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Molecular cloning of the papillary renal cell carcinoma-associated translocation (X;1)(p11;q21) breakpoint

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Abstract. A combination of Southern blot analysis on a panel of tumor-derived somatic cell hybrids and fluorescence in situ hybridization techniques was used to map YACs, cosmids and DNA markers from the Xp11.2 region relative to the X chromosome breakpoint of the renal cell carcinoma-associated t(X;1)(p11;q21). The position of the breakpoint could be determined as follows: Xcen–OATL2–DXS146–DXS255–SYP–t(X;1)–TFE3–OATL1–Xpter. Fluorescence in situ hybridization experiments using TFE3-containing YACs and cosmids revealed split signals indicating that the corresponding DNA inserts span the breakpoint region. Subsequent Southern blot analysis showed that a 2.3-kb *Eco*RI fragment which is present in all TFE3 cosmids identified, hybridizes to aberrant restriction fragments in three independent t(X;1)-positive renal cell carcinoma DNAs. The breakpoints in these tumors are not the same, but map within a region of approximately 6.5 kb. Through preparative gel electrophoresis an (X;1) chimaeric 4.4-kb *Eco*RI fragment could be isolated which encompasses the breakpoint region present on der(X). Preliminary characterization of this fragment revealed the presence of a 150-bp region with a strong homology to the 5′ end of the mouse TFE3 cDNA in the X-chromosome part, and a 48-bp segment in the chromosome 1-derived part identical to the 5′ end of a known EST (accession number R93849). These observations suggest that a fusion gene is formed between the two corresponding genes in t(X;1)(p11;q21)-positive papillary renal cell carcinomas.

Renal cell carcinomas (RCCs) form a very heterogeneous group of tumors, both on a histologic and a cytogenetic level. These tumors can be subdivided into clear cell type nonpapillary and chromophilic tumors with a papillary growth pattern, commonly referred to as papillary tumors. Whereas in nonpapillary carcinomas abnormalities involving the short arm of chromosome 3 prevail, these appear to be absent in papillary carcinomas. In contrast, recurrent numerical aberrations involving mainly chromosomes 7, 17 and the Y chromosome are detected (Kovacs et al., 1991; van den Berg et al., 1993), although some of these alterations may not be tumor-specific, since they also occur in the surrounding normal kidney tissue (Dal Cin et al., 1992; Elfving et al., 1995; van den Berg et al., 1996).

In addition, a t(X;1)(p11;q21) and variants thereof have repeatedly been described in a subset of papillary renal cell carcinomas (de Jong et al., 1986; Tomlinson et al., 1991; Meloni et al., 1993; Tonk et al., 1995; Dijkhuizen et al., 1995; Zhao et al., 1995; Hernandez-Marti et al., 1995). Despite the limited amount of data on the histologic details of these tumors, there is growing evidence that this translocation is specific for a subset of papillary RCCs, i.e. those chromophilic tumors showing some clear cell-like features, due to the deposition of fat and glycogen (Thoenes et al., 1986; Meloni et al., 1993; Tonk et al., 1995; Dijkhuizen et al., 1995). Since this translocation is sometimes the sole cytogenetic anomaly present (de Jong et al., 1986), the gene(s) involved in this translocation may play a crucial role in tumor development. Therefore, we set out to clone the t(X;1) via detailed mapping of the breakpoint within Xp11 and the subsequent isolation and characterization of a chimaeric genomic fragment.

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Materials and methods

Tumor cell lines and somatic cell hybrids

Two primary renal cell cultures (Cl89-12117 and Cl89-17872) and one primary tumor (REN11-TT) (Tonk et al., 1995), all male-derived, were used in this study. Cl89-12117 contains the t(X;1) as the only abnormality, whereas Cl89-17872 also shows numerical aberrations in addition to the t(X;1) (Meloni et al., 1993). The karyotype of REN11-TT reads as follows: 49,Y,t(X;1)(p11;q21),+der(X)t(X;1)(p11;q21),+5,–16,+17,+18. REN11-N represents the corresponding normal tissue. Cl89-12117-derived somatic cell hybrids were obtained as described before (Geurts van Kessel et al., 1983; Sinke et al., 1993). Briefly, after fusion of A3 or Wg3h hamster cell lines and the t(X;1)-positive Cl89-12117 renal carcinoma cells, a panel of somatic cell hybrids was isolated in which the reciprocal translocation chromosomes segregate. WgRe5 was chosen as the der(X) hybrid, A3Re3A as the der(1) hybrid (Sinke et al., 1993). As controls, two hybrid lines containing a normal X chromosome (578, Wieacker et al., 1984) or a normal chromosome 1 (GM13.139, Coriell Repository) as the only human constituent, and parental hamster (A3) and mouse (A9) cell lines were included. HL60 is a myeloid leukemia cell line. G1 is a Grawitz tumor from an unrelated patient, NK1 the corresponding normal tissue, and NK2 normal renal tissue from another unrelated patient with renal cancer.

DNA probes and Southern blot analysis

Probes were labeled by random priming and hybridized at 65 °C in 0.5 M sodium phosphate buffer, 1 mM EDTA, and 7 % SDS. Due to the presence of repetitive sequences, 14E6 and 4.4-kb probes were preannealed for 3–5 h at 65 °C in 0.12 M sodium phosphate buffer in the presence of 100–250 µg total human DNA or hybridime (HT Biotechnology). Washes were performed in 40 mM sodium phosphate, 0.1 % SDS. Hybridizations of the library filters were performed in the same manner. Washes were performed stepwise, starting with 100 mM phosphate, 0.5 % SDS, 1 mM EDTA which was decreased to 40 mM, 0.1 % SDS.

Genomic DNA was isolated using standard protocols by proteinase K, SDS treatment followed by phenol, chloroform extractions and ethanol precipitation. After digestion and size selection on an agarose gel, the DNA was blotted onto Hybond N-Plus (Amersham) or Genescreen Plus (Dupont) membranes.

FISH

YAC and cosmid probes were labeled with digoxigenin-11-dUTP (Boehringer Mannheim), and centromere probes (X-specific alphoid sequence probe pBamX5) with Cy3-dCTP (BDS/KIMTEC) using a nick translation kit (Life Technologies). After coprecipitation with a 50-fold excess of Cot-1 DNA and heating for 10 min at 80 °C, repetitive sequences present in the YACs were preannealed for 30 min at 37 °C in 7 µl of FDST (50 % deionized formamide, 10 % dextran sulphate, 2 × SSC, 1 % [v/v] Tween-20, pH 7.0). Chromosome slides were treated as described before (Dijkhuizen et al., 1995). After hybridization (50 % formamide, 2 × SSC) which was performed at 37 °C for at least 48 h, and washes (50 % formamide, 2 × SSC; 2 × SSC), immunochemical detection was carried out using FITC-conjugated sheep-antidigoxigenin (1:20; Boehringer Mannheim). The slides were mounted in anti-fade medium (1.4 % w/v diazobicyclo-(2,2,2)-octane (DABCO, Merck) containing DAPI (0.5 µg/ml, Sigma) for counterstaining of the chromosomes. Slides were analyzed under a Zeiss Axiophot epifluorescence microscope equipped with appropriate filters. Digital images were captured as described before (Dijkhuizen et al. 1995) using the processing software program BDS-image (Oncor).

Construction of the genomic slice library

100 µg of genomic DNA from the Cl89-12117 tumor cells was digested, divided over 10 slots, and run on a 1 % agarose gel. A vertical slice corresponding to one lane was blotted onto Hybond N-plus Membrane (Amersham, UK) and screened for the location of the breakpoint fragment. Three slices of 1.5, 2.5, and 1.5 mm were cut out of the gel, and the DNA was subsequently purified using standard methods. About 200 ng of this DNA was rerun on a gel and checked for the presence of the breakpoint fragment via Southern blot hybridizations. The DNA fragments were cloned in a lambda-ZAP vector (Stratagene) and packaged using Gigapack Gold packaging extract (Stratagene).

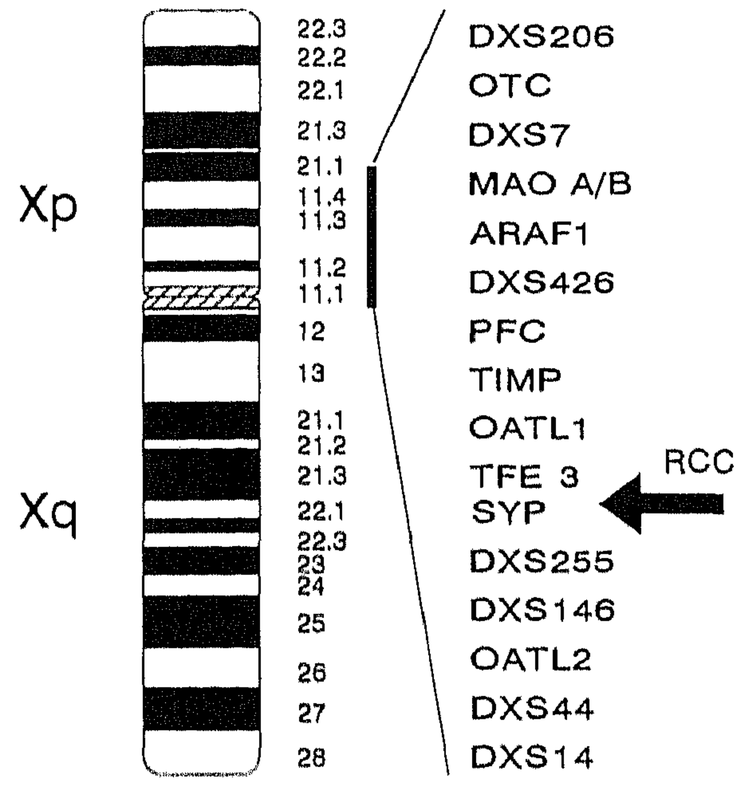

Fig. 1. Schematic map of the papillary RCC-associated t(X;1) region within Xp11. The position of the breakpoint is indicated by an arrow.

Sequence and computer analysis

DNA sequences were analyzed on an automated DNA sequencer (ABI 373A) using the Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems). Searches for homology were performed using the FASTA programs.

Results and discussion

Mapping of the X-chromosome breakpoint

Based on previous studies by us and other groups (Suijkerbuijk et al., 1993; Sinke et al., 1993; Dijkhuizen et al., 1995; Shipley et al., 1995), the papillary RCC-associated t(X;1) (p11.2;q21) breakpoint on the X chromosome was mapped in a region containing the ornithine aminotransferase pseudogene clusters OATL1 and OATL2. Initial FISH experiments showed a split signal in t(X;1)-positive tumor cells when using an OATL2 YAC as a probe (Suijkerbuijk et al., 1993). However, we found that this observation resulted from the presence of (low-)repetitive sequences on both sides of the breakpoint (Dijkhuizen et al., 1995; Shipley et al., 1995). By using Southern blot analysis on a panel of tumor-derived somatic cell hybrids and FISH on t(X;1)-positive tumor cells, we have mapped additional YACs from this genomic region relative to the Xp11 breakpoint. pTAK8/DXS146- and M27β/DXS255-positive YACs (Fig. 1) mapped to der(X), as well as YACs from a contig extending approximately 1 Mb telomeric to M27β (Fisher et al., 1995). Three different SYP-containing cosmids (SYP A, G, and H), all mapped to der(X) as determined by both FISH and Southern blot analysis. This observation was confirmed by the hybridization of a 7-kb genomic SYP fragment to der(X) as shown in Fig. 2A. In contrast, a 1.9-kb TFE3 cDNA clone that we isolated, designated cp13 (unpublished results), mapped to der(1) (Fig. 2B), indicating that the position of the breakpoint must be located between the latter two markers (Fig. 1). FISH data with TFE3 YACs (TFE3/2; 100 kb and TFE3/3; 240 kb; Fisher et al., 1995) were inconsistent. TFE3/2 mainly mapped to der(X), whereas TFE3/3 appeared to give a

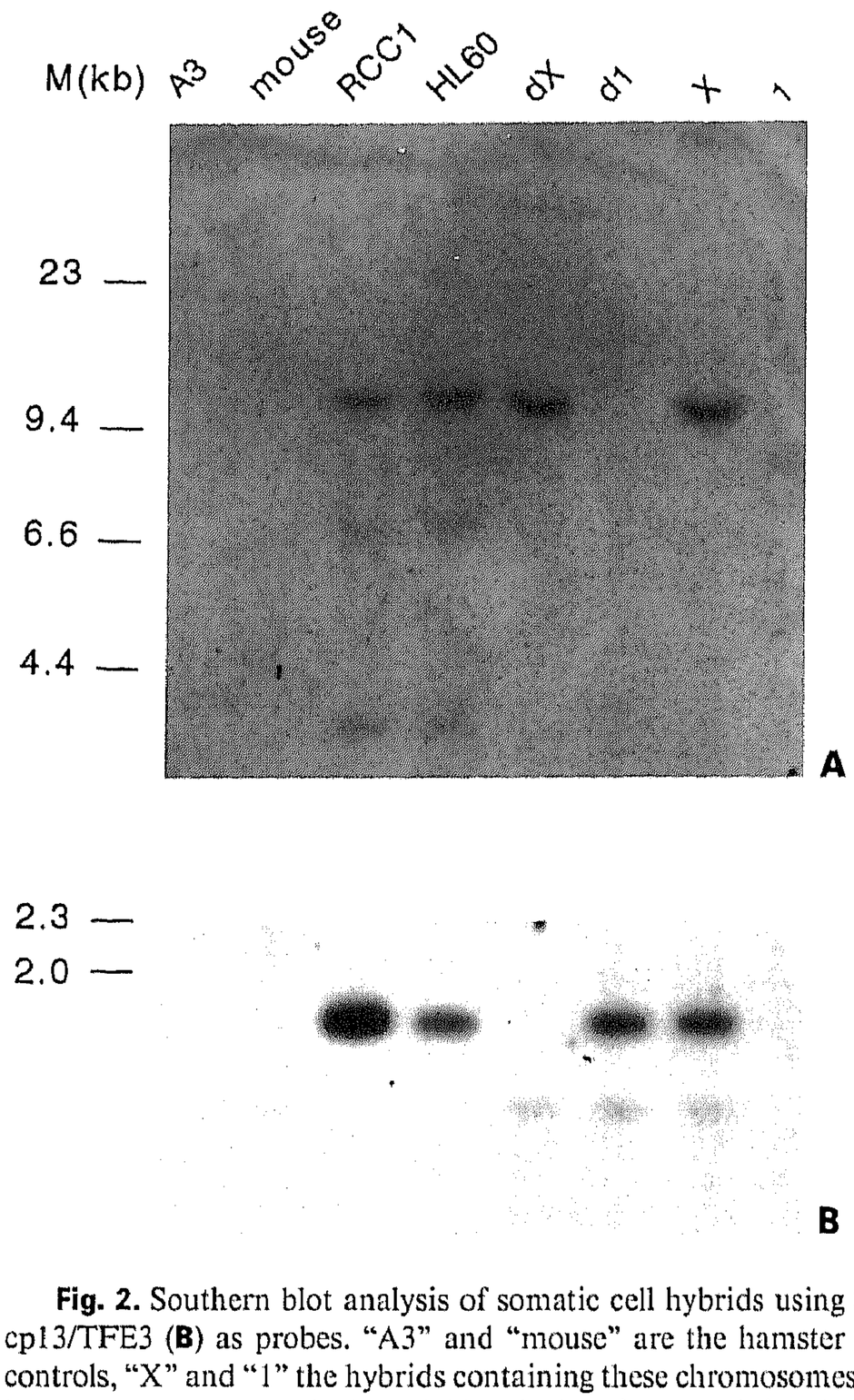

Fig. 2. Southern blot analysis of somatic cell hybrids using SYP (**A**) or cp13/TFE3 (**B**) as probes. "A3" and "mouse" are the hamster and mouse controls, "X" and "1" the hybrids containing these chromosomes as the only human component, and "d" and "d1" the hybrids containing the der(X) and der (1), respectively. RCC1 is the Cl89-12117 renal cancer cell containing the t(X;1). The myeloid leukemia cell line HL60 was used for a total human DNA control. Lambda *Hin*dIII DNA was used as a molecular size marker. The restriction enzymes used were: *Eco*RI/*Hin*dIII (**A**) and *Xba*I/*Hin*dIII (**B**).

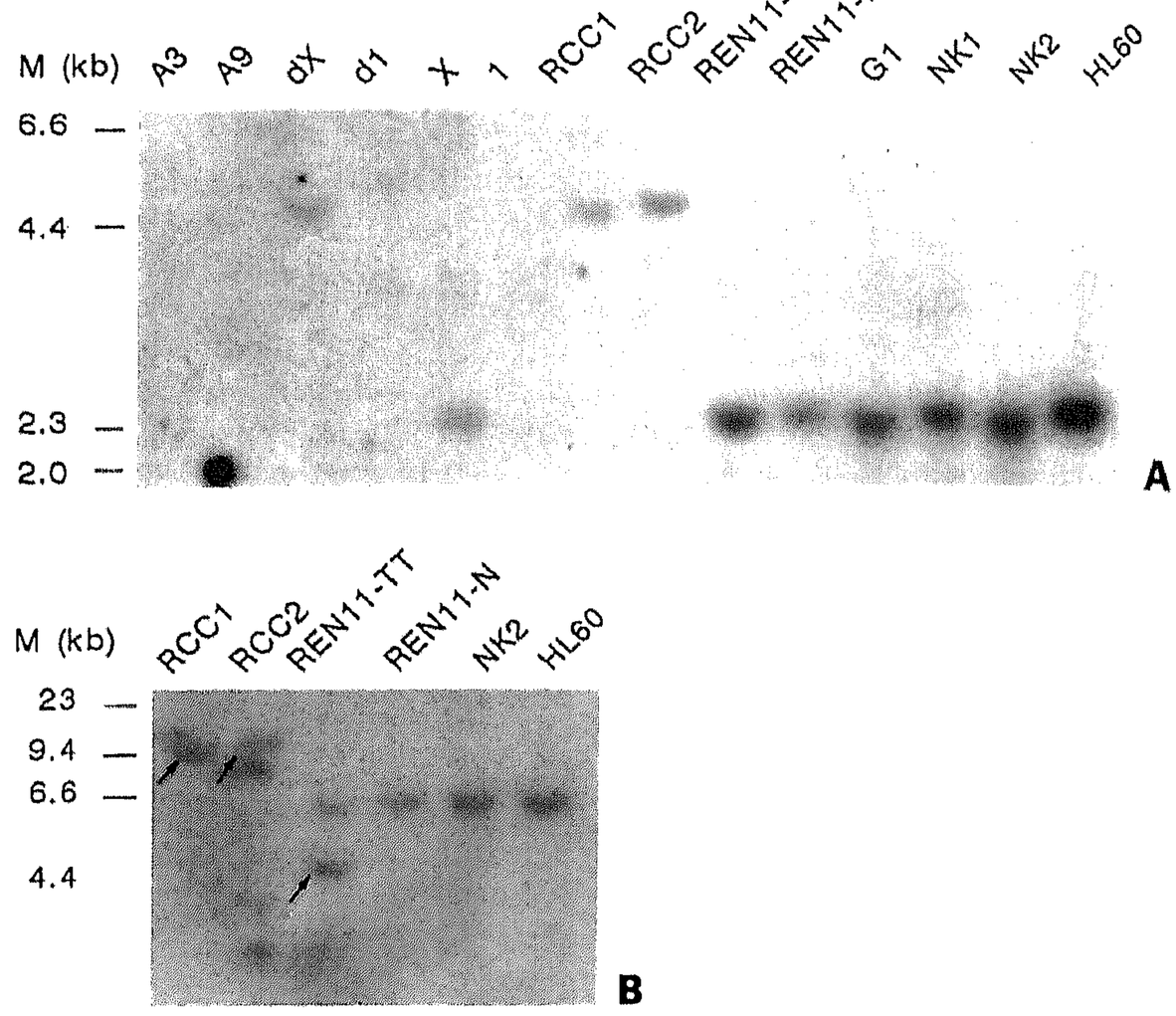

Fig. 3. Southern blot analysis using 14E6 as a probe showing aberrantly hybridizing bands in three t(X;1)-positive tumor DNAs. A3 and A9 are the parental rodent controls, "X" and "1" the hybrids containing these chromosomes as the only human component, "dX" and "d1" the hybrids containing the der(X) and der (1), respectively, RCC1 and RCC2 the Cl89-12117 and Cl89-17872 renal cancer cells containing the t(X;1), REN11-TT the third (X;1)-positive RCC, REN11-N normal kidney tissue from the same patient, G1 Grawitz tumor 1, NK normal kidney tissue, isolated from a patient with renal cancer, and HL60 a myeloid leukemia cell line. Genomic digests were (**A**) *Eco*RI, (**B**) *Bgl*II. Lambda *Hin*dIII was used as a molecular size marker.

split signal. However, the strength of the signals did not rise significantly above background level. Based on these preliminary results, an X-chromosome cosmid library (LL0XNC01) was screened with cp13 and several overlapping TFE3 cosmids were isolated. Subsequent FISH analysis using these cosmids as probes resulted in a pattern similar to that of the TFE3 YACs, i.e. some cosmids showed a split signal, indicating that these YACs and cosmids may indeed span the t(X;1) breakpoint region.

Cloning of the breakpoint fragment

Subclones of the TFE3 cosmids were used to screen Southern blots containing DNA from three independent t(X;1) positive tumors (Cl89-12117, C189-17872, REN11-TT) as well as Cl12117-derived somatic cell hybrids and controls. A 2.3-kb *Eco*RI fragment (14E6) was isolated which recognized aberrant *Eco*RI bands of 4.4 and 4.5 kb in the C189-12117 and Cl89-17872 tumor cells, respectively, (Fig. 3A). The corresponding aberrant 4.4-kb fragment was also present in the tumor-derived der(X) containing somatic cell hybrid. In tumor REN11-TT no aberrantly hybridizing *Eco*RI fragments were detected using this probe.

However, in other genomic digests (*Hin*dIII, *Bgl*II), an aberrant band was also detected in the third case, REN11-TT, next to the normal X-chromosome fragment which is also present in the X-only containing somatic cell hybrid and the corresponding normal tissue REN11-N, suggesting that some normal tissue must have been present in this primary tumor specimen (Fig. 3B). These results indicate that the Xp11 breakpoints in these three cases differ slightly, but all map within a genomic region of approximately 6.5 kb at maximum.

In order to clone the aberrant 4.4-kb *Eco*RI fragment, a preparative gel was made using *Eco*RI-digested genomic DNA from the Cl89-12117 tumor cells. A vertical slice of this gel was blotted onto nylon membrane and screened with 14E6 to locate the position of the 4.4-kb band. Three horizontal slices surrounding this location were cut out, followed by purification of the DNA. A small amount thereof was rerun on a gel, and checked for the presence of the 4.4-kb band by hybridization, after which a genomic slice library was constructed in an *Eco*RI-digested dephosphorylated lambda-ZAP vector (Stratagene). Screening of approximately 10^6 recombinant clones of this library with 14E6 yielded several positive plaques. After in vivo excision, plasmids were obtained containing the aberrant