milan criteria through diagnosis of liver cirrhosis in this study. Both ethanol consumption and diabetes mellitus can activate fibroblasts,[41,42] which are crucial components of the tumor microenvironment promoting the growth and invasion of cancer cells.[43,44] Thus, alcohol intake and diabetes mellitus may be associated with the clinical progression of NBNC-HCC through the tumor microenvironment.

Then, we created a decision tree algorithm to identify the clinical feature profiling associated with the staging of NBNC-HCC; the reproducibility of this model was confirmed by the independent validation datasets. Serum AFP level was selected for the initial classification, and serum DCP level was selected for the third division, creating groups 3 and 4. Although it is still unclear why the serum AFP level was associated with the Milan criteria to a greater extent than the serum DCP level, an association of the serum AFP level with the pathological features of HCC is a possible explanation. The AFP level is related to the number of HCC, whereas the DCP level is more specific to vascular invasion.[45–47] In this study, the staging of HCC was evaluated by using the Milan criteria, which include number and size of HCC but not vascular invasion,[26] explaining why serum AFP level was selected for the initial classification.

Diagnosis of liver cirrhosis was selected for the second division in the decision tree algorithm. Although liver cirrhosis is a well-known major risk factor for the development of HCC,[5,10,12,25,34,42] our result indicates that liver cirrhosis may suppress the progression of NBNC-HCC. We do not have any data accounting for the association between diagnosis of liver cirrhosis and suppression of the NBNC-HCC progression, the following is, however, a possible explanation for this contradiction. HCC surveillance may be performed more often in patients with liver cirrhosis than in those without liver cirrhosis,[12,25] so HCC could be identified at an early stage in patients with liver cirrhosis.

A limitation of this study is that a relationship between progression of NBNC-HCC and non-alcoholic steatohepatitis (NASH) was not evaluated. The reason is that NASH-related HCC is often diagnosed as cryptogenic cirrhosis-related HCC because of reduction of hepatic triglycerides according to the progression of NASH, so-called "burned-out NASH".[48] However, NASH is deeply involved in the development of HCC and a major reason for the increase in number of NBNC-HCC patients.[8,49,50] Recently, visceral fat accumulation is also reported to be an independent risk factor for HCC recurrence after curative treatment.[51] Thus, further study will be focused on a relationship between the progression of NBNC-HCC and NASH.

In conclusion, data mining disclosed complex associations of risk factors and clinical feature profiling associated with the staging of NBNC-HCC.

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A Study Design
B Data Collection
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D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

The incidence of hepatocellular carcinoma associated with hepatitis C infection decreased in Kyushu area

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Summary

Background: The incidence of hepatocellular carcinoma (HCC) in Japan has still been increasing. The aim of the present study was to analyze the epidemiological trend of HCC in the western area of Japan, Kyushu.

Material/Methods: A total of 10,010 patients with HCC diagnosed between 1996 and 2008 in the Liver Cancer study group of Kyushu (LCSK), were recruited for this study. Cohorts of patients with HCC were categorized into five year intervals. The etiology of HCC was categorized to four groups as follows; B: HBsAg positive, HCV-RNA negative, C: HCV-RNA positive, HBsAg negative, B+C: both of HBsAg and HCV-RNA positive, non-BC: both of HBsAg and HCV-RNA negative.

Results: B was 14.8% (1,485 of 10,010), whereas 68.1% (6,819 of 10,010) had C, and 1.4% (140 of 10,010) had HCC associated with both viruses. The remaining 1,566 patients (15.6%) did not associate with both viruses.
Cohorts of patients with HCC were divided into six-year intervals (1996–2001 and 2002–2007). The ratio of C cases decreased from 73.1% in 1996–2001 to 64.9% in 2002-2007. On the other hand, B and -nonBC cases increased significantly from 13.9% and 11.3% in 1996-2001 to 16.2% and 17.6% in 2002-2007, respectively.

Conclusions: The incidence of hepatocellular carcinoma associated with hepatitis C infection decreased after 2001 in Kyushu area. This change was due to the increase in the number and proportion of the HCC not only nonBC patients but also B patients.

key words: **hepatitis virus • hepatocellular carcinoma • Japan**

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Background

The three leading causes of death in Japan are malignancy neoplasms, cardiovascular diseases, and cerebrovascular diseases. Since 1981, malignant neoplasms have been the leading cause of death in Japan. For the last 30 years, liver cancer has been the third leading cause of death from malignant neoplasms in men. In women, liver cancer has ranked fifth during the past decade [1]. Hepatocellular carcinoma (HCC) accounts for 85% to 90% of primary liver cancers[2] and the age-adjusted HCC mortality rate has increased in recent decades in Japan [3]. Similarly, a trend of increasing rates of HCC has been reported from several developed countries in North America, Europe and Asia [4,5]. HCC often develops in patients with liver cirrhosis caused by hepatitis B virus (HBV), hepatitis C virus (HCV), excessive alcohol consumption, or nonalcoholic fatty liver disease. Of the hepatitis viruses which cause HCC, HCV is predominant in Japan [6–9].

Although the age-adjusted incidence of HCC has increased in Japan, sequential changes in etiology of HCC patients between 2001 and 2008 are not fully understood [10]. To clarify factors affecting epidemiological changes in Japanese HCC patients, especially the recent trend of HCC, we analyzed the epidemiological trend of HCC in the western area of Japan, Kyushu area.

Material and Methods

Patients

A total of 10,010 patients with HCC diagnosed between 1996 and 2008 in the Liver Cancer study group of Kyushu (LCSK), were recruited for this study. The diagnosis of HCC was based on AFP levels and imaging techniques including ultrasonography (USG), computerized tomography (CT), magnetic resonance imaging (MRI), hepatic angiography (HAG), and/or tumor biopsy. The diagnostic criteria for HCC were either a confirmative tumor biopsy or elevated AFP (≥20 ng/mL) and neovascularization in HAG and/or CT.

Etiology of HCC

A diagnosis of chronic HCV infection was based on the presence of HCV-RNA detected by polymerase chain reaction (PCR), whereas diagnosis of chronic HBV infection was based on the presence of hepatitis B surface antigen (HBsAg). The etiology of HCC was categorized to four groups as follows; **B:** HBsAg positive, HCV-RNA negative, **C:** HCV-RNA positive, HBsAg negative, **B+C**: both of HBsAg and HCV-RNA positive, **nonBC:** both of HBsAg and HCV-RNA negative.

Statistical analysis

The data were analyzed by the Mann-Whitney test for the continuous ordinal data, the x2 test with Yates' correction and the Fisher exact test for the association between two qualitative variables. The standard deviation was calculated based on the binomial model for the response proportion. P<0.05 was considered statistically significant.

Results

Clinical features of the studied patients

A total of 10,010 patients with HCC were diagnosed at our study group from 1996 to 2008. Table 1 show that the proportion of patients diagnosed with **B** was 14.8% (1,485 of 10,010), whereas 68.1% (6,819 of 10,010) had **C**, and an additional 1.4% (140 of 10,010) had HCC associated with both viruses. The remaining 1,566 patients (15.6%) did not associate with both viruses. In analysis of patients in HCC by category, the median age of patients at diagnosis of **B** was 57 years old significant younger than other types HCC(**C: 69, nonBC: 70,.B+C 65 years old**).

As shown in Figures 1 and 2, the number and ratio of **B** cases remained unchanged from1996 to 2001 and thereafter increased and plateaued, whereas **C** rapidly increased from 1996 to 2000 and thereafter decreased and plateaued. In addition, the number and ratio of the **nonBC** cases has increased continued gradually and continued in this study period.

Change of etiology in patients with HCC during the period 1996–2007 with 6-years intervals

Cohorts of patients with HCC were divided into six-year intervals (1996–2001 and 2002–2007). Table 2 show that the incident rate of **C** decreased significantly from 73.1% in 1996-2001 to 64.9% in 2002-2007 (1996–2001 vs. 2002–2007, p=<0.001). On the other hand, the incident rate of **B** and -**nonBC** increased significantly from 13.9% and 11.3% in 1996-2001 to 16.2% and 17.6% in 2002-2007, respectively. Not only the incident rate but also number of **B** and -**nonBC** became larger in same 6 years periods.

Table 3 shows that male/female ratio of **C** and -**nonBC** decreased significantly from 2.2 and 4.0 in 1996-2001 to 1.8 and 2.7 in 2002-2007, respectively (p<0.001). The ratio became clearly smaller, indicates an increase in female patients with **C** and **nonBC.** On the other hand, the male/female ratio of **B** patients did not significantly change during the period. The median age at diagnosis of **B, C,** and **nonBC** in six-year intervals were significant increase from 56 to 58, from 67 to 71 and from 68 to 71 years of age during the period.

Discussion

Our study was the twenty-three major liver center-based study designed to examine the sequential change in the background of HCC patients during the past 13 years, 1996–2008. More than 80% of our patients had chronic HBV or HCV infections. During this observation period, the number and proportion of HCC-C reached a peak in 2000 and thereafter decreased and became stabilized. Previous studies from Japan reported that the proportion of the HCC patients with HCV infection had been increased and reached a plateau in the period of 1981–2001 [1,3,10–12]. However, in our study, the number and proportion of the HCC patients with HCV infection cases decreased in 2001–2008. The reason may be explained as follows; interferon therapy for chronic hepatitis C may have been associated with a decreased incidence of HCC [13–17]. Oral supplementation with a oral branched-chain amino acids has been useful in the prevention HCC [18]. Finally, the chronically HCV-infected

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Table 1. The characteristic of HCC patients during the period of 1996–2008.

Age (y.o.)	B		C		nonB		B+C		Total
	Male	Female	Male	Female	Male	Female	Male	Female	
0–	1	0	0	1	0	0	0	0	**2**
10–	4	1	0	0	0	2	0	0	**7**
20–	6	2	1	0	1	1	0	0	**11**
30–	31	5	4	0	11	3	2	0	**56**
40–	204	22	130	12	32	15	12	0	**427**
50–	507	66	728	145	167	32	31	6	**1,682**
60–	287	118	1836	741	411	102	35	13	**3,543**
70–	140	64	1775	947	483	133	22	14	**3,578**
80–	9	18	271	214	97	65	1	4	**679**
90–	0	0	9	5	9	2	0	0	**58**
Total	**1,189**	**296**	**4,754**	**2,065**	**1,211**	**355**	**103**	**37**	**10,010**
	1,485 (4.8%)		6,819 (68.1%)		1,566 (15.6%)		140 (1.4%)		
Median	57	63	67	70	68	70	61	68	**67**
	57		69		70		65		
Mean	56	64	68	71	69	71	62	68	**67**
	58		68		68		63		
Range	1–87	14–89	27–94	0–93	28–96	17–90	36–82	55–82	**0–96**
	1–89		0–94		17–96		36–82		

Age: B vs. C p≤0.001; B vs. B+C p≤0.001; B vs. nonBC p≤0.001; C vs. BC p≤0.001; C vs. nonBC p=0.043; BC vs. nonB+C p≤0.001. IQR – interquartile range; SD – standard deviation.

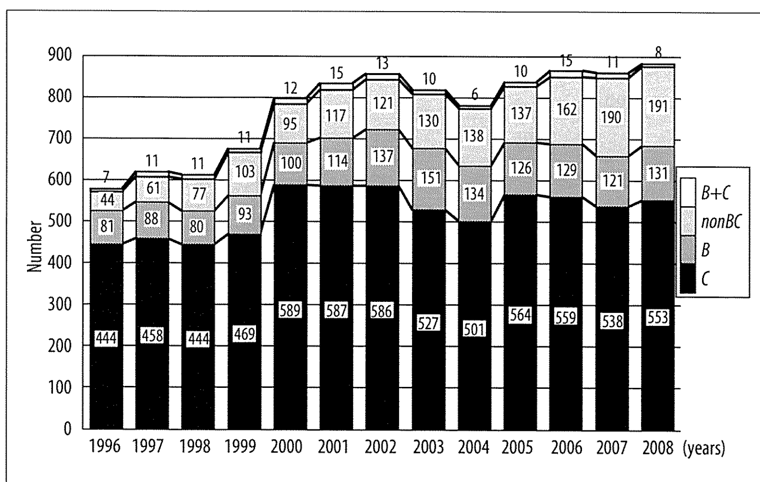

Figure 1. Sequential changes in the number of HCC patients categorized by etiology during the period 1996–2008.

population is aging in Japan. Yoshizawa et al. reported that age-specific prevalence for the presence of HCVAb among ~300,000 voluntary blood donors from Hiroshima in 1999 clearly increased with the age, reaching the highest proportion of 7% in individuals who were more than 70 years old [10,19]. In this study, the median age of the HCC patients with HCV infection steadily increased from 67 to 71 years of age during the studied period. In a word, HCV infected people become older with years in Japan and they were regarded as a high risk for HCC.

The prevalence rate of HBV in Kyushu area has been reported to be higher than other area in Japan [1]. In Kyushu area, 95% of patients with chronic HBV infection had HBV genotype C except for Okinawa [20]. HBV genotype C is thought to be associated with higher incidence of HCC

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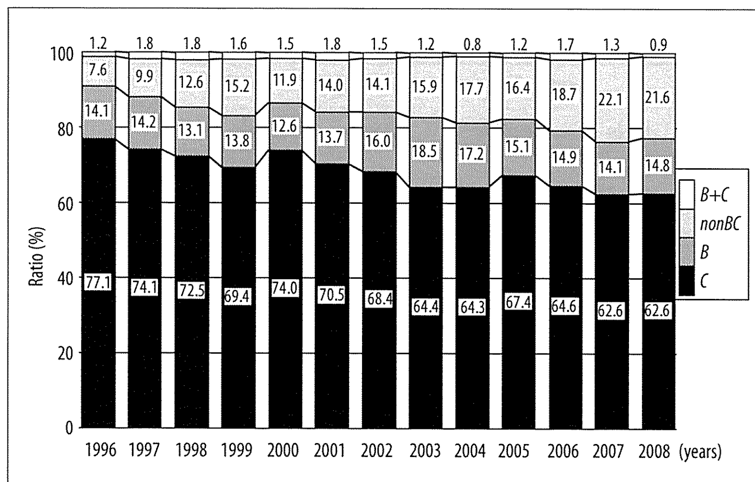

Figure 2. Sequential changes in the ratio of HCC patients categorized by etiology during the period 1996–2008.

Table 2. Change of etiology in patients with HCC during the period 1996–2007 with 6-years intervals.

Period		1996–2001	2002–2007	P value
Number		3,023	4,173	
Sex	Male	2,162	2,849	
	Female	861	1,324	
Ratio (male/female)		2.5	2.2	0.003
Age (y.o.) (IQR)		66 (14)	69 (12)	<0.001
Hepatitis virus (%)	B	13.9	16.2	
	C	73.1	64.9	
	B+C	1.7	1.3	
	nonBC	11.3	17.6	0.001

QR – interquartile range.

Table 3. The median age and male/female ratio of HCC patients during the period of 1996–2007.

Period		1996–2001	2002–2007	P value
B				
Age (y.o.) (IQR)		56 (14)	58 (15)	0.001
Sex	Male	331	519	
	Female	88	157	
Ratio (male/female)		3.8	3.3	0.391
C				
Age (y.o.) (IQR)		67 (9)	71 (11)	<0.001
Sex	Male	1,524	1,753	
	Female	687	955	
Ratio (male/female)		2.2	1.8	0.002
nonBC				
Age (y.o.) (IQR)		68 (12)	71 (13)	<0.001
Sex	Male	273	534	
	Female	69	201	
Ratio (male/female)		4.0	2.7	0.012

QR – interquartile range.

compared with other HBV genotypes [21]. In the present study, the incident rate of HCC patients with HBV infection became larger in this study period. To explain this change, we must consider from two viewpoints. The one is that the number of patients with HCC caused by HCV infection decreased, the other is that the proportion of chronic HBV infected patients who have reached the age of developing HCC is relatively high as described below.

Nationwide health survey for HBsAg in the over 40 years of age population had been done between 2002 and 2006 in Japan. This survey reports indicated that the average HBsAg prevalence was 1.2% in the total Japanese population patients with chronic HBV infection [10] and the age-specific prevalence of HBsAg was higher in the group aged between 50 (1.4%) and 55 years (1.5%). In the HCC patients with HBV genotype C, the mean age was 55 years in Japan [20]. This overlap between age-specific prevalence and hepatocellular carcinogenic age would be associated with the increase of HCC patients with HBV infection. Nucleoside analogue reverse transcriptase inhibitor (NARTI) therapy effectively reduces the incidence of HCC in chronic hepatitis B patients [22,23]. However, Interferon therapy for chronic hepatitis C started from 1992, whereas NARTI therapy for HBV started from 2000 in Japan [24,25]. Hence, HBV associated HCC will probably decrease in Japan during the next 10 to 20 years.

The survey of HCC patients associated with nonBC infection in Japan was conducted by Inuyama Hepatitis Research Group from 1995 to 2003. The ratio of HCC patients with nonBC accounted 9.3% [1]. In the present study, the ratio of HCC patients with nonBC was 14.1%. Furthermore, the number and the proportion of HCC patients with nonBC have been gradually increasing in the periods. The current two studies account for the increase in number and proportion of HCC patients with nonBC. First, Lai et al. reported

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