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Chapter 1

Genetics of Renal Tumors

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1. Introduction

Kidney and urinary tract cancers accounted for a total of 16936 cases and 6764 deaths in 2007 in Japan (Matsuda et al., 2012), which is roughly 2% of all cancers. Renal cell carcinoma (RCC) is the most common type of kidney cancer, and is classified into three major subtypes, clear cell RCC, papillary RCC and chromophobe RCC, representing 80, 10, and 5% of all RCCs, and the majority of renal tumors are sporadic although 2-4% are hereditary (Hagenkord et al., 2011).

A number of genes have been studied in association with renal tumors, including those involved in tumorigenesis, and the progression and outcome of the cancer, by means of mutational searches, gene expression profiling, proteomics/metabolomics and pathological/clinical studies. The genes can be classified into several categories, such as familial, sporadic, epigenetic and quantitative, depending on the timing of their expression, and the factors affecting their effects, such as microRNA (miRNA) and metabolites have emerged. Since tumorigenesis is believed to be initiated with genetic/epigenetic modulations of at least several genes, but not a single gene alone, the balance among these cancer-related genes is considered to be more important than the contribution of a dramatic change caused by a single gene. Thus, an extensive and competitive search for oncogenes and tumor suppressor genes based on the search for their mutations was immediately accompanied by the search for interacting proteins/factors at the mutation sites. This indicates that lineages of gene functions, or signaling pathways, are important to understanding tumorigenesis, as well as the progression and outcome of the cancer. Although such pathways are not fully understood, it is important to summarize the latest knowledge of genes and their functions in terms of the coordinated functions of genes to achieve a basic understanding of cancer and to use the information obtained for diagnostics/therapeutics.

Here, we summarize and discuss the genes associated with renal tumors (Section 2) and then show one of them, *Kank1*, from gene-function networks or signaling pathways (Section 3). We

also discuss a methodology for collecting information on multiple gene functions with a simple pathological system (Section 4).

2. Genes associated with renal tumors

While kidney cancer ranked 9th in 2002 in the European Union and the United States (Baldewijns et al., 2008), its mortality rate was not high in Japan (12th in 2002 and 2007: Matsuda et al., 2012). Although this difference could be attributable to risk factors such as smoking, hypertension and long-term dialysis, there might be a contribution of genes associated with the cancer. In spite that RCC shows a poor survival rate (less than 19%) for patients with metastasis, molecular pathological tests, such as those dividing good and poor prognosis groups, have not been established (Stewart et al., 2011). A lack of such effective tests may be one of the reasons why the mortality rate in Japan has been gradually increasing from 1.8% (2002) to 2.0% (2007).

A large majority of RCC cases are sporadic and only 2-4% are hereditary. There are cases where gene expression profiling cannot distinguish between them (Beroukhim et al., 2009), suggesting common genetic factors between them. Several genes are known to be associated with RCC, such as *VHL*, *TSC1* and *TSC2*, which play different roles in the mechanism of cancer and so have different advantages in diagnostics/therapeutics. The information about genes can be categorized by the levels of genomics, transcriptomics, proteomics and others including metabolomics, and used to understand the mechanism of cancer, to support diagnostic or therapeutic processes. In this section, we focus on the roles and merits of these genes.

2.1. Genes associated with tumorigenesis

Since a majority of sporadic cancers originate from a recessive mutation that causes a loss of function of a particular type of gene, loss of heterozygosity (LOH) is an important step in the disabling of a functional gene (or a wild-type allele) to give a mutated and cancer phenotype. Such genes are termed tumor suppressor genes, and so far, more than 100 have been reported (Fearon, 2002; Polinsky, 2007). Among them, twenty well-characterized genes showed both familial and sporadic phenotypes (Sherr, 2004). Since a cancer phenotype can be revealed by morphological changes, growth stimulation, gaining immortality and/or others, there are quite a few functions associated with tumor suppressor genes. Thus, it is easier to examine tumorigenesis in association with genomic status, mutations and/or epigenetic modifications, by analyzing the loci specific to RCC.

2.1.1. VHL gene

The gene best known to be associated with RCC is the von Hippel-Lindau (*VHL*) gene, whose inactivation accounts for nearly 100% of hereditary cases and sporadic clear cell RCC cases (Baldewijns et al., 2008). This gene was found by positional cloning from the locus associated with the VHL disease, a familial syndrome accompanying cancer in the eye, brain, spinal cord,

kidney, pancreas and adrenal glands. The *VHL* gene encodes the 30-kDa protein VHL, 213 amino acid residues long, and is implicated in the regulation of hypoxia-inducible factors (HIFs) (Maher et al., 2011). The VHL protein forms a complex with elongin B, elongin C and cullin-2, and the complex has ubiquitin ligase E3 activity and is involved in the ubiquitination and degradation of HIFα, the α subunits of transcription factors HIF-1 and HIF-2, which form a dimer with HIFβ and regulate the transcription of hypoxia-inducible genes such as those for VEGF (vascular endothelial growth factor), PDGF (platelet derived growth factor) and TGFα (transforming growth factor α) (Kondo and Kaelin, 2001; Kaelin, 2009; Fig. 1). However, the cancer found in VHL disease is sporadic and the lifetime risk of RCC in VHL disease patients is about 70% (Maher et al., 2011). So, it is reasonable to assume that additional genes are involved in RCC and the mutations in *VHL* are not the definitive cause of RCC, which is one of the reasons to explore new genes and genetic loci (see below). Meanwhile, the status of the *VHL* gene is important for the treatment of VHL disease and kidney cancer patients. HIF-responsive gene products, such as VEGF and PDGF, activate the angiogenesis of tumors and therefore are good therapeutic targets. Inhibitors of VEGF and PDGF, sunitinib and sorafenib, have been approved by the US Food and Drug Administration (Kaelin, 2009).

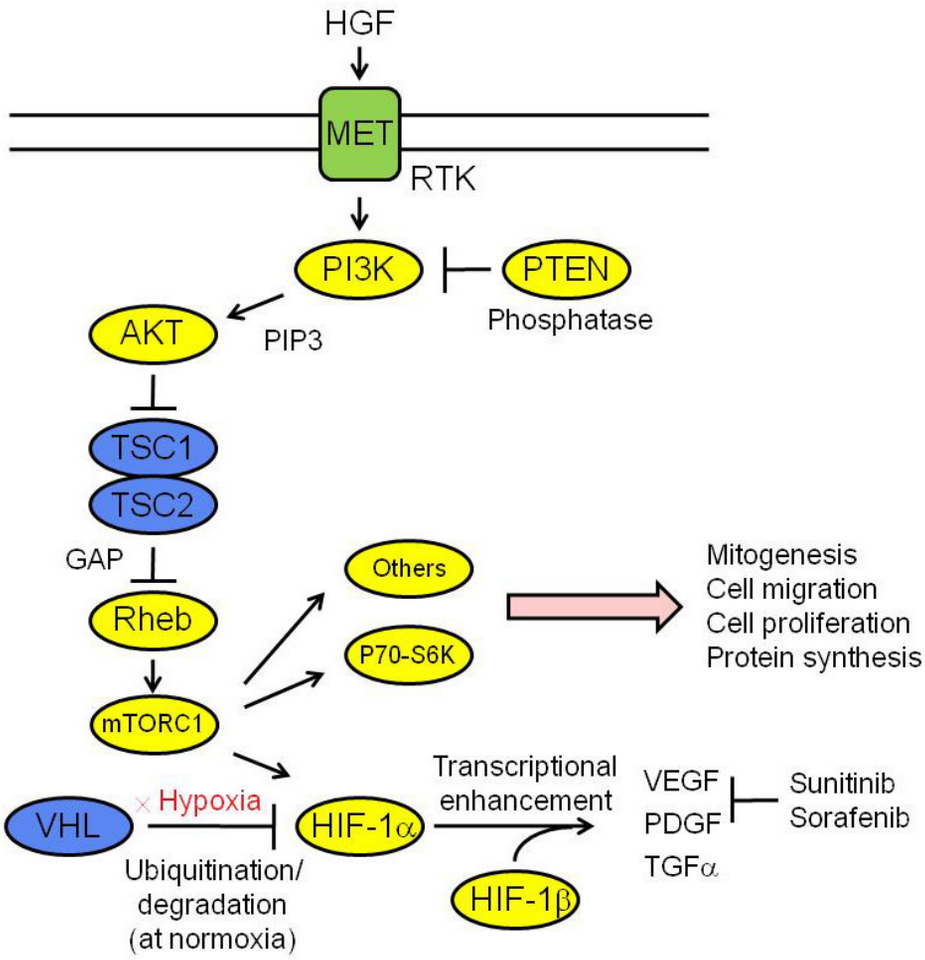

Figure 1. Summary of signal transduction pathways associated with RCC.

2.1.2. MET gene

The *MET* protooncogene was found in hereditary papillary RCC without mutations in the *VHL* gene (Schmidt et al., 1997). *MET* encodes a membrane receptor (MET) for hepatocyte growth factor (HGF). MET has tyrosine kinase activity, and HGF activates this kinase activity and initiates signaling for mitogenesis and migration (Fig. 1). While aberrantly active MET triggers tumor growth, angiogenesis and metastasis, such cases are relatively rare (~5%) among sporadic papillary RCC, suggesting other genes to play a major role in the tumorigenesis (Baldewijns et al., 2008).

2.1.3. TSC1/TSC2 genes

Two tumor suppressor genes, *TSC1* and *TSC2*, were found in a study of tuberous sclerosis complex (TSC), which is known to develop into various types of RCCs, including clear cell RCC, papillary RCC and chromophobe RCC (Borkowska et al., 2011). The TSC1 and TSC2 proteins form a heterodimer and inhibit the mammalian target of rapamycin (mTOR; a key signaling mediator for cell growth), by inactivating a small GTPase Rheb (an activator of mTOR) as a GTPase-activating protein (GAP) (Linehan et al., 2010; (Fig. 1). However, mutations are not frequently found in sporadic RCC (Parry et al., 2001) and therefore its role is not completely clear yet.

2.1.4. PBRM1 gene

Several genes, *UTX* (or *KDM6A*), *JARID1C* (or *KDM5C*) and *SETD2*, were found in close association with clear cell RCC by a recent technology of the next-generation sequencing (Dalgliesh et al., 2010). As these genes are related with the methylation status of lysine residues of hitone H3, further mutation studies were conducted to identify a SWI/SNF chromatin remodeling complex gene, *PBRM1*, to be frequently (over 40%) mutated in clear cell RCC (Varela et al., 2011). *PBRM1* is mapped to chromosome 3p21 and encodes the BAF180 protein, a chromatin targeting subunit of a SWI/SNF chromatin remodeling complex, which regulates replication, DNA repair and cell proliferation/differentiation. Knock-down of this gene enhanced colony formation and migration of cancer cells, suggesting this gene to be a tumor suppressor gene. Further studies are needed to reveal a mechanism of cancer involving *PBRM1* and to find its clinical application.

2.1.5. Genes related to hereditary renal cancer syndromes

Approximately 2-4% of RCC cases are hereditary and some genes have been identified as the genes responsible for hereditary renal cancer (HRC) syndromes (Verine et al., 2010). Apart from the genes already mentioned above (*VHL*, *MET*, *TSC1* and *TSC2*), several more genes have been described in association with HRC syndromes, including *FH* and *FLCN* genes. *FH* is the gene responsible for a HRC syndrome, hereditary leiomyomatosis and renal cell cancer (HLRCC), in which affected individuals often develop cutaneous and uterine leiomyoma and an aggressive form of papillary RCC (Linehan et al., 2004). The *FH* gene encodes an enzyme (FH) catalyzing the conversion of fumarate to malate in the tricarboxylic acid (Krebs) cycle.

From the analysis of their mutations, this gene is considered as a tumor suppressor gene (Sudarshan et al., 2007). Although the mechanism that leads *FH* alterations to cancer is not clearly understood, there is a link between fumarate dysregulation and impaired HIF hydroxylation (Isaacs et al., 2005).

FLCN, on the other hand, is the gene responsible for Birt-Hogg-Dubé (BHD) syndrome, which is a rare autosomal dominant disease including kidney tumors, predominantly chromophobe RCC. Mutations in this gene were found in approximately 80% of BHD kindreds and loss of expression of this gene were frequently found in kidney tumors from BHD patients, suggesting this gene to be a tumor suppressor gene (Baldewijns et al., 2008).

2.1.6. Other genes

Several genes were recently implicated in association with RCC, including *BAP1*, *SETD2* and *NF2*, by means of advanced technologies such as the next-generation sequencing, a microarray-based analysis and a mouse transgene analysis. *BAP1* plays a role of a tumor suppressor and encodes a nuclear deubiquitinase, which is inactivated in 15% of clear cell RCC cases (Peña-Llopis et al., 2012). Mutations in *BAP1* anticorrelates with those in another tumor suppressor gene, *PBRM1*, and these mutations comprise a subtype of clear cell RCC (70% of all clear cell RCC cases). The BAP1 protein may work with host cell factor-1 (HCF-1), a scaffold protein, to regulate transcription factors and suppress cell proliferation.

SETD2 was found by the analysis of accumulated transcripts containing premature termination codons and encodes a histone methyltransferase, which is responsible for trimethylation of the lysine residue at position 36 of histone H3 and may play a role in suppressing tumor development (Duns et al., 2012).

NF2 was identified as a tumor suppressor gene by the analysis of knock-out mice (Morris and McClatchey, 2009). The mice developed kidney tumors in 6-10 months with characteristics of hyperactive epidermal growth factor receptor (EGFR) signaling. Merlin, the *NF2* gene product, was implicated in suppressing tumorigenesis by inhibiting hyperactivated EGFR signaling.

2.2. Genes implicated in diagnostic markers and therapeutic targets

The recurrence of RCC is 20 to 40%, depending on the stage and grade of tumor (Chin et al., 2006). So, it is important to understand the genes (and their products) associated with progression/metastasis to predict the outcome of cancer. The classification of RCC subtypes is apparently not possible by a single marker, but could be done using combinations of markers such as vimentin, epithelial cell adhesion molecule (EpCAM), glutathione S-transferase α (GSTα), carbonic anhydrase II (CA II), cytokeratin 7 (CK7) and cluster of differentiation 10 (CD10) (Stewart et al., 2011).

Important prognostic markers for RCC represent specific cellular signaling pathways, such as the VHL and mTOR pathways. The VHL pathway gives several well-studied markers, such as VHL, HIFs, VEGF and carbonic anhydrase 9 (CAIX), although their ap-

plicability is sometimes questionable (Stewart et al., 2011). HIF-responsive gene products are potential markers representing angiogenesis (VEGF, PDGF, SDF, CXCR4, TGFβ and CTGF), glucose uptake and metabolism (HK2 and PDK4), pH control (CAIX and CAXII), invasion/metastasis (MMP1, SDF, CXCR4 and c-Met), and proliferation and survival (TGFα) (Smaldone and Maranchie, 2009).

Another pathway for potential makers is the mTOR pathway (Fig. 1). The main cascade of this pathway is PI3K/AKT/mTOR, which mediates signals by activating phosphoinositide 3-kinase (PI3K) through kinases such as receptor tyrosine kinases to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which further activates AKT via phosphorylation and phospho-AKT activates mTOR complex 1 (mTORC1) through inhibition of the TSC1/TSC2 complex (Allory et al., 2011). Then, mTORC1 phosphorylates proteins such as P70-S6 kinase and activates protein synthesis and cell proliferation. Importantly, HIF-1α expression is dependent on mTORC1 signaling (Toschi et al., 2008). Potential markers in this pathway include P70-S6 kinase, PTEN (a phosphatase that decreases PIP3) and phospho-AKT.

2.3. Mutation sites and LOH loci

A comprehensive analysis of RCC genomes has been done through genomic (Hatano et al., 2001; Cifola et al., 2008), transcriptomic (Takahashi et al., 2001; Takahashi et al., 2003; Cifola et al., 2008) and proteomic/metabolic (Perroud et al., 2006; Raimondo et al., 2012) approaches. We used a genome-subtraction technique, or the in-gel competitive reassociation method (Kiyama et al., 1995; Rodley et al., 2003), for cloning the sites of LOH that occurred in a RCC genome by subtracting normal DNA from cancer DNA of the same patient (Hatano et al., 2001). The minimum size of LOH (caused by hemizygous deletions) detected by this method was roughly 50 kb. This resolution was made possible by *Mse*I, which recognizes TTAA, a sequence appearing frequently in human genomic DNA, and completely digests genomic DNA to sizes mostly below 1 kb. Such a high resolution has not been used even in recent genome-wide association studies (see Jacobs et al., 2012, for example). A total of 187 clones were mapped on the chromosomes and a total of 44 candidate regions, where at least two clones were mapped within 5 Mb, were selected and analyzed for mapping the sites of LOH in 61 cancer cases (Table 1). Among them, we found interesting LOH sites at 5q32-q34, 6q21-q22, 8p12 and 9p24, whose frequencies are relatively high among RCC and whose lengths are less than ~10 Mb (Hatano et al., 2001; Sarkar et al., 2002; Fig. 2). A tumor suppressor gene, *Kank1*, was found at 9p24 after extensive analysis of the LOH site by examining the loss of function upon its mutation; the loss of expression of the gene at mRNA and protein levels in RCC, and the loss of suppression of tumor growth in renal tumor cells (Sarkar et al., 2002).

2.4. Chromosomal abberations in RCC

Chromosomal abberations are often observed in RCC (Ross et al., 2012). Deletions of chromosome 3p, where the *VHL* gene resides, are found in most sporadic and familial clear cell RCCs. Distinctive abnormalities were reported for papillary RCC, where, in contrast to clear cell RCC, most of the tumors are characterized by trisomy of chromosomes 7 and 17 along with loss of Y, while the 3p arm is intact. In Xp11.2 RCC, the gene fusion was observed between the *TFE3*

gene on the X chromosome and either of *ASPL* (17q25), *PRCC* (1q21), *PSF* (1q34), *NonO* (Xq12) and *CLTC* (17q23) (Kuroda et al., 2012). All of the gene fusions result in overexpression of the TFE3 protein, a transcription factor. Among them, the translocation of t(X;17)(p11.2q25), which fuses the *ASPL* and *TFE3* genes, is most frequently observed. Meanwhile, there are some unclassified cases, such as those where trisomy 7/17 in areas typical of papillary RCC and both trisomy 7/17 and 3p loss in areas with clear cell RCC were observed (Ross et al., 2012).

2.5. Exploration of new genetic markers

Even though a number of genetic markers have been reported, they are not currently used for the diagnosis of RCC. As discussed in Section 1, this is because understanding a single gene or a few genes is not enough for a diagnosis of sufficient reliability. For diagnosing more complex and more specific states of diseases or disease phenotypes, groups of markers that are able to more accurately distinguish the phenotypes are needed. Such markers should be derived from the direct process of the disease and therefore would represent the signal transduction that occurs within the cell. There are several new technologies which might open the door to a more comprehensive understanding of RCC especially at the level of cellular signaling: array-based genome-wide association studies, microRNA (miRNA) studies and next-generation sequencing-based expression profiling.

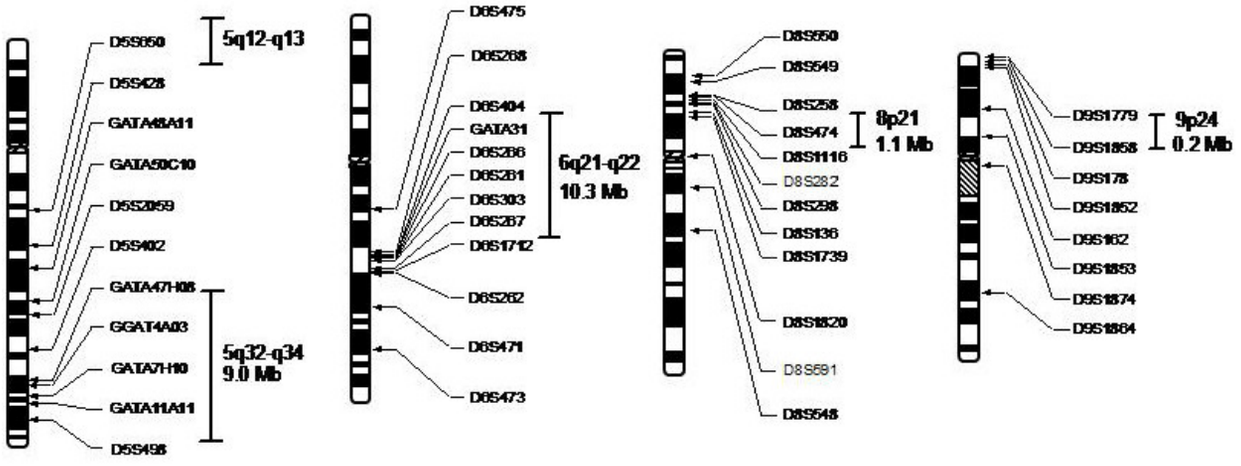

Figure 2. Significant LOH regions in RCC. LOH regions of less than ~10 Mb were identified as the minimum overlapping regions of LOH by subtraction cloning of mutated regions followed by the quantitative allelic analysis of over 60 RCC cases using microsatellite markers (see Hatano et al., 2001; Sarkar et al., 2002).

Location	LOH	(%)
3p22-p23	15/16	(93.8)
3p21.2-p21.3	21/23	(91.3)
3q13.3-q21	13/31	(41.9)
5q12-q13.1	10/31	(32.3)
5q13.1-q14	7/28	(25.0)
5q23.3	10/27	(37.0)
5q31.3-q32	7/28	(25.0)
5q32-q34	14/30	(46.7)
6q22.3	9/34	(26.5)
7p12-p14	5/32	(15.6)
7q11.23	7/33	(21.2)
7q22.1-q31.2	5/38	(13.2)
8p12	5/29	(17.2)
8q13	6/34	(17.6)
9p21-p22	5/20	(25.0)
9p12-q11	6/33	(18.2)
9q31	8/41	(19.5)
11q13.3-q14.1	5/28	(17.9)
11q22.3	2/17	(11.8)
14q11.2-q12	9/26	(34.6)
14q13-q21	10/34	(29.4)
14q24.1-q31	12/36	(33.3)
14q32.1-q32.3	7/22	(31.8)
15q23	5/28	(17.9)
18p11.1-q11.2	8/35	(22.9)
18q22	7/39	(17.9)
Xq26-q28	3/4	(75.0)

Table 1. Summary of LOH at significant locations among 44 sites. For details, see Hatano et al. (2001) and Sarkar et al. (2002). Only the loci in which more than 10 % of RCC patients had LOH are shown. The LOH analysis is applicable to only female patients for the cluster at Xq26-q28. There were a total of 44 clusters containing more than two of 187 clones analyzed within 5 Mb. The locations of the clusters other than those shown above are as follows: 1p31.1, 1p13.3-p22.3, 1p13.3-q12, 1q12-p21.1, 2p21-p22, 2p12-q11.2, 4p14, 4p13.3-p21.1, 4q22, 4q32, 10p14-p15.1, 10p12.1-p12.2, 12q13.3-q15, 13q13-q14.1, 13q14.2-q14.3, 16q12.1-q12.2, and 20p11.2-p12.

Recent advances in high-resolution genomic arrays have enabled us to analyze 1,000 or more disease cases efficiently, and thus to give statistically significant loci associated with the diseases. Such an approach was applied to the study of RCC. A genome-wide association study

based on more than 5,000 RCC cases revealed two loci, 2p21 and 11q13.3, to be associated with RCC susceptibility (Purdue et al., 2011). Although the authors claimed these sites to be previously unidentified, both of the loci were actually identified in 2001 (Hatano et al., 2001; Table 1). While the association is statistically significant, the frequencies among RCC cases are not very high (less than 20%), and therefore, it is doubtful that these sites alone can be used for diagnosis. The candidate genes in these loci which contribute to the association are *EPAS1* encoding hypoxia-inducible factor-2α (HIF2α) and *SCARB1* encoding a scavenger receptor. While HIF2α was known to be associated with RCC though it has not yet been used clinically, SCARB1 is new and its association with RCC may indicate a new signaling pathway. The array-based genome-wide association technique was also applied to the study of copy-number variations (Krill-Burger et al., 2012).

The study of miRNA is rapidly providing as new information about disease phenotypes. MiRNA, a group of short non-coding RNA with lengths of 19-22 nucleotides, differs from mRNA in that it has a role in gene function, and, while information about mutations is important for mRNA, quantity is mostly emphasized for miRNA. So, while there are cases where mRNA bearing a mutation without a change in its quantity contributes to a disease phenotype, there would be few such cases for miRNA. Naturally, the linkage of a disease to a genomic location reveals in most cases a mutation in a gene. This may indicate that miRNA contributes to quantitative change as a group as a result of changes in transcriptional efficiency caused by alterations to the transcriptional machinery or genomic location/status, or by epigenetic modifications. In contrast to mRNA, however, the quantity of miRNA can be controlled rapidly and specifically, and thus, miRNA could be more advantageous for the rapid control of the amount of specific proteins, which is important in signal transduction. Such cases were reported for TGFβ, WNT, Notch and EGF signaling in association with homeostasis, cancer, metastasis, fibrosis and stem cell biology (Inui et al., 2010), and VHL-signaling and VEGF-signaling in association with RCC (Fendler et al., 2011). Several miRNAs were reported to be induced or repressed by VHL-induced hypoxia in RCC and regulate the PI3K/AKT/mTOR pathway and Wnt signaling/β-catenin pathway to control cell proliferation, tumorigenesis and other cellular functions (Redova et al., 2011).

Next-generation sequencing technology was applied to genome-wide expression profiling of miRNA related to clear cell RCC (Osanto et al., 2012). By analyzing 22 RCCs, 100 miRNA differentially expressed between clear cell RCC and matched normal tissues were found. While the biological relevance of these novel miRNAs is unknown, they may be potential diagnostic markers or targets for therapeutics.

3. Kank family genes and renal tumors

3.1. Structure of *Kank*-family genes

The human *Kank1* gene was found as a candidate tumor suppressor gene for renal tumors at 9p24, and encodes a protein containing ankyrin-repeats at the C-terminus and coiled-coil motifs near the N-terminus (Sakar et al., 2002). Based on domain and phylogenetic analyses,

Kank2, *Kank3* and *Kank4* were found to form a family with *Kank1* (Zhu et al., 2008). Five repeats of the ankyrin-repeat motif comprise the basic structure of all Kank proteins (Fig. 3A). In addition, each Kank protein contains different combinations of four types of coiled-coil motifs. They also have a conserved region close to the N-terminus, named the KN-motif (Zhu et al., 2008; Fig. 3A), which contains a leucine-rich region and an arginine-rich region.

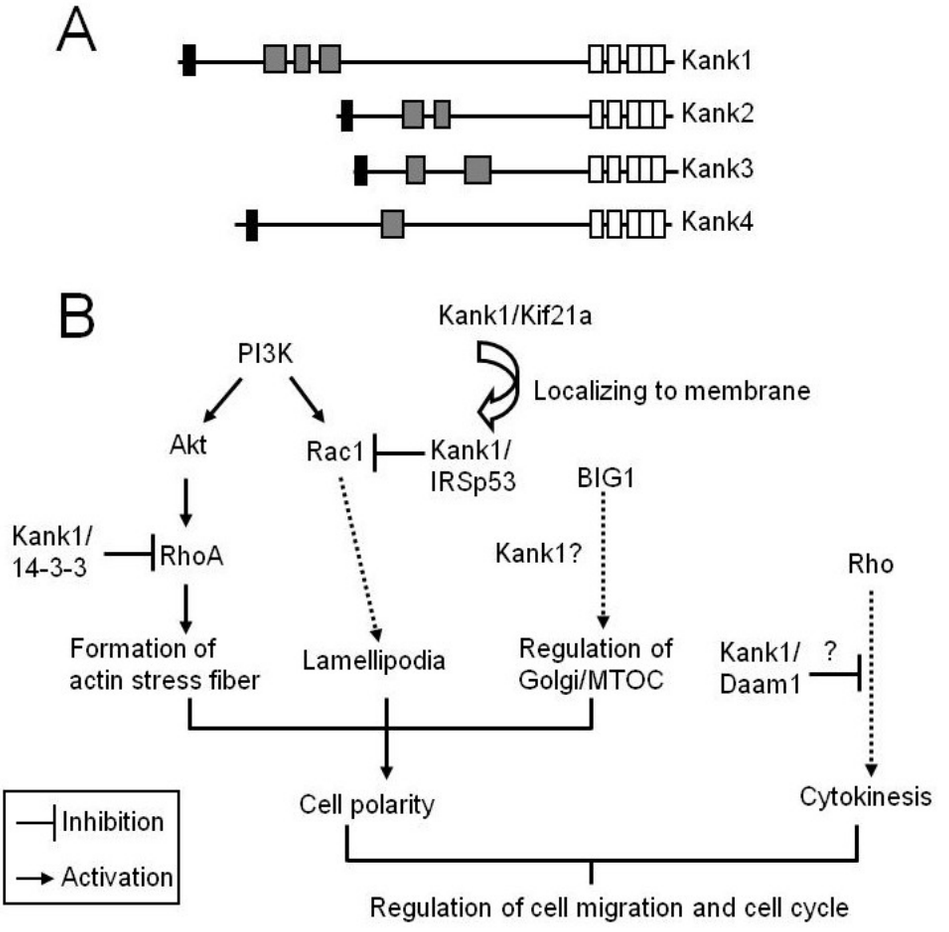

Figure 3. (A) Schematic structure of human Kank family proteins. Black boxes indicate the Kank N-terminal (KN) motif. Gray boxes indicate coiled-coil motifs. White boxes indicate the ankyrin-repeat (ANK) motifs. (B) A hypothetical model of Kank1 functions. Kank1 is transported to areas of membrane ruffling, such as lamellipodia, through association with Kif21a. Kank1 regulates RhoA and Rac1 activities through interaction with 14-3-3 in PI3K/Akt signaling and IRSp53 in Rac1 signaling, respectively. These interactions negatively regulate the formation of actin stress fibers and lamellipodia, and finally decrease cell migration. Kank1 and BIG1 may exist in a multimolecular complex that affects Golgi/MTOC orientation and regulates cell polarity during directed migration. Kank1 may inhibit Rho activation by binding to Rho-regulating proteins, like Daam1, which may result in negative regulation of cytokinesis.

Yeast-two hybrid or mass-spectrometrical studies have shown that Kank1 can directly bind to several proteins, such as 14-3-3 proteins, insulin receptor substrate (IRS) p53, Kif21a and Disheveled-associated activator of morphogenesis 1 (Daam1). Kank1 binds to IRSp53 and Daam1 at its coiled-coil domain (Kakinuma et al., 2011). In addition, there is a 14-3-3-binding

motif, serine at position 167, located between the first and second coiled-coil motifs. Kif21a is a unique protein found to interact with the ankyrin-repeat domain of Kank1 (Kakinuma et al., 2008 & 2009; Roy et al., 2009; Suzuki et al., unpublished data). Although the function of the KN-motif is not clear, it contains several potential motifs for a nuclear localization signal (NLS) and nuclear export signal (NES). These signals may contribute to nucleo-cytoplasmic shuttling of Kank1, and further affect the subcellular distribution of β-catenin (Wang et al., 2006; Previdi et al., 2010).

3.2. Functions of *Kank*-family genes

Some studies have demonstrated that *Kank*-family genes are related to various cell functions. VAB-19, an ortholog of the Kank1 protein in *C. elegans*, was reported to occur with components of an epidermal attachment structure. It plays an antagonistic role in the regulation of actin cytoskeleton and halts basement membrane opening associated with cell invasion and tissue remodeling (Ding et al., 2003; Ihara et al., 2011). The deletion of *Kank1* is associated with parent-of-origin-dependent inheritance of familial cerebral palsy (Lerer et al., 2005). There have also been reports that *Kank1* was fused with the gene for platelet-derived growth factor receptor β (PDGFRβ) and that the fusion protein was a vital regulator of hematopoietic cell proliferation (Medves et al., 2010 & 2011). Meanwhile, *Kank1* expression was down-regulated in patients with polycythemia vera, suggesting this gene to be related to myeloproliferative disorders (Kralovics et al., 2005). Some studies have described about the functions of other *Kank*-family members. Kank2, found as a novel podocyte-associated protein, may contribute to the regulation of actin dynamics in podocyte foot processes in the renal filter physiology and diseases (Xu et al., 2011). In addition, NBP, an ortholog of Kank3 in zebrafish, interacts with Numb, an adaptor protein implicated in various basic cellular processes, through the PTB domain, which is well conserved among vertebrate *Kank* genes. In embryogenesis, NBP accumulates at the cell periphery during gastrulation and, later in the development, is concentrated at the basal poles of differentiated cells. These findings suggest a role for NBP in regulating cell adhesion and tissue integrity (Boggetti et al., 2012).

Kank1 may contribute to several regulatory activities, such as regulation of the actin cytoskeleton, cell migration and the cell cycle through interactions with the proteins described above (Sakar et al., 2002; Kakinuma et al., 2008 & 2009; Roy et al., 2009; Suzuki et al., unpublished data; summarized in Fig. 3B). Kank1 regulates the Rac1-dependent formation of lamellipodia and the activity of RhoA, resulting in the inhibition of cell migration. This function is mediated through two binding partners of Kank1, 14-3-3 and IRSp53. Kank1 binds to the Akt-phosphrylation motif of 14-3-3θ, 14-3-3γ, 14-3-3η and 14-3-3ε. Interaction between these two proteins is enhanced by growth factors such as insulin and epidermal growth factor (EGF) (Kakinuma et al., 2008). This interaction regulates the activation of RhoA through the PI3K/Akt signaling pathway. When a 14-3-3 binding motif is phosphorylated by Akt, 14-3-3 is separated from an activation complex for RhoA, and binds to Kank1 resulting in the inhibition of RhoA activities, and thereby decreases the formation of actin stress fibers and inhibition of cell migration (Kakinuma et al., 2008). The coiled-coil domain of IRSp53, which

is the site for the interaction with active Rac1, binds to Kank1. Endogenous Kank1 and IRSp53 are co-localized at the site of membrane protrusions such as lamellipodia, which are needed for cell migration. Overexpression of Kank1 inhibits the formation of lamellipodia induced by active Rac1 in NIH3T3 cells, and knockdown of Kank1 enhances the formation. Therefore, Kank1 negatively regulates membrane protrusions at the leading edge of cells, by inhibiting the association between active Rac1 and IRSp53 (Roy et al., 2009). Taken together, Kank1 regulates cell migration through inhibition of IRSp53 in Rac1 signaling and inactivation of RhoA activity through PI3K/Akt signaling (Fig. 3B). As the Kank1 locus shows loss of heterozygosity in RCC and the expression of the Kank1 gene is suppressed in RCC, Kank1 may contribute to the malignant transformation of cells such as metastasis.

Kank1 regulates cell migration by inhibiting Rac1 signaling and RhoA activity as described above. To fulfill this function, Kank1 needs to be located at the leading edge of cells and affect the neighboring membrane. Because Kank1 has no membrane-targeting motif or membrane protein to associate with, some proteins may help transport Kank1 to the site of membrane ruffling. Kank1 interacts with the third and fourth coiled-coil domains of KIF21a, a member of the Kif4-class superfamily of kinesin motors that acts as a plus-end kinesin motor (Marszalek et al., 1999; Kakinuma et al., 2009), at its ankyrin-repeat domain. Overexpression of Kif21a or one of the Kif21a mutants (R954W) enhances the translocation of Kank1 to the membrane. In contrast, knockdown of Kif21a decreases the amount of Kank1 at the membrane (Yamada et al., 2005; Kakinuma et al., 2009). Although the mechanisms involved need further study, translocation of Kank1 mediated by Kif21a may affect cell migration (Fig. 3B). Kank1 is also functionally associated with a protein, brefeldin A-inhibited guanine nucleotide-exchange 1 (BIG1), a binding partner of Kif21a. Although there is no direct interaction between these two proteins, they may exist in a multimolecular complex that maintains the orientation of the Golgi/microtubule-organizing center (MTOC) and regulates cell polarity during directed migration. Furthermore, a protein complex containing BIG1, Kif21a and Kank1 may contribute to directed transport along microtubules (Li et al., 2011).

Overexpression of Kank1 suppresses the cell cycle and cell growth (Sakar et al., 2002). We observed that the overexpression of Kank1 blocked cytokinesis and generated binucleated cells. We also found co-localization of endogenous Kank1 with Rho, a key molecule required in cytokinesis for regulating the constriction of the contractile ring, at the contractile ring during cytokinesis of NIH3T3 cells (Kamijo et al., 2006; Li et al., 2010; Kakinuma et al., 2011; Suzuki et al., unpublished data). The coiled-coil domain of Kank1 binds to another protein, Daam1 (Suzuki et al., unpublished data). Daam1 belongs to a novel protein family containing formin homology domains and has been implicated in the regulation of cell polarity associated with the Wnt/Frizzled/Rho signaling pathway (Jantsch-Plunger V et al., 2000; Kosako H et al., 2000). Although the mechanism is still not clear, Kank1 may block cytokinesis by regulating Rho activity through the interaction with Daam1 (Fig. 3B). Therefore, it may reveal a new mechanism of regulation of cytokinesis and tumor suppression.

3.3. *Kank*-family genes and renal tumors

The *Kank1* gene was found at 9p24 by a comprehensive analysis of human chromosomes for loss of heterozygosity (LOH) in RCC (Sakar et al., 2002). Kank1 family proteins localize at the area of cytoplasma in renal tubular cells and glandular cells of some digestive and endocrine organs (Roy et al., 2005). Kank family genes show different expression patterns at the mRNA and protein levels in normal and tumor kidney tissues and some kidney tumor cell lines (Zhu et al., 2008; Wang et al., 2005). Loss of expression of *Kank1* in RCC was confirmed by Western blotting, RT-PCR and immunohistochemical analyses (Sakar et al., 2002, Roy et al., 2005). In addition, immunostaining in RCC showed decreased expression of Kank1 in high grade tumors (Zhu et al., 2011). Therefore, the *Kank* family genes may be related to renal carcinoma, and function as tumor suppressors.

A growth inhibitory effect of Kank1 has been reported. Overexpression of *Kank1* in HEK293 cells resulted in cell cycle arrest at G_0/G_1. On the other hand, growth suppression of tumor cells was caused by *Kank1* gene expression using nude mice abdominally injected with HEK293 cells stably expressing *Kank1* (Sakar et al., 2002). These findings demonstrated that Kank1 can regulate the growth of cells and can also regulate the abnormal growth of cancer cells. Kank1 may exert its growth inhibitory effect by regulating Rho activity mediated via its association with Daam1, resulting in abnormal nuclear division, and thus blocking the cytokinesis of cancer cells (Suzuki et al., unpublished data). According to recent studies, Kank1 can negatively regulate the formation of actin stress fibers and cell migration (Kakinuma et al., 2008; Roy et al., 2009). When cells need to control migration, Kank1 could be transferred to the leading edge of the moving cells' membranes, mediated by Kif21a, and co-localized with IRSp53. Kank1 may bind to IRSp53 competing with active Rac1, and thus inhibits integrin-induced cell spreading and the formation of lamellipodia. Simultaneously, Kank1 may inactivate RhoA, which is controlled by binding with 14-3-3, inhibit the formation of actin stress fibers and ultimately inhibit cell migration. Loss of expression of Kank-family proteins may enhance cell migration in renal cell carcinoma. Since enhancement of cell migration is related to metastasis, Kank-family proteins might be related to the malignancy of renal cell carcinoma.

According to studies to date, the Kank1 protein may act as a tumor suppressor through inhibition of cell migration and cell cycle. These functions are facilitated by several proteins interacting with Kank1, including 14-3-3, IRSp53, Kif21a and Daam1. Further studies of the interactions of these proteins will help us to understand clearly the role of Kank family proteins in tumorigenesis.

3.4. Clinical study of *Kank1* gene in renal cancer patients

3.4.1. Genetic and clinical characteristics of renal tumors

Kidney cancer accounts for about 4% of adult cancers, with an estimated 64,770 new cases annually in the US (Siegel et al., 2012). Of kidney cancers, 92% are pathologically diagnosed as RCC. This "RCC" has interesting and unique characteristics when investigated from a clinical view point. Although 95% of patients with T1-T2 RCC survived 5 to 10 years, among

those with metastatic disease the 5 year survival rate was 26% (DeCastro and McKiernan, 2008). Renal cancer is resistant to conventional chemotherapeutic agents and also to radiation therapy. Many cancer-related genes have been found in renal cancer, including a multi-drug resistance gene (Walsh et al., 2009), anti-apoptotic genes (Bilim et al., 2009), and radiation resistant components (Kransny et al., 2010). The most characteristic genomic structure in renal cancer is the *VHL*-related hypoxia-inducible factor gene and its cascades shown in hereditary RCC and sporadic RCC cases (Linehan et al., 2011). The down-regulation in expression of *Kank1*, our main theme, was also found from the study of renal cancer and normal renal tubular cells (Sarkar et al., 2002), as we mentioned in other sections. The current WHO classification of RCC in 2004 (Deng and Melamed, 2012) follows the earlier Heidelberg and Rochester classifications, recognizing the heterogeneity of RCC, and describes distinct types of RCC with unique morphologic and genetic characteristics. The most popular histological type, clear cell RCC, accounts for 80 % of all RCC cases. Compared with clear cell RCC, papillary RCC (10%) and chromophobe RCC (5%) are more benign. Collecting duct (bellini) (1%) type or other rare sarcomatous types of RCCs are more aggressive (Deng and Melamed, 2012). However, once metastasis occurs, papillary and choromophobe RCCs are more resistant to immunological and new molecular targeting agents than clear cell RCC (Chowdhury et al., 2011). These clinical features characterize the complexity of the clinical categorization of RCC.

Kank1 was found by a genome subtraction method among the genes at 9p24 susceptible to RCC (Sarkar et al., 2002). A devoted study revealed that *Kank1* belongs to a four-member family, has splice variants, and plays a role in cell migration, intracellular transport and cell division, suggesting that *Kank1* has a kind of tumor suppressor function (Kakinuma et al., 2009). In this section, the expression of the Kank1 protein in renal cancer specimens resected from RCC patients is indicated using immunohistochemical methods, and the relationship between the expression and tumor pathology, patient status, and clinical outcomes is examined.

3.4.2. Expression of Kank1 protein in renal cancer and autologous normal kidney

1. Expression of *Kank1* in RCC

We tried to find a RCC-related gene at 9p24, which lead to the discovery of *Kank1*. Of nine ESTs analyzed in the 9p24 region, only three (WI-17492, WI-12779 and WI-19184) were expressed in the kidney. The *Kank1* gene was associated with WI-12779. This *Kank1*-associated EST lost its expression in six out of eight cancer cases. Kank1 expression was examined in 5 matched normal kidney and cancer pairs by Western blotting using an anti-Kank1 antibody, which was obtained as mentioned below. Reduced or loss of Kank1 expression in cancer was observed in all 5 cases.

2. Immunohistochemical study of *Kank1* expression in RCC and the relationship between its expression and clinical-pathological outcomes

One hundred and five formalin-fixed paraffin-embedded slides including normal renal tubular cells and RCC were subjected to immunohistological staining for Kank1 with a monoclonal antibody. An anti-Kank1 (total Kank1) antibody was generated by a previously

reported method (Roy et al., 2005). In brief, amino acids 406 to 580 of the Kank1 protein were fused in-frame with the glutathione S-transferase gene in the vector pGEX. After induction of the fusion protein in *E. coli*, it was purified and used to immunize mice. A mouse hybridoma cell producing an anti-Kank1 antibody was selected and amplified for further use.

The histological subtypes of RCC analyzed here were as follows; 92 clear cell RCCs, 11 papillary RCCs, 5 chromophobe RCCs and 7 other histological types. We compared all histological subtypes with clear cell RCC. The evaluation of positivity of staining was done by two independent examiners, who decided that the sample was positive when more than 30 % of cells were stained with the antibody, weakly positive (±) when 5 to 30 % cells were stained, and negative when less than 5 % cells were stained. The 2004 WHO histological classification (Eble et al., 2004), 2002 TNM classification (Edge et al., 2010) and Fuhrman nuclear grade (Fuhrman et al., 1982) were used in this study. Kaplan-Meyer cause-specific survival was determined and statistical difference in positivity was evaluated by the Kluskal-Wallis test using Stat View™ software following the instructions.

Representative examples of positive and negative staining for the Kank1 protein in clear cell RCC and positive staining in normal renal tubular cells are indicated in Fig. 4. Normal renal tubules usually expressed Kank1. Of 92 clear cell RCCs, Kank1 was positive in 47 cases (52%). Kank1 was weakly positive (less than 30% of cells) in 14 cases (15%). Kank1 was negative in 29 cases (33%). The results grouped by clinical outcome (clear cell RCC) and histology are summarized in Table 2. There was no relation or special tendency between the staining results and clinical results on Kank1 expression. Kank1 was expressed in 87.5% of other histological RCC subtypes.

		Kank1		
		(+)	**(±)**	**(-)**
Clear cell	Alive without cancer	29	11	18
	Alive with cancer	7	1	4
	Dead	11	2	7
Others	16 alive, 7 dead	21	2	1

Table 2. Immunohistological staining of Kank1 antibody classified by clinical outcome (clear cell RCC) and histological subtypes. Sums of the numbers of patients do not match all the evaluated numbers due to inavailabilty of follow-up to judge the clinical outcome.

There were no differences in the survival curves for clear cell RCC among the groups (Fig. 5). However, when the positivity rate was evaluated among the groups divided by the Furman nuclear grade, a highly malignant grade of clear cell RCC showed high Kank1 positivity ($p < 0.05$), while the others did not (Table 3). In clear cell RCC, 42% of grade 1 tumors were Kank1 negative, while 80% of grade 3 tumors were Kank1 positive. In other histological types, there was no apparent difference among nuclear grades (most of them showed Kank1). When subdivided by pathological T stages, higher T stages of clear cell RCC showed a tendency to

express Kank1 ($p = 0.07$) (Table 4). Other factors such as patient's age, gender and the size of the tumor (largest diameter) had no relation to the expression of Kank1 in clear cell and other RCCs (data not shown).

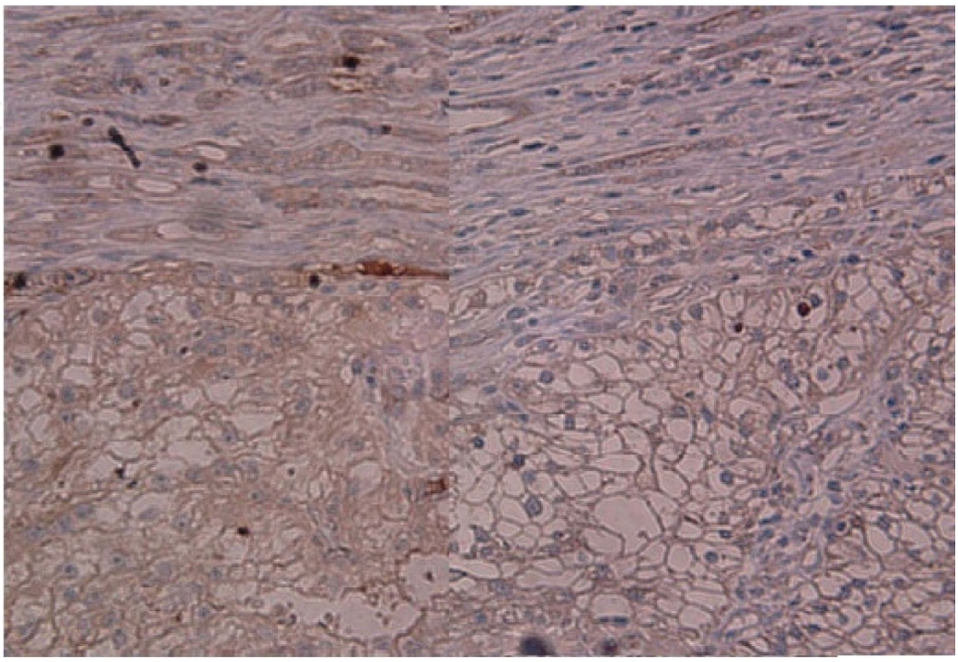

Figure 4. Immunohistochemical analysis of Kank1 protein in clear cell RCC. There was a case of positive staining of Kank1 protein in both normal renal tubular cells (upper left) and clear cell RCC (lower left), while another case indicates negative staining of Kank1 in clear cell RCC (lower right) while it was positive in the normal renal cells (upper right) (reduced from 40× images).

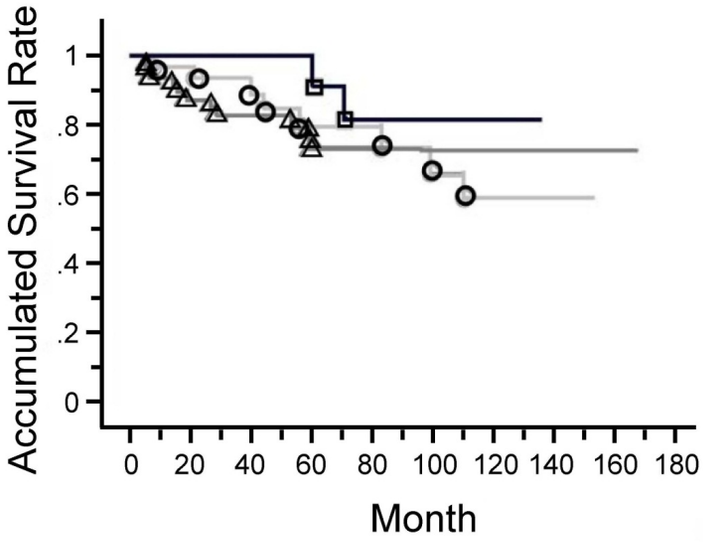

Figure 5. Kaplan-meyer's overall survival curve of RCC patients classified by Kank1 positivity (○ psitive; △ weakly positive; □ negative). None of these survival curves showed statistical differences.

		Kank1		
		(+)	(±)	(-)
Clear cell RCC	grade 1	9	6	11
	grade 2	31	6	19
	grade 3	8	2	0
Others	grade 1	5	0	0
	grade 2	12	1	1
	grade 3	5	1	0

Table 3. Results of Kank1 staining classified by histological grade.

		Kank1		
		(+)	(±)	(-)
Clear cell RCC	pT1	27	10	17
	pT2	5	2	8
	pT3	14	2	4
	pT4	2	0	0
Others	pT1	11	1	1
	pT2	5	0	0
	pT3	5	0	0
	pT4	1	1	0

Table 4. Results of Kank1 staining classified by pathological stage.

3.4.3. Meaning of Kank1 expression and clinical outcome

Many RCC cells showed inactivation of the *Kank1* gene as shown here. This inactivation presumably occurs at the early stage of carcinogenesis in normal renal tubular cells. Because hemizygous methylation of *Kank1* was observed in many cancer cells (Sarkar et al., 2002), inactivation of *Kank1* could be caused in both alleles by an epigenetic modification such as methylation, rather than by mutations.

Concerning the genetic abnormality of RCC, mutations in the *VHL* gene are most prevalent especially in clear cell RCC (Arai and Kanai, 2011). While *VHL* mutations can be found quite often in sporadic clear cell RCC, they are not significant in other RCC histological subtypes or benign oncocytoma. *VHL* mutations affect the activation of hypoxia-inducible factors, and

investigation of this pathway will contribute to a new molecular targeting therapy for RCC (Suwaki et al., 2011). The difference in *VHL* mutations among the RCC histological subtypes suggests a difference in carcinogenesis for each histological subtype, though the origin of the cancer is always a renal tubular cell.

Given that the alteration of *Kank1* expression occurred at the early stage of carcinogenesis, our findings that *Kank1* expression differed among the histological subtypes of RCC might reflect a difference in cancer development (Kim et al., 2005). In clear cell RCC, the loss of *Kank1* expression occurred at a high rate in the lower grade tumors, and the expression was reoccurred as the malignant grade increased. Although the reason for this is not clear, it is presumed that epigenetic modifications such as methylation might have been removed when the malignant grade increased, and consequently, the expression reoccurred (Kisseljova and Kisseljov, 2005). There was no difference in *Kank1* expression between the samples obtained from the groups of patients who survived or not (Table 2). This may reflect the fact that histological grade does not necessarily contribute to clinical outcome, but clinical stage (i.e. the presence of metastasis) is more crucial to obtaining a good prognosis (RCC patients diagnosed at the early stage have more than a 90% five year survival rate) (Lane and Kattan, 2008). The discordance of T stage (tumor size) and the malignant grade on *Kank1* expression could also be supposed for the same reason. A similar result was found for the expression of *CDKN2A* encoding a growth suppressor protein, which is located at 9p21 and close to *Kank1* (9p24) (unpublished data). Although the loss of *Kank1* expression resulted in increased proliferation and poor differentiation in *in vitro* study (Sarkar et al., 2002), our results about the *in vivo* expression of *Kank1* in clinical cases proved that reduced expression does not necessarily reflect a high grade malignancy or poor clinical outcome. These contradictory experimental and clinical results are very interesting, because they suggest that malignant transformation of a normal renal tubular cell has many genetic alterations and clinical outcome is contributed to by many factors in RCC.

4. Prospect of using Kank family genes in genetic diagnosis and gene therapy for renal tumors

4.1. Future diagnostics for RCC

The lack of clinical impact of the current diagnostic markers for RCC apparently requires progress in methodology, biology and pathology (Stewart et al., 2011). The progress in methodology needs the quality of the methods to satisfy the specificity, stability and biological relevance of the markers for diagnosis. For this, sufficient numbers, tens to thousands, of markers would be needed and such markers could be obtained only through cellular signaling analyses. There are quite a number of potential protein and genetic markers for diagnosis and therapeutic targets of RCC based on the information of signal transduction (see Section 2), and more information would be added in the future. While sampling is easier for DNA and RNA-based assays, protein assays such as immunohistochemistry and more advanced mass-spectrometry techniques have problems of contamination and degradation/modification at

sampling and processing. In immunohistochemisty, protein cross-linking at the preparation steps disturbs antibody binding. Sampling of homogenously expressed proteins is crucial for the stability of assays, but would not be possible for most sampling cases as the tissue itself is not homogenous. However, diagnosis even for such cases could be possible with markers sufficiently distinguishing heterogenously expressed proteins in different parts of the diseased tissue. In all cases, a statistical significance analysis should be included as a standard evaluation step for quality control of multi-marker systems such as DNA microarrays (Shi et al., 2010).

Biologically relevant markers will be made available in the future based on the analysis of signal transduction, because, as shown in Fig. 1 (Section 2), there are a number of markers available even within a single signaling pathway and there are sufficient numbers of different pathways affected by the disease, which will contribute to the stability of assays. As discussed, the VHL and mTOR pathways have drawn much attentions to prognosis/diagnosis and therapeutic targets for RCC, but there are more pathways such as the Myc and FLCN pathways and pathways related to VEGF, PDGF and TGFα, and some are specific to subtypes of RCC (Linehan et al., 2010; Allory et al., 2011).

Meanwhile, pathologically relevant markers will also be made available in the future, although the situation is different from other technologies due to the technical limit in the number of markers to examine simultaneously.

4.2. A new fluorescence-based immunohistochemical technique

One obstacle to improving immunohistochemistry is the availability of markers. Immunostaining is a relatively simple technique and thus can be used in unequipped laboratories and hospitals, because the preparation, storage and handling of samples are relatively simple. However, ordinary immunostaining is based on single-dye (or single-marker) colorimetric techniques such as the alkaline phosphatase-based method. This is because of a lack of multi-dye (or multi-marker) colorimetric techniques due to expensive devices and, especially, inavailability of stable fluorescent dyes. Fluorescent dyes have been used in many technologies although this has not happened yet in immunohistochemistry because of the lack of their sufficient stability. Stable fluorescent dyes are thus needed for progress in immunohistochemistry.

We reported applications of a new fluorescent dye, Fluolid, for DNA microarray assays and immunohistochemistry (Zhu et al., 2011). Fluolid dyes, including Fluolid-Orange, show stability against heat and excess light compared with other dyes (Fig. 6) and thus can be stored for more than a year without losing fluorescence (data not shown). So, multi-color immunohistochemistry with stable fluorescent dyes will change the pathological diagnostics in several ways: long-term storage of stained sections, simultaneous multi-marker detection and handling of fluorescently stained sections. Heat and light stable fluorescent dyes will enable us to store fluorescently stained sections at room temperature for a long time, which will be important for follow-up studies by microdissection of specific regions.

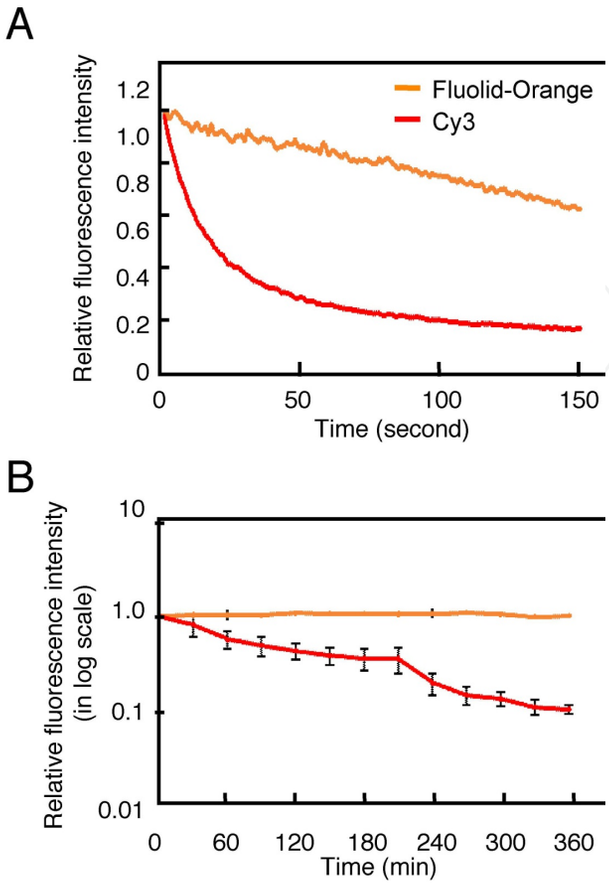

Figure 6. Stability of fluorescently labeled IgG. (A) Photostability of Fluolid-Orange- or Cy3-labeled IgG under irradiation for up to 150 sec with a laser beam at 488 nm. (B) Heat stability. Fluolid-Orange- or Cy3-labeled IgG was left in an environment of 100ºC and fluorescence was measured every 30 min. For details, see Zhu et al. (2011).

4.3. Future therapeutics

As discussed in Section 4.1, future diagnosis will be based on sufficient numbers of protein markers possibly obtained from signal transduction pathways, which will give a statistically significant decision even for cases where no decisive markers, such as disease-causing mutations or constitutive active proteins, are available. In the case of future therapeutics, multiple targets will also be considered to be an effective strategy. Signal transduction-based targeted therapeutics have already been developed for some diseases and drugs such as imatinib or Gleevec/Glivec, a small molecule inhibitor against activated tyrosine kinase activity by the Bcr-Abl fusion gene used for the treatment of chronic myelogenous leukemia, are available (Radford, 2002). Other monoclonal antibody-based drugs such as trastuzumab or Herceptin, which blocks a growth factor receptor HER2/neu (c-erbB-2) to treat breast cancer, and panitumumab or Vectibix, which blocks HER1 to treat colorectal cancer, have been developed based on signal-transduction. Although these drugs are effective, continuous use will sometimes generate drug-resistant cancer (Schenone et al., 2011). So, treatment with multiple targeting drugs will be important in future therapeutics and the same is true for the matched diagnostics about multiple targets.

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