

Abbreviations

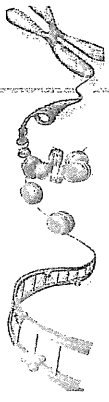
BAC	bacterial artificial chromosome
BAMCA	BAC array-based methylated CpG island amplification
CIMP	CpG island methylator phenotype
DNMT	DNA methyltransferase
HBV	hepatitis B virus
HCC	hepatocellular carcinoma

HCV	hepatitis C virus
LOH	loss of heterozygosity
PanIN	pancreatic intraductal neoplasia
PCNA	proliferating cell nuclear antigen
RCC	renal cell carcinoma
UC	urothelial carcinoma

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DNA methylation profiles in precancerous tissue and cancers: carcinogenetic risk estimation and prognostication based on DNA methylation status

Alterations in DNA methylation, which are associated with DNA methyltransferase abnormalities and result in silencing of tumor-related genes and chromosomal instability, are involved even in precancerous changes in various organs. DNA methylation alterations also account for the histological heterogeneity and clinicopathological diversity of human cancers. Therefore, we have analyzed DNA methylation on a genome-wide scale in clinical tissue samples. Our approach using the bacterial artificial chromosome array-based methylated CpG island amplification method has revealed that DNA methylation alterations correlated with the future development of more malignant cancers are already accumulated at the precancerous stage in the kidney, liver and urinary tract. DNA methylation profiles at precancerous stages are basically inherited by the corresponding cancers developing in individual patients. Such DNA methylation alterations may confer vulnerability to further genetic and epigenetic alterations, generate more malignant cancers, and thus determine patient outcome. On the basis of bacterial artificial chromosome array-based methylated CpG island amplification data, indicators for carcinogenetic risk estimation have been established using liver tissue specimens from patients with hepatitis virus infection, chronic hepatitis and liver cirrhosis or histologically normal urothelia, and for prognostication using biopsy or surgically resected specimens from patients with renal cell carcinoma, hepatocellular carcinoma and urothelial carcinoma. Such genome-wide DNA methylation profiling has now firmly established the clinical relevance of translational epigenetics.

KEYWORDS: bacterial artificial chromosome array-based methylated CpG island amplification DNA methylation DNA methyltransferase precancerous condition prognostication risk estimation

DNA methylation, a covalent chemical modification resulting in the addition of a methyl group at the carbon 5 position of the cytosine ring in CpG dinucleotides, is one of the most consistent epigenetic changes occurring in human cancers [1–3]. 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 [4–6]. DNA methylation is a stable modification inherited throughout successive cell divisions, and is essential for X-chromosome inactivation, genome imprinting, silencing of transposons and other parasitic elements, and proper expression of genes [7].

Human cancer cells show a drastic change in DNA methylation status, specificity in the overall DNA hypomethylation and regional DNA hypermethylation [1–3]. In 1995, when the *RB* and *VHL* genes were the only tumor suppressor genes known to be silenced by DNA methylation, we showed for the first time that the E-cadherin tumor suppressor gene is silenced by DNA methylation around the promoter region [8], and

proposed the universality of a ‘two-hit’ mechanism involving DNA hypermethylation and loss of heterozygosity during carcinogenesis. The list of tumor-related genes whose levels of expression are altered owing to DNA hypo- or hypermethylation has been increasing [9]. At this point, some explanation is necessary regarding the mechanisms whereby tumor-related genes whose DNA methylation status is altered during carcinogenesis are selected. One, but likely not the only, explanation for such selection is polycomb binding, in which CpG-rich sequences targeted by the polycomb complex in normal embryonic stem cells consequently form a bivalent domain carrying both ‘activating’ H3K4 methylation and ‘inactivating’ H3K27 methylation [10]. This bivalent state is converted to a primary active or repressive chromatin conformation after differentiation cues have been received. During carcinogenesis, such modifications may render the genes vulnerable to errors, resulting in aberrant DNA methylation [11].

On the other hand, DNA hypomethylation induces chromosomal instability through decondensation of heterochromatin and enhancement

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of chromosomal recombination during carcinogenesis [12]. For example, in hepatocellular carcinoma (HCC) [13] and urothelial carcinoma (UC) [14], DNA hypomethylation of pericentromeric satellite regions is correlated with copy number alterations on chromosomes 1 and 9, where such regions are found abundantly. A DNA methyltransferase (DNMT), DNMT3b, is required for DNA methylation of pericentromeric satellite regions in early mouse embryos [15]. We have demonstrated the possibility that an inactive splice variant, DNMT3b4 is upregulated, competes with DNMT3b3, the major splice variant in normal liver tissue, for targeting to pericentromeric satellite regions, and may lead to chromosomal instability through induction of DNA hypomethylation in such regions during hepatocarcinogenesis [16].

Translational epigenetics has now come of age [17–19], and the empirical analysis of DNA methylation status in clinical tissue samples with reference to the clinicopathological parameters of human cancers is becoming increasingly important for the diagnosis, prevention and therapy of cancers [20–22].

DNA methylation alterations during multistage carcinogenesis

Accumulating evidence suggests that alterations of DNA methylation may play a significant role even at precancerous stages in association with chronic inflammation, persistent infection with viruses and other pathogenic microorganisms, such as hepatitis B virus (HBV) or hepatitis C virus (HCV) [23–25], Epstein–Barr virus [26], human papillomavirus [27], and *Helicobacter pylori* [28] and cigarette smoking [29]. In the 1990s, we frequently observed DNA hypermethylation on C-type CpG islands, which are generally methylated in a cancer-specific but not age-dependent manner [30], and DNA methylation alterations at ‘hot spots’ of loss of heterozygosity in HCCs, even in samples of noncancerous liver tissue showing chronic hepatitis or liver cirrhosis, which are widely considered to be precancerous conditions, in comparison with normal liver tissue samples [23–25]. These findings [23] represented some of the earliest reports of DNA methylation alterations at the precancerous stage. Silencing of the E-cadherin gene, which encodes a Ca²⁺-dependent cell–cell adhesion molecule, in samples of noncancerous liver tissue showing chronic hepatitis or cirrhosis may result in heterogeneous E-cadherin expression, which is associated with small focal areas where hepatocytes show only slight

E-cadherin immunoreactivity [31]. Silencing of the E-cadherin gene may participate even in the very early stage of hepatocarcinogenesis through loss of intercellular adhesiveness and destruction of tissue morphology. Expression levels of mRNA for DNMT1, the major and best known DNMT, are significantly higher in 48 samples of noncancerous liver tissue showing chronic hepatitis or cirrhosis than in normal liver tissue, and are even higher in 67 samples of HCCs [32]. The incidence of DNMT1 overexpression in 53 samples of HCCs is significantly correlated with poorer tumor differentiation and portal vein involvement [33]. 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 [33].

Ductal carcinomas of the pancreas frequently develop after chronic damage owing to pancreatitis. At least a proportion of peripheral pancreatic ductal epithelia with an inflammatory background may be at precancerous stages. We conducted an immunohistochemical analysis of DNMT1 in 48 samples of peripheral pancreatic duct epithelia showing no remarkable histological findings without an inflammatory background, 54 samples of peripheral pancreatic duct epithelia with an inflammatory background, 188 samples of another precancerous lesion, pancreatic intraepithelial neoplasia (PanIN), and 220 areas of invasive ductal carcinoma from surgical specimens resected from 100 patients (5, 24, 49 and 22 patients at Stage I to II, III, IVa and IVb, respectively) [34]. DNA methylation status of the *p14*, *p15*, *p16*, *p73*, *APC*, *bMLH1*, *MGMT*, *BRCA1*, *GSTP1*, *TIMP-3*, *CDH1* and *DAPK-1* genes was also analyzed in tissue samples during pancreatic carcinogenesis. To examine DNA methylation status in tiny tissue samples of peripheral pancreatic duct epithelia without or with an inflammatory background, avoiding any contamination with surrounding acinar cells and/or lymphocytes, we employed a method combining tissue microdissection with agarose bead-based bisulfite conversion followed by nested methylation-specific PCR: preheated low-melting agarose was mixed with the harvested microdissected tissue samples and the mixtures were pipetted into chilled mineral oil to form agarose beads. The beads with the tissue were incubated with proteinase K followed by bisulfite conversion. After neutralization with hydrochloric acid, the beads were used directly for nested methylation-specific PCR. This method also allowed us to examine the DNA methylation

status in PanIN and ductal carcinoma, avoiding contamination by the abundant desmoplastic stroma. The incidence of DNMT1 protein expression [34] and the average number of methylated tumor-related genes [35] increased with progression from peripheral pancreatic ductal epithelia with an inflammatory background, to PanIN, to well differentiated ductal carcinoma, and finally, to a poorly differentiated ductal carcinoma, in comparison with normal peripheral pancreatic duct epithelia without an inflammatory background. DNMT1 overexpression in ductal carcinomas of the pancreas is significantly correlated with the extent of invasion to the surrounding tissue, an advanced stage, poorer patient outcome [34], and accumulation of DNA methylation of tumor-related genes [35]. Although the maintenance activities of DNMT1 are related to its *in vitro* preference for hemimethylated substrates [36], excessive amounts of DNMT1 in comparison to those of proliferating cell nuclear antigen, which targets DNMT1 to replication foci [37], may participate in *de novo* methylation of CpG islands [38]. In fact, significant correlation between DNMT1 overexpression and accumulation of DNA methylation of specific genes in cell lines, mouse models and clinical tissue samples of various cancers has also been reported by other groups [39–43]. However, other groups have stated that they did not find such significant correlations [44–46]. Therefore, the participation of DNMT1 overexpression in accumulation of DNA methylation of tumor-related genes remains a controversial issue.

Genome-wide DNA methylation analysis

Recently, it has become possible to analyze DNA methylation on a genome-wide scale. The fact that DNA methylation alterations are associated with multistage carcinogenesis has prompted us to perform such genome-wide analysis in tissue specimens. The methods most commonly used to read methylated cytosines are a DNA methylation-sensitive restriction enzyme-based approach, such as the well-established technique of methylated CpG island amplification [47], affinity-based approaches, whereby antimethylcytosine antibody or methyl-binding domain proteins are used to enrich methylated fractions of genomic DNA, and bisulfite conversion of non-methylated cytosines to thymidine through hydrolytic deamination. These strategies for revealing methylated cytosines have been applied mainly to array platforms [48], and the resolution of the microarrays has been markedly

improved [49,50]. In addition to the introduction of new-generation sequencing technologies for bisulfite-converted genomic sequencing [48], a high-throughput technique without bisulfite conversion has been developed based on single-molecule, real-time DNA sequencing [51]. These new technologies will be able to efficiently reveal genome-wide DNA methylation profiles in tissue samples.

At the present time, many researchers in the field of cancer epigenetics use mainly promoter arrays to identify the genes that are silenced by DNA methylation in cancer cells. However, the promoter regions of specific genes are not the only target of DNA methylation alterations in human cancers. DNA methylation status in genomic regions that do not directly participate in gene silencing, such as the edges of CpG islands, may be altered at precancerous stages before the alterations of the promoter regions themselves occur [52]. 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 owing to a process of long-range epigenetic silencing, has recently attracted attention [53]. Therefore, we have recently employed bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA; FIGURE 1) [54]. Test or reference DNA was first digested with the methylation-sensitive restriction enzyme *SmaI* and subsequently with the methylation-insensitive *XmaI*. Adapters were ligated to the *XmaI*-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, respectively, and precipitated together with ethanol in the presence of Cot-I DNA. The mixture was applied to a custom-made array harboring approximately 4500 BAC clones located throughout chromosomes 1 to 22, X and Y.

Even though the resolution of BAMCA is inferior to the abovementioned newly developed high-resolution arrays, BAMCA has an ability for detecting any tendency for coordinated regulation of DNA methylation at multiple CpG sites in individual large regions of chromosomes, because any region covered by one BAC contains multiple *SmaI* sites. In fact, we validated this ability by the quantitative evaluation of DNA methylation status at each *SmaI* site on representative BAC clones by pyrosequencing: when almost all *SmaI* sites on

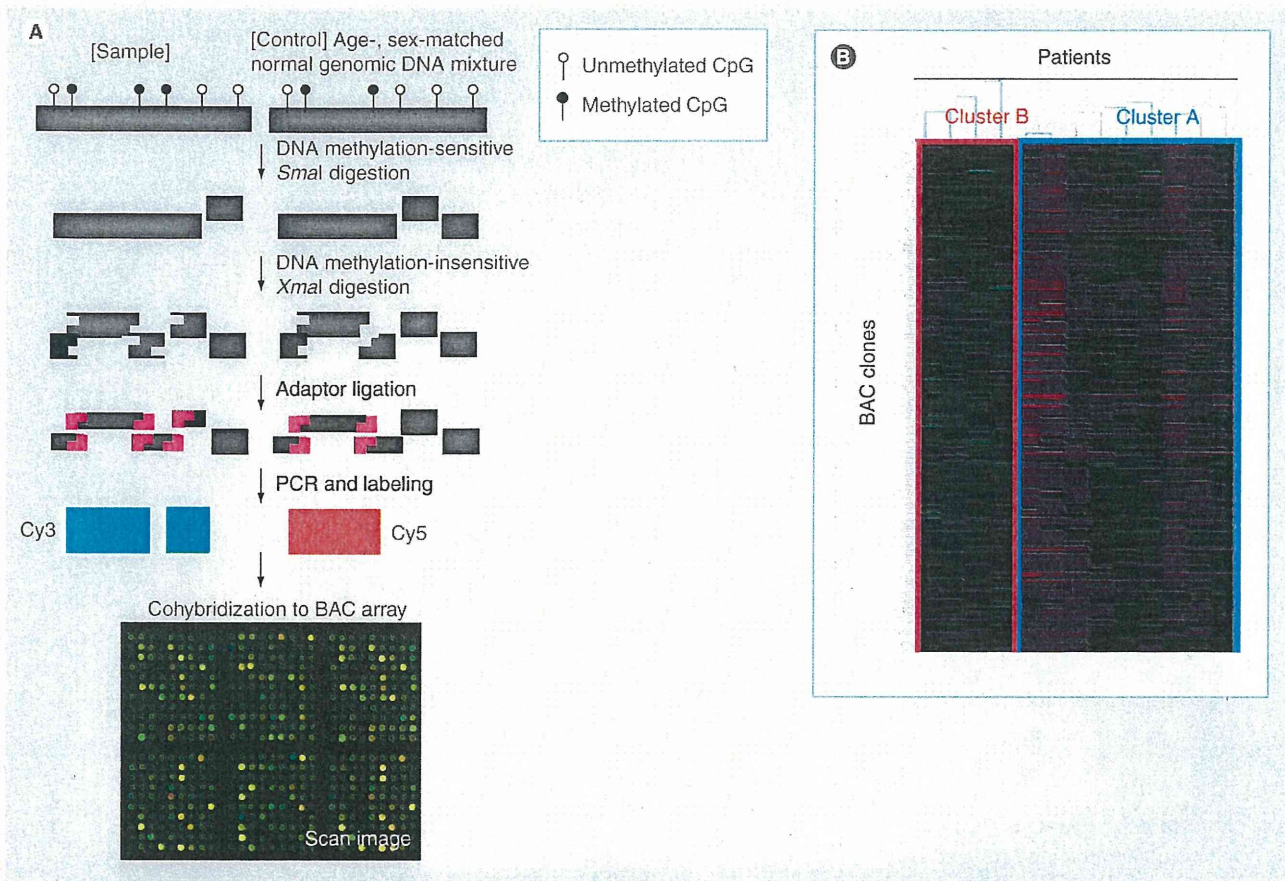


Figure 1. Bacterial artificial chromosome array-based methylated CpG island amplification in tissue samples.

(A) BAC array-based methylated CpG island amplification (BAMCA) method and an example of a scan image. **(B)** An example of unsupervised 2D hierarchical clustering analysis of patients with cancers based on BAMCA data. Patients with cancers are frequently clustered into subclasses associated with distinct DNA methylation profiles and distinct clinicopathological parameters. BAC: Bacterial artificial chromosome.

a BAC clone simultaneously showed lower or higher DNA methylation rates in comparison with those of normal tissues by pyrosequencing, the signal ratio on the BAC clone demonstrated by BAMCA showed DNA hypo- or hyper-methylation, respectively [55]. BAMCA has also been used by other groups to identify tumor-related genes whose expression levels are regulated by DNA methylation in human cancer cells [56–58].

Genome-wide DNA methylation profiles in precancerous stages are inherited by cancers & determine the tumor aggressiveness

With respect to renal cell carcinomas (RCCs), DNA methylation profiling of more than 800 genes has been reported in both Von Hippel Lindau-related and -unrelated RCCs, and it is known that genes linked to TGF- β or ERK/Akt signaling are preferentially methylated in papillary RCCs in comparison to clear cell RCCs [59]. However, precancerous

conditions in the kidney have rarely been described because of the lack of any remarkable histological changes, or association with chronic inflammation and persistent infection with viruses or other pathogenic microorganisms. The DNA methylation status of non-cancerous renal tissues obtained from patients with RCCs has not been analyzed in detail. When BAMCA methods were applied to 51 samples of noncancerous renal tissue obtained from patients with clear cell RCCs, many BAC clones showed DNA hypo- or hyper-methylation in comparison to normal renal tissue samples from patients without any primary renal tumors [60]. From the viewpoint of DNA methylation, we can consider that noncancerous renal tissue from patients with RCCs is already at precancerous stages, showing genome-wide DNA methylation alterations [60].

We then performed unsupervised 2D hierarchical clustering analysis based on BAMCA data for noncancerous renal tissue samples. The

patients with RCCs were clustered into two subclasses, clusters A_{NK} and B_{NK} [60]. Tumors developing in individual patients belonging to cluster B_{NK} were clinicopathologically more aggressive than those in cluster A_{NK} . The overall survival rate of patients in cluster B_{NK} was significantly lower than that of patients in cluster A_{NK} . Aggressiveness of the corresponding tumors and even patient outcome may thus be determined by DNA methylation profiles at precancerous stages.

Renal cell carcinomas are usually well demarcated, being surrounded by a fibrous capsule, and hardly ever contain a fibrous stroma. Therefore, we were able to obtain cancer cells of high purity from fresh surgical specimens, avoiding contamination with both noncancerous epithelial cells and stromal cells. When we analyzed 51 samples of RCC (30, 16 and 5 patients at Stage I to II, III and IV, respectively), more BAC clones showed DNA hypo- or hyper-methylation, and its degree was increased in comparison with samples of noncancerous renal tissue obtained from patients with RCCs. Unsupervised 2D hierarchical clustering analysis based on BAMCA data for RCCs was able to group patients into two subclasses, clusters A_{TK} and B_{TK} [60]. Clinicopathologically aggressive tumors were accumulated in cluster B_{TK} . The overall survival rate of patients in cluster B_{TK} was significantly lower than that of patients in cluster A_{TK} .

All patients who were grouped in cluster B_{NK} on the basis of BAMCA data for noncancerous renal tissue were included in cluster B_{TK} on the basis of BAMCA data for the RCCs themselves [60]. The majority of the BAC clones significantly discriminating cluster B_{NK} from cluster A_{NK} also discriminated cluster B_{TK} from cluster A_{TK} . Among BAC clones characterizing both clusters B_{NK} and B_{TK} , where the average signal ratio of cluster B_{NK} was higher than that of cluster A_{NK} , the average signal ratio of cluster B_{TK} was also higher than that of cluster A_{TK} without exception (FIGURE 2A). Among BAC clones characterizing both clusters B_{NK} and B_{TK} , where the average signal ratio of cluster B_{NK} was lower than that of cluster A_{NK} , the average signal ratio of cluster B_{TK} was also lower than that of cluster A_{TK} without exception (FIGURE 2A). Comparison between the signal ratios of each BAC clone characterizing both clusters B_{NK} and B_{TK} revealed that the DNA methylation status of the noncancerous renal tissue was basically inherited by the corresponding RCC in each individual patient (FIGURE 2B) [60].

In noncancerous renal tissue showing no remarkable histological changes and consisting mainly of renal tubules with specialized functions, no progenitor cell is able to gain a growth advantage, and clonal expansion is unable to occur. Therefore, the distinct DNA methylation profiles of cluster B_{NK} cannot be established through the selection of one of a number of random DNA methylation profiles in noncancerous renal tissue, and instead may be established through distinct target mechanisms. Since the DNA methylation profiles in cluster B_{TK} are shared by phenotypically similar patients, who all suffer from clinicopathologically aggressive tumors and show a poor outcome, DNA methylation alterations in at least a proportion of the BAC regions characterizing cluster B_{TK} cannot be passenger changes. DNA methylation alterations of BAC regions characterizing cluster B_{TK} may significantly participate in carcinogenesis, since the DNA methylation profiles in cluster B_{NK} were established at very early stages of carcinogenesis and inherited during progression of the cancers themselves as cluster B_{TK} . At least a proportion of DNA methylation alterations at precancerous stages may be 'epigenetic gatekeepers' [2], which allow time for further epigenetic and genetic alterations.

In fact, when the DNA methylation status on CpG islands of the *p16*, *hMLH1*, *VHL* and *THBS-1* genes, and the methylated in tumor (MINT)-1, -2, -12, -25 and -31 clones were examined by bisulfite modification in the same cohort, genome-wide DNA methylation alterations consisting of both hypo- and hyper-methylation revealed by BAMCA in cluster B_{TK} were frequently associated with accumulation of regional DNA hypermethylation on these CpG islands [61]. For comparison with BAMCA data, we also examined copy number alterations by array-based comparative genomic hybridization using the same BAC array in the same cohort. By unsupervised hierarchical clustering analysis based on copy number alterations, RCCs were clustered into the two subclasses, clusters GA_{TK} and GB_{TK} [62]. Copy number alterations were accumulated in the cluster GB_{TK} . Loss of chromosome 3p and gain of 5q and 7 were frequent in both clusters GA_{TK} and GB_{TK} , whereas loss of 1p, 4, 9, 13q and 14q was frequent only in cluster GB_{TK} [62]. Clear cell RCCs showing higher histological grades, vascular involvement, renal vein tumor thrombi and higher pathological stages were accumulated in cluster GB_{TK} [62]. The recurrence-free and overall survival rates of patients in cluster GB_{TK} were

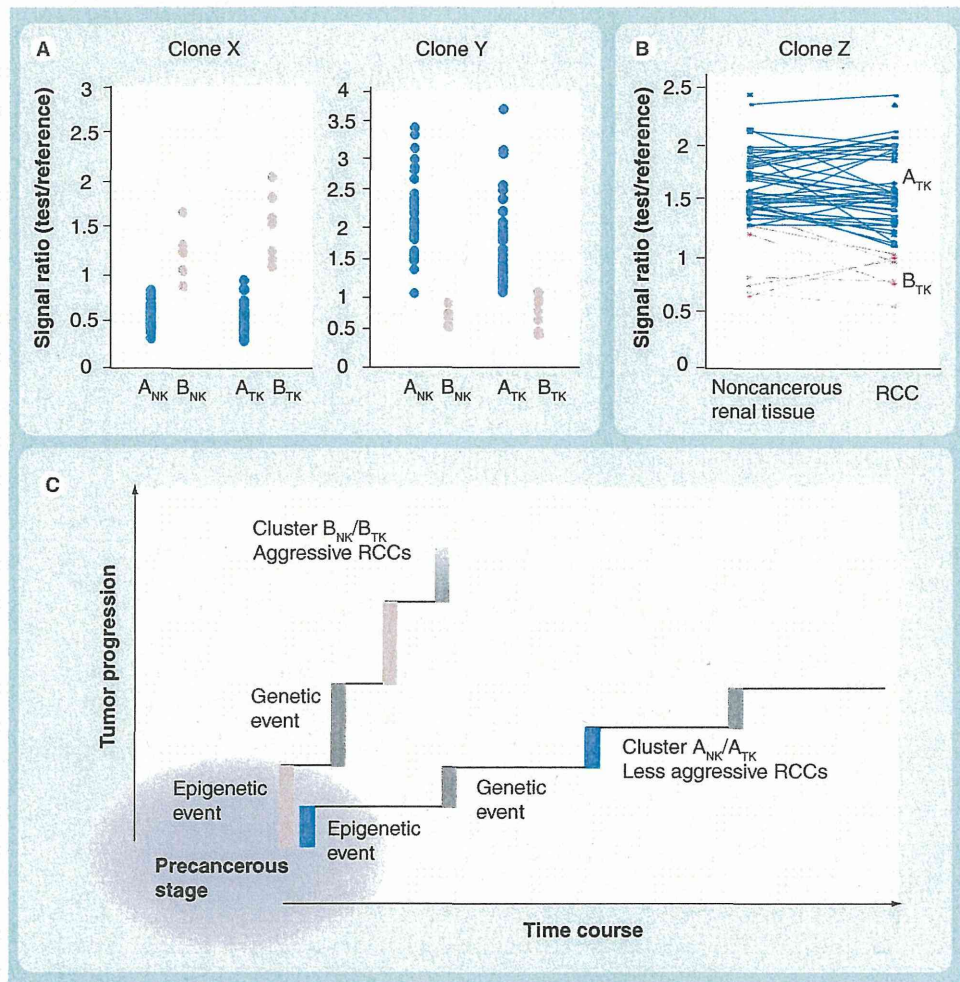


Figure 2. DNA methylation profiles in precancerous conditions and renal cell carcinomas. (A) Correlation between DNA methylation profiles of precancerous conditions and those of RCCs. Cluster B_{NK} was completely included in cluster B_{TK}. The majority of the bacterial artificial chromosome (BAC) clones, 724 in all, significantly discriminating cluster B_{NK} from cluster A_{NK}, also discriminated cluster B_{TK} from cluster A_{TK}. In 311 of the 724 BAC clones, where the average signal ratio of cluster B_{NK} was higher than that of cluster A_{NK}, such as clone X, the average signal ratio of cluster B_{TK} was also higher than that of cluster A_{TK} without exception. In 413 of the 724 BAC clones, where the average signal ratio of cluster B_{NK} was lower than that of cluster A_{NK}, such as clone Y, the average signal ratio of cluster B_{TK} was also lower than that of cluster A_{TK} without exception. (B) Scattergram of the signal ratios in samples of noncancerous renal tissue and RCCs for all patients examined for a representative BAC clone, clone Z. The DNA methylation status of the noncancerous renal tissue was basically inherited by the corresponding RCC in individual patients. (C) Significance of DNA methylation alterations at precancerous stages. Regional DNA hypermethylation of C-type CpG islands and copy number alterations were accumulated in cluster B_{TK}. In other words, DNA methylation alterations in precancerous conditions, such as DNA methylation profiles corresponding to cluster B_{NK}, may not occur randomly but may be prone to further accumulation of epigenetic and genetic alterations, thus generating more malignant cancers, such as the RCCs in patients belonging to cluster B_{TK}. RCC: Renal cell carcinoma.

significantly lower than those of patients in cluster GA_{TK} [62]. Multivariate analysis revealed that genetic clustering was a predictor of recurrence-free survival, and was independent of histological grade and pathological stage [62]. A subclass of cluster B_{TK} based on BAMCA data was completely included in cluster GB_{TK}

showing accumulation of copy number alterations. Genetic and epigenetic alterations are not mutually exclusive during renal carcinogenesis, and particular DNA methylation profiles may be closely related to chromosomal instability. DNA methylation alterations at precancerous stages, which may not occur randomly but may

foster further epigenetic and genetic alterations, can generate more malignant cancers and even determine patient outcome (FIGURE 2C).

Even though high-throughput detection technologies have recently been developed, the dynamics of DNA methylation at repetitive sequences and gene bodies still remain to be determined [7]. However, our BAC array-based methods do not focus only on specific promoter regions and CpG islands, and have successfully identified the BAC regions including non-unique sequences in which coordinated DNA methylation alterations have clinicopathological impact. Evaluation of the correlation between the DNA methylation status of identified repetitive sequences and gene bodies, and the clinicopathological diversity of cancers may provide new insights into the roles of DNA methylation during multistage carcinogenesis.

Carcinogenetic risk estimation based on DNA methylation profiles

With respect to HCCs, the results of analysis of DNA methylation status of CpG islands of multiple genes have been reported. Some groups have attempted to use DNA methylation profiles as molecular markers of early HCCs or as prognostic indicators for patients with HCCs [63,64]. Since BAMCA detects DNA methylation alterations that are regulated in a coordinated manner at multiple CpG sites in individual large regions of chromosomes, BAMCA may be able to identify unique diagnostic indicators that may be overlooked by other technologies. We then applied the BAMCA method to multistage hepatocarcinogenesis. HCCs are known to be medullary tumors without a fibrous stroma. Therefore, we were able to obtain cancer cells of high purity from fresh surgical specimens, avoiding any contamination with stromal cells. In samples of noncancerous liver tissue obtained from patients with HCCs, many BAC clones showed DNA hypo- or hyper-methylation in comparison with normal liver tissue from patients without HCCs. Patients showing DNA hypo- or hyper-methylation on more BAC clones in their samples of noncancerous liver tissue frequently developed metachronous or recurrent HCCs after hepatectomy [65], suggesting that DNA methylation alterations at precancerous stages may not occur randomly but tend to lead to the development of more malignant HCCs.

The effectiveness of surgical resection for HCC is limited, unless the disease is diagnosed early at the asymptomatic stage. Therefore, surveillance at precancerous stages is a priority. To

reveal the baseline liver histology, microscopy examination of liver biopsy specimens is performed for patients with hepatitis virus infection prior to interferon therapy. Therefore, carcinogenetic risk estimation using such liver biopsy specimens would be advantageous for close follow-up of patients who are at high risk of HCC development.

Inflammation itself can induce drastic DNA methylation alterations at the chronic hepatitis stage. Although a proportion of such alterations do participate in progression to HCC, the remaining inflammation-associated DNA methylation alterations may diminish after the hepatitis stage has passed and as HCC develops. DNA methylation alterations associated only with inflammation and not with carcinogenesis cannot be regarded as indicators for carcinogenetic risk estimation in patients who are being followed up owing to chronic hepatitis. Therefore, to estimate the degree of carcinogenetic risk based on DNA methylation profiles, we focused on BAC clones for which DNA methylation status was altered at the chronic hepatitis and liver cirrhosis stages and were inherited by HCCs from such precancerous stages. Among them, a bioinformatics approach identified the top 25 BAC clones for which DNA methylation status was able to discriminate 15 samples of noncancerous liver tissue from patients with HCCs in the learning cohort from 10 samples of normal liver tissue with sufficient sensitivity and specificity. We established the criteria for carcinogenetic risk estimation by combining the cutoff values of signal ratios for the 25 BAC clones (FIGURE 3A). Based on these criteria, the sensitivity and specificity for diagnosis of noncancerous liver tissue samples obtained from patients with HCCs in the learning cohort as being at high risk of carcinogenesis were both 100% [65]. Our criteria enabled diagnosis of additional noncancerous liver tissue samples obtained from patients with HCCs in the validation cohort ($n = 50$) as being at high risk of carcinogenesis with a sensitivity and specificity of 96% [65].

The number of BAC clones satisfying the criteria in noncancerous liver tissue samples showing chronic hepatitis obtained from patients with HCCs was not significantly different from that in noncancerous liver tissue samples showing cirrhosis obtained from patients with HCCs. In addition, the average number of BAC clones satisfying the criteria was significantly lower in samples of liver tissue obtained from patients who were infected with HBV or HCV, but who had never developed HCCs, than that in noncancerous liver tissue samples obtained from

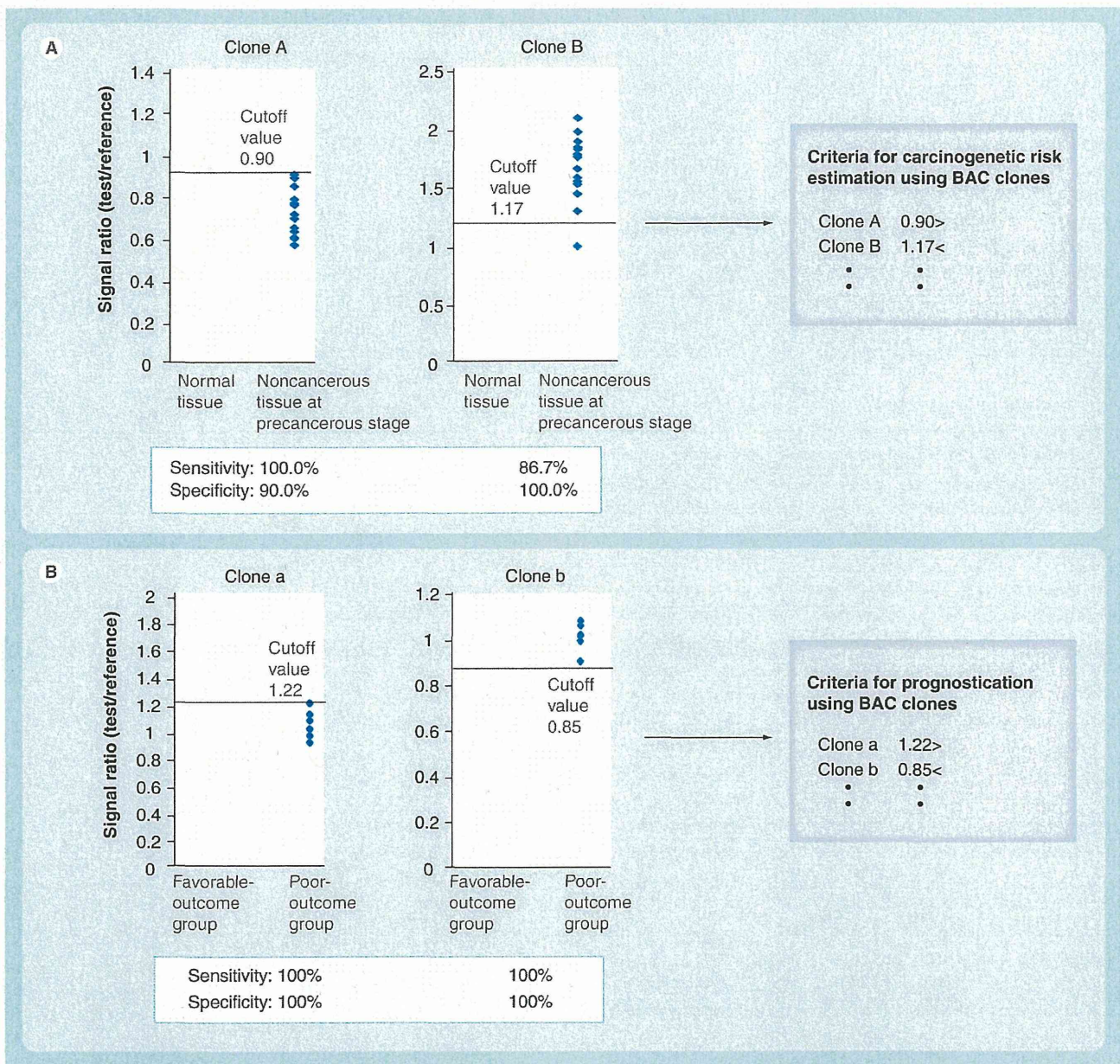


Figure 3. Carcinogenetic risk estimation and prognostication of patients with cancers based on DNA methylation status. (A) Carcinogenetic risk estimation based on DNA methylation status. Examples of scattergrams of the signal ratios in normal tissue samples and noncancerous tissue samples at precancerous stages for representative BAC clones, clone A and clone B. Using the cutoff values in each panel, noncancerous tissue samples at precancerous stages were discriminated from normal tissue samples with sufficient sensitivity and specificity. Based on a combination of the cutoff values for the selected BAC clones, the criteria for carcinogenetic risk estimation were established. (B) Prognostication of patients with cancers based on DNA methylation status. Examples of scattergrams of the signal ratios in the favorable-outcome group and poor-outcome group for representative BAC clones, clone a and clone b. Using the cutoff values in each panel, patients belonging to the poor-outcome group were discriminated from those belonging to the favorable-outcome group. Based on a combination of the cutoff values for the selected BAC clones, the criteria for prognostication were established. BAC: Bacterial artificial chromosome.

patients with HCCs. Therefore, our criteria may be applicable for classifying liver tissue obtained from patients who are followed up because of hepatitis virus infection, chronic hepatitis or liver cirrhosis into that which may generate HCCs and that which will not [65].

Next, we quantitatively examined the DNA methylation status at each *XmaI/SmaI* site on the 25 BAC clones by pyrosequencing. The sensitivity and specificity of the criteria revised by pyrosequencing have been successfully improved by using CpG sites having the largest diagnostic

impact on each BAC clone. It has been validated that screening by BAMCA, which has an ability for detecting any tendency for coordinated regulation of DNA methylation at multiple CpG sites in the entire BAC region, followed by revision using pyrosequencing, is a promising approach for carcinogenetic risk estimation. Pyrosequencing can be performed using a very small amount of degraded DNA extracted from liver biopsy specimens. In other words, unless another approach such as pyrosequencing is used to validate BAMCA data, risk assessment of liver biopsy specimens based only on BAMCA is premature. We now intend to validate the reliability of such risk estimation prospectively using liver biopsy specimens obtained prior to interferon therapy from a large cohort of patients with HBV or HCV infection.

Urothelial carcinomas are clinically remarkable because of their multicentricity owing to the 'field effect', whereby carcinogenic agents in the urine cause malignant transformation of multiple urothelial cells [66]. 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 (FIGURE 4A) [66]. Superficial papillary carcinomas usually remain noninvasive, although patients need to undergo repeated urethroscoposcopy because of recurrences. By contrast, the clinical outcome of nodular invasive carcinoma is poor. In our previous study, accumulation of DNA methylation on C-type CpG islands associated with DNMT1 overexpression was observed even in noncancerous urothelia obtained from patients with UCs, and was further increased especially in nodular invasive carcinomas [67,68]. These previous data suggest that carcinogenetic risk estimation of UCs based on DNA methylation status might be a promising strategy.

We carefully took the tissue specimens from the surface of elevated UC lesions to avoid contamination with noncancerous urothelial and stromal cells. Principal component analysis based on BAMCA data revealed that stepwise DNA methylation alterations from 17 samples of noncancerous urothelia obtained from patients with UCs to 40 samples of UCs, in comparison with 18 samples of normal urothelia, occurred in a genome-wide manner [55]. We then performed unsupervised 2D hierarchical clustering

analysis based on BAMCA data for noncancerous urothelia. The examined patients with UCs were clustered into two subclasses, clusters A_{NU} and B_{NU} . The incidence of invasive UCs (pT2 or more) was significantly higher in patients belonging to cluster B_{NU} defined on the basis of DNA methylation status in their noncancerous urothelia in comparison to cluster A_{NU} [55]. Moreover, Wilcoxon test identified the BAC clones whose signal ratios 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). DNA methylation profiles on such BAC clones of noncancerous urothelia obtained from patients with invasive UCs were inherited by the invasive UCs themselves (FIGURE 4B) [55]. DNA methylation alterations that were correlated with the development of more malignant invasive cancers were already accumulated in noncancerous urothelia.

To estimate the degree of carcinogenetic risk based on DNA methylation profiles in noncancerous urothelia, 83 BAC clones whose signal ratios discriminated noncancerous urothelia obtained from patients with UCs from normal urothelia with a sensitivity and specificity of 75% or more than 75% were identified. We established the criteria for carcinogenetic risk estimation by combining the cutoff values of the signal ratios for these 83 BAC clones [55]. We are currently attempting to develop a methodology for assessing the tendency of DNA methylation in the 83 BAC regions in urine samples to make such risk estimation applicable to 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.

Approximately 10–30% of patients with UCs of the renal pelvis and ureter develop intravesical metachronous UCs after nephroureterectomy [69]. Therefore, such patients need to undergo repeated urethroscoposcopy examinations to detect intravesical metachronous UCs. To decrease the need for invasive urethroscoposcopy examinations and assist close follow-up of such patients after nephroureterectomy, indicators for intravesical metachronous UCs have been needed. Since such metachronous UC originates from the noncancerous urothelium of the urinary bladder, we focused on the DNA methylation status of noncancerous urothelia, which may be exposed to the same carcinogens

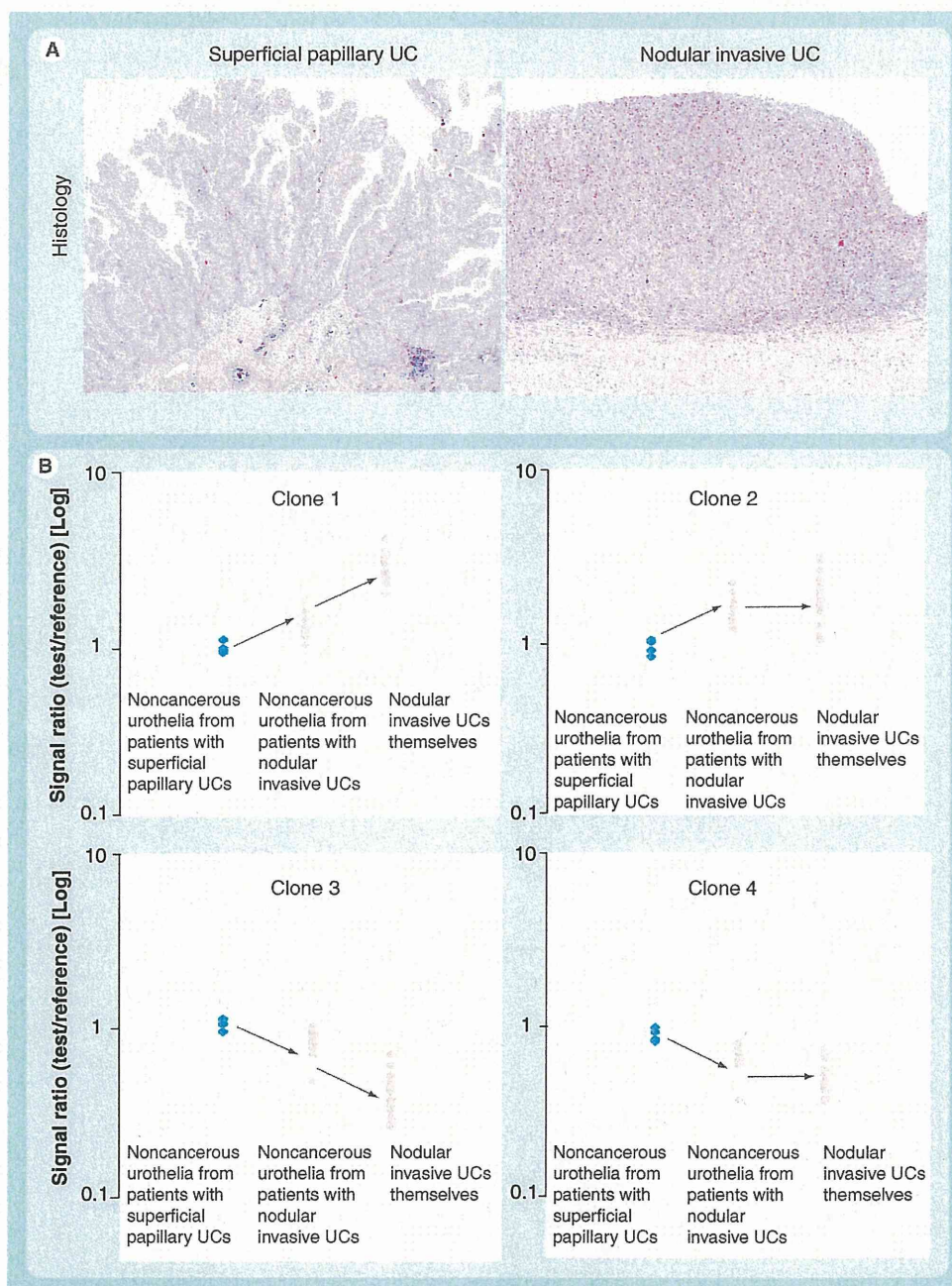


Figure 4. Significance of DNA methylation alterations at precancerous stages during urothelial carcinogenesis. For legend please see facing page.

in the urine, obtained by nephroureterectomy from patients with UCs of the renal pelvis or ureter. Unsupervised 2D hierarchical clustering analysis based on BAMCA data for noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter was able to group the examined patients into two subclasses, clusters A_{NP} and B_{NP} . The patients in cluster B_{NP} frequently developed intravesical metachronous UCs, whereas none belonging to cluster A_{NP} did so [55], indicating that DNA methylation

profiles of noncancerous urothelia obtained by nephroureterectomy from patients with UCs of the renal pelvis or ureter are correlated with the risk of intravesical metachronous UC development. We identified nine BAC clones whose signal ratios discriminated noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter who developed intravesical metachronous UC after nephroureterectomy from noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter who did

Figure 4. Significance of DNA methylation alterations at precancerous stages during urothelial carcinogenesis. (A) Histological features of superficial papillary UC and nodular invasive UC. Superficial papillary carcinomas usually remain noninvasive, although patients need to undergo repeated urethrocystoscopic resection because of recurrences. By contrast, the clinical outcome of nodular invasive carcinomas is poor. **(B)** Scattergrams of the signal ratios in noncancerous urothelia obtained from patients with superficial UCs, noncancerous urothelia obtained from patients with invasive UCs, and invasive UCs themselves. Wilcoxon test revealed that the signal ratios of 131 bacterial artificial chromosome (BAC) clones differed significantly between noncancerous urothelia obtained from patients with superficial UCs and noncancerous urothelia obtained from patients with invasive UCs. 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 invasive UCs themselves were even higher than (42 BAC clones such as clone 1), or not significantly different from (25 BAC clones such as clone 2), 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 invasive UCs themselves were even lower than (38 BAC clones such as clone 3), or not significantly different from (26 BAC clones such as clone 4), those in noncancerous urothelia obtained from patients with invasive UCs without exception. Therefore, DNA methylation profiles of noncancerous urothelia obtained from patients with invasive UCs were inherited by the invasive UCs themselves. UC: Urothelial carcinoma.

not with a sensitivity and specificity of 100% [55]. Thus, after validation using other technologies such as pyrosequencing, a combination of CpG sites on the present nine BAC clones may provide an optimal indicator for the development of intravesical metachronous UC.

Prognostication of patients with cancers based on DNA methylation profiles

Some RCCs relapse and metastasize to distant organs, even if resection has been considered complete. Recently, immunotherapy and novel targeting agents have been developed for treatment of RCC. However, unless relapsed or metastasized tumors are diagnosed early by close follow-up, the effectiveness of any therapy is very restricted. Therefore, to assist close follow-up of patients who have undergone nephrectomy and are still at risk of recurrence and metastasis, prognostic indicators have been explored. Among the examined patients in the abovementioned cluster B_{TK}, 38% died owing to recurrent RCCs, whereas only 2.3% of the patients in cluster A_{TK} died. Multivariate analysis revealed that our clustering was a predictor of recurrence and was independent of histological grade, macroscopic configuration, vascular involvement and renal vein tumor thrombi [60]. We were able to set the cutoff values of the signal ratios for 14 BAC clones to determine whether or not patients in this cohort belonged to cluster B_{TK} with a sensitivity and specificity of 100% [60].

To establish criteria for prognostication of patients with HCCs, in the learning cohort, HCC samples obtained from patients who had survived more than 4 years after hepatectomy and

HCC samples obtained from patients who had suffered recurrence within 6 months and died within a year after hepatectomy were defined as a favorable-outcome group and a poor-outcome group, respectively. Wilcoxon test revealed that the signal ratios of 41 BAC clones differed significantly between the two groups (n = 19). We established the criteria for prognostication by combining the cutoff values of signal ratios for the 41 BAC clones (FIGURE 3B) [65]. Multivariate analysis revealed that satisfying the criteria for 32 or more BAC clones was a predictor of recurrence, and was independent of histological differentiation, portal vein tumor thrombi, intrahepatic metastasis and multicentricity [65]. The cancer-free and overall survival rates of patients with HCCs in the validation cohort (n = 44) satisfying the criteria for 32 or more BAC clones were significantly lower than those of patients with HCCs satisfying the criteria for less than 32 BAC clones [65]. Such prognostication using biopsy or hepatectomy specimens may be able to assist clinicians in devising therapeutic strategies for patients with insufficient liver function.

Recently, new forms of systemic chemotherapy and targeted therapy have been developed for treatment of UCs. 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. It is expected that a combination of several CpG islands of tumor-related genes would be useful as epigenetic markers for prognostication of UCs [70]. In addition, when we applied BAMCA to UCs, unsupervised 2D hierarchical clustering analysis based on BAMCA data for UCs was

able to group the examined patients into two subclasses, clusters A_{TU} and B_{TU} . Among the patients belonging to cluster B_{TU} , 19% suffered recurrence after surgery, whereas none belonging to cluster A_{TU} did so [55]. Wilcoxon test revealed that the signal ratios of 20 BAC clones in UCs differed significantly between the patients who suffered recurrence after surgery and the patients who did not. The criteria for a combination of the 20 BAC clones were able to discriminate patients who suffered recurrence after surgery from patients who did not with a sensitivity and specificity of 100%, whereas a high histological grade, invasive growth (pT2 or more) and vascular or lymphatic involvement were incapable of such complete discrimination [55]. The reliability of such prognostication will need to be validated in a prospective study.

Future perspective

The incidence of DNA methylation alterations is generally high in various organs during multistage carcinogenesis. Since even subtle alterations of DNA methylation profiles at the precancerous stage are stably preserved on DNA

double strands by covalent bonds, and these can be detected using highly sensitive methodology. Therefore, they may be better diagnostic indicators than mRNA and protein-expression profiles, which can be easily affected by the micro-environment of cancer cells or precursor cells. Genome-wide DNA methylation profiling can provide indicators for carcinogenetic risk estimation and prognostication using samples of urine, sputum and other body fluids, and also biopsy and surgically resected specimens. However, exploitation of diagnostic indicators can never be regarded as optimal, and it is expected that ongoing technical innovation and prospective validation will lead to further improvements of diagnostic sensitivity and specificity.

Patients with cancers are frequently clustered into subclasses showing both distinct genome-wide DNA methylation profiles and distinct clinicopathological characteristics (FIGURE 1B). Such clustering of cancers may provide clues for clarification of the molecular mechanisms establishing the distinct DNA methylation profiles of each cluster and the identification of target molecules for prevention and therapy in

Introduction

- Human cancer cells show a drastic change in DNA methylation status, that is overall DNA hypomethylation and regional DNA hypermethylation.
- DNA methylation alterations are known to result in altered expression of tumor-related genes and chromosomal instability in human cancers.

DNA methylation alterations during multistage carcinogenesis

- DNA methylation alterations play a significant role even at the precancerous stage, especially in association with chronic inflammation and persistent infection with viruses, such as hepatitis B virus or hepatitis C virus.
- DNA methyltransferase 1 overexpression in cancers is frequently correlated with accumulation of DNA methylation of tumor-related genes and poorer patient outcome.

Genome-wide DNA methylation analysis

- For genome-wide analysis, microarray platforms are used in combination with DNA methylation-sensitive restriction enzyme-based or antimethyl-cytosine antibody affinity techniques, and new generation sequencing technologies are also being introduced.
- Bacterial artificial chromosome array-based methylated CpG island amplification (BAMCA) may be suitable for overviewing the DNA methylation tendency of individual large regions among all chromosomes.

Genome-wide DNA methylation profiles at precancerous stages are inherited by cancers & determine tumor aggressiveness

- Distinct DNA methylation profiles in noncancerous tissue at the precancerous stage is basically inherited by the cancer developing in each individual patient.
- DNA methylation alterations at the precancerous stage, which may not occur randomly but may foster further epigenetic and genetic alterations, can generate more malignant cancers and even determine patient outcome.

Carcinogenetic risk estimation based on DNA methylation profiles

- On the basis of BAMCA data, criteria for estimation of the risk of hepatocellular carcinoma and urothelial carcinoma development have been established.

Prognostication of patients with cancers based on DNA methylation profiles

- On the basis of BAMCA data, criteria for the prognostication of patients with renal cell carcinomas, hepatocellular carcinomas and urothelial carcinomas have been established.

Future perspective

- Genome-wide DNA methylation profiling can provide indicators for carcinogenetic risk estimation and prognostication using samples of body fluids and tissue specimens.
- Based upon genome-wide DNA methylation profiling, translational epigenetics has come of age.

patients belonging to each cluster. Based upon genome-wide DNA methylation profiling, translational epigenetics has clearly come of age.

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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- 66 Indicators for carcinogenetic risk estimation in chronically diseased liver, and prognostication of patients with hepatocellular carcinomas, have been established based on BAMCA data.

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 multi-stage 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 carcinogenic 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 urethroscopic 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 carcinogenic 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 \pm 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 \pm 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 \pm 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- μ g 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 hyperme-

thylation, 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 , $P = 4.37e-9$). 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 , $P = 7.35e-9$). 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 (pT_a 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

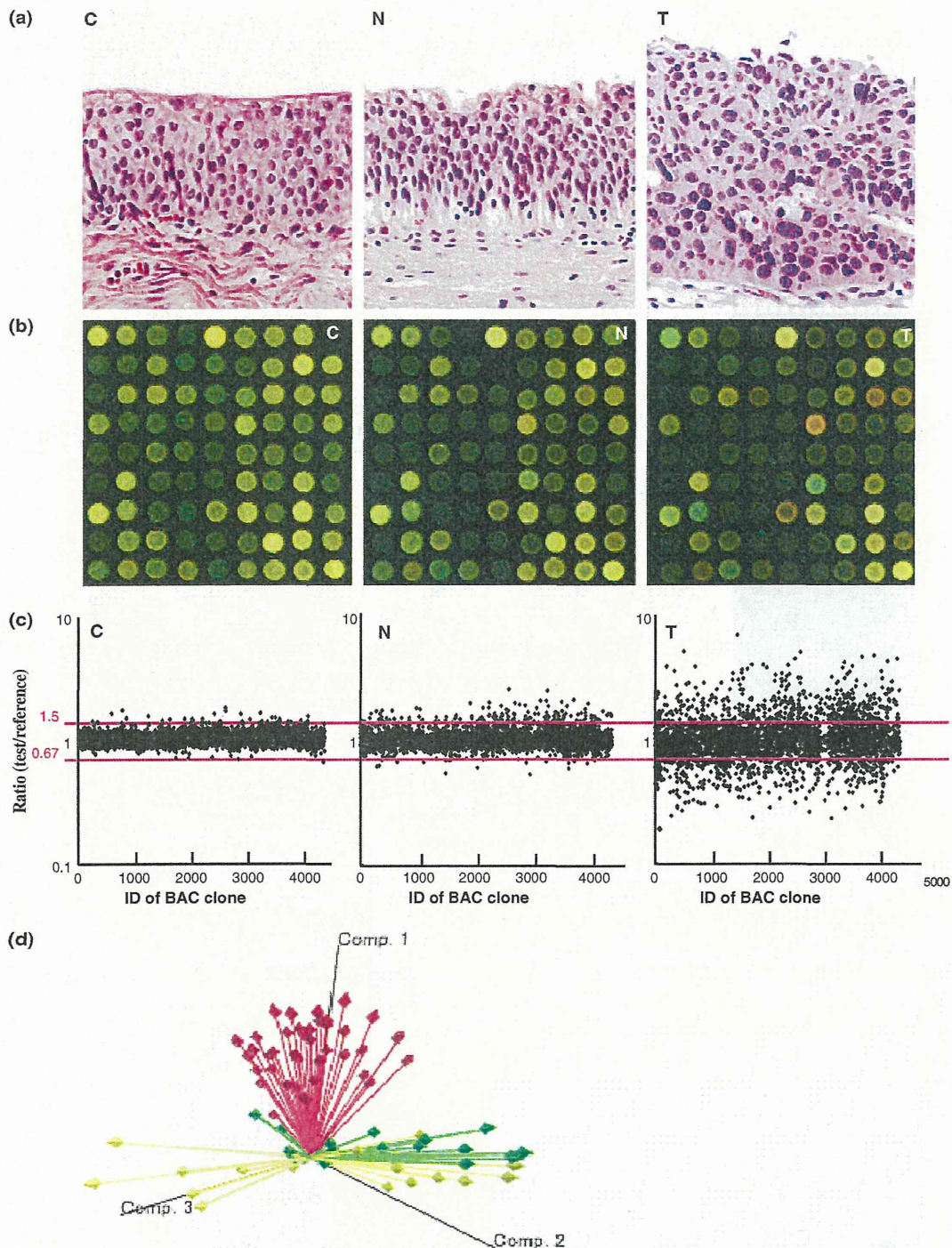


Fig. 1. DNA methylation alterations during multistage urothelial carcinogenesis. (a) Microscopic view of normal urothelium obtained from a patient without urothelial carcinoma (UC) (C), noncancerous urothelium obtained from a patient with UC (N), and UC (T). N shows no remarkable histological changes in comparison to C, that is no cytological or structural atypia is evident. Hematoxylin–eosin staining. Original magnification, $\times 20$. (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.