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Characterization of a 3;6 Translocation Associated with Renal Cell Carcinoma

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Abstract

The most frequent cause of familial clear cell renal cell carcinoma (RCC) is von Hippel–Lindau disease and the *VHL* tumor suppressor gene (TSG) is inactivated in most sporadic clear cell RCC. Although there is relatively little information on the mechanisms of tumorigenesis of clear cell RCC without *VHL* inactivation, a subset of familial cases harbors a balanced constitutional chromosome 3 translocation. To date nine different chromosome 3 translocations have been associated with familial or multicentric clear cell RCC; and in three cases chromosome 6 was also involved. To identify candidate genes for renal tumorigenesis we characterized a constitutional translocation, t(3;6)(q22;q16.1) associated with multicentric RCC without evidence of *VHL* target gene dysregulation. Analysis of breakpoint sequences revealed a 1.3-kb deletion on chromosome 6 within the intron of a 2 exon predicted gene (NT_007299.434). However, RT-PCR analysis failed to detect the expression of this gene in lymphoblast, fibroblast, or kidney tumor cell lines. No known genes were disrupted by the translocation breakpoints but several candidate TSGs (e.g., EPHB1, EPHA7, PPP2R3A, RNF184, and STAG1) map within close proximity to the breakpoints.

INTRODUCTION

Although only 2% of renal cell carcinoma (RCC) cases are familial, the identification of familial RCC genes (e.g., the *VHL* gene) has provided important insights into the pathogenesis of sporadic RCC. Thus, somatic inactivation of the *VHL* tumor suppressor gene (TSG) is found in ~60% of sporadic clear cell RCC (Kim and Kaelin, 2004; Maher, 2004). *VHL* inactivation leads to stabilization of the HIF-1 and HIF-2 transcription factors and overexpression of downstream target genes, including VEGF, cyclin D1, and carbonic anhydrase IX (CA-IX) (Maxwell et al., 1999; Zatyka et al., 2002; Staller et al., 2003). *VHL* mutations are rare in nonclear cell RCC, but there is also evidence for a non *VHL* dependent pathway of renal tumorigenesis in clear cell RCC. Thus, a subset of sporadic cases do not have *VHL* inactivation and linkage to *VHL* has been excluded in kindreds with familial non *VHL* clear cell RCC (Teh et al., 1997; Woodward et al., 2000).

A subgroup of patients with familial clear cell RCC harbors a constitutional chromosome 3 translocation. Nine different chromosome 3 translocations have been associated with RCC susceptibility. In seven cases the translocations t(1;3)(q32; q13), t(2;3)(q33;q21), t(2;3)(q35;q21), t(3;4)(p13; p16), t(3;6)(q12;q15), t(3;8)(p13;q24), and t(3;8) (p14.1; q24.23) (see Melendez et al., 2003 and references within) were associated with familial clear cell RCC and in two cases, individuals with a familial t(3;6) (p13;q25.1) (Kovacs et al., 1989) and t(3;6)(q23; q16.2) (Subramonian et al., 1998), developed multicentric clear cell RCC. Cloning of the translocation breakpoint has, in some cases, led to the identification of novel RCC susceptibility genes. Thus characterization of the t(3;8)(p14;q24) breakpoints revealed that the *FHIT* and *TRC8* genes were disrupted (Ohta et al., 1996; Gemmill et al., 1998). Subsequently, loss of *FHIT* expression has been shown to occur in a variety of human cancers, including RCC, and *FHIT* has been demonstrated to have TSG activity (Pekarsky et al., 2002). *TRC8* has also been proposed as a candidate TSG (Gemmill et al., 2002). In another family, a t(1;3)(q32;q13) associated with familial RCC was characterized and two candidate TSGs, *NORE1A*, and *LSAMP*, were disrupted (Chen et al., 2003). Both genes are silenced by promoter methylation in a subset of sporadic RCC (Chen et al., 2003; Morris et al., 2003). However, for many RCC-associated constitutional chromosome 3 translocations, characterization of the translocation breakpoints has led to the identification of genes that were not implicated in the pathogenesis of RCC or the breakpoints were found not to directly disrupt known genes (Druck et al., 2001; Bodmer et al., 2002, 2003). In such cases susceptibility to RCC may result from chromosome 3 instability (predisposing to loss of 3p which contains several TSGs) or because the translocations have long-range effects on gene expression. Thus, in disorders such as aniridia and campomelic dysplasia, translocations many hundreds of kilobases away from *PAX6* or *SOX9*, respectively, may alter gene expression and cause disease (Crolla and Van Heyningen, 2002). Here we report the molecular characterization of a t(3;6)(q22;q16.2) associated with multicentric RCC (Subramonian et al., 1998).

MATERIALS AND METHODS

Cell Line

An EBV-transformed lymphoblastoid cell line was established from the proband. Cells were maintained at 37°C under 5% CO₂ and cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FCS, 0.1 mM nonessential amino acids, 1% glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Genomic DNA was prepared using a Nucleon Kit (Amersham, Little Chalfont, Buckinghamshire, UK). SKRC39 cells were also cultured under the same conditions.

Chromosome Preparation and Flow Sorting

Derivative chromosomes, der(3) and der(6) were flow sorted from the patient's cell line and used as templates for whole genome amplification (Genomiphi), following the manufacturer's instructions (Amersham) (Fiegler et al., 2003). The amplified DNAs were used in array painting experiments and as templates for further PCRs.

CGH Array

Array CGH was carried out as described previously (Gribble et al., 2005). About 450 ng of genomic DNA from the cell line containing the translocation was labeled and hybridized against 450 ng pooled male DNA (DNA from 20 individuals).

Array Painting

The experimental procedures used for array painting were essentially as described (Fiegler et al., 2003). Briefly, the two derivative chromosomes were separated by flow sorting, amplified, and 450 ng of each were differentially labeled and hybridized onto DNA arrays.

Construction of BAC Contigs and Fluorescent In Situ Hybridization Analyses

Nine 3q22.2-3q22.3 and twelve 6q16.1 BAC clones were obtained from the BACPAC Resource Center (Children's Hospital, Oakland Research Institute, Oakland, CA). The clones were selected based on information in the BAC clone mapping databases and the human genome sequence draft database. BAC DNA was prepared using Phase-Prep BAC DNA Kit (Sigma, Gillingham, UK) and then labeled directly by nick translation according to the manufacturer's instructions (Vysis), with Spectrum Green or Spectrum Red-dUTPs (Vysis, Downers Grove, IL). Probes were blocked with Cot-1 DNA to suppress repetitive sequences. Metaphase spreads obtained from the patient's cell line and two normal controls were hybridized overnight at 37°C with labeled probes. Chromosome 3 and 6-specific centromeric probes were included in hybridization mixture (Vysis). After posthybridization washes the chromosomes were counterstained with DAPI in Vectashield antifade solution (Vector Laboratories, Peterborough, UK).

BAC Clones used for the Construction of BAC Contigs and FISH Analyses

The 9 BAC clones from 3q22.2-3q22.3 are RP11-192L3, RP11-1079L18, RP11-73J21, RP11-354M3, RP11-767H24, RP11-452N24, RP11-219P9, RP11-1096B15, and RP11-93K21. The 12 BAC clones from 6q16.1 are RP11-777M22, RP11-461I4, RP11-244K16, RP11-960B10, RP11-119L14, RP11-25909, RP11-419I4, RP11-630M20, RP11-654K3, RP11-44P5, RP11-939I18, and RP11-243P16.

STS-PCR and Amplification of Breakpoint Sequences

Sequence information derived from the two breakpoint spanning BACs was used to design primers to amplify regions along the sequences of interest. Flow sorted der(3) and der(6) DNA, as well as genomic DNA were used as templates. This approach enabled the region containing the breakpoints to be narrowed until it was possible to PCR across the breakpoint. Breakpoint fragments generated using pairs of primers from the different chromosomes were then sequenced using an ABI BigDye Cycle Sequencing Kit (Perkin-Elmer, Rotkreuz, Switzerland) and analyzed using an ABI Prism377 DNA sequencer (Perkin-Elmer).

RESULTS

Clinical Case Report

A 49-years-old man presented with hematuria and loin pain. Renal ultrasound and CT scans demonstrated bilateral multicentric RCC (Subramonian et al., 1998). A left partial nephrectomy was performed and histopathological examination revealed a well-differentiated clear cell RCC. Two months later, a radical right nephrectomy was performed for the two large (4.5 cm) tumors in the right kidney. Histopathological examination demonstrated clear cell RCC with occasional areas of papillary cancer. Subsequently, he developed three new RCCs in his residual left kidney and a left nephrectomy was performed 6 years after the first operation. At Clinical Genetics assessment there was no evidence of VHL disease or tuberose sclerosis, and no family history of cancer. *VHL* gene analysis demonstrated no abnormality, but cytogenetic analysis revealed a constitutional t(3;6)(q22;q16.2). The translocation was not present in his father and two siblings (his mother had died and was not available for analysis).

Mapping and Characterization of Translocation Breakpoints

Mapping of translocation breakpoints by Array Painting—Array Painting using a 1-Mb resolution BAC array was performed on derivative chromosomes flow sorted from a lymphoblastoid cell line. DNA from the two derivative chromosomes was differentially labeled and cohybridized. The ratios of fluorescence intensity were plotted against the distance of the clones along the chromosome from the p termini. Fluorescence ratio profiles are shown in Figure 1. Sharp transitions in ratio values define the breakpoint region. Thus Array Painting mapped the breakpoints to an approximately 0.9-Mb interval between BAC clones RP11-91O5 and RP11-311K2 on chromosome 3, and an approximately 1.6-Mb region between clones RP11-21G12 and RP11-58A9 on chromosome 6. Array CGH analysis was also performed and did not detect any genomic imbalances.

Refining translocation breakpoints by FISH analysis—Human genome sequence and BAC mapping data were used to construct in silico (a) a contig of 9 BAC clones (See Materials and Methods section) spanning the 0.9-Mb region containing the t(3) breakpoint and, (b) a contig of 12 BAC clones covering the t(6) breakpoint region. BAC clones were hybridized to metaphase spreads from patient and normal lymphoblast cells. The presence of split signals within the patient metaphase spreads identified two breakpoint-spanning BACs, RP11-354M3 on chromosome 3 and RP11-44P5 on chromosome 6 (Fig. 1).

Characterization and sequencing of translocation breakpoints—To map more precisely the t(3;6) breakpoints, primers were designed for STSs within the two breakpoint spanning BACs. PCR amplification was then performed using amplified DNA from each derivative chromosome and this approach enabled the region containing the breakpoints to be narrowed to within ~300 bp on chromosome 3, and to within ~1.7 kb on chromosome 6. We then attempted to PCR across the breakpoints (Fig. 2) and generated fragments of ~1.1 kb for der(3) and ~0.5 kb for der(6). Sequencing of these fragments revealed a 1.3-kb deletion on chromosome 6, a repeat motif comprising tandem tetranucleotide repeats (CTAT)_{n1}(CCAT)_{n2}, in which *n1* was reduced, as compared with the available genome sequence, from 13 to 7 and *n2* was reduced from 7 to 5, and an 8 bp loss at the immediate breakpoint site. The breakpoints did not directly disrupt any known genes, although the chromosome 6 breakpoint was located within the intron of a 3 exon predicted gene (NT_007299.434). RT-PCR analysis, however, failed to detect expression of this gene in lymphoblast, fibroblast or kidney tumor cell lines.

DISCUSSION

We have characterized the breakpoints of a 3;6 translocation associated with multiple RCC. Multicentric RCC is rare, occurring in ~2% of all cases (Blute et al., 2003). Although bilateral RCC is characteristic of inherited RCC susceptibility, it may occur sporadically in older individuals (mean 65 years) so the early age at diagnosis and multiple tumors ($n = 6$) in our patient, coupled with the known association between constitutional chromosome 3 translocations and RCC, is consistent with a causal association between the t(3;6) and multicentric RCC. It is intriguing that chromosome 6 is the most commonly involved partner in chromosome 3 translocations associated with RCC, although there is no evidence of common breakpoints: t(3;6)(q12;q15) (Eleveld et al., 2001), t(3;6)(p13;q25.1) (Kovacs et al., 1989), and t(3;6)(q23;q16.2) (Subramonian et al., 1998), perhaps suggesting that the translocation might predispose to renal neoplasia by instability of the derivative chromosomes and/or long-range effects on gene expression and not by direct disruption of specific genes. Clinically our case resembles the German family with a t(3;6)(p13;q25.1) in three consecutive generations in which the only translocation carrier aged >50 years of age developed multiple bilateral renal-cell carcinomas (Kovacs et al., 1989).

Characterization of RCC-associated translocations has led to the identification of several genes that are disrupted by the breakpoints (e.g., *FHIT*, *TRC8*, *DIRC1*, *DIRC2*, *DIRC3*, *LSAMP*, and *NORE1* (Gemmill et al., 1998; Druck et al., 2001; Bodmer et al., 2002, 2003; Chen et al., 2003). However, the role of *DIRC1*, *DIRC2*, and *DIRC3* in renal tumorigenesis requires confirmation and, as in our case, there are several cases where no transcribed genes appeared to be disrupted by the breakpoints. Thus, analysis of a t(3;8)(p14.2;q24.2) revealed a 5-kb microdeletion at the chromosome 3 breakpoint but no genes were disrupted by either breakpoint (Rodriguez-Perales et al., 2004). Evidence that RCC-associated translocations might cause instability of the translocated chromosome is provided by the observation that in some tumors associated with a constitutional chromosome 3 translocation, the derivative 3 was lost in the tumor and a somatic *VHL* mutation was also detected (such that the tumor was homozygous for *VHL* mutation/loss, a 3-hit model of tumorigenesis) (Schmidt et al., 1995; Bodmer et al., 1998; Kanayama et al., 2001). However, in other cases tumors may not show *VHL* inactivation, implicating a *VHL*-independent pathway of tumorigenesis (Valle et al., 2005). In our case molecular genetic analysis of tumor material for *VHL* mutations failed because of the poor quality DNA but immunostaining for CA-IX expression (Mandriota et al., 2002) showed no evidence of increased expression in the two RCC studied, providing indirect evidence for a possible *VHL*-independent mechanism for tumorigenesis.

Balanced translocations have been reported to produce long range (e.g., up to 1.3 Mb) “position effects” on gene expression (Crolla and Van Heyningen, 2002; Velagaleti et al., 2005). Hence genes in the vicinity of the translocation might be implicated in the pathogenesis of RCC. The known genes closest to the chromosome 3 and 6 breakpoints are *PPP2R3A*, *PCCB*, *STAG1*, *RNF184*, and *EPHB1* and *EPHA7*, respectively. *PCCB* encodes the beta subunit of propionyl-CoA carboxylase (germline *PCCB* mutations cause propionicacidemia) and we could find little evidence to implicate *PCCB* in tumorigenesis. *PPP2R3A* is a regulatory subunit of protein phosphatase 2A (PP2A), a heterotrimeric serine/threonine phosphatase (Hendrix et al., 1993). PP2A is a candidate TSG and inactivation of the regulatory PR65/A subunit occurs frequently in human tumors (Janssens et al., 2005). Hence, it can be hypothesized that the t(3;6) might lead to altered *PPP2R3A* expression and disturbances in PP2A function. Ephrins are the ligands for the Ephrin receptors that form a large family of receptor tyrosine kinases and play a key role in cell–cell interactions and cell migration. Ephrins and their receptors are frequently overexpressed in human cancers (Surawska et al., 2004). Interestingly, *EPHA7* was the closest known gene to the

chromosome 6 breakpoint, and *EPHB1* mapped ~500 kb from the chromosome 3 breakpoint. The *EPHB1* ephrin receptor interacts with the adaptor molecule Grb7 and regulates integrin-mediated attachment and migration (Han et al., 2002; Huynh-Do et al., 2002). *EPHA7* is highly expressed in the kidney vasculature and plays a key role in normal brain development by regulating apoptosis of neural progenitor cells (Hafner et al., 2004; Depaepe et al., 2005). These observations suggest that a possible relationship between RCC and *EPHB1* and *EPHA7* would merit further investigation. While our experimental studies were in progress, RNF184 (ring finger protein 184) was listed as an unannotated gene (*FLJ10546*); however, after these studies were completed it was attributed a putative role in ubiquitin ligase function. In view of the role of VHL in an E3 ubiquitin ligase complex, the possible role of RNF184 in renal tumorigenesis may also merit further investigation. Finally, the *STAG1* gene product (Stromal antigen-1, also known as SA-1), associates with the cohesin proteins, SMC1, SMC3 (CSPG6), and SCC1 (RAD21) to form one of the two characterized cohesin complexes in mammalian somatic cells. The cohesin complex has a key role in chromatid segregation and mitotic DNA replication (Uhlmann, 2004) and the STAG component of cohesin complexes may be implicated in transcriptional regulation (Lara-Pezzi et al., 2004). Subunits of the cohesin complexes have been linked to cancer susceptibility. Thus SMC1 is an effector of the ataxia telangiectasia mutated/Nijmegen breakage syndrome pathway and forms part of the DNA damage response network (Kim et al., 2002; Yazdi et al., 2002) and RAD21 expression is induced by BRCA1 (Atalay et al., 2002) and mutations in human RAD21 gene have been related with the radiosensitivity in some cancer patients (Severin and Leong, 2001). Thus, we suggest that further studies are indicated to evaluate the role of genes close to the translocation breakpoints (e.g., PPP2R3A, PCCB, STAG1, RNF184, *EPHB1*, and *EPHA7*) in the pathogenesis of familial and sporadic RCC and, possibly, other tumor types (Kemp et al., 2006).

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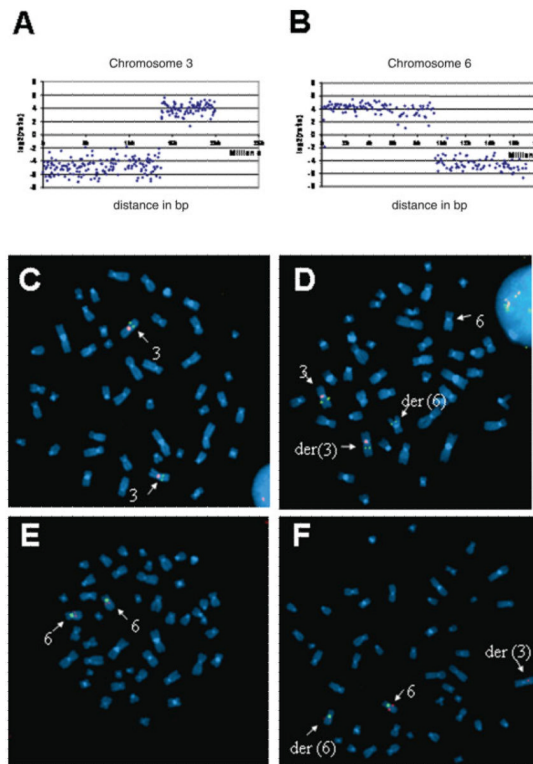


Figure 1.

Panels A and B: Array Painting analysis demonstrating changes in hybridization signals at translocation breakpoints on chromosomes 3 and 6. Panels C–F: metaphase spreads prepared from lymphoblast cell line established from normal control (C and E) and patient with t(3;6) translocation (D and F). C and D show chromosome 3 identified using chromosome 3 specific centromeric probe, labeled with spectrum-orange. Normal and t(3;6) spreads were hybridized with a spectrum-green labeled chromosome 3 BAC probe, RP11-354M3, that contained the breakpoint (as shown by the split signal). E and F show corresponding images for chromosome 6. The breakpoint mapped within RP11-44P5 as shown by the split signal. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

