potential of the tumor, and the majority of recurrences in the early phase represent recurrence by metastasis, while the later phase of recurrences most often represent secondary de novo tumors whose malignant potential has not yet increased during the process of multistep carcinogenesis. This contention is further supported by the observed association of elevated tumor marker levels with a higher frequency of extrahepatic recurrence.

Two different underlying mechanisms are thought to contribute to postoperative HCC recurrence. In theory, recurrence by metastasis takes place in the early period after surgery, whereas recurrence in the late phase largely represents a new primary lesion.[37,38] Likewise, it can be hypothesized that (1) metastatic recurrence exhibits similar tumor characteristics to the primary lesion, while de novo lesions are independent of the primary tumors in terms of the marker expression profile, and (2) tumor marker levels in recurrent tumors in the early phase show a close relationship with those before hepatectomy, while this relationship becomes obscure in recurrent tumors in the late phase. Chronological alterations in the correlation coefficients (Figs. 3 and 4) support this hypothesis. Moreover, this correlation was stronger for AFP than for DCP across all the study groups. This observation suggests that the increased AFP values both before hepatectomy and at the time of recurrence are at least partially accounted for by the background liver diseases.

A limitation of this investigation is that all of the study patients underwent curative liver resections. They would therefore be supposed to exhibit relatively well-preserved liver function, despite the presence of cirrhosis, from the viewpoint of screening. Likewise, they would be expected to have relatively early stage of HCC as compared with patients undergoing transcatheter arterial embolization, from the standpoint of prediction of response to therapies.

In conclusion, although DCP might be more accurate than AFP for the differentiation of HCC from nonmalignant chronic liver disease, the two markers are complementary to each other. The levels of both markers increased with tumor growth, but no specific association of either with any specific pathological entities was noted. The observed relationship between the preoperative marker values and the values measured at the time of recurrence may serve as a basis for predicting the pattern of recurrence of HCC, i.e., recurrence by metastasis or de novo secondary lesions.

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miR-124 and *miR-203* are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma

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MicroRNAs (miRNAs) are a class of small non-coding RNAs that, in general, negatively regulate gene expression. They have been identified in various tumor types, showing that different sets of miRNAs are usually deregulated in different cancers. Some miRNA genes harboring CpG-islands undergo methylation-mediated silencing, a characteristic of many tumor-suppressor genes. To identify such miRNAs in hepatocellular carcinoma (HCC), we first examined the methylation status of 43 loci containing CpG-islands around 39 mature miRNA genes in a panel of HCC cell lines and non-cancerous liver tissues as controls. Among 11 miRNA genes frequently methylated in HCC cell lines but not in non-cancerous liver tissues, 3 miRNA genes, i.e. *miR-124*, *miR-203*, and *miR-375*, were selected as silenced miRNAs through CpG-island methylation by comparing methylation and expression status and evaluating restored expression after treatment with 5-aza-2'-deoxycytidine. In primary tumors of HCC with paired non-tumorous liver tissues, only *miR-124* and *miR-203* showed frequent tumor-specific methylation, and their expression status was inversely correlated with methylation status. Ectopic expression of *miR-124* or *miR-203* in HCC cells lacking their expression inhibited cell growth, with direct down-regulation of possible targets, *cyclin-dependent kinase 6* (*CDK6*), *vimentin* (*VIM*), *SET and MYND domain containing 3* (*SMYD3*), and *IQ motif containing GTPase activating protein 1* (*IQGAP1*) or *ATP-binding cassette, sub-family E, member 1* (*ABCE1*), respectively. Our results suggest that *miR-124* and *miR-203* are novel tumor-suppressive miRNAs for HCC epigenetically silenced and activating multiple targets during hepatocarcinogenesis.

Key Words: hepatocellular carcinoma • miRNA • DNA methylation • CpG-island • tumor-suppressor gene

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Genome-wide DNA methylation profiles in liver tissue at the precancerous stage and in hepatocellular carcinoma

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To clarify genome-wide DNA methylation profiles during hepatocarcinogenesis, bacterial artificial chromosome (BAC) array-based methylated CpG island amplification was performed on 126 tissue samples. The average numbers of BAC clones showing DNA hypo- or hypermethylation increased from noncancerous liver tissue obtained from patients with hepatocellular carcinomas (HCCs) (N) to HCCs. N appeared to be at the precancerous stage, showing DNA methylation alterations that were correlated with the future development of HCC. Using Wilcoxon test, 25 BAC clones, whose DNA methylation status was inherited by HCCs from N and were able to discriminate 15 N samples from 10 samples of normal liver tissue obtained from patients without HCCs (C) with 100% sensitivity and specificity, were identified. The criteria using the 25 BAC clones were able to discriminate 24 additional N samples from 26 C samples in the validation set with 95.8% sensitivity and 96.2% specificity. Using Wilcoxon test, 41 BAC clones, whose DNA methylation status was able to discriminate patients who survived more than 4 years after hepatectomy from patients who suffered recurrence within 6 months and died within a year after hepatectomy, were identified. The DNA methylation status of the 41 BAC clones was correlated with the cancer-free and overall survival rates of patients with HCC. Multivariate analysis revealed that satisfying the criteria using the 41 BAC clones was an independent predictor of overall outcome. Genome-wide alterations of DNA methylation may participate in hepatocarcinogenesis from the precancerous stage, and DNA methylation profiling may provide optimal indicators for carcinogenetic risk estimation and prognostication.

Key words: bacterial artificial chromosome array-based methylated CpG island amplification; hepatocellular carcinoma; multistage carcinogenesis; precancerous condition; prognostication

Alteration of DNA methylation is one of the most consistent epigenetic changes in human cancers.[1,2] It is known that DNA hypomethylation results in chromosomal instability as a result of changes in the chromatin structure, and that DNA hypermethylation of CpG islands silences tumor-related genes in cooperation with histone modification in human cancers.[3,4]

With respect to hepatocarcinogenesis, we have shown that alterations of DNA methylation at multiple chromosomal loci can be detected even in noncancerous liver tissue showing chronic hepatitis or cirrhosis, which are widely considered to be precancerous conditions, but not in normal liver tissue, using classical Southern blotting analysis.[5] This was one of the earliest reports of alterations of DNA methylation at the precancerous stage. Multiple tumor-related genes, such as the *E-cadherin*[6,7] and *hypermethylated-in-cancer (HIC)-1*[8] genes, are silenced by DNA hypermethylation in hepatocellular carcinomas (HCCs). DNA methyltransferase (DNMT) 1 expression is significantly higher even in noncancerous liver tissue showing chronic hepatitis or cirrhosis than in the normal liver tissue and is even higher in HCCs.[9,10] DNMT1 overexpression is also correlated with poorer tumor differentiation, portal vein involvement and intrahepatic metastasis of HCCs and poorer patient outcome.[11] On the other hand, overexpression of DNMT3b4, an inactive splice variant of DNMT3b, may lead to chromosomal instability through induction of DNA hypomethylation in pericentromeric satellite regions during hepatocarcinogenesis.[12]

Because aberrant DNA methylation is one of the earliest molecular events during hepatocarcinogenesis and also participates in malignant progression,[13,14] it may be possible to estimate the future risk of developing more malignant HCCs on the basis of DNA methylation status. However, only a few previous studies focusing on HCCs have used recently developed array-based technology for assessing genome-wide DNA methylation status,[15] and such studies have focused mainly on identification of tumor-related genes that are silenced by DNA methylation. DNA methylation profiles, which could become the optimum indicator for carcinogenetic risk estimation and prediction of patient outcome, should therefore be further explored during hepatocarcinogenesis using array-based approaches.

In this study, to clarify genome-wide DNA methylation profiles during multistage hepatocarcinogenesis, we performed bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA)[16–18] using a microarray of 4,361 BAC clones[19] in the normal liver tissue obtained from patients without HCCs, noncancerous liver tissue obtained from patients with HCCs, and in HCCs themselves.

Material and methods

Patients and tissue samples

As a learning cohort, 15 samples of the noncancerous liver tissue (N1 to N15) and 19 primary HCCs (T1 to T19) were obtained from surgically resected specimens from 16 patients who underwent partial hepatectomy at the National Cancer Center Hospital, Tokyo, Japan. The patients comprised 13 men and 3 women with a mean (±SD) age of 64.9 ± 7.4 years. Of these, 7 were positive for hepatitis B virus (HBV) surface antigen (HBs-Ag), 8 were positive for anti-hepatitis C virus (HCV) antibody (anti-HCV) and 1 was negative for both. Histological examination of the noncancerous liver tissue samples revealed findings compatible with chronic hepatitis in 5 and cirrhosis in 9 and no remarkable histological findings in 1.

Additional Supporting Information may be found in the online version of this article.

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For the comparison, 10 normal liver tissue samples (C1 to C10) showing no remarkable histological findings were also obtained from 10 patients without HCCs who were both HBs-Ag- and anti-HCV-negative. The patients comprised 7 men and 3 women with a mean age of 58.4 ± 9.7 years. Nine patients underwent partial hepatectomy for liver metastases of primary colon cancers, and 1 patient did so for liver metastases of gastrointestinal stromal tumor of the stomach.

In addition, for the comparison, 7 liver tissue samples (V1 to V7) were obtained from 7 patients who were positive for HBs-Ag or anti-HCV, but who had never developed HCCs. The patients comprised 4 men and 3 women with a mean age of 62.4 ± 5.2 years. Three patients underwent partial hepatectomy for liver metastases of primary colon or rectal cancers, and 1 patient did so for liver metastases of gastric cancer. Three patients underwent partial hepatectomy for cholangiocellular carcinomas.

As a validation cohort, 26 normal liver tissue samples (C11 to C36) showing no remarkable histological features were obtained from 26 patients without HCCs who were both HBs-Ag- and anti-HCV-negative. Twenty-four noncancerous liver tissue samples (N16 to N 39) and 25 primary HCCs (T20 to T44) were obtained from surgically resected specimens from 24 patients who underwent partial hepatectomy were added. The patients from whom C11 to C36 were obtained comprised 21 men and 5 women with a mean age of 59.9 ± 10.9 years. The patients with HCCs from whom N16 to N 39 and T20 to T44 were obtained comprised 22 men and 2 women with a mean age of 61.6 ± 11.4 years. Of the 24 patients with HCCs from whom N16 to N 39 and T20 to T44 were obtained, 5 were positive for HBs-Ag, 16 were positive for anti-HCV and 3 were negative for both. Histological examination of N16 to N 39 revealed findings compatible with chronic hepatitis and cirrhosis in 16 and 8 samples, respectively.

This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan.

BAMCA

High molecular weight DNA from fresh-frozen tissue samples was extracted using phenol-chloroform followed by dialysis. Because DNA methylation status is known to be organ specific, the reference DNA for analysis of the developmental stages of HCCs should be obtained from the liver and not from other organs or peripheral blood. Therefore, a mixture of normal liver tissue DNA obtained from 5 male patients (C37 to C41) and 5 female patients (C42 to C46) was used as a reference for analyses of male and female test DNA samples, respectively.

DNA methylation status was analyzed by BAMCA using a custom-made array (MCG Whole Genome Array-4500) harboring 4,361 BAC clones located throughout chromosomes 1 to 22 and X and Y,[19] as described previously.[16–18] Briefly, 5-μg aliquots of test or reference DNA were first digested with 100 units of methylation-sensitive restriction enzyme *Sma* I and subsequently with 20 units of methylation-insensitive *Xma* I. Adapters were ligated to *Xma* I-digested sticky ends, and polymerase chain reaction (PCR) was performed with an adapter primer set. Test and reference PCR products were labeled by random priming with Cy3- and Cy5-dCTP (GE Healthcare, Buckinghamshire, UK), respectively, and precipitated together with ethanol in the presence of Cot-1 DNA. The mixture was applied to array slides and incubated at 43°C for 72 hr. Arrays were scanned with a GenePix Personal 4100A (Axon Instruments, Foster City, CA) and analyzed using GenePix Pro 5.0 imaging software (Axon Instruments) and Acue 2 software (Mitsui Knowledge Industry, Tokyo, Japan). The signal ratios were normalized in each sample to make the mean signal ratios of all BAC clones 1.0.

Statistics

Differences in the average number of BAC clones that showed DNA methylation alterations between groups of samples were analyzed using the Mann-Whitney *U* test or the Kruskal-Wallis test. Correlations between DNA methylation alterations in noncancerous liver tissue samples and the incidence of metachronous development and recurrence of HCCs were analyzed using the chi-squared test. Differences at $p < 0.05$ were considered significant. BAC clones whose signal ratios yielded by BAMCA were significantly different between groups of samples were identified by Wilcoxon test ($p < 0.01$). A support vector machine algorithm and a leave-one-out cross-validation were used to identify BAC clones by which the cumulative error rate for discrimination of sample groups became minimal. Two-dimensional hierarchical clustering analysis of noncancerous liver tissue samples and the BAC clones, and such analysis of HCCs and the BAC clones, were performed using the Expressionist software program (Gene Data, Basel, Switzerland). Survival curves of patient groups with HCCs were calculated by the Kaplan-Meier method, and the differences were compared by the log-rank test. The Cox proportional hazards multivariate model was used to examine the prognostic impact of DNA methylation status, histological differentiation, portal vein tumor thrombi, intrahepatic metastasis and multicentricity. Differences at $p < 0.05$ were considered significant.

Results

Genome-wide DNA methylation alterations during multistage hepatocarcinogenesis

Figures. 1*a* and 1*b* show examples of scanned array images and scattergrams of the signal ratios (test signal/reference signal), respectively, for normal liver tissue from a patient without HCC (Panel C), and both noncancerous liver tissue (Panel N) and cancerous tissue (Panel T) from a patient with HCC. In all normal liver tissue samples, the signal ratios of 97% of the BAC clones were between 0.67 and 1.5 (red bars in Fig. 1*b*). Therefore, in noncancerous liver tissue obtained from patients with HCCs and HCCs, DNA methylation status corresponding to a signal ratio of less than 0.67 and more than 1.5 was defined as DNA hypomethylation and DNA hypermethylation of each BAC clone compared with normal liver tissue, respectively.

In samples of noncancerous liver tissue obtained from patients with HCCs, many BAC clones showed DNA hypo- or hypermethylation (Panel N of Fig. 1*b*). In the learning cohort, all 9 patients (100%) showing DNA hypo- or hypermethylation on 70 or more than 70 BAC clones in their noncancerous liver tissue samples developed metachronous or recurrent HCCs after hepatectomy, whereas only 2 (30%) of the 6 patients showing DNA hypo- or hypermethylation on less than 70 BAC clones in their noncancerous liver tissue samples did so ($p = 0.0235$).

In HCCs themselves, more BAC clones showed DNA hypo- or hypermethylation, and the degree of DNA hypo- or hypermethylation, *i.e.*, deviation of the signal ratio from 0.67 or 1.5, was increased (Panel T of Fig. 1*b*) in comparison with noncancerous liver tissue obtained from patients with HCCs. The average numbers of BAC clones showing a signal ratio of less than 0.67 ($p = 0.0000063$) and more than 1.5 ($p = 0.00000052$) were increased significantly relative to normal liver tissue, to noncancerous liver tissue obtained from patients with HCCs, and to HCCs (Table I).

There were no significant differences in the number of BAC clones showing DNA hypo- or hypermethylation in samples of normal liver tissue obtained from male and female patients without HCCs (66.0 ± 30.1 and 98.7 ± 55.9, $p = 0.362$) and noncancerous liver tissue (111.2 ± 68.4 and 60.7 ± 46.9, $p = 0.279$) and cancerous tissue (521.5 ± 255.8 and 626.7 ± 329.0, $p = 0.539$) obtained from male and female patients with HCCs, respectively. 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 in both noncancerous liver tissue (108.3 ± 80.5 and 98.4 ± 60.0, $p = 1.000$) and cancerous tissue (475.6 ± 323.8 and 497.0 ± 247.8, $p = 0.689$), Wilcoxon test ($p < 0.01$) identified BAC clones in which DNA methylation status differed significantly between HBV- and

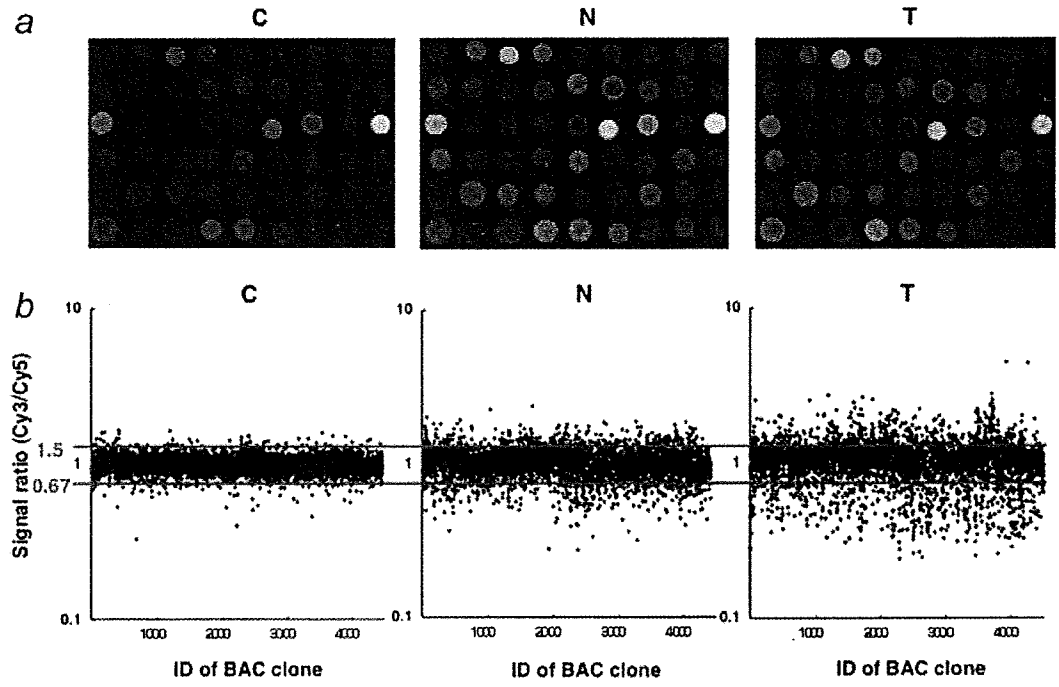

FIGURE 1 – Genome-wide DNA methylation alterations during multistage hepatocarcinogenesis. (*a*) Scanned array images yielded by BAMCA in normal liver tissue obtained from a patient without HCC (C) and noncancerous liver tissue (N) and cancerous tissue (T) obtained from a patient with HCC. (*b*) Scattergrams of the signal ratios yielded by BAMCA. In all C samples, the signal ratios of 97% of BAC clones were between 0.67 and 1.5 (red bars). In N and T, DNA methylation status corresponding to a signal ratio of less than 0.67 and more than 1.5 was defined as DNA hypomethylation and DNA hypermethylation on each BAC clone compared with C, respectively. Even in N, many BAC clones showed DNA hypo- or hypermethylation. In T, more BAC clones showed DNA hypo- or hypermethylation, and the degree of DNA hypo- or hypermethylation, *i.e.*, deviation of the signal ratio from 0.67 or 1.5 was increased in comparison with N.

TABLE I – GENOME-WIDE DNA METHYLATION ALTERATIONS DURING MULTISTAGE HEPATOCARCINOGENESIS

Tissue samples	Average number of BAC clones (mean ± SD)					
	Signal ratio <0.67 (DNA hypomethylation)	*p*	Signal ratio >1.5 (DNA hypermethylation)	*p*	Signal ratio <0.67 or >1.5(DNA hypo- or hypermethylation)	*p*
Normal liver tissue samples obtained from patient without HCCs (C, $n = 10$)	39.9 ± 20.8	0.0000063[1]	38.9 ± 24.9	0.00000052[1]	75.8 ± 39.3	0.00000061[1]
Noncancerous liver tissue samples obtained from patient with HCCs (N, $n = 15$)	61.2 ± 46.8	0.000102[2]	39.9 ± 27.3	0.0000026[2]	101.1 ± 66.5	0.0000065[2]
HCCs (T, $n = 19$)	278.9 ± 167.7	–	228.9 ± 125.7	–	507.8 ± 281.9	–

p values <0.05, which indicate significant differences.
[1]Kruskal-Wallis test among C, N and T.–[2]Mann-Whitney *U* test between N and T.

HCV-positive patients with HCCs in noncancerous liver tissue (18 BAC clones) and cancerous tissue (15 BAC clones), respectively.

DNA methylation profiles discriminating noncancerous liver tissue obtained from patients with HCCs from normal liver tissue

The above findings indicating accumulation of clinicopathologically significant genome-wide DNA methylation alterations in noncancerous liver tissue prompted us to estimate the degree of carcinogenetic risk based on DNA methylation profiles. Wilcoxon test ($p < 0.01$) revealed that the signal ratios of 512 BAC clones differed significantly between normal liver tissue samples and noncancerous liver tissue samples obtained from patients with HCCs. To omit potentially insignificant BAC clones associated only with inflammation and/or fibrosis and focus on BAC clones for which DNA methylation status was inherited by HCCs from the precancerous stage, we defined Groups I, II, III and IV. Group I: BAC clones in which the average signal ratio of noncancerous liver tissue obtained from patients with HCCs was higher than that of normal liver tissue and the average signal ratio of HCCs was even higher than that of noncancerous liver tissue obtained from patients with HCCs (41 BAC clones), Group II: BAC clones in which the average signal ratio of noncancerous liver tissue obtained from patients with HCCs was higher than that of normal liver tissue and the average signal ratio of HCCs did not differ from that of noncancerous liver tissue obtained from patients with HCCs (146 BAC clones), Group III: BAC clones in which the average signal ratio of noncancerous liver tissue obtained from patients with HCCs was lower than that of normal liver tissue and the average signal ratio of HCCs was even lower than that of noncancerous liver tissue obtained from patients with HCCs (40 BAC clones), and Group IV: BAC clones in which the average signal ratio of noncancerous liver tissue obtained from patients with HCCs was lower than that of normal liver tissue and the average

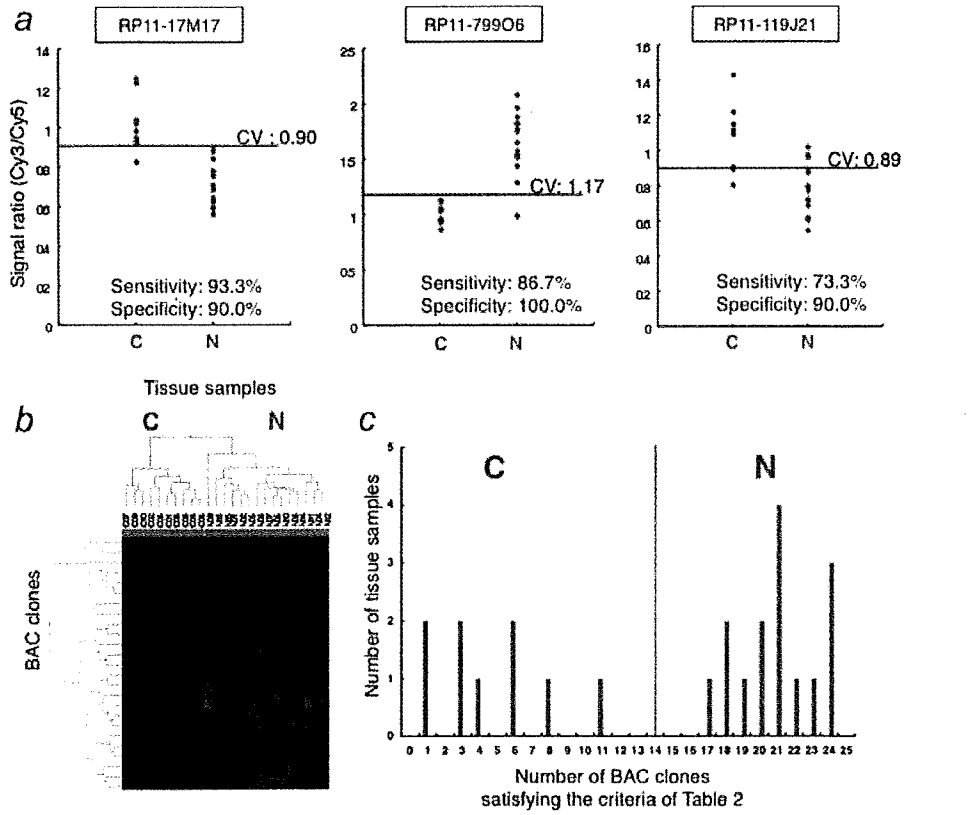

FIGURE 2 – DNA methylation profiles discriminating noncancerous liver tissue obtained from patients with HCCs from normal liver tissue. (*a*) Scattergrams of the signal ratios in normal liver tissue samples (C1 to C10) and noncancerous liver tissue samples obtained from patients with HCCs (N1 to N15) 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

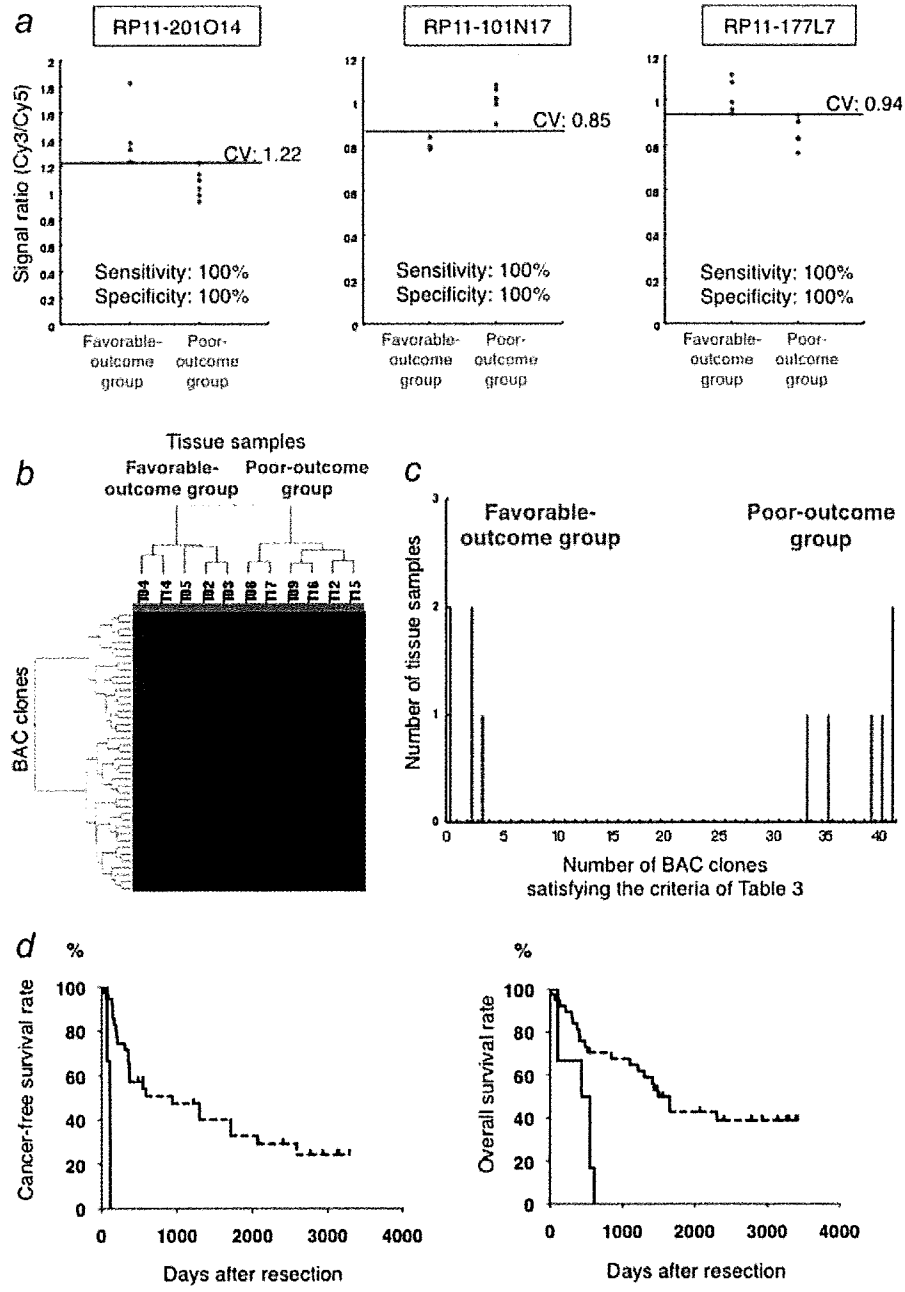

FIGURE 3 – DNA methylation profiles in HCCs associated with patient outcome. (*a*) Scattergrams of the signal ratios in HCCs from patients who survived more than 4 years after hepatectomy (favorable-outcome group, $n = 5$) and HCCs from patients who suffered recurrence within 6 months and died within 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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The Value of Anatomical Liver Sectionectomy for Patients With a Solitary Hepatocellular Carcinoma From 2 to 5 cm in Greatest Diameter

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Background: The value of anatomical liver sectionectomy for patients with a solitary hepatocellular carcinoma (HCC) from 2 to 5 cm in greatest diameter has not been clarified.

Methods: We retrospectively studied 454 patients with a solitary HCC from 2 to 5 cm in greatest diameter who underwent initial curative hepatectomy from 1991 to 2006. We separated the liver into three segments according to the Glissonean pedicle ramification (Takasaki's liver segments). Takasaki's segment is recognized as a section in the International Hepato-Pancreato-Biliary Association Brisbane 2000 terminology. Outcomes after sectionectomy (n = 143) and partial sectionectomy (n = 311) for patients with a solitary HCC from 2 to 5 cm in greatest diameter were examined.

Results: The 5-year recurrence-free survival rate and survival rate after sectionectomy (42% and 81%, respectively) were significantly better than those after partial sectionectomy (27%: $P = 0.0023$, and 66%: $P = 0.0006$, respectively). Multivariate analysis showed sectionectomy to be a significant independent prognostic factor for recurrence-free survival and overall survival in patients with a solitary HCC from 2 to 5 cm in greatest diameter ($P = 0.0217$, and $P = 0.0085$, respectively).

Conclusions: Anatomical liver sectionectomy prevents intrahepatic recurrence of HCC and prolongs survival in patients with a solitary HCC from 2 to 5 cm.

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KEY WORDS: **anatomical liver resection; liver sectionectomy; HCC; surgical outcomes**

INTRODUCTION

Radio-frequency ablation (RFA) is considered as one of the options of treatment for small hepatocellular carcinoma (HCC) less than 5 cm in greatest diameter [1–3]. However, local recurrence rates are reported to be higher after RFA than after surgery [4–8]. HCC 2–5 cm in greatest diameter has about 20% of microscopic vascular invasion and intrahepatic metastasis around the tumor [9]. Therefore, RFA might be insufficient for curative treatment for HCC over 2 cm in greatest diameter [10]. On the contrary, the optimal surgical extent for small HCC has not been clarified [11,12]. The value of anatomical liver sectionectomy in patients with a solitary HCC from 2 to 5 cm in greatest diameter has not been clarified in detail.

METHODS

We retrospectively studied 454 patients with a solitary HCC from 2 to 5 cm in greatest diameter who underwent initial curative hepatectomy from 1991 to 2006 at Tokyo Women's Medical University Hospital, Tokyo. We separated the liver into three segments according to the Glissonean pedicle ramification (Takasaki's liver segments) [13–18]. Takasaki's left segment, middle segment and right segment correspond to the left liver, the right anterior and the right posterior section, respectively, in the International Hepato-Pancreato-Biliary Association (IHPBA) Brisbane 2000 terminology (Fig. 1) [19]. Systematized hepatectomy was classified into sectionectomy or larger resection and partial sectionectomy. Sectionectomy refers to resection of one of Takasaki's segments. Outcomes after sectionectomy (n = 143) and partial sectionectomy (n = 311) for patients with a solitary HCC from 2 to 5 cm in greatest diameter were examined. The selection between sectionectomy and partial segmentectomy was made on the basis of liver function, tumor size, and tumor location. There was a tendency to select partial sectionectomy in cases with severe liver cirrhosis, minute tumors, or tumors located at the liver's surface.

Gender, age, hepatitis B surface antigen, anti-hepatitis C, serum alpha-fetoprotein level (AFP), liver cirrhosis, the Child-Pugh class, indocyanine green 15 min retention test ($ICGR_{15}$), greatest dimension, microscopic portal vein invasion, microscopic satellite nodules and the surgical margin were examined.

Cumulative survival rates of patients were calculated with the Kaplan–Meier method and survival rates were compared by means of the log-rank test. The duration of survival was defined as the time from liver surgery to the date of death or last contact. The median follow up was 44 months (ranging from 12 days to 206 months). To compare the subgroups, the chi-square test or *t*-test was used. Differences were considered significant when $P < 0.05$.

RESULTS

The rate of liver cirrhosis and $ICGR_{15}$ are significantly higher in patients after partial sectionectomy than in patients after sectionectomy ($P < 0.0001$, $P < 0.0001$; respectively). The tumor size is significantly

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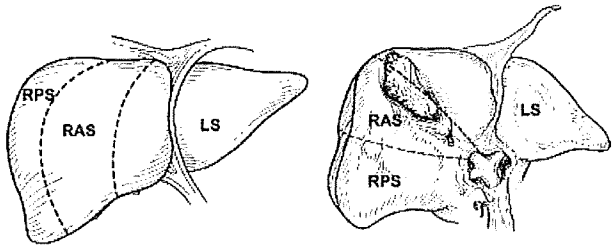

Fig. 1. The boundaries of each section. LS, left section (the left liver); RAS, right anterior section; RPS, right posterior section.

larger in patients after sectionectomy than in patients after partial sectionectomy ($P < 0.0001$). The rate of pathological portal vein invasion and intrahepatic metastasis in patients after sectionectomy were not significantly different from those in patients after partial sectionectomy ($P = 0.15$, $P = 0.19$, respectively; Table I).

The 5-year recurrence-free survival rate and survival rate in patients after sectionectomy were significantly better than those in patients after partial sectionectomy ($P = 0.0023$, $P = 0.0006$, respectively; Figs. 2 and 3). Univariate analysis showed HBs antigen, HCV antibody, cirrhosis, $ICGR_{15}$, surgical procedure and intrahepatic metastasis to be significant prognostic factors for recurrence-free survival and overall survival (Table II). Multivariate analysis showed sectionectomy to be a significant independent prognostic factor for recurrence-free survival and overall survival in patients with a solitary HCC from 2 to 5 cm in greatest diameter ($P = 0.0217$, $P = 0.0085$, respectively; Table III).

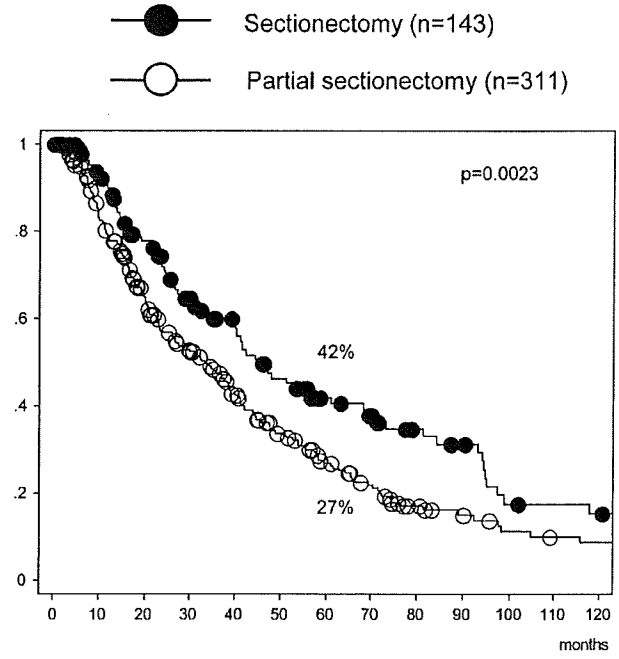

Fig. 2. The 5-year recurrence-free survival rate in patients after sectionectomy was significantly better than that in patients after partial sectionectomy ($P = 0.0023$).

DISCUSSION

HCC spreads to the liver parenchyma through the portal vein. Portal vein invasion is reported to be a significant prognostic factor for survival after surgery. The rate of portal vein invasion increases as the tumor size increases. The effectiveness of RFA decreases and the local recurrence rate increases when HCC is over 2.5 cm in greatest diameter [6,10]. Serum AFP levels, tumor location, vascular invasion and satellite nodules are also considered before RFA therapy. Abnormal findings in these factors also suggest spreading of the tumor to the liver parenchyma around the tumor. Therefore, anatomical hepatectomy should be performed for patients with HCC over 2 cm in greatest diameter or with other abnormal factors. Some surgeons prefer sub-segmentectomy for small HCC. However, the definition of sub-segmentectomy is vague and the extent of sub-segmentectomy is different in each case. Therefore, a standard of anatomical systematic hepatectomy is needed.

Takasaki et al. reported the Glissonean pedicle approach in the liver in 1986 [13–17]. The liver is divided into three segments according to the ramification of the portal pedicles at the hepatic hilus. These pedicles can be divided outside of the liver without liver dissection. Takasaki's segmentectomy is the most fundamental systematized hepatectomy because the portal vein ramification at the hepatic hilus always consists of three divisions in every case. We also reported that we should perform Takasaki's segmentectomy when solitary HCC less than 5 cm in greatest diameter shows extranodular growth [9]. Simple nodular HCC with extranodular growth means that the tumor has portal vein invasion or satellite nodules around the tumor. The surgical outcomes are poorer in patients with HCC with extranodular growth after partial segmentectomy. Partial segmentectomy is insufficient treatment for HCC with portal vein invasion and satellite nodules. This means that

TABLE I. Patients' Characteristics

	Sectionectomy, n = 143	Partial sectionectomy, n = 311	*P*-value
Sex (male)	116 (81%)	235 (76%)	0.19
Age (years, mean ± SD)	64 ± 9	64 ± 8	0.85
HBV	28 (20%)	58 (19%)	0.81
HCV	89 (62%)	211 (68%)	0.24
Cirrhosis (present)	45 (31%)	168 (54%)	<0.0001
$ICGR_{15}$, (%, mean ± SD)	13 ± 8	19 ± 12	<0.0001
Child-Pugh (A)	131 (92%)	259 (83%)	0.0178
AFP (ng/ml, mean ± SD)	1,493 ± 5,123	715 ± 4,009	0.08
Size (cm, mean ± SD)	3.5 ± 0.8	3.1 ± 0.8	<0.0001
Portal vein invasion (present)	31 (22%)	50 (16%)	0.15
Intrahepatic metastasis (present)	17 (12%)	25 (8%)	0.19
Surgical margin (<5 mm)	8 (6%)	35 (11%)	0.06

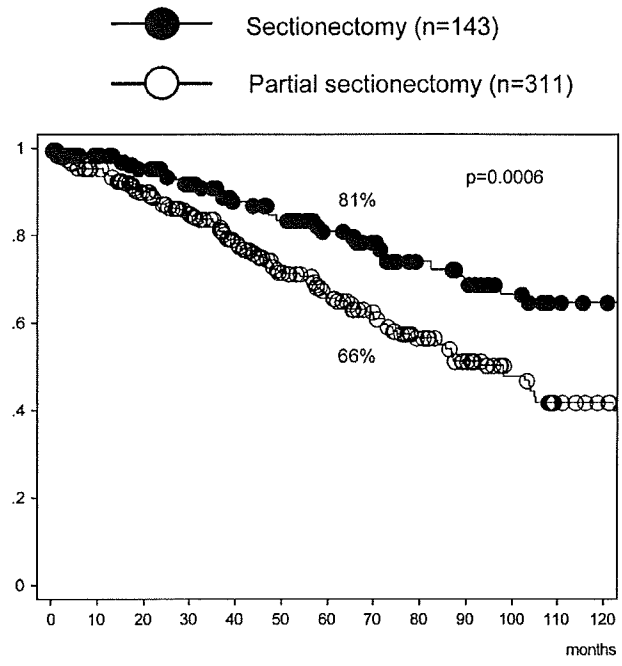

Fig. 3. The 5-year survival rate in patients after sectionectomy was significantly better than that in patients after partial sectionectomy ($P = 0.0006$).

HCC with portal vein invasion and satellite nodules spreads through the section (Takasaki's segment) containing the tumor.

Other studies have also shown that anatomical liver resection affected tumor recurrence and the overall survival [20,21]. However, the extent of resection for HCC remains controversial. The number of patients in each group (anatomical resection vs. limited resection) was smaller than 50 and the surgical procedures were variable. All reports have been retrospective analyses, which include selection bias and potential omitted variables which might contribute to error.

The surgical margin should also be considered. The results of univariate analysis showed that the surgical margin influenced the overall survival, but not disease free survival. The surgical margin is strongly related with liver cirrhosis and liver dysfunction. Therefore, multivariate analysis did not show the surgical margin to be an independent prognostic factor. Several reports have shown that the surgical margin did not affect hepatic recurrence after surgery [22,23].

The staging system of the International Union Against Cancer (UICC) includes HCC with portal vein invasion and satellite nodules in T2 HCC [24]. Therefore, sectionectomy (Takasaki's segmentectomy) should be performed for T2 HCC [18]. However, portal vein invasion and satellite nodules around the tumor cannot be seen on preoperative imaging. A simpler T-stage definition is needed. Tumor size is the simplest factor for predicting spreading of the tumor. In cancer of other organs, such as breast cancer, the tumor size is a factor in the staging system. Our data suggest that solitary HCC 2–5 cm in greatest diameter spreads through the section containing the tumor, which implies that the extent of tumor spreading in HCC 2–5 cm in greatest diameter is similar to that in T2 HCC in the UICC staging system. Solitary HCC 2–5 cm in greatest diameter should be included in T2 HCC even if no vascular invasion or satellite nodules are detected. It would facilitate discussion of the treatment options for HCC if the treatment algorism could be matched to the tumor size.

TABLE II. Univariate Analysis of Risk Factors for the 5-Year Recurrence-Free Survival Rate and Survival Rate

		5-year disease free survival	*P*-value	5-year survival	*P*-value
HBs antigen	Negative vs. positive	29%/42%	0.0026	67%/84%	0.0117
HCV antibody	Negative vs. positive	45%/25%	<0.0001	77%/67%	0.0095
Cirrhosis	Absent vs. present	40%/23%	<0.0001	79%/62%	0.0001
$ICGR_{15}$	<17% vs. >17%	39%/20%	<0.0001	80%/56%	<0.0001
Child-Pugh class	A vs. B	34%/20%	0.0021	75%/42%	<0.0001
Alfa-fetoprotein	<1,000 ng/ml vs. >1,000 ng/ml	30%/38%	0.33	70%/82%	0.0283
Surgical procedure	Sectionectomy vs. partial sectionectomy	42%/27%	0.0023	81%/66%	0.0006
Portal vein invasion	Absent vs. present	31%/33%	0.99	73%/61%	0.17
Intrahepatic metastasis	Absent vs. present	33%/25%	0.0436	73%/53%	0.0315
Surgical margin	<5 mm vs. >5 mm	21%/33%	0.37	50%/73%	0.0285

HBs, hepatitis B surface antigen; HCV, hepatitis C virus; $ICGR_{15}$, indocyanine green retention rate at 15 min.

TABLE III. Multivariate Analysis Using Cox's Proportional Hazard Model

		Relative risk	95% CI	*P*-value
Recurrence				
HCV	Positive	1.442	1.066–1.950	0.0175
Cirrhosis	Present	1.426	1.088–1.868	0.0101
Intrahepatic metastasis	Present	1.599	1.077–2.374	0.0200
Surgical procedure	Sectionectomy	0.722	0.547–0.953	0.0217
Survival				
Child-Pugh	A	0.502	0.316–0.797	0.0035
Intrahepatic metastasis	Present	1.971	1.191–3.262	0.0083
Surgical procedure	Sectionectomy	0.569	0.374–0.866	0.0085

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Display Settings: Abstract

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Right hepatectomy for hepatocellular carcinoma in patients with an indocyanine green retention rate at 15 minutes of 10% or higher.

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BACKGROUND/AIMS: Liver failure after right hepatectomy for hepatocellular carcinoma (HCC) in patients with an indocyanine green retention rate at 15 min (ICGR(15)) of 10% or higher remains a controversial issue. METHODS: Between 1995 and 2004, 98 patients with an ICGR(15) of 10% or higher were scheduled to undergo right hepatectomy or tri-sectionectomy for HCC. The hepatic resection volume (HR) excluding the tumor was measured using computed tomography. The allowable HR (AHR) was determined in each patient with a logarithmic graph based on the ICGR(15) and the %HR. Liver failure and mortality were evaluated between 54 patients with HR <or= AHR (low-risk group) and 44 patients with HR > AHR (high-risk group). RESULTS: The number of patients with liver failure was significantly lower in the low-risk group (2%) than in the high-risk group (23%, p = 0.0021). No mortality was observed in the low-risk group, while mortality was seen in the high-risk group (11%, p = 0.016). Multivariate analysis showed that the high-risk group was identified as a significant predictor of liver failure (p = 0.011). CONCLUSIONS: In patients with an ICGR(15) of 10% or higher, determination of AHR is useful to predict liver failure prior to right hepatectomy or tri-sectionectomy.

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MeSH Terms, Substances

LinkOut - more resources

肝臓　50巻3号　105-121（2009）　

＜総　説＞

肝疾患の造影超音波診断―その変遷と新しい展開―

飯島　尋子*

はじめに

肝腫瘍診断におけるスクリーニング法としての超音波診断法は低侵襲，安価で簡便，その診断能の高さから揺るぎのないものである．超音波造影剤の主成分はマイクロバブルであり，生体組織と気体との音響インピーダンスが大きく異なるため，境界で大きな散乱（エコー）が発生することにより画像化される．経静脈性超音波造影剤は，わが国で1999年に使用可能となった．その後，長年にわたって新世代の造影剤の発売が待たれたが，2007年1月にそれが実現した．これにより，肝腫瘍においては腫瘍内血流や血管新生，Kupffer細胞の多寡，また基礎疾患となる慢性肝疾患における血管の変化は肝内門脈末梢枝のレベルまで描出できるようになった．造影超音波検査は空間，時間分解能が高いこと，また一番の利点は，リアルタイム性に富み身体的負担が少ないことである．肝腫瘍においては，迅速かつ正確な診断が患者予後や治療方針の決定においてきわめて重要であり，造影剤を使用することは，治療ガイドや治療効果判定など大きな武器になる．

消化器肝臓領域で使用できる経静脈性超音波造影剤は，シェルを持たないLevovistを第1世代の超音波造影剤とすると，シェルをもつ造影剤は第2世代の造影剤といえる．この第2世代の造影剤は，全世界では何種類かが臨床応用されている．欧州，中国ではSonoVue，カナダではDefinityが使用されている．わが国で使用できる第2世代の造影剤であるSonazoidは，これらの造影剤より若干高い音圧で使用され，マクロファージすなわちKupffer細胞に貪食される特徴を有する．Sonazoidによる造影超音波診断は，この点を応用し肝腫瘍の分化度診断や鑑別診断，さらには肝機能診断へと発展することが期待されている．

他の画像診断法の発展も著しく特にCTでは多列化により高い客観的診断能が得られている．また，本年発売になったEOBプリモビストは，肝細胞特異性を有するMRI用肝臓造影剤であり，血流診断に続き肝細胞に特異的に取り込まれ，特にdysplastic noduleなどの前癌病変と肝細胞癌との鑑別に期待が寄せられる．このように肝臓領域では機器の進歩もさることながら造影剤の進歩も著しい．

本稿では，造影超音波検査のたどってきた道程と現状そして今後の展望につき私見を含めて概説したい．

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1. 造影超音波検査法の変遷

造影超音波検査は，1969年に心臓カテーテルを用い直接インドシアニングリーンを心腔内に注入し，弁の逆流診断を行ったのが最初である[1]．その後，血流を視覚的に観察し心臓内のシャントや弁逆流の証明など主に循環器領域を中心とし研究が行われてきた[2)~7)]．超音波造影の映像がマイクロバブル由来であると報告されたのは1980年頃である．インドシアニングリーンや生理食塩水の中に含まれるマイクロバブルが超音波のエコー源となっていることを確認し，造影剤の改良が必要であることを指摘している[8]．その後もしばらく心筋染影，心機能診断を主体に循環器領域での研究が盛んに行われた[9)~11)]．肝腫瘍診断では，これらのマイクロバブルを応用した方法で1986年に松田らがCO2マイクロバブルを作成し血管造影時に肝動脈から動注による方法を開発した[12]．本法は，DSAを凌駕し血流検出感度では血管造影CTに近似した所見が得られるようになった[13)~16)]．しかし，その診断法も血管造影に伴うもので侵襲的検査法であった．1990年代にEcohvistが右心系造影の目的で市販され，術中門脈造影などの有用性が報告されたが，バブルが不安定で普及しなかった[17]．ほぼ同時期に日本と米国でAlbnexが市販された．しかし半減期が短時間で左心系と右心系での圧の変化による気泡の消失が多くいずれの造影剤も普及しなかった[18)~21)]．Levovistは，Ecohvistにパルミチンサンを加えることにより気泡の安定性が増加した[22)~24)]．心腔造影の目的では相前後してOptisonやImagentが市販された．Imagentは，現在は製造中止となっている．1996

Table 1 Pharmacokinetic Classification of Ultrasound Contrast Agent

Transpulmonary blood pool
Short half-life (＜5 min. after a bolus injection)
Albunex
Levovist (SHU 508A)
Longer half-life (5 min. after a bolus injection)
Optison (FSO 69)
SonoVue (BR1)
Definity (DMP 115)
Sonazoid (NC100100)
Transpulmonary with Organ-specific phase (liver, spleen)
Levovist (SHU 508A)
Sonazoid (NC100100)

年に腹部領域ではLevovistが欧州とカナダで市販されドプラ法で臨床使用され肝腫瘍診断や転移巣の検出に優れることが報告された[25)~28)]．Levovistは，数分間にわたって全身血管系を循環するため，右心腔，左心腔のみならず種々の臓器および血管における造影が可能となり脳血管障害の診断や腎動脈血流障害，乳腺腫瘍，超音波内視鏡など広く使用されるに至ったが，ドプラ法の感度不足なども指摘されている[29)~43)]．その後，心腔造影ではImagentが，腹部領域では2001年にカナダでDefinityが，同年欧州ではSonoVueが市販，中国では2006年からSonoVueが市販され肝腫瘍診断の有用性が報告されるようになった[44)~48)]．韓国では，2008年SonoVueが市販された．わが国に限ると，1999年9月Levovistが市販され肝腫瘍をはじめ血流診断や肝癌治療効果判定などに使用され映像方法や機器の発展につながった．その後2007年1月からSonazoidが認可され，その臨床応用が報告されつつある．Sonazoidは，1980年代にNycomed社が開発を始め，日本では1998年から開発が開始された．2001年から第3相試験を開始し2003年から肝細胞癌に対するラジオ波凝固療法に対する効果判定の有効性を検討する探索試験を開始し，2007年市販されるに至った．この造影剤は世界に先駆け発売され超音波診断においては画期的なことである．

映像方法も，造影剤の進歩とともに発展している．Levovistは，高音圧系の造影剤であり，治験の段階ではドプラ増強剤としての効果により診断するものであった[25)~28)49)~51)]．ドプラ手法や，間歇送信によるフラッシュエコーによりマイクロバブルを検出する方法であり，血流検出においては有用であった[52)~59)]．しかしカラードプラでの診断は，血流検出能の向上はあるが類洞レベルの血流表示には限界があり実際の臨床では造影CTや造影MRIに比較して劣っていると言わざるを得なかった[60)]．その後Bモードで非線形シグナルを映像化したティッシュハーモニックイメージが開発された[61)~64)]．本法を応用し微小気泡からの非線形散乱を映像化する技術である造影ハーモニックイメージングが開発され組織のperfusion imageが得られるようになり，肝腫瘍血流が詳細に検出できるようになった[65)66)]．さらに肝実質相では，Loss of correlation（LOC）を応用した手法により高音圧で気泡を崩壊させバブルからのシグナルを映像化することにより診断する技術が開発され[67)68)]，また，LevovistがKupffer細胞に貪食されることを利用した肝実質イメージによる診断法も発展し肝癌においては，分化度診断また発育過程，転移性肝癌の検出，肝細胞癌の治療域の判定，さらに肝機能診断への応用が可能となった[69)~75)]．しかしその手技の煩雑さ画像の読影，機器条件の難しさから広く普及するには至らなかった．

2. 造影剤の性質と種類

超音波は簡便，リアルタイムしかも非侵襲であることが利点である．それに使用する造影剤も，副作用がなく非侵襲でなければならない．

(1) 薬理動態

超音波造影剤は，その薬理動態により肺を通過しblood-pool agentとして使用されるものとblood-pool agentとして使用された後，臓器特異性を有しマクロファージに貪食されるものに分類される（Table 1）．LevovistとSonazoidはともにblood-pool agentとして診断に寄与した後，マクロファージに貪食され臓器特異性を有する[76)~78)]．SonoVueやDefinityは，blood-pool agentの性質が主体である[79)~81)]．一方，シェル（膜）をもつものと持たないものにも分類される．SonazoidやSonoVueは難溶性のガスを内包し脂質の膜に覆われている[78)80)]．一方Levovistはシェルを持たず，空気をガスとしている[24)]．

造影剤は，静脈投与された後，右心系から肺循環を経て左心系ならび末梢循環を通過し再循環する．気泡径に比して充分大きな血管内を流れる気泡からの信号を映像化すると，いわゆる血管造影となる．心腔造影や実質臓器の比較的太い（血管径が200 μm以上）動脈や静脈の評価に使われる[79)81)]．一方，毛細血管に近い太さの血管を通る時に映像化すると，実質が均一に染影