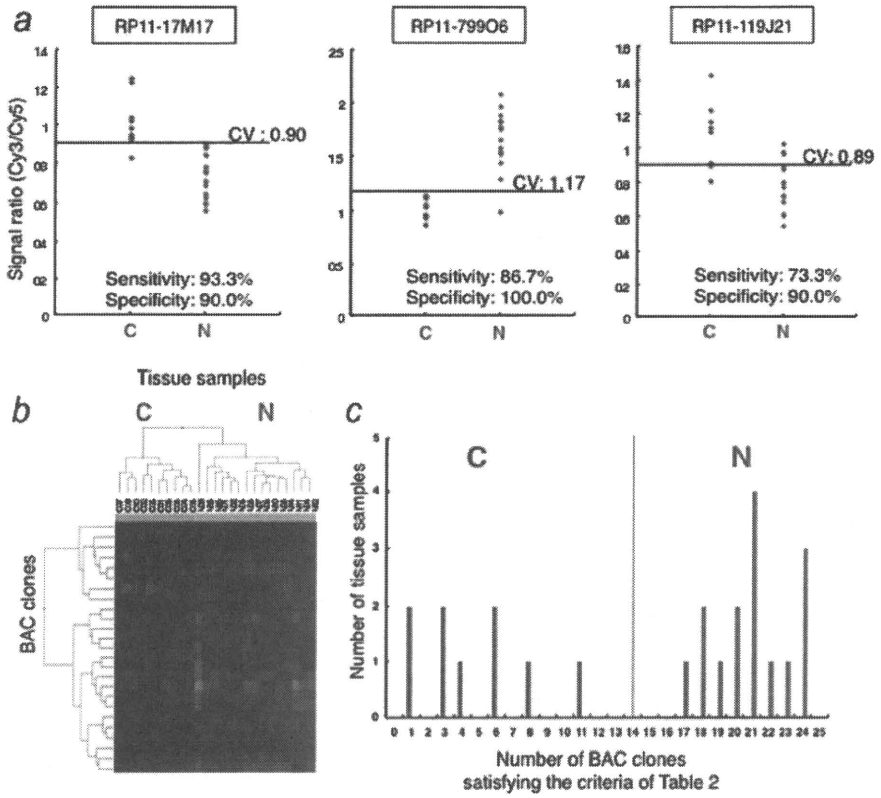

FIGURE 2 – DNA methylation profiles discriminating noncancerous liver tissue obtained from patients with HCCs from normal liver tissue. (*a*) Scattergrams of the signal ratios in normal liver tissue samples (C1 to C10) and noncancerous liver tissue samples obtained from patients with HCCs (N1 to N15) in the learning cohort on representative BAC clones, RP11-17M17, RP11-799O6 and RP11-119J21. Using the cutoff values (CV) described in each panel, noncancerous liver tissue samples obtained from patients with HCCs (N) in the learning cohort were discriminated from normal liver tissue samples (C) with sufficient sensitivity and specificity. (*b*) By 2-dimensional hierarchical clustering analysis using the 25 BAC clones selected by the process described in the Results section, normal liver tissue samples (C1 to C10) and noncancerous liver tissue samples obtained from patients with HCCs (N1 to N15) in the learning cohort were subclassified into the different subclasses without any error. The cluster trees for tissue samples and BAC clones are shown at the top and left of the panel, respectively. (*c*) Histogram showing the number of BAC clones satisfying the Table II criteria in samples C1 to C10 and N1 to N15. On the basis of this histogram, we established the following criteria: when the noncancerous liver tissue satisfied the criteria in Table II for 14 (green bar) or more than 14 BAC clones, it was judged to be at high risk of carcinogenesis.

signal ratio of HCCs did not differ from that of noncancerous liver tissue obtained from patients with HCCs (131 BAC clones). From the 512 BAC clones, 358 (Groups I, II, III and IV), in which the DNA methylation status was inherited by HCCs from noncancerous liver tissue, were selected. From the 358 BAC clones, the first 40 were identified by spot ranking analysis using the support vector machine algorithm for discrimination of noncancerous liver tissue obtained from patients with HCCs from normal liver tissue. Figure 2*a* shows scattergrams of the signal ratios in normal liver tissue samples and noncancerous liver tissue samples obtained from patients with HCCs on representative examples of the 40 BAC clones. Using the cutoff values described in each panel, noncancerous liver tissue obtained from patients with HCCs in the learning cohort was discriminated from normal liver tissue with sufficient sensitivity and specificity (Fig. 2*a*). From the 40 BAC clones, 25, for which such discrimination was performed with a sensitivity or specificity of 70% or more than 70%, were selected (Supporting Information Table S1). The cutoff values of the signal ratios for the 25 BAC clones, and their sensitivity and specificity, are shown in Table II. Two-dimensional hierarchical clustering analysis using the 25 BAC clones is shown in Figure 2*b*: 10 normal liver tissue samples (C1 to C10) and 15 noncancerous liver tissue samples obtained from patients with HCCs (N1 to N15) in the learning cohort were subclassified into different subclasses without any error. The number of BAC clones satisfying the criteria listed in Table II in noncancerous liver tissue samples showing chronic hepatitis (20.6 ± 1.8) was not significantly different from that showing cirrhosis (21.3 ± 2.4, $p = 0.542$) in the learning cohort.

A histogram showing the number of BAC clones satisfying the criteria listed in Table II for samples C1 to C10 and N1 to N15 is shown in Figure 2*c*. On the basis of this figure, we finally established the following criteria: when noncancerous liver tissue satisfied the criteria of Table II for 14 or more BAC clones (green bar in Fig. 2*c*), it was judged to be at high risk of carcinogenesis, and when noncancerous liver tissue satisfied the criteria of Table II for less than 14 BAC clones, it was judged not to be at high risk of carcinogenesis. Based on these criteria, both the sensitivity and specificity for diagnosis of noncancerous liver tissue samples obtained from patients with HCCs in the learning cohort as being at high risk of carcinogenesis were 100%.

To confirm these criteria, an additional 50 liver tissue samples were analyzed by BAMCA as a validation study (Supporting Information Figure S1). Twenty-three of 24 validation samples satisfying the criteria of Table II for 14 or more BAC clones were noncancerous liver tissue samples obtained from patients with HCCs (N16 to N36 and N38), and 24 of 26 validation samples satisfying the criteria of Table II for less than 14 BAC clones were normal

TABLE II – 25 BAC CLONES WHICH COULD DISCRIMINATE NONCANCEROUS LIVER TISSUES (N) FROM NORMAL LIVER TISSUES (C)

BAC clone ID	Location	Cutoff value	DNA methylation status[1]	Sensitivity (%)	Specificity (%)
RP11-104J13	1p35-1p36	1.01	C>N	93.3	70.0
RP11-52I2	1p34-1p35	1.00	C<N	80.0	60.0
RP11-29M22	1p11-1p12	1.11	C<N	86.7	90.0
RP11-21K1	2q37.2	1.00	C>N	86.7	70.0
RP11-109B15	5q33	1.04	C<N	66.7	90.0
RP11-88B24	6q26	0.95	C>N	80.0	70.0
RP11-112B7	7p13-7p14	1.00	C>N	80.0	70.0
RP11-48D21	8p11.2	1.00	C>N	80.0	90.0
RP11-120E20	11p15.4-11p15.5	0.90	C>N	73.3	100.0
RP11-334E6	11q23	1.00	C>N	86.7	80.0
RP11-17M17	11q25	0.90	C>N	93.3	90.0
RP11-319E16	12p13.32a	1.00	C>N	80.0	90.0
RP11-1100L3	12q13.13c-12q13.13d	1.04	C<N	86.7	80.0
RP11-799O6	12q13.3a-12q13.3b	1.17	C<N	86.7	100.0
RP11-119J21	12q24.33	0.89	C>N	73.3	90.0
RP11-332N6	14q11.2b	0.95	C>N	86.7	100.0
RP11-529E4	14q12c	1.00	C>N	93.3	50.0
RP11-89M4	16p13.2-16p13.3	1.20	C<N	86.7	100.0
RP11-215M5	15q15-15q21.1	1.00	C<N	86.7	70.0
RP11-348B12	19p13	1.00	C<N	80.0	80.0
RP11-134G22	20p11.2-20p12	1.01	C>N	80.0	90.0
RP11-328M17	22q13.2-22q13.33	0.93	C>N	86.7	100.0
RP11-354I12	22q13.31-22q13.33	1.00	C>N	93.3	80.0
RP11-55J11	22q13.2-22q13.33	1.00	C>N	80.0	70.0
RP11-480M11	Xq27.1-Xq28	0.90	C>N	80.0	90.0

[1]C>N, when the signal ratio was lower than the cutoff value, the tissue sample was considered to be at high risk for carcinogenesis; C<N, when the signal ratio was higher than the cutoff value, the tissue sample was considered to be at high risk for carcinogenesis.

liver tissue samples (C11 to C31, 33, 34 and 36). That is, our criteria enabled diagnosis of noncancerous liver tissue samples obtained from patients with HCCs in the validation set as being at high risk of carcinogenesis with a sensitivity of 95.8% and a specificity of 96.2%. The number of BAC clones satisfying the criteria listed in Table II in noncancerous liver tissue samples showing chronic hepatitis (17.6 ± 2.5) was not significantly different from that showing cirrhosis (19.4 ± 1.8, $p = 0.128$) in the validation cohort.

In addition, the average number of BAC clones satisfying the criteria in Table II was significantly lower in 7 samples of liver tissue obtained from patients who were infected with HBV or HCV, but who had never developed HCCs (V1 to V7, 13.14 ± 4.78), than that in N1 to N39 (19.21 ± 2.67, $p = 0.00419$).

Association of HCC DNA methylation profiles with patient outcome

To establish criteria for prognostication of patients with HCCs, in the learning cohort, 5 of 19 HCC samples obtained from patients who had survived more than 4 years after hepatectomy and 6 of 19 HCC samples from patients who had suffered recurrence within 6 months and died within a year after hepatectomy were defined as a favorable-outcome group and a poor-outcome group, respectively. Wilcoxon test ($p < 0.01$) revealed that the signal ratios of 41 BAC clones (Supporting Information Table S1) differed significantly between the favorable-outcome group ($n = 5$) and the poor-outcome group ($n = 6$). Figure 3*a* shows scattergrams of the signal ratios in samples from the favorable- and poor-outcome groups for representative examples of the 41 BAC clones. Using the cutoff values described in Figure 3*a* and Table III for the 41 BAC clones, samples from the poor-outcome group were discriminated from favorable-outcome group samples with sufficient sensitivity and specificity (Fig. 3*a* and Table III). Two-dimensional hierarchical clustering analysis using the 41 BAC clones is shown in Figure 3*b*: 5 HCCs in the favorable-outcome group and 6 HCCs in the poor-outcome group were subclassified into different subclasses without any error (Fig. 3*b*). A histogram showing the number of BAC clones satisfying the criteria in Table III is shown in Fig. 3*c*. In all 19 HCCs in the learning cohort, multivariate analysis revealed that satisfying the criteria in Table III for 32 or more BAC clones was a predictor of overall patient outcome and was independent of parameters that are already known to have prognostic impact,[20] such as histological differentiation, portal vein tumor thrombi, intrahepatic metastasis and multicentricity (Table IV).

To confirm these criteria, an additional 25 HCC samples were analyzed by BAMCA as a validation study, and then evaluated based on the criteria in Table III. All 44 HCCs were divided into 2 groups according to the number of BAC clones satisfying the criteria (32 or more BAC clones *vs.* less than 32 BAC clones). The period covered ranged from 11 to 3,413 days (mean, 1,349 days). The cancer-free and overall survival rates of patients with HCCs satisfying the criteria in Table III for 32 or more BAC clones was significantly lower than that of patients with HCCs satisfying the criteria in Table III for less than 32 BAC clones (Fig. 3*d*, $p = 0.000000002$ and $p = 0.0013$, respectively).

Discussion

Although many researchers in the field of cancer epigenetics use promoter arrays to identify the genes that are methylated in cancer cells,[21–23] we used a BAC array[19] in this study. The efficiency of identification of specific genes that are silenced by DNA methylations around the promoter regions and may become a target of therapy may be generally lower using the BAMCA approach than with conventional promoter array-based analysis. However, the promoter regions of specific genes are not the only target of DNA methylation alterations in human cancers. DNA methylation status in genomic regions not directly participating in gene silencing, such as the edges of CpG islands, may be altered at the precancerous stage before the alterations of the promoter regions themselves occur.[24] Moreover, aberrant DNA methylation of large regions of chromosomes, which are regulated in a coordinated manner in human cancers due to a process of long-range epigenetic silencing, has recently attracted attention.[25] BAMCA methods may be suitable for overviewing the DNA methylation status of individual large regions among all chromosomes and for

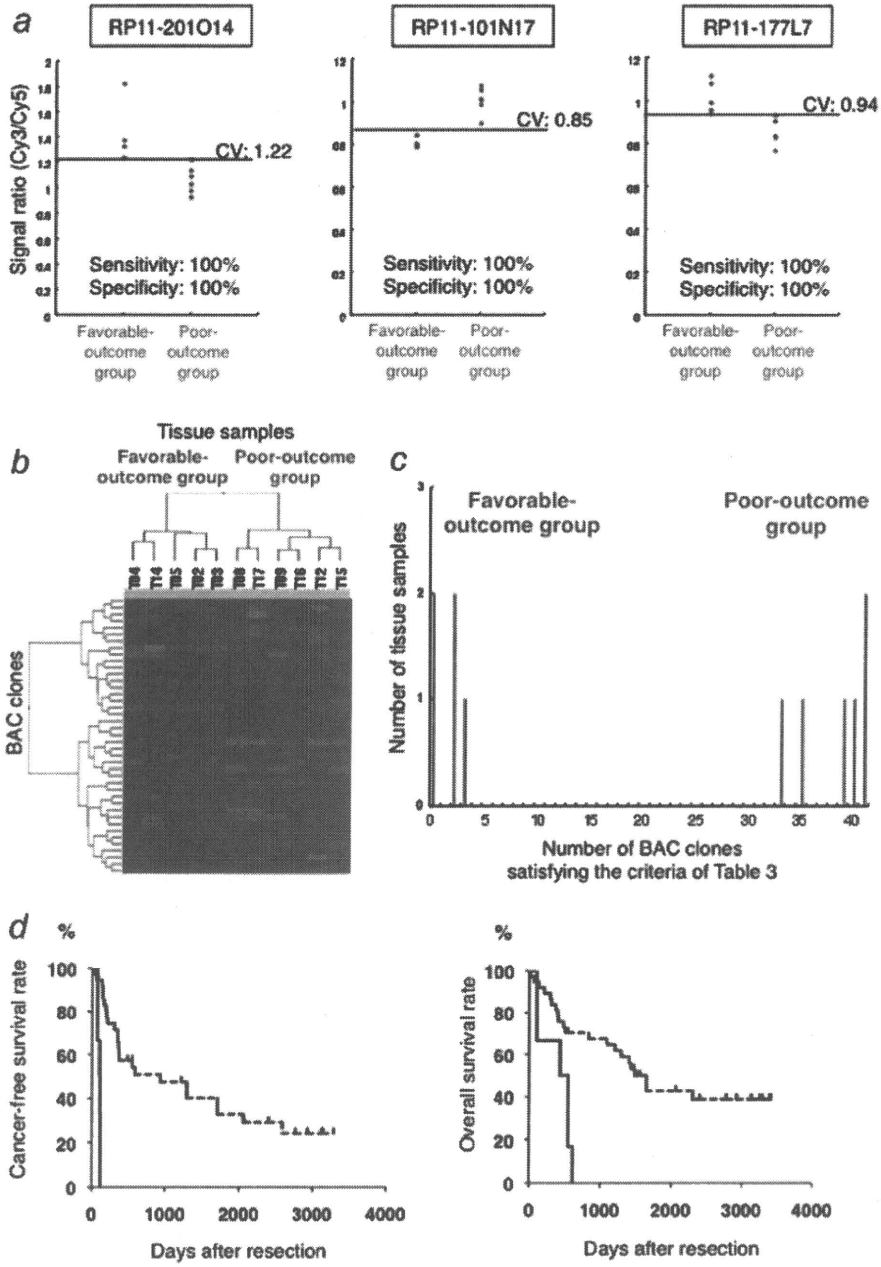

FIGURE 3 – DNA methylation profiles in HCCs associated with patient outcome. (*a*) Scattergrams of the signal ratios in HCCs from patients who survived more than 4 years after hepatectomy (favorable-outcome group, $n = 5$) and HCCs from patients who suffered recurrence within 6 months and died within a year after hepatectomy (poor-outcome group, $n = 6$) in the learning cohort for representative BAC clones, RP11-201O14, RP11-101N17 and RP11-177L7. Using the described cutoff values (CV), the poor-outcome group was discriminated from the favorable-outcome group with 100% sensitivity and specificity. (*b*) By 2-dimensional hierarchical clustering analysis using the 41 BAC clones selected by Wilcoxon test, HCCs in the favorable-outcome group and those in the poor-outcome group in the learning cohort were subclassified in the different subclasses without any error. The cluster trees for tissue samples and BAC clones are shown at the top and left of the panel, respectively. (*c*) Histogram showing the number of BAC clones satisfying the Table III criteria in HCCs of the favorable- and poor-outcome groups in the learning cohort. (*d*) Kaplan-Meier survival curves of all patients with HCCs (T1 to T44). The cancer-free (left panel, $p = 0.000000002$) and overall (right panel, $p = 0.0013$) survival rates of patients with HCCs satisfying the Table III criteria for 32 or more than 32 BAC clones (solid lines) were significantly lower than that of patients with HCCs satisfying the Table III criteria for less than 32 BAC clones (broken lines).

TABLE III – 41 BAC CLONES WHICH COULD DISCRIMINATE HCCS IN POOR-OUTCOME GROUP (P) FROM THOSE IN FAVORABLE-OUTCOME GROUP (F)

BAC clone ID	Location	Cutoff value	DNA methylation status[1]	Sensitivity (%)	Specificity (%)
RP11-89K16	1p35	1.50	F<P	83.3	100.0
RP11-201O14	1p34.3-1p36.13	1.22	F>P	100.0	100.0
RP11-156K6	1p31.1-1p31.3	1.15	F>P	100.0	80.0
RP11-553K8	1q31.2-1q31.3	1.16	F>P	100.0	100.0
RP11-89E10	1q31.3	0.91	F<P	100.0	100.0
RP11-180L21	2p16-2p21	1.29	F>P	100.0	80.0
RP11-90B13	2p14-2p15	1.13	F>P	83.3	100.0
RP11-449B19	2q11.2	0.75	F<P	100.0	80.0
RP11-30M1	2q32.3	1.10	F<P	100.0	100.0
RP11-89B13	2q32.3-2q33.1	1.11	F>P	83.3	80.0
RP11-255O19	3p24.3-3p25	1.08	F>P	100.0	100.0
RP11-421F9	3p24.2a	0.97	F>P	83.3	100.0
RP11-122D19	3p21.2	0.99	F<P	100.0	80.0
RP11-36K8	4q22	0.91	F>P	83.3	100.0
RP11-101N17	4q26	0.85	F<P	100.0	100.0
RP11-177L7	4q32	0.94	F>P	100.0	100.0
RP11-13O14	4q34-4q35	0.88	F<P	83.3	100.0
RP11-88H16	5p14	0.85	F<P	100.0	100.0
RP11-91G9	5q22-5q23	1.45	F<P	83.3	100.0
RP11-79K22	6q16	0.98	F<P	83.3	100.0
RP11-126B8	7q21.3	1.06	F>P	100.0	100.0
RP11-89P11	7q35	0.83	F>P	83.3	100.0
RP11-88N8	8q21.11d	1.02	F>P	100.0	100.0
RP11-85C21	9q33.3-9q34.2	0.95	F<P	83.3	100.0
RP11-714M16	10q26.11-10q26.3	1.00	F<P	100.0	100.0
RP11-48A2	10q26.2	0.69	F<P	100.0	80.0
RP11-206I1	11p11.2	1.20	F<P	100.0	100.0
RP11-35F11	11q12	1.30	F<P	100.0	80.0
RP11-158I9	11q23	1.04	F>P	83.3	100.0
RP11-74I8	12q13	1.13	F<P	100.0	100.0
RP11-167B4	16p13.3	0.97	F>P	83.3	100.0
RP11-368N21	16p11.2-16p12	1.10	F>P	83.3	100.0
RP11-303G21	16q12.1b	0.80	F>P	83.3	100.0
RP11-151M19	16q22	1.05	F>P	100.0	100.0
RP11-135N5	17p13.2	1.00	F>P	100.0	100.0
RP11-398A1	17q11.2d	1.00	F>P	100.0	100.0
RP11-15A1	19q13	1.08	F>P	83.3	100.0
RP11-697B10	19q13.3	0.90	F>P	83.3	100.0
RP11-79A3	19q13.3	1.05	F<P	100.0	100.0
RP11-29H19	20q12	1.00	F>P	100.0	100.0
RP11-36N5	22q11.2	1.15	F>P	83.3	100.0

[1]F>P, when the signal ratio was lower than the cutoff value, the tissue sample was considered to have been obtained from a patient with poor prognosis; F<P, when the signal ratio was higher than the cutoff value, the tissue sample was considered to have been obtained from a patient with poor prognosis.

identifying reproducible indicators for carcinogenetic risk estimation and prognostication. In fact, we have successfully obtained optimal indicators for carcinogenetic risk estimation and prognostication of renal cell carcinomas[26] and urothelial carcinomas (data will be published elsewhere) by BAMCA using the same array as that used in this study.

Our previous studies indicated that alterations of DNA methylation are one of the earliest events of multistage hepatocarcinogenesis and participate in malignant progression of HCCs.[5,7-14,27-29] However, since in previous studies we examined DNA methylation status on only a restricted number of CpG islands or chromosomal loci, it has not yet been clarified whether DNA methylation status on only restricted regions is simply altered at the precancerous stage, or whether genome-wide alterations of DNA methylation status have certain clinicopathological significance. As shown in Panel N of Figure 1*b*, genome-wide DNA methylation alterations (both hypo- and hypermethylation) were confirmed even in noncancerous liver tissue samples obtained from patients with HCCs. The number of BAC clones showing DNA methylation alterations and the degree of DNA methylation alterations were found to increase stepwise from the precancerous stage to the HCC stage (Fig. 1*b* and Table I). This study revealed that alterations of DNA methylation during multistage hepatocarcinogenesis occur in a genome-wide manner. Genome-wide DNA methylation alterations may participate in multistage hepatocarcinogenesis potentially through the induction of chromosomal instability and silencing of tumor-suppressor genes. DNA methylation alterations in noncancerous liver tissue were correlated with the future development of HCCs, suggesting that DNA methylation alterations at the precancerous stage may not occur randomly but are prone to further accumulation of genetic and epigenetic alterations.

Although mass vaccination against HBV has been initiated, this will not have a major impact for many years, as the age at presentation of HBV is older than 50 years mainly in Asia and Africa.[30] The spread of HCV in Japan that occurred in the 1950s and 1960s has resulted in a rapid increase in the incidence of HCC since 1980. In other countries including the United States, where HCV infection spread more recently, an increase in the incidence of HCC is imminent.[31] Although there were no significant differences in the number of BAC clones showing DNA hypo- or hypermethylation between HBV- and HCV-positive patients with HCCs, Wilcoxon test identified BAC clones in which DNA methylation status differed significantly between HBV- and HCV-positive patients with HCCs in both noncancerous liver tissue and cancerous tissue, suggesting that the HBV-related carcinogenetic

TABLE IV – MULTIVARIATE ANALYSIS OF CLINICOPATHOLOGICAL PARAMETERS AND DNA METHYLATION PROFILES ASSOCIATED WITH OVERALL OUTCOME IN PATIENTS WITH HCCS

Parameters	Hazard ratio (95% CI)	χ^2	p
Histological differentiation			
Well differentiated	1 (Reference)	0.031	0.8594
Moderately or poorly differentiated	0.817 (0.088-7.616)		
Portal vein tumor thrombi			
Negative	1 (Reference)	2.095	0.1478
Positive	4.474 (0.588-34.033)		
Intrahepatic metastasis[1]			
Negative	1 (Reference)	0.090	0.7647
Positive	1.248 (0.292-5.336)		
Multicentricity[1]			
Negative	1 (Reference)	1.499	0.2209
Positive	0.328 (0.055-1.955)		
The criteria of Table 3			
Satisfying for less than 32 BAC clones	1 (Reference)	4.997	0.0254
Satisfying for 32 or more BAC clones	4.466 (1.202-16.585)		

CI, confidence interval.
[1]In patients with multiple lesions, whether the lesions other than the main tumor from which tissue samples were obtained for this study were intrahepatic metastases of the main tumor or second primary lesions was judged by microscopic observation of hepatectomy specimens based on the previously described criteria.[35]

pathway may result in distinct DNA methylation profiles. These findings are in accordance with a previous report showing that HBV-related proteins can induce DNA methylation alterations.[32]

The effectiveness of surgical resection for HCC is limited, unless the disease is diagnosed early at the asymptomatic stage. Therefore, surveillance at the precancerous stage will become a priority. To reveal the baseline liver histology, microscopic examination of liver biopsy specimens is performed in patients with HBV or HCV infection prior to interferon therapy.[33,34] Therefore, carcinogenetic risk estimation using such liver biopsy specimens will be advantageous for close follow-up of patients who are at high risk of HCC development. Because even subtle alterations of DNA methylation profiles at the precancerous stage are stably preserved on DNA double strands by covalent bonds, they may be better indicators for risk estimation than mRNA and protein expression profiles that can be easily affected by the microenvironment of precursor cells.

The present genome-wide analysis revealed DNA methylation profiles that were able to discriminate noncancerous liver tissue obtained from patients with HCCs from normal liver tissue and diagnose it at high risk of HCC development in the learning set. The sensitivity and specificity in the validation set were 95.8 and 96.2%, respectively, and the criteria listed in Table II were validated. For carcinogenetic risk estimation using liver biopsy specimens obtained prior to interferon therapy, DNA methylation profiles actually associated with carcinogenesis should be discriminated from those associated with inflammation and/or fibrosis. Therefore, we first omitted potentially insignificant BAC clones associated only with inflammation and/or fibrosis and focused on BAC clones for which DNA methylation status was inherited by HCCs from the precancerous stage (Groups I, II, III and IV). In fact, it was confirmed that there were no significant differences in the number of BAC clones satisfying the criteria in Table II between noncancerous liver tissue samples showing chronic hepatitis and noncancerous liver tissue samples showing cirrhosis, not only in the learning set ($p = 0.542$) but also in the validation set ($p = 0.128$), indicating that our criteria were not associated with the degree of inflammation or fibrosis. In addition, the average numbers of BAC clones satisfying the criteria in Table II were significantly lower in liver tissue of patients without HCCs (V1 to V7) than in noncancerous liver tissue of patients with HCCs (N1 to N39), even though the patients from whom V1 to V7 were obtained were infected with HBV or HCV. Therefore, our criteria not only discriminate noncancerous liver tissue obtained from patients with HCCs from normal liver tissue but may also be applicable for classifying liver tissue obtained from patients who are followed up because of HBV or HCV infection, chronic hepatitis or cirrhosis into that which may generate HCCs and that which will not. Our criteria are applicable to both patients with chronic hepatitis and liver cirrhosis, although liver cirrhosis is known to show a more pronounced tendency to lead to HCC development than chronic hepatitis.[20] We intend to validate the reliability of such risk estimation prospectively using liver biopsy specimens obtained prior to interferon therapy from a large cohort of patients. On the basis of the present data, we now consider it justifiable to propose that clinicians can apply a portion of biopsy cores for this type of prospective study.

Because a sufficient quantity of good-quality DNA can be obtained from liver biopsy specimens, PCR-based analyses focusing on individual CpG sites are not always required. Although cut-off values should be modified for widely available standardized reference DNA, array-based analysis that overviews aberrant DNA methylation in each BAC region is immediately applicable to routine laboratory examinations. Moreover, because DNA methylation status of CpG sites is often regulated in a coordinated manner in each individual large region on chromosomes,[13,14,25] an overview of the DNA methylation tendency (hypo- or hypermethylation) in the whole BAC region can be a more reproducible diagnostic indicator than one focusing on individual CpG sites.

The present genome-wide analysis revealed DNA methylation profiles that were able to discriminate a poor-outcome group from a favorable-outcome group. Correlation between the DNA methylation profiles and both cancer-free and overall survival rates of patients with HCCs (Fig. 3*d*) validated the criteria in Table III. Prognostication based on our criteria may be promising for supportive use during follow-up after surgical resection, because multivariate analysis revealed that our criteria can predict overall patient outcome independently of parameters observed in hepatectomy specimens that are already known to have prognostic impact.[20] Such prognostication using liver biopsy specimens obtained before transarterial embolization, transarterial chemoembolization and radiofrequency ablation may be advantageous even to patients who undergo such therapies. The reliability of such prognostication needs to be validated again prospectively in surgically resected specimens or biopsy specimens.

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Favorable Long-Term Surgical Outcomes of Hepatocellular Carcinoma in Patients With Hepatitis B Envelope Antibody

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Background: Surgical outcome of patients with hepatocellular carcinoma (HCC) in relation to the serum hepatitis B envelope (HBe) has not been clarified in detail.
Methods: We retrospectively studied 732 patients with HCC within the Milan criteria who underwent hepatectomy from 1991 through 2005. Serum hepatitis B surface antigen (HBs-Ag) and hepatitis C virus (HCV) antibody were routinely performed preoperatively, and 92 patients were only positive for HBs-Ag. Serum HBe antibody (HBe-Ab) was further examined, and surgical outcomes after hepatectomy were compared with those of 70 patients who were positive for HBe-Ab and 15 patients who were negative for HBe-Ab.
Results: The 5- and 10-year survival rates were significantly greater in patients who were positive for HBe-Ab (90% and 80%, respectively) than in patients who were negative for HBe-Ab (61% and 37%: $P = 0.0004$, respectively). The 5-year recurrence-free survival rate was significantly greater in patients who were positive for HBe-Ab (53%) than in patients who were negative for HBe-Ab (21%: $P = 0.0054$). Multivariate analysis showed that positive HBe-Ab was an independent prognostic factor for survival ($P = 0.0045$) and recurrence-free survival ($P = 0.004$).
Conclusions: Favorable long-term surgical outcomes were achieved in patients with HCC within the Milan criteria who were positive for HBe-Ab.
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KEY WORDS: **hepatocellular carcinoma, hepatitis B envelope, hepatoctomy**

INTRODUCTION

Worldwide, most hepatocellular carcinomas (HCC) develop in patients with chronic hepatitis or liver cirrhosis caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infection. Higher HBV viral loads are strongly associated with the development of HCC and recurrence of HCC after surgery, and serum hepatitis B envelope antigen (HBe-Ag) is a simple surrogate marker of HBV-DNA [1–5]. Moreover, in patients with HBV-related small HCC, higher rates of intrahepatic recurrence of HCC and poorer survival in patients who were positive for HBe-Ag than in those who were negative for HBe-Ag have also been reported [6,7]. However, there have been no previous reports of surgical outcomes of HCC within Milan criteria in relation to the HB envelope. Therefore, there might be a difference in outcome after hepatectomy for HCC within the Milan criteria in relation to the HB envelope.

MATERIALS AND METHODS

Between 1991 and 2005, 1,188 patients with HCC underwent initial curative hepatectomy for HCC at our institute. Of these, 732 patients were given diagnoses of HCC within the Milan criteria (550 patients were solitary HCC of 5 cm or less and 182 patients were 2 or 3 HCCs of 3 cm or less) on histopathological examination. Laboratory tests, serum hepatitis B surface antigen (HBs-Ag), and HCV-antibody were routinely performed preoperatively in all patients, and 92 patients (13%) were found to be positive for HBs-Ag, 522 (71%) positive for HCV, 7 (1%) positive for both HBs-Ag and HCV, and 111 (15%) negative for either HBs-Ag or HCV. Serum HBe-Ag and HBe-Ab were further examined preoperatively in 85 of 92 patients with HBs-Ag, showing 70 patients to be positive for HBe-Ab and 15 patients to be negative for HBe-Ab. The remaining seven patients were excluded from this study because HBe-Ag or HBe-Ab was not examined. We retrospectively compared the results with the surgical outcomes of 70 patients who were positive for HBe-Ab and 15 patients who were negative for HBe-Ab. Five hundred twenty-two patients with HCV, 7 patients who were positive for both HBs-Ag and HCV, and 111 patients who were negative for either HBs-Ag or HCV were excluded from this study.

There were 67 men (79%) and 18 women (21%), and the mean age was 55 years (range, 22–76 years). Serum aspartate aminotransferase (AST), Child–Pugh class, indocyanine green retention rate at 15 min ($ICGR_{15}$), Platelet count, and serum level of alpha-fetoprotein (AFP) were examined preoperatively. Serum hepatitis B viral load was examined in our hospital from 1997. However, serum hepatitis B viral load was not examined routinely, therefore hepatitis B viral load was assessed in 31 of 85 patients with positive HBs-Ag. All patients underwent hepatectomy, and all surgical procedures were systematized hepatectomy with the Glissonean pedicle transection method [8,9]. The choice of resection was made on the basis of the tumor size, tumor type, and liver function ($ICGR_{15}$). In most of the patients with small and simple nodular HCC without daughter lesions, segmentectomy or smaller resection was performed. However, in patients with large and simple nodular HCC, sectionectomy or larger resection was performed considering functional liver reserve. In patients with HCC with daughter lesions within the section,

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sectionectomy or larger resection was performed. However, in patients with HCC with daughter lesions and poor functional liver reserve, segmentectomy or smaller resection was performed. Pathologically, macroscopic tumor type, portal or hepatic vein invasion, intrahepatic metastasis, and multicentric occurrence were examined. The terminology of liver resection was determined based on the Terminology Committee of the International Hepato-Pancreato-Biliary Association in 2000 [10]. Pathological findings were evaluated according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer of the Liver Cancer Study Group of Japan [11].

Follow-up

After surgery, patients were followed up every 4–12 weeks at the outpatient department of our institution. Ultrasonography or computed tomography was performed once every 3–4 months. Intrahepatic recurrence was defined clinically as the appearance of a new lesion with radiological features typical of HCC, as confirmed by the above two imaging methods or biopsy specimens. Survival duration was defined as the time from hepatic resection to the date of death or last contact.

Statistical Analysis

Because of sample size limitations, patients with small nodular type with indistinct margins were combined with those with simple nodular type. Patients with simple nodular with extranodular growth type, confluent multinodular type, and massive type were combined into a single group. Categorical variables were assessed using the chi-square test and continuous variables were assessed using the unpaired *t*-test or Fisher's test. The overall survival and recurrence-free survival rates among the patients were calculated by the Kaplan–Meier method and compared with the log-rank test. Univariate prognostic factors were entered into multivariate analysis (Cox's proportional hazard model) to identify independent predictors of survival or recurrence. *P*-values <0.05 were taken to indicate the statistical significance. We used Stat View (version 4.5 Hulinks, Tokyo, Japan) for statistical analysis.

RESULTS

Patient characteristics in relation to HB envelope are shown in Table I. The mean AST, $ICGR_{15}$, platelet count, and AFP did not differ between patients who were positive for HBe-Ab and those who were negative for HBe-Ab. Serum hepatitis B viral loads were assessed in 31 of 85 patients with positive HBs-Ag, and there was no significant difference between patients who were positive for HBe-Ab and those who were negative for HBe-Ab. The number of cases of Child–Pugh class did not differ between groups, nor did the number of cases of cirrhosis, macroscopic tumor type, portal invasion, intrahepatic metastasis, multicentric occurrence, or surgical procedure.

The median patient follow-up was 72 months (ranging from 0.6 to 209 months). No patient died after surgery in hospital. The overall survival rate and recurrence-free survival rate for 85 patients with HCC within the Milan criteria and HBs-Ag were 86% and 48%, at 5 years, respectively. The overall survival curves and recurrence-free survival curves in relation to the HBe-Ab are shown in Figures 1 and 2. The 5- and 10-year survival rates were significantly greater in patients who were positive for HBe-Ab (90% and 80%, respectively) than in patients who were negative for HBe-Ab (61% and 37%: $P=0.0004$, respectively). The 5-year recurrence-free survival rate was significantly greater in patients who were positive for HBe-Ab (53%) than in patients who were negative for HBe-Ab (21%: $P=0.0054$).

Outcomes were compared considering subgroups of the Milan criteria. In patients with a single HCC <5 cm or less, the 5-year survival rate was significantly greater in patients who were positive for HBe-Ab (90%) than in patients who were negative for HBe-Ab (64%: $P=0.0008$), and the 5-year recurrence-free survival rate was tended to be greater in patients who were positive for HBe-Ab (58%) than in patients who were negative for HBe-Ab (31%: $P=0.09$). In patients with 2 or 3 HCCs <3 cm or less, the 5-year survival rate was tended to be greater in patients who were positive for HBe-Ab (92%) than in patients who were negative for HBe-Ab (50%: $P=0.37$), and the 5-year recurrence-free survival rate was significantly greater in patients who were positive for HBe-Ab (29%) than in patients with negative for HBe-Ab (14%: $P=0.0178$).

Outcomes were compared among groups with Child–Pugh class A. In patients with Child–Pugh class A, the 5-year survival rate and recurrence-free survival rate were significantly greater in patients who were positive for HBe-Ab (93% and 55%, respectively) than in patients with negative for HBe-Ab (57%: $P<0.0001$ and 23%: $P=0.0054$, respectively). However, in patients with Child–Pugh class B, assessment was not possible because of the limited sample size of patients who were positive for HBe-Ab ($n=3$) and negative for HBe-Ab ($n=2$).

The univariate analysis of prognostic factors and multivariate analysis by Cox's proportional hazard model are summarized in Tables II and III. The univariate prognostic factors were entered into a multivariate model to identify independent predictors of survival and recurrence-free survival. Multivariate analysis showed that positive

TABLE I. Patients Characteristics in Relation to the HB Envelope

		Positive for HBe-Ab (n = 70)	Negative for HBe-Ab (n = 15)	*P*-value
Serum hepatitis B viral load	Present	15 of 28 patients assessed	3 of 3 patients assessed	0.10
Sex	Male	57 (81%)	10 (67%)	0.20
Age (year)	Mean ± SD	56 ± 10	52 ± 11	0.06
AST (U/l)	Mean ± SD	39 ± 18	41 ± 16	0.82
$ICGR_{15}$ (%)	Mean ± SD	12 ± 8	14 ± 14	0.44
Platelet count ($\times 10^4/\mu l$)	Mean ± SD	14 ± 5	13 ± 7	0.41
log AFP (ng/ml)	Mean ± SD	1.8 ± 1.2	2.2 ± 1.4	0.14
Child–Pugh	A	67 (96%)	13 (87%)	0.21
Cirrhosis	Present	37 (53%)	10 (67%)	0.33
Surgical procedure	Sectionectomy	42 (60%)	10 (67%)	0.63
Macroscopic type	Simple nodular	49 (70%)	13 (87%)	0.19
Portal invasion	Present	11 (16%)	4 (27%)	0.31
Intrahepatic metastasis	Present	4 (6%)	2 (13%)	0.29
Multicentric occurrence	Present	12 (17%)	4 (27%)	0.47

HBe-Ab, hepatitis B envelope antibody; AST, aspartate aminotransferase; ICGR15, indocyanine green retention rate at 15 min; log AFP, logarithmic alpha-fetoprotein; sectionectomy, sectionectomy or larger resection.

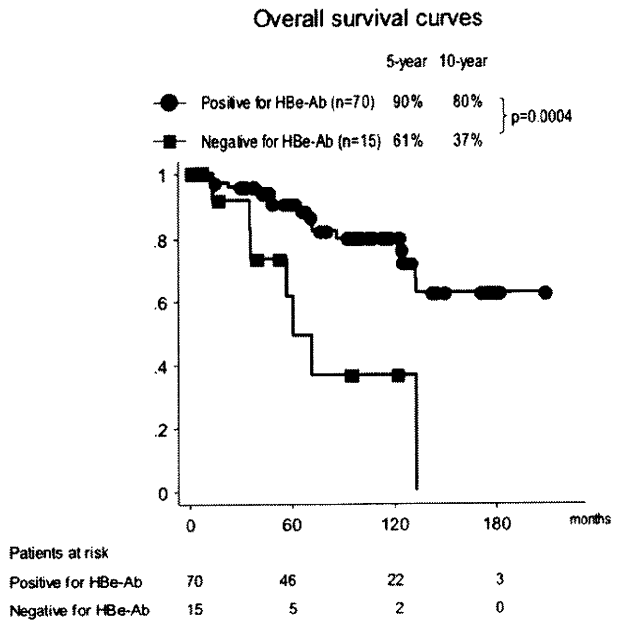

Fig. 1. The overall survival curves of patients with HCC within the Milan criteria in relation to the HB envelope are shown.

HBe-Ab was an independent prognostic factor for survival and recurrence-free survival in patients with HCC who met the Milan criteria.

DISCUSSION

It is well known that positive HBe-Ab means seroconversion in patients with HBV, and activity or progression of chronic hepatitis differs between patients who are positive for HBe-Ab (negative for HBe-Ag) and negative for HBe-Ab (positive for HBe-Ag) even in patients with HBs-Ag. Therefore, the higher carcinogenesis of HCC or poorer prognosis in patients who are positive for HBe-Ag than in patients who are negative for HBe-Ag has been reported [1]. Furthermore, poorer surgical outcomes after hepatectomy for small HCC in patients who are positive for HBe-Ag than in patients who are negative for HBe-Ag have been reported [6,7] However, there have been no previous reports of surgical outcomes of HCC within Milan criteria in relation to the HB envelope. In this study, lower risk of recurrence and longer survival were seen in patients who were positive for HBe-Ab than in patients who were negative for HBe-Ab. Moreover, multivariate analysis showed positive HBe-Ab to be a significant independent prognostic factor for survival and recurrence-free survival in patients with HBV-related HCC within the Milan criteria.

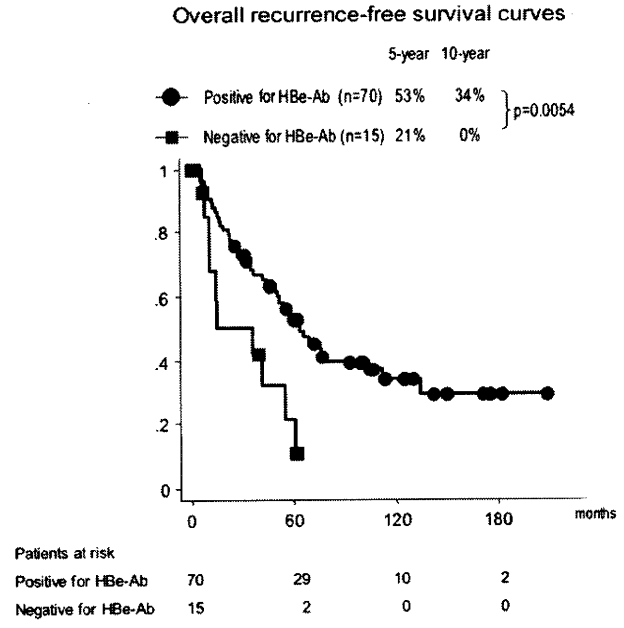

Fig. 2. The overall recurrence-free survival curves of patients with HCC within the Milan criteria in relation to the HB envelope are shown.

Liver transplantation in patients with a solitary HCC of 5 cm or less or 2 or 3 HCCs of 3 cm or less has obtained better results with regard to both survival and tumor-free survival rates [12–16]. Greater survival rate and recurrence-free survival rate after liver transplantation for patients with HCC within the Milan criteria than after hepatectomy have been reported [17–19]. However, Poon et al. also reported that, although recurrence after hepatectomy was higher than after liver transplantation, there was no significant difference in the long-term survival after resection and transplantation for patients with HBV-related HCC within the Milan criteria [15]. In the present study, favorable surgical outcomes, not only 5-year survival (90%) but also 10-year survival (80%) were achieved in patients with HBV-related HCC within the Milan criteria and positive HBe-Ab. Hepatectomy is therefore recommended in patients with HCC within the Milan criteria and positive HBe-Ab.

Recently, practice guidelines for the treatment of HCC have been published by American Association for the Study of Liver Disease (AASLD) [20,21] and in Japan [22]. According to the AASLD, surgical resection is recommended for patients who have a single HCC, Child–Pugh class A, and no cirrhotic or cirrhosis but still well-preserved liver function (normal bilirubin and hepatic vein pressure gradient <10 mmHg). According to the Japanese guidelines, liver resection is recommended for patients who have a single HCC or 2 or 3 HCCs with the liver damage categorized into class A or B. There is no consideration of either HBV or HCV infection in both algorithms. Routine tests for HB envelope are required to consider the treatment strategy for HBV-related HCC within the Milan criteria, because greater long-term survival after hepatectomy can be expected in patients with HBV-related HCC within the Milan criteria and positive HBe-Ab.

There have been reports regarding the effects of antiviral drugs on the prevention of initial HCC and recurrence of HCC after treatment. Kubo et al. reported that 24 patients with high-serum HBV DNA concentrations who underwent liver resection for HBV-related HCC and postoperative lamivudine therapy had improved tumor-free survival rates after liver resection [4]. In the present study, we did not compare outcomes in relation to the antiviral drug because the study period was long, from 1991 to 2005, and postoperative antiviral therapy was recently performed in only six patients from 2000. However, antiviral therapy after hepatectomy for patients with HCC and negative HBe-Ab is required because shorter survival and higher risk of recurrence after hepatectomy were seen in patients who were negative for HBe-Ab than in patients who were positive for HBe-Ab.

There have been only a few reports on outcomes after hepatectomy for small HCC in relation to the HB envelope [6,7]. In the present study, favorable long-term surgical outcomes were achieved in patients with HBV-related HCC within the Milan criteria and positive HBe-Ab. Multivariate analysis showed that positive HBe-Ab was an independent prognostic factor for survival and recurrence-free survival. Routine tests for HB envelope are required for patients with

TABLE II. Factors Associated With Survival Rate and Recurrence-Free Survival Rate of Patients by Univariate Analysis

	Number	5-year survival (%)	*P*-value	5-year recurrence-free survival (%)	*P*-value
Sex					
Male	67	87	0.34	53	0.0437
Female	18	85		26	
Age (years)					
<55	45	84	0.44	42	0.20
≥55	40	89		55	
Hepatitis B envelope antibody					
Positive	70	90	0.0004	53	0.0054
Negative	15	61		21	
Serum hepatitis B viral load[a]					
Present	15	100	0.09	43	0.58
Absent	16	79		46	
AST (U/l)					
<40	44	90	0.95	51	0.17
≥40	41	83		45	
$ICGR_{15}$ (%)					
<12	52	87	0.99	51	0.19
≥12	33	86		44	
Platelet count ($\times 10^4/\mu l$)					
≤14	49	81	0.06	43	0.06
>14	36	93		56	
Child–Pugh class					
A	80	88	0.0306	51	<0.0001
B	5	50		0	
Cirrhosis					
Present	47	81	0.21	45	0.18
Absent	38	93		45	
log AFP (ng/ml)					
≤1.9	50	82	0.08	45	0.24
>1.9	35	93		53	
Surgical procedure					
Sectionectomy	52	90	0.33	57	0.0235
Partial sectionectomy	33	80		33	
Macroscopic type					
Simple nodular	62	87	0.33	47	0.99
Others	23	84		53	
Portal invasion					
Present	15	77	0.41	49	0.90
Absent	70	88		48	
Intrahepatic metastasis					
Present	6	80	0.10	20	0.0089
Absent	79	87		50	
Multicentric occurrence					
Present	16	85	0.86	22	0.0019
Absent	69	86		54	

C.I., Confidence interval; HBe Ab, hepatitis B envelope antibody.
AST, aspartate aminotransferase; ICGR15, indocyanine green retention rate at 15 min; log AFP, logarithmic alpha-fetoprotein; sectionectomy, sectionectomy or larger resection.
[a]Thirty-one patients were assessed.

TABLE III. Multivariate Analysis Using Cox's Proportion Hazards Model

Risk factor			Relative risk	95% CI	*P*-value
Survival	Hepatitis B envelope antibody	Positive	0.250	0.096–0.651	0.0045
Recurrence	Hepatitis B envelope antibody	Positive	0.323	0.149–0.697	0.0040
	Intrahepatic metastasis	Present	5.546	1.821–16.862	0.0026

CI, confidence interval.

HBV-related HCC, and hepatectomy is recommended in patients with HCC within the Milan criteria and positive HBe-Ab.

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Original Paper

Specific Characteristics of Scirrhous Hepatocellular Carcinoma

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ABSTRACT

Background/Aims: Scirrhous hepatocellular carcinoma has been defined within general hepatocellular carcinoma. To define scirrhous hepatocellular carcinoma, the present study identified the specific characteristics as compared with general hepatocellular carcinoma.

Methodology: The scirrhous hepatocellular carcinoma was defined when almost all areas of the tumor were occupied with scirrhous structures. It was identified 14 patients with scirrhous hepatocellular carcinoma and 300 patients with general hepatocellular carcinoma (control), all underwent hepatectomy from 1988 to 1994. It was compared the clinical background of the patients, prognosis, and pathological features, which included immunohistological staining using Hepatocyte Paraffin 1.

Results: All scirrhous hepatocellular carcinoma showed whitish, hard forms resembling intrahepatic cholangiocarcinoma. The rates of average ICGR15, positive hepatitis C virus antibody, and microscopic invasion to the bile duct were 12%, 33%, and 20%, respectively, each significantly different than general hepatocellular carcinoma. The cumulative 10-year survival rate of scirrhous hepatocellular carcinoma and general hepatocellular carcinoma were 70% and 31%, respectively. With immunohistological staining, 43% of scirrhous hepatocellular carcinoma showed negative staining for Hepatocyte Paraffin 1.

Conclusions: The scirrhous hepatocellular carcinoma has specific characteristics such as lower rates of hepatitis C virus infection, better prognosis, and different histological findings; therefore, scirrhous hepatocellular carcinoma should be strictly classified under a new category.

INTRODUCTION

According to the Histological Typing of Tumors of the Liver (WHO International Histological Classification of Tumors) (1), scirrhous hepatocellular carcinoma (HCC) is described as "areas with abundant fibrosis stroma separating cords of tumor cells". "Scirrhous" is defined as a histological type of HCC indicating a specific area of a tumor, not defining the whole area of HCC (1). The structure of a scirrhous carcinoma could be detected partially in a HCC, mixed with other structures. Scirrhous HCC generally shows a macroscopic finding resembling mass-forming type of intrahepatic cholangiocarcinoma (ICC) (2-4). However, accumulated studies of scirrhous HCC are not sufficient because of its rare occurrence and vague definition. To strictly define scirrhous HCC, the present study attempted to identify specific characteristics of scirrhous HCC and compared its clinicopathological features with general HCC (2).

METHODOLOGY

In the present study, scirrhous HCC was defined as a carcinoma with almost all areas of the tumor occupied with scirrhous structures, characterized by abundant fibrosis stroma separating cords of tumor cells. The pathological diagnosis was confirmed by reviewing the available hematoxylin and eosin (H&E) stained slides by a pathologist. As for the general HCC which served as the control, the nonscirrhous, simple nodular type HCC was macroscopically selected. The simple nodular HCC was defined as a macroscopic type of HCC in the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (Liver Cancer Study Group of Japan) (2). The present study compared the clinical background, the pathological findings, and the prognosis between the scirrhous HCC and general HCC. It was performed immunohistological staining in scirrhous HCC using hepatic differentiation marker Hepatocyte Paraffin 1 (Hep), biliary differentiation marker Cytokeratin 19 (CK 19), MUC 1 glycoprotein (MUC 1), and epithelial membrane antigen (EMA).

Samples sections were fixed in formalin. Paraffin embedded cell block tissues were deparaffinized and hydrated in a series of xylene, graded alcohols, and water. To improve the staining methods for an-

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tigen retrieval, we microwave heated the samples in 10mM citrate buffer at pH 6.0. The endogenous peroxidase was blocked by immersing the samples in 3% hydrogen peroxide. The sections were then washed in a phosphate–buffered saline (PBS) solution. The primary antibody was applied before an overnight incubation at 4°C. The mouse monoclonal antibody was applied before an overnight incubation at 4°C. The mouse monoclonal antibody Hep (clone OCH1E5.2.10; DAKO, Carpenteria, CA) was used at a dilution of 1:50. The mouse monoclonal antibody CK 19 (clone RCK108; DAKO) was used at a dilution of 1:200. The monoclonal antibody Muc 1 (clone Ma695; DAKO) was used at a dilution of 1:1000. The mouse monoclonal antibody EMA (clone E29; DAKO) was used at a dilution of 1:300. After another wash with PBS, biotinylated goat antimouse secondary antibody (DAKO) was applied at room temperature for 40 minutes. The sections were washed again and treated for 40 minutes with streptavian conjugated to horseradish peroxidase (DAKO). The colorizing agent diaminobenzidine was used subsequently. The sections were counterstained with hematoxylin. PBS, instead of the primary antibody, was used as a negative control.

Normal hepatocytes around tumor cells were strongly stained for Hep with cytoplasmic, diffuse, and granular patterns. When more than 50% of the total tumor showed positive staining for Hep, the staining was graded as 2+. When 5 to 50% of the tumor showed positive staining for Hep, the staining was graded as 1+. When almost all (>95%) of the tumor showed negative staining for Hep, the staining was graded as -. CK 19, MUC 1, EMA staining patterns were graded similarly. Because Hep is a very useful marker for diagnosing HCC in that HCC shows more than 90% positive staining for Hep (5-9), the immunostaining for Hep in the 300 general HCC cases was not perform.

The present study was a retrospective study by which we examined the data of the patients from the database. Informed consents were not obtained from the patients at the time of study; however, all of our patients gave written consents at the time of treatment, stating that anonymous clinical data of the patients could be used for research purposes at a later date.

Statistical Analysis

Chi-square test and Student's t test were applied to compare for differences between groups. The survival curves were analyzed by Kaplan-Meier method and then were compared by log-rank test. For all tests, a p-value of less than 0.05 was considered significant.

RESULTS

Clinical background

From 1988 to 1994, 589 HCC were curatively resected in our hospital. Out of these HCC, 300 were general HCC, 14 (2.4%) were scirrhous HCC. Among

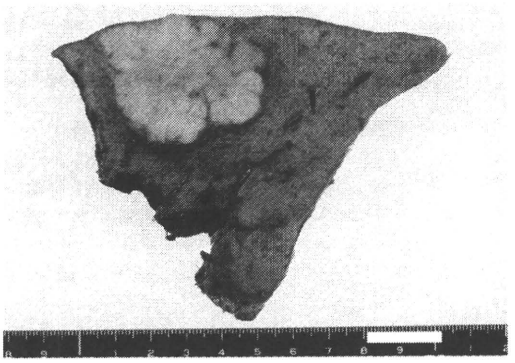

FIGURE 1
Typical macroscopic finding of scirrhous HCC

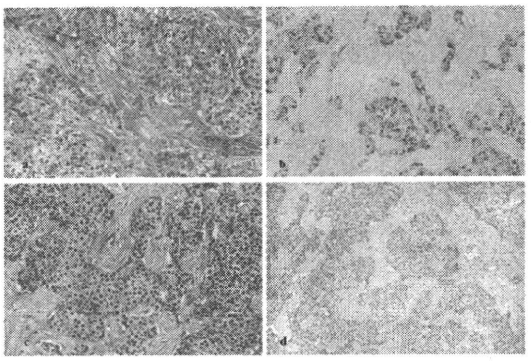

FIGURE 2
Immunohistological finding of scirrhous HCC with Hep positive staining (a, H&E staining; b, Hep staining) and Hep negative staining (c, H&E staining; d, Hep staining)

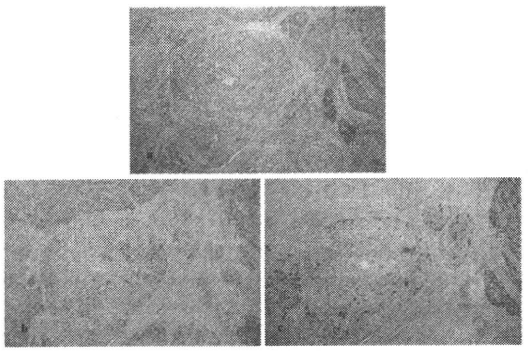

FIGURE 3
Immunohistological finding of scirrhous HCC with both Hep and CK 19 positive staining (a, H&E staining; b, Hep staining 2+; c, CK 19 staining 1+)

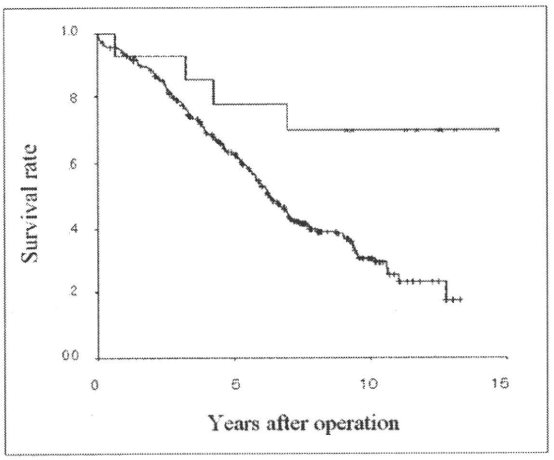

FIGURE 4
The survival curve of scirrhous HCC (xx; n=14) and general HCC (++; n=300). 10–year survival of general HCC=31%; 10–year survival of scirrhous HCC=70% (p=0.0095)

TABLE 1 Clinicopathological Characteristics of General HCC (n=300) and Scirrhous HCC (n=14)

	General HCC	Scirrhous HCC	p - value
Male (n)	230 (77%)	11 (79%)	NS
Age (yr; mean±SD)	61.3±7.8	60.2±9.0	NS
ICG $_R$15 (%; mean±SD)	18.8±10.2	12.3±6.5	<0.001
HBsAg (+) (n)	60 (20%)	2 (14%)	NS
HCV (+) (n)	216 (72%)	5 (33%)	<0.01
LC (n)	171 (57%)	5 (33%)	NS
Size (cm; mean±SD)	3.4±2.4	3.2±1.9	NS
Vp (n)	46 (15%)	3 (20%)	NS
Vv (n)	7 (2.3%)	0 (0%)	NS
B (n)	7 (2.3%)	3 (20%)	<0.01
IM (n)	40 (13%)	4 (27%)	NS
N (n)	4 (1.3%)	0 (0%)	NS
Log10(AFP) (mean±SD)	1.69±0.96	1.83±0.91	NS

ICG $_R$15: indocyanine green retention at 15 minutes; LC: liver cirrhosis; Vp: portal vein invasion; Vv: hepatic vein invasion; B: bile duct invasion; IM: intrahepatic metastasis; N: lymph node metastasis; AFP: alpha-fetoprotein

the scirrhous HCC, the mean rate of ICGR15 was 12%, 2 (14%) were hepatitis B surface antigen (HBsAg) positive, 5 (33%) were hepatitis C virus antibody (anti-HCV) positive, and 5 (33%) showed liver cirrhosis. Of the total scirrhous HCC, 7 (50%) had single HCC, and the other 7 (50%) had synchronous multiple HCC, of which one was scirrhous HCC and the rest were nonscirrhous HCC. 2 samples (14%) showed previous transcatheter arterial embolization (TAE), but the other 12 samples (86%) were primary HCC showing no previous treatment. No patients showed hypercalcemia in the blood examination.

Macroscopic finding (Figure 1)

All scirrhous HCC showed a whitish, hard form with slightly lobulated margin, which were obviously different than the general HCC. They rather resembled the mass-forming type of ICC (2). 6 cases (43%) also showed a macroscopic central scar, which resembled the finding of focal nodular hyperplasia (FNH) or fibrolamellar HCC.

Microscopic finding

For scirrhous HCC, the rate of bile duct invasion was 21% (3/14). The formation of a capsule was detected in 6 cases (43%), and all 6 cases showed infiltration to the capsule. The differentiation was determined as moderate in 13 cases (93%) and poor in 1 case (7%); none of the samples showed well differentiated cells.

Comparison between scirrhous HCC and general HCC (Table 1)

In the present study was compared the clinical and pathological findings of scirrhous HCC and general HCC. We found the rate of mean ICGR15 and the rate of anti-HCV positive significantly lower in the scirrhous HCC, whereas the rate of microscopic invasion to the bile duct was significantly higher in the scirrhous HCC ($p < 0.01$).

Immunohistological finding (Figure 2, 3)

In the immunohistological staining for Hep, scirrhous HCC showed predominantly positive (2+) in 57% (8/14), predominantly negative (1+) in 29% (4/14), and completely negative (-) in 14% (2/14) of the cases. 21% (3/14) of the cases also showed partial positive staining for the biliary marker CK 19 (2 cases of Hep 2+/CK 19 1+, 1 case of Hep -/CK 19 2+), and 14% (2/14) cases showed partial positive staining 1+ for MUC1 and EMA.

Postoperative prognosis (Figure 4)

For scirrhous HCC, the 5-year survival rate and no recurrent survival rate were 71% (10/14) and 43% (6/14), and the 10-year survival rate and no recurrent survival rate were 50% (7/14) and 21% (3/14), respectively. The recurrent patterns were all intrahepatic recurrence with no distant metastasis. The intrahepatic recurrent rate within 5 years of scirrhous HCC was 50% (7/14). For the general HCC, the 5-year survival rate and no recurrent survival rate were 20% (59/300) and 2% (6/300), and the 10-year survival rate and no recurrent survival rate were 0.7% (2/300) and 0% (0/300), respectively. The intrahepatic recurrent rate within 5 years was 77% (232 /300). The postoperative 5-year and 10-year survival rates and the intrahepatic recurrent rate of scirrhous HCC were all better than that of general HCC. With the Kaplan-Mayer method, we found the cumulative 5-year survival rate of scirrhous HCC and general HCC 77% and 63%, respectively, and the cumulative 10-year survival rate were 70% and 31%, respectively (Log Rank test, $p < 0.01$).

DISCUSSION

According to the Liver Cancer Study Group of Japan, the frequency of scirrhous HCC is comparatively rare, namely 1.3% in all surgical resected cases and 1.1% in autopsies (10). Other reports on scirrhous HCC have shown a lower rate of liver cirrhosis and comparatively good prognosis (3,11). However, there has been no definite agreement in other clinical characteristics of scirrhous HCC, such as the rate of HBs antigen positive, AFP, and hypercalcemia (3,11). In the clinical data, scirrhous type HCC showed significantly lower rate of HCV infection, liver cirrhosis, and lower data of ICGR15 compared with general HCC ($p<0.01$). The relatively good prognosis of scirrhous HCC is probably caused by the less liver damage from lower HCV infection rate and the lower intrahepatic multicentric occurrence rate after a hepatectomy. Therefore, there was a gradual widening gap in the 5 to 10 years survival curves between the two groups. Iha *et al.* (4) reported a significant lower rate of recurrence in 12 resected cases of scirrhous HCC compared with 307 cases of non-scirrhous HCC. In the Histological Typing of Tumors of the Liver (WHO International Histological Classification of Tumors), scirrhous HCC

is described as the most common tumor found after radiation therapy, chemotherapy, or infarction (1), but our result showed that schirrhous HCC could occur predominantly without previous treatments of TAE or radiation. Almost all (86%) patients in the present study had no previous treatment.

The pathological results showed interesting features of scirrhous HCC, which should be distinguished from general HCC. These features include 1) the macroscopic characteristics resembling ICC, 2) higher rate of microscopic invasion to the bile duct, 3) higher rate in the loss of positive staining for Hep, and 4) partial positive staining for CK 19, MUC1, EMA. These features suggest some similarities between scirrhous HCC and ICC. The similarity in their macroscopic form was probably because of the common abundant fibrous components. Also, in terms of microscopic invasion to the bile duct, there was a great difference between scirrhous HCC and resected ICC (21% vs 52%) (10). The high frequency of lymph node metastasis and the poor prognosis of ICC also clearly segregate itself from scirrhous HCC (10). Yamamoto *et al.* (12) reported HCC with a central scar and a scalloped tumor margin resembling focal nodular hyperplasia in macroscopic appearance. These scalloped HCC showed a good surgical outcome. A definite difference between the scalloped HCC and our scirrhous HCC was that the scalloped HCC was defined on a macroscopic finding, and our scirrhous HCC was defined on a microscopic finding. However, because both HCC showed similar macroscopic finding, there might be some overlapping characteristics between scalloped HCC and scirrhous HCC (12). In general, Hep is recognized as a very useful immunostaining antibody for HCC, where HCC showed more than 90% positive staining for Hep (5-9). Microscopic examination revealed that the antigen of Hep might be the mitochondria in the hepatocytes (5), and reduced differentiation of HCC showed lower rate of positive staining for Hep (13,14). Therefore, the high frequency of Hep negative staining in our study might suggest the loss of hepatic antigen and reduced differentiation in scirrhous HCC. In the immunostaining for the biliary markers (CK 19, EMA, MUC 1), 21% of scirrhous HCC showed partial positive staining suggesting partial existence of biliary differentiation in scirrhous HCC. Matsuura *et al.* (15) also reported a significantly higher expression of cytokeratin 7 and a lower expression of Hep in scirrhous HCC than the "ordinary" HCC, which by definition is a similar carcinoma as the general HCC. Based on the less damaged liver, the loss of hepatic differentiation, the existence of partial biliary differentiaion, and the tendency of the microscopic invasion to the bile duct, we suggest that scirrhous HCC could be placed in a special pathological position between general HCC and ICC. In the various histological changes of HCC, the mechanisms in the formation of abundant fibrous stroma and occurrence of scirrhous HCC are not clear. These factors need to be examined further from many aspects to further clarify the origin and the position of scirrhous HCC.

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Identification of Novel Immunohistochemical Tumor Markers for Primary Hepatocellular Carcinoma; Clathrin Heavy Chain and Formiminotransferase Cyclodeaminase

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Early diagnosis of hepatocellular carcinoma (HCC) greatly improves its prognosis. However, the distinction between benign and malignant tumors is often difficult, and novel immunohistochemical markers are necessary. Using agarose two-dimensional fluorescence difference gel electrophoresis, we analyzed HCC tissues from 10 patients. The fluorescence volumes of 48 spots increased and 79 spots decreased in tumor tissues compared with adjacent nontumor tissue, and 83 proteins were identified by mass spectrometry. Immunoblot confirmed that the expression of clathrin heavy chain (CHC) and Ku86 significantly increased, whereas formiminotransferase cyclodeaminase (FTCD), rhodanese, and vinculin decreased in tumor. The protein expression in tumor and nontumor tissues was further evaluated by immunostaining. Interestingly, CHC and FTCD expression was strikingly different between tumor and nontumor tissues. The sensitivity and specificity of individual markers or a combination for the detection of HCC were 51.8% and 95.6% for CHC, 61.4% and 98.5% for FTCD, and 80.7% and 94.1% for CHC+FTCD, respectively. Strikingly, the sensitivity and specificity increased to 86.7% and 95.6% when glypican-3, another potential biomarker for HCC, was used with FTCD. Moreover, CHC and FTCD were useful to distinguish early HCC from benign tumors such as regenerative nodule or focal nodular hyperplasia, because the sensitivity and specificity of the markers are 41.2% and 77.8% for CHC, 44.4% and 80.0% for FTCD, which is comparable with those of glypican-3 (33.3% and 100%). The sensitivity significantly increased by combination of these markers, 72.2% for CHC+FTCD, and 61.1% for CHC+glypican-3 and FTCD+glypican-3, as 44.4% of glypican-3 negative early HCC were able to be detected by either CHC or FTCD staining. ***Conclusion:*** **Immunostaining of CHC and FTCD could make substantial contributions to the early diagnosis of HCC.** (HEPATOLOGY 2008;48:519-530.)

Abbreviations: 2-DE, two dimensional electrophoresis; 2D-DIGE, two dimensional fluorescence difference gel electrophoresis; CHC, clathrin heavy chain; eHCC, early hepatocellular carcinoma; FNH, focal nodular hyperplasia nodules; FTCD, formiminotransferase cyclodeaminase; HCC, primary hepatocellular carcinoma; Ku86, 82-kDa ATP-dependent DNA helicase II; LRN, large regenerative nodule; mRNA, messenger RNA.

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Primary hepatocellular carcinoma (HCC) is a major health problem worldwide.[1,2] It is known that HCC develops from a chronic inflammatory liver disease due to hepatitis B virus and hepatitis C virus infection; therefore, HCC shows especially high prevalence in Asia and Africa, where the rate of hepatitis C virus infection is high.[3] In Japan, HCC has been ranked as the third most common cancer causing death.[4] Screening tests are serological and radiological. Alpha-fetoprotein, lens culinaris agglutinin-reactive fraction of alpha-fetoprotein, and serum protein induced by vitamin K absence or antagonist-II are the most commonly used diagnostic markers for HCC, although their sensitivity and specificity are not high enough and are inadequate for identifying early stage HCC.[5,6] The radiological test most widely used for surveillance is ultrasonography. Although ultrasound is able to detect small nodules of smaller than 2 cm, biopsy of these lesions is recommended for the diagnosis of HCC if the vascular profile on dynamic imaging is not characteristic of HCC.[7] Such small masses range from benign nodules to malignant HCCs, and it is difficult, even for experienced pathologists, to distinguish dysplasia and well-differentiated HCC, especially when the lesion is small; therefore, development of new immunocytochemical markers is needed to diagnose early HCC.

Recently, the human genome project has been completed, and the genome database published. Moreover, high-throughput analysis of proteins has become possible by the development of tandem mass spectrometry technology. The breakthrough of this proteome technology enabled comparative studies of comprehensive protein expression and the identification of protein. As for HCC, proteome analysis using two-dimensional electrophoresis (2-DE), two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), and liquid chromatography have recently been reported.[8-10] Although a number of proteins have been identified as candidate markers for HCC,[11,12] none have been applied in the clinical setting; therefore, a more comprehensive and sophisticated approach is mandatory to find novel proteins associated with HCC. Oh-ishi et al.[13] developed agarose 2-DE, which uses agarose gel in the first dimension. This method not only allows for large-scale quantitative comparisons of protein expression but is also able to resolve high-molecular-mass proteins larger than 150 kDa that are difficult to resolve with immobilized pH gradient (IPGs). We have previously identified several novel proteins with altered expression in primary colorectal cancer and esophageal cancer using agarose 2-DE or agarose 2D-DIGE.[14,15] These techniques appear to have advantages of adequate sensitivity, high reproducibility, and a wide dynamic range.

In this study, we aimed to identify novel biomarkers useful for the diagnosis of HCC. For that purpose, we compared protein expressions between HCC and adjacent nontumor tissues using the agarose 2D-DIGE method. Differentially expressed proteins were validated by immunoblot or immunostaining and were further evaluated for their potential as novel immunohistochemical markers.

Materials and Methods

The following details can be found in the Supplementary Information 1: protein extraction, fluorescent dye (CyDye) labeling, agarose 2D-DIGE, enzymatic in-gel digestion of proteins, identification of proteins, and quantification of messenger RNA (mRNA).

Human Tissue Samples. Ten HCC tissues were obtained at resection in the Department of General Surgery, Chiba University Hospital. The clinical features of these 10 cases are summarized in Table 1. Written informed consent was obtained from each patient before surgery. Excised samples were obtained within 1 hour after the operation from the tumor and adjacent non-tumor tissue. All excised tissues were immediately placed in liquid nitrogen and stored at −80°C until analysis.

Immunoblotting. Protein extracts were separated by electrophoresis on 10% to 20% polyacrylamide gradient gel. Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) in a tank transfer apparatus (Bio-Rad, Hercules, CA), and the membranes were blocked with 5% skim milk in phosphate-buffered saline. Anti-clathrin heavy chain (CHC) mouse monoclonal antibody (BD Biosciences Pharmingen) diluted 1:4000, anti-82 kDa adenosine triphosphate-dependent DNA helicase II (Ku86) mouse monoclonal antibody (COSMO BIO Co., Ltd, Tokyo, Japan) diluted 1:4000, anti-vinculin mouse monoclonal antibody (Upstate Biotechnologies, NY) diluted 1:8000, anti-formiminotransferase cyclodeaminase (FTCD) rabbit polyclonal

Table 1. Clinical Features of Patients with HCC

No.	Age	Sex	Virus	Size (mm)	Adjacent Tissue	AJCC Stage
1	69	Male	–	70 × 70	Normal	III
2	65	Male	HCV	60 × 45	LC	III
3	76	Male	–	55 × 45	CH	III
4	80	Male	HCV	30 × 38	LC	II
5	58	Female	–	45 × 40	LC	II
6	61	Male	HCV	35 × 32	CH	II
7	65	Male	HCV	25 × 16	LC	II
8	75	Male	HCV	25 × 23	CH	I
9	75	Male	HCV	25 × 20	LC	II
10	79	Male	HCV	110 × 90	CH	III

HCV, hepatitis C virus; LC, liver cirrhosis; CH, chronic hepatitis.

antibody (Abcam, Cambridge, UK) diluted 1:2000, and anti-thiosulfate sulfurtransferase (rhodanese) rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:1000 in blocking buffer were used as primary antibodies. Goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) diluted 1:3000, and rabbit antigoat IgG horseradish peroxidase (Cappel, West Chester, PA) diluted 1:500 in blocking buffer were used as secondary antibodies. Antigens on the membrane were detected with enhanced chemiluminescence detection reagents (GE Healthcare).

Immunohistochemistry. From 20 HCC specimens (five well-differentiated, 10 moderately differentiated, and five poorly differentiated HCC), paraffin-embedded blocks of tumor and adjacent nontumor tissue were collected in the Department of General Surgery, Chiba University Hospital. Four-μm sections from paraffin tissue were fixed on slide glasses. In addition, tissue arrays (CA3, CSN3, CS3; SuperBio-Chips, Seoul, Korea) were used for immunohistochemistry, which contained 83 tumor (14 well differentiated, 40 moderately differentiated, 11 poorly differentiated, and 18 unclassified HCC) and 68 nontumor liver tissues. Two adenoma specimens were obtained from the Division of Clinical Investigation, National Hospital Organization, Chiba Medical Center. Three large regenerative nodules (LRN), five focal nodular hyperplasia nodules (FNH), and 18 early HCC (eHCC) specimens were obtained from the Institute of Gastroenterology, Tokyo Women's Medical University Hospital. Tissues were deparaffinized in xylene and rehydrated by reducing the concentration of ethanol (100%, 100%, and 70%, 5 minutes each). Antigents were unmasked with microwave irradiation for 5 minutes in pH 6.0 citric buffer three times. Primary antibodies were diluted as follows. Anti-CHC antibody diluted 1:200, anti-FTCD antibody diluted 1:200, anti-rhodanese antibody diluted 1:100, and anti-Glypican-3 antibody (Biomosaics, Burlington, VT) diluted 1:100 in blocking buffer. EnVision + system (DAKO Japan, Kyoto, Japan) was used to visualize tissue antigens. Tissue sections were counterstained with hematoxylin for 1 minute. Protein expression was scored as negative (0), weak (1), moderate (2), and strong (3). Two pathologists evaluated immunohistochemical staining of the samples. The results of the evaluation agreed in 96.0% of cases. When the results were discordant, the judgment was made by the other investigator.

Results

Identification of Altered Expressed Proteins in Human HCC Tissue. To search for novel biomarkers useful for the diagnosis of HCC, we used the agarose 2D-DIGE method to explore proteins differently expressed between HCC and adjacent nontumor tissues. Each nontumor sample was labeled with Cy3, each cancer sample was labeled with Cy5, and pooling aliquots were labeled with Cy2, respectively. These labeled proteins were mixed and separated in the same 2D gel (Fig. 1A). Protein spots that were increased or decreased in tumor tissues were displayed as red or green, respectively. These spots were detected and quantitated with DeCyder imaging analysis software, and then statistical analysis was performed across the 10 gels. The fluorescence volumes of 48 spots increased and 79 spots decreased in cancer tissues compared with adjacent nontumor tissue (Student t test, $P < 0.05$). To identify the proteins, 500 μg whole-cell lysates of HCC or nontumor tissues (Table 1; cases 1 and 2) were separated by conventional agarose 2-DE, and proteins were visualized by Coomassie blue staining (Fig. 1B). We carefully compared the DIGE image with Coomassie blue staining gels and picked altered protein spots manually. A total of 101 (83 proteins) of 127 spots were identified by mass spectrometry (Tables 2 and 3). The expression of these identified proteins was differentially expressed in most of the 2D-DIGE gel (Tables 2 and 3). Although many have previously been reported as differentially expressed proteins in HCC, which we were able to reproduce using a proteomic approach, a few were further tested for their clinical use. Moreover, most down-regulated proteins were related to detoxification and metabolism, which probably reflect liver dysfunction accompanying the development of HCC. Thus, we made an attempt to find proteins that could be potential diagnostic markers for HCC.

Validation of Differentially Expressed Protein Between Tumor and Nontumor Tissues. Although 2-DE is a powerful technique, multiple proteins may be included in one spot, leading to misinterpretation of the results. Therefore, to confirm the difference of protein expression between tumor and nontumor tissues, validation using other methods is essential. Thus, immunoblot analyses of several proteins with commercially available antibodies were performed to confirm the differential protein expression in tumor tissues. CHC and Ku86 were up-regulated, whereas FTCD, rhodanese, and vinculin were down-regulated in most tumor tissues (Fig. 2). It is interesting to note that a ladder of smaller bands below full-length vinculin was observed and one of the bands around 60 kDa, which might be cleaved products of vinculin, was stronger in nontumor tissues than in tumor tissues.

Quantification of mRNA Levels. Differentially expressed proteins are commonly regulated at the transcrip-

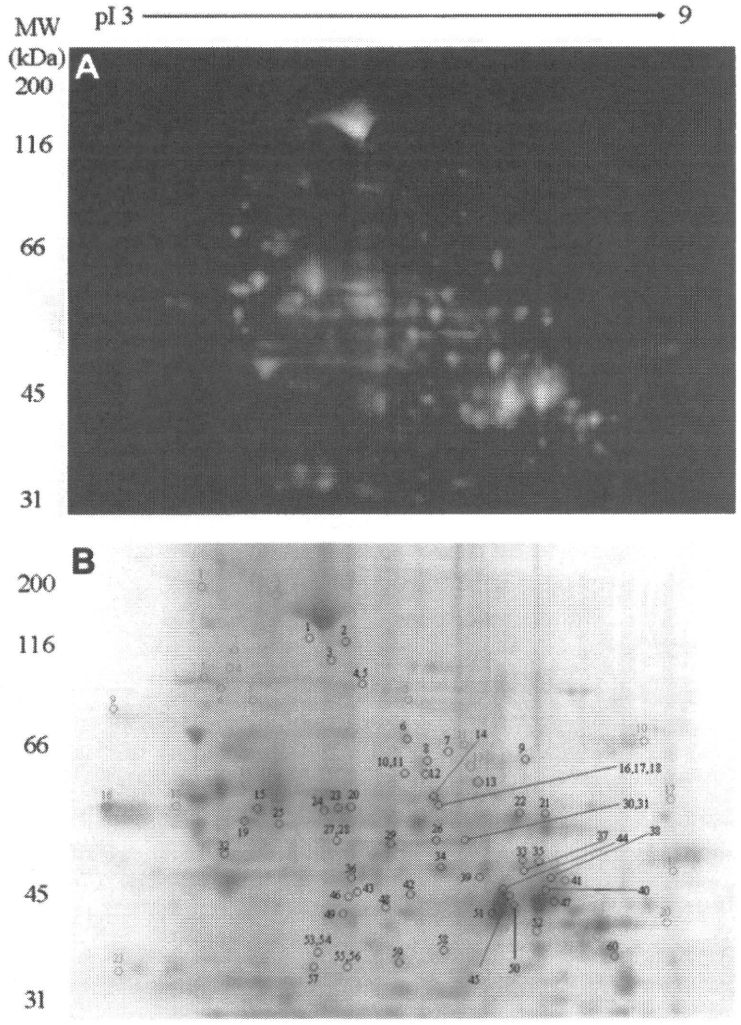

Fig. 1. Proteome analysis of HCC tissues using agarose 2D-DIGE and agarose 2-DE. Whole-cell lysates were prepared from matched samples of tumor tissue, adjacent nontumor tissue, and pooling aliquots (internal control). (A) Increased protein spots in tumor tissues are displayed in red (Cy5), and decreased protein spots in tumor tissues are displayed in green (Cy3). (B) Conventional agarose 2-DE patterns were visualized by Coomassie Blue staining. Protein spots cut from this gel were identified by mass spectrometry and are shown in red circles (up-regulated in HCC) or black circles (down-regulated in HCC). 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis.

tional level or through translational and posttranslational modifications. To explore the mechanisms leading to the changes of protein expression, we examined the mRNA level of the proteins by quantitative reverse transcription polymerase chain reaction. The mRNA levels of FTCD, rhodanese, and vinculin were decreased in most tumor tissues, consistent with the changes of protein expression. In contrast, CHC and Ku86 mRNA levels did not correlate with their protein expression levels (Fig. 3); therefore, overexpression of CHC and Ku86 in tumor tissues does not occur at the transcriptional level.

Immunohistochemical Analysis. Although there was no bias in the cellularity of tumor and adjacent nontumor tissues, whole tissue sections included nonhepatic parenchymal cells, and the altered protein expression in our 2-DE analysis may emanate from such nonhepatocyte components. Thus, the differential protein expression in HCC was also validated by immunohistochemistry to examine the localization of identified proteins. Paraffin-embedded tumor tissue and adjacent nontumor tissues of all 20 cases were stained with antibodies that were used in immunoblot analysis (Fig. 4). CHC has been reported to localize in the plasma membrane and the cytoplasmic face of intracellular organelles. Although no staining of CHC was observed in nontumor tissues, tumor cells showed scattered staining in the cytoplasm and plasma membrane (Fig. 4A). Bile duct, endothelial cell, and Kupffer cells were also positively stained. FTCD showed strong and uniform staining in the cytoplasm of nontumor tissue compared with faint staining in the cytoplasm of tumor cells (Fig. 4B). Rhodanese showed a mixture of scattered and strong staining in the cytoplasm of nontumor tissue, whereas tumor tissue was scarcely stained (Fig. 4C). These results confirmed the differential expression of proteins between tumor and nontumor tissues.

Clinical Application. Discrimination of well-differentiated HCC and nontumor tissues within a cirrhotic liver is often difficult even for experienced pathologists, and additional immunohistochemical markers are

Table 2. Protein Expression in HCC and Adjacent Nontumorous Tissue

			Protein Increased in Tumor Tissue						
No	Database Accession No.	Protein Name	Average Mass	Homogeneity Rate (%)	T-test	Score	Coverage (%)	Fold Increase	References*
T1	gi-2506872	Fibronectin precursor	262,586	80	0.035	73.8	3.2	1.53	(1)†
T2	gi-4758012	Clathrin heavy chain 1	191,595	89	<0.001	42.2	3.1	2.26	
T3	gi-19913410	Major vault protein	99,248	100	<0.001	82.4	8.6	1.73	(2)
T4	gi-2804273	Alpha actinin 4	102,250	78	0.008	88.6	10.3	1.36	
T5	gi-4507677	Tumor rejection antigen (gp96)	92,450	90	0.022	167.4	20.2	1.67	(3)†
T6	gi-6005942	Valosin-containing protein	89,247	90	0.028	128.2	18.4	1.49	(4)
T7	gi-34304590	Heat shock 90kDa protein 1 beta	83,194	100	0.002	51.9	7.0	2.13	(5)†
T8	gi-10863945	82-kDa ATP-dependent DNA helicase II (Ku86)	82,686	100	0.020	53.0	8.0	3.04	
T9	gi-4506077	Protein kinase C substrate 80K-H isoform 1	59,278	78	<0.001	52.5	10.7	1.69	
T10	gi-862457	Enoyl-CoA hydratase	82,888	80	0.021	43.0	6.8	2.09	(6)‡
T11	gi-4557385	Complement component 3 precursor	187,027	80	0.014	88.1	5.3	1.71	(7)
T12	gi-4389275	Albumin complex with myristic/triiodobenzoicacid	66,017	100	0.001	127.0	17.1	1.43	
T13	gi-37267	Transketolase	67,732	88	0.036	70.0	9.7	2.67	(8)†
T14	gi-129379	60kDa Heat shock protein, mitochondrial precursor	60,998	83	0.004	139.4	21.2	1.88	(5)†
T15	gi-576554	Antithrombin III variant	52,673	75	0.041	30.4	8.4	1.53	(9)†
T16	gi-4757900	Calreticulin precursor	48,123	100	0.017	83.3	15.4	1.52	(10)†
T17	gi-2506774	Keratin, type II cytoskeletal 8 (Cytokeratin 8)	53,623	88	0.008	150.2	32.5	2.29	
T18	gi-4504505	Hydroxysteroid (17-beta) dehydrogenase 4	79,668	100	0.015	57.2	8.3	2.09	(11)†
T19	gi-24497583	Aldo-ketoreductase family 1, member C3	36,835	90	0.038	114.3	25.7	1.86	(12)
T20	gi-4504447	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2	35,987	88	0.008	40.6	12.3	1.44	
T21	gi-21735621	Mitochondrial malate dehydrogenase precursor	35,485	88	0.037	53.3	18.7	1.28	(13)
T22	gi-5031765	11-Beta-hydroxysteroid dehydrogenase 1	32,382	88	0.037	21.3	4.2	1.28	
T23	gi-30584583	Homo sapiens tyrosine 3-monooxygenase	29,250	90	<0.001	93.5	37.2	2.16	

*The references details can be found in Supplementary Information 2.
†Previously reported to be up-regulated in HCC.
‡Previously reported to be down-regulated in HCC.

needed. Although the expression level of CHC and FTCD was strikingly different between tumor and nontumor tissues, analysis of 10 cases is not enough to consider CHC and FTCD as potential histological markers for HCC. Also the histology of nontumor tissues of the 10 cases was variable. To validate the usefulness of CHC and FTCD staining for the diagnosis of HCC, we obtained a commercial tissue array of HCC in which the degree of tumor differentiation and clinicopathological features had been proven (Table 4). The expression level of CHC and FTCD was scored as 0, 1, 2, or 3 by the staining intensity of the proteins. Most HCC tissues showed strong CHC expression (score 3) and negative to weak (score 0, 1) FTCD expression (43 of 83 cases and 51 of 83 cases). In contrast, most non-HCC tissues showed negative to moderate CHC expression (score 0, 1, 2) and moderate to strong FTCD (score 2, 3) expression (65 of 68 cases and 67 of 68 cases) (Table 5A). The sensitivity and specificity for the diagnosis of HCC using CHC expression level above were 51.8% and 95.6%, whereas those using FTCD expression level were 61.4% and 98.5%, respectively. If the combination of CHC and FTCD expression levels were used, the sensitivity and specificity for the diagnosis of HCC were 80.7% and 94.1%, respectively (Table 5B). Interestingly, CHC and FTCD expression level in tumor tissues correlates with tumor differentiation (well-differentiated HCC, 21.4%, 28.6%; moderately differentiated HCC, 52.5%, 15.0%; poorly differentiated HCC, 72.7%, 9.1%, respectively) (Table 5C). CHC and FTCD expression levels did not correlate with other clinicopathological features (age, sex, stage, and tumor size) (data not shown). These results indicated that immunostaining of CHC and FTCD could contribute to the pathological diagnosis of HCC.

Glypican-3 has been reported as a promising marker in the distinction between HCC and nonmalignant hepatocellular lesions.[16,17] Therefore, we compared the diagnostic value of CHC and FTCD for HCC with that of glypican-3 and also examined whether the combination of the three potential markers can improve the diagnostic accuracy of HCC. The sensitivity and specificity of glypican-3 were 62.7% and 97.1%, respectively, which were comparable with those of CHC or FTCD (Table 5B). Strikingly, the sensitivity and specificity increased to