mophobe RCCs and oncocytomas was significantly different from that of clear cell RCCs, suggesting that histological subtype-specific DNA methylation alterations have already occurred, even in apparently normal renal tissue (table 1). Although we analyzed samples of non-tumorous renal cortex tissue as well as tumorous tissue using normal renal cortex tissue as a reference for the comparison of all histological subtypes (table 1), chromophobe RCCs and oncocytomas are considered to be derived from the intercalated cells of the collecting duct. We also examined DNA methylation status in non-tumorous renal medulla tissue obtained from patients with chromophobe RCCs using a mixture of normal renal medulla DNA as a reference. The numbers of BAC clones showing DNA methylation alterations in renal medulla tissue did not differ significantly from those in renal cortex tissue obtained from individual patients with chromophobe RCCs (online supplementary table 1, www.karger.com/doi/10.1159/000322072). Therefore, we were able to observe differences of DNA methylation status between non-tumorous renal tissue from patients with chromophobe RCCs and that from patients with clear cell RCCs even when we used tissue samples of the renal medulla and cortex, which are the tissues of origin of chromophobe RCCs and clear cell RCCs, respectively. It is possible that the DNA methylation status of non-tumorous renal tissue obtained from patients with papillary RCCs was not different from that of clear cell RCCs (table 1), because papillary RCCs themselves showed DNA methylation profiles similar to those of clear cell RCCs (fig. 2).

It is known that patients with chromophobe RCCs and oncocytomas generally show a more favorable outcome than patients with clear cell RCCs [32]. Since patients with chromophobe RCCs and oncocytomas showed DNA methylation alterations in non-tumorous renal tissue that differed from those of patients with more aggressive clear cell RCCs (table 1), we evaluated the correlation between the DNA methylation status of non-tumorous renal tissue and patient outcome. Surprisingly, patients with accumulation of DNA methylation (DNA hypo- or hypermethylation on ≥250 BAC clones) in their non-tumorous renal tissue showed a poorer outcome than patients without such accumulation (DNA hypo- or hypermethylation on <250 BAC clones; fig. 4b). Although one cannot easily conclude that DNA methylation alterations in non-tumorous renal tissue are correlated with histological subtype (chromophobe RCCs and oncocytomas vs. clear cell RCCs) or patient outcome (favorable outcome vs. poorer outcome), or both, the present study including various histological subtypes indicated that DNA methylation status was not simply altered in precancerous conditions, but that significant DNA methylation profiles determining the histological subtypes of future developing renal tumors and/or patient outcome are already established at the precancerous stage.

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Research Article

Diagnosis and Prognostication of Ductal Adenocarcinomas of the Pancreas Based on Genome-Wide DNA Methylation Profiling by Bacterial Artificial Chromosome Array-Based Methylated CpG Island Amplification

Masahiro Gotoh,[1] Eri Arai,[1] Saori Wakai-Ushijima,[1] Nobuyoshi Hiraoka,[1] Tomoo Kosuge,[2] Fumie Hosoda,[3] Tatsuhiro Shibata,[3] Tadashi Kondo,[4] Sana Yokoi,[5,6] Issei Imoto,[5] Johji Inazawa,[5] and Yae Kanai[1]

[1] *Pathology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[2] *Hepatobiliary and Pancreatic Surgery Division, National Cancer Center Hospital, Tokyo 104-0045, Japan*
[3] *Cancer Genomics Project, National Cancer Center Research Institute, Tokyo 104-0045, Japan*
[4] *Proteome Bioinformatics Project, National Cancer Center Research Institute, Tokyo 104-0045, Japan*
[5] *Department of Molecular Cytogenetics, Medical Research Institute and School of Biomedical Science, Tokyo Medical and Dental University, Tokyo 113-8510, Japan*
[6] *Cancer Genome Center, Chiba Cancer Center Research Institute, Chiba 260-8717, Japan*

Correspondence should be addressed to Yae Kanai, ykanai@ncc.go.jp

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To establish diagnostic criteria for ductal adenocarcinomas of the pancreas (PCs), bacterial artificial chromosome (BAC) array-based methylated CpG island amplification was performed using 139 tissue samples. Twelve BAC clones, for which DNA methylation status was able to discriminate cancerous tissue (T) from noncancerous pancreatic tissue in the learning cohort with a specificity of 100%, were identified. Using criteria that combined the 12 BAC clones, T-samples were diagnosed as cancers with 100% sensitivity and specificity in both the learning and validation cohorts. DNA methylation status on 11 of the BAC clones, which was able to discriminate patients showing early relapse from those with no relapse in the learning cohort with 100% specificity, was correlated with the recurrence-free and overall survival rates in the validation cohort and was an independent prognostic factor by multivariate analysis. Genome-wide DNA methylation profiling may provide optimal diagnostic markers and prognostic indicators for patients with PCs.

1. Introduction

It is known that DNA hypomethylation results in chromosomal instability as a result of changes in chromatin structure and that DNA hypermethylation of CpG islands silences tumor-related genes in cooperation with histone modification in human cancers [1–5]. The incidence of DNA methylation alterations is generally high in cancers of various organs, and particular DNA methylation profiles are significantly associated with poorer tumor differentiation, tumor aggressiveness, and poor prognosis [6–8]. Moreover, unlike alterations of mRNA and protein expression, which can be easily affected by the microenvironment of cancer cells, DNA methylation alterations are stably preserved on DNA double strands by covalent bonds and can be detected using highly sensitive methodology. Therefore, alterations of DNA methylation can become optimal diagnostic markers of cancers and prognostic indicators for affected patients.

With regard to pancreatic carcinogenesis, we have reported that accumulation of DNA methylation of tumor-related genes [9] is associated with overexpression of DNA methyltransferase (DNMT) 1 [10], the major DNMT, even

in peripheral pancreatic duct epithelia with an inflammatory background, in comparison with normal peripheral pancreatic duct epithelia. Ductal adenocarcinomas of the pancreas frequently develop after chronic damage due to pancreatitis, and at least a proportion of peripheral pancreatic duct epithelia with an inflammatory background are at the precancerous stage [11]. The average number of methylated tumor-related genes and the incidence of DNMT1 overexpression increase progressively with the progression of another precancerous lesion, pancreatic intraductal neoplasia [12], to well-differentiated ductal adenocarcinoma, and finally to poorly differentiated ductal adenocarcinoma, suggesting that DNA methylation alterations participate in multistage pancreatic carcinogenesis [9, 10]. However, even though we and other groups have examined the DNA methylation status of several specific tumor-related genes [9, 13–17], only a few previous studies have employed recently developed array-based technology for analysis of DNA methylation in ductal adenocarcinomas of the pancreas [18, 19]. To our knowledge, no diagnostic criteria have yet been established for pancreatic cancers on the basis of such genome-wide DNA methylation profiling.

In the present study, in order to obtain diagnostic markers and prognostic indicators of ductal adenocarcinomas of the pancreas, we performed bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) [20–22], which is a technique suitable for overviewing the DNA methylation tendency of individual large regions among all chromosomes [23, 24], in samples of normal pancreatic tissue obtained from patients without ductal adenocarcinomas (C), noncancerous pancreatic tissue obtained from patients with ductal adenocarcinomas (N), and cancerous tissue (T).

2. Materials and Methods

2.1. Patients and Tissue Samples. Ninety-one T-samples were obtained from surgically resected specimens from patients with ductal adenocarcinomas who underwent pancreatectomy at the National Cancer Center Hospital, Tokyo, Japan, between 2003 and 2008. From 33 of the 91 patients, N-samples were also obtained from the same surgically resected specimens. Microscopic examination of the histological specimens taken from a region immediately adjoining that from which N-samples had been obtained revealed various degrees of chronic pancreatitis, but no contaminating cancer cells. Fifteen C-samples were obtained from patients without ductal adenocarcinomas who underwent pancreatectomy for metastasis of renal cell carcinoma (1 patient), adenocarcinoma of the gallbladder (3 patients), adenocarcinoma of the papilla of Vater (6 patients), serous cystadenoma (1 patient), mucinous cystadenoma (1 patient), solid-pseudopapillary neoplasm (1 patient), endocrine tumor (1 patient) of the pancreas, and lymphoplasmacytic pancreatitis (1 patient). The total samples were randomly divided into a learning cohort (8 C-, 17 N-, and 46 T-samples) and a validation cohort (7 C-, 16 N-, and 45 T-samples). In the learning cohort, patients from whom C-, N-, and T-samples were obtained comprised 5 men and 3 women with a mean age of 69.6 ± 8.1 (mean ± SD) years, 6 men and 11 women with a mean age of 67.6 ± 10.1 years, and 28 men and 18 women with a mean age of 64.2 ± 10.8 years, respectively. In the validation cohort, the patients from whom C-, N-, and T-samples were obtained comprised 3 men and 4 women with a mean age of 62.9 ± 18.2 years, 11 men and 5 women with a mean age of 65.0 ± 8.7 years, and 27 men and 18 women with a mean age of 64.6 ± 9.7 years, respectively. The clinicopathological parameters of patients who provided T-samples in both the learning and validation cohorts are summarized in Table 1. This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan, and was performed in accordance with the Declaration of Helsinki, 1995. All patients gave their informed consent prior to their inclusion in this study.

2.2. BAMCA. High-molecular-weight DNA from fresh frozen tissue samples was extracted using phenol-chloroform, followed by dialysis. DNA methylation status was analyzed by BAMCA using a custom-made array (MCG Whole Genome Array-4500) harboring 4361 BAC clones located throughout chromosomes 1 to 22, X and Y [25], as described previously [23, 26, 27]. Briefly, a mixture of normal pancreatic tissue DNA obtained from 8 C-samples in the learning cohort was used as a reference for all analyses of test DNA samples in both the learning and validation cohorts. Five-microgram aliquots of test or reference DNA were first digested with 100 units of methylation-sensitive restriction enzyme *Sma* I (NEB, Ipswich, MA) and subsequently with 20 units of methylation-insensitive *Xma* I (NEB). 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-I DNA (Invitrogen, Carlsbad, CA). The mixture was applied to array slides and incubated at 43°C for 63 h. Arrays were scanned with a GenePix Personal 4100A (Molecular Devices, Sunnyvale, CA) and analyzed using GenePix Pro 5.0 imaging software (Molecular Devices) 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. The reproducibility of BAMCA data was confirmed in representative samples by the duplicate study (data not shown).

2.3. Statistics. BAC clones whose signal ratios obtained by BAMCA differed significantly between the groups of samples were identified by Wilcoxon test. Survival curves of patient groups were calculated by the Kaplan-Meier method, and the differences were compared using the Log-rank test. The Cox proportional hazards multivariate model was used to examine the prognostic impact of DNA methylation status, surgical margin status (R0 versus R1 or R2) [28] and lymph node metastasis. Differences at $P < .05$ were considered significant.

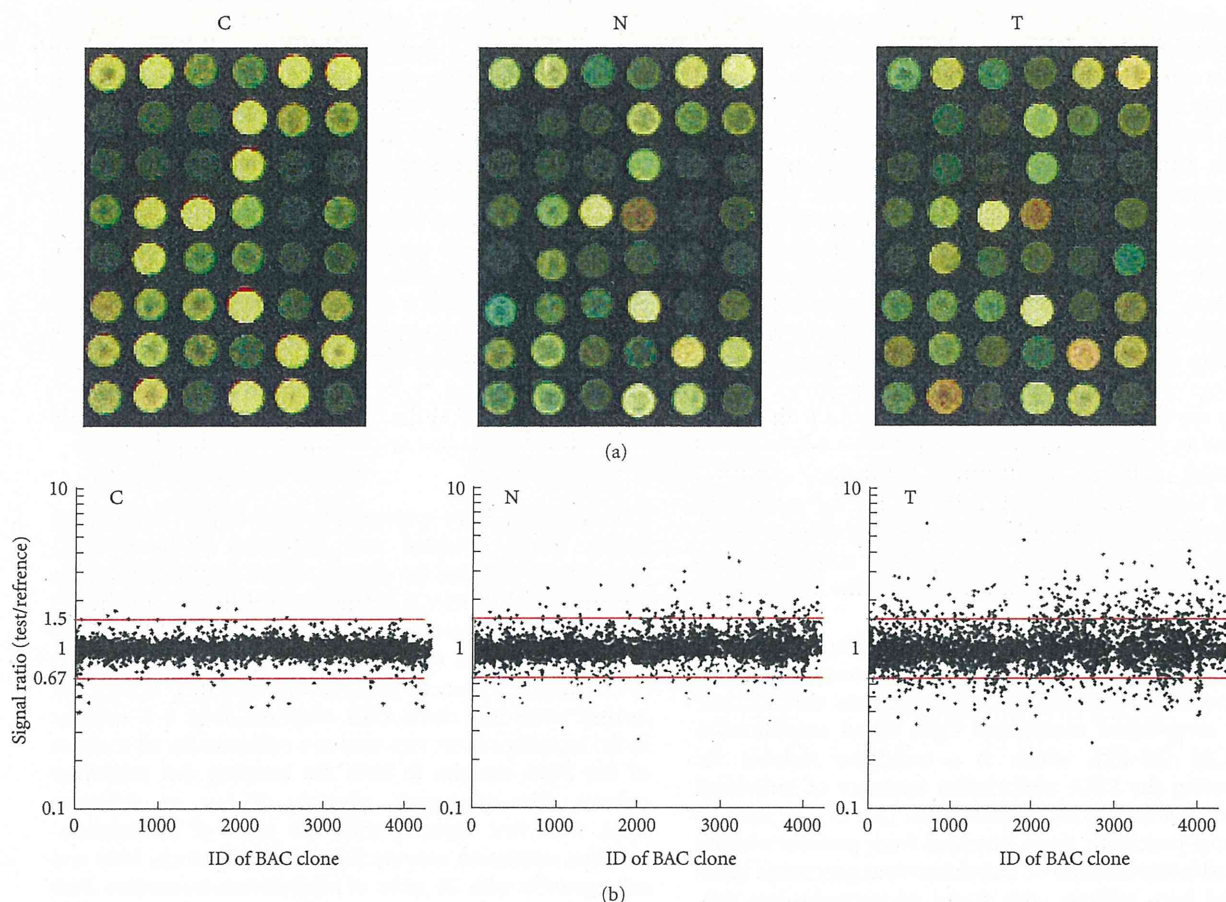

FIGURE 1: Genome-wide DNA methylation analysis by BAMCA. (a) Representative examples of scanned array images in a sample of normal pancreatic tissue obtained from a patient without ductal adenocarcinoma of the pancreas (C) and samples of both noncancerous pancreatic tissue (N) and cancerous tissue (T) obtained from a single patient with ductal adenocarcinoma of the pancreas. Test and reference DNA labeled with Cy3 and Cy5 was cohybridized, respectively. (b) Representative examples of scattergrams of the signal ratios (test signal/ reference signal) in each C-, N-, and T-sample. In all C-samples, the signal ratios of 97% of the BAC clones were between 0.67 and 1.5 (red lines). Therefore, in N- and T samples, DNA methylation status corresponding to a signal ratio of less than 0.67 and more than 1.5 was defined as DNA hypo- and hypermethylation on each BAC clone relative to C-samples, respectively. In N-samples, many BAC clones showed DNA hypo- or hypermethylation. In T-samples, more BAC clones showed DNA hypo- or hypermethylation, and the degree of DNA hypo- or hypermethylation, that is, deviation of the signal ratio from 0.67 or 1.5, was increased in comparison with N-samples.

3. Results

3.1. Genome-Wide DNA Methylation Alterations in Tissue Samples. Figure 1 shows representative examples of scanned array images and scattergrams of the signal ratios (test signal/ reference signal) for a C-sample, a N-sample, and the corresponding T-sample. In all C-samples, the signal ratios of 97% of the BAC clones were between 0.67 and 1.5 (red lines in Figure 1(b)). Therefore, in N- and T-samples, DNA methylation status corresponding to a signal ratio of less than 0.67 and more than 1.5 was defined as DNA hypo- and hypermethylation of each BAC clone relative to C-samples, respectively, as in our previous studies [23, 26, 27]. In N-samples, many BAC clones showed DNA hypo- or hypermethylation (Figure 1(b)). In T-samples, more BAC clones showed DNA hypo- or hypermethylation, and the degree of DNA hypo- or hypermethylation, that is, deviation of the signal ratio from 0.67 or 1.5, was increased in comparison with N-samples (Figure 1(b)).

3.2. Establishment of Criteria for Diagnosis of Ductal Adenocarcinomas of the Pancreas Based on DNA Methylation Profiles. Wilcoxon test ($P < .01$) revealed that the average signal ratios of 331 BAC clones (Supplementary Table SI available at doi:10.1155/2011/780836) in T-samples differed significantly from those in both C- and N-samples. Figure 2(a) shows scattergrams of the signal ratios for representative examples of the 331 BAC clones: RP11-88P10 and RP11-424K7 were able to discriminate T-samples from both C- and N-samples with 100% specificity (the ratio of the number of true negatives to the number of true negatives and false

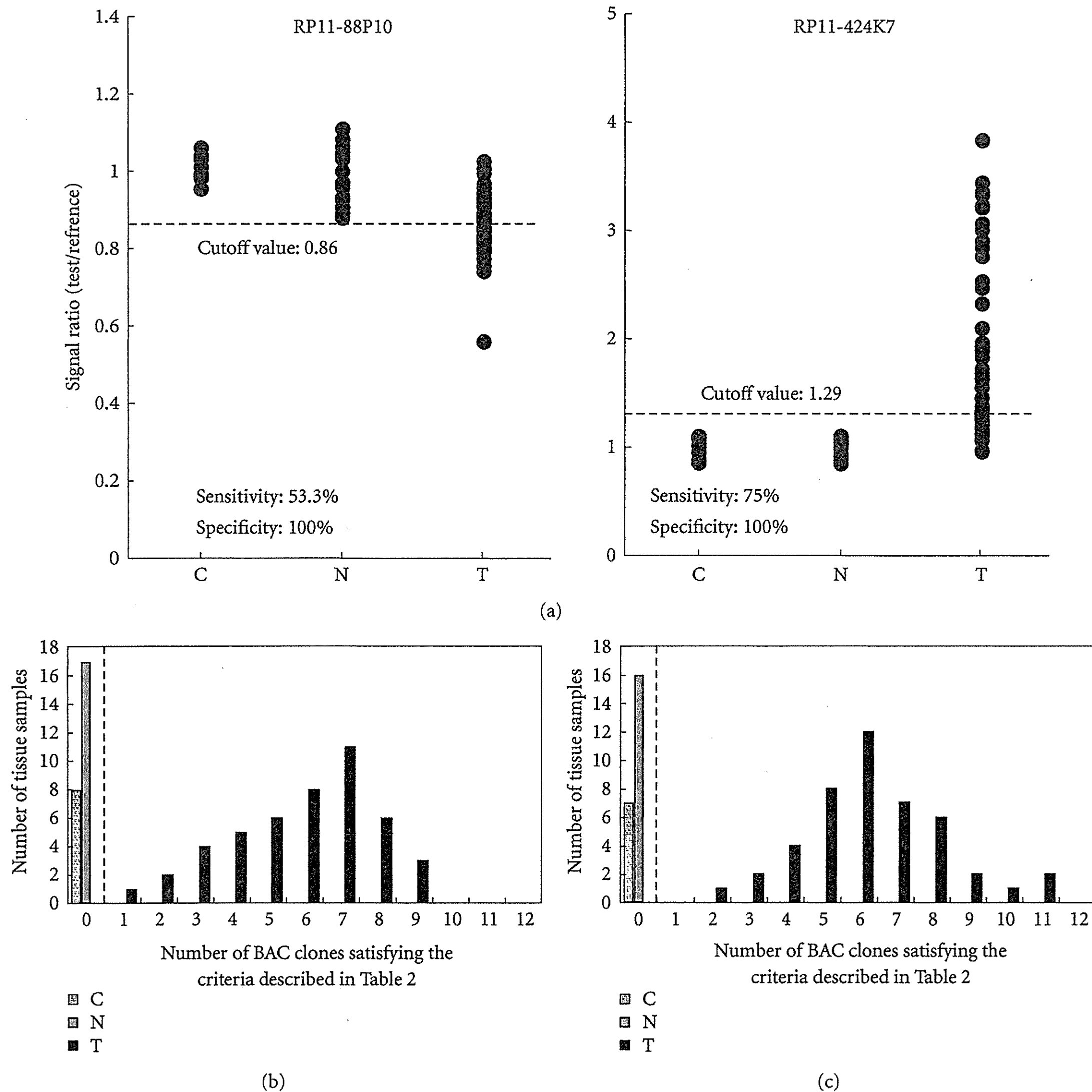

Figure 2: Establishment of criteria for diagnosis of ductal adenocarcinomas of the pancreas. (a) Scattergrams of the signal ratios in samples of normal pancreatic tissue obtained from patients without ductal adenocarcinomas (C), noncancerous pancreatic tissue obtained from patients with ductal adenocarcinomas (N) and cancerous tissue (T) on representative BAC clones, RP11-88P10 and RP11-424K7. Using the cutoff values indicated by the dotted lines, T-samples were discriminated from both C- and N-samples in the learning cohort with 100% specificity. (b) Histogram showing the number of BAC clones satisfying the criteria listed in Table 2 in the learning cohort ($n = 71$). C-, N- and T-samples are indicated by empty, shaded, and filled columns, respectively. Based on this histogram, we established the following criteria: when the tissue samples satisfied the criteria listed in Table 2 for 1 or more BAC clones (dotted line), they were judged to be cancerous tissue, and when tissue samples did not satisfy the criteria for any BAC clone, they were judged not to be cancerous tissue. Based on these criteria, both the sensitivity and specificity for diagnosis of T-samples in the learning cohort as being cancerous were 100%. (c) Validation of the above criteria using 68 additional tissue samples in the validation cohort. All 45 validation samples satisfying the criteria in Table 2 for 1 or more BAC clones (dotted line) were T-samples (filled columns), and all 23 validation samples not satisfying the criteria in Table 2 for any BAC clone were C- (empty column) or N- (shaded column) samples. Both the sensitivity and specificity for diagnosis of T-samples in the validation cohort as being cancerous were again 100%.

positives) using cutoff values of 0.86 and 1.29 (dotted lines in Figure 2(a)), respectively, (specificity was calculated as the ratio of the number of C- and N-samples showing signal ratios of 0.86 or more than 0.86 and 1.29 or less than 1.29 relative to the total number of C- and N-samples, resp.). The cutoff values of the signal ratios and sensitivities (the ratios of the number of true positives to the number of true positives and false negatives) of 12 BAC clones for which such discrimination was performed with 100% specificity are shown in Table 2. Genes located on the 12 BAC clones

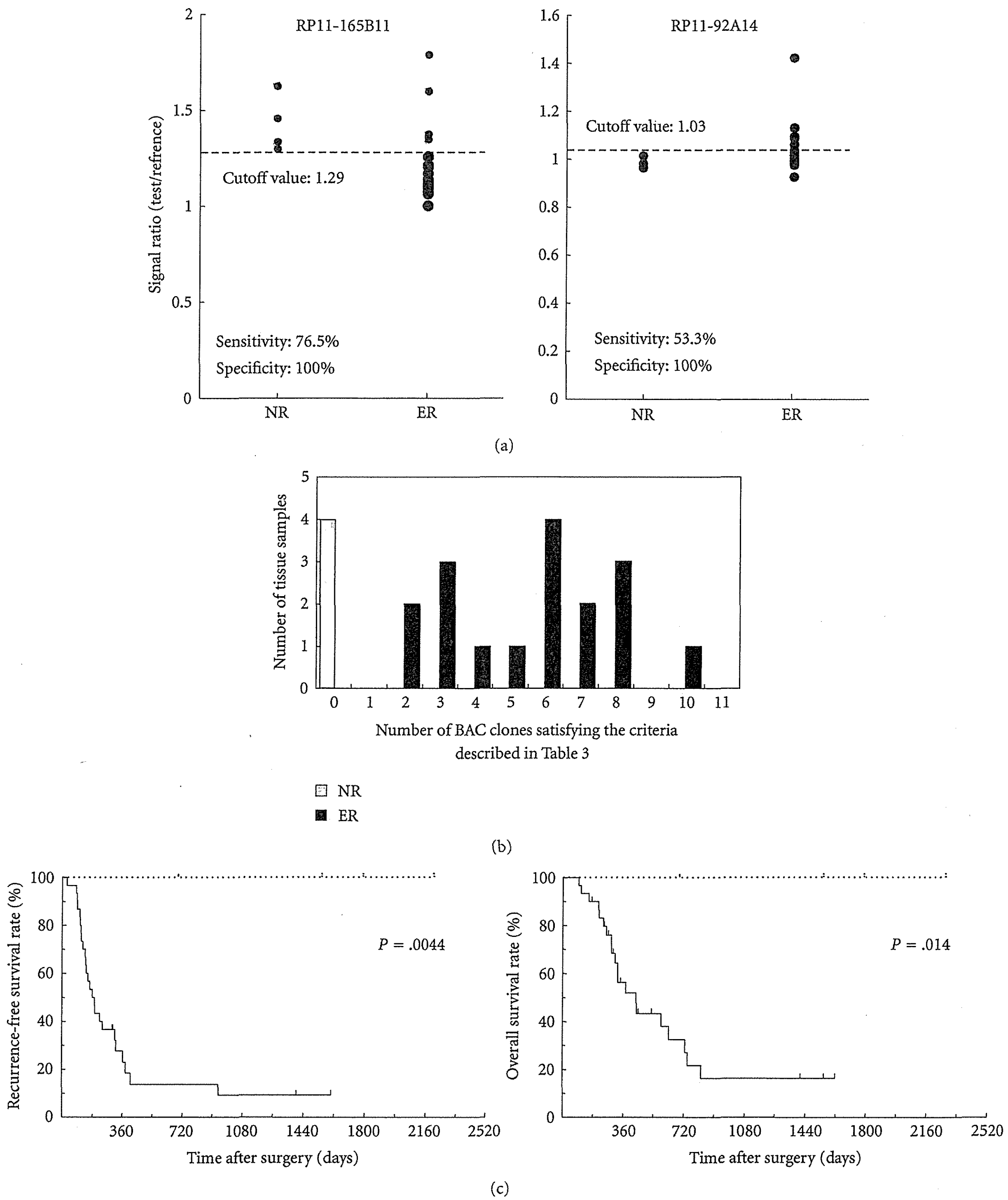

Figure 3: Establishment of criteria for prognostication of patients with ductal adenocarcinomas of the pancreas. (a) Scattergrams of the signal ratios in samples of cancerous tissue obtained from patients in the no-relapse group (NR, $n = 4$) and early-relapse group (ER, $n = 17$) who had not undergone adjuvant chemotherapy with gemcitabine after surgery on representative bacterial artificial chromosome (BAC) clones, RP11-165B11 and RP11-92A14. Using the cutoff values indicated by the dotted lines, patients belonging to the ER-group were discriminated from those belonging to the NR-group in the learning cohort with 100% specificity. (b) Histogram showing the number of BAC clones satisfying the criteria listed in Table 3 for patients belonging to the NR- (shaded column) and ER- (filled columns) groups in the learning cohort. (c) Kaplan-Meier survival curves of 34 patients who had not undergone adjuvant chemotherapy with gemcitabine after surgery in the validation cohort. Both the recurrence-free and overall survival rates of 29 patients satisfying the criteria listed in Table 3 for 2 or more BAC clones (solid lines) were significantly lower than those of 5 patients satisfying the criteria listed in Table 3 for less than 2 BAC clones (dotted lines). Log-rank test ($P = .0044$ and $P = .014$, resp.).

TABLE 1: Clinicopathological parameters of patients with ductal adenocarcinomas of the pancreas.

Clinicopathological parameters	Number of patients	
	Learning cohort	Validation cohort
Greatest diameter of the tumor		
2.0 cm or less	2	1
More than 2.0 cm, but no more than 4.0 cm	29	29
More than 4.0 cm	15	15
Histological classification		
Well differentiated adenocarcinoma	2	4
Moderately differentiated adenocarcinoma	35	30
Poorly differentiated adenocarcinoma	6	9
Adenosquamous carcinoma	2	1
Mucinous noncystic carcinoma	1	1
Lymphatic vessel invasion		
Negative	0	0
Positive	46	45
Venous invasion		
Negative	0	0
Positive	46	45
Lymph node metastasis		
Negative	13	9
Positive	33	36
Status of the surgical margin		
Negative (R0*)	27	33
Positive (R1 or R2*)	19	12
Total	46	45

*defined in [28].

are summarized in Supplementary Table SII. A histogram showing the number of BAC clones satisfying the criteria listed in Table 2 in 8 C-samples, 17 N-samples, and 46 T-samples in the learning cohort is shown in Figure 2(b). Based on this histogram, we finally established the following criteria: when tissue samples satisfied the criteria in Table 2 for 1 or more BAC clones, they were judged to be ductal adenocarcinomas, and when tissue samples did not satisfy the criteria for any BAC clone, they were judged not to be ductal adenocarcinomas. Based on these criteria, both the sensitivity and specificity for diagnosis of T-samples in the learning cohort as ductal adenocarcinomas were 100% (sensitivity was calculated as the ratio of the number of T-samples satisfying the criteria in Table 2 for 1 or more BAC clones to the total number of T-samples, and specificity was calculated as the ratio of the number of C- and N-samples not satisfying the criteria in Table 2 for any BAC clone relative to the total number of C- and N-samples).

To confirm these criteria, 68 additional tissue samples in the validation cohort were analyzed. Forty-five samples satisfying the criteria listed in Table 2 for 1 or more BAC clones were all T-samples, and the other 23 samples not satisfying the criteria listed in Table 2 for any BAC clone were all C- or N-samples (Figure 2(c)). Our criteria enabled diagnosis of T-samples in the validation cohort as ductal adenocarcinomas with 100% sensitivity and specificity.

3.3. Establishment of Criteria for Prognostication of Patients with Ductal Adenocarcinomas of the Pancreas Based on DNA Methylation Profiles. To establish criteria for prognostication, 21 patients who had not undergone adjuvant chemotherapy with gemcitabine in the learning cohort were divided into two groups: 4 patients who had not suffered relapse for more than 4 years after pancreatectomy and 17 patients who had suffered relapse within 18 months after pancreatectomy were defined as the no-relapse group and early-relapse group, respectively. The period covered ranged from 215 to 1,846 days (mean, 823 days). Wilcoxon test ($P < .05$) revealed that the average signal ratios of 64 BAC clones differed significantly between T-samples obtained from the no-relapse group and those from the early-relapse group.

Figure 3(a) shows scattergrams of the signal ratios for representative examples of the 64 BAC clones: RP11-165B11 and RP11-92A14 were able to discriminate T-samples from patients belonging to the early-relapse group from those belonging to the no-relapse group with 100% specificity (the ratio of the number of true negatives to the number of true negatives and false positives) using cutoff values of 1.29 and 1.03 (dotted lines in Figure 3(a)), respectively, (specificity was calculated as the ratio of the number of T-samples from patients belonging to the no-relapse group showing signal ratios of 1.29 or more than 1.29 and 1.03 or less than 1.03 relative to the total number of T-samples from patients belonging to the no-relapse group, resp.). The cutoff values of the signal ratios and sensitivities (the ratios of the number of true positives to the number of true positives and false negatives) of 11 BAC clones for which such discrimination was performed with 100% specificity are shown in Table 3. Genes located on the 11 BAC clones are summarized in Supplementary Table SII. A histogram showing the number of BAC clones satisfying the criteria listed in Table 3 in 4 T-samples from the no-relapse group and 17 T-samples from the early-relapse group in the learning cohort is shown in Figure 3(b). Based on these criteria (2 or more BAC clones versus less than 2 BAC clones listed in Table 3), both the sensitivity and specificity of discrimination of patients belonging to the early-relapse group from those belonging to the no-relapse group in the learning cohort were 100% (sensitivity was calculated as the ratio of the number of T-samples from patients belonging to the early-relapse group satisfying the criteria in Table 3 for 2 or more BAC clones relative to the total number of T-samples from patients belonging to the early-relapse group, and specificity was calculated as the ratio of the number of T-samples from

Table 2: Twelve BAC clones that were able to discriminate samples of cancerous tissue from samples of normal pancreatic tissue obtained from patients without ductal adenocarcinomas and samples of noncancerous pancreatic tissue obtained from patients with ductal adenocarcinomas in the learning cohort with 100% specificity.

BAC clone ID	Location	Cutoff value (CV)	DNA methylation status*	Sensitivity (%)	Specificity (%)
RP11-121D3	3p26.3	1.46	CV<	43.5	100
RP11-89G4	5q31.1	0.80	CV>	37.0	100
RP11-177M14	6q23.2	1.45	CV<	67.4	100
RP11-92I18	10q11.23	1.34	CV<	67.4	100
RP11-36H11	11p13-11p12	0.56	CV>	26.7	100
RP11-91M21	12q24.21	1.49	CV<	53.3	100
RP11-458A21	14q13.3	1.29	CV<	72.7	100
RP11-88P10	15q12	0.86	CV>	53.3	100
RP11-424K7	16q12.1	1.29	CV<	75.0	100
RP11-2O22	19q13.31	1.16	CV<	33.3	100
RP11-149O7	20p12.3	1.22	CV<	31.1	100
RP11-79G10	20q12	1.16	CV<	35.6	100

*CV>, when the signal ratio was lower than the cutoff value, the tissue sample was considered to be cancerous; CV <, when the signal ratio was higher than the cutoff value, the tissue sample was considered to be cancerous.

Table 3: Eleven BAC clones that were able to discriminate patients belonging to the early-relapse group from those belonging to the no-relapse group in the learning cohort with 100% specificity.

BAC clone ID*	Location	Cutoff value (CV)	DNA methylation status**	Sensitivity (%)	Specificity (%)
RP11-101J8	1q23.1	0.98	CV<	47.1	100
RP11-137N24	1q25.1	1.08	CV<	58.8	100
RP11-180L21	2p21	0.97	CV<	37.5	100
RP11-91K8	3q22.1	0.84	CV<	41.2	100
RP11-89E2	4q28.2	0.99	CV<	58.8	100
RP11-81B23	5p14.3	0.99	CV>	50.0	100
RP11-373P23	10q21.1	1.04	CV<	29.4	100
RP11-666F17	12p11.23	1.15	CV>	58.8	100
RP11-165B11	16p13.13	1.29	CV>	76.5	100
RP11-236B14	19q13.33	0.87	CV>	52.9	100
RP11-92A14	21q21.1	1.03	CV<	53.3	100

*CV>, when the signal ratio was lower than the cutoff value, the sample of cancerous tissue was considered to originate from a patient who would suffer early relapse; CV<, when the signal ratio was higher than the cutoff value, the sample of cancerous tissue was considered to originate from a patient who would suffer early relapse.

Table 4: Multivariate analysis of clinicopathological parameters and DNA methylation profiles associated with recurrence-free and overall survival in patients with ductal adenocarcinomas of the pancreas.

	Recurrence-free survival			Overall survival		
Parameters	Hazard ratio (95% CI*)	χ^2	P	Hazard ratio (95% CI)	χ^2	P
Status of the surgical margin						
Negative (R0**, $n = 60$)	1			1		
Positive (R1 or R2 **, $n = 31$)	1.072 (0.645–1.782)	0.071	.7898	1.452 (0.804–2.619)	1.531	.2159
Lymph node metastasis						
Negative ($n = 22$)	1			1		
Positive ($n = 69$)	1.621 (0.878–2.995)	2.383	.1227	1.477 (0.709–3.073)	1.086	.2973
The criteria in Table 3						
Satisfying for less than 2 BAC clones ($n = 10$)	1			1		
Satisfying for 2 or more BAC clones ($n = 81$)	18.694 (2.559–136.555)	8.331	.0039	12.136 (1.660–88.711)	6.051	.0139

* CI, confidence interval; ** defined in [28].

patients belonging to the no-relapse group satisfying the criteria in Table 3 for less than 2 BAC clones relative to the total number of T-samples from patients belonging to the no-relapse group).

To confirm these criteria, 34 additional T-samples obtained from patients who had not undergone adjuvant chemotherapy with gemcitabine after surgery in the validation cohort were analyzed. The period covered ranged from 92 to 2,274 days (mean, 612 days). Both the recurrence-free and overall survival rates of 29 patients satisfying the criteria listed in Table 3 for 2 or more BAC clones were significantly lower than those of 5 patients satisfying the criteria listed in Table 3 for less than 2 BAC clones (Figure 3(c), $P = .0044$ and $P = .014$, resp.).

Moreover, multivariate analysis in all 91 patients with ductal adenocarcinomas revealed that satisfying the criteria listed in Table 3 for 2 or more BAC clones was a prognostic parameter for both recurrence-free and overall survival that was independent of surgical margin positivity (R1 or R2) [28] and lymph node metastasis at the time of surgery, which are known to have a prognostic impact [29–33] (Table 4).

4. Discussion

Ductal adenocarcinoma of the pancreas, one of the most lethal of all human cancers, is now a common cause of cancer mortality in the United States and Japan [34]. Due to its aggressive growth behavior with early local spread into the surrounding tissues mostly along neural sheaths, peritoneal dissemination, and liver and lymph node metastasis, the prognosis remains poor. Surgical treatment still provides the only possibility of cure [35]. Although advances in preoperative diagnostic imaging have made it possible to detect tumors at an early stage when they are still resectable, diagnosis using pancreatic biopsy and/or specimens of pancreatic juice is indispensable before surgery. In general, pancreatic biopsy yields only a small amount of tissue, and in pancreatic juice specimens, the cellular morphology is not well preserved due to degeneration. Therefore, molecular diagnosis is advantageous for supporting the histological and/or cytological assessment of such specimens. DNA methylation profiles, which are stably preserved on DNA double strands by covalent bonds, even after degeneration of cellular morphology, may become diagnostic markers in pancreatic biopsy and/or pancreatic juice specimens.

We have previously established diagnostic criteria for cancers of the kidney [26], liver [27] and urinary tract [23] based on genome-wide DNA methylation profiles using the BAC array-based approach, BAMCA, which can assess DNA methylation status not only on promoter regions of specific tumor-related genes but also on genomic regions in which DNA hypomethylation affects chromosomal instability. Moreover, during human carcinogenesis, DNA methylation status is frequently altered in a coordinated manner, through processes such as long-range epigenetic silencing [36], in large chromosome regions. Since BAMCA is suitable for overviewing the DNA methylation tendency of individual large regions among all chromosomes [23, 24], we again employed this method to establish diagnostic criteria for ductal adenocarcinomas of the pancreas.

The results of BAMCA for C-samples reflected the DNA methylation profiles of normal peripheral pancreatic duct epithelia (the origin of ductal adenocarcinomas), acinar cells and islet cells. In N-samples, BAMCA revealed DNA hypo- or hypermethylation on many BAC clones in comparison to C-samples (Figure 1(b)). Microscopic observation of N-samples revealed lymphocytes and fibroblasts associated with various degrees of chronic pancreatitis, which is considered to be one of the precancerous conditions for ductal adencarcinomas [11]. Our previous studies using microdissection and immunohistochemistry revealed accumulation of DNA hypermethylation of tumor-related genes associated with DNMT1 overexpression, even in peripheral pancreatic duct epithelia at the precancerous stage [9, 10]. Therefore, the results of BAMCA for N-samples may reflect the DNA methylation profiles of peripheral pancreatic duct epithelia at the precancerous stage, lymphocytes, fibroblasts, acinar cells, and islet cells. In order to diagnose ductal adenocarcinomas in tissue samples, cancer-specific DNA methylation profiles should be discriminated from those of normal or precancerous peripheral pancreatic duct epithelia, lymphocytes, fibroblasts, acinar cells, and islet cells. Therefore, we identified 12 BAC clones whose DNA methylation status was able to discriminate T-samples from both C- and N-samples.

In both the learning and validation cohorts, the criteria combining the 12 BAC clones were able to diagnose T-samples as ductal adenocarcinomas with a sensitivity and specificity of 100%. Our criteria may be advantageous for supporting the histological diagnosis of tiny tissue samples obtained by pancreatic biopsy. Discrimination of cancer cells from exfoliated noncancerous epithelial cells and lymphocytes using the 12 BAC clones may be applicable for diagnosis using specimens of pancreatic juice. Development of methodology for assessing DNA methylation status on the 12 BAC clones in fewer cells may be more advantageous for clinical application, as we have already established a methodology for quantification of DNA methylation levels on specific CpG sites in a very small quantity of genomic DNA for estimation of carcinogenetic risk in patients with chronic liver diseases (unpublished data). Development of this methodology means that if DNA methylation alterations on the 12 BAC clones are not observed in circulating blood cells, our criteria may become applicable for noninvasive diagnosis of pancreatic cancers based on serum markers that differ from the widely used carbohydrate antigen 19-9, whose serum levels are also elevated in patients with chronic pancreatitis [37].

Even when resection with curative intent is performed for patients with pancreatic cancers, the rate of disease recurrence is high and the survival rate after surgery is poor. As surgical resection alone has limitations, development of nonsurgical treatments, including adjuvant therapy, is needed in order to improve the prognosis of patients with pancreatic cancers. Although previous studies have suggested the efficacy of adjuvant chemotherapy [38], it should be carried out carefully, paying close attention to adverse reactions [39]. In order to help decide the indications

for such adjuvant chemotherapy after surgery, prognostic indicators should be explored. The criteria listed in Table 3 were able to discriminate the early-relapse group from the no-relapse group with 100% sensitivity and specificity in the learning cohort. Significant correlation between DNA methylation status on the 11 BAC clones and the recurrence-free and overall survival rates of patients with ductal adenocarcinomas in the validation cohort validated the criteria. Multivariate analysis revealed that our criteria were able to predict recurrence-free and overall patient outcome independently of parameters that had been reported to be significantly prognostic in many previous studies, such as surgical margin positivity (R1 or R2) [28] and lymph node metastasis. Therefore, prognostication based on our criteria may be promising for supportive use during followup after surgical resection in patients with ductal adenocarcinomas of the pancreas. Since histological heterogeneity is frequently observed even in a ductal adenocarcinoma of the pancreas from a single patient, the consistency of BAMCA data for multiple T-samples obtained from a single tumor should be carefully confirmed in a prospective validation study before clinical application of the prognostic criteria.

5. Conclusions

BAMCA revealed genome-wide DNA methylation alterations in ductal adenocarcinomas of the pancreas. Criteria combining the DNA methylation status on 12 BAC clones were able to discriminate T-samples from both C- and N-samples and to diagnose T-samples as ductal adenocarcinomas, with 100% sensitivity and specificity in both the learning and validation cohorts. Satisfying the criteria using 11 BAC clones was able to predict the recurrence-free and overall survival of patients with ductal adenocarcinomas independently of surgical margin positivity (R1 or R2) [28] and lymph node metastasis. Genome-wide DNA methylation profiling may provide optimal diagnostic markers for pancreatic cancers and prognostic indicators for affected patients.

Abbreviation

BAC: Bacterial artificial chromosome
BAMCA: BAC array-based methylated CpG island amplification
DNMT: DNA methyltransferase.

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Conflict of Interests

There is no potential conflict of interests to disclose.

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Review Article

Genome-wide DNA methylation profiles in precancerous conditions and cancers

Yae Kanai[1]

Pathology Division, National Cancer Center Research Institute, Tokyo, Japan

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Alterations of DNA methylation, which result in chromosomal instability and silencing of tumor-related genes, are among the most consistent epigenetic changes observed in human cancers. Analysis of tissue specimens has revealed that DNA methylation alterations participate in multistage carcinogenesis, even from the early and precancerous stages, especially in association with chronic inflammation and/or persistent viral infection, such as chronic hepatitis or liver cirrhosis resulting from infection with hepatitis B or C virus. DNA methylation alterations can account for the histological heterogeneity and clinicopathological diversity of human cancers. Overexpression of DNA methyltransferase 1 is not a secondary result of increased cell proliferative activity, but is significantly correlated with accumulation of DNA hypermethylation in CpG islands of tumor-related genes. Alteration of DNA methyltransferase 3b splicing may result in chromosomal instability through DNA hypomethylation in pericentromeric satellite regions. Genome-wide analysis of DNA methylation status has revealed that the DNA methylation profile at the precancerous stage is basically inherited by the corresponding cancers developing in individual patients. DNA methylation status is not simply altered at the precancerous stage; rather, DNA methylation alterations at the precancerous stage may confer vulnerability to further genetic and epigenetic alterations, generate more malignant cancers, and thus determine patient outcome. Therefore, genome-wide DNA methylation profiling may provide optimal indicators for carcinogenetic risk estimation and prognostication, and thus provide an avenue for cancer prevention and therapy on an individual basis. (*Cancer Sci* 2010; 101: 36–45)

DNA methylation, a covalent chemical modification resulting in addition of a methyl group at the carbon five position of the cytosine ring in CpG dinucleotides, is one of the most consistent epigenetic changes observed in human cancers.[1] DNMTs transfer methyl groups from *S*-adenosylmethionine to cytosines.[2] The preference of DNMT1, a major and well-known DNMT, for hemimethylated over unmethylated substrates *in vitro*,[3] and its targeting of replication foci by binding to PCNA,[4,5] are believed to allow copying of the DNA methylation pattern on the parental strand to the newly synthesized daughter DNA strand. Thus, DNMT1 has been recognized as a ''maintenance'' DNMT,[6] whereas DNMT3a and DNMT3b show *de novo* DNA methylation activity.[7] DNA methylation normally promotes a highly condensed heterochromatin structure associated with deacetylation of histones H3 and H4, loss of histone H3, lysine 4 (H3K4) methylation, and gain of H3K9 and H3K27 methylation.[8] When methyl-CpG-binding proteins, such as MeCP2[9,10] and MBD2,[11] bind to methylated CpG dinucleotide, their transcriptional repression domain recruits a co-repressor complex containing histone deacetylases. However, histone methyltransferases, such as G9A[12] and SUV39H1,[13] are required to recruit DNMTs. DNA methylation is a stable modification inherited throughout consecutive cell divisions, being essential for the normal development and function of adult organs, particularly for X-chromosome inactivation, genome imprinting, silencing of transposons and other parasitic elements, and proper expression of genes.[14]

Reduction of DNMT1 activity in genetically engineered animals alters the number of tumors or the timing of tumor development, suggesting a causal relationship between DNA methylation alterations and tumorigenesis.[15,16] In 1995, when the *RB* and *VHL* genes were the only tumor suppressor genes known to be silenced by DNA methylation, we showed that the E-cadherin tumor suppressor gene is silenced by DNA methylation around the promoter region.[17] The list of tumor-related genes whose expression levels are altered due to DNA hypo- or hypermethylation is increasing.[18–22] Transcriptionally repressive chromatin modifications within the promoters of tumor-related genes silenced by DNA methylation are known to resemble the chromatin modifications of these genes in normal embryonic stem cells, for example, polycomb complex binding and H3K27 methylation.[23] These genes also have an active marker, H3K4 methylation, in normal stem cells, and this bivalent state is converted to a primary active or repressive chromatin conformation after differentiation cues have been received.[23] During carcinogenesis, such modifications may render the genes vulnerable to errors, resulting in aberrant DNA methylation.[24] DNA hypomethylation induces chromosomal instability through decondensation of heterochromatin and enhancement of chromosomal recombination during carcinogenesis.[25] Translational epigenetics have come of age,[26,27] and empirical analysis of DNA methylation status in clinical tissue samples in connection with the clinicopathological diversity of human cancers is assuming increasing importance for the diagnosis, prevention, and therapy of cancers.[28,29]

Alterations of DNA methylation during multistage carcinogenesis

Alterations of DNA methylation at the precancerous stage. DNA methylation alterations play a key role in the early steps of human carcinogenesis. In the 1990s, although LOH on chromosome 16 was frequently detected by classical Southern blotting in HCCs that were poorly differentiated, large in size, and associated with metastasis,[30] only a few of the molecular events occurring in the earlier stage of hepatocarcinogenesis were known. Since DNA methylation alterations may be correlated with chromosomal instability, we examined the DNA methylation status on chromosome 16 using Southern blotting

[1]To whom correspondence should be addressed.
E-mail: ykanai@ncc.go.jp

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with a DNA methylation-sensitive restriction enzyme. DNA methylation alterations at multiple loci on chromosome 16, compared to normal liver tissue samples, were frequently revealed even in samples of non-cancerous liver tissue showing chronic hepatitis or liver cirrhosis,[31,32] which are widely considered to be precancerous conditions,[33] indicating that DNA methylation alterations are a very early event during multistage hepatocarcinogenesis. This was one of the earliest reports of DNA methylation alterations at the precancerous stage.[31]

DNA hypermethylation around the promoter region of the E-cadherin tumor suppressor gene (16q22.1), which encodes a Ca^{2+}-dependent cell-cell adhesion molecule,[34] has been detected even in samples of non-cancerous liver tissue showing chronic hepatitis or cirrhosis.[35] Heterogeneous E-cadherin expression in such non-cancerous liver tissue, which is associated with small focal areas of hepatocytes showing only slight E-cadherin immunoreactivity, might be due, at least partly, to DNA hypermethylation.[35] Reduction of E-cadherin expression due to DNA methylation around the promoter region may participate even in the very early stage of hepatocarcinogenesis through loss of intercellular adhesiveness and destruction of tissue morphology.

Studies of LOH by PCR using microsatellite markers have been reported, using specimens microdissected from precancerous lesions in several organ types. Whether aberrant DNA methylation precedes chromosomal instability during hepatocarcinogenesis was re-examined using microdissected specimens obtained from lobules, pseudo lobules or regenerative nodules in non-cancerous liver tissue from patients with HCCs by bisulfite modification. Although no degree of DNA methylation of any of the examined C-type CpG islands, which are generally methylated in a cancer-specific but not age-dependent manner, was ever detected in normal liver tissue from patients without HCCs, DNA hypermethylation of such islands was frequently found, even in microdissected specimens of non-cancerous liver tissue showing no remarkable histological changes obtained from patients with HCCs in which LOH was never detected.[36] Thus it was confirmed that aberrant DNA methylation is an earlier event preceding chromosomal instability during hepatocarcinogenesis.

As another example of inflammation-associated carcinogenesis, ductal carcinomas of the pancreas frequently develop after chronic damage due to pancreatitis. At least a proportion of peripheral pancreatic ductal epithelia with an inflammatory background may be at the precancerous stage. When the DNA methylation status of the *p14*, *p15*, *p16*, *p73*, *APC*, *hMLH1*, *MGMT*, *BRCA1*, *GSTP1*, *TIMP-3*, *E-cadherin*, and *DAPK-1* genes was examined, the average number of methylated tumor-related genes and the incidence of DNA methylation of at least one gene were increased in peripheral pancreatic ductal epithelia with an inflammatory background and in another precancerous lesion, PanIN, in comparison with normal peripheral pancreatic duct epithelia.[37]

UCs of the urinary bladder, renal pelvis, and ureter are clinically remarkable because of their multicentricity and tendency to recur (Fig. 1a).[38] A possible mechanism for such multiplicity is the ''field effect.'' Even non-cancerous urothelia showing no remarkable histological changes obtained from patients with UCs can be considered precancerous, because they may have been exposed to carcinogens in the urine. When the DNA methylation status of multiple C-type CpG islands was examined, the average number of methylated C-type CpG islands was increased in non-cancerous urothelia showing no remarkable histological changes obtained from patients with UCs, in comparison with normal urothelia obtained from patients without UCs.[39]

Cigarette smoking is another background factor associated with alterations of DNA methylation during multistage carcinogenesis. DNA hypermethylation at the D17S5 locus, where the *HIC (hypermethylated-in-cancer)-1* tumor suppressor gene was identified, is observed even in non-cancerous lung tissue, which may contain progenitor cells for cancers, obtained from patients with non-small-cell lung cancers. The incidence of DNA hypermethylation in non-cancerous lung tissue obtained from patients with non-small-cell lung cancers is significantly correlated with both smoking history and the extent of pulmonary anthracosis, as an index of the cumulative effects of smoking.[40] Thus, DNA methylation alterations are frequently found even at the precancerous stage in various organs, especially in association with chronic inflammation[41,42] and/or persistent infection with viruses[43–45] or other pathogenic microorganisms, and with cigarette smoking.

DNA methyltransferase 1 overexpression and regional DNA hypermethylation. With respect to the molecular backgrounds of DNA methylation alterations,[46] it has been reported that levels of DNMT1 mRNA expression are significantly higher in samples of non-cancerous liver tissue showing chronic hepatitis or cirrhosis than in normal liver tissue, and are even higher in HCCs.[47,48] The incidence of DNMT1 overexpression in HCCs is significantly correlated with poorer tumor differentiation and portal vein involvement.[49] Moreover, the recurrence-free and overall survival rates of patients with HCCs showing DNMT1 overexpression are significantly lower than those of patients with HCCs that do not.[49]

As mentioned above, at least a proportion of peripheral pancreatic ductal epithelia with an inflammatory background may be at the precancerous stage. The incidence of DNMT1 protein expression increases with progression from peripheral pancreatic ductal epithelia with an inflammatory background, to PanIN, to well-differentiated ductal carcinoma, and finally to poorly differentiated ductal carcinoma of the pancreas, in comparison with normal peripheral pancreatic duct epithelia.[50] DNMT1 overexpression in ductal carcinomas of the pancreas is significantly correlated with the extent of invasion to the surrounding tissue, an advanced stage, and poorer patient outcome.[50] The average number of methylated tumor-related genes in microdissected specimens of peripheral pancreatic ductal epithelia with an inflammatory background, PanIN, and ductal carcinoma was significantly correlated with the level of DNMT1 protein expression examined immunohistochemically in precisely microdissected areas.[37]

Expression levels of DNMT1 mRNA and protein are significantly correlated with poorer differentiation and the CIMP, a cancer phenotype characterized by accumulation of DNA methylation of C-type CpG islands,[51,52] in stomach cancers,[53] but no such association has been observed for the expression of DNMT2, DNMT3a, or DNMT3b.[54] Epstein–Barr virus infection in stomach cancers is significantly associated with marked accumulation of DNA methylation of C-type CpG islands and overexpression of DNMT1 protein.[53] *Helicobacter pylori* infection, another etiologic factor for stomach carcinogenesis, has also been reported to strongly promote regional DNA hypermethylation[55] but is not correlated with DNMT1 expression levels.[53]

It is debatable whether increased DNMT1 expression is due to an increase in the proportion of dividing cells or to an acute increase of DNMT1 expression per individual cancer cell. Immunohistochemical examinations have clearly revealed that the incidence of nuclear DNMT1 immunoreactivity is already higher in non-cancerous urothelia showing no remarkable histological changes obtained from patients with UCs, which may already be exposed to carcinogens in the urine but in which the PCNA labeling index had not yet increased, compared to that in normal urothelia from patients without UCs, indicating that

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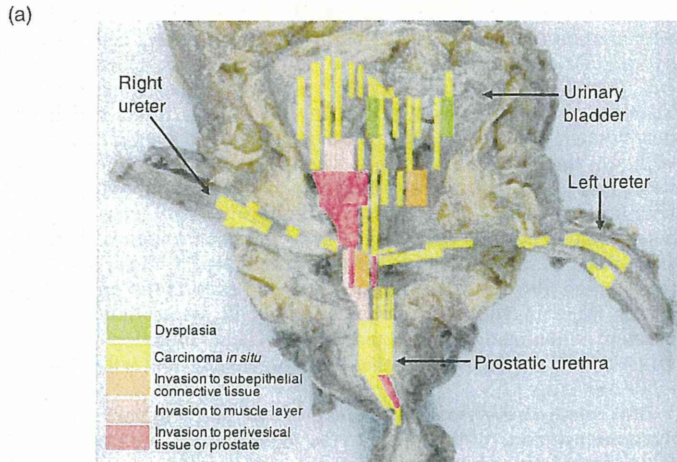
(a)



(b)

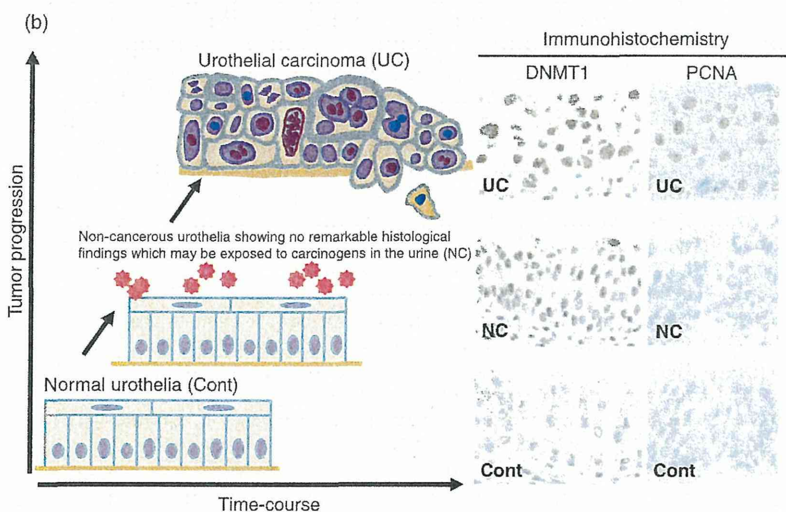


Fig. 1. Overexpression of DNA methyltransferase (DNMT) 1 protein during multistage urothelial carcinogenesis. (a) Specimen obtained by radical cystectomy for multiple urothelial carcinomas (UCs) of the urinary bladder, bilateral ureters, and prostatic urethra. UCs are clinically remarkable because of their multicentricity and tendency to recur: synchronously or metachronously multifocal UCs often develop in individual patients.[38] A possible mechanism for such multiplicity is the "field effect." Even non-cancerous urothelia showing no remarkable histological changes obtained from patients with UCs can be considered precancerous, because they may be exposed to carcinogens in the urine. (b) Immunohistochemical examination for DNMT1 and proliferating cell nuclear antigen (PCNA) in tissue specimens. The incidence of nuclear DNMT1 immunoreactivity had already increased in non-cancerous urothelia showing no remarkable histological changes obtained from patients with UCs (NC), where the PCNA labeling index had not yet increased, compared to that in normal urothelia obtained from patients without UCs (Cont), indicating that DNMT1 overexpression preceded any increase of cell proliferative activity.[56] The intensity of nuclear DNMT1 immunoreactivity was further increased in UCs.[56]

DNMT1 overexpression preceded increased cell proliferative activity (Fig. 1b).[56] The incidence of nuclear DNMT1 immunoreactivity showed a further and progressive increase in dysplastic urothelia, and during transition to UCs (Fig. 1b).[56] Among all examined microdissected specimens of non-cancerous urothelia showing no remarkable histological changes from patients with UCs, or dysplastic urothelia and UCs, accumulation of DNA methylation of C-type CpG islands was significantly correlated with the level of DNMT1 protein expression.[39]

Thus DNMT1 overexpression participates not only in the precancerous stage but also in the malignant progression of various cancers, and has a prognostic impact on patients. DNMT1 overexpression is frequently associated with CIMP of cancers. Although the maintenance activities of DNMT1 are related to its *in vitro* preference for hemimethylated substrates, excessive amounts of DNMT1 in comparison to PCNA may participate in *de novo* methylation of CpG islands. The molecular mechanisms that target DNMT1 to unmethylated substrates in cancers need to be clarified.

Splicing alteration of DNMT3b and DNA hypomethylation in pericentromeric satellite regions. DNA hypomethylation in pericentromeric satellite regions is known to result in centromeric decondensation and enhanced chromosome recombination. In HCCs[57] and UCs,[58] DNA hypomethylation of these regions is correlated with copy number alterations on chromosomes 1 and 9, respectively, where satellite regions are rich. DNMT3b is required for DNA methylation of pericentromeric satellite regions in early mouse embryos, and germline mutations of the *DNMT3b* gene have been reported in patients with immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome, a rare recessive autosomal disorder characterized by DNA hypomethylation of pericentromeric satellite regions.[59] The major splice variant of DNMT3b in normal liver tissue samples is DNMT3b3, which possesses the conserved catalytic domains.[60] DNMT activity of human DNMT3b3 has been confirmed *in vitro*.[61] In contrast, DNMT3b4 lacks the conserved catalytic domains, although it retains the *N*-terminal domain required for targeting to heterochromatin sites. Samples of normal liver tissue show only a trace level of DNMT3b4 expression.[60] The levels of DNMT3b4 mRNA expression and the ratio of DNMT3b4 mRNA to DNMT3b3 in samples of non-cancerous liver tissue obtained from patients with HCCs, and in HCCs themselves, are significantly correlated with the degree of DNA hypomethylation in pericentromeric satellite regions.[60] DNA demethylation on satellite 2 has been observed in DNMT3b4-transfected human epithelial 293 cells.[60] As DNMT3b4 lacking DNMT activity competes with DNMT3b3 for targeting to pericentromeric satellite regions, DNMT3b4 overexpression may lead to chromosomal instability through induction of DNA hypomethylation in such regions.

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Furthermore, the growth rate of DNMT3b4 transfectants is approximately double that of mock-transfectants soon after the introduction of DNMT3b4, when chromosomal instability may not yet have accumulated.[62] Genes implicated in interferon signaling including signal transducer and activator of transcription (STAT) 1, which acts as an effector of interferon signaling, are upregulated in DNMT3b4 transfectants,[62] suggesting that DNMT3b may act to maintain the DNA methylation status of not only pericentromeric satellite regions but also specific genes, probably in cooperation with DNMT1, in cancer cells.

Genome-wide DNA methylation profiling

DNA methylation profiles in precancerous conditions are inherited by cancers. The above findings that DNA methylation alterations are associated with multistage carcinogenesis have prompted us to carry out genome-wide DNA methylation analysis of tissue specimens. Recently, analysis on a genomic-wide scale has become possible using DNA methylation-sensitive restriction enzyme-based or anti-methyl-cytosine antibody affinity techniques that enrich methylated and unmethylated fractions of genomic DNA.[63,64] These fractions can then be hybridized to DNA microarrays or sequenced. Ultra-high-throughput DNA sequencing technologies are being introduced for the direct sequencing of enriched, methylated fragments or for bisulfite-converted genomic sequencing.[65]

We have used BAMCA.[66–69] Many researchers in this field use the promoter arrays to identify genes that are methylated in cancer cells. 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.[70] Genomic regions in which DNA hypomethylation affects chromosomal instability may not be contained in promoter arrays. Moreover, aberrant DNA methylation of large chromosome regions, which are regulated in a coordinated manner in human cancers due to a process of long-range epigenetic silencing, has recently attracted attention.[71] Therefore, we used a BAC array that may be suitable, not for focusing on specific promoter regions, but for overviewing the DNA methylation status of individual large regions among all chromosomes.

When BAMCA methods were applied to samples of non-cancerous renal tissue obtained from patients with clear cell RCCs, many BAC clones showed DNA hypo- or hypermethylation in comparison to normal renal tissue samples from patients without any primary renal tumors.[72] RCCs are usually well demarcated and covered by a fibrous capsule, and hardly ever contain fibrous stroma between cancer cells (Fig. 2a). We were therefore able to obtain cancer cells of high purity from surgical specimens, avoiding contamination with either non-cancerous epithelial cells or stromal cells (Fig. 2a). Therefore, the DNA methylation alterations observed in samples of non-cancerous renal tissue from patients with RCCs cannot be attributable to contamination during sampling. Moreover, DNA methylation alterations in non-cancerous renal tissue did not depend on the distance from the RCC itself to the site from which the non-cancerous renal tissue samples were taken. Because of the lack of any remarkable histological changes or any association with chronic inflammation and persistent infection with viruses or other pathogenic microorganisms, precancerous conditions in the kidney have rarely been described. However, from the viewpoint of DNA methylation, we can consider that non-cancerous renal tissue from patients with RCCs is already at the precancerous stage, showing genome-wide DNA methylation alterations.

We then carried out two-dimensional unsupervised hierarchical clustering analysis based on BAMCA data for non-cancerous renal tissue samples. The patients with RCCs were clustered into two subclasses, clusters A_N and B_N (Fig. 2a). The corresponding RCCs of patients in Cluster B_N showed more frequent macroscopically evident renal vein tumor thrombi, microscopically evident vascular involvement, and higher pathological TNM stages than those in Cluster A_N.[72] The overall survival rate of patients in Cluster B_N was significantly lower than that of patients in Cluster A_N (Fig. 2a).[72] Tumor aggressiveness and even patient outcome might thus be determined by DNA methylation profiles at the precancerous stage.

In RCCs themselves, more BAC clones showed DNA hypo- or hypermethylation, and its degree was increased in comparison with samples of non-cancerous renal tissue obtained from patients with RCCs. Two-dimensional unsupervised hierarchical clustering analysis based on BAMCA data for RCCs was able to group patients into two subclasses, Clusters A_T and B_T (Fig. 2a). RCCs in Cluster B_T more frequently showed renal vein tumor thrombi, vascular involvement, and higher pathological TNM stages than those in Cluster A_T.[72] The overall survival rate of patients in Cluster B_T was significantly lower than that of patients in Cluster A_T (Fig. 2a).[72]

Patients who were grouped in Cluster B_N on the basis of BAMCA data for non-cancerous renal tissue were also grouped in Cluster B_T on the basis of BAMCA data for RCC themselves. That is, Cluster B_N was completely included in Cluster B_T (Fig. 2b).[72] The majority of the BAC clones significantly discriminating Cluster B_N from Cluster A_N also discriminated Cluster B_T from Cluster A_T.[72] Among BAC clones characterizing both clusters B_N and B_T, where the average signal ratio of Cluster B_N was higher than that of Cluster A_N, the average signal ratio of Cluster B_T was also higher than that of Cluster A_T without exception (Fig. 2b). Among BAC clones characterizing both clusters B_N and B_T, where the average signal ratio of Cluster B_N was lower than that of Cluster A_N, the average signal ratio of Cluster B_T was also lower than that of Cluster A_T without exception (Fig. 2b). Comparison between the signal ratios of each BAC clone characterizing both clusters B_N and B_T in non-cancerous renal tissue and those in the corresponding RCCs for all patients revealed that the DNA methylation status of the non-cancerous renal tissue was basically inherited by the corresponding RCC in each individual patient (Fig. 2b).[72]

In non-cancerous renal tissue showing no remarkable histological changes and consisting mainly of renal tubules with specialized functions, no progenitor cell is able to gain a growth advantage, and clonal expansion is unable to occur. Therefore, the distinct DNA methylation profile of Cluster B_N, which is clinicopathologically valid, cannot be established through the selection of one of a number of random DNA methylation profiles in non-cancerous renal tissue in patients with clear cell RCCs, and instead may be established through distinct target mechanisms. As the DNA methylation profiles in Cluster B_T are shared by phenotypically similar patients, who all suffer from clinicopathologically aggressive tumors and show a poor outcome, DNA methylation alterations in at least a proportion of the BAC regions characterizing Cluster B_T cannot be passenger changes. It is clear that cancer itself can induce alterations in DNA methylation. However, DNA methylation alterations of BAC regions characterizing Cluster B_T may significantly participate in carcinogenesis, as the DNA methylation profile in Cluster B_N was established at a very early and precancerous stage of carcinogenesis and inherited during progression of the cancers themselves as Cluster B_T. At least a proportion of DNA methylation alterations at the precancerous stage may be ''epigenetic gatekeepers''[21] and which allow time for further epigenetic and genetic alterations including genetic gatekeeper mutations (Fig. 3).

In fact, when the DNA methylation status of C-type CpG islands was examined,[73] the average number of methylated

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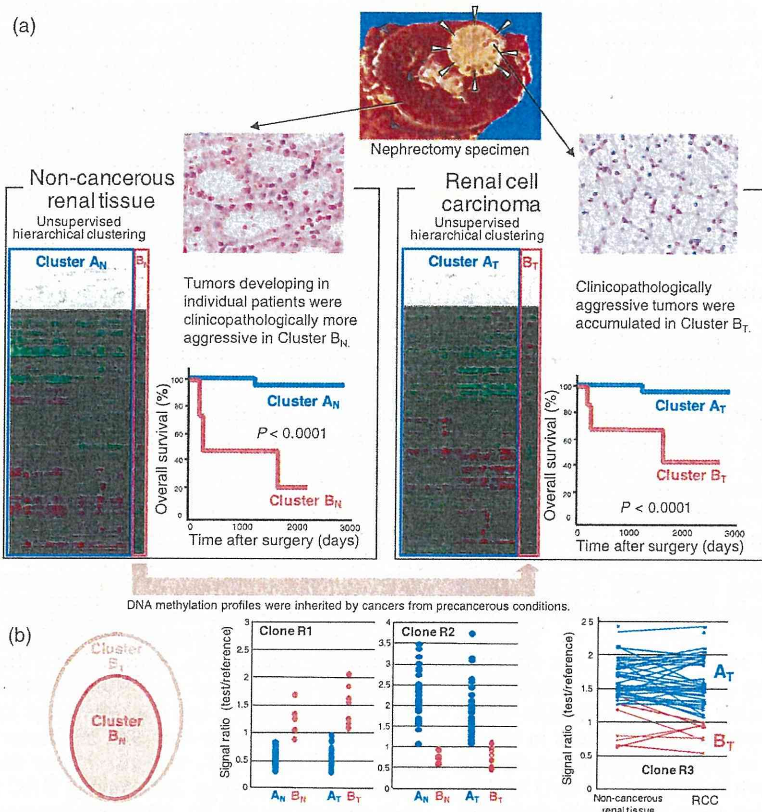

Fig. 2. DNA methylation profiles in precancerous conditions and renal cell carcinomas (RCCs). (a) Bacterial artificial chromosome array-based methylated CpG island amplification (BAMCA) data for tissue samples obtained from patients with RCCs (arrowheads). Using unsupervised hierarchical clustering analysis based on BAMCA data for samples of their non-cancerous renal tissue, patients with RCCs were clustered into two subclasses, Clusters A_N and B_N.[72] Clinicopathologically aggressive RCCs were accumulated in Cluster B_N, and the overall survival rate of patients in Cluster B_N was significantly lower than that of patients in Cluster A_N.[72] Using unsupervised hierarchical clustering analysis based on BAMCA data for their RCCs, patients were clustered into two subclasses, Clusters A_T and B_T.[72] Clinicopathologically aggressive clear cell RCCs were accumulated in Cluster B_T, and the overall survival rate of patients in Cluster B_T was significantly lower than that of patients in Cluster A_T.[72] (b) Correlation between DNA methylation profiles of precancerous conditions and those of RCCs. Cluster B_N was completely included in Cluster B_T (left panel). The majority of the bacterial artificial chromosome (BAC) clones, 724 in all, significantly discriminating Cluster B_N from Cluster A_N, also discriminated Cluster B_T from Cluster A_T.[72] In 311 of the 724 BAC clones, where the average signal ratio of Cluster B_N was higher than that of Cluster A_N, such as Clone R1 in the middle panel, the average signal ratio of Cluster B_T was also higher than that of Cluster A_T without exception.[72] In 413 of the 724 BAC clones, where the average signal ratio of Cluster B_N was lower than that of Cluster A_N, such as Clone R2 in the middle panel, the average signal ratio of Cluster B_T was also lower than that of Cluster A_T without exception.[72] As shown in the scattergram of the signal ratios in non-cancerous renal tissue samples and RCCs for all examined patients for a representative BAC clone, Clone R3, the DNA methylation status of the non-cancerous renal tissue was basically inherited by the corresponding RCC in individual patients (right panel).[72]

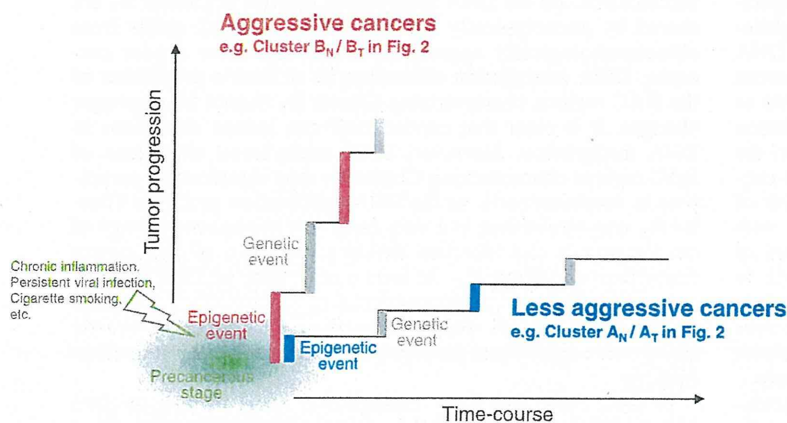

Fig. 3. Significance of DNA methylation alterations at the precancerous stage. Chronic inflammation, persistent infection with viruses or other pathogenic microorganisms, cigarette smoking, exposure to chemical carcinogens, and other unknown factors may participate in the establishment of particular DNA methylation profiles, such as Cluster B_N in Fig. 2. Such DNA methylation alterations in precancerous conditions may not occur randomly, but may be prone to further accumulation of epigenetic and genetic alterations (regional DNA hypermethylation of C-type CpG islands and copy number alterations were accumulated in Cluster B_T in Fig. 2),[72] thus generating more malignant cancers, such as the renal cell carcinomas in patients belonging to Cluster B_T.

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CpG islands was significantly higher in Cluster B_T based on BAMCA than in Cluster A_T. The frequency of CIMP in Cluster B_T was significantly higher than that in Cluster A_T. Genome-wide DNA methylation alterations consisting of both hypo- and hypermethylation revealed by BAMCA in Cluster B_T were associated with regional DNA hypermethylation of C-type CpG islands. For comparison with their DNA methylation status, we also examined copy number alterations by array-based comparative genomic hybridization. By unsupervised hierarchical clustering analysis based on copy number alterations, RCCs were clustered into the two subclasses, clusters A_{TG} and B_{TG}. Loss of chromosome 3p and gain of chromosomes 5q and 7 were frequent in both clusters A_{TG} and B_{TG}. Loss of chromosomes 1p, 4, 9, 13q, and 14q was frequent only in Cluster B_{TG}, and not in Cluster A_{TG}.[74] RCCs showing higher histological grades, renal vein tumor thrombi, vascular involvement and higher pathological TNM stages were accumulated in Cluster B_{TG}. The recurrence-free and overall survival rates of patients in Cluster B_{TG} were significantly lower than those of patients in Cluster A_{TG}.[74] A subclass of Cluster B_T based on BAMCA data was completely included in Cluster B_{TG} showing accumulation of copy number alterations. Genetic and epigenetic alterations are not mutually exclusive during renal carcinogenesis, and particular DNA methylation profiles may be closely related to chromosomal instability. DNA methylation alterations at the precancerous stage, which may not occur randomly but may foster further epigenetic and genetic alterations, can generate more malignant cancers and even determine patient outcome (Fig. 3).

Carcinogenetic risk estimation and prognostication based on DNA methylation status. In samples of non-cancerous liver tissue obtained from patients with HCCs, many BAC clones show DNA hypo- or hypermethylation in comparison with normal liver tissue from patients without HCCs (Fig. 4a).[75] 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 is a priority for patients with HBV or HCV infection. To reveal the baseline liver histology, microscopic examination of liver biopsy specimens is carried out in patients with HBV or HCV infection prior to interferon therapy.[76,77] Carcinogenetic risk estimation using such liver biopsy specimens is advantageous for close follow-up of patients who are at high risk of HCC development. To establish an indicator for carcinogenetic risk estimation, 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 altered at the precancerous stage in comparison to normal liver tissue and was inherited by HCCs themselves from the precancerous stage (Fig. 4b). Among the BAC clones studied, a bioinformatics approach further identified the top 25 for which DNA methylation status was able to discriminate non-cancerous liver tissue from patients with HCCs in the learning cohort from normal liver tissue with sufficient sensitivity and specificity.[75] By two-dimensional hierarchical clustering analysis using these 25 BAC clones, samples of normal liver tissue and samples of non-cancerous liver tissue obtained from patients with HCCs in the learning cohort were successfully subclassified into different subclasses without any error (Fig. 4c). The criteria established using a combination of the DNA methylation status of the 25 BAC clones (Fig. 4d) diagnosed non-cancerous liver tissue from patients with HCCs in the learning cohort as being at high risk of carcinogenesis with a sensitivity and specificity of 100%.[75] The sensitivity and specificity in the validation cohort were both 96%, and thus our criteria were successfully validated.[75]

It was confirmed that there were no significant differences in the number of BAC clones satisfying our criteria between samples of non-cancerous liver tissue showing chronic hepatitis and samples of non-cancerous liver tissue showing cirrhosis, indicating that our criteria were not associated with the degree of inflammation or fibrosis.[75] In addition, the average numbers of BAC clones satisfying our criteria were significantly lower in liver tissue samples from patients with HBV or HCV infection but without HCCs than in samples of non-cancerous liver tissue obtained from patients with HCCs.[75] Therefore, our criteria may be applicable for classifying liver tissue samples obtained from patients who are being followed up because of HBV or HCV infection, chronic hepatitis, or cirrhosis into those that may generate HCCs and those that will not. 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 with HBV or HCV infection.

To establish criteria for prognostication of patients with HCCs, in the learning cohort, patients who had survived more than 4 years after hepatectomy and 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 revealed that the signal ratios of 41 BAC clones differed significantly between the two groups.[75] Two-dimensional hierarchical clustering analysis using the 41 BAC clones successfully subclassified HCCs in the favorable-outcome group and the poor-outcome group into different subclasses without any error (Fig. 5a). We also established cut-off values for the 41 BAC clones that allowed discrimination of samples between the poor-outcome and favorable-outcome groups with sufficient sensitivity and specificity (Fig. 5b). Multivariate analysis revealed that satisfying our criteria 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 significance,[75] such as histological differentiation, and presence of portal vein tumor thrombi, intrahepatic metastasis, and multicentricity.[33] The cancer-free and overall survival rates of patients with HCCs satisfying the criteria for 32 or more BAC clones in the validation cohort were significantly lower than those of patients with HCCs satisfying the criteria for less than 32 BAC clones (Fig. 5c).[75] Such prognostication using liver biopsy specimens obtained before transarterial embolization, transarterial chemoembolization, and radiofrequency ablation may be advantageous even for patients who undergo such therapies.

As mentioned above, even non-cancerous urothelia showing no remarkable histological changes obtained from patients with UCs may be exposed to carcinogens in urine. In fact, genome-wide DNA methylation profiles of non-cancerous urothelia obtained from patients with nodular invasive UCs showing an aggressive clinical course were inherited by the nodular invasive UCs themselves, suggesting that DNA methylation alterations that were correlated with the development of more malignant invasive cancers had already accumulated in non-cancerous urothelia.[78] These findings prompted us to estimate the degree of carcinogenetic risk based on DNA methylation profiles in non-cancerous urothelia. We were able to identify BAC clones for which DNA methylation status was able to completely discriminate non-cancerous urothelia from patients with UCs from normal urothelia and diagnose them as having a high risk of urothelial carcinogenesis.[78] If it were possible to identify individuals who are at high risk of urothelial carcinogenesis, then strategies for the prevention or early detection of UCs, such as smoking cessation or repeated urine cytology examinations, might be applicable.

In order to start adjuvant systemic chemotherapy immediately in patients who have undergone total cystectomy and are still at high risk of recurrence and metastasis of UCs, prognostic indicators have been explored. Subclassification based on unsupervised two-dimensional hierarchical clustering analysis using BAMCA data for UCs was significantly correlated with recurrence after surgery due to metastasis to pelvic lymph nodes or

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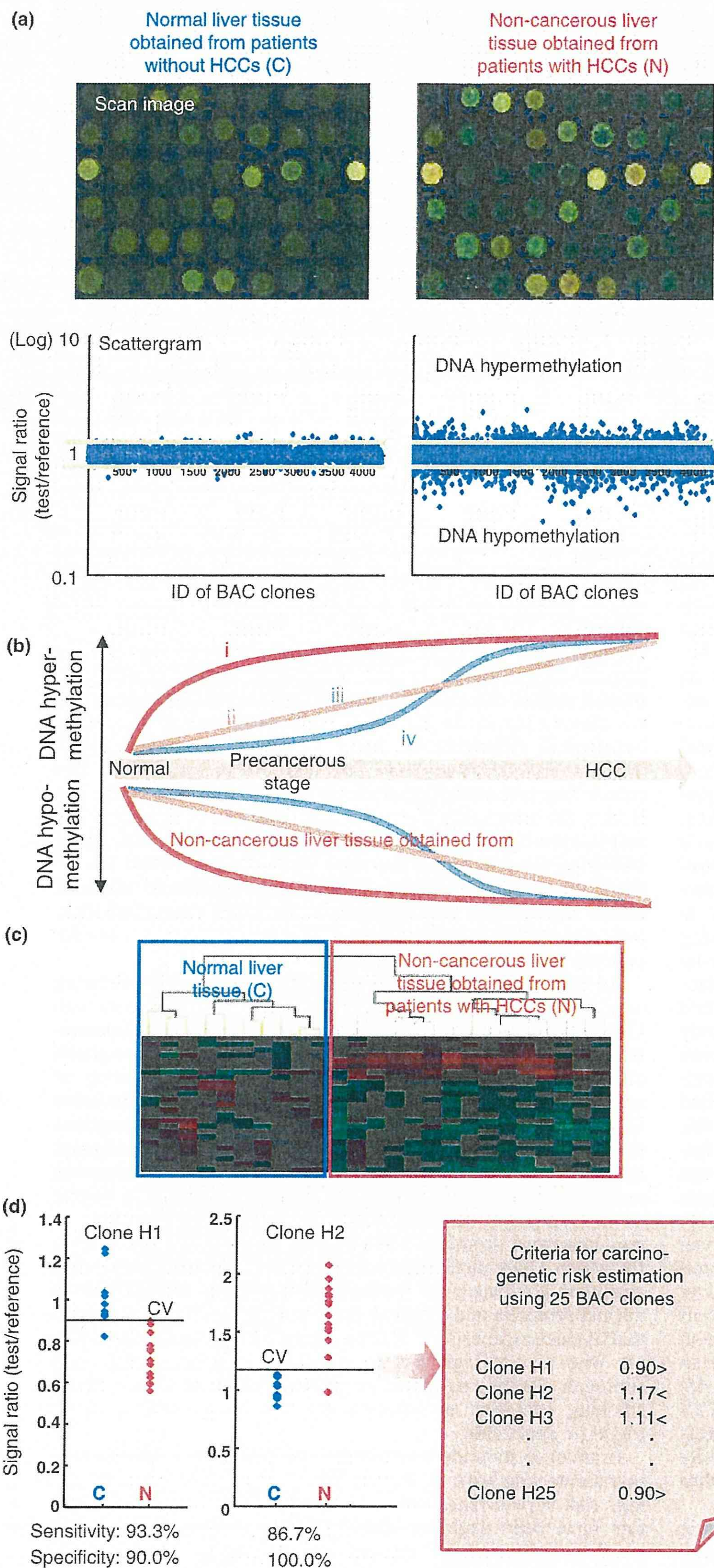

Fig. 4. Risk estimation of hepatocellular carcinoma (HCC) development based on DNA methylation status. (a) Examples of scan images and scattergrams of signal ratios in normal liver tissue obtained from patients without HCCs (C) and non-cancerous liver tissue obtained from patients with HCCs (N). In N samples, many bacterial artificial chromosome (BAC) clones showed DNA hypo- or hypermethylation compared to C samples.[75] (b) Four patterns of DNA methylation alterations seen in BAC clones during multistage hepatocarcinogenesis: (i) DNA methylation alterations occurred at the chronic hepatitis and liver cirrhosis stage, and DNA methylation status did not alter in HCCs from the chronic hepatitis and liver cirrhosis stage; (ii) DNA methylation alterations occurred at the chronic hepatitis and liver cirrhosis stage and further altered in HCCs; (iii) although DNA methylation alterations occurred at the chronic hepatitis and liver cirrhosis stage, the DNA methylation status returned to normal in HCCs; and (iv) DNA methylation alterations occurred only in HCCs. In order to establish criteria for carcinogenetic risk estimation, we focused on BAC clones whose DNA methylation status was inherited by HCCs from the precancerous stage (groups i and ii), whereas group iii may only reflect inflammation and/or fibrosis, and group iv may participate only in the malignant progression stage. (c) Two-dimensional hierarchical clustering analysis using BAC clones that were selected as the top 25 for which DNA methylation status was able to discriminate N from C with sufficient sensitivity and specificity by Wicoxon test and the support vector machine algorithm.[75] C and N samples in the learning cohort were successfully subclassified into different subclasses without any error.[75] (d) Scattergrams of the signal ratios in C and N samples in the learning cohort for representative BAC clones, Clone H1 and Clone H2. Using the cut-off values (CV) in each panel, N samples in the learning cohort were discriminated from C samples with sufficient sensitivity and specificity.[75] Based on a combination of DNA methylation status for the 25 BAC clones, the criteria for carcinogenetic risk estimation were established. Using these criteria, the sensitivity and specificity for diagnosis of N samples in the learning cohort as being at high risk of carcinogenesis were both 100%.[75] The sensitivity and specificity in the validation cohort were both 96%, and thus the criteria were successfully validated.[75]

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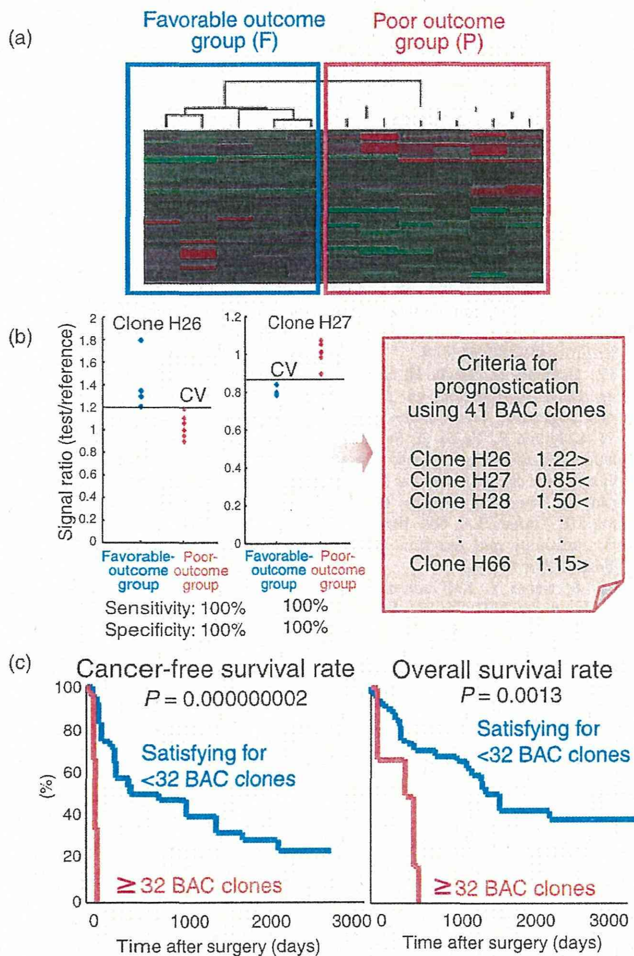

Fig. 5. Prognostication of patients with HCC development based on DNA methylation status. (a) Two-dimensional hierarchical clustering analysis using 41 bacterial artificial chromosome (BAC) clones selected as those for which DNA methylation status was able to discriminate a poor-outcome group (P), who suffered recurrence within 6 months and died within a year after hepatectomy, from a favorable-outcome group (F), who survived for more than 4 years after hepatectomy, with sufficient sensitivity and specificity by Wilcoxon test.[75] F and P patients in the learning cohort were successfully subclassified into different subclasses without any error.[75] (b) Scattergrams of the signal ratios in F and P patients in the learning cohort for representative BAC clones, Clone H26 and Clone H27. Using the cut-off values (CV) in each panel, P patients in the learning cohort were discriminated from F patients with 100% sensitivity and specificity.[75] Based on a combination of the DNA methylation status of the 41 BAC clones, criteria for prognostication were established. (c) The cancer-free and overall survival rates of patients with HCCs in the validation cohort. Patients with HCCs satisfying the criteria for 32 or more BAC clones showed significantly poorer outcome than patients with HCCs satisfying the criteria for less than 32 BAC clones.[75]

distant organs.[78] These data prompted us to establish criteria for predicting recurrence of UCs based on DNA methylation status, and we successfully identified BAC clones for which DNA methylation status completely discriminated patients who suffered recurrence from patients who did not, whereas high histological grade, invasive growth, and vascular or lymphatic involvement were unable to achieve such complete discrimination.[78]

It is well known that patients with UCs of the renal pelvis and ureter frequently develop metachronous UC in the urinary bladder after nephroureterectomy. Therefore, such patients need to undergo repeated urethrocystoscopic examinations for detection of intravesical metachronous UCs. To decrease the need for such invasive urethrocystoscopic examinations, indicators for intravesical metachronous UCs are needed. DNA methylation profiles of non-cancerous urothelia obtained by nephroureterectomy from patients with UCs of the renal pelvis or ureter, which may be exposed to the same carcinogens in the urine as non-cancerous urothelia from which metachronous UCs originate, were correlated with the risk of intravesical metachronous UC development.[78] In non-cancerous urothelia from nephroureterectomy specimens, we are able to identify BAC clones for which DNA methylation status was able to completely discriminate patients with UCs of the renal pelvis or ureter who developed intravesical metachronous UCs from patients who did not.[78] After prospective validation, combination of such BAC clones may be an optimal indicator for the development of intravesical metachronous UC.

Perspective

On the basis of DNA methylation profiling, translational epigenetics has clearly come of age. The incidence of DNA methylation alterations is generally high during multistage carcinogenesis in various organs. DNA methylation alterations are stably preserved on DNA double strands by covalent bonds, and these can be detected using highly sensitive methodology. Therefore, they may be better diagnostic indicators than mRNA and protein expression profiles, which can be easily affected by the microenvironment of cancer cells or precursor cells. Genome-wide DNA methylation profiling can provide optimal indicators for carcinogenetic risk estimation and prognostication using samples of urine, sputum, and other body fluids, and also biopsy and surgically resected specimens.

However, most of the recently developed detection technologies such as promoter arrays, CpG-island arrays and high-throughput sequencing are sequence-based methods and cannot comprehensively measure the DNA methylation status of repetitive sequences and gene bodies. The dynamics of DNA methylation at such non-unique sequences still remain to be determined.[79] Our BAC array-based methods do not focus only on specific promoter regions and CpG islands, and have successfully identified the chromosomal regions in which coordinated DNA methylation alterations have clinicopathological impact. Evaluation of the correlation between the methylation status of each CpG site in selected BAC clones and the clinicopathological diversity of cancers may provide new insights into the roles of DNA methylation during multistage carcinogenesis. Subclassification of cancers based on DNA methylation profiling may provide clues for clarification of distinct target mechanisms and molecules for prevention and therapy in patients belonging to specific clusters.

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