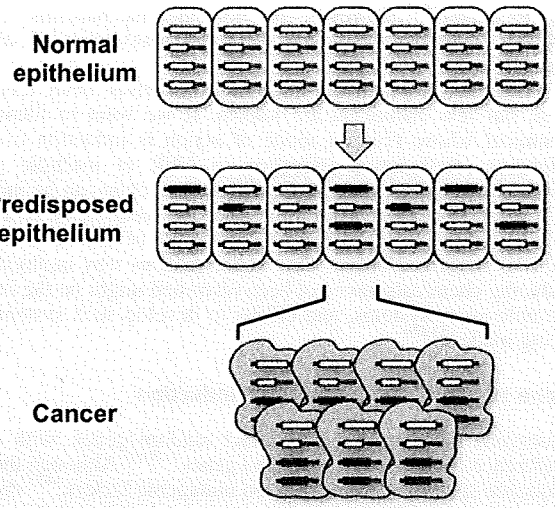

Fig. 1. Epigenetic field for cancerization and clonal selection in cancer. Normal epithelium consists of cells with little aberrant methylation. By exposure to inducers of methylation, specific genes are methylated in minor fractions of cells. A cancer develops from one of the cells that has already accumulated silencing of driver genes. From the viewpoint of assessment of an effect of an inducer, analysis of non-cancerous tissues provides overall information on the genes methylated, and that of a cancer provides information on the genes stochastically methylated in the very precursor cell and driver genes.

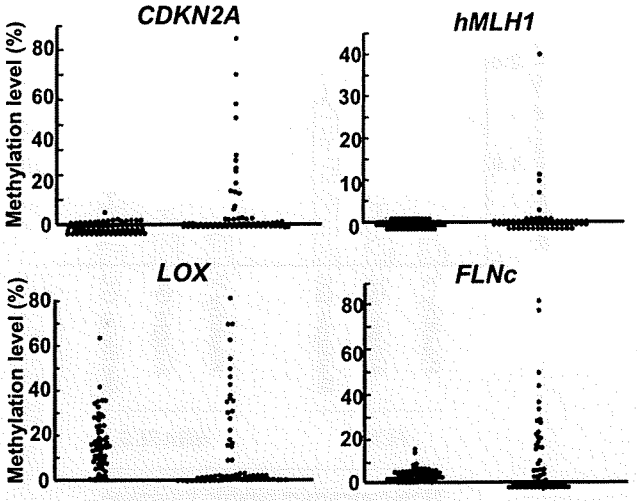

Fig. 2. Distribution patterns of methylation in non-cancerous and cancer tissues. Methylation levels, which reflect fractions of cells with the methylation, were quantified in 66 paired samples of non-cancerous and cancer tissues of gastric cancer patients (modified from Enomoto *et al.*[39]). They showed a unimodal distribution in non-cancerous tissues, and a "bimodal" distribution, namely zero or positive, in cancer tissues. This finding supports the idea that methylation in a non-cancerous tissue reflects events in many cells in the tissue whereas that in a cancer tissue mostly reflects only events in its single precursor cell.

can be affected by the overall exposure level to its inducers and by the susceptibility of individual genes to undergo an alteration. In actual analysis, the proportion of target cells, such as content of epithelial cells in a sample with epithelial and stromal cells, also affects the fraction of cells with an alteration.

In contrast, in cancer tissues, an alteration responsible for clonal growth (driver) is present in all the cancer cells. Even if an alteration is not a driver, if the alteration has taken place before the clonal growth started, it is present in all the cancer cells. In actual analysis, cancer samples contain a large contamination of non-cancer cells, and the fraction of cells with the alteration is mainly determined by the fraction of cancer cells in a sample. If an alteration is induced after initiation of clonal growth, it can be present in a fraction of cancer cells, and its overall fraction is determined by the fraction within cancer cells and by the fraction of cancer cells within a sample.

These theoretical considerations were substantiated by actual measurement of cells with methylation of specific genes in non-cancerous and cancer tissues of gastric cancer patients (Fig. 2) and esophageal cancer patients.[39,40] The methylation level, which reflects the fraction of DNA molecules with methylation and thus the fraction of cells with the methylation, shows a unimodal distribution in non-cancerous tissues, especially for the weak tumor-suppressor gene *LOX* and the marker gene *FLNc*.[41] It shows a "bimodal" distribution, namely zero or positive, in cancer tissues, especially for the tumor-suppressor genes *CDKN2A* and *MLH1*.

Rare presence of mutations in non-cancerous tissues. Adjacent non-cancerous tissues are often used as a control for cancer tissues, and are regarded not to have detectable levels of mutations. To detect accurately such low levels of mutations in non-cancerous tissues, transgenic animals in which rare mutations can be quantified by selectable mutations of a marker gene have been developed.[42,43] Using these transgenic animals and various carcinogenic factors, mutation frequencies of a specific marker gene in non-cancerous tissues have been shown to be $\sim 10^{-5}$/cell, and to be 10^{-3}/cell, even in a tissue heavily exposed to a mutagenic compound.[44] This very low frequency of mutations in non-cancerous tissues gives a rationale for the routine use of such tissues as a control.

DNA methylation in non-cancerous tissues and aging. Once the situation goes to DNA methylation, many investigators noticed that trace amounts of DNA with methylation are present in non-cancerous tissues of cancer patients. However, it is usually difficult to distinguish whether such methylation is a simple drift or fluctuation without any biological or pathological meaning or something associated with cancer development. A pioneering work by Issa *et al.* analyzed the correlation between age and levels of methylation, and convincingly showed that aging is one factor that induces DNA methylation.[45]

Association between methylation accumulation and cancer risk: Epigenetic field for cancerization. We systematically collected gastric tissue samples from healthy individuals and gastric cancer patients (non-cancerous part) in an age-matched manner.[46] Methylation levels of eight CGI in various positions against TSS were accurately quantified. Methylation levels in non-cancerous gastric tissues of gastric cancer patients were in the range 0.2–8.2%, and were much higher than those in gastric mucosae of healthy individuals. This showed that very high levels of methylation can be present in non-cancerous tissues, different from mutations. The finding also suggested that accumulation of methylation is related to gastric cancer risk. Subsequently, gastric mucosae of patients with multiple gastric cancers were shown to have higher methylation levels than those of patients with a single gastric cancer (Fig. 3).[47] These discoveries clearly demonstrated that methylation levels in gastric mucosae correlate with gastric cancer risk.

A higher incidence or level of methylation in non-cancerous tissues of cancer patients than that in the corresponding tissues of healthy individuals was also observed for liver,[48] colon,[49] esophageal,[50] and renal[51] cancers. In these types of cancers, accumulation of methylation is likely to be involved in the formation of a field for cancerization (Fig. 1).[52] The gene inactivated by methylation of its promoter CGI in non-cancerous

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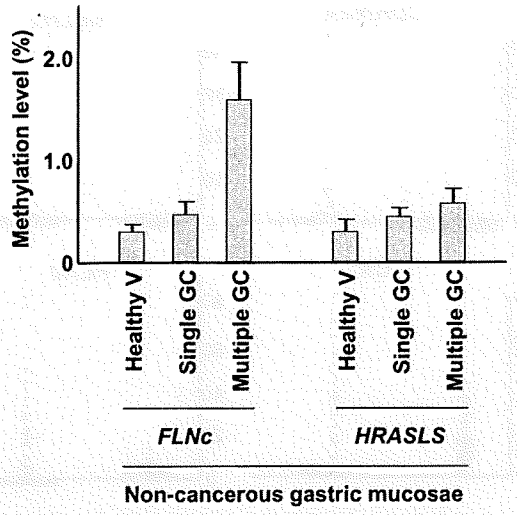

Fig. 3. Correlation between methylation level and cancer risk. Methylation levels of two marker genes (*FLNc* and *HRASLS*) were quantified in gastric mucosae of healthy individuals (healthy V), non-cancerous gastric mucosae of patients with a single gastric cancer (single GC), and non-cancerous gastric mucosae of patients with multiple gastric cancers (multiple GC) (modified from Nakajima *et al.*[47]). This showed that accumulation levels of specific genes in non-cancerous gastric mucosae can correlate with gastric cancer risk. Taken together with the findings in other types of cancers, quantification of methylation levels in normal-appearing tissues is a promising cancer risk marker that reflects one's own life history.

tissues might be a weak tumor-suppressor gene that does not induce cellular transformation by itself, such as *SFRP1*,[53] or might be a passenger that is methylated in parallel with tumor-suppressor genes.

Inducers of methylation in contrast with those of mutations

Epidemiology indicates that cancer is mainly caused by environmental factors,[54] and identification of inducers of aberrant DNA methylation, in addition to those of mutations, is critically important. However, only limited information is available for the inducers of aberrant methylation.[55]

Inducers of mutations. Clarification of inducers of mutations, namely mutagens, constitutes a large field of science, and comprehensive description is beyond the scope of this article. Simplistically, mutations are induced by exogenous mutagenic factors, such as chemicals and radiation, and endogenous factors, such as oxygen radicals.[56] Mutagenic chemicals are contained in diverse sources, including tobacco smoke, overcooked food, and many synthetic chemicals.

Inducers of DNA methylation. To identify inducers of aberrant methylation in humans, analysis of non-cancerous tissues is important because the methylation level in non-cancerous tissues reflects how potently the methylation was induced by a factor (Fig. 1). Aging was the first factor that was identified to promote accumulation of DNA methylation,[45] and quantification of methylation in non-cancerous colonic tissues contributed to the identification.

Afterwards, the presence of methylation in colonic mucosae of patients with ulcerative colitis indicated that chronic inflammation is an important inducer of methylation.[57,58] The importance of chronic inflammation was further supported by the presence of methylation in non-cancerous liver tissue of patients with hepatitis,[48] in inflammatory reflux esophagitis,[59] and in non-cancerous gastric tissue of individuals infected by *Helicobacter pylori*.[46] However, the molecular mechanisms of how chronic inflammation induces aberrant methylation are almost unknown.

There can be chemicals that induce aberrant DNA methylation, but few chemicals are known. If we want to identify a chemical whose primary mode of action is induction of gene silencing, methylation induction in NFR of multiple genes should be demonstrated. Methylation of an exon can be induced as a result of gene expression change, and methylation of a NFR of a specific gene can be induced as a result of loss of its expression, as described below. One of the reasons why methylation-inducing chemicals have not been identified might be the lack of suitable assay systems, and efforts to develop such systems are being made.[55,60]

Gene specificity in methylation induction

Mutations are considered to affect random genes, with some preference for actively transcribed genes.[18,61] Although there is sequence specificity depending on mutagenic factors,[62] there is little gene specificity. Many investigators thought that DNA methylation would have a similar nature in random target genes, but it has now been shown that there is strong target gene specificity in methylation induction.

Presence of target gene specificity in methylation induction. It was initially found that specific CGI are methylated in specific tumor types, and the presence of gene specificity for methylation induction was indicated.[27,37] However, analysis of a cancer tissue reveals only events in its single precursor cell, and the information obtained is very stochastic. Analysis of a panel of cancers can reflect events in the precursor cells of the cancers, but the number of precursor cells analyzed is still limited to the number of cancers analyzed.

In order to avoid selection bias by gene function, and to analyze as many cells as possible, analysis of a non-cancerous tissue is advantageous. We analyzed methylation of a panel of genes in gastric mucosae with and without *H. pylori* infection, and showed that specific genes are methylated in gastric mucosae with *H. pylori* infection.[63] We also analyzed the methylation levels of a panel of genes in esophageal mucosae, and found that specific genes are methylated in correlation with smoking history.[40] These showed that specific inducers of aberrant DNA methylation induce methylation of specific genes. The presence of a ''methylation fingerprint'' of individual methylation inducers suggests that the fingerprint can be used as a marker for past exposure to specific carcinogenic factors in our lives.

Molecular mechanisms of target gene specificity. As a molecular mechanism for gene specificity, low transcription was suggested in pioneering studies that used an exogenously introduced gene and endogenous genes demethylated by a demethylating agent.[64,65] Analysis of selected genes in embryonic stem cells, along with normal adult tissue, and cancer cells revealed that genes marked with trimethylation of histone H3 lysine 27 (H3K27me3) in embryonic stem cells are likely to become methylated in cancers.[66–68] The finding was further supported by a genome-wide analysis of genes with H3K27me3 in cancer cells and corresponding normal cells.[19]

In addition to these factors that confer susceptibility to DNA methylation, the presence of RNA polymerase II (pol II), active or stalled, in NFR was shown to confer resistance to DNA methylation.[34] Although the presence of active histone modifications also confers resistance, the effect of active histone modifications was overridden by the presence of pol II in multivariate analysis, suggesting that the presence of pol II is the final effector that protects NFR from DNA methylation. Taken all together, DNA methylation of NFR is protected by the presence of pol II regardless of transcription levels, and promoted by the

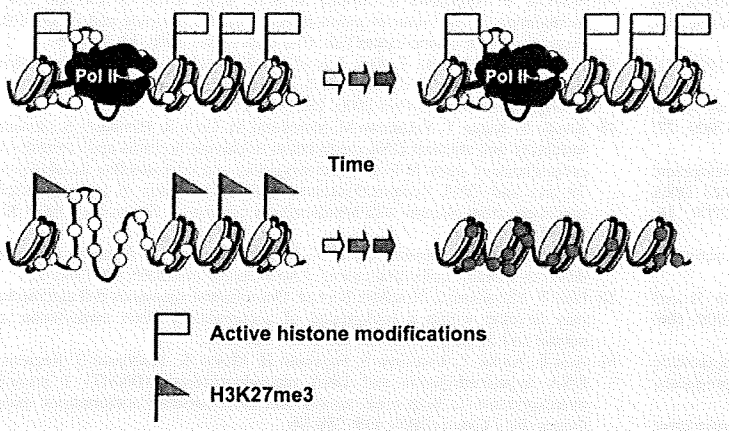

Fig. 4. Determinants of methylation destiny. Genes with RNA polymerase II (pol II), active or stalled, are resistant to DNA methylation, and genes with H3K27me3 are susceptible to DNA methylation. The presence of pol II is associated with the presence of active histone modifications, even if a gene is not actively transcribed. Open and closed circles show unmethylated and methylated CpG sites, respectively.

presence of H3K27me3 (Fig. 4). Once DNA methylation is induced in susceptible NFR, the H3K27me3 mark almost disappears[19] or decreases to a very low level.[69]

Reversibility of alterations

One of the major differences, or most important difference, between mutations and DNA methylation is reversibility. Physiologically, epigenetic modifications undergo dynamic changes during development, differentiation, and reprogramming.[70,71] In somatic cells the demethylating agents 5-azacytidine and 5-aza-2′-deoxycytidine have long been used in the laboratory.[72] Now these agents have come into clinics and are showing very promising effects in hematological malignancies.[73] The detailed pharmacological mechanisms and usage are summarized in the reviews cited above.

Future perspectives

Now, unique characteristics of DNA methylation are clear, but many questions still remain. Are there any chemicals that induce aberrant methylation of NFR directly, not as a result of gene expression changes? How does chronic inflammation induce aberrant DNA methylation? Do we know enough about the determinants of gene specificity?

At the same time, the biomedical application of DNA methylation is becoming more promising. The large number of genes methylated in a cancer increases the chance of successful identification of methylation biomarkers to predict patient prognosis and response to therapeutics. Cancer-specific methylation can be used for detection of cancer cells. The presence of an epigenetic field for cancerization in normal-appearing tissues can be used as a cancer risk marker, which reflects one's own life history. The deep involvement of chronic inflammation in methylation induction indicates that suppression of components involved in the induction can be utilized as a target of cancer prevention. The methylation fingerprint can be used in epigenetic epidemiology.

Mutations have not been considered as a cause of disorders that involve irreversible alteration of cellular functions, such as neurodegenerative disorders, diabetes, immunological disorders, and renal disorders. This was because mutations are rare events and cannot affect as many cells as the function of a tissue is affected as a whole. However, methylation can be induced in many more cells in a tissue, and genes affected are specific. This suggests that a critical gene can be inactivated in a significant fraction of cells, and raises the possibility that aberrant DNA methylation is causally involved in chronic disorders other than cancers.

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Genome-wide DNA methylation profiles in urothelial carcinomas and urothelia at the precancerous stage

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To clarify genome-wide DNA methylation profiles during multistage urothelial carcinogenesis, bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) was performed in 18 normal urothelia obtained from patients without urothelial carcinomas (UCs) (C), 17 noncancerous urothelia obtained from patients with UCs (N), and 40 UCs. DNA hypo- and hypermethylation on multiple BAC clones was observed even in N compared to C. Principal component analysis revealed progressive DNA methylation alterations from C to N, and to UCs. DNA methylation profiles in N obtained from patients with invasive UCs were inherited by the invasive UCs themselves, that is DNA methylation alterations in N were correlated with the development of more malignant UCs. The combination of DNA methylation status on 83 BAC clones selected by Wilcoxon test was able to completely discriminate N from C, and diagnose N as having a high risk of carcinogenesis, with 100% sensitivity and specificity. The combination of DNA methylation status on 20 BAC clones selected by Wilcoxon test was able to completely discriminate patients who suffered from recurrence after surgery from patients who did not. The combination of DNA methylation status for 11 BAC clones selected by Wilcoxon test was able to completely discriminate patients with UCs of the renal pelvis or ureter who suffered from intravesical metachronous UC development from patients who did not. Genome-wide alterations of DNA methylation may participate in urothelial carcinogenesis from the precancerous stage to UC, and DNA methylation profiling may provide optimal indicators for carcinogenetic risk estimation and prognostication. (*Cancer Sci* 2010; 101: 231–240)

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] Accumulating evidence suggests that alterations of DNA methylation are involved even in the early and the precancerous stages.[6,7] On the other hand, in patients with cancers, aberrant DNA methylation is significantly associated with poorer tumor differentiation, tumor aggressiveness, and poorer patient outcome.[6,7] Therefore, alterations of DNA methylation may play a significant role in multistage carcinogenesis.

With respect to urothelial carcinogenesis, we have reported accumulation of DNA methylation on C-type CpG islands in a cancer-specific but not age-dependent manner, and protein overexpression of DNA methyltransferase (DNMT) 1, a major DNMT, even in noncancerous urothelia with no apparent histological changes obtained from patients with urothelial carcinomas (UCs).[8,9] Moreover, accumulation of DNA methylation on C-type CpG islands associated with DNMT1 protein overexpression was more frequently evident in aggressive nodular invasive UCs[8–10] resulting in poorer patient outcome than in superficial papillary UCs, which usually remain noninvasive even after repeated urethrocystoscopic resection.[11,12] Since aberrant DNA methylation is one of the earliest molecular events during urothelial carcinogenesis and also participates in tumor aggressiveness, it may be possible to estimate the future risk of developing more malignant UCs. However, only a few previous studies focusing on UCs[13] have employed recently developed array-based technology for assessing genome-wide DNA methylation status,[14–16] and such studies have focused on identification of tumor-related genes that are silenced by DNA methylation.[13] DNA methylation profiles, which could become the optimum indicators for carcinogenetic risk estimation and prognostication of UCs, should therefore be explored using array-based approaches.

In this study, in order to clarify genome-wide DNA methylation profiles during multistage urothelial carcinogenesis, we performed bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA)[17–19] using a microarray of 4361 BAC clones[20] in normal urothelia obtained from patients without UCs, noncancerous urothelia obtained from patients with UCs, and UCs themselves.

Materials and Methods

Patients and tissue samples. Seventeen samples of noncancerous urothelia (N1–N17) and 40 samples of UCs (T1–T40) of the urinary bladder, ureter, and renal pelvis were obtained from specimens that had been surgically resected by radical cystectomy (12 patients) or nephroureterectomy (28 patients) at the National Cancer Center Hospital, Tokyo, Japan. The patients comprised 31 men and nine women whose mean age was 69.03 ± 9.77 (mean ± SD) years (range, 49–85 years). Microscopic examination revealed no remarkable histological changes in the noncancerous urothelia. The patients from whom noncancerous urothelia were obtained comprised 11 men and six women with a mean age of 70.41 ± 9.33 (mean ± SD) years (range, 49–85 years). There were 17 superficial UCs (two pTa and 15 pT1 tumors) and 23 invasive UCs (six pT2, 16 pT3, and one pT4 tumor) according to the criteria proposed by World Health Organization classification.[21] For comparison, 18 samples of normal urothelia obtained from patients without UCs (C1–C18) were used. Fourteen, three, and one patient underwent nephrectomy for renal cell carcinoma, nephrectomy for retroperitoneal sarcoma around the kidney, and partial cystectomy for urachal carcinoma, respectively. The patients from whom normal urothelia were obtained comprised 13 men and five women with a mean age of 61.17 ± 15.16 (mean ± SD) years (range, 27–82 years). This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan and has

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been performed in accordance with the Declaration of Helsinki in 1995. All patients gave their informed consent prior to their inclusion in this study.

BAMCA. High-molecular-weight DNA from fresh frozen tissue samples was extracted using phenol-chloroform, followed by dialysis. Because DNA methylation status is known to be organ-specific,[22] the reference DNA for analysis of the developmental stages of UCs should be obtained from the urothelium, and not from other organs or peripheral blood. Therefore, a mixture of normal urothelial DNA obtained from 11 male patients (C19–C29) and six female patients (C30–C35) without UCs was used as a reference for analyses of male and female test DNA samples, respectively. DNA methylation status was analyzed by BAMCA using a custom-made array (MCG Whole Genome Array-4500) harboring 4361 BAC clones located throughout chromosomes 1–22, X and Y,[20] as described previously.[17–19] 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 the 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, Buckinghamshire, UK), respectively, 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. Arrays were scanned with a GenePix Personal 4100A (Axon Instruments, Foster City, CA, 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 of all BAC clones 1.0.

Statistics. Differences in the average number of BAC clones that showed DNA methylation alterations (DNA hypo- and hypermethylation) between groups of samples were analyzed using the Mann–Whitney *U*-test. Differences at $P < 0.05$ were considered significant. Principal component analysis based on BAMCA data was performed using the Expressionist software program (Gene Data, Basel, Switzerland). Unsupervised two-dimensional hierarchical clustering analysis of tissue samples and the BAC clones were performed using the Expressionist software program. Correlations between the subclassification of patients yielded by unsupervised hierarchical clustering analysis and clinicopathological parameters of UCs were analyzed using the χ^2-test. Differences at $P < 0.05$ were considered significant. BAC clones whose signal ratios yielded by BAMCA were significantly different between groups of samples were identified by Wilcoxon test ($P < 0.01$).

Results

Genome-wide DNA methylation alterations during multistage urothelial carcinogenesis. Figure 1(b,c) shows examples of scanned array images and scattergrams of the signal ratios (test signal/reference signal), respectively, for normal urothelium from a patient without UC (panel C), and both noncancerous urothelium (panel N) and cancerous tissue (panel T) from a patient with UC. In all normal urothelia (C1–C18), the signal ratios of 97% of the BAC clones were between 0.67 and 1.5 (red bars in Fig. 1c). Therefore, in noncancerous urothelia obtained from patients with UCs and UCs, DNA methylation status corresponding to a signal ratio of less than 0.67 and more than 1.5 was defined as DNA hypomethylation and DNA hypermethylation of each BAC clone compared to normal urothelia, respectively, as in our previous study.[23] In noncancerous urothelia obtained from patients with UCs, many BAC clones showed DNA hypo- or hypermethylation (panel N of Fig. 1c). In UCs themselves, 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 (panel T of Fig. 1c) in comparison with noncancerous urothelia obtained from patients with UCs. The average number of BAC clones showing DNA hypomethylation increased significantly from noncancerous urothelia obtained from patients with UCs (24.53 ± 31.48) to UCs (236.78 ± 92.78, $P = 4.37e\text{-}9$). The average number of BAC clones showing DNA hypermethylation increased significantly from noncancerous urothelia obtained from patients with UCs (29.18 ± 39.84) to UCs (289.13 ± 82.42, $P = 7.35e\text{-}9$). Principal component analysis based on BAMCA data (signal ratios) revealed progressive DNA methylation alterations from normal urothelia, to noncancerous urothelia obtained from patients with UCs, and to UCs (Fig. 1d).

Clinicopathological significance of DNA methylation alterations in noncancerous urothelia obtained from patients with UCs. In order to clarify the clinicopathological significance of DNA methylation alterations in noncancerous urothelia obtained from patients with UCs, unsupervised two-dimensional hierarchical clustering analysis based on BAMCA data (signal ratios) for noncancerous urothelia was performed. Seventeen patients with UCs were clustered into two subclasses, Clusters A_N and B_N, which contained nine and eight patients, respectively, based on the DNA methylation status of the noncancerous urothelia (Fig. 2a). All eight patients (100%) belonging to Cluster B_N suffered from invasive UCs (pT2 or more), whereas five (55.6%) of the patients belonging to Cluster A_N did so ($P = 0.0311$).

The Wilcoxon test ($P < 0.01$) revealed that the signal ratios of 131 BAC clones differed significantly between noncancerous urothelia obtained from patients with superficial UCs (pTa and pT1) and noncancerous urothelia obtained from patients with invasive UCs (pT2 or more). If the average signal ratios in noncancerous urothelia obtained from patients with invasive UCs were significantly higher than those in noncancerous urothelia obtained from patients with superficial UCs (67 BAC clones), the average signal ratios in the invasive UCs themselves were even higher than (42 BAC clones, e.g. RP11-79K14 and RP11-29C11 in Fig. 2b) or not significantly different from (25 BAC clones, e.g. RP11-3A9 and RP11-73G16 in Fig. 2b) those in noncancerous urothelia obtained from patients with invasive UCs, without exception. If the average signal ratios in noncancerous urothelia obtained from patients with invasive UCs were significantly lower than those in noncancerous urothelia obtained from patients with superficial UCs (64 BAC clones), the average signal ratios in the invasive UCs themselves were even lower than (38 BAC clones, e.g. RP11-210F15 and RP11-368O13 in Fig. 2b) or not significantly different from (26 BAC clones, e.g. RP11-442N24 and RP11-65C22 in Fig. 2b) those in noncancerous urothelia obtained from patients with invasive UCs, without exception, that is DNA methylation status of the 131 BAC clones in noncancerous urothelia obtained from patients with invasive UCs was inherited by the invasive UCs themselves.

DNA methylation profiles discriminating noncancerous urothelia obtained from patients with UCs from normal urothelia. Our finding that DNA methylation alterations in noncancerous urothelia were correlated with the development of UCs, as described above, prompted us to estimate the degree of carcinogenetic risk based on DNA methylation profiles in noncancerous urothelia. We attempted to establish criteria for indicating that noncancerous urothelia obtained from patients with UCs, and not normal urothelia, were at high risk of carcinogenesis.

The Wilcoxon test ($P < 0.01$) revealed that the signal ratios on 201 BAC clones differed significantly between normal urothelia obtained from patients without UCs and noncancerous urothelia obtained from patients with UCs. Figure 3(a) shows

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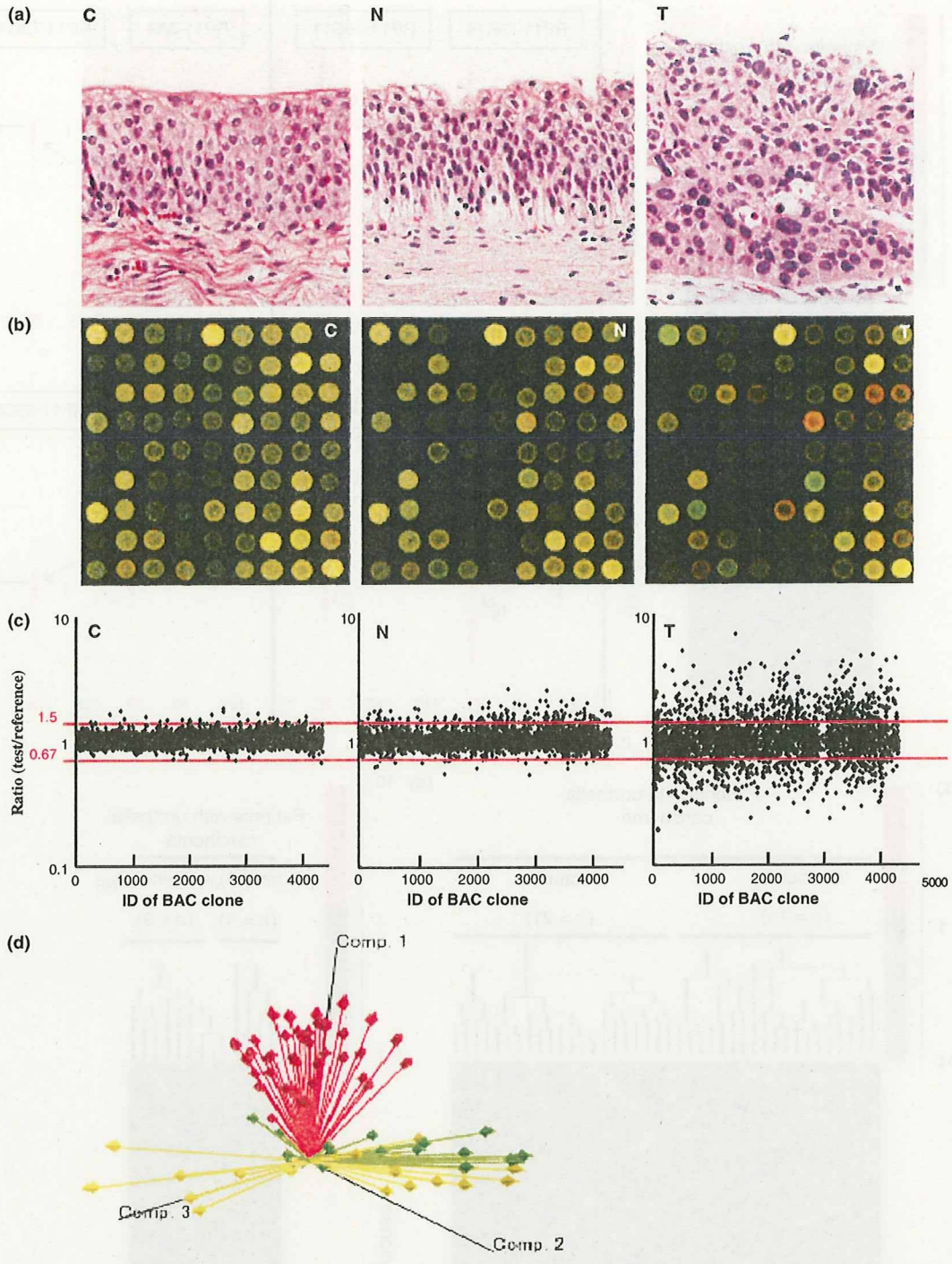

Fig. 1. DNA methylation alterations during multistage urothelial carcinogenesis. (a) Microscopic view of normal urothelium obtained from a patient without urothelial carcinoma (UC) (C), noncancerous urothelium obtained from a patient with UC (N), and UC (T). N shows no remarkable histological changes in comparison to C, that is no cytological or structural atypia is evident. Hematoxylin–eosin staining. Original magnification, ×20. (b) Scanned array images obtained by bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) in C, N, and T. Co-hybridization was done with test and reference DNA labeled with Cy3 and Cy5, respectively. (c) Scattergrams of the signal ratios (test signal/reference signal) obtained by BAMCA in C, N, and T. In all 18 normal urothelia (C1–C18), the signal ratios of 97% of the BAC clones were between 0.67 and 1.5 (red bars). Therefore, in N and T, DNA methylation status corresponding to a signal ratio of less than 0.67 and more than 1.5 was defined as DNA hypomethylation and DNA hypermethylation on each BAC clone compared to C, respectively. Even though N did not show any marked histological changes in comparison to C (panels C and N in [a]), many BAC clones showed DNA hypo- or hypermethylation. In T, more BAC clones showed DNA hypo- or hypermethylation, whose degree, that is deviation of the signal ratio from 0.67 or 1.5, was increased in comparison to N. (d) Principal component analysis based on BAMCA data (signal ratios). Progressive alterations of DNA methylation status from normal urothelia (yellow arrows) to noncancerous urothelia obtained from patients with UCs (green arrows), and to UCs (red arrows) were observed.

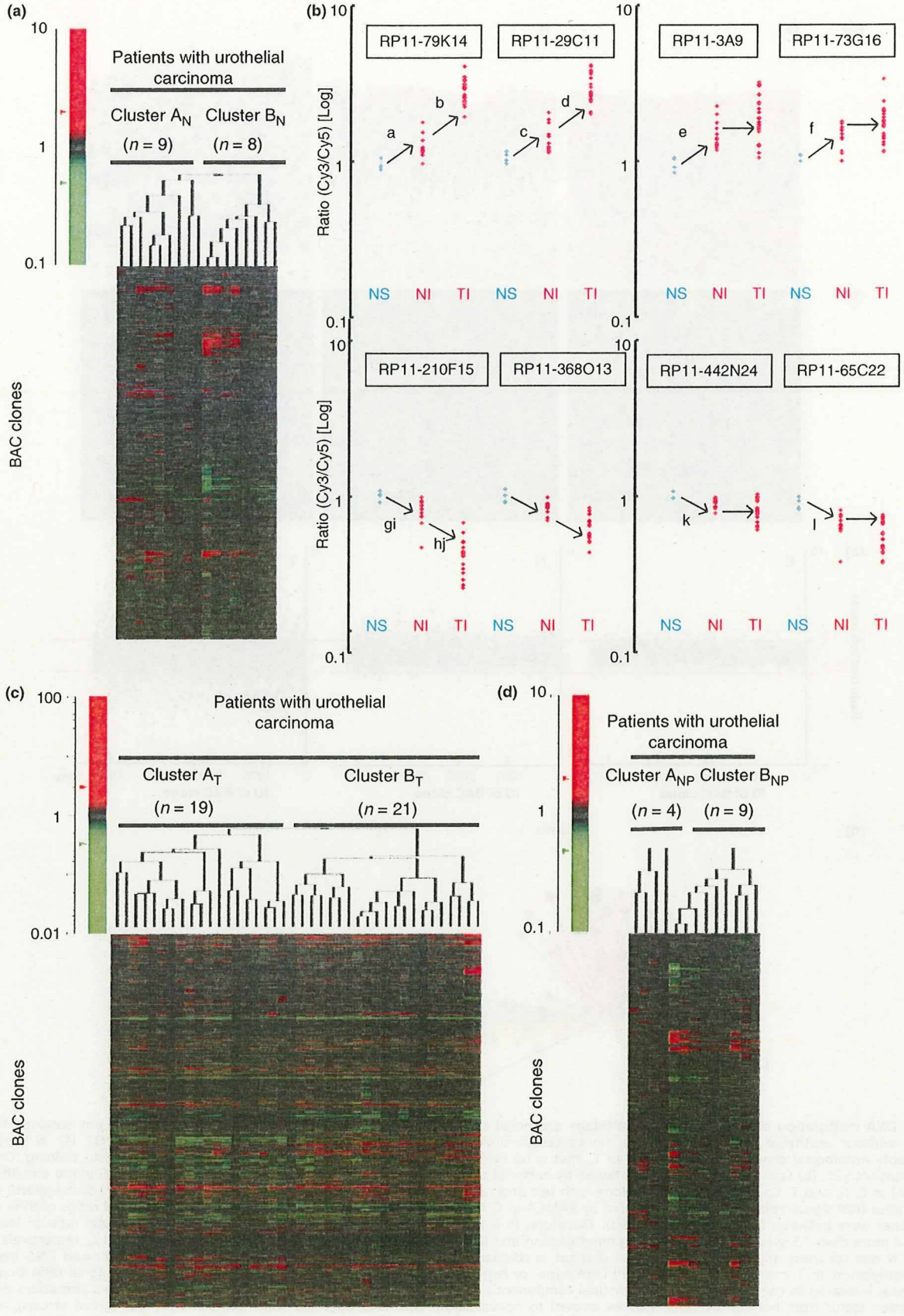

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Fig. 2. Correlations between DNA methylation status and clinicopathological parameters. (a) Unsupervised two-dimensional hierarchical clustering analysis based on bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) data (signal ratios) in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs). The signal ratio is shown in the color range map. Seventeen patients with UCs were hierarchically clustered into two subclasses, Clusters A_N (n = 9) and B_N (n = 8). Eight patients (100%) belonging to Cluster B_N developed invasive UCs (pT2 or more), whereas five patients (55.6%) belonging to Cluster A_N did so (P = 0.0311). (b) Scattergrams of the signal ratios in tissue samples. NS, noncancerous urothelia obtained from patients with superficial UCs. NI, noncancerous urothelia obtained from patients with invasive UCs. TI, invasive UCs. If the average signal ratios in NI were significantly higher than those in NS, the average signal ratios in TI themselves were even higher than (BAC clones RP11-79K14 and RP11-29C11), or not significantly different from (BAC clones RP11-3A9 and RP11-73G16), those in NI without exception. If the average signal ratios in NI were significantly lower than those in NS, the average signal ratios in TI themselves were even lower than (BAC clones RP11-210F15 and RP11-368O13), or not significantly different from (BAC clones RP11-442N24 and RP11-65C22), those in NI without exception. [a]P = 0.001680673, [b]P = 9.23504e-7, [c]P = 0.002197802, [d]P = 3.64223e-6, [e]P = 0.000840336, [f]P = 0.007692306, [g]P = 0.004395604, [h]P = 8.31509e-6, [i]P = 0.004395604, [j]P = 1.10173e-5, [k]P = 0.005882353, [l]P = 0.001098901. (c) Unsupervised two-dimensional hierarchical clustering analysis based on BAMCA data (signal ratios) in UCs. Forty patients with UCs were hierarchically clustered into two subclasses, Clusters A_T (n = 19) and B_T (n = 21). All four patients with recurrence belonged to Cluster B_T. (d) Unsupervised two-dimensional hierarchical clustering analysis based on BAMCA data (signal ratios) for noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter. Thirteen patients with UCs of the renal pelvis or ureter were hierarchically clustered into two subclasses, Clusters A_{NP} (n = 4) and B_{NP} (n = 9). All four patients who developed intravesical metachronous UC belonged to Cluster B_{NP}.

scattergrams of the signal ratios in normal urothelia and noncancerous urothelia obtained from patients with UCs for representative examples of the 201 BAC clones. Using the cut-off values described in Figure 3(a), noncancerous urothelia obtained from patients with UCs were discriminated from normal urothelia with sufficient sensitivity and specificity (Fig. 3a). From the 201 BAC clones, 83 for which such discrimination was performed with a sensitivity and specificity of 75% or more than 75% were selected (Table S1). The cut-off values of the signal ratios for the 83 BAC clones, and their sensitivity and specificity, are shown in Table S1.

A histogram showing the number of BAC clones satisfying the criteria listed in Table S1 for 18 normal urothelia (C1–C18) and 17 noncancerous urothelia obtained from patients

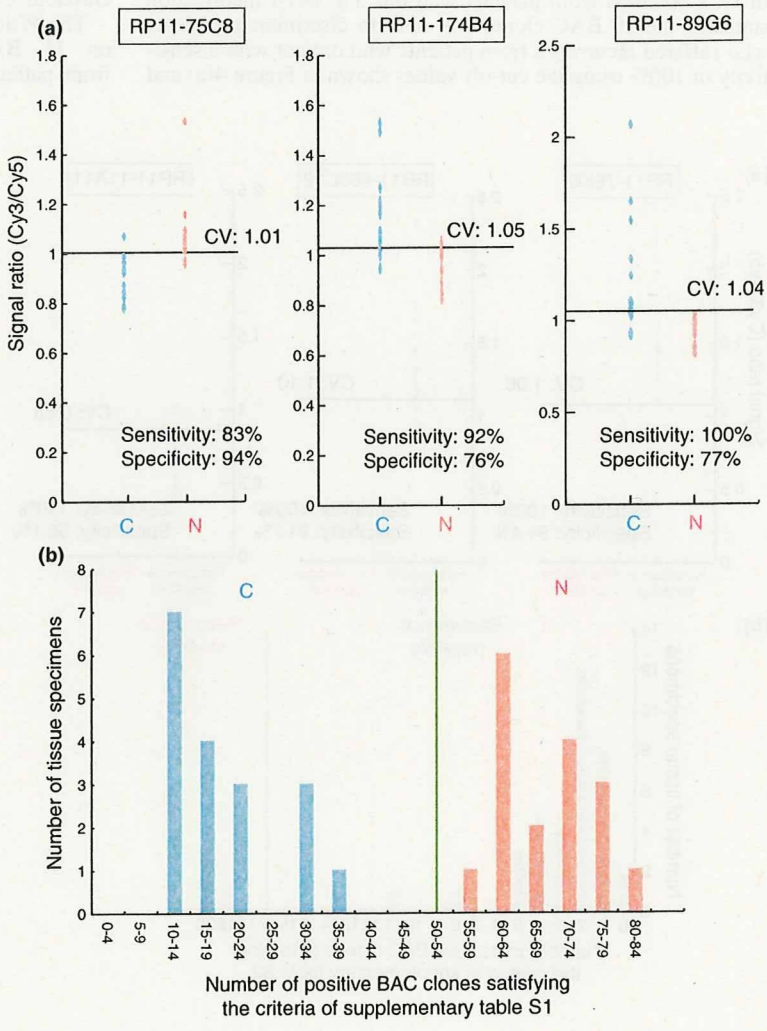

Fig. 3. DNA methylation profiles discriminating noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) (N) from normal urothelia (C). (a) Scattergrams of the signal ratios in C and N on representative bacterial artificial chromosome (BAC) clones, RP11-75C8, RP11-174B4, and RP11-89G6. Using the cut-off values (CV) described in each panel, N in this cohort were discriminated from C with sufficient sensitivity and specificity. (b) Histogram showing the number of BAC clones satisfying the criteria listed in Table S1 in samples C1–C18 and N1–N17. Based on this histogram, we established a criterion that when the noncancerous urothelia satisfied the criteria in Table S1 for 50 (green bar) or more than 50 BAC clones, they were judged to be at high risk of carcinogenesis.

with UCs (N1–N17) is shown in Figure 3(b). Based on this figure, we finally established the following criteria: when noncancerous urothelia satisfied the criteria in Table S1 for 50 or more BAC clones (green bar in Fig. 3b), they were judged to be at high risk of carcinogenesis, and when noncancerous urothelia satisfied the criteria in Table S1 for less than 50 BAC clones, they were judged not to be at high risk of carcinogenesis. Based on these criteria, both the sensitivity and specificity for diagnosis of noncancerous urothelia obtained from patients with UCs in this cohort as being at high risk of carcinogenesis were 100%.

Association of DNA methylation profiles in UCs with recurrence. Unsupervised two-dimensional hierarchical clustering analysis based on BAMCA data (signal ratios) for UCs was able to group 40 patients into two subclasses, Clusters A_T and B_T, which contained 19 and 21 patients, respectively (Fig. 2c). Four patients (19.0%) belonging to Cluster B_T suffered recurrence after surgery (metastasis to the pelvic lymph nodes in three, and metastasis to the lung and bone in one), whereas none (0%) belonging to Cluster A_T did so ($P = 0.0449$). The mean observation period was 29.8 ± 28.0 months (mean ± SD). These data prompted us to establish criteria for predicting recurrence of UCs based on DNA methylation status.

The Wilcoxon test ($P < 0.01$) revealed that the signal ratios on 20 BAC clones in UCs differed significantly between the patients who suffered recurrence after surgery and patients who did not. Figure 4(a) shows scattergrams of the signal ratios in UCs obtained from patients who suffered recurrence and those in UCs obtained from patients who did not. DNA methylation status of the 20 BAC clones was able to discriminate patients who suffered recurrence from patients who did not with a sensitivity of 100% using the cut-off values shown in Figure 4(a) and Table S2. A histogram showing the number of BAC clones satisfying the criteria listed in Table S2 for all 40 UCs is shown in Figure 4(b). Satisfying the criteria in Table S2 for 17 or more BAC clones (green bar in Fig. 4b) discriminated patients who suffered recurrence from patients who did not with a sensitivity and specificity of 100%, whereas high histological grade,[21] invasive growth (pT2 or more), and vascular or lymphatic involvement were unable to achieve such complete discrimination (data not shown).

Association of DNA methylation profiles in noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter with intravesical metachronous UC development. It is well known that patients with UCs of the renal pelvis and ureter frequently suffer from metachronous UC development in the urinary bladder after nephroureterectomy.[24,25] Since such metachronous UC originates from the noncancerous urothelium of the urinary bladder, we focused on the DNA methylation status of noncancerous urothelia obtained by nephroureterectomy from patients with UCs of the renal pelvis or ureter. Unsupervised two-dimensional hierarchical clustering analysis based on BAMCA data (signal ratios) for noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter was able to group 13 patients into two subclasses, Clusters A_{NP} and B_{NP}, which contained four and nine patients, respectively (Fig. 2d). Four (44%) of the patients in Cluster B_{NP} developed intravesical metachronous UCs, whereas none (0%) belonging to Cluster A_{NP} did so. These data prompted us to establish criteria that could predict the development of intravesical metachronous UC based on DNA methylation status.

The Wilcoxon test ($P < 0.01$) revealed that the signal ratios on 11 BAC clones in noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter differed

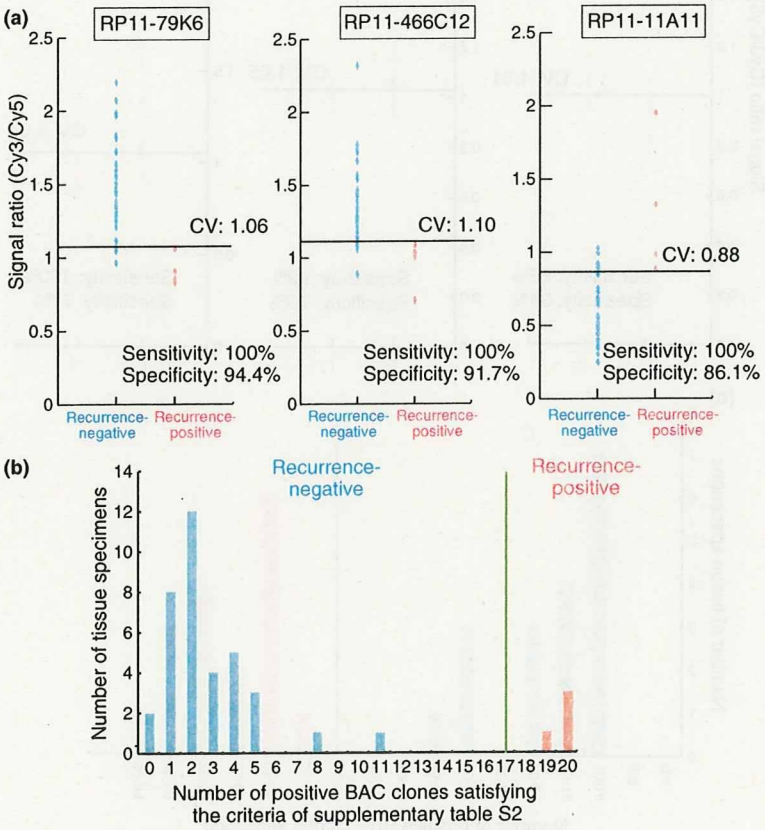

Fig. 4. DNA methylation profiles in urothelial carcinomas (UCs) associated with recurrence. (a) Scattergrams of the signal ratios in UCs from patients who did not develop recurrence ($n = 36$) and UCs from patients who developed recurrence ($n = 4$) on representative bacterial artificial chromosome (BAC), clones, RP11-79K6, RP11-466C12, and RP11-11A11. Using the cut-off values (CV) described in each panel, recurrence-positive patients were discriminated from recurrence-negative patients with 100% sensitivity. (b) Histogram showing the number of BAC clones satisfying the criteria listed in Table S2 in all 40 patients with UCs. Satisfying the criteria in Table S2 for 17 (green bar) or more than 17 BAC clones discriminated recurrence-positive patients from recurrence-negative patients with a sensitivity and specificity of 100%, whereas high histological grade (21), invasive growth (pT2 or more), and vascular or lymphatic involvement were unable to achieve such complete discrimination (data not shown).

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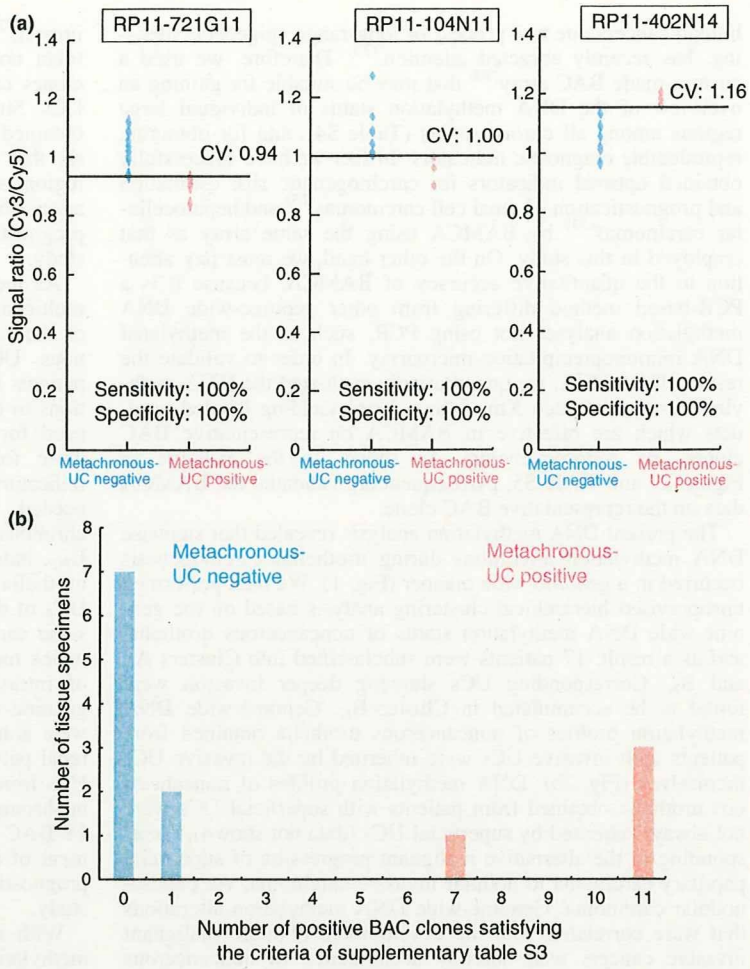

Fig. 5. DNA methylation profiles in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) of the renal pelvis or ureter associated with intravesical metachronous UC development. (a) Scattergrams of the signal ratios in noncancerous urothelia obtained from patients who did not develop intravesical metachronous UCs (n = 9) and noncancerous urothelia obtained from patients who developed intravesical metachronous UCs (n = 4) on representative bacterial artificial chromosome (BAC) clones, RP11-721G11, RP11-104N11, and RP11-402N14. Using the cut-off values (CV) described in each panel, metachronous UC-positive patients were discriminated from metachronous UC-negative patients with 100% sensitivity and specificity. (b) Histogram showing the number of BAC clones satisfying the criteria listed in Table S3 in all 13 patients with UCs of the renal pelvis or ureter from whom noncancerous urothelia were obtained. Patients who were negative and positive for metachronous UC were confirmed to show a marked difference in the DNA methylation status of the 11 BAC clones.

significantly between patients who developed intravesical metachronous UC after nephroureterectomy and patients who did not. DNA methylation status of nine of the 11 BAC clones was able to discriminate patients who suffered from intravesical metachronous UC development from patients who did not with a sensitivity and specificity of 100% using the cut-off values shown in Figure 5(a) and Table S3. A histogram showing the number of BAC clones satisfying the criteria listed in Table S3 for 13 noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter is shown in Figure 5(b).

Discussion

Urothelial carcinomas are clinically remarkable because of their multicentricity: synchronously or metachronously multifocal UCs often develop in individual patients. A possible mechanism for such multiplicity is the ''field effect,'' whereby carcinogenic agents in the urine cause malignant transformation of multiple urothelial cells.[26] Even noncancerous urothelia showing no remarkable histological features obtained from patients with UCs can be considered to be at the precancerous stage, because they may be exposed to carcinogens in the urine. On the other hand, UCs are classified as superficial papillary carcinomas or nodular invasive carcinomas according to their configuration. Superficial papillary carcinomas usually remain noninvasive, although patients need to undergo repeated urethrocystoscopic resections because of recurrences. In contrast, the clinical outcome of nodular invasive carcinomas is poor.[11,12]

In our previous study, accumulation of DNA methylation on C-type CpG islands associated with DNMT1 protein overexpression was observed even in noncancerous urothelia obtained from patients with UCs.[8,9] Aberrant DNA methylation was further increased, especially in nodular invasive carcinomas.[8–10] These previous data suggested that carcinogenetic risk estimation and prognostication of UCs based on DNA methylation status might be a promising strategy. Although optimal diagnostic indicators have never been explored using array-based genome-wide DNA methylation analysis, alterations of DNA methylation on several CpG islands in UCs have been reported separately.[27–31]

Many researchers in the field of cancer epigenetics have used promoter arrays to identify the genes that are methylated in cancer cells.[14–16] 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.[32] 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.[33] Therefore, we used a custom-made BAC array[20] that may be suitable for gaining an overview of the DNA methylation status of individual large regions among all chromosomes (Table S4), and for obtaining reproducible diagnostic indicators. In fact we have successfully obtained optimal indicators for carcinogenetic risk estimation and prognostication of renal cell carcinomas[23] and hepatocellular carcinomas[34] by BAMCA using the same array as that employed in this study. On the other hand, we must pay attention to the quantitative accuracy of BAMCA, because it is a PCR-based method differing from other genome-wide DNA methylation analyses not using PCR, such as the methylated DNA immunoprecipitation-microarray. In order to validate the results of BAMCA, we quantitatively evaluated the DNA methylation status of each Xma I/Sma I site yielding labeled products which are effective in BAMCA on representative BAC clones, by pyrosequencing. As shown in the example in Figure S1 and Table S5, pyrosequencing validated the BAMCA data on the representative BAC clone.

The present DNA methylation analysis revealed that stepwise DNA methylation alterations during urothelial carcinogenesis occurred in a genome-wide manner (Fig. 1). We then performed unsupervised hierarchical clustering analysis based on the genome-wide DNA methylation status of noncancerous urothelia, and as a result, 17 patients were subclassified into Clusters A_N and B_N. Corresponding UCs showing deeper invasion were found to be accumulated in Cluster B_N. Genome-wide DNA methylation profiles of noncancerous urothelia obtained from patients with invasive UCs were inherited by the invasive UCs themselves (Fig. 2b). DNA methylation profiles of noncancerous urothelia obtained from patients with superficial UCs were not always inherited by superficial UCs (data not shown), corresponding to the alternative malignant progression of superficial papillary carcinoma to nodular invasive carcinoma, via papillonodular carcinoma. Genome-wide DNA methylation alterations that were correlated with the development of more malignant invasive cancers were already accumulated in noncancerous urothelia, suggesting that DNA methylation alterations at the precancerous stage may not occur randomly but are prone to further accumulation of genetic and epigenetic alterations and generate more malignant cancers.

The present genome-wide analysis revealed DNA methylation profiles that were able to completely discriminate noncancerous urothelia obtained from patients with UCs from normal urothelia and diagnose them as having a high risk of urothelial carcinogenesis with a sensitivity and specificity of 100%. We are currently attempting to develop methodology for assessing the tendency for DNA methylation in the 83 BAC regions in urine samples with a view to application for screening of healthy individuals. If it proves possible to identify individuals who are at high risk of urothelial carcinogenesis, then strategies for the prevention or early detection of UCs, such as smoking cessation or repeated urine cytology examinations, might be applicable.

Even after surgery with curative intent, some UCs relapse and metastasize to lymph nodes or distant organs.[35] Recently, new systemic chemotherapy and targeted therapy have been developed for treatment for UCs.[36] In order to start adjuvant systemic chemotherapy immediately in patients who have undergone surgery and are still at high risk of recurrence and metastasis, prognostic indicators have been explored. The present genome-wide analysis revealed DNA methylation profiles that were able to discriminate patients who suffered recurrence after surgery from patients who did not with a sensitivity and specificity of 100% (Fig. 4b), whereas a high histological grade,[21] invasive growth (pT2 or more), and vascular or lymphatic involvement, which are known to have a prognostic impact,[37,38] were incapable of such complete discrimination (data not shown). Therefore, a combination of the 20 BAC clones can have significant prognostic value for patients with UCs. Since a sufficient quantity of good-quality DNA can be obtained from each surgical specimen, our array-based analysis that overviews aberrant DNA methylation of each BAC region is immediately applicable to routine laboratory examinations for prognostication after surgery. The reliability of such prognostication will need to be validated in a prospective study.

As mentioned above, UCs are remarkable because of their multicentricity. Approximately 10–30% of patients with UCs of the renal pelvis and ureter develop intravesical metachronous UCs after nephroureterectomy.[24,25] Therefore, such patients have to undergo repeated urethrocystoscopic examinations to detect intravesical metachronous UCs. To decrease the need for invasive urethrocystoscopic examinations and assist close follow-up of such patients after nephroureterectomy, indicators for intravesical metachronous UCs have been needed. All of our patients who developed intravesical metachronous UCs after nephroureterectomy belonged to Cluster B_{NP}, indicating that DNA methylation profiles of noncancerous urothelia obtained by nephroureterectomy from patients with UCs of the renal pelvis or ureter, which may be exposed to the same carcinogens in the urine as noncancerous urothelia from which metachronous UCs originate, are correlated with the risk of intravesical metachronous UC development. The present genome-wide analysis revealed DNA methylation profiles that were able to completely discriminate patients with UCs of the renal pelvis or ureter who developed intravesical metachronous UCs from patients who did not, in noncancerous urothelia from nephroureterectomy specimens. A combination of the present 11 BAC clones may be an optimal indicator for the development of intravesical metachronous UC. The reliability of such prognostication will again need to be validated in a prospective study.

With respect to background factors of genome-wide DNA methylation alterations during urothelial carcinogenesis, smoking history did not correlate significantly with the numbers of BAC clones showing DNA hypo- or hypermethylation in noncancerous urothelia obtained from patients with UCs and in UCs, or with clustering (Cluster A_N *vs* Cluster B_N and Cluster A_T *vs* Cluster B_T) (Table S6). In addition, immunohistochemically examined DNMT1 protein expression levels did not correlate significantly with the numbers of BAC clones showing DNA hypo- or hypermethylation in noncancerous urothelia obtained from patients with UCs and in UCs, or with clustering (Cluster A_N *vs* Cluster B_N and Cluster A_T *vs* Cluster B_T) (Table S7), indicating that expression levels of DNMT1 did not by themselves simply determine DNA methylation profiles. However, our previous study revealed remarkable protein overexpression of DNMT1 in noncancerous urothelia obtained from patients with UCs as compared to normal urothelia.[8] Therefore, undefined cofactors may recruit DNMT1 or other proteins regulating DNA methylation status to aberrant target sequences and may participate in DNA methylation alterations in noncancerous urothelia obtained from patients with UCs. Further studies are needed to elucidate molecular mechanisms of DNA methylation alterations in such noncancerous urothelia.

Moreover, when the DNA methylation status for CpG islands of *p16*, human MutL homologue 1 (*hMLH1*), thrombospondin-1 (*THBS-1*), and death-associated protein kinase (*DAPK*) genes and the methylated in tumor (MINT)-1, -2, -12, -25, and -31 clones were examined in noncancerous urothelia obtained from patients with UCs and in UCs by methylation-specific PCR and combined bisulfite restriction enzyme analysis as in our previous study,[9,39] the incidence of DNA

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methylation on each CpG island and the average number of methylated CpG islands did not correlate significantly with the numbers of BAC clones showing DNA hypo- or hypermethylation in noncancerous urothelia obtained from patients with UCs and in UCs, or with clustering (Cluster A_N *vs* Cluster B_N and Cluster A_T *vs* Cluster B_T) (Table S8). Therefore, molecular mechanisms for alterations of genome-wide DNA methylation profiles may differ from those for regional DNA hypermethylation on CpG islands.

Although BAMCA mainly provides an overview of the DNA methylation status of individual large regions among all chromosomes as mentioned above, it may also be able to identify genes for which expressions are regulated by DNA methylation, since there are promoter regions of specific genes including CpG islands on BAC clones showing clinicopathologically significant DNA hypo- or hypermethylation (Table S4). Expression levels and the DNA methylation status of these genes, as well as the functions of the proteins coded by such genes, will be examined in a future investigation. If further studies identify tumor-related genes for which expression levels are regulated by DNA methylation among such candidates, these tumor-related genes may serves as targets for epigenetic prevention and therapy, along with the molecules causing alterations of genome-wide DNA methylation profiles.

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Supporting Information

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

Fig. S1. Examples of bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) data validation by pyrosequencing.

Table S1. Eighty-three bacterial artificial chromosome (BAC) clones that were able to discriminate noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) (N) from normal urothelia (C) with a sensitivity and specificity of 75% or more.

Table S2. Twenty bacterial artificial chromosome (BAC) clones that were able to discriminate urothelial carcinomas (UCs) in patients who developed recurrence (Pos) from those in patients who did not (Neg).

Table S3. Eleven bacterial artificial chromosome (BAC) clones that were able to discriminate noncancerous urothelia in patients with urothelial carcinomas (UCs) of the renal pelvis or ureter who developed intravesical metachronous UC (Pos) from those in patients who did not (Neg).

Table S4. Genes, CpG islands in the promoter regions, and repeat elements of bacterial artificial chromosome (BAC) clones in Tables S1, S2, and S3.

Table S5. Primer sets for validation study by pyrosequencing.

Table S6. Correlation between smoking history and DNA methylation status in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) and UCs.

Table S7. Correlation between protein expression levels of DNA methyltransferase (DNMT) 1 and DNA methylation status in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) and UCs.

Table S8. Correlation between regional DNA hypermethylation on CpG islands and the results of bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) and UCs.

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Intraductal carcinosarcoma with a heterologous mesenchymal component originating in intraductal papillary-mucinous carcinoma (IPMC) of the pancreas with both carcinoma and osteosarcoma cells arising from IPMC cells

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ABSTRACT

Carcinosarcoma of the pancreas is extremely rare and its histogenesis is still unclear. This is a report on a 64-year-old female patient with an intraductal carcinosarcoma arising from intraductal papillary-mucinous carcinoma (IPMC) in the pancreas tail. The carcinosarcoma grew as a polypoid mass within the main pancreatic duct. Histologically, the tumour consisted of adenocarcinoma covering the luminal surface of the lesion with minimal stromal invasion, and osteosarcoma occupying the stroma. Immunohistochemical and gene mutation analyses revealed that both the carcinomatous and sarcomatous tumour cells of the carcinosarcoma, as well as the IPMC cells, expressed TP53 and had identical mutations in *KRAS* and *TP53* genes, indicating that these two neoplastic components of the carcinosarcoma shared a common tumorigenesis and arose from the IPMC. This is the first report of a carcinosarcoma originating in IPMC. These findings imply that carcinosarcoma with a heterologous mesenchymal component is of ductal origin.

INTRODUCTION

Carcinosarcoma of the pancreas is a very rare tumour and only several cases have been reported hitherto.[1–6] These cases were diagnosed as carcinosarcoma histopathologically and immunohistochemically on the basis of the presence of both malignant epithelial and malignant mesenchymal components. Only two of the reported cases showed heterologous mesenchymal components.[1 2] The histogenesis of this tumour is still unclear, although there have been several hypotheses that it originates from epithelial cells, mesenchymal cells, undifferentiated precursor cells or stem cells. It has been difficult to assess its histogenesis, because pancreatic carcinosarcoma is extremely rare and is usually advanced at the time of diagnosis.

Here we present the first reported case of pancreatic intraductal carcinosarcoma with a heterologous mesenchymal component (osteosarcoma), which is located in an intraductal papillary-mucinous carcinoma (IPMC). This case is thought to be important for considering the histogenesis of pancreatic carcinosarcoma with a heterologous mesenchymal component.

CASE REPORT

A 64-year-old Japanese woman who attended our hospital for a health check-up was found by abdominal ultrasonography to have a cystic tumour in the tail of the pancreas. She had no symptoms and all clinical and laboratory data were normal. The serum concentrations of tumour markers were elevated (CA19-9, 87 U/ml; carcinoembryonic antigen (CEA), 2.7 ng/ml). She had been treated for diabetes mellitus for 11 years. Abdominal CT revealed a 2 cm cystic mass in the pancreatic tail (figure 1A). Within the cyst, there were irregular and solid nodules with calculus. The tail of the pancreas had been totally replaced by the tumour. No lymphadenopathy, ascites, liver metastasis or mass in the soft tissues was found. Distal pancreatectomy was performed under a preoperative diagnosis of invasive carcinoma originating in IPMC. The operation was uneventful, and 12 months after surgery, the patient is well without any tumour recurrence or metastasis.

PATHOLOGICAL AND GENETIC FINDINGS

A grossly elastic, hard, solid, spherical mass measuring 35×21×14 mm was present in the tail of the pancreas. At the cut surface, there was a papillary-to-polypoid projection located in the main and branch pancreatic ducts, which were cystically dilated and filled with clear yellowish mucinous fluid (figure 1B). These intraductal lesions were surrounded by yellowish-grey solid and nodular components of the tumour from the side of the pancreatic tail.

Histologically, the tumour comprised an intraductal neoplasm and a derivative invasive carcinoma (figure 2). The luminal surface of the dilated pancreatic ducts was covered with atypical mucin-secreting columnar epithelial cells showing papillary growth (figure 2A,B), indicating a diagnosis of IPMC. No ovarian-type stroma was evident. It was noteworthy that biphasic histological features were found in the polypoid lesion in the main pancreatic duct, which consisted of papillary proliferation of adenocarcinoma covering the luminal surface of the projecting mass with infrequent and minimal stromal invasion and an osteosarcoma occupying the stroma. The osteosarcoma showed invasive growth, but its extension was limited to the stroma of the IPMC, which was not beyond the duct wall

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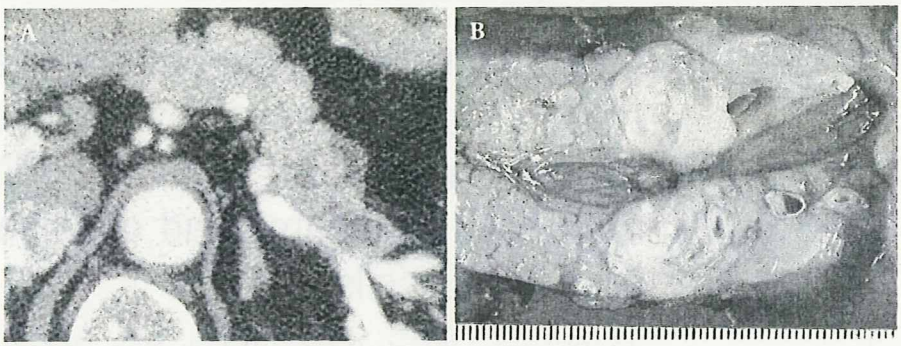

Figure 1 (A) Abdominal CT image showing a 2 cm cystic mass in the pancreatic tail. (B) Fresh cut view of the body and tail of the pancreas.

(figure 2C–E). The osteosarcoma was characterised by a dense proliferation of malignant spindle-shaped and pleomorphic cells with mononucleated and multinucleated giant cells that had atypical and bizarre nuclei and formed osteoid and bone (figure 2E). Occasional infiltration of osteoclast-like multinucleated giant cells without nuclear atypia was evident. This intraductally proliferating mixed epithelial and mesenchymal tumour was diagnosed as carcinosarcoma, which seemed to have originated in the IPMC. Formation of osteoid and/or bone is rare but possible in cases of undifferentiated carcinoma with osteoclast-like giant cells, although the osteoid and/or bone is a result of reactive stromal metaplasia without any atypia in such cases.[7]

In addition to the intraductal tumour, IPMC cells had infiltrated beyond the duct wall and reached the surrounding stroma, showing a marked desmoplastic reaction at the side of the pancreatic tail bearing the tumour (figure 2B). The infiltrating cancer cells proliferated with poorly formed glands and solid to nested growth, indicating poorly differentiated adenocarcinoma. The infiltrating adenocarcinoma formed a nodular mass measuring 25×21×14 mm, although the invasive adenocarcinoma was not connected to the intraductal carcinosarcoma.

Immunohistochemical examination revealed expression of cytokeratins (AE1/AE3 and CK7) and vimentin, which

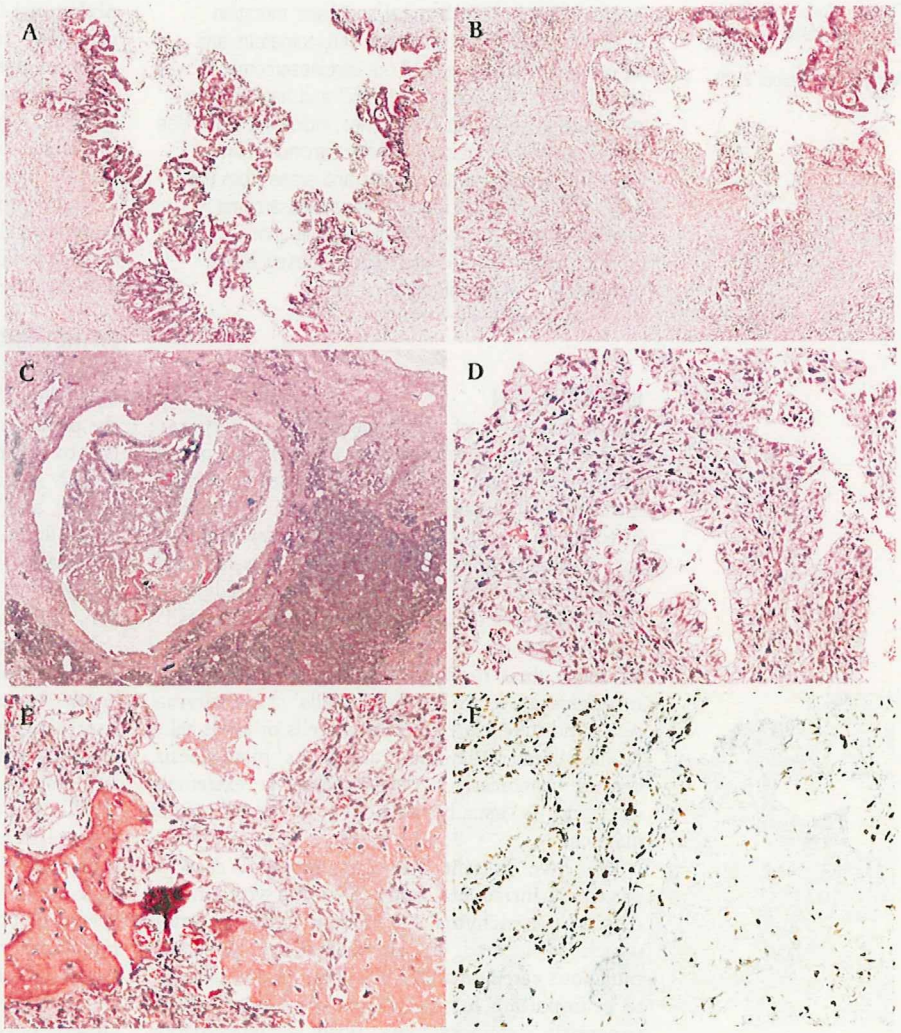

Figure 2 (A, B) Histopathological features of intraductal papillary-mucinous carcinoma (IPMC) (A) and invasive adenocarcinoma arising from IPMC (B). (C–E) Histopathological features of intraductal carcinosarcoma originating in IPMC. (C) A very-low-power view of the polypoid lesion in the main pancreatic duct. (D, E) Mid-power view of the polypoid lesion. (F) Immunohistochemical expression of TP53 in intraductal carcinosarcoma originating in IPMC.

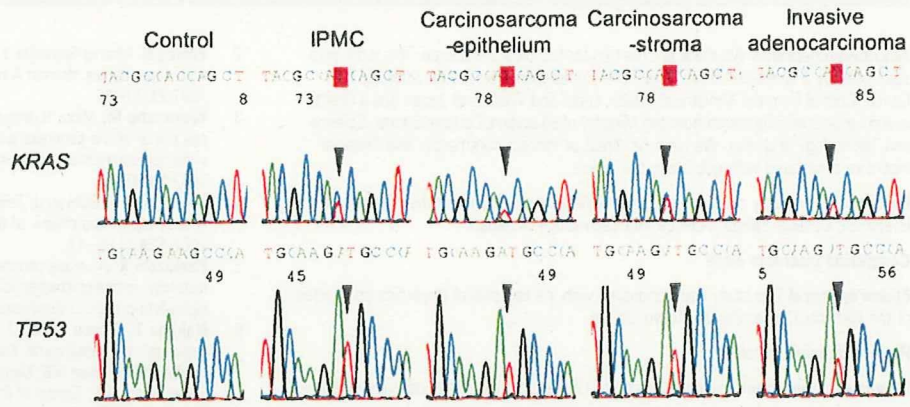

Figure 3 Mutations of *KRAS* and *TP53* genes in each tumour component. All four tumour components examined (epithelial and mesenchymal tumour cells in the carcinosarcoma, intraductal papillary-mucinous carcinoma (IPMC) cells and invasive adenocarcinoma cells), harboured identical *KRAS* and *TP53* mutations. The sequences were read with reverse primers. Triangles indicate locations of point mutations.

confirmed the epithelial and mesenchymal components of tumour cells detected histologically. TP53 was expressed in the nuclei of most of the intraductal and invasive epithelial tumour cells as well as the mesenchymal tumour cells (figure 2F). CD68 antigen was expressed in some of the multinucleated giant cells without nuclear atypia. These CD68-positive osteolclast-like giant cells did not express TP53.

Four distinct tumour components (epithelial and mesenchymal tumour cells in the carcinosarcoma, IPMC cells and invasive adenocarcinoma cells) were separately laser-microdissected and analysed for *KRAS* and *TP53* mutations. DNA samples extracted from the microdissected tissues were subjected to PCR with a pair of specific primers to amplify exon 1 of *KRAS* or exon 4 of *TP53*, and isolated PCR products were sequenced bidirectionally. The analysis revealed identical *KRAS* (G35A mutation in exon 1) and *TP53* (T337A mutation in exon 4) mutations in all four tumour components examined (figure 3). Non-neoplastic pancreatic parenchyma adjacent to the tumour exhibited wild-type sequences, confirming the somatic nature of the mutations.

DISCUSSION

Carcinosarcoma is a biphasic tumour consisting of an intimate admixture of malignant epithelial and mesenchymal components identifiable on the basis of their morphological, immunohistochemical and sometimes ultrastructural features. Nine cases of carcinosarcoma of the pancreas have been reported,[1–6] including two with heterologous mesenchymal components; one of the latter cases exhibited leiomyosarcoma, chondrosarcoma and rhabdomyosarcoma,[1] and the other showed malignant nerve sheath tumour as heterologous mesenchymal components.[2] No case of either carcinosarcoma arose from intraductal papillary-mucinous neoplasm (IPMN) and all were found at an advanced stage, with an average tumour diameter of 9.6 cm (range 2.5–19 cm).

To our knowledge, the present case of carcinosarcoma with a heterologous mesenchymal component originating in IPMN is the first of its kind to have been reported. Immunohistochemical and gene mutation analyses revealed that both the carcinomatous and sarcomatous tumour cells in the carcinosarcoma as well as the IPMC cells expressed TP53 and had common mutations in *KRAS* and *TP53* genes, indicating that these two neoplastic components of the carcinosarcoma had a common origin, IPMC. This case provides new findings supporting the hypothesis that carcinosarcoma with a heterologous mesenchymal component is of ductal origin and arises from IPMN.

The histogenesis of carcinosarcoma is still controversial, but the previously proposed hypotheses have now been combined as the following: (1) it is a combination tumour in which carcinomatous and sarcomatous elements arise from a multipotential stem cell; (2) it is a collision tumour in which two independent neoplasia, carcinoma and sarcoma, develop; (3) it is a carcinoma showing metaplastic changes to sarcoma components. The definition of carcinosarcoma in the WHO histological classification differs according to the organ in which the tumour develops. A mixed epithelial and mesenchymal tumour with heterologous mesenchymal components is defined as carcinosarcoma in the histological classification of tumours of many organs, including the colon and rectum, gallbladder and extrahepatic bile ducts, and lung.[8] In contrast, a mixed epithelial and mesenchymal tumour, regardless of the presence of heterologous mesenchymal components, is defined as carcinosarcoma in the histological classification of tumours of the breast and female genital tract. It is thought that most, but not all, of the mesenchymal components in carcinosarcoma of the female genital tract arise from the carcinoma through metaplastic change and that the small population of the carcinosarcoma left is formed by collision of carcinoma and sarcoma.[9] The present case suggests that carcinosarcoma of the pancreas arises from a carcinoma with metaplastic changes, although the WHO histological classification of pancreatic tumours includes no specific category for carcinosarcoma.[8] According to the previous report,[2] a sarcomatous component is speculated to arise from ovarian-type stroma characteristic of mucinous cystic neoplasm. In such a case, carcinosarcoma should be formed as a collision tumour.

In summary, we have presented a case of pancreatic intraductal carcinosarcoma originating in IPMC. Our morphological, immunohistochemical and genetic findings suggest that the carcinosarcoma with a heterologous mesenchymal component was ductal in origin.

Take-home messages

- Carcinosarcoma of the pancreas is extremely rare and its histogenesis is still unclear.
- We describe an intraductal carcinosarcoma arising from intraductal papillary-mucinous carcinoma (IPMC) in the pancreas tail.
- Both the epithelial component (adenocarcinoma) and heterologous mesenchymal component (osteosarcoma) of the carcinosarcoma, as well as the IPMC, expressed TP53 and had identical mutations in *KRAS* and *TP53* genes, indicating that these two neoplastic components of the carcinosarcoma shared a common tumorigenesis and arose from the IPMC. These findings imply that carcinosarcoma with a heterologous mesenchymal component is of ductal origin.

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Competing interests None.

Ethics approval This study was conducted with the approval of the ethics committee of the National Cancer Center, Tokyo, Japan.

Patient consent Obtained.

Provenance and peer review Not commissioned; not externally peer reviewed.

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Review Article

Genome-wide DNA methylation profiles in precancerous conditions and cancers

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

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

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

Alterations of DNA methylation during multistage carcinogenesis

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

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

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

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

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

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

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

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

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

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

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