Table 8. Adjusted hazard ratio (HR) and 95% confidence interval (CI) of anastomotic recurrence by bile duct resection margin status in patients with a positive bile duct resection margin

	No. of recurrent cases	Person-days	Adjusted HR†	95% CI
Bile duct resection margin status				
R1 (is)	3	22 029	1.00	
R1	14	25 237	4.48	1.09, 18.5

†Adjusted for operation day and type of surgical resection. R1 (is): resection margin with intraductal carcinoma component, R1: resection margin with subepithelial invasive component.

patients with an intraductal carcinoma component infrequently developed large tumors and infrequently showed deep invasion into the bile duct wall and venous, lymphatic and perineural involvement in the main tumor nodule (Table 1). Furthermore, patients with an intraductal carcinoma component were infrequently at the advanced stage when diagnosed (Table 1). During the preparation of this paper, Nakanishi *et al.*[29] reported that cases of extrahepatic bile duct carcinoma with intraepithelial spread showed a more differentiated histological grade, less deep invasion, infrequent portal vein or hepatic invasion and a better prognosis than cases without such spread. In addition, we demonstrated statistically significant inverse correlations between the intraductal carcinoma component on the one hand and lymphatic and perineural involvement and TNM stage on the other, whereas Nakanishi *et al.* failed to show such correlations. Moreover, we demonstrated an inverse correlation between the intraductal carcinoma component and tumor aggressiveness by both univariate and multivariate analyses in a larger cohort than that reported by Nakanishi *et al.*[29], whereas that report performed only univariate analysis.

We have revealed that human cancer cell lines showing wide intraepithelial spreading in mouse inoculation models show strong adhesiveness to extracellular matrix proteins, which are components of the basement membrane of epithelial tissues, *in vitro*.[30] The expression patterns of cell-matrix adhesion molecules, such as integrins, in human cancer cell lines showing wide intraepithelial spreading in mouse inoculation models differ from those in human cancer cell lines that do not show such spreading.[30] In addition, it has been confirmed that there is a similar difference in the expression pattern of cell-matrix adhesion molecules between cancer cells showing, and not showing, such spreading in surgically resected clinical samples.[30] Cell-matrix adhesion molecules generally participate in cancer-stromal interactions and determine the invasiveness of human cancers.[31] Therefore, it is feasible that cancer cells showing wide intraepithelial spreading also show strong adhesiveness to the basement membrane of cancer nests and a less invasive tendency. This may be the reason why an intraductal carcinoma component was inversely correlated with aggressiveness in the main tumor nodule in this study. Although the molecular mechanism responsible for such an inverse correlation in biliary tract carcinoma needs to be further clarified, the presence of an intraductal carcinoma component may become an indicator of lower tumor aggressiveness. In fact, patients with an intraductal carcinoma component showed a significantly better prognosis than patients without such a component, irrespective of whether all patients from the present cohort or only patients who underwent complete resection were examined. The correlation between an intraductal carcinoma component and a favorable prognosis is consistent with the similar correlation observed in ductal carcinoma of the pancreas: the presence of an intraductal carcinoma component is reportedly a significantly good prognostic parameter for patients with invasive ductal carcinoma of the pancreas after surgical resection.[32–34]

On the other hand, the presence of an intraductal carcinoma component was significantly correlated with a positive bile duct resection margin (Table 4). Recently, Wakai *et al.* have reported that invasive carcinoma at the ductal resection margin appears to have a strong impact on patient survival, whereas residual carcinoma *in situ* does not, after surgical resection for extrahepatic bile duct carcinoma,[14] although they did not mention any background factors for the difference in prognostic impact between invasive carcinoma and carcinoma *in situ* at the ductal resection margin. We also defined two types of positive bile duct resection margin: R1 (is) (positive with only an intraductal carcinoma component) and R1 (positive with a subepithelial invasive component). An R1 bile duct resection margin was more frequently associated with poorer prognosis than a negative bile duct resection margin, whereas an R1 (is) bile duct resection margin was not more frequently associated with poorer prognosis than a negative bile duct resection margin (Tables 6 and 7). As in other malignancies, tumor recurrence after radical surgery for biliary tract carcinoma leads to eventual death.[26] Although Wakai *et al.* did not perform statistical analysis about anastomotic recurrence, the risk of anastomotic recurrence at the bile duct resection margin was significantly higher (Table 8), and the period until such recurrence was significantly shorter (Fig. 3), in patients with an R1 bile duct resection margin than in patients with an R1 (is) bile duct resection margin. Therefore, at least part of the difference in prognostic impact between R1 (is) and R1 bile duct resection margins is attributable to the difference in the risk of anastomotic recurrence.

However, the incidence of anastomotic recurrence in patients whose bile duct resection margin was positive [R1 (is) and R1] was only 26% (17 of 66 cases); some patients may die because of 'local recurrence' or distant metastasis before anastomotic recurrence becomes clinically obvious. Sakamoto *et al.* have proposed that anastomotic recurrence should be distinguished from 'local recurrence' derived from perineural invasion around the hepatic artery and/or involved regional nodes.[35] It is self-evident that all patients with an R1 (is) bile duct resection margin possess an intraductal carcinoma component that is inversely correlated with tumor aggressiveness, including perineural and lymphatic involvement and clinical stage. The intraductal carcinoma component may be partly responsible for the difference in prognostic impact between R1 (is) and R1 with reference not only to 'anastomotic recurrence' but also to 'local recurrence' derived from perineural invasion and/or involved regional nodes.

We analyzed both the intraductal carcinoma component and resection margin status in the same cohort and examined in detail the inconsistency of less tumor aggressiveness and a positive surgical margin in patients with intraductal carcinoma components. Surgeons are frequently required to decide the resection area based on the results of intraoperative histological diagnosis of bile duct resection margin status using frozen sections, and generally intend to achieve a negative bile duct resection margin. If the frozen section diagnosis is R1, the risk of death will be actually reduced if surgeons make efforts to achieve a negative bile duct resection margin. However, if the frozen section diagnosis is R1 (is), an intraductal carcinoma component is present and the prognosis for such patients will be

favorable. Moreover, if surgeons perform additional resection to achieve a negative bile duct resection margin, then the prognosis for patients whose initial bile duct resection margin is R1 (is) may not be improved significantly. Therefore, surgeons should not be persistent in trying to achieve a negative surgical margin when the intraoperative frozen section diagnosis is R1 (is), and can choose a safe surgical procedure to avoid postoperative complications.

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Correction

The authors would like to draw the reader's attention to errors in the following article:

Ojima H, Kanai Y *et al.* Intraductal carcinoma component as a favorable prognostic factor in biliary tract carcinoma. *Cancer Science* 2009; **100**: 62–70.

On page 63, the fourth sentence of the paragraph of "Univariate analysis of correlation between an intraductal carcinoma component and clinicopathological parameters" should read "Tumor size ($P = 0.003$), depth of invasion ($P < 0.0001$), venous involvement ($P < 0.0001$), lymphatic involvement ($P = 0.0006$), perineural involvement ($P < 0.0001$), the pathological assessment of the primary tumor (pT) ($P < 0.0001$), and pathological TNM stage ($P < 0.0001$) were each inversely correlated with presence of an intraductal carcinoma component:".

In Table 1, the fifth line should be corrected to followings.

	No. of the cases		*P* for difference*
	Intraductal carcinoma component		
	Negative ($n = 135$)	Positive ($n = 79$)	
Tumor size (cm)			0.003
<2	11	24	
2–3	43	16	
3–4	32	13	
≥4	49	26	

In Table 2, the sixth line should be corrected to followings.

	No. of deaths	Person-days	Crude death rate†	Crude HR	95% CI	*P* for trend
Tumor size (cm)						
<2	13	53 441	24.3	1.00		<0.01
2–3	33	80 951	40.8	2.02	1.06, 3.84	
3–4	30	52 249	57.4	2.47	1.28, 4.73	
≥4	55	73 372	75.0	3.12	1.70, 5.72	

On page 66, the third sentence of the paragraph of "Univariate analysis of correlations between bile duct resection margin status and clinicopathological parameters" should read "Location of the main tumor nodule ($P = 0.0004$), tumor size ($P = 0.004$), histological type ($P = 0.008$) and venous involvement ($P = 0.009$) were each significantly correlated with bile duct resection margin status (Table 5)".

In Table 5, the fifth line should be corrected to followings.

	No. of the cases			*P* for difference*
	Proximal side ductal resection margin			
	Negative ($n = 148$)	R1 (is) ($n = 21$)	R1 ($n = 45$)	
Tumor size (cm)				0.004
<2	22	10	3	
2–3	43	2	14	
3–4	31	4	10	
≥4	52	5	18	

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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

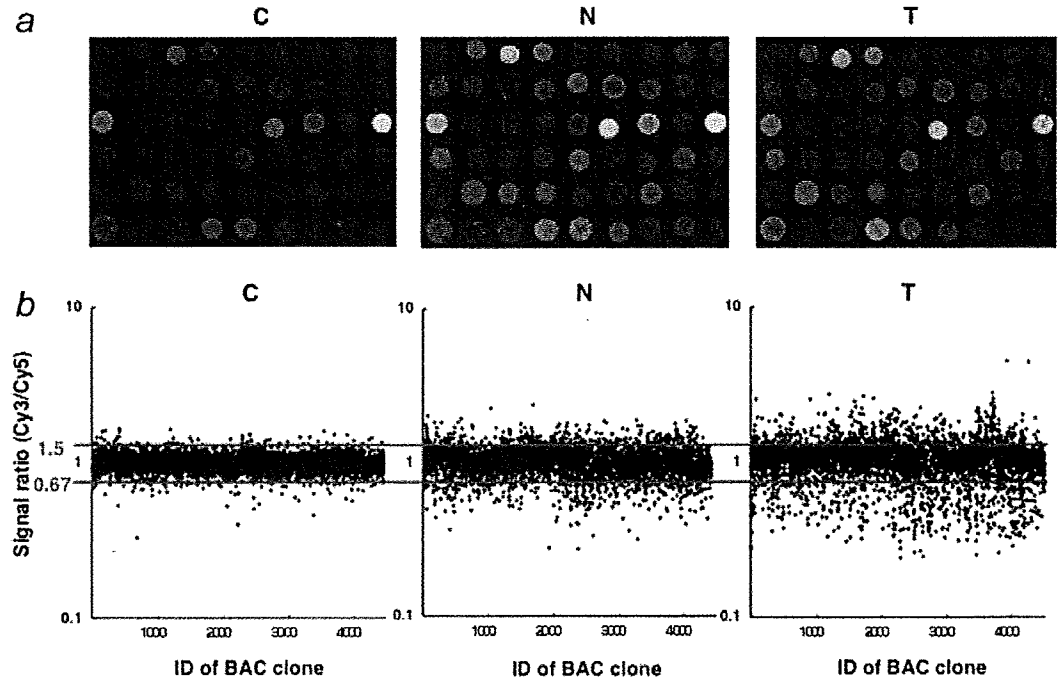

FIGURE 1 – Genome-wide DNA methylation alterations during multistage hepatocarcinogenesis. (*a*) Scanned array images yielded by BAMCA in normal liver tissue obtained from a patient without HCC (C) and noncancerous liver tissue (N) and cancerous tissue (T) obtained from a patient with HCC. (*b*) Scattergrams 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

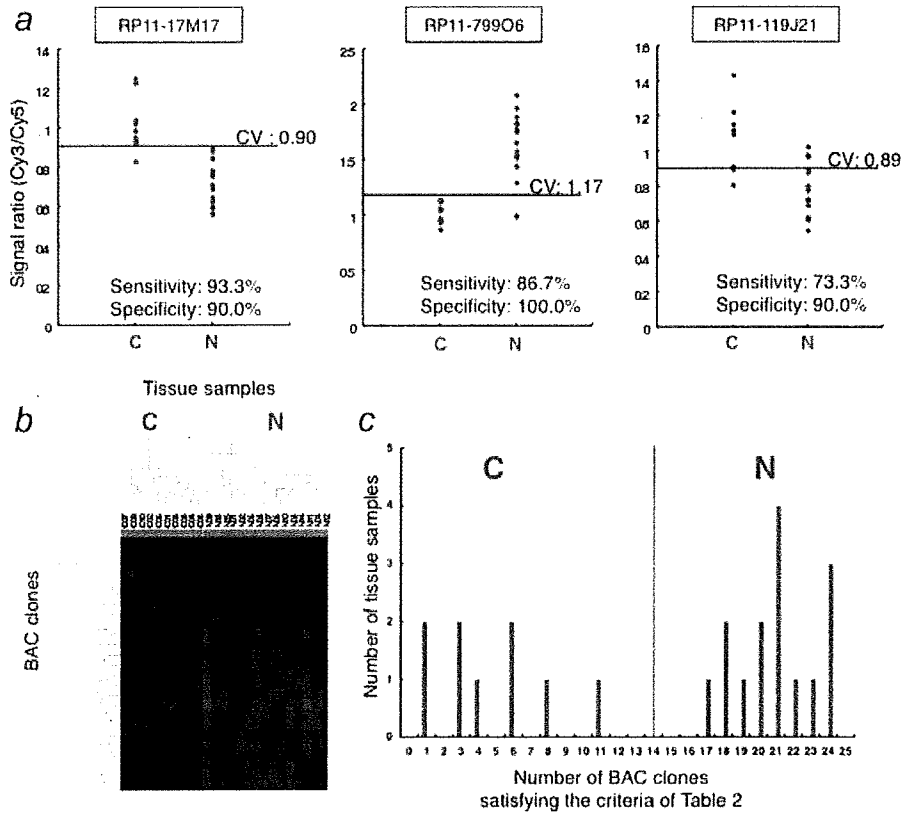

FIGURE 2 – DNA methylation profiles discriminating noncancerous liver tissue obtained from patients with HCCs from normal liver tissue. (*a*) Scattergrams of the signal ratios in normal liver tissue samples (C1 to C10) and noncancerous liver tissue samples obtained from patients with HCCs (N1 to N15)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

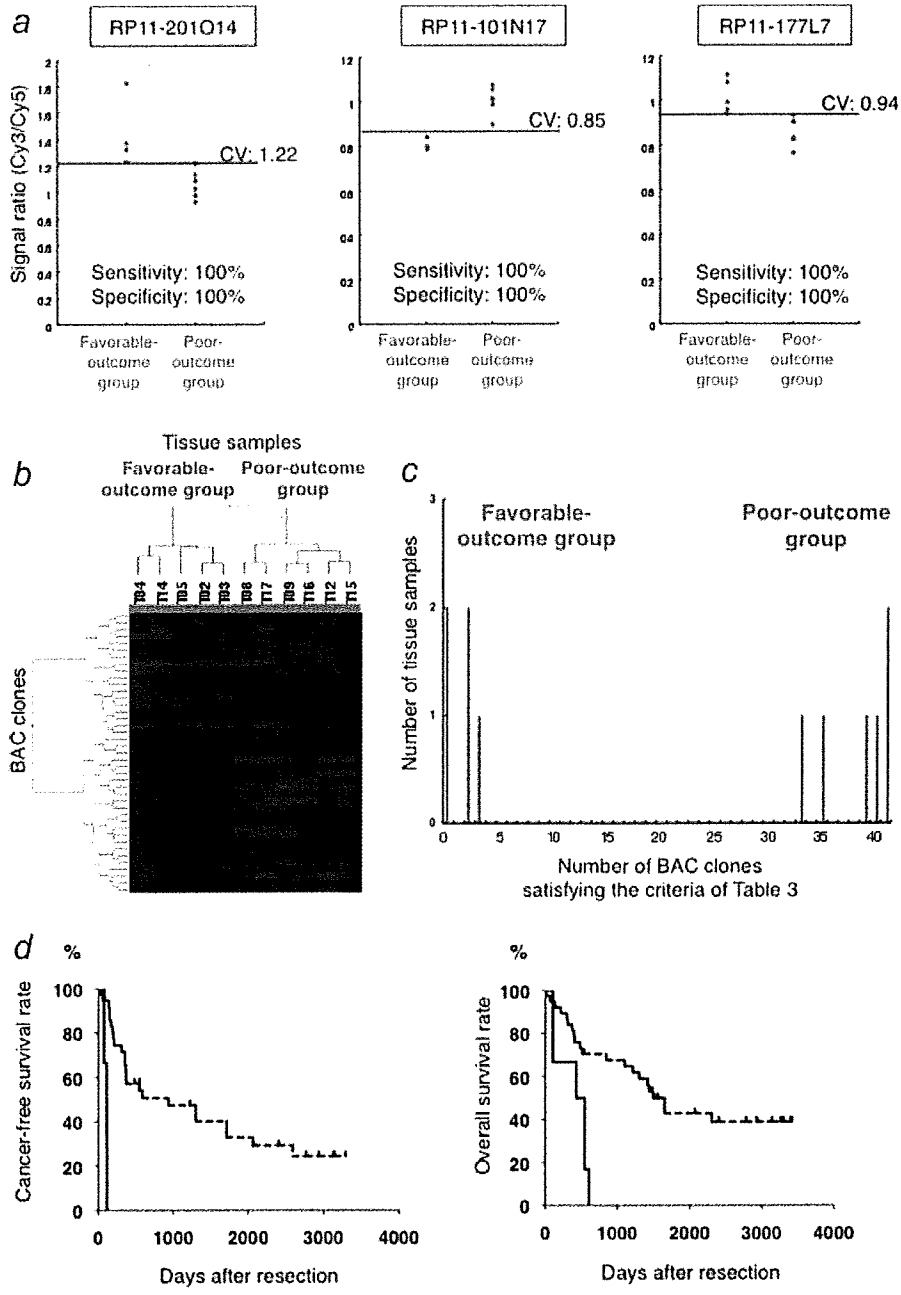

FIGURE 3 – DNA methylation profiles in HCCs associated with patient outcome. (*a*) Scattergrams of the signal ratios in HCCs from patients who survived more than 4 years after hepatectomy (favorable-outcome group, $n = 5$) and HCCs from patients who suffered recurrence within 6 months and died within 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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Review Article

Molecularly targeted therapy for hepatocellular carcinoma

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Accumulated understanding of the molecular pathways regulating cancer progression has led to the development of novel targeted therapies. Hepatocellular carcinoma (HCC) remains a highly lethal disease that is resistant to conventional cytotoxic chemotherapy and radiotherapy. Unlike conventional chemotherapy, molecular-targeted agents offer the potential advantages of a relatively high therapeutic window and use in combination with other anticancer strategies without overlapping toxicity. It is hoped that these drugs will become valuable therapeutic tools within the multimodal approach to treating cancer. A recent clinical trial revealed an oral multikinase inhibitor, sorafenib, as the first agent that has demonstrated improved overall survival in patients with advanced HCC. The present review summarizes molecular abnormalities of HCC with a focus on clinical studies, and current status as well as problems of the targeted strategies for HCC. (*Cancer Sci* 2009; 100: 1–8)

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide accounting for 500 000–600 000 deaths per year,[1,2] and the incidence is still increasing.[3] Although the primary curative treatment for HCC is surgical resection, there has been limited improvement in the availability of alternative treatments in the last decade.[4] A major obstacle for the treatment of HCC is the high frequency of tumor recurrence after curative resection. In fact, effective palliative treatment is hindered by the fact that HCC is frequently resistant to conventional chemotherapy and radiotherapy.[4] Moreover, the existing conventional chemotherapeutics are more or less non-selective cytotoxic drugs with significant systemic side effects. Importantly, as most patients with HCC have compromised liver function aggressive medical therapy regimens can not be applied. Thus, usually no effective therapy can be offered to these patients.[2,4] There is an urgent need to develop novel treatments for recurrent and advanced HCC.

Numerous studies on molecular abnormalities in HCC progression have revealed the crucial roles of such molecules in cell proliferation, as well as survival not only of cancer cells but also angiogenic or stromal cells.[5,6] Among the key pathways in the pathogenesis of HCC, this review focuses on the pathological processes including vascular endothelial growth factor (VEGF)-dependent tumor angiogenesis, epidermal growth factor receptor (EGFR), and insulin-like growth factor (IGF)-dependent tumor cell proliferation and survival in HCC (Fig. 1). Furthermore, several intracellular factors essential for hepatocarcinogenesis are demonstrated (Figs 2,3), coupled with molecularly targeted agents in clinical trials (Table 1).

VEGF and VEGF receptor family

In vivo tumor progression requires various host factors as well as tumor factors; in particular, neovascularization is one of the most important host factors.[7] HCC is well known as one of the tumors to present with typical neovascularization. A dramatic alteration in the arterial hypervascularity is observed in moderately to poorly differentiated-type HCC, but the new blood vessels are so irregular that the flow is often stagnated.[7,8] VEGF is one of the most potent growth factors of the vascular endothelial cells, as well as one of the critical effectors on progenitor cells.[9] The VEGF ligand family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, PIGF-1, PIGF-2, and VEGF-E derived from a virus gene (Fig. 1a).[10] VEGF-A and VEGF-B have spliced variants. The currently known VEGF genes and polypeptides belong to a family of structurally and functionally related growth factors, which also includes the platelet-derived growth factors (PDGF) that mainly function in the vascular mural cells (pericytes). In *Drosophila*, PEGF and VEGF-like factors share a single receptor. The human VEGF receptor (VEGFR) family consists of VEGFR-1/Flt-1, VEGFR-2/KDR/Flk-1, and VEGFR-3/Flt-4. Although either VEGFR-1 or VEGFR-2 regulates angiogenesis and vasculogenesis, VEGFR-3 is mainly related to lymphoangiogenesis.[11] We first found a close relationship between VEGF expression and the vascularity of HCC tumors compared that of non-cancerous liver tissue from a clinical specimen.[12] The expression of VEGF protein was found to correlate with clinicopathological factors such as proliferation, vascular invasion, and tumor multiplicity.[13] VEGF expression was reported to associate with not only invasion and metastasis of HCC,[14] but also postoperative recurrence.[15]

Expression of VEGF is regulated by micro-environmental and genetic alterations in cancer cells. Hypoxia is a key micro-environmental factor of angiogenesis, and hypoxia-inducible factors (HIF) are known to stimulate VEGF expression.[16,17] The upregulation of VEGF in HCC is controlled by transcriptional levels as well as the mRNA stability of VEGF.[18] In addition, the *p53* tumor-suppressor and *HBx* genes might regulate VEGF expression in HCC.[19,20] Furthermore, we previously identified angiopoietin-2–Tie2 signaling as another angiogenic pathway essential for HCC progression.[9,21] These angiopoietin-2 signals also require VEGF activation in the angiogenic switch.[22]

It should be mentioned that the receptors VEGFR-1/Flt-1 and VEGFR-2/KDR/Flk-1 have been identified on HCC cells.[23] These findings suggest that VEGF might function in the migration of endothelial cells, as well as in HCC cells per se, indicating a possibly novel mechanism for HCC progression.[24] Recent studies revealed critical roles of VEGFR-1-expressing hematopoietic cells in formation of the premetastatic niche.[25,26] VEGF signaling functions not only in angiogenesis but also in cancer invasion and metastasis.[27] Given that the VEGF and VEGFR pathways are required for the pathogenesis and progression of HCC, it is

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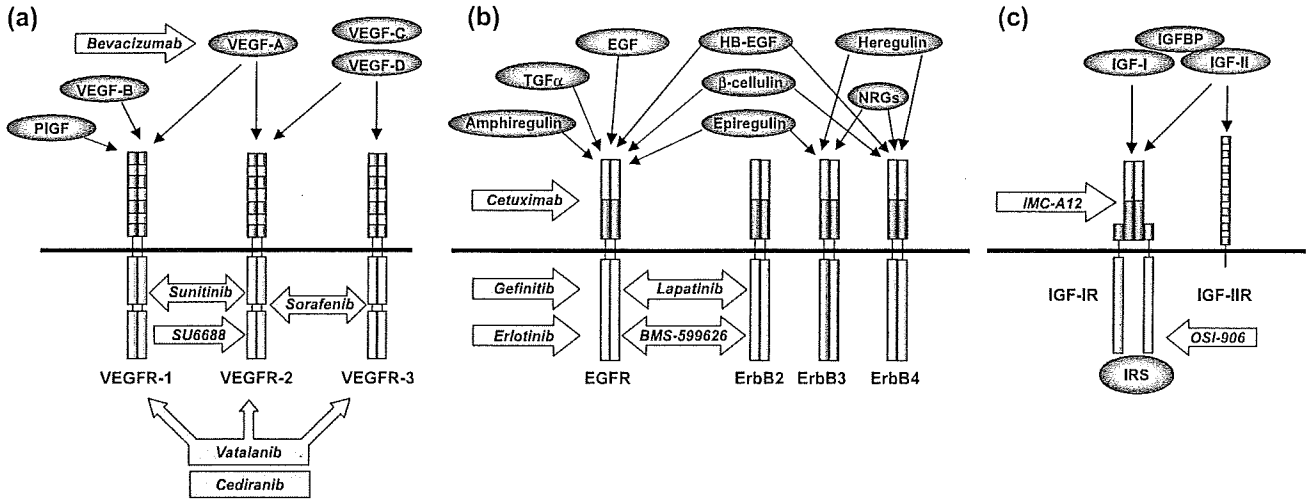

Fig. 1. Molecular targets in the (a) epidermal growth factor (EGF) and EGF receptor (EGFR) family, (b) vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) family, and (c) insulin-like growth factor (IGF) and IGF receptor (IGFR). Targeted agents are indicated by arrows.

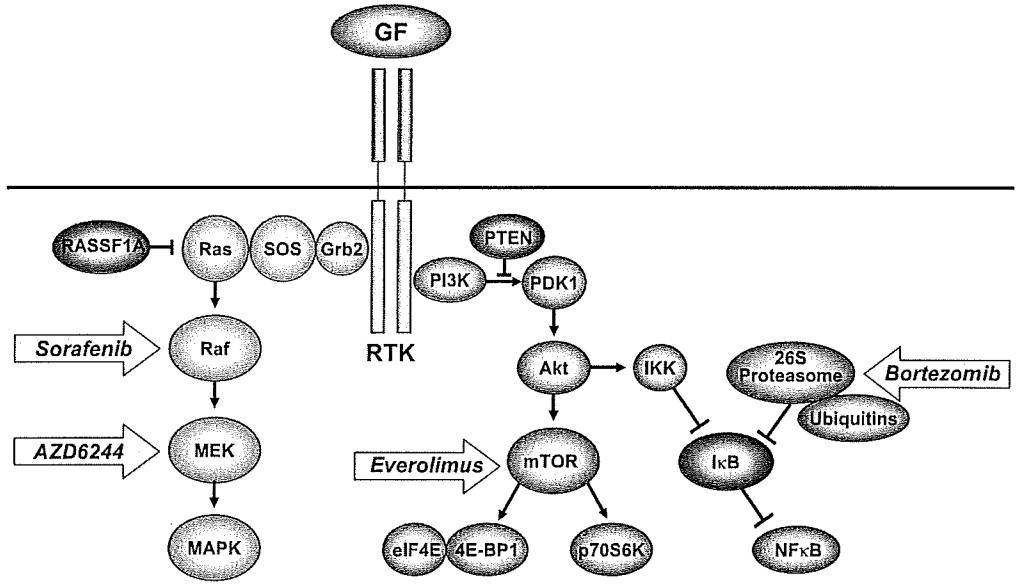

Fig. 2. Molecular targets in mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) signal transduction pathways stimulated by receptor tyrosine kinases (RTK). Targeted agents are indicated by arrows. GF, growth factor; SOS, sun of sevenless; MEK, MAPK/extracellular regulated kinase kinase; PTEN, phospharase and tensin homologue; PDK, phosphoinositide-dependent kinase; IKK, IκB kinase; mTOR, mammalian target of rapamycin.

possible that inhibitors of VEGF signaling are promising therapeutic agents for HCC treatment.

Most of these compounds can be broadly classified into two main categories: small-molecule kinase inhibitors and monoclonal antibodies. Sorafenib (Nexavar, BAY43-9006) is a unique multitargeting small molecule that inhibits the receptor tyrosine kinases (RTK) VEGFR-2, VEGFR-3, Flt-3, PDGF receptor (PDGFR), and fibroblast growth factor receptor (FGFR)-1, as well as Raf serine-threonine kinase in the signal transduction pathway Ras–Raf–mitogen-activated protein kinase/extracellular regulated kinase (MEK) –mitogen-activated protein kinase (MAPK)[28] (Figs 1a,2). In a recent phase III trial, the Sorafenib HCC Assessment Randomized Protocol (SHARP), 602 patients with advanced HCC who had received no prior systemic therapy were evaluated and randomized to receive either sorafenib ($n = 299$) or placebo ($n = 303$).[1] The median overall survival was 10.7 months in sorafenib-treated patients compared with 7.9 months in patients who received placebo, indicating a 44% increase in overall survival (hazard ratio, 0.69; $P < 0.0001$). The median time to radiological progression was 5.5 months in sorafenib-treated patients compared with 2.8 months in patients who received placebo (hazard ratio, 0.58; $P < 0.0001$). The overall incidence of treatment-related adverse events was 80% in the sorafenib group and 52% in the placebo group. Grade 3 drug-related adverse events included diarrhea (8% in the sorafenib group vs 2% in the placebo group, $P < 0.001$), and hand or foot skin reaction (8% in the sorafenib group vs <1% in the placebo group, $P < 0.001$). Grade 3 or 4 laboratory abnormalities occurred with grade 3 hypophosphatemia (11% in the sorafenib group vs 2% in the placebo group, $P < 0.001$) and grade 3 or 4 thrombocytopenia

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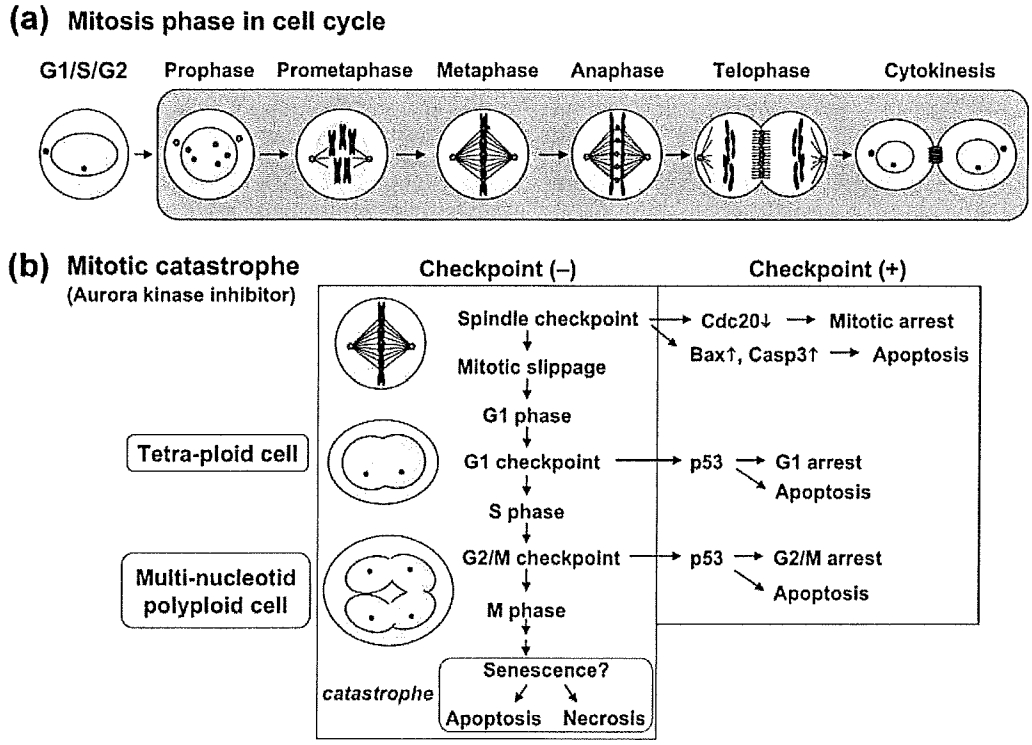

Fig. 3. (a) The mitosis phase of the cell cycle. Localization of Aurora kinases is shown. Green spots, the centrosome protein Aurora kinase A; red spots, the chromosomal passenger Aurora kinase B. (b) The concept of mitotic catastrophe, induced by inhibition of Aurora kinases in cancer cells. Under abnormalities in each checkpoint system, p53-independent death is induced as senescence-like polyploidy without successfully completing mitosis.

Table 1. Molecularly targeted agents for hepatocellular carcinoma

Agent	Classification	Function	Clinical trial
Sorafenib (Nexabar, BAY43-9006)	Small molecule compound	VEGFR2, VEGFR3, PDGFR-β tyrosine kinase, Raf serine/threonine kinase inhibitor	Phase III
Sunitinib (Sutent, SU11248)	Small molecule compound	VEGFR1, VEGFR2, PDGFR, Flt-3, c-KIT tyrosine kinase inhibitor	Phase II
SU6688 (TSU-68)	Small molecule compound	VEGFR2, PDGF R-β, FGFR tyrosine kinase inhibitor	Phase II
Vatalanib (PTK787/ZK222584)	Small molecule compound	VEGFR1, VEGFR2, VEGFR3, PDGFR-β, c-KIT tyrosine kinase inhibitor	Phase II
Cediranib (AZD2171)	Small molecule compound	VEGFR1, VEGFR2, VEGFR3, PDGFR, c-KIT tyrosine kinase inhibitor	Phase II
Bevacizumab (Avastin)	Monoclonal antibody	VEGF-A neutralization	Phase II
Gefintinib (Iressa, ZD1839)	Small molecule compound	EGFR/ErbB1/Her1 tyrosine kinase inhibitor	Phase II
Erlotinib (Tarceva, OSI774)	Small molecule compound	EGFR/ErbB1/Her1 tyrosine kinase inhibitor	Phase II
Lapatinib (Tykerb, GW572016)	Small molecule compound	EGFR/ErbB1/Her1 and ErbB2/Her2/Neu tyrosine kinase inhibitor	Phase II
BMS-599626	Small molecule compound	EGFR/ErbB1/Her1 and ErbB2/Her2/Neu tyrosine kinase inhibitor	Phase II
Cetuximab (Erbitux, GW572016)	Monoclonal antibody	EGFR/ErbB1/Her1 neutralization	Phase II
AZD6244 (ARRY-142886)	Small molecule compound	MEK serine-threonine tyrosine kinase inhibitor	Phase II
IMC-A12	Monoclonal antibody	IGF-IR neutralization	Phase II
Everolimus (RAD001)	Small molecule compound	mTOR serine-threonine kinase inhibitor	Phase I and II
Sirolimus (Rapamune)	Small molecule compound	mTOR serine-threonine kinase inhibitor	Phase I
Bortezomib (Velcade)	Small molecule compound	Proteasome inhibitor	Phase I and II
PXD101 (Belinostat)	Small molecule compound	HDAC inhibitor	Phase I and II
PI-88	Small molecule compound	Heparanase inhibitor	Phase III

(4% in the sorafenib group vs <1% in the placebo group, $P = 0.006$).

Sunitinib (Sutent, SU11248) is another oral inhibitor that targets RTK of the split-kinase domain family, including VEGFR-1, VEGFR-2, PDGFR-α, PDGFR-β, c-Kit, Flt-3, and RET.[29] The phase II studies have examined the tolerability and efficacy of sunitinib in patients with advanced HCC.[30] Of the 37 patients enrolled, one patient had a confirmed partial response (PR), and 39% patients had stable disease (SD) as their best response. Grade 3 and 4 toxicities included thrombocytopenia (43%), neutropenia (24%), central nervous system symptoms (24%), asthenia (22%), and hemorrhage (14%). Dose reductions were required in 27%

patients. Four patients developed grade 5 events, including ascites, edema, bleeding, drowsiness, and hepatic encephalopathy.

Vatalanib (PTK787/ZK222584) is an oral pan-VEGFR inhibitor with activity against PDGFR-β and c-Kit (Fig. 1a).[31] Preclinical studies suggested anti-angiogenic and angiogenesis-independent effects on HCC growth arrest.[32] In a phase I study of vatalanib in 18 patients with unresectable HCC, nine patients had a best response of SD, and nine patients had progressive disease (PD).[33] Cediranib (AZD2171) is another potent pan-VEGFR inhibitor with activity against PDGFR and c-Kit.[34] According to a phase II study of cediranib in patients with advanced HCC, 28 patients were accrued, and 19 patients were evaluable for toxicity.[35] Of these, 16 patients (84%) developed grade 3 toxicity. Fatigue, hypertension, and anorexia accounted for the majority of adverse events. A high rate of refusal of further treatment was encountered and apparently was related to the high rate of grade 3 fatigue. A dose reduction of cediranib was planned in an ongoing study. SU6668 (TSU-68) is a potent RTK inhibitor against VEGFR-2, PDGFR-β, and FGFR.[36] According to preliminary data from a Japanese phase I and II study in 15 HCC patients, one patient had PR, seven patients had SD (two patients for over 12 months), and seven patients had PD.[37] Tumor necrosis was observed in eight patients. The adverse events were hypoalbuminemia, diarrhea, abdominal pain, fever, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) elevation.

Bevacizumab (Avastin), a recombinant, humanized monoclonal antibody that targets VEGF, has emerged as an important therapeutic agent in several malignancies.[38] In addition to its direct antiangiogenic effects, bevacizumab may enhance chemotherapy administration by normalizing tumor vasculature.[39] Several studies have explored the use of bevacizumab either as a single agent or in combination with cytotoxic or molecularly targeted agents in patients with HCC. In a phase I study in 25 patients using single-agent bevacizumab, two patients had a PR, and 18 patients had SD. The median time to progression was 6.5 months.[40] In a phase II study using single-agent bevacizumab,[41] among the 24 patients who were evaluable for efficacy, three patients had a PR and seven patients had SD that lasted at least 16 weeks. The combination of bevacizumab with cytotoxic agents also was evaluated. In a recent phase II study, bevacizumab in combination with gemcitabine and oxaliplatin (GEMOX) was used as treatment for patients with advanced HCC.[42] This regimen had moderate antitumor activity in HCC with an overall response rate of 20% in evaluable patients. An additional 27% patients had SD with a median duration of 9 months. The median overall survival was 9.6 months, the median progression-free survival was 5.3 months, and the progression-free survival rate at 3 and 6 months approached 70 and 48%, respectively. Bevacizumab-related side effects, including hypertension, bleeding, and proteinuria, were generally manageable. The encouraging results from that early study should be confirmed cautiously by an independent study in the future.

EGFR/ErbB family

EGFR/ErbB1/Her1 is a member of a RTK family that also includes ErbB2/Her2/Neu, ErbB3/Her3, and ErbB4/Her4 (Fig. 1b).[43] EGFR/ErbB1 binds not only epidermal growth factor (EGF) but also transforming growth factor (TGF)-α, amphiregulin, HB-EGF, β-cellulin, and epiregulin. Although heregulins, epiregulin, and NRG have been identified as ligands of ErbB3/Her3, and heregulins, epiregulin, HB-EGF, β-cellulin, and NRG have been identified as ligands of ErbB4/Her4, no ligands have been identified for ErbB2/Her2/Neu. Either EEGFR/ErbB1 or ErbB2/Her2/Neu is overexpressed in various cancers. The binding of ligand to EGFR/ErbB1 leads to homodimerization or heterodimerization with ErbB2/Her2/Neu or ErbB3/Her3, resulting in tyrosine kinase activation and self-phosphorylation (not in ErbB2/Her3). Activation of RTK transduces the MAPK and phosphatidylinositol-3 kinase (PI3K) signaling pathways. The MAPK signals mainly stimulate cell proliferation, and the PI3K signals activate Akt anti-apoptotic pathways. Overexpression of TGF-α has been observed in the early stages of hepatocarcinogenesis, and is associated with upregulation of VEGF.[44]

The crucial role of EGFR in HCC proliferation has provided the rationale for targeting and interrupting this key signaling network. Gefinitib (Iressa, ZD1839) is an oral tyrosine kinase inhibitor that selectively suppresses EGFR and not other tyrosine kinases such as ErbB2 or VEGFR (Fig. 1).[45] According to the clinical trials for non-small lung cancers, the effects of gefitinib correlate with EGFR mutations in cancer cells.[46,47] In a phase II clinical trial of 31 patients with HCC, one patient had PR and seven patients had SD, but there were no clear effects using single administration as the median overall survival was 6.5 months and the median progression-free survival was 2.8 months.[48] The criterion for second-stage accrual was not met, and the authors concluded that gefitinib as a single agent was not active in patients with advanced HCC. Erlotinib (Tarceva, OSI774) is another oral EGFR-selective inhibitor, and two phase II clinical studies have evaluated its safety and efficacy in patients with advanced HCC.[49] In the study by Philip *et al.*, 3 of 38 patients had PR and 12 patients had progression-free survival at 6 months.[50] The median overall survival for this cohort was 13 months. In another report by Thomas *et al.* 17 of 40 patients achieved progression-free survival at 16 weeks and the progression-free survival rate at 24 weeks was 28%.[51] No PR or complete response (CR) was observed in that study, but the median overall survival was 25 weeks. Lapatinib (Tykerb, GW572016), a selective dual inhibitor of both EGFR and ErbB2 tyrosine kinases, also demonstrated modest activity in HCC in a preliminary report.[52] Among the first 17 patients with advanced HCC, two patients had a confirmed PR and an additional eight patients had SD. However, the progression-free survival was only 2.3 months in this cohort. For another dual inhibitor, BMS-599626, a phase II clinical trial is ongoing in patients with HCC.

Cetuximab (Erbitux, IMC-C225), a chimeric monoclonal antibody against EGFR, has attracted attention in colorectal cancer as a K-Ras biomarker.[53,54] Cetuximab was also tested in two phase II studies in patients with advanced HCC. Zhu *et al.* reported five patients with SD in 30 patients with advanced HCC.[55] The median overall survival was 9.6 months, and the median progression-free survival was 1.4 months. Gruenwald *et al.* reported their preliminary experience of cetuximab in a similarly designed study in patients with HCC.[56] Of the 32 patients who were enrolled, 27 patients were evaluable for efficacy. No responses were observed, and the median time to progression for all patients was 8 weeks. The combination of cetuximab with GEMOX was evaluated in a phase II study.[57] Of the 43 patients who were enrolled, 35 patients were evaluable for efficacy, with a response rate of 23%. Given the known antitumor activity of GEMOX in prior phase II studies and the lack of activity of cetuximab as a single agent, the relative contribution of cetuximab to this regimen remains to be defined. The axis of TGF-α–EGFR signaling might be an attractive therapeutic target as frequent mutations have not been found in downstream molecules, such as Ras and Raf family members, in HCC.

Insulin-like growth factor and IGF receptor family

There is compelling evidence that both of the insulin-like growth factors IGF-I and IGF-II and their receptor IGF-1R are involved in the development and progression of cancer[58] (Fig. 1c). Interaction of IGF-I and IGF-II with IGF-1R plays a pivotal role in the tumorigenesis, proliferation, and spread of

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many cancers by promoting cell-cycle progression, preventing apoptosis, and regulating and maintaining the tumorigenic phenotype. A wide variety of tumors (including HCC) show abnormal or enhanced expression of IGF and IGF-1R, which has been correlated with disease stage, reduced survival, development of metastases, and tumor dedifferentiation.[59] We have previously identified insulin receptor substrate (IRS)-1, the main substrate of IGF-1R/insulin receptor (IR),[60,61] as an overexpressed molecule with significant roles in hepatocarcinogenesis.[62,63] Interestingly, serine phosphorylated IRS-1 protein by TNF-α is converted into an inhibitor of IGF-1R/IR.[64] Obesity and diabetes are clearly associated with an increased risk of HCC, and this seems to be due to alterations in the IGF signaling systems.

Several approaches have demonstrated the therapeutic potential of interfering with IGF-1R-mediated signaling *in vitro* and *in vivo*. Specific IGF receptor (IGFR) antibodies have also been shown to suppress prostate and breast cancer cell growth in a recent preclinical study.[65] The most advanced clinical anti-IGFR antibody is *IMC-A12*, which is currently being tested in a phase II trial for HCC. Importantly, IGFR inhibition appears to be well tolerated in the preliminary clinical studies conducted so far.[66] Safety is important, as IGFR-based inhibition has long been regarded as a high-risk intervention because of the high homology of IGF-1R with the related IR, and there is a fear that IGF-1R-tyrosine kinase inhibitors in particular might also block IR, which could lead to insulin resistance and overt diabetes.[67] However, the current *in vivo* studies did not confirm this apprehension, resulting in growing interest in anti-IGFR-based therapies. OSI-906, an IGF-1R tyrosine kinase inhibitor, is currently being tested in phase I trials for solid tumors including pancreatic cancer. The most promising IGF- and IGFR-targeted agents are currently under intense investigation in preclinical and early clinical trials.[68]

Intracellular signaling pathways

Activated RTK stimulate several intracellular signal transduction pathways, including Ras–Raf–MEK–MAPK and PI3K–Akt–mammalian target of rapamycin (mTOR) (Fig. 2).[69] In a series of specific phosphorylation events, the adaptor protein Grb2 stimulates sun of sevenless (SOS), leading to the activation of Ras, which is farnesylated and localized under the cell membrane. Farnesyl transferase inhibitors have been used in clinical trials of pancreatic cancer treatments, usually with mutations in the K-ras oncogene.[70] In spite of the low incidence of Ras gene mutations in HCC, silencing of the *RASSF1A* gene (a member of the Ras inhibitor family) with DNA methylation was found frequently in human HCC.[71] Inactivation of the Ras inhibitor might result in persistent activation of the downstream pathway during hepatocarcinogenesis. The activated form of Ras then stimulates Raf serine-threonine kinase. As mentioned above, Raf kinase is one of the targets of the multikinase inhibitor sorafenib.[1,28] Activated Raf kinase phosphorylates MEK kinases, which activate the extracellular regulated kinases Erk1/2 of the MAPK family. A MEK kinase inhibitor, AZD6244 (ARRY-142886), has been evaluated for HCC treatment in a phase II clinical trial. Once activated, Erk1/2 translocates to the nucleus where it acts as a regulator of gene expression, including those for proteins involved in cell cycle progression, apoptosis resistance, and cellular motility.[72]

The PI3K–Akt–mTOR pathway has emerged as a contributor to hepatocarcinogenesis.[73] PI3K consists of p85 adaptor and p110 kinase subunits. After association with the intracellular domain of several RTK or specific substrates such as IRS-1, PI3K phosphorylates phosphatidylinositol 3,4,5-trisphosphate (PIP3) to generate phosphatidylinositol 4,5-bisphosphate (PIP2), which transduces phosphoinositide-dependent kinase (PDK), which in turn activates the serine-threonine kinase Akt.[72] PIP3 is dephosphorylated by phospharase and tensin homologue (PTEN), a tumor suppressor, which reverses this pathway. Once activated, Akt regulates multiple cellular target proteins, including mTOR, IκB kinase (IKK), Bad, and Gsk3. The mTOR protein regulates phosphorylation of the p70 S6 serine-threonine kinase and the translational repressor protein 4E-BP1.[74] Both proteins regulate the translation of proliferative and angiogenic factors, such as c-myc, cyclin-D1, and HIF1-α, and are indirectly involved in the expression of VEGF.[75] The mTOR inhibitors temsirolimus (CCI-779) and everolimus (RAD001) have been developed as rapamycin derivates.[76,77] A phase I clinical trial using rapamycin and bevacizumab and a phase I and II clinical trial using everolimus are ongoing in patients with HCC.[76]

Another downstream protein of Akt, IKK, provokes subsequent activation of the transcription factor nuclear factor (NF)-κB.[78] NF-κB promotes cell survival by activating transcription of normally repressed target genes by binding the specific inhibitor IκB, which sequesters the NF-κB p50–p65 heterodimer in the cytoplasm.[79] Inhibition is reversed in response to several intracellular stimuli, resulting in targeted, ubiquitin–proteasome-mediated degradation of IκB.[80] The proteasome is a 26S multiprotein complex that consists of a 19S regulatory subunit and a 20S catalytic subunit. Ubiquitinated proteins are recognized by the 19S unit, which results in the liberation of ubiquitin chains that are recycled and the formation of a denatured protein that is transferred to the outer ring or the 20S core unit. Bortezomib (Velcade, PS-341) is a potent and selective inhibitor of the 20S proteasome.[81] The actions of bortezomib are pleiotropic and include inhibition of NF-κB activation by preventing IκB degradation. 26S proteasome-mediated protein degradation is a central metabolic and regulatory process in cell physiology. Apart from its role in scavenging damaged proteins, the 26S complex is an important regulator of cell life and fate. For instance, specific ubiquitination of key proteins, such as cyclins A, B, D, and E, eEF2-kinase, c-Myc, Notch, c-Jun, p21WAF/CIP1, p27, p53, topoisomerases I and II, the apoptosis modulators XIAP, Bik/NBK, Bad and Bid, the transcription coactivator β-catenin, and the NF-κB regulator IκB, targets these proteins toward proteasome degradation.[80] Through its regulation of protein turnover, the 26S proteasome is thus involved in cell-cycle progression, apoptosis, and other processes like angiogenesis and cell motility that are important in cancer progression. With the unique and independent anti-tumor effects of bortezomib, combination therapy with other cytotoxic or molecular-targeting agents is expected. Bortezomib received approval for second-line therapy of patients with progressive multiple myeloma,[80] and a phase I and II study for HCC is ongoing.[82] Because abnormalities of intracellular signal transduction pathways should play essential roles in carcinogenesis and cancer progression, further studies should be carried out.

Aurora kinases and mitotic catastrophe

Cell-cycle checkpoints are pivotal mechanisms safeguarding genomic stability. Cells that harbor defects in checkpoints are predisposed to genomic instability and neoplastic transformation. Of all the different checkpoint controls, the most important one is the mitotic spindle checkpoint, which is considered the primary defense against aneuploidy and ensures accurate chromosome segregation to produce genetically identical daughter cells (Fig. 3a).[83] Among spindle checkpoint kinases, the Aurora family of serine-threonine kinases has recently emerged as a key mitotic regulator required for genomic stability.[84] Aberrant expression of the Aurora kinase family has been reported in a variety of solid tumors. In mammals, the Aurora family consists of three members: A, B, and C. Aurora kinase A plays important roles in centrosome maturation and separation, and acentrosomal and centrosomal spindle assembly. Recent studies revealed that

Aurora kinase A also controls spindle axis orientation during asymmetric division of stem cells. Abnormalities of asymmetric division were detected in *Drosophila* that had a mutation in the mitotic kinase Aurora A, resulting in massive overproliferation in tumors.[85] Thus, the cell fate of cancer stemness might be regulated by Aurora kinase.

Aurora kinase B, another member of the family, is a chromosomal passenger protein that regulates accurate chromosomal segregation, cytokinesis, protein localization to the centromere and kinetochore, correct microtubule–kinetochore attachments, and regulation of the mitotic checkpoint.[86] Recently, we identified overexpression of Aurora kinase B as the only independent factor predictive of aggressive recurrence of HCC, based on analysis of genome-wide microarray profiling on clinical samples.[87,88] It is of interest that Aurora kinase B overexpression is closely correlated with genetic instability of HCC. Several small-molecule inhibitors of Aurora kinases have been developed as potential anticancer agents, including ZM447439,[89] hesperadin,[90] VX-680,[91] PHA-680632,[92] and MLN 8054.[93] Aurora kinase inhibitors such as AT9283 and AZD1152[94] are currently undergoing phase I clinical evaluation as treatments for malignancies.[95]

Aurora kinase B, in particular, may be a suitable anticancer target as its inhibition rapidly results in catastrophic mitosis with senescence.[95,96] During mitotic karyokinesis under downregulation of Aurora kinases, a process termed micronucleation occurs in the cancer cells. Thus, unable to maintain G_2 arrest, they enter mitosis and after being arrested for several hours at metaphase, they eventually die without successfully completing mitosis. This process is known as p53-independent cell death or mitotic catastrophe (Fig. 3b).[96] In our studies, a selective Aurora kinase B inhibitor induced *in vitro* polyploidy of human HCC cells, resulting in mitotic catastrophe. Our preclinical studies using the Aurora kinase B inhibitor revealed remarkable anti-tumor effects on HCC models *in vivo*. The inhibitor was well tolerated within the dose range required to elicit a potent and durable effect in mice. Specific inhibition of Aurora kinases is a promising novel therapeutic approach for the treatment of HCC. Further studies and clinical trials of Aurora inhibitors will confirm their significance in HCC therapeutics.

Conclusion

The concept of targeted therapies that specifically inhibit molecular abnormalities has emerged as a promising approach for the innovative and effective medical treatment of various cancers, including HCC. In this regard, sorafenib must throw new light and impact on studies of molecularly targeted agents in HCC.[6] However, although the SHARP study revealed positive and landmark results, the benefits of sorafenib were reported to be relatively modest in patients with HCC.[1] Furthermore, biomarkers to predict its effects are poorly understood. Future research should continue to unravel the mechanism of hepatocarcinogenesis and to identify key relevant molecular targets for therapeutic intervention. The advantages of molecular targeting are being explored in combination treatments as well as adjuvant or neoadjuvant therapies with surgical resection, liver transplantation, radiofrequency and transarterial chemoembolization.[4] We are only now at the beginning of the history of finding novel treatments for HCC.

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