A Novel Ankyrin-Repeat-containing Gene (Kank) Located at 9p24 Is a Growth Suppressor of Renal Cell Carcinoma*  

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By a combination of genome subtraction and comprehensive analysis of loss of heterozygosity based on mapping hemizygous deletions for a potential tumor-related locus, a minimum overlapping region of deletions at 9p24 the size of 165 kb was identified and found to harbor a new potential tumor suppressor gene for renal cell carcinoma, the Kank gene. Kank (for kidney ankyrin repeat-containing protein) contains four ankyrin repeats at its C terminus. Expression of the gene was suppressed in 6 of 8 or 6 of 10 cancer tissues examined by reverse transcription-PCR or Western blotting, respectively, and in several kidney tumor cell lines due to methylation at Cpg sites in the gene. Epigenetic methylation or imprinting seemed to be the first hit, which was followed by a second hit of deletion, resulting in loss of function in many of these deletion cases. Expression of this gene in expression-negative HEK293 cells induced growth retardation at G0/G1 as well as morphological changes.

Renal cell carcinoma (RCC) is the most common malignancy in the adult kidney and shows genetic rearrangement in a number of chromosomes (1–5). Among them, chromosome 3 shows a very high incidence of loss of heterozygosity (LOH); and several established or candidate tumor suppressor genes, including the VHL, FHIT, and RASSF1A genes, are located on this chromosome (6–8). The VHL gene is responsible for a hereditary cancer syndrome, the von Hippel-Lindau syndrome, and accounts for 30–90% LOH on chromosome 3 in sporadic clear cell RCC. On the other hand, other chromosomes show LOH at various frequencies, generally ranging from 20 to 50%; and several candidate genes were mapped. Tuberous sclerosis patients develop RCC, and the TSC-2 gene located on chromosome 16 may be responsible for this type of RCC (9). However, details of the mechanism of RCC formation are unknown for most of these established or candidate tumor suppressor genes, and a number of genes are associated with RCC.

Loss of function of recessive tumor suppressor genes proceeds through inactivation of the genes for both alleles (10). This process, so-called “two hits,” involves genetic as well as epigenetic mutations. On the other hand, an increasing amount of evidence supports loss of function by methylation in the promoter and/or exon 1 regions (11–13). Such genes might not accompany a genetic mutation at a very high frequency and thus show a relatively low incidence of deletions, which are needed for mapping the sporadic tumor-related loci with microsatellite markers. This indicates that a tumor suppressor gene can be found even in the loci showing relatively low frequencies of homozygous or hemizygous deletions. As a comprehensive method for searching and cloning the sites of hemizygous methylation is not available, mapping hemizygous deletions for the tumor-related loci is still an efficient strategy.

We previously performed a comprehensive search for LOH sites in 44 (mostly clear cell-type) RCC cases, and a total of 27 candidate loci showing frequencies of LOH of at least 10% were found (14). Among them, LOH on chromosome 9 showed as a relatively frequent event in RCC (25%). We report here a candidate tumor suppressor gene and a mechanism of LOH for this gene.

EXPERIMENTAL PROCEDURES  

Cell Culture, Matched cDNA, and Antibody—RCC cell lines were obtained from American Type Culture Collection. The experimental procedure for the primary culture of cells has been described previously (15). The normal cells retained the characteristics of renal tubular cells. The matched normal and tumor cDNA pairs for screening ESTs were purchased from CLONTECH. For generating anti-Kank antibody, PCR-amplified cDNA corresponding to amino acids 406–580 of the Kank protein was fused in-frame with the glutathione S-transferase gene in the vector pGEX (Amersham Biosciences); and after induction of the fusion protein in Escherichia coli cells with isopropyl-β-D-thiogalactopyranoside, the protein was purified using glutathione-Sepharose beads (Amersham Biosciences) and used to immunize rabbits. The immunized serum was affinity-purified on a column prepared with the same antigenic glutathione S-transferase fusion protein coupled to Sepharose.

Immunostaining—The Kank cDNA was cloned into pcDNA3.1(+) (referred to here as pCMV-vec; Invitrogen) to obtain pCMV-Kank, which was used for transfection. HEK293 cells grown on coverslips were transfected with pCMV-Kank using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. The following day, the cells were fixed with cold (−20 °C) methanol, washed with PBS, and incubated with anti-Kank antibody for 1 h at room temperature. Fluorescein isothiocyanate-labeled rabbit IgG was used for visualization of the Kank protein under a fluorescence confocal microscope (LSM-410, Carl Zeiss, Inc.).

Immunoblotting and Immunoprecipitation—To prepare cell extracts, cells were washed three times with PBS and lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 140 mM NaCl, 10% glycerol, 1% Nonidet P-40, 100 mM NaF, 200 mM Na2VO4, 1 mM phenylmethylsulfonil fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml chymotrypsin for 15 min on ice. The lysates were centrifuged for 15 min at 4 °C and used for...
immunoprecipitation. Cell lysates and immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk and then probed with anti-Kank antibody. Alkaline phosphatase-conjugated rabbit IgG (Promega) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Invitrogen) were used for detection of Kank.

LOH Analysis—LOH analysis using GeneScan Version 3.1 (Applied Biosystems, Inc.) was performed as described previously (14). For LOH analysis of patient R6, genomic DNA was extracted from primary culture cells derived from normal and tumor tissues.

RT-PCR Analysis—The RT reaction was performed with 1 μg of total RNA using 5 pmol of oligo(dT) at 37 °C for 1 h in the presence of 200 units of Moloney murine leukemia virus reverse transcriptase (New England Biolabs Inc.). An aliquot (1 μl) of the RT product was amplified by PCR (30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s). Primer sequences were as follows for ESTs: WI-17492, TCAGTCAAG-GTCACTCATATATA (forward) and TTGTGCTGTCGACAT (reverse); WI-12779, AAGTAAATGTGACAGGTAAA AAGG (forward) and CTGACAGCATTMTYCA GCT TTT G (reverse); and WI-19184, GAATTCCTCCTCCCTGTGC (forward) and AAACCGG-CACAATCCACAC (reverse). Primer sequences were as follows for KIAA0172: GTGGAGACCAGGACAAGCAAG (forward) and TCCAGAGGGGGAGTTGAGCCT (reverse) for the 5′-region and the 3′-region. Primer sequences were as follows for WI-12779: AAGTAAATGTGACAGGTAAA AAGG (forward) and TTGTGCTGTCGACAT (reverse). Primer sequences were as follows for KIAA0172: GTGGAGACCAGGACAAGCAAG (forward) and TCCAGAGGGGGAGTTGAGCCT (reverse) for the 5′-region and the 3′-region.

Methylation Analysis—Genomic DNA was treated with sodium bisulfite as described (16). The treated DNA (30 ng) was amplified by PCR using primers (GCAG-TGTGGACGCTCTCAG) and Cr (TCCACAGACCTCCAGCACTC). The cDNA was prepared from RET primary culture cells and amplified using primers Cr and Cr under identical conditions. The PCR products were purified with QIAGEN spin columns and sequenced in both directions using primers Cr and Cr. To confirm the sequences, PCR products were cloned into the pGEM-T vector (Promega), and clones were selected at random and sequenced using the same primers (data not shown).

Cell Growth Assay—pCMV-Kank (5 μg) was transfected into HEK293 cells using LipofectAMINE 2000. The empty vector (pCMV-vec) was transfected as a control. The transfected cells were cultured to 5 × 10^6 cells on triplicate plates in two independent experiments. After selection with Geneticin (500 μg/ml) for 2 weeks, the colonies were stained, and enumerated. HEK293 colonies were isolated after Geneticin selection to establish stable cell lines, and their Kank gene expression was confirmed by RT-PCR.

Cell Cycle Analysis—HEK293 cells were grown in six-well culture dishes, and 2 μg of pCMV-vec or pCMV-Kank was transfected. Twenty-four hours after transfection, the cells were serum-starved for 12 h and then supplemented with 10% fetal calf serum before being trypsinized at the indicated time intervals. The cells were fixed in 70% ethanol, digested with RNase (0.02 μg/μl), stained with 50 μg/ml propidium iodide, and analyzed using a BD Pharmingen FACScan with ModFit software.

Tumor Study with Mice—HEK293 cells stably expressing pCMV-Kank were collected and resuspended in PBS. Then, 5 × 10^6 cells in a volume of 300 μl were injected at one or two sites on the flanks of 3- to 4-week-old male athymic BALB/c mice. The growth of the injected tumor cells was assessed by measuring the tumor volume in three dimensions twice every 10 days.

RESULTS

Localization of an RCC-related Gene at 9p24—A genome subtraction method, the in-gel competitive reassociation method, which was applied to normal and tumor DNAs, has superiority over conventional methods in detecting deletions of <1 Mb and also in detecting hemizygous deletions (17-20). We previously reported 27 potential tumor-related loci using a library obtained by subtraction of tumor tissue DNA from normal tissue DNA of the same patient (14). By extensive analysis of LOH in RCC tissues using microsatellite markers, one of the loci on chromosome 9 was found to show a relatively high incidence of LOH (25%, 5 of 20 cases) (14). The minimum overlapping region of LOH at this 9p24 locus was further narrowed down to a region 165 kb in size (Fig. IA). We extensively searched for genes and ESTs in and around this region. Of seven ESTs mapped to this region, only three (WI-17492, WI-12779, and WI-19184) were expressed in the kidney. These ESTs were further examined by RT-PCR analysis using matched normal and cancer cDNA pairs (Fig. 1B). The results indicated that WI-17492 showed a very high incidence (75%, six of eight cases) of reduced expression in tumor cDNA samples (pairs 1–3 and 5–7) compared with the other ESTs. Despite potential contamination of the normal kidney cells or the connective tissue cells in the tumor samples, giving higher tran-
scription backgrounds, this observation was notable compared with other ESTs. WI-12779 is an EST derived from the 3'-region of the 4792-bp KIAA0172 cDNA (obtained from the Kazusa DNA Research Institute) (21). When KIAA0172 expression was examined, no trace of transcription at either the 5'- or 3'-region of the cDNA was found in the embryonic kidney cell line HEK293 or in the kidney tumor cell lines G-402 and VMRC-RCZ (Fig. 1C).

Structure and Characterization of the Kank Gene—KIAA0172 has an open reading frame that encodes a potential polypeptide of 1194 amino acids with an estimated molecular mass of 130 kDa (Fig. 2A). We determined the complete nucleotide sequence of this cDNA by determining the cap site and promoter activity (data not shown). There are several interesting motifs, three coiled-coil motifs at the N terminus and 164-amino acid long ankyrin repeats at the C terminus, both of which might act as protein-protein interaction sites. We named the gene product Kank for kidney ankyrin repeat-containing protein from its protein structure and its characterization in kidney tumors. This ankyrin repeat motif is widely found in proteins involved in signal transduction, structural proteins, and many others (22). This protein was endogenously detected in the cytosolic part of VMRC-RCW cells by immunostaining (Fig. 2, B and C) and as a unique band by immunoprecipitation followed by Western blotting (Fig. 2E, lane 2). Expression of Kank cDNA using a cytomegalovirus promoter (pCMV-Kank) gave identical results both for localization (Fig. 2D) and size (Fig. 2E, lane 4) as observed for the in vivo product, confirming the gene structure and subcellular localization.

Loss of Expression of the Kank Gene in RCC—Loss of expression of this candidate gene in RCC was first examined by Western blotting using the tumor and normal tissues from the same individuals (Fig. 3). Of 10 pairs of tissues, loss of expression was observed in tumors from six patients (patients 52, 64, 123, R6, 122, and 51) (Fig. 3A). Therefore, the frequency of loss of expression obtained by Western blotting (60%) roughly matched that obtained by RT-PCR using matched cDNA pairs (Fig. 1B). We also confirmed loss of expression in several tumor cell lines by Western blotting (Fig. 3B).

Loss of expression of the Kank gene in RCC was then examined using R6N and R6T primary culture cells, which were derived from the normal (R6N) and RCC (R6T) tissues of the same patient (Fig. 4). Loss of expression of the Kank gene in the original tumor tissue was demonstrated by Western blotting (Fig. 3A). LOH analysis using the microsatellite marker D9S1779 showed that one of the two alleles was apparently deleted in the tumor, giving a low value (0.55) compared with the original peak ratio (1.06) (Fig. 4A). Although one allele was retained in the tumor cells, its expression was markedly decreased (Fig. 4B). This loss of expression was confirmed in the original tumor, whereas its expression was observed in the cytosolic part of the normal renal tubular cells (Fig. 4C). We also examined the allele-specific expression of this gene using a single nucleotide polymorphic site 1428 nucleotides from the cap site (Fig. 4D). Whereas R6N genomic DNA showed both G and C at this position, cDNA from the same cells showed only...
G, confirming that only the allele containing G at nucleotide 1428 is active. We therefore concluded that this candidate gene is closely related to RCC.

Silencing Kank Gene Expression by Allele-specific Methylation—To understand the mechanism of loss of expression of the Kank gene, we first searched for mutations. However, there was no case of nonsense mutation in 60 RCC (mostly clear cell-type) samples, suggesting that loss of expression of the gene was not due to null mutation. Because the majority of the first exon was part of the CpG island, which contained potential methylation sites, we examined the methylation status (using sodium bisulfite) of six CpG sites in an extensively (G/H11001 C)-rich region located close to the promoter and the first ATG codon (Fig. 5A). A total of 10 matched normal and tumor DNA pairs from the RCC patients showing LOH at this locus in the tumor and HEK293 and G-402 cells were used, and the results for six CpG sites in six clones or alleles analyzed are summarized in Fig. 5B. Although these six sites showed various degrees of methylation in normal and tumor tissues and the established cell lines, a complete absence of methylation was observed only in the normal tissues, but not in the tumor.

**Fig. 4.** Relationship between LOH and loss of expression of the Kank gene in RCC primary culture cells. A, LOH analysis of the DNA from R6N and R6T primary culture cells, derived from normal (R6N) and tumor (R6T) tissues of RCC patient R6, respectively. LOH was determined by GeneScan using marker D9S1779. The ratios of the shaded areas (N1 and N2 for the normal tissues and T1 and T2 for the tumor tissues) representing the allelic status were calculated for both the normal and tumor DNAs and are indicated. B, RT-PCR analysis of Kank (W1-12779) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA in the primary culture cells. The PCR products were resolved on 1.5% agarose gels. C, immunostaining of the original cancer (T) and normal kidney (N) tissues from patient R6 using anti-Kank antibody (upper panels). Rhodamine-conjugated anti-rabbit IgG was used as a secondary antibody. The same sections were stained with Hoechst 33258 (lower panels) to visualize the nuclei. D, allele-specific expression of the Kank gene determined using a single nucleotide polymorphism. The single nucleotide polymorphic site located at nucleotide 1428 from the cap site is boxed. Chromatograms of the region containing the single nucleotide polymorphism (G or C) are shown for R6N genomic DNA and cDNA.

**Fig. 5.** Methylation analysis of the Kank gene. A, the CpG island in exon 1. The shaded box indicates the region containing the six CpG sites analyzed for methylation. The sequence containing the CpG sites is shown below. B, summary of the methylation status of 10 matched normal (N) and tumor (T) DNA pairs and HEK293 and G-402 cells. Open and closed boxes represent unmethylated and methylated CpG sites, respectively, and the results of the six sites (sites 1–6) shown in A are summarized. The results obtained with six randomly selected clones for each set of DNA are shown, comprising a list of 60 allelic statuses of methylation for each of the normal and tumor tissues and 12 allelic statuses for the two cell lines. Also shown in graphical representation are the numbers of alleles (or the sequenced clones) methylated or not in the matched normal and tumor DNA pairs. C, re-expression of the Kank gene by treatment with 5-aza-2'-deoxycytidine. After HEK293 and G-402 cells were cultured in the absence or presence of 10 μM 5-aza-2'-deoxycytidine ([5-Aza-C]), the Kank gene transcript was analyzed by RT-PCR. G3PDH, glyceraldehyde-3-phosphate dehydrogenase. D, microscope images of HEK293 cells with or without 5-aza-2'-deoxycytidine treatment. E, growth retardation of HEK293 cells by 5-aza-2'-deoxycytidine treatment. On day 9 of culture, the cells were trypsinized, and the viable cells were counted after trypan blue staining. The values represent the means ± S.D. from three independent experiments.
tissues or established cell lines. Interestingly, we observed some degree of methylation even in the normal tissues, which represented almost half the cases (35 of 60 alleles examined by cloning) (see graph in Fig. 5B). In contrast, there was no case of non-methylation in the tumors. Furthermore, both the R6N and R6T primary culture cells showed various degrees of methylation, indicating a complex allelic status for methylation. There was no case of non-methylation in R6T cells, whereas R6N cells contained both methylated and unmethylated alleles.

Thus, the active allele was deleted, and the inactive allele with methylation was retained at the tumor formation in most cases (9 of 10 cases) (Fig. 5B). This indicates that, in RCC showing LOH by deletion of the \textit{Kank} locus, one allele was already affected before the second event of deletion. We are now investigating whether this predisposed methylation is due to imprinting or other mechanisms.

We also examined whether methylation of the gene is a cause of gene silencing (Fig. 5C). After HEK293 and G-402 cells were

![Image](http://www.jbc.org/)

**Fig. 6.** \textbf{Effect of \textit{Kank} expression on tumor cell growth.} A, colony formation assay. HEK293 cells were transfected with an empty vector (pCMV-vec) or with a plasmid containing \textit{Kank} cDNA with the cytomegalovirus promoter (pCMV-Kank) (upper panels). The numbers of colonies were counted in four quadrants, and the total colony number for pCMV-vec was taken as 100% (middle panel). Changes in cell morphology after transfection of the \textit{Kank} gene into HEK293 cells are shown (lower panels). Cells were observed under a light microscope after being washed with PBS. B, cell cycle analysis comparing pCMV-vec- and pCMV-Kank-transfected HEK293 cells. The percentages of cells in G0/G1 are indicated along with the times after transfection. C, growth rates for the stable HEK293 cell lines expressing the vector or the \textit{Kank} gene. The indicated numbers of cells were spread on culture dishes at day 0, and their numbers were counted after the indicated days of culture. D, tumor formation in nude mice. Stable clones containing pCMV-vec and pCMV-Kank (pCMV-vec1 and pCMV-Kank12, respectively) were injected into BALB/c nude mice (a total of six sites for pCMV-vec and four sites for pCMV-Kank). Arrows indicate the positions of the injected tumors. Tumor volumes (±S.D.) were determined twice every 10 days. E, Western blot analysis of \textit{Kank} gene expression in the mouse tumors. The tumors grown in the mice shown in D were subjected to Western blot analysis using anti-\textit{Kank} or anti-\textbf{β}-actin antibody.
whether there is any growth suppression upon expression of the Kank gene in HEK293 cells (Fig. 6). The Kank expression vector (pCMV-Kank) or the empty vector (pCMV-vec) was transfected into HEK293 cells; and after 15 days of culture, the numbers of colonies were counted (Fig. 6A). The colony numbers of pCMV-Kank-transfected cells were ~20% of the control value, indicating a growth suppression effect by the gene. We also observed morphological changes in the cells upon expression of the Kank gene: the cells enlarged and became flattened, and the periphery became unclear, probably due to slow growth and structural changes within the cells. Cell cycle analysis using a flow cytometer indicated that, after transfection of pCMV-Kank, the cells showed growth arrest at G0/G1, as the population of cells at these stages increased gradually, starting from 6 h after serum addition and reaching 62.3% after 24 h (Fig. 6B). There was no such change for the pCMV-vec-transfected cells. Changes in the growth rates (approximately half of the control) for three HEK293 cell lines permanently expressing the Kank gene were also observed (Fig. 6C). Finally, we confirmed that growth suppression was caused by Kank gene expression using nude mice injected with HEK293 cells stably expressing either Kank or the empty vector (Fig. 6D). There were clear differences in tumor growth between these constructs: all pCMV-vec-expressing cells showed aggressive tumor growth starting 40 days after injection, whereas no growth (three of four cases) or moderate growth (one of four cases) was observed for the pCMV-Kank-expressing cells. There was a low level of expression of the Kank gene in the tumor showing moderate growth compared with Kank-expressing stable HEK293 cells (Fig. 6D).

**Effect of Kank on β-Actin Distribution**—We established several G-402 cell lines expressing the Kank gene. They generally showed dramatic morphological changes with retarded growth, such as shown in Fig. 7, which were similar to the changes shown in Fig. 6A. When one of these cell lines and the control G-402 cell lines stably expressing the vector were immunostained with anti-Kank or anti-β-actin antibody (fluorescein isothiocyanate; green). The cells were then washed with PBS and stained with 4,6-diamidino-2-phenylindole (DAPI) (blue).

**Phase contrast**

**Phase contrast**

**G-402-vec**

**G-402-Kank**

![Subcellular localization of Kank](http://www.jbc.org/)

**DISCUSSION**

Haploinsufficiency is one of the prerequisites for recessive tumor suppressor genes, where serial hits for both alleles are needed for loss of function (23). Inherited cancers show the first hit as a germ-line mutation, and the second hit results in loss of function of the gene, as explained by the hypothesis of Knudson (24). On the other hand, sporadic cancers have more difficulty in assessing whether the gene acts as a tumor suppressor because the first hit is recognized mainly after the second hit, which results in tumor formation. In this case, one cannot see the process of cancer formation, as the first-hit state in a single cell is hard to observe and is mainly seen after the second hit, which is often complicated by additional mutations in many other genes, sometimes accompanied by more dynamic chromosomal rearrangements. Thus, one can try to reconstruct the process retrospectively. A third case, loss of function by methylation, is becoming more evident, as this includes epigenetic changes sometimes observed in many cells (25–28). However, there is no report so far of imprinted tumor suppressor genes, where allele-specific and hemizygous methylation in the normal cells followed by loss of heterozygosity is expected for the same gene. Because the numbers of epigenetic alterations in sporadic cancers, expected to be a few to hundreds (29), cannot be explained by somatic mutations alone (usually on the order of 10^-5 in frequency or less), methylation and other
mechanisms with even higher frequencies might have contributed. The very high frequency (9 of 10 cases) of allele-specific methylation of the Kank gene in normal tissues, which was followed by deletion of the normal allele, indicates a crucial role for this gene in the tumorigenesis of RCC.

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Shubhashish Sarkar, Badal Chandra Roy, Naoya Hatano, Teiichiro Aoyagi, Kazuo Gohji and Ryoiti Kiyama

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