rofibromin 2 (NF2) gene, encoding marlin protein that is similar to the ERM (ezrin, radixin, moesin) family members that link cytoskeletal components and the cell membrane, have been reported in clear cell RCCs. Since none of the samples of clear cell RCC with the *NF2* mutation harbored a *VHL* mutation, it has been suggested that somatic *NF2* mutations may account for a proportion of cases in this subset [15].

Genetic clustering of clear cell RCCs

Since the genetic backgrounds of RCCs have not been fully understood to date, we have analyzed copy number alterations by array-comparative genomic hybridization (CGH) using a custom-made bacterial artificial chromosome (BAC) array (MCG Whole Genome Array-4500) harboring 4361 BAC clones throughout chromosomes 1 to 22 and X and Y clones [16] in clinical tissue samples (**Figure 2A**), and clarified the genetic clustering of clear cell RCCs [17]. RCC is usually enclosed within a fibrous capsule and well demarcated, and hardly ever contains fibrous stroma between the cancer cells. Therefore, we were able to obtain cancer cells of high purity from surgical specimens, avoiding contamination with both non-cancerous epithelial cells and stromal cells. By unsupervised hierarchical clustering analysis of RCCs based on array-CGH data, clear cell RCCs were clustered into the two subclasses, Clusters A_{TG} and B_{TG} (**Figure 2B**). In clear cell RCCs, the average number of BAC clones on which loss or gain was detected was significantly higher in Cluster B_{TG} than in Cluster A_{TG}. In both clusters, loss or gain of an entire chromosome or an entire chromosome arm was frequent. Loss of chromosome 3p and gain of chromosomes 5q and 7 were frequent in both Clusters A_{TG} and B_{TG}. On the other hand, loss of chromosome 1p, 4, 9, 13q or 14q was frequent only in Cluster B_{TG}, but not in Cluster A_{TG} (**Figure 2C**). Gain on 1q31-ter, 3q and 8q was frequent only in Cluster B_{TG}, whereas loss at the same loci was observed in Cluster A_{TG}, although the frequency was rather low. The present genome-wide analysis indicated that loss of chromosome 3p and gain of 5q and 7 may be copy number aberrations that are indispensable for the development of clear cell RCCs, regardless of genetic clustering [17]. Additional loss of chromosome 1p, 4, 9, 13q or 14q may promote the genetic pathway to Cluster B_{TG} [17].

On the basis of microscopic examination of the entire tumor mass, the presence or absence of vascular involvement was evaluated in the examined clear cell RCCs. Macroscopic observation revealed the presence or absence of renal vein tumor thrombi. Clear cell RCCs in Cluster B_{TG} showed significantly higher histological grades and more frequently showed vascular involvement, renal vein tumor thrombi and higher pathological tumor-node-metastasis (TNM) stages than those in Cluster A_{TG}. Thus, accumulated genetic alterations may play a significant role in the more malignant potential of clear cell RCCs belonging to Cluster B_{TG}.

Even if resection has been considered complete, some RCCs relapse and metastasize to distant organs and can lead to death in middle-aged adults belonging to the working population. Unless relapsed or metastasized tumors are diagnosed early by close follow-up, the effectiveness of any adjuvant therapy is very restricted. Therefore, to assist the close follow-up of patients who have undergone nephrectomy and are still at risk of recurrence and metastasis, prognostic indicators should be explored. Recurrence or metastasis was observed in 40% of patients who underwent curative resection in Cluster B_{TG}, but in only 9% of patients who did so in Cluster A_{TG} [17]. The recurrence-free survival rate of patients in Cluster B_{TG} was significantly lower than that of patients in Cluster A_{TG}. Twenty-four% of the patients in Cluster B_{TG} died as a result, whereas none of the patients in Cluster A_{TG} died [17]. The overall survival rate of patients in Cluster B_{TG} was also significantly lower than that of patients in Cluster A_{TG} (**Figure 2D**). Multivariate analysis revealed that genetic clustering was a predictor of recurrence-free survival, and was independent of histological grade and pathological TNM stage. In addition, a sufficient quantity of good-quality DNA was obtainable from each nephrectomy specimen. Therefore, use of a mini-array harboring a set of BAC clones that can effectively discriminate Cluster B_{TG} after nephrectomy may be a promising method of prognostication.

Epigentic Alterations in RCCs

Epigenetics and cancers

In addition to genetic events, human cancer cells show drastic epigenetic alterations. DNA methylation, a covalent chemical modification

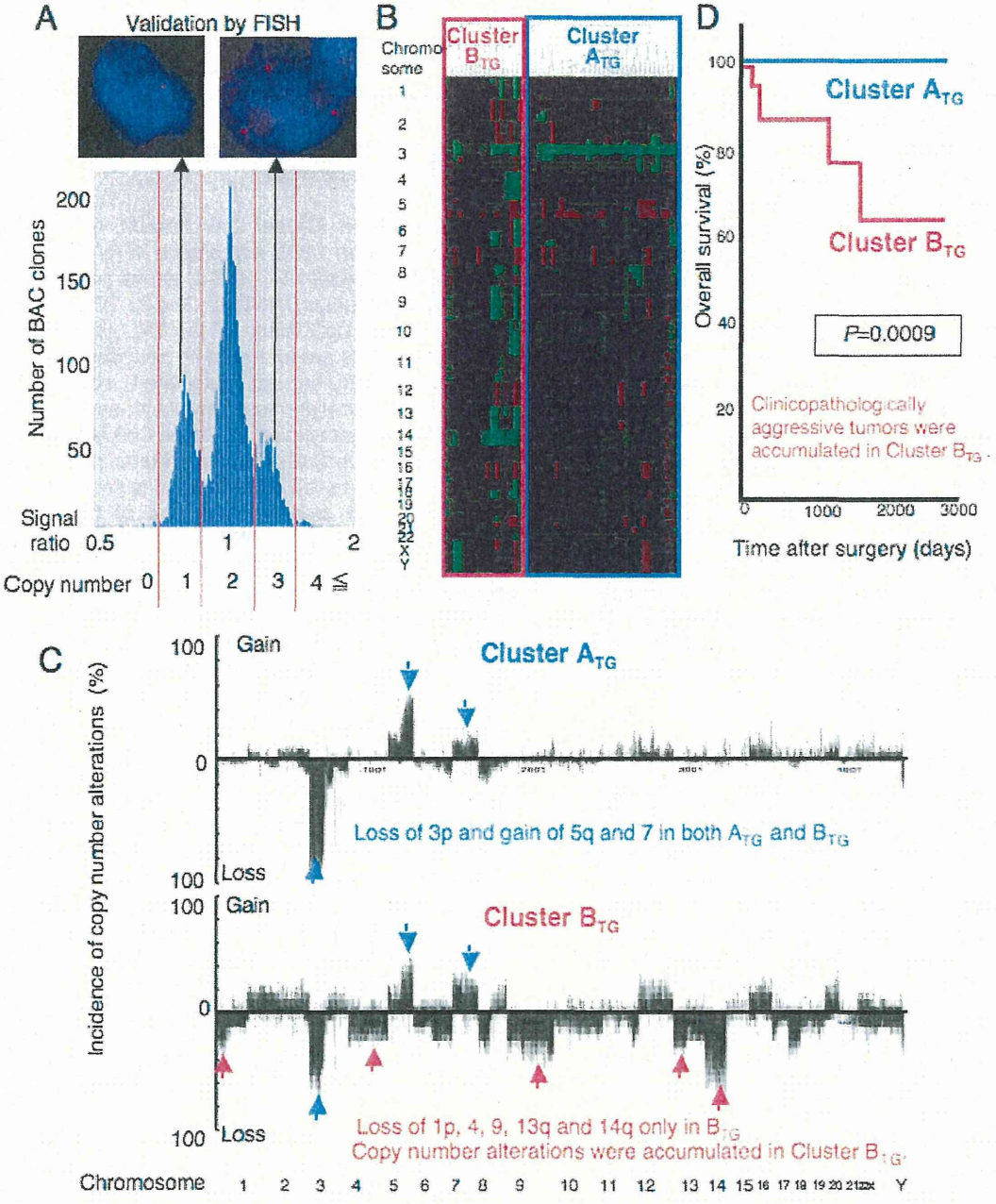

Figure 2. Genetic clustering of clear cell renal cell carcinomas (RCCs). An example of a histogram of the signal ratios (test signal/reference signal) afforded by array-CGH in a clear cell RCC. The thresholds of the signal ratios for copy numbers of 0, 1, 2, 3 and 4 or more were determined from the troughs (red bars) between the distinct peaks. **A.** FISH analysis using the same clone validated the results of array-CGH (ref. 17). **B.** Unsupervised hierarchical clustering analysis based on array-CGH data. Clear cell RCCs were grouped into Clusters A_{TG} and B_{TG} (ref. 17). **C.** Distinct copy number profiles in Clusters A_{TG} and B_{TG}. Loss of chromosome 3p and gain of 5q and 7 may promote the development of RCCs belonging to Cluster A_{TG} and showing a favorable outcome. When loss of 1p, 4, 9, 13q or 14q is added, more malignant RCCs in Cluster B_{TG} may develop (ref. 17). **D.** Kaplan-Meier survival curves based on genetic clustering of clear cell RCCs (Clusters A_{TG} and B_{TG}). None of the patients in Cluster A_{TG} died as a result, and the overall survival rate of patients in Cluster B_{TG} was significantly lower than that of patients in Cluster A_{TG} (Log-rank test, ref. 17).

resulting in 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 [18-20]. DNA methyltransferases (DNMTs) transfer methyl groups from S-adenosylmethionine to cytosines. DNA methylation normally promotes a highly condensed heterochromatin structure associated with deacetylation of histones H3 and H4. In addition, methylation of histone H3 lysine 4 (H3K4), H3K36 and H3K79 is connected with transcriptional activation, whereas methylation of H3K9, H3K27 and H4K20 has been connected with transcriptional repression [21]. 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 [22]. In human cancer cells, DNA hypomethylation induces chromosomal instability through decondensation of heterochromatin and enhancement of chromosomal recombination [23]. On the other hand, DNA hypermethylation of CpG islands around the promoter regions silences tumor-suppressor genes [24].

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 infection with viruses or other pathogenic microorganisms, such as hepatitis B or C viruses, Epstein-Barr virus, human papillomavirus and *Helicobacter pylori* [25-27]. For example, we have observed frequent regional DNA hypermethylation and/or DNMT1 overexpression in non-cancerous liver tissues showing chronic hepatitis or liver cirrhosis with hepatitis virus infection obtained from patients with hepatocellular carcinomas (HCCs) [28-32], and in non-cancerous pancreatic tissues showing chronic pancreatitis obtained from patients with pancreatic cancer [33,34]. Unlike cancers derived from such organs, renal tumors are not usually generated from a background of persistent viral infection and/or chronic inflammation. Although several factors such as smoking and obesity have been reported to be possible risk factors for renal tumors as mentioned above, pathologists hardly ever observe any histological change corresponding to such risk factors in non-tumorous renal tissue. Therefore, precancerous conditions in the kidney have been rarely described. Therefore we attempted to clarify the role of DNA methylation alterations during renal carcinogenesis.

Regional DNA hypermethylation in precancerous conditions and RCCs

We focused on C-type CpG islands of the *CDKN2A, human MutL homologue 1 (hMLH1)* and *thrombospondin-1 (THBS-1)* genes and the methylated in tumor (MINT)-1, -2, -12, -25 and -31 clones and CpG island of the *VHL* gene. C-type CpG islands are known to be methylated in a cancer-specific, but not age-related, manner. The cancer phenotype associated with accumulation of DNA methylation on C-type CpG islands is defined as the CpG-island methylator phenotype (CIMP), and such accumulation is generally associated with frequent silencing of tumor-related genes due to DNA hypermethylation only, or a two-hit mechanism involving DNA hypermethylation and loss of heterozygosity in human cancers of various organs [35]. Bisulfite conversion has been carried out using genomic DNA, and this process converts unmethylated cytosine residues to uracil, whereas methylated cytosine residues remain unchanged [36]. The DNA methylation status on CpG islands was determined by methylation-specific PCR (MSP) or combined bisulfite restriction enzyme analysis (COBRA). MSP is based on the principle that the DNA sequences of methylated and unmethylated genomic regions differ after bisulfite conversion and can thus be distinguished by sequence-specific PCR primers. In COBRA, bisulfite-modified DNA was amplified by PCR using primers designed to amplify methylated and unmethylated genomic regions equally. The amplified fragments were digested with restriction enzymes that cleave DNA only if the CpG sites in their recognition sequences are methylated.

Even in non-tumorous renal tissues showing no remarkable histological changes obtained from patients with renal tumors, the average number of methylated CpG islands was significantly higher than in normal renal tissues obtained from patients without any primary renal tumor, regardless of patient age [37]. Stepwise accumulation of DNA methylation on CpG islands from normal renal tissues, to non-tumorous renal tissues showing no remarkable histological changes obtained from patients with renal tumors, and to renal tumors has been clearly

shown. Although precancerous conditions in the kidney have been rarely described, as mentioned above, from the viewpoint of altered DNA methylation, we have shown that it is possible to recognize the presence of precancerous conditions even in the kidney [37]. In other words, regional DNA hypermethylation may participate in the early and precancerous stage of multistage renal tumorigenesis.

In renal tumors, the *CDKN2A* and *THBS-1* genes seem to be hot spots of regional DNA hypermethylation during multistage renal tumorigenesis. The incidence of DNA methylation on the MINT 2 clone was low in renal cancers, even though this clone is one of the hot spots of regional DNA hypermethylation in HCCs. The incidence of DNA methylation on the MINT 25 clone was, if anything, high even in normal renal tissues, although it was never observed in normal liver tissues, indicating that MINT 25 may be normally methylated in a renal tissue-specific manner. Thus the DNA methylation profiles of both normal and tumorous tissues tended to be organ-specific.

In clear cell RCCs, correlations between the average number of methylated CpG islands and tumor clinicopathological parameters were evaluated. Clear cell RCCs were classified into three groups on the basis of macroscopic configuration: single nodular type [type 1], single nodular with extranodular growth type [type 2], and contiguous multinodular type [type 3] RCCs [37]. These criteria for macroscopic configuration follow those that have already been established for HCCs: type 2 or 3 HCCs show poorer histological differentiation and a higher incidence of portal vein involvement and intrahepatic metastasis than type 1 HCCs. Patients with types 2 and 3 HCCs show poorer prognosis than those with type 1 [38]. With respect to clear cell RCCs, accumulation of DNA methylation on CpG islands was significantly correlated with a type 2 or 3 macroscopic configuration, higher histological grade, an infiltrating growth pattern and vascular involvement [37], suggesting that regional DNA hypermethylation is continuously involved in multistage renal tumorigenesis from precancerous conditions to malignant progression. The recurrence-free and overall survival rates of patients with RCCs showing accumulated DNA methylation on 3 or more CpG islands was significantly lower than that of patients with RCCs not showing this feature, indicating that regional DNA hypermethylation may be a biological predictor of patient prognosis. In addition to the above-mentioned genetic clustering, analysis of DNA methylation status in nephrectomy specimens may become a useful tool for prognostication of individual clinical cases.

Surprisingly, the average number of methylated CpG islands in non-tumorous renal tissues obtained from patients with histological grade 3 clear cell RCCs was significantly higher than that in equivalent tissue obtained from patients with histological grade 1 or 2 RCCs [25,37]. These data suggest that precancerous conditions showing regional DNA hypermethylation may generate more malignant RCCs.

Genome-wide DNA methylation profiling in precancerous conditions and RCCs

In order to further clarify the significance of DNA methylation alterations during renal carcinogenesis, we performed genome-wide DNA methylation analysis using BAC array-based methylated CpG island amplification (BAMCA) [39-41] in tissue samples. 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. Genomic regions in which DNA hypomethylation affects chromosomal instability may not be contained in promoter arrays or CpG island arrays. Moreover, aberrant DNA methylation of large regions of chromosomes, which are regulated in a coordinated manner due to a process of long-range epigenetic silencing, has recently attracted attention in human cancers [42]. Therefore, we again used a custom-made BAC array MCG Whole Genome Array-4500, which may be suitable, not for focusing on specific promoter regions or individual CpG sites, but for overviewing the DNA methylation tendency of individual large regions among all chromosomes [43]. Briefly, test or reference DNA was first digested with the methylation-sensitive restriction enzyme Sma I and subsequently with 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, respectively and applied to the custom-made BAC array. We validated the ability for detecting any tendency for coordinated regulation of DNA methylation at multiple CpG sites in individual large regions of chromosomes of BAMCA by quantitative evaluation of DNA methylation status at each Sma I site on representative BAC clones by pyrosequencing [44].

Non-tumorous renal tissue obtained from patients with papillary RCCs, chromophobe RCCs and oncocytomas did not show any histological changes when compared with both non-tumorous renal tissue obtained from patients with clear cell RCCs and normal renal tissue obtained from patients without any primary renal tumor. However, the average numbers of BAC clones showing DNA hypo- or hypermethylation in non-tumorous renal tissue obtained from patients with chromophobe RCCs and oncocytomas were significantly smaller than the average number in non-tumorous renal tissue obtained from patients with clear cell RCCs [45]. In non-tumorous renal tissue from all examined patients with renal tumors (clear cell RCCs, papillary RCCs, chromophobe RCCs and oncocytomas), biphasic accumulation of DNA methylation alterations was evident. Among such patients, the recurrence-free survival rate of patients showing DNA hypo- or hypermethylation on more BAC clones in their non-tumorous renal tissue was significantly lower than that of patients showing DNA hypo- or hypermethylation on fewer BAC clones [45]. Significant DNA methylation profiles determining the histological subtype (chromophobe RCCs and oncocytomas vs clear cell RCCs) of future developing renal tumors and/or patient outcome (favorable outcome vs poorer outcome) may be already established at the precancerous stage.

In samples of non-cancerous renal tissue from patients with clear cell RCCs, many BAC clones already showed DNA hypomethylation or DNA hypermethylation relative to normal renal tissues. In clear cell RCCs themselves, more BAC clones showed DNA hypomethylation or DNA hypermethylation, the degree of which was increased in comparison with non-cancerous renal tissue samples obtained from patients with clear cell RCCs [46]. In samples of non-cancerous renal tissue from patients with clear cell RCCs, which were already at the precancerous stage with accumulation of DNA methylation on C-type CpG islands in spite of an absence of marked histological changes as mentioned above, genome-wide DNA methylation alterations (both hypo- and hypermethylation) were also confirmed by BAMCA.

We then performed two-dimensional unsupervised hierarchical clustering analysis based on the genome-wide DNA methylation status (signal ratios by BAMCA) of the non-cancerous renal tissue samples. On the basis of the DNA methylation profiles of their non-cancerous renal tissue samples, the patients with clear cell RCCs were clustered into two subclasses, Clusters A_{NM} and B_{NM}. The corresponding clear cell RCCs of patients in Cluster B_{NM} showed more frequent macroscopically evident multinodular (type 3) growth, vascular involvement and renal vein tumor thrombi, and higher pathological TNM stages than those in Cluster A_{NM} [46]. Our Clusters A_{NM} and B_{NM} in precancerous tissue can be considered clinicopathologically valid, as 60% of the patients in Cluster B_{NM} died of recurrent RCC, compared with only 2% of the patients in Cluster A_{NM} [46]. The overall survival rate of patients in Cluster B_{NM} was significantly lower than that of patients in Cluster A_{NM} (**Figure 3A**). DNA methylation alterations at the precancerous stage may even determine the outcome of patients with clear cell RCCs.

Two-dimensional unsupervised hierarchical clustering analysis based on BAMCA data (signal ratios) for clear cell RCCs was able to group patients into two subclasses, Clusters A_{TM} and B_{TM}. Clear cell RCCs in Cluster B_{TM} showed more frequent vascular involvement and renal vein tumor thrombi, and also higher pathological TNM stages than those in Cluster A_{TM} [46]; 37.5% of the patients in Cluster B_{TM} died due to RCC recurrence, compared with only 2.3% of the patients in Cluster A_{TM} [46]. The overall survival rate of patients in Cluster B_{TM} was significantly lower than that of patients in Cluster A_{TM}. Multivariate analysis revealed that our clustering was a predictor of recurrence and was independent of histological grade, macroscopic configuration, vascular involvement or presence of renal vein tumor thrombi. Patients belonging to Cluster B_{TM} were completely discriminated from patients belonging to Cluster A_{TM} based on the DNA methylation status of 14 BAC clones. In other words, the DNA methylation status of the 14 BAC clones was able to determine whether or not patients belonged to Cluster B_{TM}, a signifi-

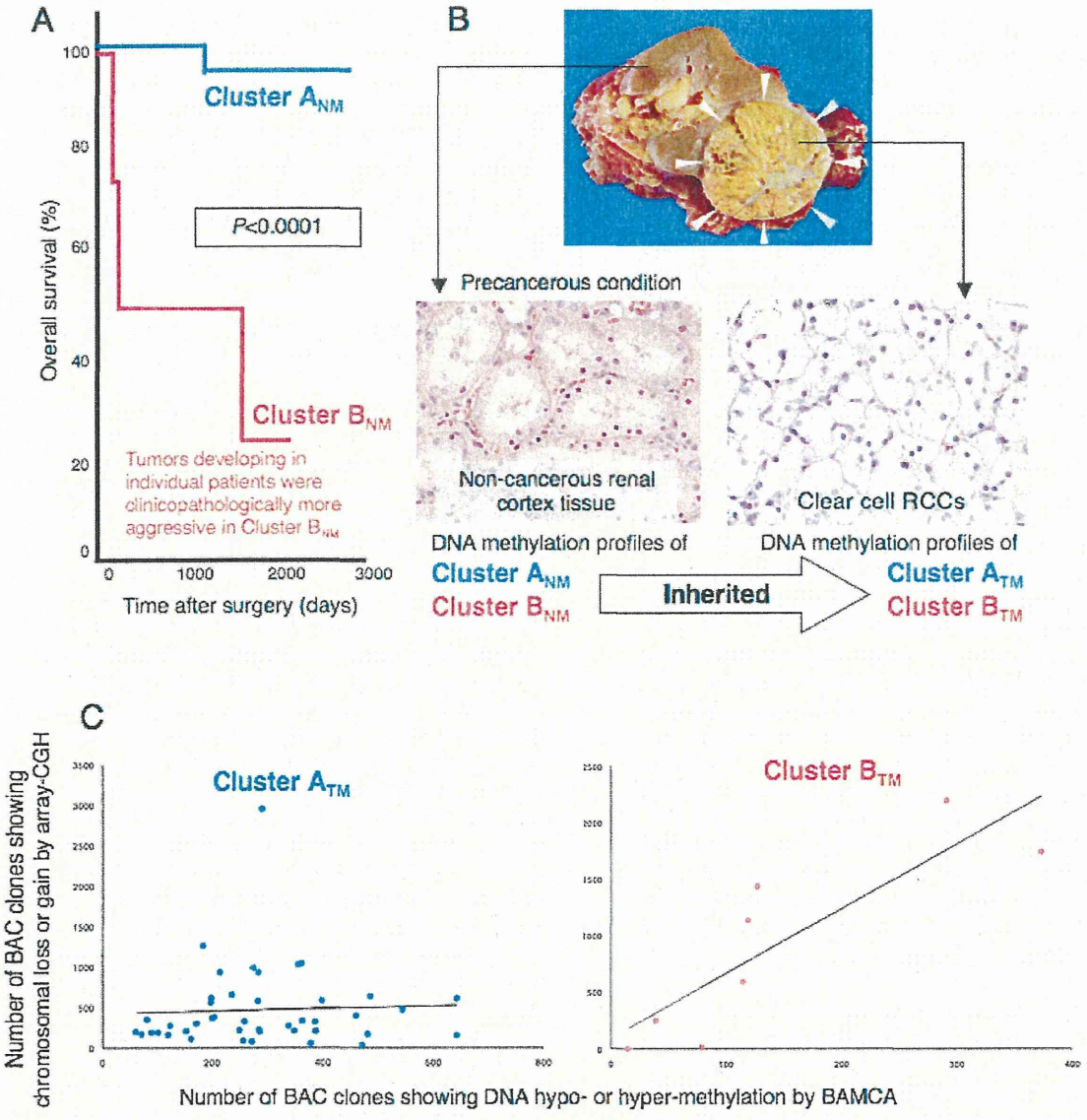

Figure 3. DNA methylation profiles in precancerous conditions and clear cell renal cell carcinomas (RCCs). **A.** Genome -wide DNA methylation profiles in the non-cancerous renal tissue were significantly correlated with clinicopathological parameters of clear cell RCCs developing in individual patients, and also outcome, indicating that DNA methylation alterations at the precancerous stage may generate more malignant cancers and even determine outcome (ref. 46). **B.** DNA methylation profiles in the non-cancerous renal tissue (Clusters A_{NM} and B_{NM}, see text) were basically inherited by the corresponding clear cell RCCs developing in individual patients as the DNA methylation profiles of Clusters A_{TM} and B_{TM}, respectively (ref. 46). **C.** In Cluster B_{TM}, the number of clones showing copy number alterations by array-CGH was significantly correlated with that of DNA hypo- and hypermethylation by BAMCA in the same patient, whereas no such significant correlations were observed in Cluster A_{TM}, suggesting that particular DNA methylation profiles may be closely related to chromosomal instability (unpublished data).

cant prognostic indicator, with a sensitivity and specificity of 100% using the appropriate cutoff value of signal ratios [46]. The use of DNA methylation status on such BAC clones as an indicator may be a promising approach for prognostication of clear cell RCCs.

Significance of DNA methylation alterations at the precancerous stage

When we compared the DNA methylation profiles of non-cancerous renal tissue and those of the corresponding clear cell RCC, Cluster B_{NM} was completely included in Cluster B_{TM}. Wilcoxon test revealed that the signal ratios of 1143 BAC clones in non-cancerous renal tissue differed significantly between Clusters A_{NM} and B_{NM} and that the signal ratios of 1111 BAC clones in clear cell RCCs differed significantly between Clusters A_{TM} and B_{TM}. Among the 1143 BAC clones significantly discriminating Cluster B_{NM} from Cluster A_{NM}, 724, i.e. the majority, also discriminated Cluster B_{TM} from Cluster A_{TM}. In 311 of these 724 BAC clones, in which the average signal ratio of Cluster B_{NM} was higher than that of Cluster A_{NM}, the average signal ratio of Cluster B_{TM} was also higher than that of Cluster A_{TM} without exception. In 413 of the 724 BAC clones showing a lower average signal ratio of Cluster B_{NM} than that of Cluster A_{NM}, the average signal ratio of Cluster B_{TM} was also lower than that of Cluster A_{TM} without exception [46]. When we examined each of the representative BAC clones characterizing both Clusters B_{NM} and B_{TM}, the BAMCA signal ratio in the non-cancerous renal tissue was at almost the same level as that in the corresponding clear cell RCC developing in each individual patient. Accordingly, we concluded that the genome-wide DNA methylation profiles of non-cancerous renal tissue are basically inherited by each corresponding clear cell RCC (**Figure 3B**).

As mentioned above, we examined DNA methylation status on CpG islands for the *CDKN2A, hMLH 1, VHL* and *THBS 1* genes, and the methylated in tumor (MINT)-1, -2, -12, -25 and -31 clones were examined in the same clear cell RCCs. The average number of methylated CpG islands was significantly higher in Cluster B_{TM} (2.75±1.67) than in Cluster A_{TM}. The frequency of CIMP in Cluster B_{TM} (62.5%) was significantly higher than that in Cluster A_{TM} (16%). Genome-wide DNA methylation alterations consisting of both hypo- and hypermethylation of DNA revealed by BAMCA in Cluster B_{TM} are associated with regional DNA hypermethylation on CpG islands [37,46]. Moreover, a subclass of Cluster B_{NM} and B_{TM} based on BAMCA data is completely included in Cluster B_{TG} showing accumulations of copy number alterations [17,46]. Therefore, epigenetic and genetic alterations are not mutually exclusive during renal carcinogenesis. Particular DNA methylation profiles at the precancerous stage may be closely related to, or may be prone to chromosomal instability (**Figure 3C**). DNA methylation alterations in precancerous conditions, which may not occur randomly but are prone to further accumulation of epigenetic and genetic alterations, can generate more malignant cancers and even determine the outcome for individual patients.

Tumor-related genes silenced by DNA hypermethylation in RCCs

Somatic *VHL* mutations occur in 50-80% of sporadic clear cell RCCs [47]. Alternative mechanisms of *VHL* inactivation have therefore been explored, and Herman et al. have demonstrated DNA hypermethylation of the *VHL* gene in 19% of examined tumors [48]. In a renal cancer cell line, treatment with a DNA demethylating agent, 5-aza-2'-deoxycytidine, resulted in re-expression of the *VHL* gene. Thus, other than the *RB* gene, the *VHL* gene became the second known example of a tumor-suppressor gene silenced by DNA methylation. The list of tumor-related genes silenced by DNA hypermethylation during renal carcinogenesis has recently been increasing. DNA methylation profiling in both VHL-related and VHL-unrelated RCCs has shown that the average number of methylated genes revealed by high-throughput Goldengate analysis in sporadic RCCs of patients with wild-type *VHL* is higher than in RCCs of patients with mutant *VHL* [49]. The *Ras association domain family member 1 (RASSF1), twist homolog 1 (TWIST1), paired-like homeodomain 2 (PITX2), cadherin 13 (CDH13), heparan sulfate (glucosamine) 3-O -sulfotransferase 2 (HS3ST2), T-cell acute lymphocytic leukemia 1 (TAL1), Wilms' tumor 1 (WT1), matrix metallopeptidase 2 (MMP2), deleted in colorectal carcinoma (DCC), islet cell autoantigen 1 (ICA1)* and *tumor suppressor candidate 3 (TUSC3) genes* are more frequently methylated in sporadic RCCs of patients with wild-type *VHL* than in RCCs of patients with mutant *VHL*, whereas only *gamma-aminobutyric acid A receptor, beta 3 (GABRB3)* is methylated more frequently in VHL-related RCCs [49].

Frequent DNA methylation of proapoptotic TP53 target genes in stomach and colorectal cancers has recently attracted attention [50]. When examined in RCCs, the *apoptotic peptidase activating factor 1 (APAF1)* and *death-associated*

protein kinase 1 (DAPK1) proapoptotic genes were frequently silenced due to DNA hypermethylation, and such DNA hypermethylation had a prognostic impact in affected patients [51]. With respect to Wnt antagonist family genes in RCCs, DNA hypermethylation and/or repressive histone modification have been observed in the *secreted frizzled-related protein 1 (SFRP1), SFRP2, SFRP5, WNT inhibitory factor 1 (WIF1)* and *dickkopf homolog 3 (DKK3)* genes. Simultaneous detection of DNA methylation of such Wnt antagonist family genes may be a useful indicator for diagnosis of RCCs [52,53].

Microarray analysis of RCC cell lines treated with 5-aza-2'deoxycytidine has revealed upregulation of the *ubiquitin carboxyl-terminal esterase L1 (UCHL1)* gene [54]. The *UCHL1* gene involved in the regulation of cellular ubiquitin levels plays important roles in different cellular processes. Significant growth inhibition in *UCHL1* transfectants suggests that UCHL-1 functions as a potential tumor suppressor gene in RCCs [55]. Moreover, silencing of the *UCHL-1* gene due to DNA hypermethylation is reportedly correlated with poor outcome in patients with RCCs [55]. Loss of transforming growth factor beta receptor III (TGFBR3) correlates with loss of TGF-beta responsiveness and dysregulated TGF-beta signaling in RCCs. However, reduced expression of the *TGFBR3* gene was shown not to be due to DNA hypermethylation of the promoter region of the *TGFBR3* gene itself, but to silencing of the transcriptional factor *GATA binding protein 3 (GATA3)* due to DNA hypermethylation resulting in reduced expression of *TGFBR3* during RCC progression [56]. In addition, silencing due to DNA methylation of a number of genes may play a role in renal carcinogenesis; these include the p53-inducible gene *14-3-3 sigma* [57], *ABCG2* which is of importance in clinical drug resistance [58], a gap junction molecule connexin 32 [59], actin-binding protein DAL-1/4.1B [60], TIMP3 which participates in cancer invasion [61], the *fragile histidine triad (FHIT)* gene which encompasses the most common human fragile site FRA3B at 3p14.2 [62], cell adhesion molecule junction plakoglobin (JUP) [63], HGF activator inhibitor HAI-2 [64], a member of the homeobox gene family *HOXB13* [65], *tissue-specific proapoptotic BH3-only protein BCL2-interacting killer (BIK)* [66], *TU3A* which was originally identified as a candidate tumor suppressor gene in RCCs [67] and XAF1 which antagonizes the anticaspase activity of X-linked inhibitor of apoptosis (XIAP) [68].

Recently, the methodology for analysis of DNA methylation on a genome-wide scale has been markedly improved [69], and the use of microarrays to which bisulfite-converted genomic DNA is applied, has become popular, achieving a resolution as good as a single CpG [70,71]. New -generation sequencing technologies have been introduced for bisulfite-converted genomic DNA or genomic DNA enriched by affinity-based approaches using anti-methyl-cytosine antibody or methyl-binding domain proteins [72]. In addition, a high-throughput technique without bisulfite conversion has been developed based on single-molecule, real-time DNA sequencing [73]. These new technologies will be able to efficiently accelerate the identification of tumor-related genes whose expression is altered due to DNA hypo- or hyper-methylation and reveal the clinical relevance of translational epigenetics.

DNA hypomethylation in RCCs

Unlike the case of DNA hypermethylation, the number of reports addressing DNA hypomethylation of specific genes or elements has been restricted to date. Carbonic anhydrase IX (CA9) is a transmembrane glycoprotein and the only known tumor-associated carbonic anhydrase that may be involved in cell proliferation and transformation. DNA hypomethylation of the *CA9* gene has been shown to participate in activation of the promoter activity in RCC cell lines and clinical tissue samples [74,75]. Transposons, proviral DNA and other parasitic elements in the mammalian genome make up the repetitive sequences in the intergenic and introgenic regions of DNA. In general, activation of parasitic elements, such as LINE-1 and HERV-K, can allow for their movement within the genome. However, activation of these parasitic elements due to DNA hypomethylation does not seem to be a major event during renal carcinogenesis [76].

Histone modifications in RCCs

Since techniques for analysis of histone modification in clinical tissue specimens have not been fully established to date, the overall picture of histone modification status in clinical samples of various cancers including RCCs is unclear. However, the results of immunohisto-

chemistry to evaluate histone methylation levels have been reported. Levels of H3K4-monomethyl, -dimethyl and -trimethyl staining were each inversely correlated with the aggressiveness of RCCs. The combined staining score for H3K4 modifications (monomethylation to trimethylation) was shown to be an independent predictor of outcome in patients with RCCs [77].

With respect to cross-talk between genetic alterations and histone modifications, a recent robust analysis of 3544 protein genes in clear cell RCCs has revealed somatic truncating mutations in the *SET domain containing 2 (SETD2)* gene, which encodes a histone H3K36 methyltransferase, and also in the *lysine-specific demethylase 5C (KDM5C/JARID1C)* gene, which encodes a histone H3K4 demethylase [15]. No mutations were found in either *SETD2* or *KDM5C* in the subset of non-clear cell RCCs. The majority of samples with truncating *SETD2* and *KDM5C* mutations had *VHL* mutations. Significant (two-fold or less) differences in the expression levels of 298 genes were noted in clear cell RCCs showing the *SETD2* mutation relative to those not showing it, whereas *KDM5C*-mutant RCCs showed significant differences in the expression levels of 18 genes relative to *KDM5C*-wild-type RCCs [15].

Perspective

Both genetic and epigenetic events appear to accumulate during renal carcinogenesis, reflecting the clinicopathological diversity of RCCs. Loss of chromosome 3p and gain of chromosomes 5q and 7 may be indispensable copy number aberrations for the development of clear cell RCCs. When loss of chromosome 1p, 4, 9, 13q or 14q is added, more malignant RCCs may develop. DNA methylation alterations play significant roles in multistage renal carcinogenesis even in early precancerous stages. Genome-wide DNA methylation profiles in precancerous conditions are basically inherited by the corresponding RCCs developing in individual patients: DNA methylation alterations at the precancerous stage may render cells prone to further epigenetic and genetic alterations, generate more malignant cancers, and even determine patient outcome. Previous attempts have been made to use genetic alterations of *VHL* and other tumor-related genes as diagnostic indicators in tissue and serum specimens [9,78]. On the other hand, DNA methylation alterations occur earlier than genetic alterations during carcinogenesis and are stably preserved on DNA double strands by covalent bonds, unlike the profiles of mRNA and protein expression, which can be easily affected by the microenvironment of cancer cells or their precursor cells. Genome-wide DNA methylation profiling may provide optimal indicators for early diagnosis of RCCs and prognostication of affected patients.

RCCs are thought to be immunogenic, and immunotherapy including the administration of interferon-alpha or interleukin (IL)-2 has been used as a standard treatment for RCCs for 20 years [79]. However, the success of immunotherapy is limited because of immuno-escape mechanisms including down-regulation of major histocompatibility complex class I antigens and secretion of immunosuppressive cytokines such as IL10. In addition to traditional surgical approaches and immunotherapy, molecular targeted therapy has recently been introduced. Since the induction of VEGF by HIF activation downstream of VHL inactivation is the most important mechanism determining the hypervascularity of RCCs [79,80], VEGF receptor inhibitors such as sunitinib, sorafenib and axitinib, and the VEGF-ligand binding agent bevacizumab, have been introduced for VEGF-targeted therapy. mTOR is another target for treatment of RCCs, and an ester of rapamycin, tersirolimus, has been introduced clinically. However, the mechanisms responsible for refractoriness to molecular targeted therapy are unclear, and the optimal administration regimen for these agents has not been defined [81]. Therefore, recently introduced agents have not accomplished complete anti-tumor effects. Further investigation of the genetic and epigenetic events occurring during renal carcinogenesis is needed to identify more key molecules for use in prevention, diagnosis and therapy.

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Abbreviations: BAC; bacterial artificial chromosome, BAMCA; BAC array-based methylated CpG island amplification, BHD; Birt-Hogg-Dube, CGH; comparative genomic hybridization, CIMP; CpG island methylator phenotype, DNMT; DNA methyltransferase, HCC; hepatocellular carcinoma, HGF; hepatocyte growth factor, HIF; hypoxia-inducible factor, HPH; HIF prolyl hydroxylase, IL; interleukin, mTOR; mammalian target of rapamycin, MINT; methylated in tumor, MSP; methylation-specific PCR, RB; retinoblastoma, RCC; renal cell carcinoma, TNM; tumor-node-metastasis, TSC; tuberous sclerosis complex, VEGF; vascular endothelial growth factor, VHL; von Hippel-Lindau.

Please address correspondence to: Yae Kanai, M.D.,Ph.D., Division of Molecular Pathology, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; Tel: 81-3-3542-2511; Fax: 81-3-3248-2463; E-mail: ykanai@ncc.go.jp

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

Eri Arai[a] Saori Wakai-Ushijima[a] Hiroyuki Fujimoto[d] Fumie Hosoda[b] Tatsuhiro Shibata[b] Tadashi Kondo[c] Sana Yokoi[e] Issei Imoto[e] Johji Inazawa[e] Setsuo Hirohashi[a] Yae Kanai[a]

[a]Pathology Division, [b]Cancer Genomics Project, and [c]Proteome Bioinformatics Project, National Cancer Center Research Institute, [d]Urology Division, National Cancer Center Hospital, and [e]Department of Molecular Cytogenetics, Medical Research Institute and School of Biomedical Science, Tokyo Medical and Dental University, Tokyo, Japan

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

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

Introduction

Accumulating evidence suggests that not only genetic but also epigenetic alterations play a significant role in human carcinogenesis. DNA methylation alterations are one of the most consistent epigenetic changes occurring during carcinogenesis in various organs: it is known that DNA hypomethylation results in chromosomal instability as a result of changes in chromatin structure, and that DNA hypermethylation of CpG islands silences tumor-related genes in cooperation with histone modification [1–5].

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Yae Kanai
Pathology Division, National Cancer Center Research Institute
5-1-1 Tsukiji, Chuo-ku
Tokyo 104-0045 (Japan)
Tel. +81 3 3542 2511, Fax +81 3 3248 2463, E-Mail ykanai@ncc.go.jp

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

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

Materials and Methods

Patients and Tissue Samples

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

BAMCA Analysis

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

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

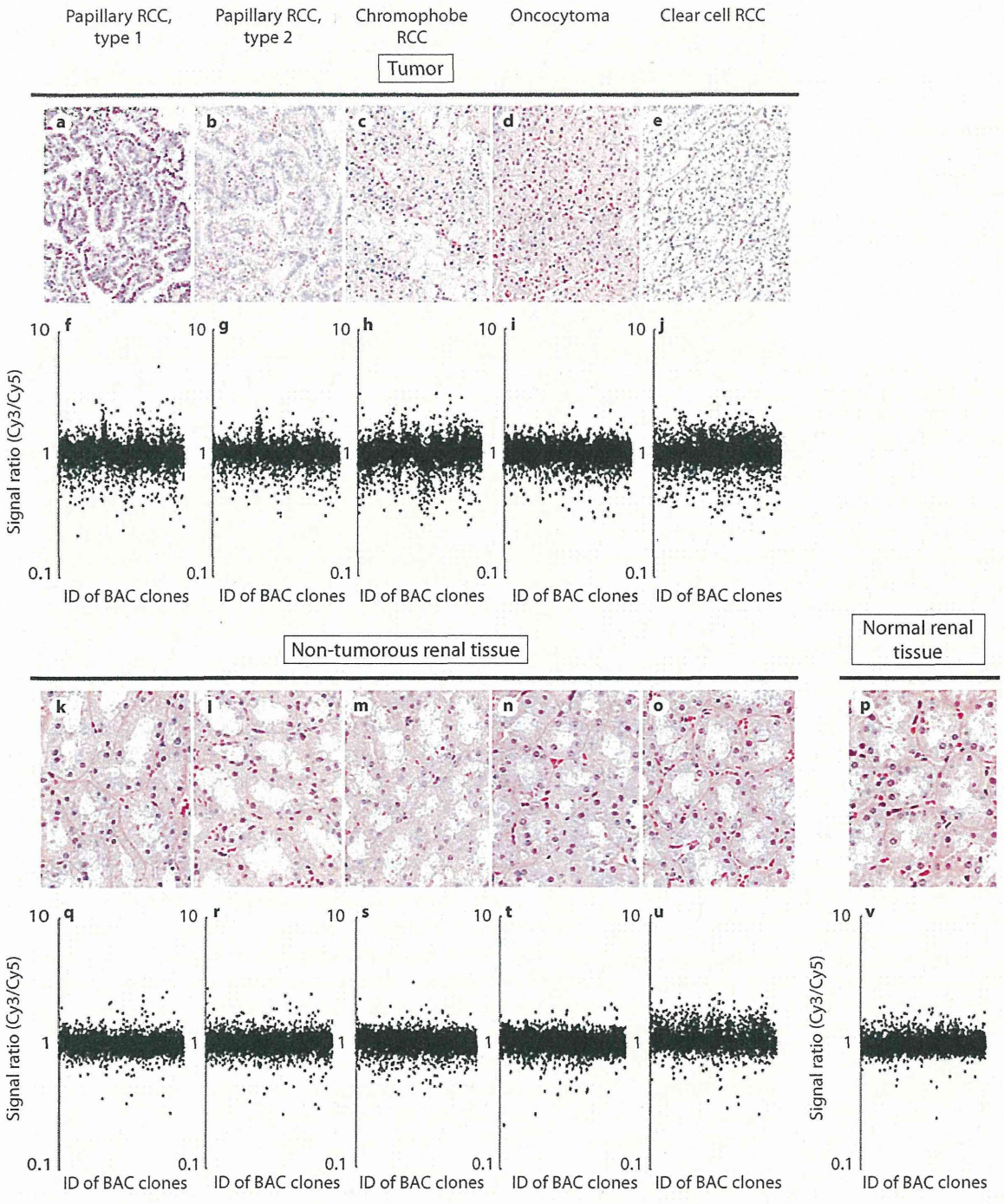

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Table 1. The average number of BAC clones showing DNA methylation alterations (DNA hypo- or hypermethylation) in tumorous tissue and non-tumorous renal tissue obtained from patients with renal tumors

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

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

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

The genome-wide DNA methylation status was analyzed by BAMCA using a custom-made array (MCG Whole Genome Array-4500) harboring 4,361 BAC clones throughout chromosomes 1–22 and X and Y [17], as described previously [19, 20]. Briefly, 5-μg aliquots of test or reference DNA were first digested with 100 units of the methylation-sensitive restriction enzyme *Sma*I and subsequently with 20 units of the methylation-insensitive *Xma*I. Adapters were ligated to *Xma*I-digested sticky ends, and PCR was performed with an adapter primer set. Test and reference PCR products were labeled by random priming with Cy3- and Cy5-dCTP (GE Healthcare, Little Chalfont, UK), respectively, using a BioPrime Array CGH Genomic Labeling System (Invitrogen, Carlsbad, Calif., USA) and precipitated together with ethanol in the presence of Cot-I DNA. The mixture was applied to array slides and incubated at 43°C for 72 h. The arrays were scanned with a GenePix Personal 4100A (Axon Instruments, Foster City, Calif., USA) and analyzed using GenePix Pro 5.0 imaging software (Axon Instruments) and Acue 2 software (Mitsui Knowledge Industry, Tokyo, Japan). The signal ratios were normalized in each sample to make the mean signal ratios for all BAC clones 1.0. In accordance with previously described criteria [9], in the tumor and the corresponding non-tumorous renal tissue, DNA methylation status corresponding to a signal ratio of <0.67 and >1.5 was defined as DNA hypomethylation and DNA hypermethylation of each BAC clone compared with normal renal tissue, respectively. In our previous study, tumorous tissue and the corresponding non-tumorous renal tissue of 51 patients with clear cell RCCs (T1–T51) were analyzed by the same BAMCA method using the same array and reference DNA (fig. 1j) [9].

Statistics

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

Results

Genome-Wide DNA Methylation Profiles of Renal Tumors

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

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

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

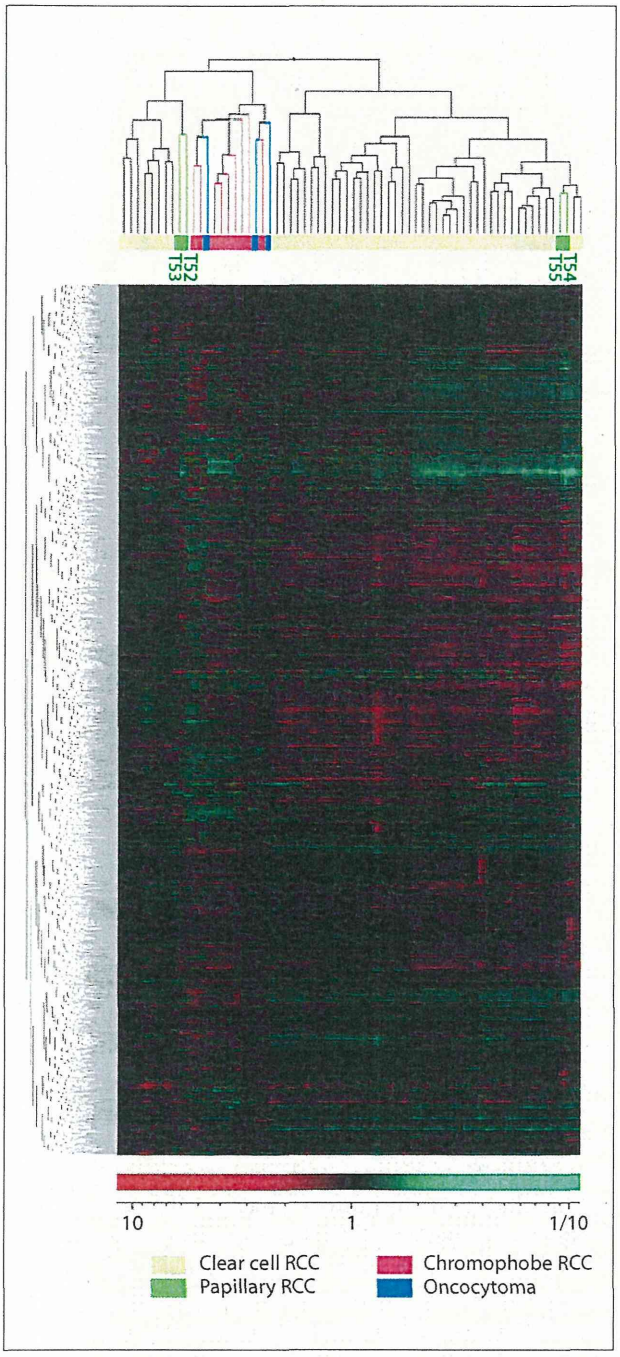


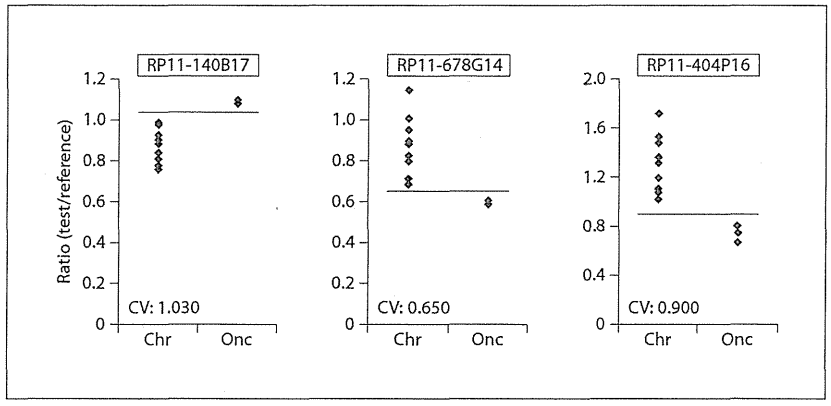
Fig. 2. Two-dimensional unsupervised hierarchical clustering analysis based on BAMCA data (signal ratios) for tumorous tissue obtained from patients with type 1 (T52 and T53) and type 2 (T54 and T55) papillary RCCs, chromophobe RCCs (T56–T65), oncocytomas (T66–T68) and clear cell RCCs (T1–T51). The signal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

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

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

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

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



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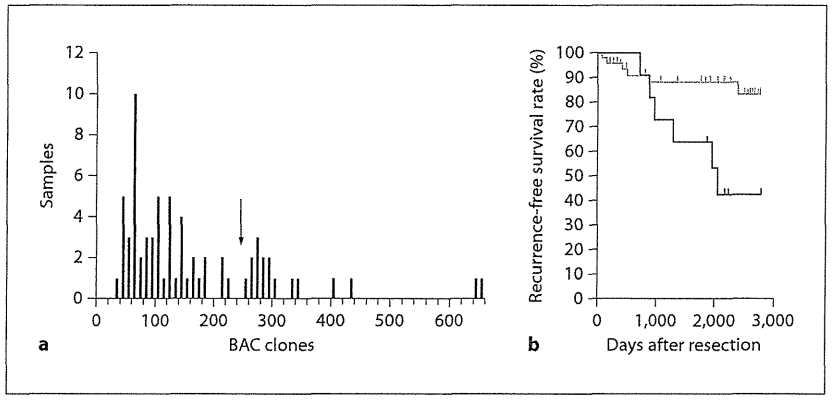

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Fig. 3. Scattergrams of the signal ratios in chromophobe RCCs (Chr, T56–T65) and 3 oncocytomas (Onc, T66–T68) for representative BAC clones, RP11-140B17, RP11-678G14 and RP11-404P16. Using the cut-off values (CV) described in each panel, chromophobe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

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

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

Discussion

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

Many researchers in the field of cancer epigenetics have used promoter arrays to identify the genes that are methylated in cancer cells [23–25]. However, the promoter regions of specific genes are not the only target of DNA methylation alterations in human cancers. DNA methylation status in genomic regions that do not directly participate in gene silencing, such as the edges of CpG islands, may be altered at the precancerous stage before the alterations of the promoter regions themselves occur [26]. Genomic regions in which DNA hypomethylation affects chromosomal instability may not be contained in promoter arrays. Moreover, aberrant DNA methylation of large regions of chromosomes, which are regulated in a coordinated manner in human cancers due to a process of long-range epigenetic silencing, has recently attracted attention [27]. Therefore, we used a custom-made BAC

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

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

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

array [17] that may be suitable not for focusing on specific promoter regions, but for overviewing the DNA methylation status of individual large regions among all chromosomes.

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

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

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