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Review Article

Genetic and epigenetic alterations during renal carcinogenesis

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Abstract: Renal cell carcinoma (RCC) is not a single entity, but comprises a group of tumors including clear cell RCC, papillary RCC and chromophobe RCC, which arise from the epithelium of renal tubules. The majority of clear cell RCCs, the major histological subtype, have genetic or epigenetic inactivation of the *von Hippel-Lindau (VHL)* gene. Germline mutations in the *MET* and *fumarate hydratase (FH)* genes lead to the development of type 1 and type 2 papillary RCCs, respectively, and such mutations of either the *TSC1* or *TSC2* gene increase the risk of RCC. Genome-wide copy number alteration analysis has suggested that loss of chromosome 3p and gain of chromosomes 5q and 7 may be copy number aberrations indispensable for the development of clear cell RCC. When chromosome 1p, 4, 9, 13q or 14q is also lost, more clinicopathologically aggressive clear cell RCC may develop. Since renal carcinogenesis is associated with neither chronic inflammation nor persistent viral infection, and hardly any histological change is evident in corresponding non-tumorous renal tissue from patients with renal tumors, precancerous conditions in the kidney have been rarely described. However, regional DNA hypermethylation on C-type CpG islands has already accumulated in such non-cancerous renal tissues, suggesting that, from the viewpoint of altered DNA methylation, the presence of precancerous conditions can be recognized even in the kidney. Genome-wide DNA methylation profiles in precancerous conditions are basically inherited by the corresponding clear cell RCCs developing in individual patients: DNA methylation alterations at the precancerous stage may further predispose renal tissue to epigenetic and genetic alterations, generate more malignant cancers, and even determine patient outcome. The list of tumor-related genes silenced by DNA hypermethylation has recently been increasing. Genetic and epigenetic profiling provides an optimal means of prognostication for patients with RCCs. Recently developed high-throughput technologies for genetic and epigenetic analyses will further accelerate the identification of key molecules for use in the prevention, diagnosis and therapy of RCCs.

Keywords: Renal cell carcinoma, copy number alteration, DNA methylation, precancerous condition, prognostication

Introduction: etiology and pathology

Worldwide about 271,000 cases of kidney cancer have been diagnosed and 116,000 persons have died because of kidney cancer [1]. In the United States, 57,000 cases of kidney cancer have been diagnosed and 14,000 persons have died. The majority of kidney cancers (80-85%) are renal cell carcinomas (RCCs) originating from the renal parenchyma. The remaining 15-20% are mainly urothelial carcinomas of the renal pelvis. Kidney cancer accounts for 2% of all adult malignancies, with a male to female ratio of 3:2 among affected patients [1]. The incidence of RCC peaks in the sixth decade of life, 80% of cases affecting the 40- to 69-year-old age group [2]. The incidence of RCC has been rising steadily each year in Europe and the United States over the last three decades. It is generally highest in Western and Eastern European countries and Scandinavia, as well as in Italy, North America, Australia and New Zealand. The lowest rates are reported in Asia and Africa. This regional variation in the incidence of RCC (more than ten-fold) suggests the strong role of environmental risk factors [3]. However, it is difficult to ascribe a definite and direct cause for this cancer. Smoking and chemical carcinogens such as asbestos and organic solvents are related to renal tumorigenesis. Obesity and hypertension and/or use of antihypertensive medication have been consistently reported to be

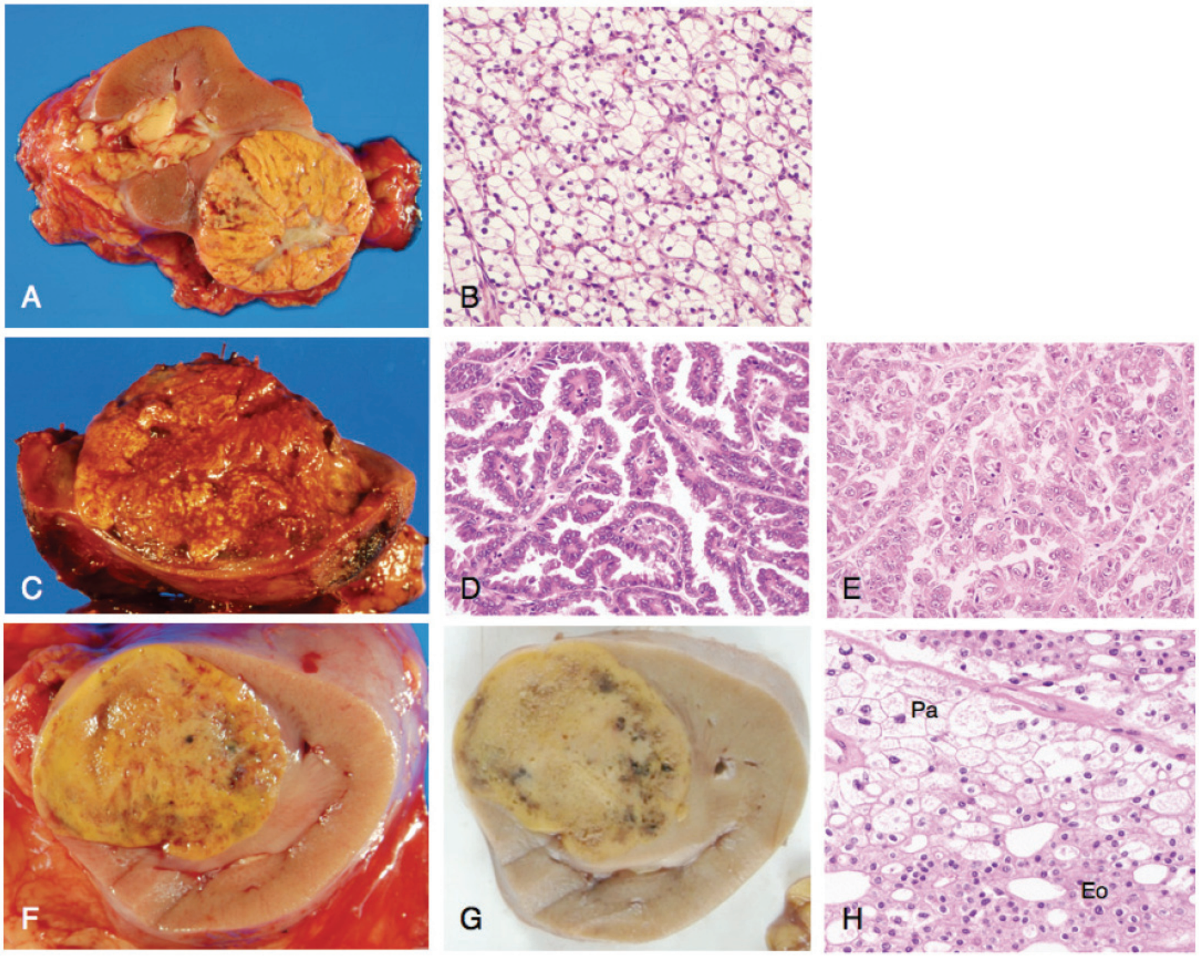

Figure 1. Macroscopic (A, C, F and G) and microscopic (B, D, E and H) views of a clear cell RCC (A and B), papillary RCCs (C, D and E) and a chromophobe RCC (F, G and H). **A.** Clear cell RCCs commonly protrude from the renal cortex as a rounded mass. Their cut surfaces are typically golden yellow, and necrosis and hemorrhage are commonly present. **B.** Clear cell RCCs typically have cytoplasm filled with lipids and glycogen and show an alveolar architecture. **C.** Papillary RCCs frequently contain areas of hemorrhage, necrosis and cystic degeneration. **D.** Type 1 papillary RCCs consist of papillae covered with a single or double layer of small cuboid cells with scanty cytoplasm. **E.** Type 2 papillary RCCs consist of papillae covered by large eosinophilic cells arranged in an irregular or pseudo-stratified manner. **F.** Chromophobe RCCs are solid circumscribed tumors with slightly lobulated surfaces. In unfixed specimens, the cut surface is homogeneously light brown or tan. **G.** Macroscopic view of the same chromophobe RCC after formalin fixation. The cut surface of chromophobe RCCs turns graysh-beige. **H.** Chromophobe RCCs consist of tumor cells with abundant eosinophilic cytoplasm (pale cells [Pa] and eosinophilic cells with a perinuclear halo [Eo]) and show mainly a solid structure.

positively associated with RCC risk [2].

RCC is not a single entity, but comprises a group of tumors that arise from the epithelium of renal tubules [4]. Clear cell RCC is the most common histological subtype (**Figure 1A**). Typically, the cells have cytoplasm filled with lipids and glycogen, are surrounded by a distinct cell membrane and contain round and uniform nuclei, and show an alveolar, acinar, cystic and solid architecture (**Figure 1B**). First, based simply on cytologic and histologic criteria, papillary RCCs (**Figure 1C**) can be divided into two 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 (**Figure 1D**), and type 2 papillary RCCs consist of papillae covered by large eosinophilic cells arranged in an irregular or pseudostratified manner (**Figure 1E**) [5]. Chromophobe RCC consists of tumor cells with abundant eosinophilic cytoplasm (pale cells and eosinophilic cells with a perinuclear halo) and show mainly a solid structure (**Figure 1F** to **1H**) [5]. Clear cell RCC and papillary RCC are derived from the proximal convoluted tubule, whereas the origin of chromophobe RCC is the distal tubule/collecting tubule. Certain inherited disorders such as von Hippel-Lindau (VHL) disease, hereditary papillary RCC and Birt-Hogg-Dube (BHD) syndrome enhance the risk of acquiring clear cell RCC, papillary RCC and chromophobe RCC, respectively [6].

Genetic alterations in RCCs

Tumor-related genes and their role in renal carcinogenesis

The World Health Organization (WHO) classification has introduced genetic alterations as a hallmark of certain histological subtypes of RCC, e.g. clear cell RCC is characterized by loss of chromosome 3p and inactivation of the *VHL* gene at 3p25.3 due to mutation or DNA methylation around the promoter region [7], although the classification of RCC is based largely on histology. The product of *VHL* is a 3-kDa protein with multiple functions, the best documented of which relates to its role as the substrate-recognition component of the E3-ubiquitin ligase complex. This complex is best known for its ability to target hypoxia-inducible factors (HIFs) for polyubiquitination and proteasomal degradation [8]. Under hypoxic conditions, HIF-1alpha and HIF-2alpha accumulate and form heterodimers with HIF-1beta and translocate to the nucleus where they induce transcription of downstream target genes including vascular endothelial growth factor (VEGF). The absence of wild-type VHL promotes inappropriate activation of downstream target genes and contributes to tumorigenesis [9]. Additionally, VHL protein has functions that are independent of HIF-1alpha and HIF-2alpha and are thought to be important for its tumor-suppressor action, assembly of the extracellular matrix, control of microtubule dynamics, regulation of apoptosis, and possibly stabilization of TP53 proteins [10].

Patients with gain-of-function germline mutations in the *MET* gene develop type 1 papillary RCC. *MET* encodes a transmembrane receptor tyrosine kinase whose ligand is hepatocyte growth factor (HGF). Activation of MET by HGF triggers tyrosine kinase activity, which facilitates several transduction cascades resulting in multiple cellular processes such as mitogenesis and migration. However, the incidence of *MET* mutations in sporadic papillary RCC is not high (about 10%) [11]. Patients with germline mutations in the *fumarate hydratase (FH)* gene develop type 2 papillary RCC [12]. VHL recognition of HIF requires hydroxylation by HIF prolyl hydroxylase (HPH), and FH activates HPH. *FH* mutation promotes tumorigenesis via HIF protein accumulation due to HPH dysfunction. Unlike the gain-of-function mutation of the *c-kit (KIT)* gene, overexpression of KIT is frequent in chromophobe RCC [13]: KIT is a type III receptor tyrosine kinase that has a role in cell signal transduction. Normally KIT is phosphorylated upon binding to its ligand, stem cell factor. This leads to a phosphorylation cascade ultimately activating various transcription factors. Such activation regulates apoptosis, cell differentiation, proliferation, chemotaxis, and cell adhesion. Although germline mutations of the *BHD* gene, which encodes folliculin, have been detected in 80% of BHD kindreds, the incidence of the mutation in sporadic chromophobe RCC is very low. Tuberous sclerosis complex (TSC) has been linked to germline inactivating mutations of either of *TSC1* (9q34) encoding hamartin or *TSC2* (16p13.3) encoding tuberin, and affected patients have an increased risk of developing renal tumors including clear cell RCC, papillary RCC and chromophobe RCC [3]. The TSC1/TSC2 protein complex inhibits mammalian target of rapamycin (mTOR) protein and is involved in signaling pathways that regulate cell growth. Although the Eker rat model with a germline insersion in the *Tsc2* gene develops dominantly inherited cancers [14], the role of TSC1 and TSC2 in human sporadic RCC is unclear.

Other known cancer genes that are frequently mutated in adult epithelial cancers, for example *RAS, v-raf murine sarcoma viral oncogene homolog B1 (BRAF), TP53, retinoblastoma (RB), cyclin-dependent kinase inhibitor 2A (CDKN2A), phosphoinositide-3-kinase, catalytic alpha polypeptide (PIK3CA), phosphatase and tensin homolog (PTEN), epidermal growth factor receptor (EGFR)* and *v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2),* make only a small contribution to clear cell RCC [15]. Recently somatic truncating mutations in the *neu-*

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

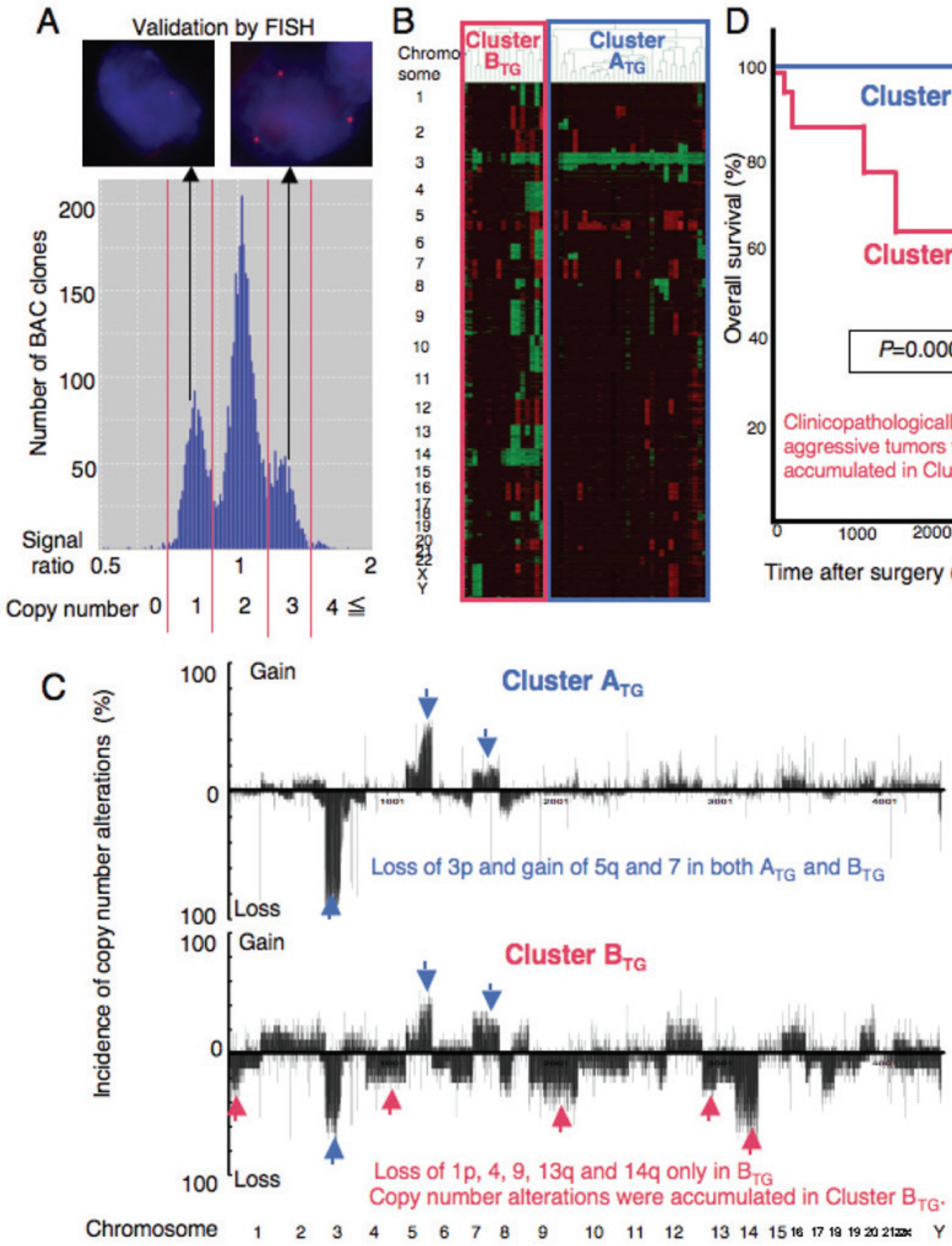

Figure 2. Genetic clustering of clear cell renal cell carcinomas (RCCs). An example of a histogram of the signal ratios (test signal/reference signal) afforded by array-CGH in a clear cell RCC. The thresholds of the signal ratios for copy numbers of 0, 1, 2, 3 and 4 or more were determined from the troughs (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-

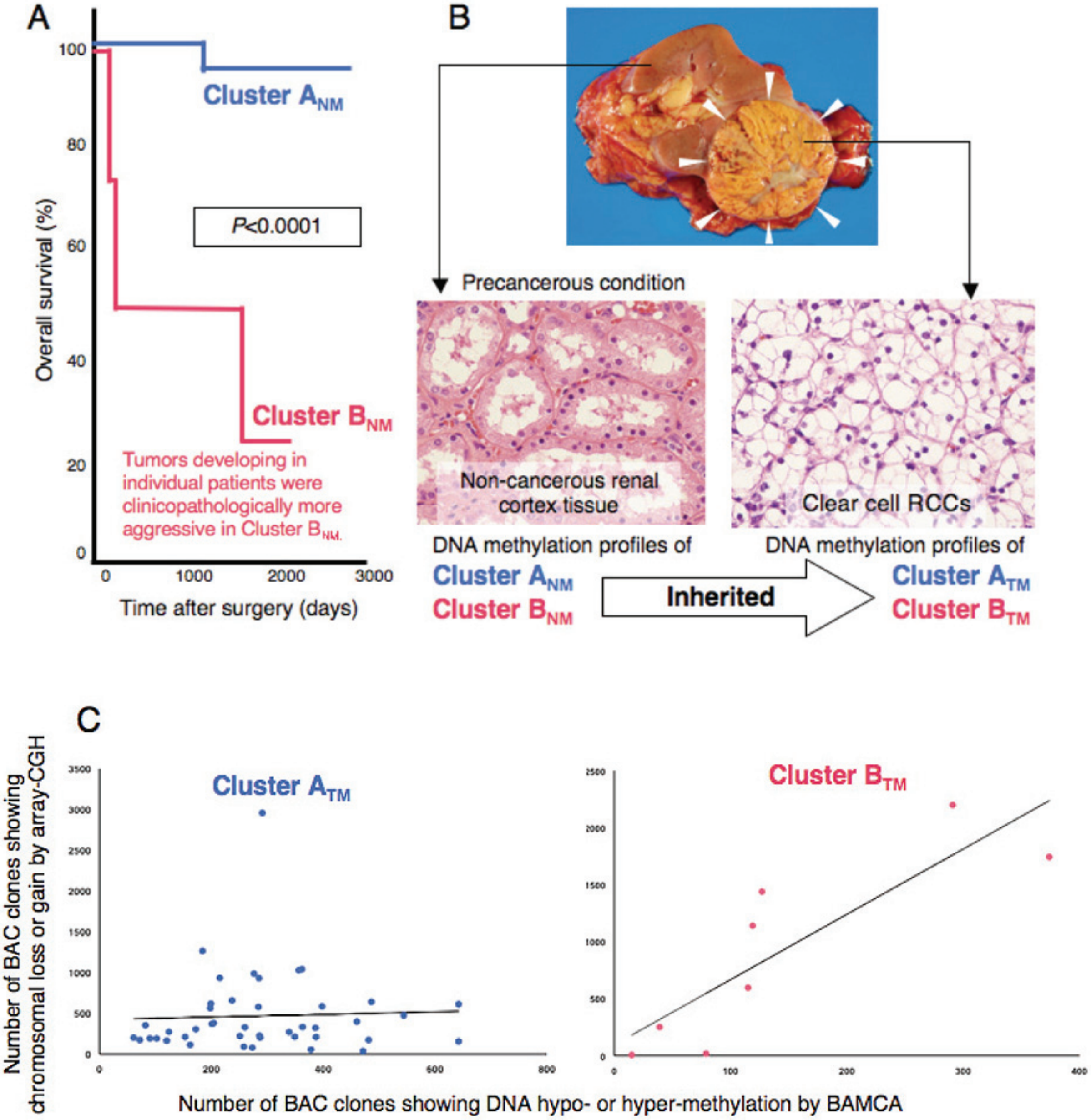

Figure 3. DNA methylation profiles in precancerous conditions and clear cell renal cell carcinomas (RCCs). A. Genome-wide DNA methylation profiles in the non-cancerous renal tissue were significantly correlated with clinicopathological parameters of clear cell RCCs developing in individual patients, 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 immunohistochemistry 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.

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