Isolation of Kidney Complementary DNAs Down-expressed in Wilms’ Tumor by a Subtractive Hybridization Approach

Estelle Austruy, Martine Cohen-Salmon, Corinne Antignac, Christophe Béroud, Isabelle Henry, Nguyen Van Cong, Laurence Brugières, Claudine Junien, and Cécile Jeanpierre

INTRODUCTION

WT⁴ is an embryonal renal malignancy with complex genetic and pathological features. The tumor arises from metanephric blastema cells that would normally differentiate into components of the nephron. Typically, three types of structures are seen in various proportions in each tumor: undifferentiated blastema, epithelial structures (tubules and glomerules), and nonnephrogenic mesenchyme (stromal cells and muscle) (1).

Most Wilms’ tumor cases are sporadic and unilateral, with no associated abnormalities. Familial and bilateral tumors represent 1–2% and 7% of cases, respectively. Predisposition to Wilms’ tumor is also associated with several rare malformation syndromes which point to molecular predisposing germline defects.

The complex syndrome WAGR is associated with a constitutional deletion of region 11p13. The WT1 gene in 11p13 has been cloned (2, 3). This gene encodes a zinc finger polypeptide which binds to the same DNA sequences as EGR-1 (early growth response 1) and has a transcriptional repression function (4). WT1 is expressed in a limited set of tissues, including developing nephrons and gonads, and in most Wilms’ tumors, except those with a homozygous deletion of 11p13 (5). Germline mutations in WT1 have also been described in patients presenting with Drash syndrome (mesangial sclerosis, male pseudohermaproditism, and WT) (6).

WBS predisposes to different embryonic tumors: Wilms’ tumor, adrenocortical carcinoma, rhabdomyosarcoma, and hepatoblastoma, hence the acronym WT2 for this second locus (7). Constitutional duplication of 11p15.5 has been described in a few WBS patients. The involvement of a gene or genes in 11p15.5 has also been demonstrated by linkage studies in families with WBS (8, 9) and by evidence of paternal disomy of 11p15.5 in sporadic cases (10, 11).

A third locus, WT3, involved in rare familial forms, is not linked to either 11p13 or 11p15 and is as yet unmapped (12–14).

As expected from Knudson’s two-hit model (15), homozygous deletions of the region 11p13 have been observed in a few sporadic tumors. However, the two different 11p WT loci (WT1 and WT2) may also be involved sequentially in the same tumor (16, 17). By analysis of Wilms’ tumor material, loss of material from specific regions of chromosomes 1 and 16q was also revealed (18–22), which indicates that other genes could be involved through their inactivation during Wilms’ tumor development. Although one of these genes could be WT3, 16q markers were shown to be unlinked to familial predisposition in five families (23).

Subtractive hybridization has proved to be an efficient method for isolation of cDNA clones encoding transcripts with differential expression between two tissues (24–28). In order to isolate kidney cDNAs possibly involved in Wilms’ tumorigenesis through the absence of their expression, we cloned a cDNA population enriched in sequences transcribed in mature kidney and absent in a genetically well characterized Wilms’ tumor. Absence of transcription can result from either mutation or deletion in the gene itself (tumor suppressor gene) or from a negative regulation of expression (28, 29). We used tumor WAGR4 developed by a patient presenting with constitutional deletion of 11p13 and in which we had demonstrated loss of alleles of 11p15 markers (17). Six different clones selected for their differential hybridization pattern, including two new expressed sequences, were characterized. Possible correlations between the level of expression of these genes and either the etiological origin of a series of Wilms’ tumors or the loss of 11p alleles were investigated.

MATERIALS AND METHODS

Normal and Tumoral Tissues. Mature kidney was obtained from extended nephrectomy. Fetal kidney samples from fetuses between 27 and 31 weeks were obtained following therapeutic abortion and in compliance with French ethical legislation. Wilms’ tumor samples were obtained from several patients with sporadic tumors (SPO1, SPO2, SPO4, SPO6, SPO7, SPO8) or bilateral tumors (BILI, BIL2) and from patients with WAGR syndrome (WAGR4) or Wiedemann-Beckwith syndrome (BW11P, BW18P). Molecular characterization of constitutional and somatic rearrangements is described elsewhere (17).

Histology and stage of the tumors were obtained from the International Society of Pediatric Oncology. The main characteristics of the tumors are described in Table 1. The 18 other Wilms’ tumors used for DNA analysis will be described elsewhere.

Rat tissues were obtained from different organs: brain; heart; skeletal muscle; stomach; liver; intestine; tongue; lung; spleen; kidney; ovary;
### KIDNEY TUMORIGENESIS

#### Table 1 RNA expression in 11 Wilms' tumors of different etiological form and stage

<table>
<thead>
<tr>
<th>Type History&lt;sup&gt;a&lt;/sup&gt;</th>
<th>WAGR4&lt;sup&gt;b&lt;/sup&gt;</th>
<th>BW11P</th>
<th>BW18P</th>
<th>BIL1</th>
<th>BIL2</th>
<th>SPO1&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SPO4</th>
<th>SPO7</th>
<th>SPO2</th>
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<td>+</td>
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<td>-</td>
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<tr>
<td>CRY2 (T68)</td>
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<td>+</td>
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<td>-</td>
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<td>-</td>
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<td>+</td>
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<tr>
<td>DL15S87E (6.2)</td>
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<td>+</td>
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<td>DL15S199E (8.4)</td>
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</tbody>
</table>

<sup>a</sup> Nomenclature of the tumors. The tumors were named as follows: WAGR4, tumor from a patient presenting with WAGR syndrome; BW11P and BW18P, tumors from patients presenting with WBS; BIL1 and BIL2, tumors from bilateral cases; SPO1, SPO2, SPO4, SPO6, SPO7, and SPO8, sporadic tumors.

<sup>b</sup> Patient with ambiguous genitalia.

<sup>c</sup> Type and histology. Minor components are indicated in brackets.

<sup>e</sup> Stage. Definition according to the International Society of Pediatric Oncology nomenclature.

<sup>f</sup> Local stage.

<sup>g</sup> Expression. Northern blot analysis of the tumors with the cDNA probes. +, level of expression in the tumor similar to that in mature kidney; ( + ), level of expression in the tumor lower than that in mature kidney; (−), residual expression; −, no expression detected. For WT1, + indicates presence of the normal transcript.

### Screening of the Library

White colonies were picked in ordered array on nitrocellulose filters (HybBond-C, Amersham), grown, and replicated as described (24). Filters were hybridized in duplicate with the radiolabeled subtracted cDNA probe. The A1 primer 517 was labeled by kinase and used as a control probe (32).

### Polymerase Chain Reaction Amplification of Inserts

Colonies were resuspended in 20 µl of water and boiled for 5 min. Five µl of supernatant were used in the amplification reaction (33). The primers were chosen in the pUC18 sequence, on each side of the cloning site, using the OLIGOTEST program (34). Two 21-mer primers, 111 base pairs apart, were used:

- 5’-CGTTGTAACCGCAGGCCAGT-3’ (direct primer)
- 5’-CACACAGGAAACAGCTATGAC-3’ (reverse primer)

Polymerase chain reaction products were run on 1.2% agarose gels. Inserts were recovered from the gel by phenol extraction.

### RNA and DNA Blot Analysis

RNA analysis was performed with 2 µg of polyA* RNA or 10 µg of total RNA (35). The RNAs were transferred onto "Hybond-C extra" membranes (Amersham). Hybridization was carried out as previously reported (35). All of the filters were hybridized with HPRT or GAPD as control probes to check for the integrity and amount of RNA in each lane (36, 37). WT13 is a cDNA probe homologous to the WT1 transcript, which was kindly provided by D. A. Haber (2).

Southern blot experiments were carried out as described previously (38).

### DNA Sequencing

Plasmid DNA was prepared by the alkaline lysis method followed by precipitation with polyethylene glycol (39). Templates were denatured with 0.2 N NaOH, neutralized with 0.5 M ammonium acetate (pH 4.6), and ethanol-precipitated. Sequencing was performed with the Sequenase kit (USB). Primers were either the universal M13 primer (−40) or the reverse primer used for polymerase chain reaction. Reaction products were run on 8% acrylamide, 7 M urea, 40-cm gels. Sequences obtained were compared to sequences from the GENBANK, EMBL, and NBRF databases, with the French BISANCE service (40).

### RESULTS

**Isolation of Differential Clones.** Of 680 ng of total mature kidney cDNA, 20% was recovered after subtraction with WAGR4 mRNA and cloned. The percentage of recombinants among white colonies was estimated at 30%, with a total number of recombinant clones of 28,000. The size of the inserts ranged between 100 and 700 base pairs.
Two different approaches were used to isolate differentially expressed clones, i.e., positive with mature kidney RNA and negative with WAGR4 RNA. In the first experiment, 18 randomly chosen inserts were analyzed by Northern blot. Three clones, T21, T68, and T70, which detected transcripts of 1.8, 1.7, and 1.0 kilobases, respectively, in mature kidney, hardly detected any signal with the WAGR4 RNA used for subtraction (Fig. 1A). Probe T70 also revealed a minor transcript of 2.9 kilobases in mature kidney (data not shown). In each case, the differential pattern of hybridization was confirmed by hybridization of the filter with HPRT (Fig. 1A). In the second experiment, we screened 400 white colonies with the subtracted cDNA probe and with the Alu probe 517. None of the 30 clones positive for the subtracted cDNA probe hybridized with the Alu probe, providing indirect evidence of the efficiency of the subtraction. Northern blot analysis confirmed a differential pattern of expression for clones 5.3, 6.2, 8.4, and 28. These clones detected mRNAs of 2.4, 0.7, 2.6, and 1.7 kilobases, respectively (Fig. 1B).

Identification of DPEP1, CRYA2, UMOD, and GPX2. A complete sequence was obtained for clone T70 (199 base pairs), clone T68 (158 base pairs), and clone 28 (139 base pairs) and confirmed by reverse sequencing. Partial sequences of 169 base pairs and 206 base pairs were obtained for clone T21 (600 base pairs) and clone 5.3 (400 base pairs), respectively. These sequences were compared with data from Genbank and EMBL databases. The partial sequence of clone T21 showed 98.8% homology with the human microsomal dipeptidase coding sequence (DPEP1) (41). Homology of 98.9% was obtained between clone T70 and the first exon of the human αB-crystallin gene (CRYA2) (42). The partial sequence of clone 5.3 showed 99.0% homology with the 3' end of the coding sequence and part of the 3' noncoding sequence of human uromodulin (UMOD) mRNA (43). Uromodulin is also known as the Tamm-Horsfall glycoprotein. Clone T68 and clone 28 were overlapping clones presenting 96.8% and 97.8% homology, respectively, with the 3' noncoding sequence of the human plasma glutathione peroxidase cDNA (GPX2) (44). Only clone T68 was used for subsequent analysis.

Expression in different tissues was investigated by Northern blot analysis (Table 2). For comparison of levels of expression in human fetal and mature kidney, Northern blots were successively hybridized with the clones and with GAPD as a control probe (Fig. 2). The cDNAs were hybridized on Southern blots with DNA from panels of somatic cell hybrids for chromosomal localization. Expri-
DISCUSSION

In order to detect kidney cDNAs down-expressed in Wilms' tumor, we used a subtractive hybridization technique. This approach was based on differential RNA expression between mature kidney and Wilms' tumor. It thus applied only to genes for which mRNA transcripts would be absent or severely reduced in the tumor, not to genes for which point mutations do not hinder transcription of mRNA. Since genetic and molecular studies on Wilms' tumor support the hypothesis that this childhood cancer arises from a variety of etiological pathways, we chose to use a single-well genetically characterized tumor for subtraction.

Six clones presenting a differential hybridization pattern with mRNA from mature kidney and WAGR4 were characterized. Four clones were identified as sequences from known genes DPEPI (clone T21), CRYA2 (clone T70), UMOD (clone 5.3), and GPX2 (clone T68). Mapping data and/or tissue-specific expression analysis using these clones were in agreement with published data for DPEPI, CRYA2, and UMOD (41-43, 47-49). Our data demonstrate that the gene coding for GPX2 maps to chromosome 5q33-qter and that it is expressed in various tissues and not restricted to placenta (44). Clone 6.2 (D11S877E) and clone 8.4 (D15S109E) correspond to new genes.

In all tumors, the expression of WT1, analyzed with probe WT33, was maintained, even in tumor WAGR4 with a constitutional deletion of 11p13 (data not shown).

Loss of Heterozygosity at the DPEPI Locus. Using a BamHI variable-number tandem repeat polymorphism detected by DPEPI (45), we demonstrated loss of allele in tumors BILI and SPO7 at locus 16q24.3 (data not shown). Analysis of 18 other Wilms' tumors was performed in order to estimate more precisely the frequency of loss of heterozygosity. A total of 5 tumors of 21 informative ones (24%) showed a loss of allele (data not shown). In order to check whether DPEPI could be directly implicated, we performed Southern blots with restriction enzymes allowing detection of genomic fragments larger than 10 kilobases. Analysis of 21 Wilms' tumors, including the same 11 tumors described here and 10 other tumors, failed to reveal DNA rearrangements of the genomic sequences homologous to DPEPI or to the other clones (data not shown).

ments concerning regional mapping of these clones are described elsewhere (38, 45, 46), and the results are summarized in Table 2.

Isolation of Two New Genes. No homology was found between sequences from clones 6.2 and 8.4 and any of the nucleotide sequences of the Genbank and EMBL databases or with the protein sequences of the NBRF database. A complete sequence of 184 base pairs was obtained for clone 6.2, with an open reading frame extending over the whole length (Fig. 3A). This clone detects a 0.7-kilobase transcript in adult kidney and pancreas and a band of lower intensity in liver and fetal kidney (Table 2; Fig. 2). Clone 6.2 (D11S877E) has been mapped to chromosome 11 in subregion 11q22.3–24.3 (Table 2) (38).

A partial sequence of 226 base pairs was obtained for clone 8.4 (400 base pairs). The longer open reading frame covered 174 base pairs (Fig. 3B). It detects a 2.4-kilobase transcript in adult kidney, brain, and testis. No expression in fetal kidney was observed (Table 2; Fig. 2). Clone 8.4 (D15S109E) was mapped to chromosome 15 (Table 2).

Decreased Expression in Wilms' Tumors. The clones were hybridized with RNA from 11 Wilms' tumors of different etiological origin and stage (Table 1; Fig. 2). Four tumors, WAG4, BILI, SPO1, and SPO4, showed an absence of expression for all six markers as well as the loss of 11p alleles. Conversely, transcription of the cDNA sequences was normal or only slightly reduced in the tumors from the two WBS patients, i.e., BW11P and BW11P. For tumors BILI, SPO2, SPO6, SPO7, and SPO8, our results appeared more heterogeneous.

<table>
<thead>
<tr>
<th>Clone symbol</th>
<th>Sequence homology</th>
<th>Locus symbol</th>
<th>Expression</th>
<th>Polymorphism</th>
<th>Assignment</th>
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<tr>
<td>T21</td>
<td>Microsomal dipeptidase</td>
<td>DPEPI</td>
<td>mK, In, Lu, Ox. (fK)</td>
<td>BamHI 16q24.3</td>
<td>16p13.11</td>
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<td>6.2</td>
<td>aß-Crystallin</td>
<td>CRYA2</td>
<td>mK. fK, Mu, He, To, (Br, Lu, St)</td>
<td>5q33-qter</td>
<td>11q22.1-23.2</td>
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<tr>
<td>8.4</td>
<td>?</td>
<td>D11S877E</td>
<td>mK, Pa (fK, Li)</td>
<td>11q23.2-24.2</td>
<td>15</td>
</tr>
</tbody>
</table>

*The abbreviations for the tissues studied are as follows: mK, mature kidney; fK, fetal kidney; Br, brain; He, heart; In, intestine; Lu, lung; Mu, muscle; Ox, ovary; Pa, pancreas. Tissues in parentheses indicate a lower expression (detected following 1 week of exposure).
KIDNEY TUMORIGENESIS

(A) clone 6.2 (D11S877E)

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<td>10</td>
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<tr>
<td>20</td>
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<td>30</td>
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<td>40</td>
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<tr>
<td>60</td>
<td>GlyYrleGluSerGlyAlaSerPheSerProSerAlaGlySerAspGlyAspLysGly</td>
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(B) clone 8.4 (D15S109E)

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<th>Amino Acid</th>
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<tr>
<td>120</td>
<td>GCCAAATAGTTAAATACCTTAAGAGTAGAGATAATACGTAGAAGTTATTTTCCAAG</td>
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</table>

Fig. 3. DNA sequence analysis of the isolated recombinants. A, total sequence of clone 6.2 (D11S877E); the predicted amino acid sequence is displayed below the nucleotide sequence. B, partial sequence of clone 8.4 (D15S109E); the part of the sequence corresponding to the largest putative open reading frame is underlined. The sequences have been deposited with EMBL accession numbers X65705 (D11S877E) and X65706 (D15S109E).

Patient SPO4 had a late onset tumor and no malformation suggestive of genetic predisposition. Interestingly, somatic loss of alleles for 11p15 markers around the WT2 locus was observed in these four tumors, suggesting a common progression mechanism. Conversely, in tumors from two patients with WBS, thus involving WT2 as a predisposing locus, expression was unaltered or only slightly reduced. These differences between the two categories of tumors could reflect pathogenetic heterogeneity of Wilms' tumor (52). This is supported by the proposal of two distinct categories of Wilms' tumor precursors: the intralobar nephrogenic rests associated with the WAGR and Drash syndromes and the perilobar nephrogenic rests commonly found with hemihypertrophy and WBS. Our results suggest that different genes could be involved in tumor progression for these two types of tumors. However, more tumors, whether or not they are hereditary, should be analyzed to confirm this hypothesis. Furthermore, no correlation between absence of expression of the six genes and stage of the tumors can yet be proposed from our study.

Two clones, DPEP1 (clone T21) and UMOD (clone 5.3), map on chromosome 16. Cytogenetic analysis has shown that this chromosome is frequently deleted in Wilms' tumors (18-21). Accordingly, we have demonstrated loss of alleles in 24% of the tumors with probe T21 (DPEP1), which maps precisely to 16q24.3. Losses of alleles in the 16q region have also been described by other authors in Wilms' tumor (22), as well as in hepatocellular, prostatic, and breast carcinomas (53). Therefore, the long arm of chromosome 16 most likely carries a tumor suppressor gene(s) involved in these different tumors.

The genes identified in this study are probably not classical tumor suppressor genes. Although their mRNAs are absent, Southern blots showed that these genes are present and not grossly rearranged in tumor cells. Different hypotheses can account for the absence of expression of these sequences in tumors. As a first hypothesis, if a gene participates in several mechanisms used by the organism to fight actively against tumor cells, its absence of expression can be related to tumor progression. Microsomal dipeptidase (DPEP1) is a zinc metalloprotease implicated in the metabolism of glutathione (54), which is known to be involved in the tumorigenesis process. Although the role of αB-crystallin (CRYA2) in kidney is not known, this protein is a small heat shock protein which might be involved in the response...
to cellular stress and could help the elimination of nascent tumor cells (55, 56). Uromodulin (UMOD) is an immunosuppressive glycoprotein which regulates the circulating activity of a number of potent cytokines, including interleukin 1 and tumor necrosis factor (43). Decreased expression of UMOD may result in uncontrolled levels of interleukin 1, tumor necrosis factor, and other lymphokines involved in cell communications and activation of protein kinases (57). Glutathione peroxidase (GPX2) is an enzyme involved in cellular detoxification, and its absence of expression might allow an increased number of mutations caused by peroxides to occur (58). The molecular and functional characterization of the two new cDNAs is in progress. The possibility that these genes might be regulated, directly or indirectly, by tumor suppressor genes like WT1 or WT2, has to be investigated. Since WT1 is significantly expressed in all tumors, it might be worthwhile to search for specific early growth response-like binding sites in their promoter sequences.

As a second hypothesis, the absence of expression of the isolated genes may reflect the absence of kidney structures specifically expressing them. DPEPI, CRYA2, and UMOD expression is limited to specific structures of the differentiated nephron. Microsomal dipeptidase (DPEPI) is located in the kidney microvillus membranes (54). CRYA2 expression in kidney is limited to specific segments of tubules, Henle’s loops, and collecting ducts (48). Kidney-specific expression of UMOD is restricted to the thick ascending limb of Henle’s loop and the early distal convoluted tubule (43). However, the pattern of expression of these genes in the different structures of fetal kidney remains unknown. The expression of GPX2 and of the two new genes in normal mature and fetal kidney structures has also to be analyzed by in situ mRNA hybridization experiments. Through in situ hybridization or quantitative RNA analysis, these different genes could be helpful for the histological characterization of tumors and hence for prognosis.

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REFERENCES

KIDNEY TUMORIGENESIS


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