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Cloning of a new familial t(3;8) translocation associated with conventional renal cell carcinoma reveals a 5 kb microdeletion and no gene involved in the rearrangement

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This study describes the molecular cloning of a familial translocation, t(3;8)(p14.2;q24.2), that segregates with the conventional renal cell carcinoma (conventional RCC). We had previously reported the family history and, through loss of heterozigosity and comparative genomic hybridization, detected the loss of the 3p chromosome arm and somatic mutation in the retained von Hippel–Lindau gene in some members of the family. With the help of array painting and sequence tagged site–PCR on flow-sorted derivative chromosomes, we have cloned the breakpoints of the translocation. We have studied the junctions on both derivative chromosomes at the genomic and expression levels. The analysis of the sequence revealed a 5 kb microdeletion at the chromosome 3 breakpoint together with a high density of repetitive motifs (Alu, short interspersed nuclear element) and an AT-rich region. Both chromosome 3 and 8 rearranged regions were very poor in gene content. We tested an expressed sequence tag, two predicted genes, one novel gene and LRIG1, a gene located more than 200 kb apart from the breakpoint on chromosome 3. None of these genes, except LRIG1, showed expression in any of the tested tissues (including normal adult and fetal kidney, sporadic kidney tumours and tumour samples from the proband's family). Taken together, all these data suggest that, rather than deregulation of specific genes that may be rearranged by the translocation, the proposed three-step model of tumour development (translocation, loss of the 3p chromosome, and mutation in a tumour suppressor gene located within that region) could be the biological mechanism that takes place in this familial form of conventional RCC.

INTRODUCTION

Renal cell carcinoma (RCC) comprises a heterogeneous group of tumours that have been divided into different subtypes based on histologic features. Conventional RCC (also known as clear cell RCC or non-papillary RCC) is the most common type (75% of all RCCs) and is thought to arise from the proximal tubule epithelium (1). Although conventional RCCs mostly occur in a sporadic form, several familial cases have been reported. The most common form of familial conventional RCC is in association with the dominantly inherited von Hippel–Lindau (VHL) cancer syndrome. The other form is composed of families that show segregation of conventional RCC with constitutive balanced translocations involving chromosome 3. Those families are: t(3;8)(p14;q24) (2), t(3;6)(p13;q25.1) (3), t(2;3)(q35;q21) (4), t(3;4)(p13;p16) (5), t(2;3)(q33;q21) (6), t(3;6)(q12;q15) (7) and t(1;3)(q32;q13.3) (8). These families are also characterized by the somatic loss of

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the derivative chromosomes that, including the short arm of chromosome 3, takes place in the kidney tumour tissue. In addition, molecular studies on tumours in these families revealed that some of them carry different VHL mutations in the allele from the remaining 'normal' chromosome 3.

The concurrent observations of loss of 3p derivative chromosomes and somatic VHL mutations has led to the proposition of a three-step model for the carcinogenesis process (9–12). The first hit would be the occurrence of a germ line chromosome 3 translocation. Non-disjunctional loss of the derivative chromosome that carries the 3p segment would represent the second step. Finally, the third step would involve a somatic mutation in the remaining 3p allele of an RCC-related tumour suppressor gene (TSG) such as VHL. However, since some of the analysed familial tumours did not carry VHL mutations, it seems likely that other/s TSGs located in the 3p arm could also be involved in the development of conventional RCC.

The existence of other putative TSGs located within 3p is supported by cytogenetic and molecular data collected from sporadic renal tumours. In fact, deletion of the short arm of chromosome 3 is the most frequent genetic change in sporadic conventional RCCs, whereas this alteration is rarely seen in papillary and chromophobe RCCs (13). Involvement of 3p in the development of conventional RCC is corroborated by the findings that conventional RCC cell lines lose their tumorigenicity upon introduction of 3p (14–17). In addition to conventional RCC, deletions of the short arm of chromosome 3 are frequent in many other cancers, including lung, ovary, testicular and breast carcinomas (18,19). Through loss of heterozygosity (LOH) studies in sporadic conventional RCCs, three common regions of allelic loss have been proposed that may contain TSGs: 3p13–14.3, 3p21.3 and 3p25. However, the smallest overlapping deletion, which has been experimentally demonstrated, occurs in ~95% of conventional RCCs at a single large region of 3p14.2–p25 (20,21). This telomeric region contains the VHL disease TSG. Again not all sporadic conventional RCCs show evidence of inactivation of the VHL gene. This suggests that VHL inactivation is not required in all conventional RCCs and that a VHL-independent pathway [involving other TSG(s) in 3p] could also exist for conventional RCC development.

Another controversial issue is the existence of genes that act as TSG because they become inactive as a result of the translocation rearrangements. This hypothesis is supported by the existence of some conventional RCC familial tumours with neither 3p derivative loss nor VHL mutation. Another strong piece of evidence suggesting the existence of breakpoint spanning genes comes from the finding of biologically significant genes that are disrupted in some of the familial translocations. These candidate genes are FHIT, TRC8, DIRC1, DIRC2, DIRC3, LSAMP and NORE1, which have been identified in four conventional RCC families (22–26). However, the role of these genes in the conventional RCC tumourigenicity remains unclear (23,25,27).

Previously, we described a Spanish family carrying a constitutional t(3;8)(p14.1;q24.23) translocation (28). All conventional RCC patients in this family are carriers of this rearrangement. Subsequent cytogenetic and molecular studies of primary tumours in this family showed loss of the derivative chromosome 8, containing the short arm of chromosome 3. The site of the 3;8 translocation breakpoint in 3p14 corresponds to the most proximal of the 3p commonly deleted regions in sporadic conventional RCCs. We speculated that deregulation of a gene(s) located at or near the translocation breakpoint may act as TSG. We decided to look for genes in both breakpoints. Here we described the positional cloning of the t(3;8)(p14.1–q24) chromosomal breakpoints. We used a strategy that combined array painting, fluorescence *in situ* hybridization (FISH) and PCR on flow sorted derivative chromosomes, long-range PCR and sequencing.

RESULTS

To initially map the translocation breakpoints we used array painting, where the flow-sorted derivative chromosomes (Fig. 1A) were DOP–PCR amplified, differentially labelled, and applied to the 1 Mb resolution BAC array developed in the Wellcome Trust Sanger Institute (29). Only clones corresponding to the sequences present in the sorted chromosomes showed fluorescence above background. The ratio profiles for chromosomes 3 and 8 are shown in Figure 1B. Sharp transitions in ratio values define the breakpoint (30). Thus, the chromosome 3 breakpoint lies between clones RP11–88H12 (AC121493) and RP11–24O17 (AC098969); and the clones that defined the chromosome 8 breakpoint are RP11–356M23 (AC021621) and RP11–172M18 (AC087711).

To allow a higher resolution mapping of the rearrangement, overlapping clones that cover the breakpoint regions were identified using the Ensembl CytoView browser (www.ensembl.org). The location of the BAC clones was verified by FISH on normal prometaphase spreads. We then hybridized these clones to metaphase spreads from the patient's cell line. We identified the clones that span both breakpoints, RP11–814M22 from chromosome 3 and two overlapping BACs (CTD–2025J6 and RP11–346A14) from chromosome 8 (Fig. 1C).

Sequence-tagged sites (STSs) located within these clones were designed generating 44 sets of primers to fine-mapping both breakpoints by polymerase chain reaction (PCR) using the amplified DNA from each derivative chromosome as template. This strategy allowed us to define the breakpoint regions more precisely. The chromosome 3 region was delimited to ~1000 bp, and the chromosome 8 region could be narrowed down to ~500 bp. Long-distance PCR was used to amplify the breakpoint regions, the primers were selected from each side of the breakpoint so that they only amplified sequences across the breakpoints and generated fragments of ~6 and ~2.5 kb for der(3) and der(8), respectively (Fig. 2). Subsequent DNA sequencing of the breakpoint fragments resulted in the identification of both breakpoints (GenBank sequence numbers AY494549 and AY494548; Fig. 2A). As expected, no product could be amplified from a normal control DNA. The sequences confirmed that the BACs detected by FISH were in fact implicated in the rearrangement. An ~5 kb microdeletion compared with the consensus chromosome 3 sequence was found at the chromosome 3 breakpoint.

We analysed the 10 kb sequence around the chromosome 3 and 8 breakpoints. Firstly, no sequence homology between the breakpoint regions was found using BLAST2 sequences

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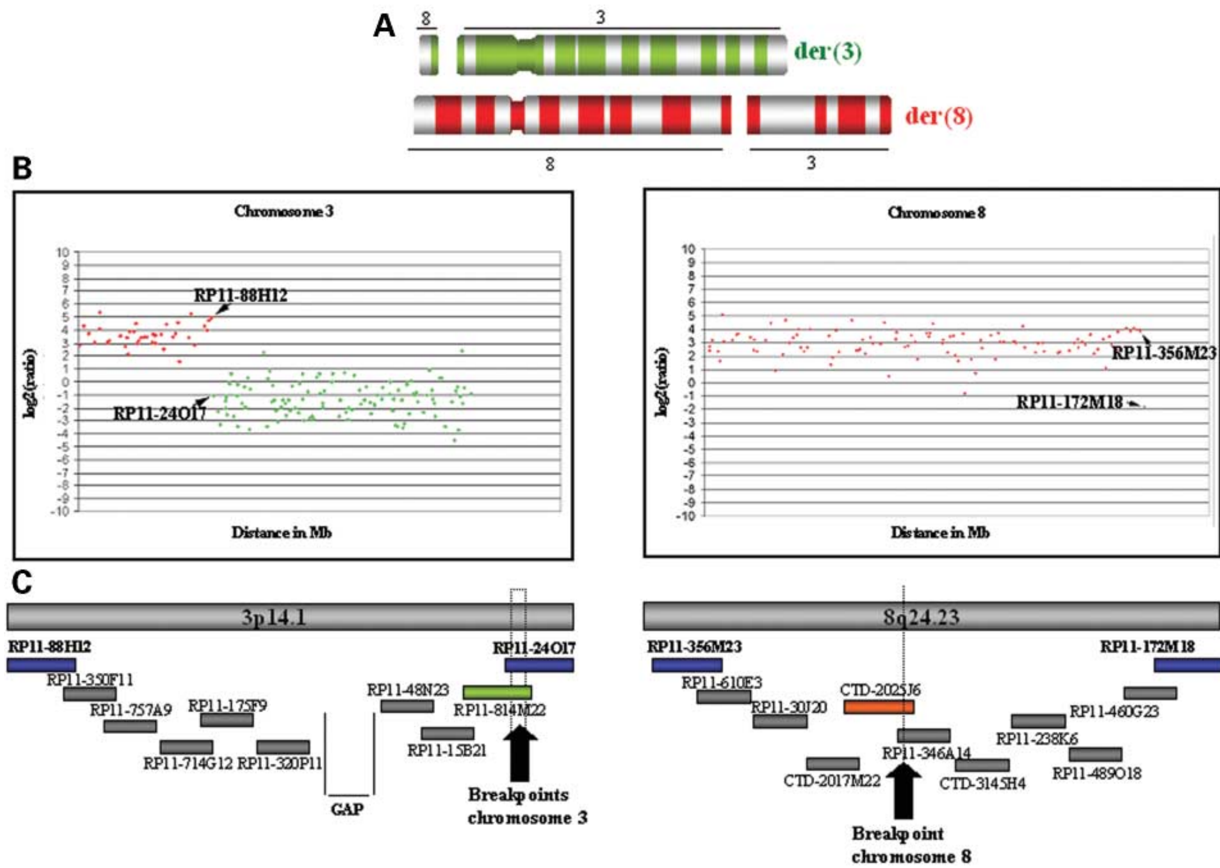

Figure 1. (**A**) Idiogram of derivative 3 and 8 chromosomes; (**B**) 1 Mb array painting profiles for the derivative chromosomes. BAC flanking the breakpoints are marked by arrows. Red dots correspond to der(8) material and green dots to der(3) DNA. (**C**) BAC contigs covering the 3p14 and 8q24 breakpoints. BACs spanning the breakpoints are indicated in red and green.

analysis. The presence of repeated sequences and recombination motifs were also analysed by repeat masker and using the Ensembl database (http://woody.embl-heidelberg.de/repeatmask and www.ensembl.org). Repetitive elements such as Alu and long interspersed nuclear element (LINE)-L2 repeats, were present (Fig. 2B) along the sequences at a higher frequency than in the mean average across the genome. This comparison was done by scanning the whole genome taking non-overlapping windows of 10 000 base pairs. We calculated the density of repeats for all chromosomes. Repetitive elements such as short interspersed nuclear element (SINE)/MIR, DNA/MER1_type and Simple repeats were present in our breakpoint regions in a frequency appreciably higher than the average across the genome. Thus, as an example, in the 10 kb sequence around the chromosome 8 breakpoint we found five simple repeats, whereas the frequency of five or more simple repeats over randomly selected contiguous sections of 10 kb was 0.034 (mean across the genome $\approx$ 1.33). In addition, we also found an AT-rich region near the microdeletion breakpoint on chromosome 3.

Both breakpoints lie in regions which apparently low content in genes. The Ensembl CytoView browser, the Human Genome Project Working Draft (UCSC, July 2003; genome.ucsc.edu) and the NCBI MapView (www.ncbi.nlm.nih.go/mapview) were used to identify described genes, expressed sequence tags (ESTs) and predicted genes on the clones around the breakpoints.

We used RT–PCR and RACE (rapid amplification of cDNA ends) analysis to study the expression of genes and ESTs that we found around both breakpoints. RT–PCR experiments were performed using total RNAs from normal peripheral blood, normal kidney and the patient's lymphoblastoid cell line. We used total RNA obtained from normal kidney tissue for the RACE analysis. We started looking for genes on the 3p14.1 band from chromosome 3. An EST (AW082148) was found close to the breakpoint (Fig. 2A), which yielded no product when we performed RT–PCR using primers localized in all three of its different exons (primers are listed in Table 1). We then studied a predicted gene (Ensembl Genome Browser, Geneid, chr3_679.1) which is directly disrupted by the translocation breakpoint. This putative gene with unknown function is composed of 7 exons and the breakpoint is located within the third intron. With the predicted mRNA sequence we designed gene specific primers to perform RACE analysis with total RNA obtained from kidney tissue. We did not obtain products for this predicted gene. We also studied a 'novel gene' that is located near the chromosome 3 breakpoint. This 396 bp gene codes for a protein similar to 60S ribosomal L21. We amplified some fragments using total RNA from peripheral blood as template, but no detectable transcripts were observed when we

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Figure 2. (**A**) Gene content of the breakpoint spanning BACs. The genetic locations of these breakpoints are included. (**B**) Schematic representation of the rearrangement showing the repetitive motifs.

tried with total RNA from normal kidney tissue. A putative candidate gene near the chromosome 3 breakpoint region is LRIG1, which encodes a transmembrane protein which, in the extracellular part, shows structural similarities to the *Drosophila* protein Kekkon-1. In *Drosophila*, this extracellular domain of Kekkon-1 functions as an inhibitor of epidermal growth factor receptor (EGFR)-mediated signalling (31,32). We designed primers for RT–PCR using as template cDNA from normal kidney, familial and sporadic kidney tumour tissues, from normal peripheral blood and from the patient's lymphoblastoid cell line. To evaluate the LRIG1 mRNA expression levels we used a semi-quantitative RT–PCR assay. We showed expression of this gene in all tissues except the patient's cell line.

The region around the 8q24 breakpoint was also examined for genes (Fig. 2A). The breakpoint spans a predicted gene (UCSC Genome browser, Genscan, NT_028251.56) with 11 exons. Neither RT–PCR nor RACE techniques could amplify a transcript from this gene. No ESTs have been localized at the breakpoint region.

Finally, we expanded our analysis by screening a cDNA library constructed from material extracted from adult kidney, foetal kidney, lung, brain, bladder, intestine and heart (BD Biosciences, Clontech). We could not find cDNA sequence transcribed from any of the genes and ESTs analysed, with the exception of the LRIG1 gene.

DISCUSSION

We have previously described a family in which a constitutional t(3;8) translocation segregates with a predisposition to clear-cell renal carcinoma. Using LOH and comparative genome hybridization (CGH) analysis in tumour tissues, we were able to demonstrate the loss of the der(8) chromosome which contains the 3p14.1-pter region of chromosome 3. We also studied the VHL gene and detected different somatic mutations that affect second and third exons in the retained copy of the gene in some of the patients in the family (28). This is in agreement with the three-step model of tumourigenesis proposed by Kovacs and Kung (9). These authors have proposed a model for conventional RCC development different from the two-hit model described by Knudson (33,34). The three hits are the constitutional translocation, the loss of the derivative chromosome in tumoural cells and the subsequent mutation in one TSG located in the retained copy of the lost chromosome region. In the case of conventional RCC, the translocations that always involve chromosome 3 could, somatically, result in random non-disjunctional loss of the derivative chromosome carrying the 3p segment. The random loss may generate a high number of renal cells retaining only one copy of the 3p segment. The tumour would result from mutation in a putative or confirmed TSG located in the

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Table 1. Description of the candidate genes studied by RT–PCR and RACE

Gene/EST	Chromosome	Status	Function	Technique	Primers	
AW082148	3	EST	Unknown	RT–PCR	Exon1-S	ccg aag gtc acg cag tta at
					Exon2-AS	tgt tcc ata ccg ttt cac tg
					Exon2-S	gtg gtt gtc agg ctt ccc ta
					Exon3-AS	tgg agt tcc gag cta agg aa
Chr3_679.1	3	Predicted gene	Unknown	RACE	Chr3-S	cat ggg ggt tgg agg gag aaa aag ca
					Chr3-AS	gca cga tgg tct ctg ggt aga gtt aag agc a
					Chr3-nested-S	tcc tgg ttc tca ttt ttc ctc tgc tct t
					Chr3-nested-AS	gca tca ggc tgg ggg ata gga ggt g
ENSG 00000177216	3	Novel gene	60S ribosomal protein L21-like?	RT–PCR	Novel-chr3-S	atg ctg ttg gca ttg ttg t
					Novel-chr3-AS	gca gct cag gct cct tcc
				RACE	Novel-chr3-S	caa aaa gga atg ccc cat aa
					Novel-chr3-AS	cag gct cct tcc caa tgg tt
NT_028251.56	8	Predicted gene	Unknown	RT–PCR	Exon1-S	gag tgt tag ccc acc atc aaa
					Exon3-AS	ttg agg ctc tag caa cac ga
					Exon5-S	tgc tat ctt att ttg cgg agt t
					Exon7-AS	gca ggt tag gga gtc aca ttc
				RACE	Chr8-S	gag cat ctg caa gag gtt gtg gtt t
					Chr8-AS	ccc atg ctt cat atc ccc acc aa
					Chr8-nested-S	gtc tcc ctc ctt tta agc ggc ata gt
					Chr8-nested-AS	gag gat tct aca tag cca gca ggg at
LRIG1	3	Known gene	Leucine-rich repeats and Ig-like domains; orthologue of mouse integral membrane glycoprotein Lig-1	RT–PCR	Lrig1-S	ggt gag cct ggc ctt atg tga ata
					Lrig1-AS	cac cac cat cct gca cct cc

remaining allele of a conventional RCC gene, frequently VHL. However, in some patients in our family and in other familial conventional RCC cases, there is no evidence of inactivation of the VHL gene, thus suggesting two alternatives: the existence of another TSG that localizes in the 3p arm, or the deregulation of the expression of one gene or genes located at or close to the breakpoint. We decided to explore the second hypothesis.

By analysing the breakpoints with array painting and FISH analysis with bacterial artificial chromosome (BAC) clones, we were able to map our chromosome 3p and 8q breakpoints within spanning clones within to the p14.1 and q24.23 cytogenetic loci, respectively. By STS–PCR and long-distance PCR we were able to clone and sequence both translocation fragments. Analysis of these sequences revealed that the chromosome 3 breakpoint coincides with an ~5 kb genomic microdeletion. The deleted region did not include any known genes. Few examples of sequence analysis across constitutional translocation breakpoints have been reported. Most of these cases involve the loss and/or gain of only a few bases (35). However, there is a case with a microdeletion of several kb associated with the translocation process (36). It seems that the mechanism generating the translocation is more complex than a simple breakage and re-annealing, and can occur by a number of different mechanisms (35).

It has been shown that gross chromosomal abnormalities such as translocations and deletions are non-randomly distributed in the human genome (37) and that this non-random distribution is strongly influenced by the local sequence environment (38,39). Several studies have documented the occurrence of various different types of repetitive sequence elements in the vicinity of breakpoint junctions (38,39). We analysed our breakpoint regions and found that they are rich in repetitive sequences especially in LINE-L2, Alu and large terminal repeats (LTR) repeats. This situation is similar to other reported familial germline translocations which are also localized in regions rich in repetitive sequences (6). We also found an AT-rich region which, as it happens in our family, has been associated with the presence of microdeletions (38).

By database searches we found that 3p and 8q breakpoint regions are poor in genes. We could identify several ‘predicted genes’ and only one EST located in the BACs that span both breakpoints. By RT–PCR and RACE analysis we were unable to detect expression in the several tissues analysed (normal adult kidney, conventional RCC tumour, lymphoblastoid patient’s cell line and normal lymphocytes). Near the chromosome 3 breakpoint we found a gene that has been proposed as a potential tumour suppressor gene, LRIG1. Its expression has been found to be down-regulated or absent in many tumour cell lines compared with corresponding normal tissues. Although the gene is located more than 150 kb away from the breakpoint, we could not exclude a putative silencing of the expression of this gene due to its repositioning after the rearrangement. Accordingly, we performed RT–PCR with LRIG1 primers in normal and tumour kidney samples from sporadic cases and our probandus’ family and we found similar levels of expression in both samples. These data did not support an essential role of this gene in the conventional-RCC development in this family.

Taking all these results together, it seems unlikely that any gene is being affected by the rearrangement process. To date, seven families with different constitutional translocations associated with conventional RCC have been described. In three of them no genes localized at the breakpoints have been described. In the rest of families, several genes have been proposed as associated with

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RCC. These genes are located at breakpoint regions, and they are: FHIT (3p14), TRC8 (8q24.1), DIRC1 (2q33), DIRC2 (3q21), DIRC3 (2q35), LSAMP (3q13.3) and NORE1 (1q32.1) (22–26). Aberrant FHIT transcripts have been found in different types of cancer, although mostly in combination with a normal transcript. Such aberrant transcripts, however, are not cancer-specific at all; they apparently occur in a variety of normal tissues. This observation makes FHIT an unlikely candidate TSG. Another interesting gene is DIRC2, which protein shows significant homology to different members of the major facilitator superfamily (MFS) of transporters. However, the role of these genes in the RCC tumourigenicity remains unclear (23,25,27). Despite the absence of a clear candidate gene, it has been demonstrated that carrying a constitutive translocation affecting chromosome 3 confers an increased risk factor for developing conventional RCC (5). The mechanism responsible for this increased risk should rely on the chromosome instability, and its likely effect on the mitotic mis-segregation of derivative chromosomes that may appear in carriers of familial reciprocal chromosome 3 translocations, particularly when the translocation breakpoints are located pericentromerically.

To summarize, our data, and the heterogeneity of previous reports about genes that are disrupted by the different translocations, are consistent with the three-step hypothesis rather than the existence of specific genes that become fused and/or rearranged in translocations-associated RCC. Owing to the clustering of repetitive sequences, it is likely that rearrangement at the breakpoint in chromosome 3 may occur frequently and it is also probable that the subsequent loss of the 3p arm confers an increased proliferative potential to the renal epithelium (11). Finally, it is possible that one of the TSGs, VHL or another yet unknown gene, located in the p arm of chromosome 3 that is constantly lost in these tumours, when mutated drives the formation of RCC.

MATERIALS AND METHODS

Cell line

An Epstein–Barr virus (EBV)-transformed lymphoblastoid cell line was established from an affected translocation carrier of the family. The cell line was cultured in RPMI-1640 medium supplemented with 15% fetal calf serum and antibiotics. High molecular weight DNA was extracted directly by using DNAzol (Molecular Research Center, Inc.).

Chromosome preparation and flow sorting

Approximately 500 der(3) or der(8) chromosomes were flow-sorted (40) from the patient's cell line and used as templates for degenerate oligonucleotide primed (DOP)–PCR as described (41). Reverse chromosome painting was used to ensure that the DOP–PCR products were derived solely from the flow-sorted reagents. DOP–PCR products from the two derivatives were differentially labelled with biotin– and digoxigenin–dUTPs by a second round of PCR cycles and hybridized to normal metaphase spreads. Only chromosomal regions comprising the derivative chromosomes showed hybridization signals. The two derivative DOP–PCR amplified DNAs were used as template for further PCRs.

Array painting and analysis

The experimental procedures used for array painting were essentially as described (30). Briefly, the two derivative chromosomes are separated by flow sorting, amplified, differentially labelled and hybridized onto the DNA array. We used the whole genome array development in the Sanger Institute (29) comprising clones selected to be spaced at ~1 Mb intervals across the human genome.

Fluorescence *in situ* hybridization and painting

Ensembl Cytoview was used to select the seventeen 3p14.1 and 8q24.23 BAC clones that covered the 1 Mb breakpoint intervals defined by array painting. Clones were purchased from the Children Hospital Oakland Research Institute (CHORI; www.chori.org/bacpac/) and from CalTech BAC (http://informa.bio.caltech.edu/) and used for FISH analysis. All BACs were labelled directly by nick translation according to the manufacter's specifications (Vysis) with SpectrumGreen– or Spectrum Orange–dUTPs (Vysis). The probes were blocked with Cot-1 DNA (Vysis, Dowers Grove, IL, USA) to suppress repetitive sequences. Metaphase spreads, obtained from the patient's cell line and a normal control, were hybridized overnight at 37°C with labelled probes. After post-hybridization washes, the chromosomes were counterstained with DAPI in antifade solution. Cell images were captured using a CCD camera (Photometrics SenSys camera) connected to a computer running the Chromofluor image analysis system (Cytovision, Applied Imaging Ltd, Newcastle, UK).

STS–PCR

Sequence information derived from the clones that spanned both breakpoints was used to design specific primers that amplified unique products of ~100 bp in size along the sequences of interest. All primers were tested on genomic and negative controls before they were used to amplify products from flow-sorted DOP–PCR products.

Long-distance PCR

Long-range PCR was used for the amplification of the translocation breakpoints. DNA from the patient's cell line and normal DNA (negative control) were used as PCR templates. We used the Expand Long Template PCR System (Roche Diagnostics, Mannheim, Germany). The primers are shown in Table 1. Breakpoint fragments were generated using pairs of primers from the different chromosomes.

The PCR fragments obtained were sequenced by using the ABI Prism system (Applied Biosystems). After the sequencing reactions, the products were electrophoresed on an ABI 3700 sequencer and analysed using ABI software. The clone sequences are entered into GenBank with accession numbers AY494549 and AY494548.

Analysis of sequences

The databases used to identify possible candidate genes altered by the rearrangement were: Ensembl (www.ensembl.org), the National Centre for Bioinformatics Institute MapViewer

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(www.ncbi.nlm.nih.gov), and UCSC Genome browser (genome.ucsc.edu). 'BLASTN' and 'BLAST 2 sequences' were also used (www.ncbi.nlm.nih.gov/BLAST).

RT–PCR

Total RNA from normal kidney tissues (BD Biosciences, Clontech), patient's derived cell line and normal lymphocytes were use as templates. cDNA synthesis was performed using Superscript-II RT (Invitrogen) with random hexamers and for the RT–PCR we used gene specific primers pairs (Table 1). The primers were designed with Primer3 (www.genome.wi.mit.edu).

For the comparative analysis of expression, semi-quantitative RT–PCR was performed and the LRG1 values were normalized to the apparent 18S rRNA levels in respective RNA samples.

RACE

Using cDNA extracted from normal human kidney, 5′ and 3′ RACE was performed with the GeneRacer Kit (Invitrogen). The cDNAs were amplified following the supplier's instructions using gene-specific primers (GSP). To increase the specificity of the product, nested PCR was carried out. The primers used are shown in Table 1. RACE products were electrophoresed and sequenced.

Bioinformatic sequence analysis

Repeat analysis of the breakpoint sequences was as described by Abeysinghe and Chuzhanova (38,39) (http://woody.embl-heidelberg.de/repeatmask, and www.ensembl.org). Comparative analysis of repetitive sequences was performed using the information obtained in the Ensembl database.

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