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Genome-Wide DNA Methylation Profiles in Renal Tumors of Various Histological Subtypes and Non-Tumorous Renal Tissues

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Key Words
Chromophobe renal cell carcinoma · Clear cell renal cell carcinoma · DNA methylation · Oncocytoma · Papillary renal cell carcinoma · Precancerous condition

Abstract
Objective: The aim of this study is to clarify genome-wide DNA methylation profiles in renal tumors of various histological subtypes. ***Methods:*** Bacterial artificial chromosome (BAC) array-based methylated CpG island amplification was performed using tissue samples of 17 patients with papillary renal cell carcinomas (RCCs), chromophobe RCCs and oncocytomas, and the results were compared with those from 51 patients with clear cell RCCs. ***Results:*** Unsupervised hierarchical clustering analysis based on DNA methylation status clustered type 1 and type 2 papillary RCCs into different subclasses. Although chromophobe RCCs and oncocytomas were clustered into the same subclass, the DNA methylation status of 21 BAC clones was able to discriminate chromophobe RCCs from oncocytomas. The number of BAC clones showing DNA methylation alteration in non-tumorous renal tissue from patients with chromophobe RCCs and oncocytomas was smaller than that from patients with clear cell RCCs. Biphasic accumulation of DNA methylation alterations was observed in non-tumorous renal tissue from all 68 patients, and patients showing such alterations on more BAC clones had a poorer outcome than patients showing them on fewer BAC clones. ***Conclusions:*** DNA methylation profiles determining the histological subtypes of renal tumors developing in individual patients and/or patient outcome may be already established in non-tumorous renal tissue at the precancerous stage.

Introduction

Accumulating evidence suggests that not only genetic but also epigenetic alterations play a significant role in human carcinogenesis. DNA methylation alterations are one of the most consistent epigenetic changes occurring during carcinogenesis in various organs: 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 [1–5].

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Although the classification of renal tumors is based largely on histology, the World Health Organization classification has introduced genetic alterations as a hallmark corresponding to the histological subtypes of renal tumors, e.g. clear cell renal cell carcinomas (RCCs), the most common histological subtype, are characterized by loss of chromosome 3p and inactivation of the *VHL* gene at 3p25.3 [6]. Moreover, we have reported the genetic clustering of clear cell RCCs based on array-comparative genomic hybridization analysis and the association between genetic clustering on the one hand and clinicopathological tumor aggressiveness or patient outcome on the other [7]. With regard to epigenetic alterations, we have revealed that non-tumorous renal tissue obtained from patients with clear cell RCCs is at the precancerous stage, showing DNA hypo- and hypermethylation in multiple chromosomal regions [8], employing recently developed array-based technology [9], although precancerous conditions in the kidney have been rarely described because non-tumorous renal tissue shows no remarkable histological changes and is unassociated with chronic inflammation and persistent infection with viruses or other pathogenic microorganisms. We have proposed 2 possible scenarios: (a) genome-wide DNA methylation profiles of non-tumorous renal tissue at the precancerous stage are inherited by the corresponding clear cell RCCs developing in individual patients, and (b) DNA methylation alterations at the precancerous stage may be prone to further accumulation of genetic and epigenetic alterations during progression [9–11]. However, to our knowledge, the results of genome-wide DNA methylation analysis have never been reported for histological subtypes of renal tumors other than clear cell RCCs, such as papillary RCCs, chromophobe RCCs and oncocytomas, though the DNA methylation status of several tumor-related genes has been reported separately in such histological subtypes [8, 12, 13].

In the present study, in order to clarify genome-wide DNA methylation profiles during multistage renal tumorigenesis, we performed bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) [14–16] using a microarray of 4,361 BAC clones [17] for papillary RCCs, chromophobe RCCs and oncocytomas, and the corresponding non-tumorous renal tissue. DNA methylation profiles of patients with renal tumors of such histological subtypes were compared with those of patients with clear cell RCCs revealed by the same method.

Materials and Methods

Patients and Tissue Samples

Tumorous tissue and corresponding non-tumorous renal tissue samples were obtained at nephrectomy from 17 patients with primary renal tumors. These patients had not received preoperative treatment and had undergone nephrectomy between 1999 and 2006 at the National Cancer Center Hospital, Tokyo, Japan. The 17 primary renal tumors were histologically subclassified into 4 papillary RCCs including 2 type 1 papillary RCCs (T52 and T53) and 2 type 2 papillary RCCs (T54 and T55), 10 chromophobe RCCs (T56–T65) and 3 oncocytomas (T66–T68) in accordance with the World Health Organization classification (fig. 1a–d) [6]. Tumors in which almost the entire area showed a papillary or tubulopapillary architecture were classified as papillary RCCs, whereas clear cell RCCs in which only a minor component showed a papillary structure were not. The DNA methylation profiles of tumorous tissue and the corresponding non-tumorous renal tissue from these 17 patients were compared with those from 51 patients with clear cell RCCs (T1–T51; fig. 1e) for whom the results obtained by BAMCA had been reported previously [9]. All the patients gave their informed consent prior to inclusion in this study, which was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan.

BAMCA Analysis

High-molecular-weight DNA from fresh frozen tissue samples was extracted using phenol-chloroform, followed by dialysis. Because the DNA methylation status is known to be organ specific [18], the reference DNA for analysis of the developmental stages of renal tumors should be obtained from the kidney, and not from other organs or peripheral blood. Therefore, a mixture of normal renal tissue DNA obtained from 6 male patients and 3 female pa-

Fig. 1. Microscopic views (**a–e**, **k–p**) and scattergrams of the signal ratios (test signal/reference signal) obtained by BAMCA (**f–j**, **q–v**) in tumorous tissue (**a–j**) and non-tumorous renal tissue (**k–o**, **q–u**) from patients with type 1 papillary RCC (**a**, **f**, **k**, **q**), type 2 papillary RCC (**b**, **g**, **l**, **r**), chromophobe RCC (**c**, **h**, **m**, **s**), oncocytoma (**d**, **i**, **n**, **t**) and clear cell RCC (**e**, **j**, **o**, **u**), and normal renal tissue obtained from a patient without any renal tumor (**p**, **v**). In type 1 (**a**) and type 2 (**b**) papillary RCCs, chromophobe RCCs (**c**), oncocytomas (**d**) and clear cell RCCs (**e**), many BAC clones showed DNA hypo- or hypermethylation (**f–j**). Non-tumorous renal tissue obtained from patients with type 1 (**k**) and type 2 papillary RCCs (**l**), chromophobe RCCs (**m**), oncocytomas (**n**) and clear cell RCCs (**o**) showed no histological changes in comparison with normal renal tissue (**p**) and could not be histologically discriminated from each other. Even in such non-tumorous renal tissue from patients with type 1 (**q**) and type 2 (**r**) papillary RCCs and clear cell RCCs (**u**), distinct DNA hypo- or hypermethylation was already evident when compared with normal renal tissue (**v**). However, the numbers of BAC clones showing DNA hypo- or hypermethylation in non-tumorous renal tissue obtained from patients with chromophobe RCCs (**s**) and oncocytomas (**t**) were significantly smaller than those in non-tumorous renal tissue obtained from patients with clear cell RCCs (**u**) (table 1).

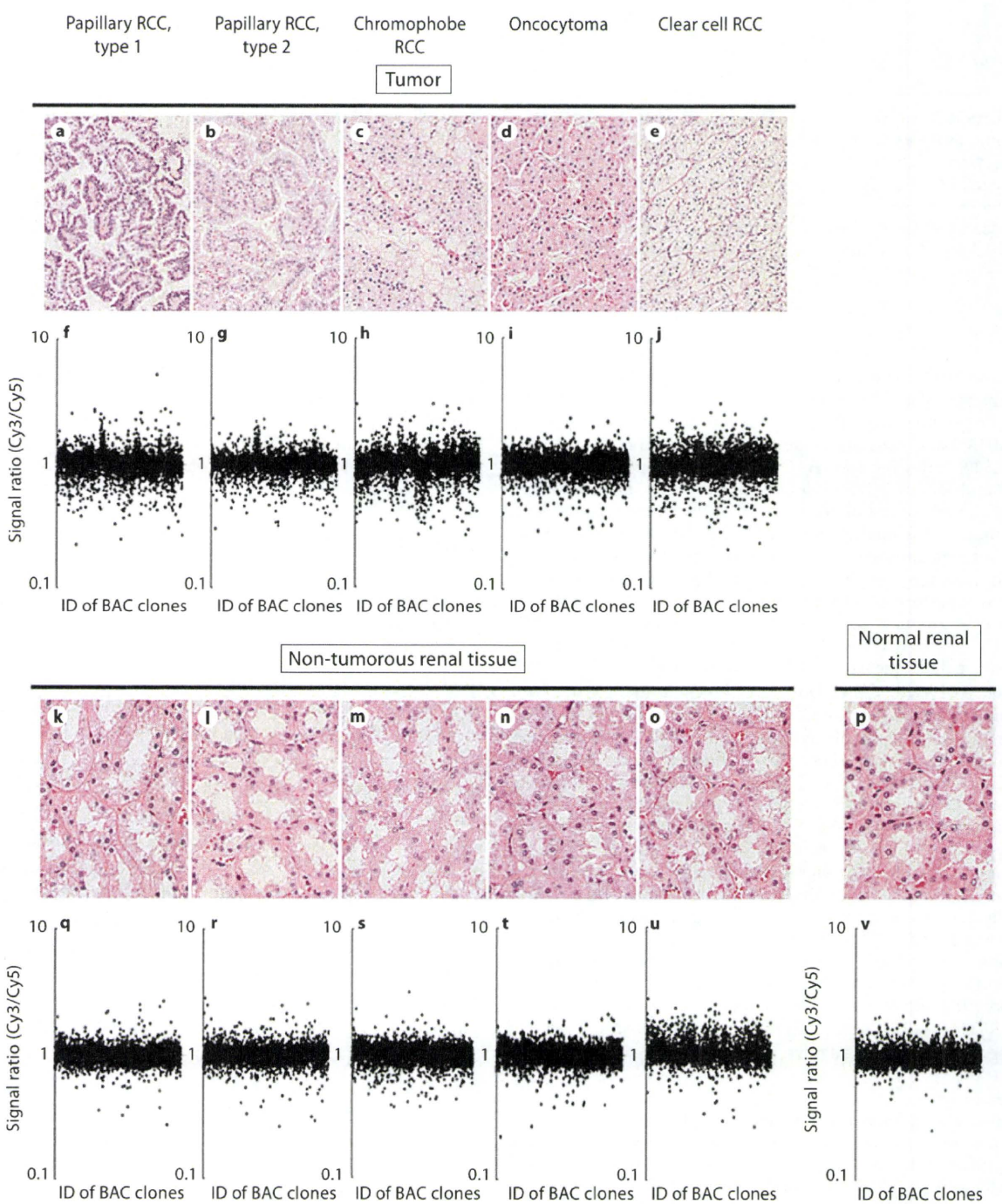

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Table 1. The average number of BAC clones showing DNA methylation alterations (DNA hypo- or hypermethylation) in tumorous tissue and non-tumorous renal tissue obtained from patients with renal tumors

	Tumor		Non-tumorous renal tissue	
	average number of BAC clones showing DNA methylation alterations	p[1]	average number of BAC clones showing DNA methylation alterations	p[1]
Papillary RCC (n = 4)	400.5 ± 249.6	0.390	108.0 ± 95.4	0.173
Chromophobe RCC (n = 10)	334.4 ± 139.7	0.167	89.0 ± 48.7	*0.041*
Oncocytoma (n = 3)	266.7 ± 205.7	0.970	54.0 ± 2.6	*0.028*
Clear cell RCC (n = 51)	265.3 ± 150.5	–	176.4 ± 138.2	–

[1] Mann-Whitney U test, compared with patients with clear cell RCCs.
p values <0.05, which indicate significant differences, are italicized.

tients without any primary renal tumor was used as a reference for analyses of male and female test DNA samples, respectively.

The genome-wide DNA methylation status was analyzed by BAMCA using a custom-made array (MCG Whole Genome Array-4500) harboring 4,361 BAC clones throughout chromosomes 1–22 and X and Y [17], as described previously [19, 20]. Briefly, 5-μg aliquots of test or reference DNA were first digested with 100 units of the methylation-sensitive restriction enzyme *Sma*I and subsequently with 20 units of the methylation-insensitive *Xma*I. Adapters were ligated to *Xma*I-digested sticky ends, and 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, Little Chalfont, UK), respectively, using a BioPrime Array CGH Genomic Labeling System (Invitrogen, Carlsbad, Calif., USA) and precipitated together with ethanol in the presence of Cot-I DNA. The mixture was applied to array slides and incubated at 43°C for 72 h. The arrays were scanned with a GenePix Personal 4100A (Axon Instruments, Foster City, Calif., USA) and analyzed using GenePix Pro 5.0 imaging software (Axon Instruments) and Acue 2 software (Mitsui Knowledge Industry, Tokyo, Japan). The signal ratios were normalized in each sample to make the mean signal ratios for all BAC clones 1.0. In accordance with previously described criteria [9], in the tumor and the corresponding non-tumorous renal tissue, DNA methylation status corresponding to a signal ratio of <0.67 and >1.5 was defined as DNA hypomethylation and DNA hypermethylation of each BAC clone compared with normal renal tissue, respectively. In our previous study, tumorous tissue and the corresponding non-tumorous renal tissue of 51 patients with clear cell RCCs (T1–T51) were analyzed by the same BAMCA method using the same array and reference DNA (fig. 1j) [9].

Statistics

Two-dimensional unsupervised hierarchical clustering analysis of the renal tumors with various histological subtypes and the BAC clones based on the signal ratios (test signal/reference signal) obtained by BAMCA were performed using the Expressionist software program (Genedata, Basel, Switzerland). Differences in the average number of BAC clones that showed DNA hypo- or hypermethylation between the histological subtypes of tumors were analyzed using the Mann-Whitney U test. BAC clones whose signal ratios differed significantly between chromophobe RCCs and oncocytomas were identified by the Wilcoxon test ($p < 0.01$). Survival curves of patient groups were calculated by the Kaplan-Meier method, and the differences were compared by the log-rank test. Differences at $p < 0.05$ were considered significant.

Results

Genome-Wide DNA Methylation Profiles of Renal Tumors

Example scattergrams of the signal ratios (test signal/reference signal) for tumorous tissue from each patient with type 1 papillary RCC, type 2 papillary RCC, chromophobe RCC and oncocytoma, respectively, are shown in figure 1f–i. The average numbers of BAC clones showing DNA hypo- or hypermethylation in papillary RCCs, chromophobe RCCs and oncocytomas were not significantly different from those in clear cell RCCs (table 1).

Figure 2 shows the results of 2-dimensional unsupervised hierarchical clustering based on the signal ratios obtained by BAMCA for 4 papillary RCCs (T52 and T55), 10 chromophobe RCCs (T56–T65), 3 oncocytomas (T66–T68) and the previously examined 51 clear cell RCCs (T1–T51). Two type 1 papillary RCCs (T52 and T53) and 2 type 2 papillary RCCs (T54 and T55) were clustered into the same subclasses and the 2 types of papillary RCCs were clustered into subclasses different from each other, and each accompanied clear cell RCCs.

All 10 chromophobe RCCs (T56–T65) and 3 oncocytomas (T66–T68) were clustered into the same subclass and excluded any tumor of other histological subtypes. On the other hand, the Wilcoxon test ($p < 0.01$) revealed that the signal ratios of 21 BAC clones differed significantly between chromophobe RCCs (n = 10) and oncocy-

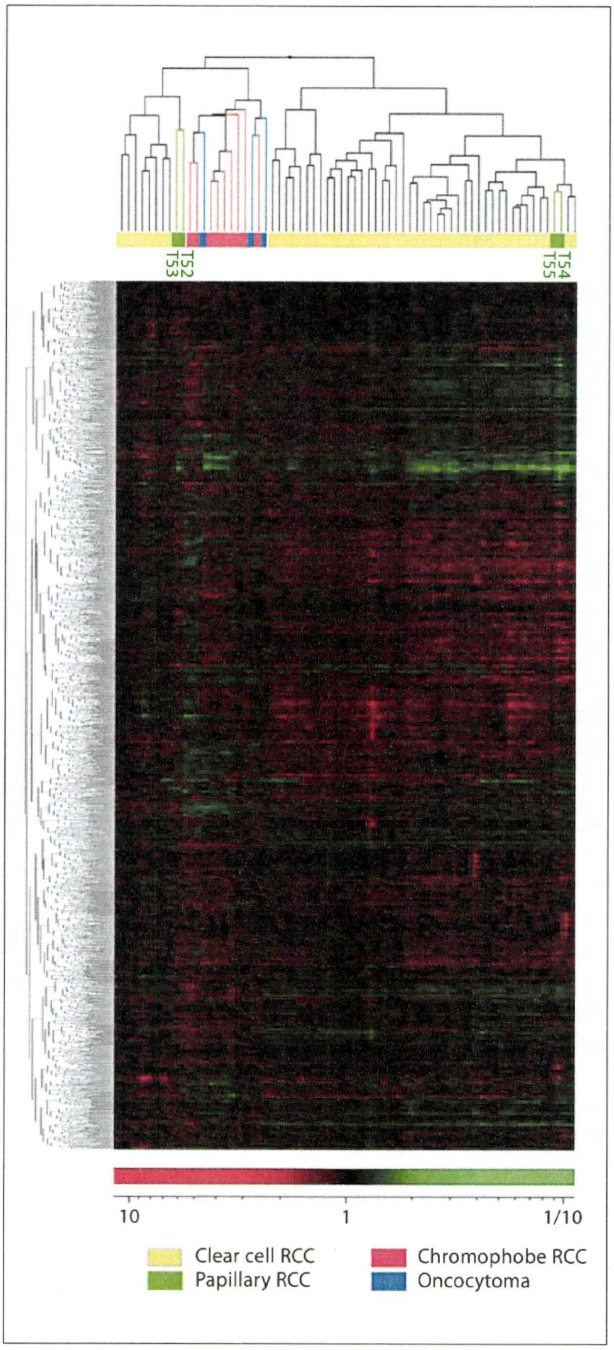


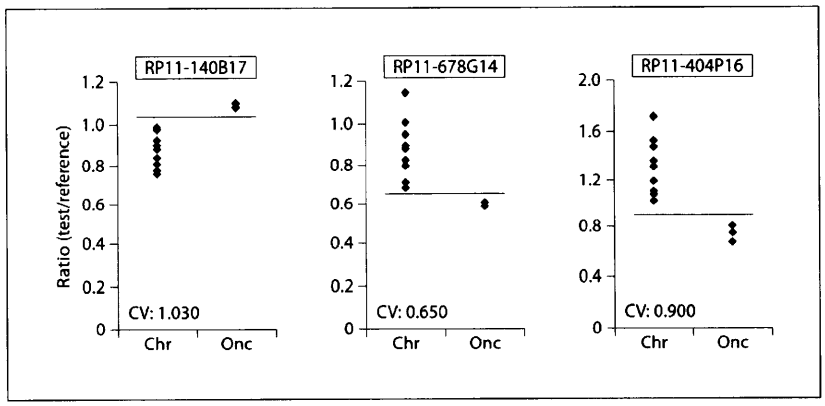
Fig. 2. Two-dimensional unsupervised hierarchical clustering analysis based on BAMCA data (signal ratios) for tumorous tissue obtained from patients with type 1 (T52 and T53) and type 2 (T54 and T55) papillary RCCs, chromophobe RCCs (T56–T65), oncocytomas (T66–T68) and clear cell RCCs (T1–T51). The signal ratio is shown as color range maps. The cluster trees for patients and BAC clones are shown at the top and left of the panel, respectively. Two type 1 papillary RCCs (T52 and T53) and 2 type 2 papillary RCCs (T54 and T55) were each clustered into the same subclasses. Type 1 and type 2 papillary RCCs were clustered into subclasses different from each other, and each was accompanied by clear cell RCCs. All 10 chromophobe RCCs and 3 oncocytomas were clustered into the same subclass, which did not include any tumor of other histological subtypes.

tomas (n = 3). Figure 3 shows scattergrams of the signal ratios in chromophobe RCCs and oncocytomas for representative examples of the 21 BAC clones. In all 21 BAC clones, using the cutoff values of the signal ratios described in figure 3 and table 2, chromophobe RCCs in this cohort were discriminated from oncocytomas with a sensitivity and specificity of 100%.

Genome-Wide DNA Methylation Profiles of Non-Tumorous Renal Tissue Obtained from Patients with Renal Tumors

In our previous study, many BAC clones showed DNA hypo- or hypermethylation even in non-tumorous renal tissue obtained from patients with clear cell RCCs (fig. 1u) when compared with normal renal tissue obtained from patients without any renal tumor (fig. 1v) [9], although non-tumorous renal tissue obtained from patients with clear cell RCCs (fig. 1o) showed no histological changes in comparison with normal renal tissue (fig. 1p). Non-tumorous renal tissue obtained from patients with papillary RCCs (fig. 1k, l), chromophobe RCCs (fig. 1m) and oncocytomas (fig. 1n) did not show any histological changes when compared with both non-tumorous renal tissue obtained from patients with clear cell RCCs (fig. 1o) and normal renal tissue (fig. 1p). Furthermore, there were no histological differences among non-tumorous renal tissue obtained from patients with papillary RCCs (fig. 1k, l), chromophobe RCCs (fig. 1m) and oncocytomas (fig. 1n). However, the average numbers of BAC clones showing DNA hypo- or hypermethylation in non-tumorous renal tissue obtained from patients with chromophobe RCCs and oncocytomas were significantly smaller than the average number in non-tumorous renal tissue obtained from patients with clear cell RCCs (table 1).

A histogram showing the numbers of BAC clones with DNA hypo- and hypermethylation in non-tumorous renal tissue from all 68 patients with renal tumors is shown in figure 4a. Biphasic accumulation of DNA methylation alterations was evident, with a trough of 250 BAC clones in non-tumorous renal tissue. Thus, the 68 patients were divided into 2 groups according to the number of BAC



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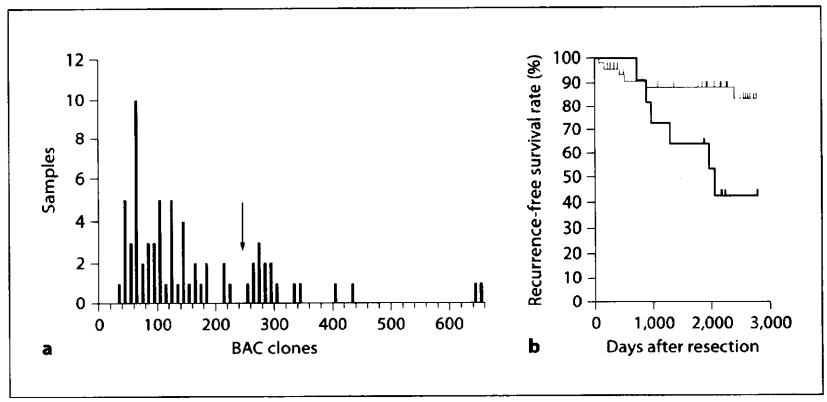

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Fig. 3. Scattergrams of the signal ratios in chromophobe RCCs (Chr, T56–T65) and 3 oncocytomas (Onc, T66–T68) for representative BAC clones, RP11-140B17, RP11-678G14 and RP11-404P16. Using the cut-off values (CV) described in each panel, chromophobe RCCs were discriminated from oncocytomas with a sensitivity and specificity of 100%.

Fig. 4. a Histogram showing the number of BAC clones with DNA hypo- or hypermethylation in non-tumorous renal tissue obtained from patients with papillary RCCs, chromophobe RCCs, oncocytomas and clear cell RCCs. Biphasic accumulation of DNA methylation alterations with a trough of 250 BAC clones (arrow) was observed in non-tumorous renal tissue. **b** Kaplan-Meier survival curves of patients with papillary RCCs, chromophobe RCCs, oncocytomas and clear cell RCCs. The period covered ranged from 63 to 2,801 days (mean 1,612). The recurrence-free survival rate of patients showing DNA hypo- or hypermethylation on ≥250 BAC clones in their non-tumorous renal tissue (n = 16, black line) was significantly lower than that of patients showing DNA hypo- or hypermethylation on <250 BAC clones in their non-tumorous renal tissue (n = 50, gray line; p = 0.0204).

clones showing DNA hypo- or hypermethylation in their non-tumorous renal tissue (≥250 BAC clones vs. <250 BAC clones). Figure 4b shows the Kaplan-Meier survival curves of 66 patients who underwent curative resection of their renal tumors. The period covered ranged from 63 to 2,801 days (mean 1,612). The recurrence-free survival rate of patients showing DNA hypo- or hypermethylation on ≥250 BAC clones in their non-tumorous renal tissue was significantly lower than that of patients showing DNA hypo- or hypermethylation on <250 BAC clones (p = 0.0204; fig. 4b).

Discussion

In tumors of many organs, an association between specific DNA methylation profiles and various histological subtypes has been reported [21, 22]. Such an association may reflect an epigenetic pathway of tumorigenesis, which is specific to each histological subtype. Although various histological subtypes of tumors occur in the kidney, to our knowledge, there has been no reported genome-wide DNA methylation analysis of such histological subtypes other than clear cell RCC.

Many researchers in the field of cancer epigenetics have used promoter arrays to identify the genes that are methylated in cancer cells [23–25]. 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 that do not directly participate 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 [26]. Genomic regions in which DNA hypomethylation affects chromosomal instability may not be contained in promoter arrays. Moreover, aberrant DNA methylation of large regions of chromosomes, which are regulated in a coordinated manner in human cancers due to a process of long-range epigenetic silencing, has recently attracted attention [27]. Therefore, we used a custom-made BAC

Table 2. Twenty-one BAC clones which were able to discriminate chromophobe RCCs (Chr) from oncocytomas (Onc)

BAC clone ID	Location	Cutoff value	DNA methylation status[1]
RP11-201O14	1p34.3–1p36.13	0.950	Chr<Onc
RP11-89O18	1p33–1p34.2	1.070	Chr<Onc
RP11-542D13	2q11.1–2q11.2	0.850	Chr<Onc
RP11-124O2	3p21.2	0.610	Chr>Onc
RP11-745L2	3q13.13d	1.135	Chr<Onc
RP11-89F1	5q32	0.950	Chr<Onc
RP11-79J23	6p21.2–6p21.3	1.070	Chr<Onc
RP11-75C8	6q21–6q22.1	0.920	Chr<Onc
RP11-10D8	7q22.1	0.780	Chr>Onc
RP11-140B17	10q25.3–10q26.13	1.030	Chr<Onc
RP11-196E1	11q23	0.910	Chr>Onc
RP11-170D9	14q11.2–14q12	0.920	Chr>Onc
RP11-91J13	14q23	1.000	Chr>Onc
RP11-397B22	16p13.3a	0.960	Chr>Onc
RP11-122P17	16q24	0.850	Chr>Onc
RP11-798B19	19p	1.050	Chr>Onc
RP11-678G14	19p12b–19p12c	0.650	Chr>Onc
RP11-46I12	19q12–19q13.1	0.650	Chr>Onc
RP11-446K10	19q13.1–19q13	0.950	Chr>Onc
RP11-10D18	20q13.1–20q13.2	0.720	Chr>Onc
RP11-404P16	Xp11.2–Xp11.3	0.900	Chr>Onc

[1] Chr<Onc = when the signal ratio was lower than the cutoff value, the tissue sample was considered to have originated from chromophobe RCC; Chr>Onc = when the signal ratio was higher than the cutoff value, the tissue sample was considered to have originated from chromophobe RCC.

array [17] 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.

Since microscopic observation frequently revealed a papillary RCC component associated with the concomitant clear cell RCC component in a single renal tumor, papillary RCCs and subclasses of clear cell RCCs may have been grouped into the same subclass in the present unsupervised hierarchical clustering (fig. 2). First, based simply on cytologic and histologic criteria, papillary RCCs were divided into 2 morphologic groups, type 1 and type 2. Type 1 papillary RCCs consist of papillae covered with a single or double layer of small cuboid cells with scanty cytoplasm, and type 2 papillary RCCs consist of papillae covered by large eosinophilic cells arranged in an irregular or pseudo-stratified manner [6]. Although type 2 papillary RCC is frequently at an advanced stage at initial diagnosis, thus resulting in poor patient survival [28], only a small number of molecular differences between type 1 and type 2 papillary RCCs, such as the level of expression of vascular endothelial growth factor receptor and copy number alterations on chromosomes 1p, 3p, 9p and 17, have been reported to date [29]. The present results (fig. 2) indicate that genome-wide DNA methylation profiles may explain the differences in background characteristics between type 1 and type 2 papillary RCCs, although further confirmation in a larger cohort will be needed.

In the present unsupervised hierarchical clustering based on BAMCA data, chromophobe RCCs and oncocytomas formed a subclass by themselves (fig. 2). Histopathological observations have underlined the similarities between chromophobe RCCs and oncocytomas. Since both of these neoplasms consist of tumor cells with abundant eosinophilic cytoplasm and mainly show a solid structure [6], differential diagnosis between them frequently becomes difficult even for experienced pathologists. Both chromophobe RCCs and oncocytomas have been described in patients with Birt-Hogg-Dubé syndrome, which is characterized by cutaneous fibrofolliculomas, renal tumors, pulmonary cysts and spontaneous pneumothorax. Moreover, such patients sometimes develop so-called hybrid oncocytic tumors with histological features similar to both chromophobe RCCs and oncocytomas [30]. On the other hand, the genetic status of chromophobe RCCs and oncocytomas differs markedly: copy number alterations on various chromosomes are frequent in chromophobe RCCs, but are rare in oncocytomas [31]. The present results indicate that similarities of genome-wide DNA methylation profiles may epigenetically cover the genetic differences between chromophobe RCCs and oncocytomas and may be able to explain the phenotypic similarities of these tumors. On the other hand, regional DNA methylation alterations on the 21 BAC clones were able to discriminate chromophobe RCCs and oncocytomas (fig. 3; table 2). Even though the overall epigenetic pathway of tumorigenesis may be shared by chromophobe RCCs and oncocytomas, there may be target chromosomal regions of DNA methylation alterations that are specific to each neoplasm. In addition to copy number status, the DNA methylation status in such chromosomal regions may become a hallmark for differential diagnosis of these morphologically similar tumors.

Surprisingly, the DNA methylation status (the number of BAC clones showing DNA hypo- or hypermethylation) of non-tumorous renal tissue obtained from chro-

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 $\geq$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

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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.

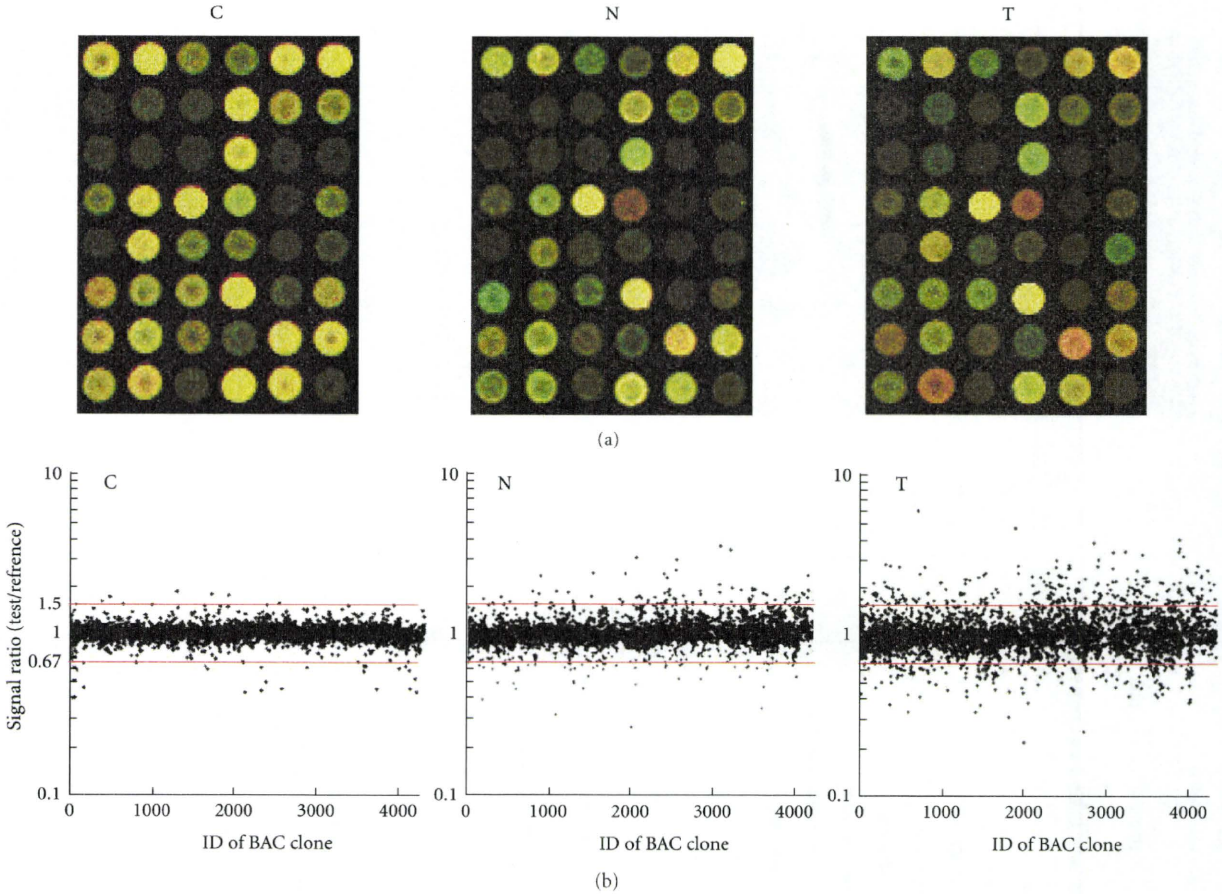

FIGURE 1: Genome-wide DNA methylation analysis by BAMCA. (a) Representative examples of scanned array images in a sample of normal pancreatic tissue obtained from a patient without ductal adenocarcinoma of the pancreas (C) and samples of both noncancerous pancreatic tissue (N) and cancerous tissue (T)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

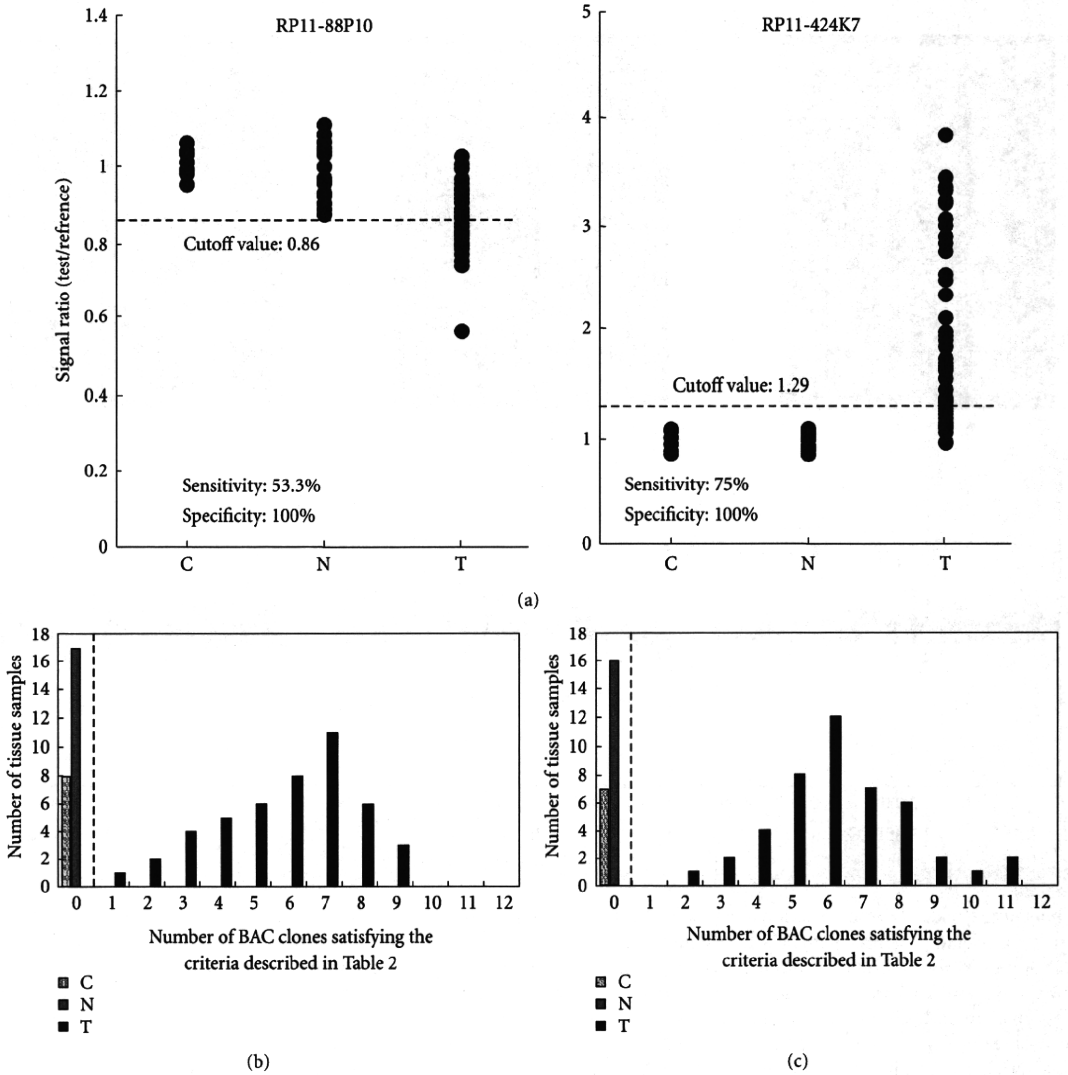

FIGURE 2: Establishment of criteria for diagnosis of ductal adenocarcinomas of the pancreas. (a) Scattergrams of the signal ratios in samples of normal pancreatic tissue obtained from patients without ductal adenocarcinomas (C), noncancerous pancreatic tissue obtained from patients with ductal adenocarcinomas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

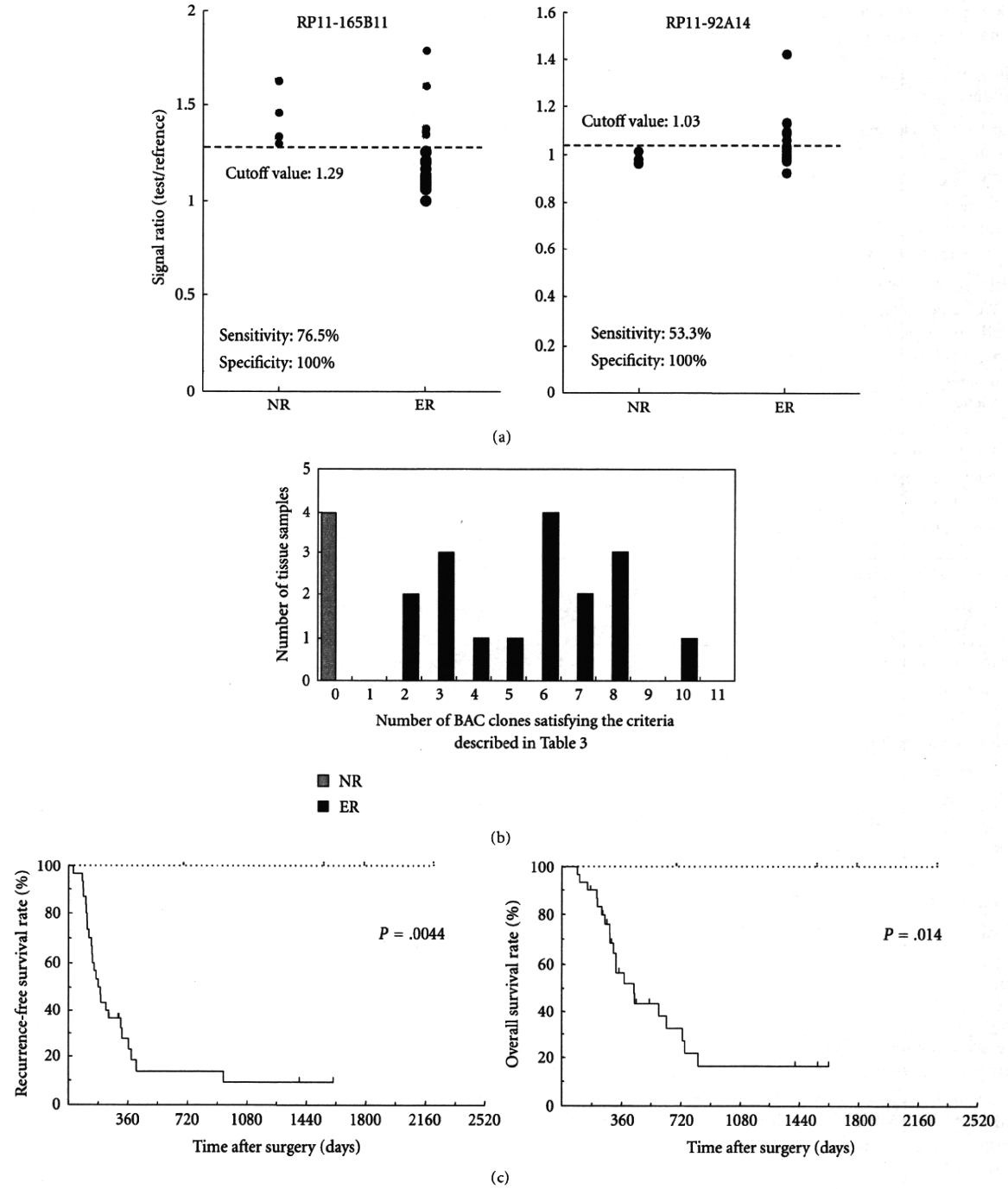

FIGURE 3: Establishment of criteria for prognostication of patients with ductal adenocarcinomas of the pancreas. (a) Scattergrams of the signal ratios in samples of cancerous tissue obtained from patients in the no-relapse group (NR, $n = 4$) and early-relapse group (ER, $n = 17$) who had not undergone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