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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

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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.

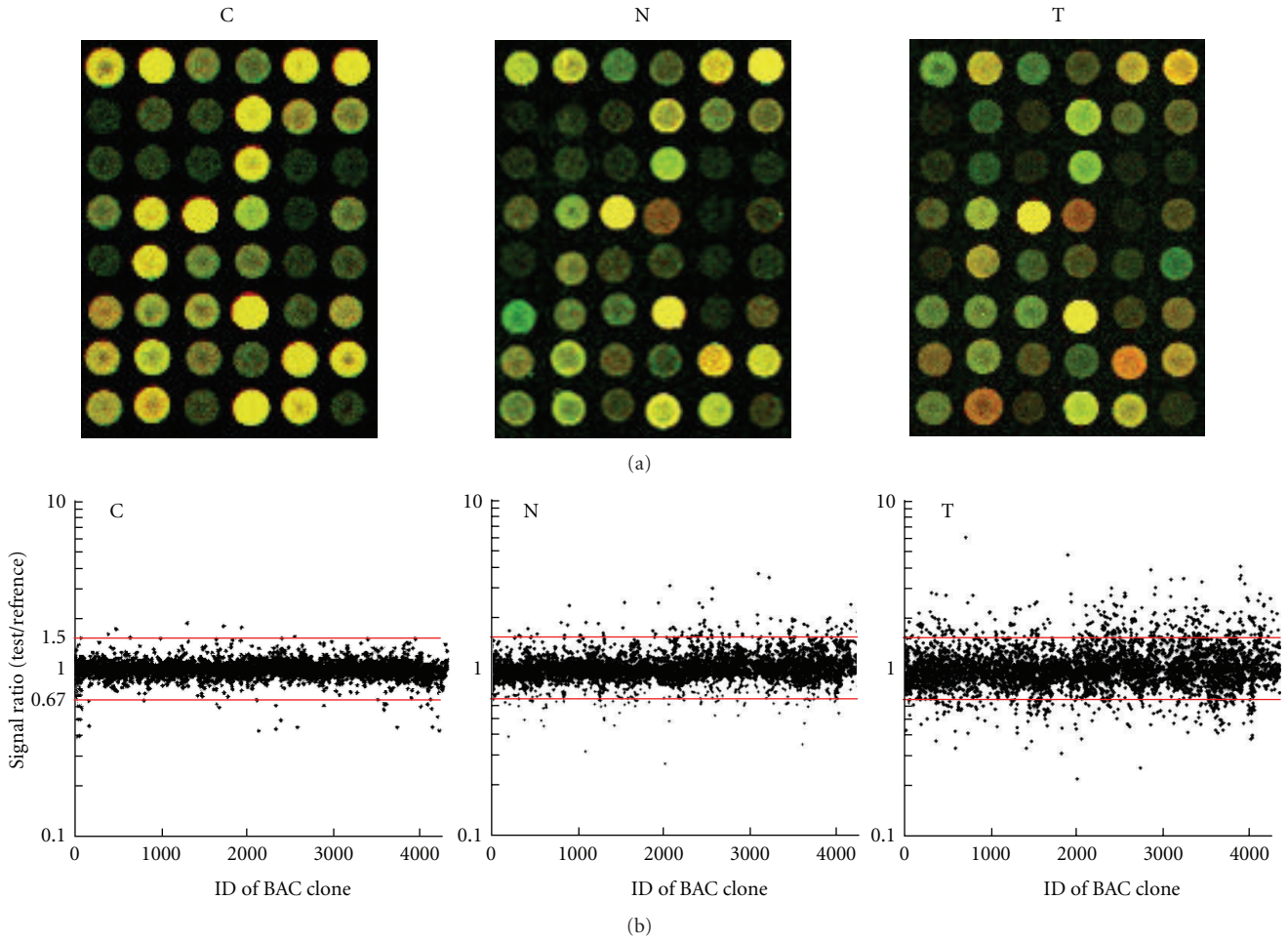

Figure 1: Genome-wide DNA methylation analysis by BAMCA. (a) Representative examples of scanned array images in a sample of normal pancreatic tissue obtained from a patient without ductal adenocarcinoma of the pancreas (C) and samples of both noncancerous pancreatic tissue (N) and cancerous tissue (T) 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

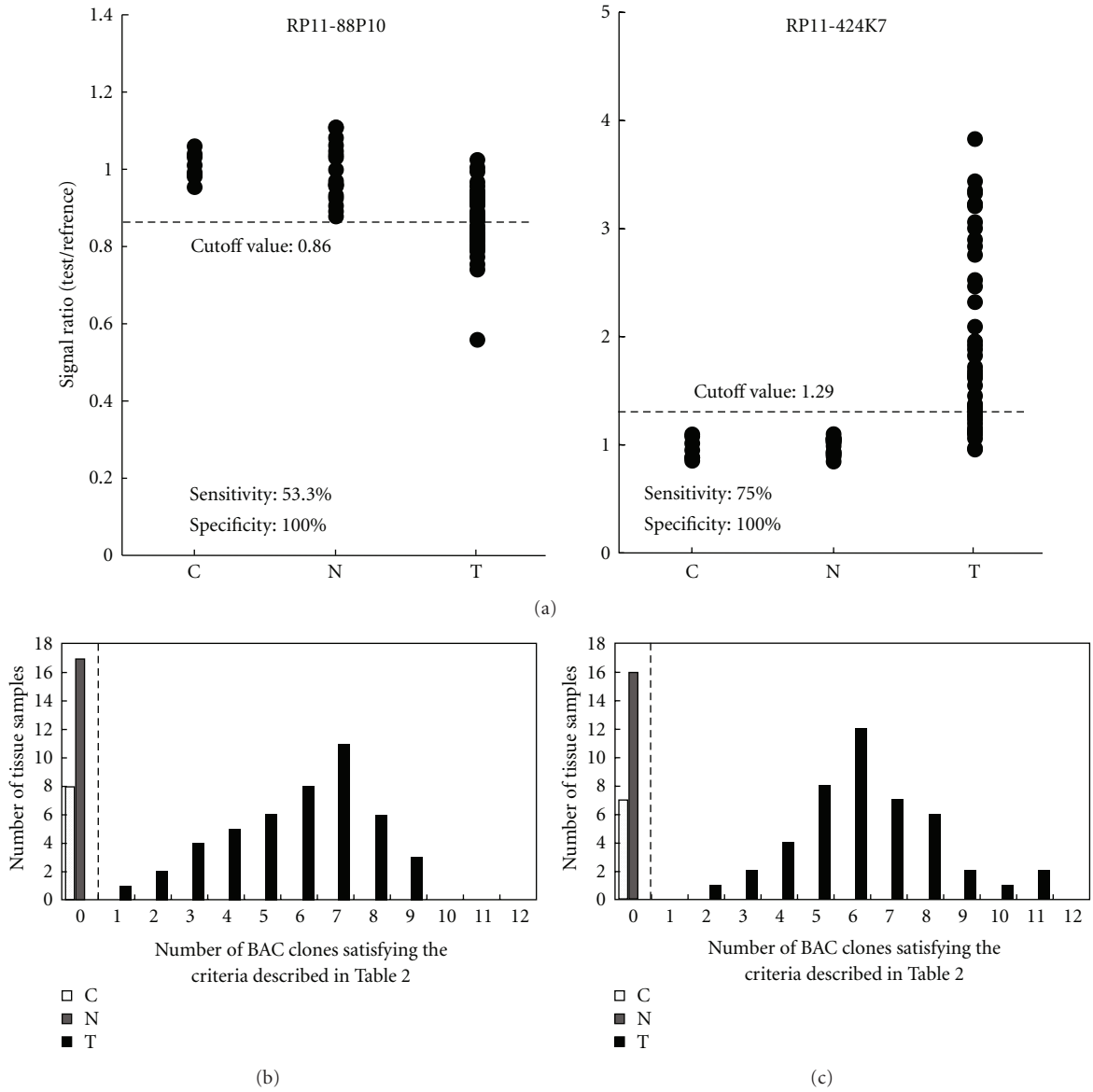

Figure 2: Establishment of criteria for diagnosis of ductal adenocarcinomas of the pancreas. (a) Scattergrams of the signal ratios in samples of normal pancreatic tissue obtained from patients without ductal adenocarcinomas (C), noncancerous pancreatic tissue obtained from patients with ductal adenocarcinomas (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

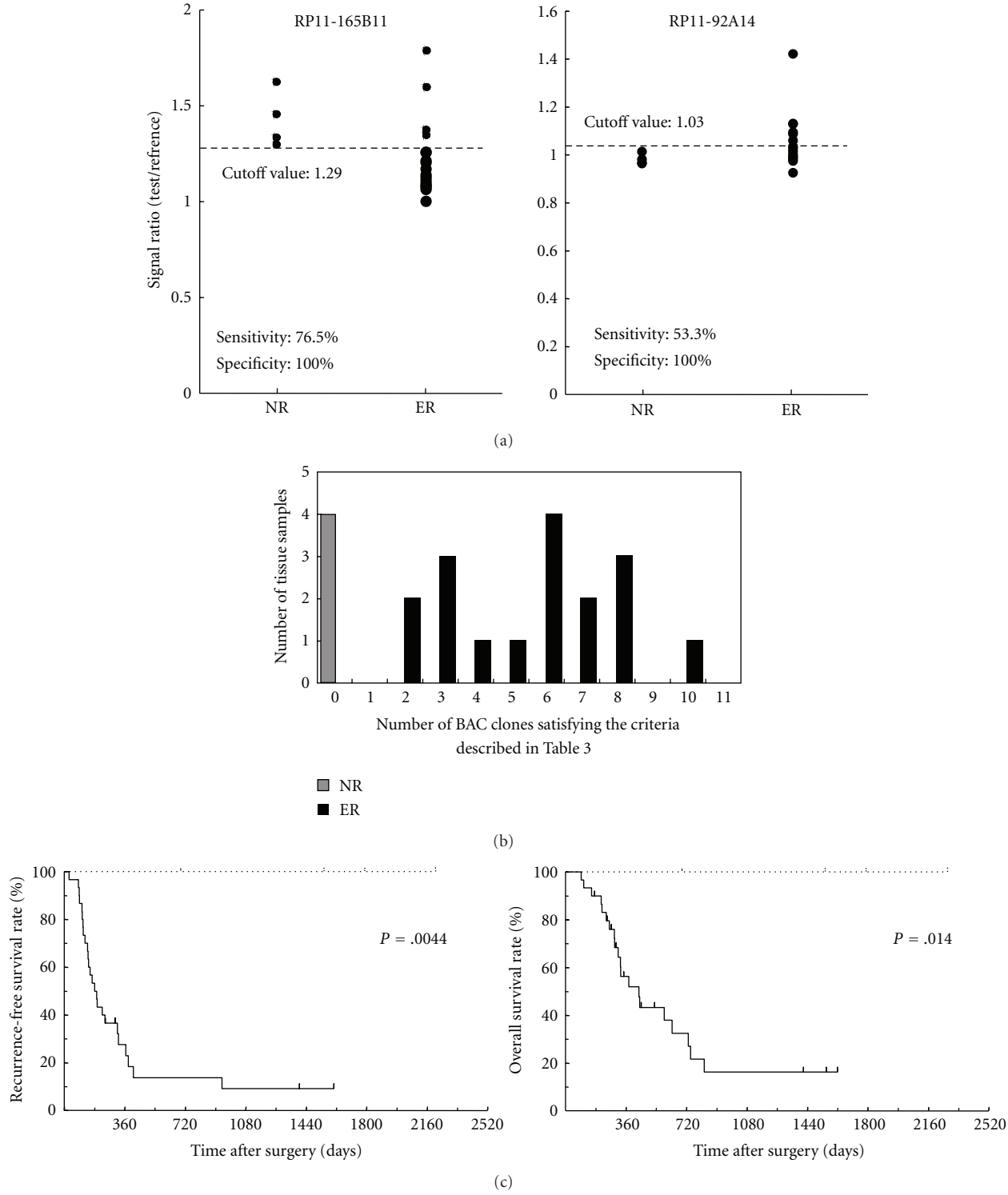

Figure 3: Establishment of criteria for prognostication of patients with ductal adenocarcinomas of the pancreas. (a) Scattergrams of the signal ratios in samples of cancerous tissue obtained from patients in the no-relapse group (NR, $n = 4$) and early-relapse group (ER, $n = 17$) who had not undergone 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 adenocarcinomas [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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