Disruption of a novel MFS transporter gene, *DIRC2*, by a familial renal cell carcinoma-associated t(2;3)(q35;q21)

Daniëlle Bodmer*, Marc Eleveld, Ellen Kater-Baats, Irene Janssen, Bert Janssen, Marian Weterman, Eric Schoenmakers, Michael Nickerson1, Marston Linehan2, Berton Zbar1 and Ad Geurts van Kessel

Department of Human Genetics, University Medical Center Nijmegen, Nijmegen, The Netherlands, 1Laboratory of Immunobiology, National Cancer Institute, Frederick Cancer and Development Center, Frederick, MD 21702 USA and 2Urologic Oncology Section Surgery Branch, National Institute of Health, Bethesda, MD 20892, USA

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Previously, we described a family with a significantly increased predisposition for renal cell cancer co-segregating with a t(2;3)(q35;q21) chromosomal translocation. Several primary tumors of the clear cell type from different family members were analyzed at a molecular level. Loss of the derivative chromosome 3 was consistently found. In addition, different somatic Von Hippel Lindau (*VHL*) gene mutations were observed in most of the tumors analyzed, even within the same patient. Based on these results a multistep tumorigenesis model was proposed in which (non-disjunctional) loss of the derivative chromosome 3 represents an early event and somatic mutation of the *VHL* gene represents a late event related to tumor progression. More recently, however, we noted that these two anomalies were absent in at least one early-stage tumor sample that we tested. Similar results were obtained in another family with renal cell cancer and t(3;6)(q12;q15), thus suggesting that another genetic event may precede these two oncogenetic steps. We speculate that deregulation of a gene(s) located at or near the translocation breakpoint may act as such. In order to identify such genes, a detailed physical map encompassing the 3q21 breakpoint region was constructed. Through a subsequent positional cloning effort we found that this breakpoint targets a hitherto unidentified gene, designated *DIRC2* (disrupted in renal cancer 2). Computer predictions of the putative *DIRC2* protein showed significant homology to different members of the major facilitator superfamily (MFS) of transporters. Based on additional *DIRC2* expression and mutation analyses, we propose that the observed gene disruption may result in haplo-insufficiency and, through this mechanism, in the onset of tumor growth.

INTRODUCTION

Hereditary renal cell carcinomas (RCCs) of the clear cell type mostly occur as a consequence of the Von Hippel Lindau (*VHL*) cancer syndrome in which patients carry a germline mutation in one allele of the *VHL* tumor suppressor gene (1). In addition, seven families have thus far been described with constitutional chromosome 3 translocations and hereditary clear cell RCC (2–7). In one of these families, in which a t(2;3)(q35;q21) translocation segregates, five RCC cases have thus far been identified in three generations (4,8,9). In all instances the patients appeared to be translocation carriers. Subsequent molecular studies on eight primary tumors in this family showed loss of the derivative chromosome 3 [der(3)] containing the short arm of chromosome 3 (3p) in seven of them and different *VHL* mutations in the remaining 3p allele in five of them (8,9). Similar observations were made in some of the other chromosome 3 translocation-positive RCC families (7,10–12). Based on these results a tumorigenesis model for the development of familial clear cell RCCs was proposed in which (non-disjunctional) loss of the short arm of chromosome 3 and somatic mutation of the remaining *VHL* allele on 3p represent critical steps (8,11). In one early-stage tumor biopsy in this family, however, neither der(3) loss nor *VHL* gene mutations were observed (9). The same was observed in several tumor biopsies in another RCC family with a t(3;6)(q12;q15) translocation (12). These findings led us to assume that the two oncogenetic steps mentioned above may be preceded by another genetic event related to tumor initiation (9,12). Deregulation of a gene(s) located at or near the translocation breakpoint(s) may act as such.

Previous positional cloning efforts have led to the identification of breakpoint-spanning genes in the familial RCC-associated...
t(3;8)(p14;q24) (13,14). At the 3p14 breakpoint the FHIT (fragile histidine triade) gene, which coincides with the common fragile site FRA3B, was identified (13). The gene encodes a hydrolase with, preferably, Ap3A as substrate (15). Aberrant FHIT transcripts and FHIT genomic lesions were frequently observed in a variety of primary tumors and tumor-derived cell lines (13,16–18). Since similar FHIT alterations were also observed in non-malignant cells (16,19–21), it was suggested that fragility of the locus itself might account for the observed anomalies (20,22). FHIT transfection studies, however, showed that replacement of FHIT in Fhit-deficient cancer cells in nude mice suppresses tumorigenicity, indicating that FHIT may function as a tumor suppressor (23). As mutant FHIT, lacking enzyme activity, can also suppress tumorigenicity, it was proposed that another function of this protein, that also this gene most likely functions as a tumor suppressor (27). These observations underline the notion that deregulation of a gene(s) located at or near familial RCC-associated translocation breakpoints may be related to tumor development (23). More recent studies reporting a reduced or even complete absence of FHIT protein expression in clear cell RCCs (24,25) are in agreement with these latter notions. In addition, the increased incidence of carcinogen-induced tumor formation in Fhit-deficient mice similar to that observed in the human Muir–Torre tumor syndrome (MTS) (26) supports such a role for FHIT. The chromosome 8 breakpoint of the 3;8 translocation also disrupts a gene, TRC9, which shows significant homology to the Drosophila patched gene (14). Interestingly, inactivation of the human homolog of this patched gene (PTCH) leads to excessive growth, indicating that also this gene most likely functions as a tumor suppressor (27). These observations underline the notion that deregulation of a gene(s) located at or near familial RCC-associated translocation breakpoints may be related to tumor development.

More recently, a new gene, DIRC1 (disrupted in renal cancer 1), was identified at the chromosome 2q33 breakpoint of a constitutional RCC-related 2;3 translocation (28). The function of this gene is unknown. At the chromosome 3 breakpoint of this translocation no genes have yet been identified.

Here we report the physical mapping and positional cloning of the t(2;3)(q35;q21) chromosomal breakpoint at 3q21 and the subsequent identification of a breakpoint-spanning gene, DIRC2, which encodes a novel member of the major facilitator superfamily (MFS) of transporters. The putative consequences of this gene disruption for RCC development are discussed.

RESULTS

Construction of a 3q21 breakpoint-spanning contig

Previously, the t(2;3)-associated breakpoints were cytogenetically assigned to bands 2q35 and 3q21, respectively (3). CEPH yeast artificial chromosome (YAC) clones from the 3q21 region were selected and positioned by fluorescence in situ hybridization (FISH) relative to the breakpoint using lymphoblastoid cell line-derived metaphase spreads. By doing so, four YACs (957g11, 766d8, 912f12 and 944d9) were mapped proximal to the 3q21 breakpoint and two YACs (790d8 and 827d3) were mapped distal to the 3q21 breakpoint (Fig. 1). In addition, three overlapping YACs (944h8, 959e4 and 822a12) were found to span the breakpoint. In Figure 2A a typical FISH experiment, with YAC 944h8 as a probe, is shown. Besides a signal on the normal chromosome 3, as expected, a split signal is seen at the breakpoint regions on both derivative chromosomes [Fig. 2A, der(2) and der(3)]. In order to confirm the breakpoint-spanning nature of this YAC, end-fragments were generated by polymerase chain reaction (PCR) between a partially degenerate oligonucleotide primer (DOP) and the vector primer, and used as probes on Southern blots containing EcoRI digested DNA extracted from translocation carrier-derived somatic cell hybrids (A3KE-11C, A3KE-34B, A3KE-1A and A3KE-1D) (8), independent monochromosomal hybrids containing human chromosomes 2 (m2) and 3 (m3), Chinese hamster A3 cells and translocation carrier-derived lymphocytes (7127). One of the end-fragments (944UL) hybridized to a 5.3 kb EcoRI fragment in lymphocytes, both cell hybrids containing chromosome 3 (Fig. 2B, lanes 7127, m3 and A3KE-1A), and the der(2) containing hybrid (Fig. 2B, lane A3KE-11C, arrow). The other end-fragment (944UR) hybridized to a 2.2 kb EcoRI fragment in lymphocytes, both cell hybrids containing chromosome 3 (Fig. 2C, lanes 7127, m3 and A3KE-1A), and the der(3) containing hybrid (Fig. 2C, lane A3KE-1D, arrow). These results thus independently confirmed the breakpoint-spanning nature of this YAC. The breakpoint could be positioned between the markers WI-5963 and D3S1760 (Fig. 1). These two markers, and two end-fragments of YACs 944h8 and 912f12, were subsequently used as probes to screen a human P1-derived artificial chromosome (PAC) library and a human chromosome 3-specific cosmid library. Five PACs (832H17, 860G10, 1173P12, 828J14 and 1040F10) and one cosmid (M1344) were selected. All shared the marker D3S1760 (Fig. 1). FISH and Southern blot analyses of the t(2;3)-derived somatic cell hybrid panel revealed that four of the selected PACs (860G10, 1173P12, 828J14 and 1040F10) and cosmid M1344 mapped distal to the 3q21 breakpoint and that PAC 832H17 was breakpoint-spanning (Fig. 1). The end-fragments of this latter PAC were again used as probes to screen the chromosome 3-specific cosmid library. Two cosmids (L1951 and O156) were isolated, of which L1951 mapped proximal and O156 mapped distal to the breakpoint. Thus, the breakpoint was narrowed down to the region between cosmid L1951 and PAC 860G10. Long range PCR between sequences of the respective PAC and cosmid inserts produced a fragment of 15 kb (Fig. 1, LR-15). Southern analysis confirmed that LR-15 was indeed breakpoint-spanning and, additionally, that the breakpoint coincides with a deletion of ~4 kb (data not shown). Through database searches two sequenced bacterial artificial chromosome (BAC) clones, RP11-90F21 and RP11-67L2 (GenBank accession nos AC011116 and AC078794, respectively), were identified (Fig. 1). Primer sets were selected and, using the t(2;3)-derived somatic cell hybrid panel, the breakpoint region could be narrowed down to ~500 bp on both sites of the deletion (Fig. 1, BP1 and BP2).

A novel gene, DIRC2, is disrupted by the 3q21 translocation breakpoint

To identify genes at/near the 3q21 breakpoint, two kidney related cDNA libraries were screened with breakpoint-spanning PAC 832H17 and breakpoint-flanking cosmid L1951. Two cDNAs were isolated, of which the 3’ ends matched with several expressed sequence tags (ESTs) from UniGene cluster Hs.11360. Alignment of the isolated cDNAs with the different EST sequences from the database revealed a consensus transcript of 1628 bp. Rapid amplification of cDNA ends (RACE) and a
modified reverse transcriptase (RT)–PCR, including Betaine and dimethyl sulfoxide (DMSO) (29), extended this cDNA sequence at the 5′ end with 420 bp (the latter with the primer sets 2–6 and 2–7; see Supplementary Material, Fig. 1). Simultaneously, database searches revealed a human cDNA for the hypothetical protein FLJ14784 (GenBank accession no. AK027690) which showed extensive homology (97–100% identity) to our transcript and extended the composite consensus sequence with another 58 bp. Alignment of the total 2106 bp cDNA sequence with that of the two sequenced breakpoint-spanning BACs (RP11-90F21 and RP11-67L2) and other overlapping BAC clones in the database, i.e. RP11-396B8, RP11-457E6 and RP11-181G12 (Fig. 1; GenBank accession nos AC010858, AC048348 and AC069581, respectively), revealed the presence of nine exons. The putative exon–intron boundaries and the sizes of the exons and introns are listed in the Supplementary Material (Table 1). An open reading frame (ORF) of 1434 bp was predicted, of which the putative translation initiation codon ATG is embedded in a strong Kozak consensus sequence (30) within the first exon (Supplementary Material, Fig. 2). The GC-rich first exon and 5′-untranslated region (5′-UTR) are indicative of a CpG island. The 3′-UTR is 535 bp long and a highly conserved polyadenylation signal sequence (AATAAA) was observed 15 nucleotides upstream of the poly(A) addition site (Supplementary Material, Fig. 2).

LR-15 contains both the exons 6 and 7 of this gene and the translocation breakpoint (BP1) was found to map 2 kb downstream from exon 7, thereby indicating that this gene is
disrupted by the translocation (Fig. 1). These results were confirmed by Southern blot analyses using cDNAs corresponding to this gene as probes on somatic cell hybrid and control DNAs (data not shown). This gene turned out to be anonymous and, therefore, we have designated it **DIRC2**.

**DIRC2** is a member of the MFS superfamily of transporters

The putative full-length **DIRC2** cDNA contains an ORF of 1434 bp encoding a predicted protein of 478 amino acids (Supplementary Material, Fig. 2). Different web-based protein prediction programs defined 12 membrane-spanning domains and cytoplasmic N- and C-termini within the predicted protein (Supplementary Material, Fig. 2). Furthermore, one putative N-glycosylation site, two putative protein kinase C phosphorylation sites, and one putative casein kinase II phosphorylation site could be predicted in different hydrophilic domains (Supplementary Material, Fig. 2). A proline-rich region was observed in the large hydrophilic region between the membrane-spanning domains 6 and 7. Database searches with the predicted amino acid sequence of **DIRC2** revealed 100 and 98% identity to the human hypothetical protein FLJ14784 (GenPept accession no. BAB55300) and a predicted protein from *Macaca fascicularis* (GenPept accession no. BAB17282), respectively. A significant homology in the amino acid region 69–427 (43% similarity) was also found with the human feline leukemia virus type C receptor (FLVCR; GenPept accession no. AAD45243). This receptor has been classified as a member of the ancient MFS of transporters, which contains carriers that transport small solutes across membranes in response to chemical-osmotic gradients (31,32). The MFS family of proteins is characterized by the presence of 12 membrane-spanning domains with a large hydrophilic region between the membrane-spanning domains 6 and 7 and a well conserved MFS-specific motif between the membrane-spanning domains 2 and 3. **DIRC2** exhibits all these characteristics (Supplementary Material, Fig. 2). Moreover, **DIRC2** shows homology to an unknown transporter in *Leishmania major* (44% similarity in amino acids 278–433; GenPept accession nos AAF34284) and a sodium-dependent inorganic phosphate cotransporter in *Arabidopsis thaliana* (50% similarity in amino acids 156–215; GenPept accession no. AAD32766).

**DIRC2** is expressed in proximal tubular cells of the kidney

To determine whether **DIRC2** is expressed in a tissue-specific manner, a cDNA probe was hybridized to a northern blot prepared from eight different normal adult human tissues. Although **DIRC2** expression levels appeared to be low in general, a 2.2 kb **DIRC2** transcript could be identified in most of these tissues, including kidney (Fig. 3A). RT–PCR on RNA extracted from proximal epithelial tubule cell lines of different species (dog, pig and mouse) was performed since clear cell RCCs are thought to develop from this part of the nephron. Since different mouse and pig ESTs showed 85–95% homology to the human **DIRC2** sequence, respectively, the primers used were designed from the human sequence. A PCR product was readily obtained from the mouse cell line using the first primer set (Fig. 3B, lane 3), whereas the second primer set yielded PCR products from the pig and dog cell lines (Fig. 3B, lanes 1 and 2). Inadequate homology of the primer sequences to the respective RNAs may explain the observed absence of signals in some of the cell lines. In addition, placenta RNA was included as a positive control for human **DIRC2** expression (Fig. 3A, lane 6 and Fig. 3B, lane p).

**DIRC2** mutation analysis in patients and tumors

To further elucidate the role of **DIRC2** in RCC development, we performed mutation analyses on tumors isolated from two affected t(2;3) family members (II:7 and III:13) (4,8,9).
PCR products spanning exons 2–8 and the coding sequence of exon 9 were analyzed by direct sequencing. Reliable sequence data could not be obtained from the first exon due to its GC-richness. One G→A sequence variant in intron 7 (31 nucleotides upstream from the intron–exon boundary of exon 8) was identified in patient III:13, but not in patient II:7 (Supplementary Material, Fig. 1). Since patient II:7 is the father of patient III:13 (4), the G→A sequence variant must be derived from the unaffected mother of III:13 and thus, is unlikely to be linked to RCC development. In addition, northern blot analysis of a renal tumor sample obtained from a t(2;3)-carrier (II:7) (4,8,9) revealed a normal DIRC2 transcript, which was also detected in an unrelated sporadic renal tumor sample, but not in the t(2;3)-positive lymphoblastoid cell line tested (Fig. 3C). No additional aberrant bands were observed in the translocation-positive tumor.

The same G→A sequence variant was also encountered in two of 10 unrelated sporadic clear cell RCC cell lines and in three of the 42 blood samples derived from RCC patients of 31 unrelated families. No other sequence variants were found in these samples. In addition, RT–PCR and northern blot analyses revealed normal DIRC2 transcripts in all sporadic RCCs that we tested (data not shown).

**DISCUSSION**

Previous molecular studies in translocation chromosome 3-positive RCC families showed that loss of the derivative chromosome containing the 3p arm and somatic mutation of the VHL gene are important steps in the development of these clear cell renal tumors (7–12). As none of these anomalies were observed in an early stage tumor in this t(2;3)-positive RCC family (9) and several tumor samples in another t(3;6)-positive RCC family (12), an additional translocation-associated oncogenic event was suggested. Therefore, we set out to analyze the t(2;3)-related 3q21 breakpoint in more detail. Through positional cloning of this breakpoint we found that an anonymous gene, designated DIRC2, was disrupted. This gene contains nine exons of which the first exon is GC-rich and, thus, is unlikely to be linked to RCC development. In addition, northern blot analysis of a renal tumor sample obtained from a t(2;3)-carrier (II:7) (4,8,9) revealed a normal DIRC2 transcript, which was also detected in an unrelated sporadic renal tumor sample, but not in the t(2;3)-positive lymphoblastoid cell line tested (Fig. 3C). No additional aberrant bands were observed in the translocation-positive tumor.

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dog and mouse, and the significant degree of homology (30–45%) of the predicted DIRC2 protein to different predicted proteins in *Leishmania major* and *Arabidopsis thaliana*, indicate that the gene has been strongly conserved throughout evolution. The hydrophilic regions of the predicted DIRC2 protein contain one glycosylation site and several phosphorylation sites which may be involved in the interaction of this protein with the extracellular and intracellular environments, respectively. A proline-rich region was also observed in the intracellular hydrophilic region between membrane-spanning domains 6 and 7. Previous studies have shown that Src homology 3 (SH3) domains from several proteins may recognize these proline-rich (P-x-x-P) motifs as binding sites (33), thereby mediating critical protein–protein interactions involved in responses to extracellular signals. The DIRC2 protein may, via this putative protein binding site, also be involved in such cellular signaling pathways. Transport systems for organic anions and cations are primarily involved in the secretion of drugs in renal tubules and, thus, play a critical role in protecting the organism against their potential toxic effects (34). Disruption of such a transporter, as is the case in the t(2;3) family, may impose stress conditions onto renal epithelial cells resulting in dysplasia and/or genomic instability. Teleologically, such a condition may result in loss of the der(3) chromosome and, thus, trigger the oncogenic process. According to the previously proposed multi-step model (8), subsequent VHL gene mutations may lead to full-blown tumor development.

To substantiate a role for DIRC2 in proximal renal tubular epithelial cells, various expression studies and mutation analyses were performed. Multiple tissue northern blot analysis showed that the DIRC2 gene is expressed in several tissues, including the kidney. Subsequent RT–PCR analyses on RNAs extracted from epithelial cells derived from the proximal kidney tubule demonstrated that this gene is also expressed in this part of the kidney. Northern blot analysis of t(2;3)-positive tumors revealed normal DIRC2 transcripts, indicating that the remaining intact chromosome 3 allele is normally transcribed. Since no additional abnormal transcripts were detected, we conclude that truncated and/or fusion transcripts are absent or expressed at very low levels. Subsequent mutation analysis on the remaining DIRC2 allele (>80% of the gene) revealed one G→A sequence variant in intron 7. As we could deduce that this variant must be derived from an unaffected family member, we conclude that it is not disease-linked. The DIRC2 gene was also normally expressed in several sporadic RCCs. Additional mutation analyses of these RCCs and blood samples of a series of unrelated RCC patients revealed no further mutations besides the G→A variant. As yet, mutations in the first exon cannot be excluded and, therefore, this option remains to be tested. The apparent hemizygous state of DIRC2 in t(2;3)-positive tumor cells leads us to suggest that, if disruption of the DIRC2 gene contributes to tumor development, it must be through a haplo-insufficiency scenario. We are currently investigating this option in further detail.

At this stage it can not be excluded that RCC initiation is caused by a position effect, whereby the translocation breakpoint deregulates another gene located upstream or downstream from DIRC2 (35). Directly proximal to the first exon of DIRC2, and thus ~80 kb proximal to the translocation breakpoint BP1, the first exon of a predicted gene was identified. In the database, several matching ESTs were found for this gene and the corresponding cDNA predicts a putative ORF encoding a protein of 484 amino acids. Subsequent database searches revealed 73% homology to PASS1 (Fig. 1), a rat protein that was previously found to be associated with the small stress protein hsp27 (GenPept accession no. AAD48846) (36). Small stress or heat shock proteins are thought to play critical roles in cellular stress responses and to render cells stress-resistant. PASS1 is most abundantly expressed in kidney and testis. In addition, two different PASS1 transcripts were encountered in kidney cells and alternative splicing has been suggested as a causative mechanism (36). Another interesting observation is that PASS1 expression affects the ability of hsp27 to protect cells against shock. This, and the finding that hsp27 levels are elevated in renal tumors as compared to normal kidney tissues (37,38), turns PASS1 into another interesting RCC candidate gene. Another gene of interest is SEMA5B, which is located ~20 kb distal to DIRC2 exon 9 (GenBank accession no. AK001234) (Fig. 1). Interestingly, two other human semaphorin genes, *SEMA3F* and *SEMA3B*, map in one of the so called tumor suppressor regions on the short arm of chromosome 3 (39–42). As such, they have been considered as candidate genes involved in tumor development. *SEMA5B* contains, besides the Sema domain (pfam01403) a plexin repeat domain (pfam01437) and four thrombospondin type 1 domains (pfam00090). These latter domains are known to bind the protein thrombospondin-1 (TSP-1) to transforming growth factor (TGF)β1 (43,44). A role for TGFβ1 in kidney cancer is suggested since elevated levels of TGFβ1 have been observed in patients with renal cell cancer (45,46) and an altered TGFβ1 signaling pathway has been observed in kidney tumors (47). The possible involvement of *SEMA5B* and PASS1 in RCC development through breakpoint related position effects remains to be investigated.

Although the translocation breakpoints of the seven RCC families with constitutional chromosome 3 translocations affect different genes, these may affect the same genetic pathway to RCC development. From these results, however, no obvious overlap in function or genetic pathway could be made between the DIRC2 gene and/or one of its neighboring genes and the other breakpoint genes, such as *FHIT*, *TRC8* or *DIRC1*. Finally, it also remains to be investigated whether additional candidate genes are located in the 2q35 breakpoint region and whether deregulation of one or more of these genes contributes to RCC development.

**MATERIALS AND METHODS**

**Patient material and cell lines**

From different family members, both carriers and non-carriers of the t(2;3)(q35;q21) translocation, Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines were established (8). From one of these cell lines (7127) somatic cell hybrids were generated after fusion with thymidine kinase-deficient Chinese hamster A3 cells. Four hybrids were selected, containing either the normal chromosomes 2 or 3 (A3KE-34B and A3KE-1A), the derivative chromosome 2 (A3KE-11C) or the derivative chromosome 3 (A3KE-1D), as described previously (8). In addition, we used independent monochromosomal human–rodent somatic cell hybrids containing the normal
chromosomes 2 or 3 as the only human constituents (Coriell Cell Repositories, Camden, NJ). Cell lines derived from the renal proximal tubules included MDCK2 (dog), LLC-PK1 (pig) and a conditionally immortalized cell line derived from a transgenic H-2Kb-ts a58 mouse (48). Mutation analyses were performed on genomic DNAs from the renal tumors from the family-members II:7 and III:13, which were both t(2;3)(q35;q21) carriers (4,8). Additionally, mutation analyses were performed on DNAs isolated from 10 sporadic RCC-derived cell lines of the clear cell subtype and blood samples collected from 42 patients in 31 unrelated families carrying renal tumors with various histologies, including renal oncocytomas, unusual papillary RCCs without germline MET mutations, and clear cell RCCs without germline VHL mutations. All human samples used for this study were obtained with informed consent.

Construction of physical YAC/PAC/BAC/cosmid map

YAC DNA isolations were performed using Zymolyase (Seikagaku). All YAC inserts were characterized using pulsed field gel electrophoresis (CHEF-DR III, Biorad). FISH analysis was performed on metaphase spreads from t(2;3)-positive EBV cell lines essentially as described previously (12). For the isolation of YAC end-fragments, a DOP-vector PCR protocol was used (49) employing the partially degenerate primer 6-MW, the left YAC vector primer UL and the right YAC vector primer UR. For nested PCR we used the left internal vector primer LS2 and the right internal vector primer RA2. Primer sequences are given in the Supplementary Material (Table 2). The DOP-vector PCR protocol was also used to isolate end-fragments from PACs. The pCYPAC2 specific primers were used as described by Wu et al. (49). A gridded total human PAC library (RPCI-5; BAC_PAC Resources, Roswell Park Cancer Institute, Buffalo, NY) (50) and a gridded chromosome 3-specific cosmid library (RessourcenZentrum/PrimärDatenbank, RZPD, Berlin) (51) were used for the selection of PACs and cosmids. DNA isolations from the isolated PACs and cosmids were performed using a plasmid purification kit (Qiagen). For Southern blot analysis, genomic DNA was isolated from the different cell lines via standard SDS/proteinase K lysis, genomic DNA was isolated from the renal tumors from the family-members II:7 and III:13, which were both t(2;3)(q35;q21) carriers (4,8). Additionally, mutation analyses were performed on DNAs isolated from 10 sporadic RCC-derived cell lines of the clear cell subtype and blood samples collected from 42 patients in 31 unrelated families carrying renal tumors with various histologies, including renal oncocytomas, unusual papillary RCCs without germline MET mutations, and clear cell RCCs without germline VHL mutations. All human samples used for this study were obtained with informed consent.

cDNA cloning and sequencing

For the search of genes in the breakpoint region, two cDNA libraries derived from normal human kidney tissue and human kidney tumor tissue that were previously constructed in our laboratory (52) were used. These cDNA libraries were screened with genomic YAC and PAC clones. Subsequent sequence analyses were performed using a Ready Reaction Dye Terminator Cycle sequencing kit (Perkin-Elmer) and run on a ABI 3700 automated sequencer (Applied Biosystems). BlastN searches against nr, dbEST, month and HTGS databases were performed at the NCBI server (http://www.ncbi.nlm.nih.gov/) (53). Sequences of the breakpoint-spanning gene were assembled with those obtained from the EST database using the Staden Genome Assembly Package (54) accessed through the Nijmegen Center of Molecular and Biomolecular Informatics (www.cmbi.nl).

To extend the 5′ end of the DIRC2 cDNA, 5′ RACE was performed with the Marathon cDNA amplification kit (Clontech) using cDNA prepared from human kidney. The cDNAs were amplified following the supplier’s manual using gene-specific primers (GSPs) 782-GSP3 and 754-GSP3N (also see Supplementary Material, Table 2 and Fig. 1). For this PCR reaction the AdvanTage cDNA PCR kit (Clontech) was used.

To extend the GC-rich first exon, RT–PCR was performed with extra Betaine and DMSO again on the Marathon Ready human kidney cDNA (Clontech) with different primers corresponding to the 5′ genomic region (primers 1–4) and cDNA specific primers corresponding to exons 2 and 3 (primers 5–7). The sequences of these primers and their positions within the DIRC2 gene are shown in the Supplementary Material (Table 2 and Fig. 1, respectively). PCR reactions were carried out in 25 µl reaction mixtures with 0.5 ng cDNA, 1× buffer (Gibco BRL), 1.5 mM MgCl₂ (Gibco BRL), 0.2 mM of each dNTP, 0.2 pmol of each primer, 0.5 U Taq polymerase (Gibco BRL), 1 M Betaine (Fluka) and 1.3% DMSO (Sigma) under standard conditions at an annealing temperature of 58°C (29).

Protein analysis

EST predictions and BlastP searches against the nr, swissprot and month databases were performed at the NCBI server (54). Transmembrane segments were identified by five different prediction programs: DAS at Stockholm University (http://www.sbc.su.se/~miklos/DAS/), TMHMM at the Technical University of Danmark (http://www.cbs.dtu.dk/services/TMHMM-1.0/), PHD-hhtm at the European Molecular Biology Laboratory (EMBL) (http://www.embl-heidelberg.de/predict-protein/), TMPred at Swiss Institute for Experimental Cancer Research (ISREC), (http://ulrec3.unilt.ch/software/TMPRED_form.html) and SOSUI at Tokyo University (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html). Protein motifs in the predicted amino acid sequence were identified by PROSITE at the Expert Protein Analysis System (ExPASy) server of the Swiss Institute of Bioinformatics (SIB) (http://ca.expasy.org/tools/scnpsit1.html).
Expression analysis

For expression analysis RNA was extracted from a t(2;3)-positive EBV lymphoblastoid cell line, a t(2;3)-positive primary renal tumor, and a sporadic clear cell renal tumor cell line. RNA from the tumors were isolated using LiCl (Sigma) (55) and RNA from the EBV cell line was isolated using RNAzol (Campro Scientific). For northern blot analysis total RNA (10 µg) was denatured using glyoxal (Sigma) during gel electrophoresis and blotted onto Hybond N+ membranes (Amersham). These blots, and northern blots containing poly(A)+ RNAs from multiple normal tissues (Clontech), were hybridized with a 1.7 kb cDNA clone of DIRC2 (IMAGE:338833) as a probe using conditions as described above for Southern blot analysis. RT–PCR was performed using 5 µg of total RNA isolated from the different proximal renal tubule cell lines (see above), Superscript-II RT (Life Sciences), random hexamer primers, and specific primer pairs. The primers ex1C and ex3B amplified exons 1–3 of the human DIRC2 gene and the primers ex5C and ex8B amplified exons 3–8. Mouse Gapdh was included as a control for the integrity of the mouse RNA. The primers amplifying this Gapdh gene were mGAPDHfor and mGAPDHrev.

The PCR reactions were performed under standard conditions as described above for Southern blot analysis.

REFERENCES


