Abnormal RNA Expression of 11p15 Imprinted Genes and Kidney Developmental Genes in Wilms’ Tumor

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Abstract

Wilms’ tumor (WT) is caused by abnormal development of embryonal kidney cells. WT cells are frequently affected by deletions or functional inactivation of maternal alleles at chromosome 11p15, which indicates that the loss of maternally expressed genes in this region plays an important role in WT pathogenesis. Maternally expressed genes indeed exist within an imprinted region at 11p15.5. Among these, BWR1C is highly expressed in fetuses but not in adult kidney, which suggests that it may fulfill an important role in kidney development. Here, we demonstrate that the lack of BWR1C expression is common in WT. Its homology with the proto-oncogene TDAG51 suggests that the loss of BWR1C expression may be relevant in WT development. In addition, the analysis of the expression of other 11p15 imprinted genes and kidney-developmentally regulated genes indicates that IGFI overexpression, inappropriate coexpression of RET and GDNF and, in some cases, down-regulation of CDKN1C may also play an important role in the pathogenesis of WT. Our results add new elements to the understanding of the biological basis of WT, which may have implications for WT diagnosis and therapy.

Introduction

WT is an example of altered development of the kidney (1). An understanding of normal kidney development seems to be essential to define the molecular basis underlying its development. In humans, kidney forms through the reciprocal epithelial-mesenchymal induction of two tissues, the ureteric bud and the metanephric mesenchyme. The metanephric mesenchyme induces the ureteric bud (which will form the collecting ducts) to branch, whereas, in response to signals from the bud, the mesenchymal cells are induced to condense around the branching buds and differentiate into the epithelial cells that form the proximal and distal tubules as well as the glomeruli of the nephron. Permanent kidneys begin to develop at day 35–37 in human embryos, and nephrogenesis is complete at approximately 36 weeks (9 months) of gestation.

WT is one of the most common pediatric solid tumors. It affects 1 in 10,000 children, usually during the first 5 years of life (2), and seems to arise from persistent clusters of mesenchymal cells (metanephric blastema), a tissue at the junction between proliferative mesenchyme and nonproliferative epithelium.

Various genes have been identified to play a role in kidney development (3), including WT1, PAX2, RET, GDNF. Among these genes, only WT1 has been directly involved in the development of WT by mutation detection (4, 5). WT1 encodes a zinc-finger (Kruppel-type) transcription factor that plays a key role in the development of kidney and gonads (6), which indicates that abnormalities in kidney development could indeed promote tumorigenesis. Despite these findings, only 10% of WTs arise as a consequence of mutations in the WT gene, which indicates that other genes are also involved in the development of this pediatric tumor.

Genetic studies indicated that WT occurs with high frequency in four different syndromes: (a) the WAGR (Wilms’, aniridia, genitourinary abnormalities, mental retardation) syndrome, (b) the Denys-Drash syndrome, (c) the Beckwith-Wiedemann syndrome, and (d) the Perlman syndrome. The association of these syndromes with various loci on the short arm of chromosome 11 indicates that at least two genes, one at 11p13 (where the WT1 gene maps) and another at 11p15, are involved in the development of WT.

The role of a gene at 11p15 in the pathogenesis of WT is also supported by the detection of LOH at this chromosomal region in about 30–50% of WTs. Furthermore, preferential loss of maternal alleles indicates that the involved gene or genes at 11p15 are imprinted with maternal expression. A number of imprinted genes with maternal expression have been identified at 11p15: (a) BWR1A (also known as ORC1T2, IMP1, ITM, and TSSC5); (b) BWR1C (also known as IGF2, RET, and GDNF); and (c) CDKN1C (7–12). Their features and some suggestive experimental evidences suggest that these genes may be involved in WT. CDKN1C, which encodes the cyclin-dependent kinase inhibitor p57KIP2, was found mutated in some cases of Beckwith-Wiedemann syndrome (13, 14), an excessive-growth genetic disorder that predisposes to WT. However, to date, no CDKN1C mutation has yet been found in WT (15, 16). In addition, mutant mice lacking this gene, although they display various proliferation and differentiation defects including renal medullary dysplasia (17), do not develop tumors. Nevertheless, a reduction of the level of CDKN1C RNA expression was observed in some WTs (18, 19), which indicates a role for this gene in some WTs by a mechanism of down-regulation. BWR1A was shown to carry mutations in some human neoplasms, including breast, lung, rhabdomyosarcoma, and WT, which suggests a role in human tumorigenesis (9, 20). However, these alterations were found at a very low frequency, which indicates that alterations in this gene cannot explain all of the tumor cases characterized by LOH at 11p15. BWR1C expression is detectable only in placenta and in fetal kidney but not in adult kidney nor in other adult tissues. Expression location and timing suggest that BWR1C plays an important role in kidney development. Because BWR1C shares a strong homology with TDAG51 (12), a pro-apoptotic protein involved in the transduction of the FAS signal (21), it has been suggested that BWR1C may transmit intracellular apoptotic signals during kidney organogenesis in response to an, as

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3 The abbreviations used are: WT, Wilms’ tumor; LOH, loss of heterozygosity; LOI, loss of imprinting.
yet, unknown stimulus. Failure of this function could allow survival of cells that should be otherwise eliminated.

Here, we report the analysis of a panel of WTs for the expression of various genes involved in kidney development and of imprinted genes at 11p15 to verify whether any of them is inappropriately expressed in tumor cells.

Materials and Methods

DNA and RNA Samples. In the course of the study, two cell lines were used, one (SK-NEP-2) derived from a WT, the other (G401) derived from a rhabdoid tumor of the kidney. In addition, we analyzed 17 primary WTs and 7 normal tissues: adult lung, kidney, brain, skeletal muscle, bladder, fetal kidney, and placenta. Tumor cell lines were obtained from the American Type Culture Collection, 7 WTs were obtained from A. Angioni (Ospedale Pediatrico del Bambino Gesù, Rome), the other 10 WTs and fetal kidney cDNA from I. Hatada (Gunma University, Maebashi, Japan). All of the normal adult tissues were obtained from the S. Anna Hospital in Ferrara. Genomic DNA and RNA were purified from cell pellets and tissues as described previously (9).

Synthesis of single-strand cDNA was obtained from 1 μg of total RNA with the use of the MoMuLV reverse transcriptase Superscript II (Life Sciences), in the dye terminator chemistry. PCR reactions were carried out with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 7–12.5% glycerol with 1 μM each primer, 50 μM each dNTP, and 50 ng of genomic DNA or 0.5–1 μl of cDNA in a 10-μl reaction containing both oligodeoxythymidylate and random primers for priming the reaction.

Oligonucleotides and PCR Detection of Gene Expression. PCR reactions were carried out with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 7–12.5% glycerol with 1 μM each primer, 50 μM each dNTP, and 50 ng of genomic DNA or 0.5–1 μl of cDNA in a 10-μl volume by using 0.5 units of Taq Polymerase (Perkin-Elmer-Cetus). PCR amplification was carried out with a denaturing step at 94°C for 15 s, annealing for 30 s, and extension at 72°C. Primers, number of PCR cycles, annealing temperatures, and extension times are reported in Table 1. For the nested PCR reactions, we used 1-μl aliquots of 1:50 dilution of the primary PCR reaction. PCR products were electrophoresed on 1.8% metaphor agarose gels. Sequence analysis was carried out using both oligodeoxynucleotidylate and random primers for priming the reaction.

Methylation Analysis. The methylation status of BWR1C was studied by Southern blot analysis (22) of HindIII-digested DNA using two DNA probes spanning the promoter and the transcribed region of the gene. To prepare the use of the MoMuLV reverse transcriptase Superscript II (Life Sciences), in the dye terminator chemistry. electrophoresed on 1.8% metaphor agarose gels. Sequence analysis was carried out in an automated Applied Biosystem sequencing station model 377, using the dye terminator chemistry.

Methylation Analysis. The methylation status of BWR1C was studied by Southern blot analysis (22) of HindIII-digested DNA using two DNA probes spanning the promoter and the transcribed region of the gene. To prepare the probes used in the Southern blot analysis, primer pairs Fe-24F (5′-ATGGC-CGGCTTCCCCAGC-3′)/395EST1 (5′-TGGGACAGCTGAAGTAGTAG-3′) and 395-Ex24F (5′-GGAGTCTCAGTGGCCACA-3′)/S648EST1R (5′-CTCGGCAACATGAAACT-3′) were used to amplify, respectively, the promoter region (probe A) and the transcribed region (probe B) of the BWR1C gene.

Results and Discussion

Reverse transcript PCR expression analysis for various genes (Fig. 1) was performed in fetal and adult kidney and in WT samples. Some of the genes, such as RET, GDNF, WT1, and PAX2, were studied because they are involved in kidney development. E-cadherin and N-CAM were analyzed because they are specifically expressed in epithelial or mesenchymal cells, respectively, thus defining a critical stage, that is, the transition from one to the other cell type in nephrogenesis. Four additional genes, CDKN1C, BWR1A, BWR1C, and IGFB2, were studied because they map at 11p15 and are imprinted. Seven WTs were analyzed for all of the genes. Three of these WTs (WT1, WT6, and WT8) exhibited loss of constitutive heterozygosity at 11p15 loci (data not shown). The cDNA from 10 additional WTs were analyzed for BWR1C and BWR1A (data not shown). Only two of the studied genes revealed expression restricted to fetal tissue: RET and BWR1C.

Among kidney developmental genes, the expression of RET and GDNF in all of the WTs was notable. It is, in fact, established that RET, a receptor tyrosine kinase, transduces an intracellular signal from GDNF, the glial-derived growth factor, a soluble extracellular factor member of the transforming growth factor β family (23, 24). Knockout mice studies indicate that the absence of RET or GDNF produce similar phenotypes, which include the absence of kidney and enteric neurons (25, 26). In fact, in the developing kidney, ureteric buds express RET and expand into the metanephric blastema in response to GDNF, which is expressed by blastema cells, which partially explains the molecular basis of the reciprocal epithelial-mesenchymal induction of the two tissues. In the absence of either RET or GDNF, the kidney does not develop. Here, we found that RET, similarly to fetal but not adult kidney, was expressed in all of the WTs, and GDNF was expressed in both fetal and adult kidney, but mRNA splicing (27, 28) in WTs showed the fetal splicing pattern. The specificity of the various splicing products was confirmed by nucleo-

<table>
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<tr>
<th>Gene</th>
<th>PCR</th>
<th>Name</th>
<th>Sequence</th>
<th>Glycerol (%</th>
<th>Annealing Temperature (°C)</th>
<th>Extension (sec)</th>
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<td>12.5</td>
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<tr>
<td>CDKN1C</td>
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<td>HKP2 1101F</td>
<td>5′-AGATCTACGCTGAAGTGAAAC-3′</td>
<td>12.5</td>
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<tr>
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<td>61</td>
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<tr>
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<tr>
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<td>NCAM-1g1F</td>
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<td>GDNF</td>
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<td>7</td>
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<td>50</td>
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Table 1 Oligonucleotide pairs used in reverse transcription PCR experiments
**ABNORMAL GENE EXPRESSION IN WTS**

![Graphs](image)

**Fig. 1. Detection of expression of various genes in WT samples (W1–W8). cDNA was prepared and specific gene segments amplified as indicated in “Materials and Methods.” B, blank; DNA, genomic DNA; AK, adult kidney; FK, fetal kidney; Lu, adult lung; Br, adult brain; Pl, placenta. Genomic DNA was present in the PCR reaction when possible amplification of contaminating DNA was suspected in the analyzed cDNA. Specificity of PCR products was confirmed by nucleotide sequencing.**

Although the expression of high levels of RET in WTs is apparently not abnormal, because fetal kidney also shows a high level of RET expression, RET expression in the developing kidney is normally limited to the tip of the ureteric bud and is not present in the metanephric blastema tissue from which WT originates. Hence, RET expression in WT cells may not be legitimate, and the simultaneous expression of its ligand GDNF may produce a growth signal. Indeed, RET activates the RAS mitogen-activated protein kinase pathway (29, 30), and mitogenesis requires Src or Src-related kinase activity (31). Constitutive expression of RET in the presence of its natural ligand GDNF may, therefore, maintain an autocrine mitogenic and/or survival loop that promotes the abnormal growth of WT cells. The finding that RET and GDNF are coexpressed in the WT cell line SK-NEP-2 but not in the kidney rhabdoid tumor cell line G401, which expresses low level of RET and does not express GDNF (Fig. 2), suggests that the same cell can express both RET and GDNF. The role of RET in human tumorigenesis is well established as a rearranged dominant transforming oncogene (32). Our finding adds a novel mechanism to maintain the RET receptor kinase constitutively active, which may be a pathogenetic event in WT.

The expression of the other two developmental-specific genes, WT1 and PAX2 was detected in all of the WTs at levels comparable with that detected in adult and fetal kidney (Fig. 1). We have not analyzed these genes further for the presence of mutations, which are known to affect the WT1 gene in about 10% of WTs.

Furthermore, the epithelial and mesenchymal-specific genes E-cadherin and N-CAM did not show any change in expression level by comparing adult kidney, fetal kidney, and WTs (Fig. 1). Although, during nephrogenesis, a change in expression from N-CAM to E-cadherin adhesion molecules occurs, reflecting the transition from mesenchymal to epithelial cells, this transition may not be detectable in Wilms’ primary tumors, which consist of various cell types, including normal nonneoplastic cells. Analysis of N-CAM and E-cadherin expression of the SK-NEP-2 cell line indicates that WT cells maintain the characteristics of proliferating mesenchymal cells (Fig. 2).

Analysis of the expression of various 11p15 imprinted genes gave heterogeneous results. BWAR1A expression was similar in all of the normal and tumor samples analyzed, and we found no mutations in 17 WT samples (data not shown). These data, together with the finding of a mutation in BWAR1A previously reported in only a single WT in 48 samples (20), indicate that BWAR1A is unlikely to be involved in a large fraction of WTs.

At variance from the all of the previous genes, which displayed a homogeneous pattern of expression in all of the WTs, a heterogeneous situation was observed for CDKN1C. In general, we found no significant differences in the level of expression in adult and fetal kidney nor in most WTs, with the exception of case W3 that displayed a marked reduction of expression. Reduction of CDKN1C RNA was previously detected in some WTs (18, 19). This suggests that the abnormal low expression of CDKN1C may be relevant in some, but not all, WTs. In fact, WTs generally express a readily detectable level of CDKN1C RNA (18, 33), and no inactivating mutations of this gene have yet been found in WTs (16).

**BWAR1C** is an exception to the rule that WTs display a fetal pattern of expression. In fact, although BWAR1C showed a pattern of expression restricted to fetal kidney, remarkably, none of the WTs analyzed showed any detectable expression of this gene, which suggests that the lack of expression of BWAR1C, a promoter of apoptosis, may be required for WT development. In addition to the 7 samples shown in Fig. 1, the analysis of an additional 10 WTs confirmed the results (data not shown). Therefore, WT, unlike fetal kidney, is characterized by the absence of BWAR1C expression. Because WT represents a tissue that largely resembles fetal metanephric blastema, this finding is surprising and may reasonably lead to the hypothesis that the absence of BWAR1C expression may be pathogenetically significant. In fact, BWAR1C is highly homologous to TDAG51, a gene that transduces a
FAS-mediated apoptotic signal. Similarly to its homologue, BWR1C could transmit an apoptotic signal in response to an, as yet, unidentified upstream stimulus. In this case, its absence could be necessary to ensure the continuous growth of WT cells. Additional studies, such as in situ hybridization or immunohistochemistry, aimed at determining the exact cellular pattern of expression of BWR1C in the developing kidney will be needed to support the hypothesis.

LOH in WTs was shown to affect the maternally derived chromosome, which suggests a mechanism for the extinction of maternally expressed imprinted genes. However, BWR1C loss of expression seems to occur independently from deletions of the normally active maternal allele. In fact, BWR1C loss of expression was found in both LOH-positive and LOH-negative WT samples, indicating that maternal alleles were shut off by a mechanism distinct from deletion. We tested whether CpG methylation could explain the dramatic reduction of BWR1C expression. However, the 5' and 3' portions of the gene appear to be nonmethylated (5' promoter region) or only very weakly methylated (3' transcribed region), showing, respectively, no differences or only very few differences in the pattern of methylation between WTs and other expressor and nonexpressor tissues (Fig. 3). This apparently abnormal BWR1C down-regulation in WT seems, therefore, to be more likely attributable to the presence or absence of tissue specific trans-acting factors that influence the expression of BWR1C. A different potential mechanism of BWR1C expression extinction could involve the conversion of maternal into paternal epigenotype. However, this mechanism should also affect the expression of other imprinted, maternally expressed genes of the region. This is clearly not the case for BWR1A, and CDKN1C down-regulation is present in only few cases.

The analysis of the paternally expressed IGF2 gene confirmed that its expression was very high (data not shown) in WTs and fetal kidney but not in adult kidney, as demonstrated previously (34). Only 18 cycles were sufficient to detect the IGF2 cDNA-PCR product in WT samples, in comparison with the 28 cycles necessary for most of the other adult normal tissues and tumors. In sample W7, one of the two informative heterozygous cases, we also observed biallelic IGF2 expression, indicating LOI (35). However, as previously indicated, LOI cannot by itself explain IGF2 overexpression. LOI was shown to occur in normal tissue before tumorigenesis (36), whereas IGF2 overexpression is limited to the tumor tissue and does not involve the nearby normal tissue (36, 37).

In conclusion, although the underlying mechanisms remain obscure, our results reveal the presence of various gene expression abnormalities that could be relevant for WT pathogenesis. These
abnormalities affect three imprinted genes at 11p15 (BWR1C, CDKN1C, and IGFP2) and two genes involved in kidney development (RET and GDNF), and they consist in: (a) overexpression of IGFP2; (b) lack of expression of BWR1C; (c) abnormal coexpression of RET and GDNF; and, in some cases, (d) very low expression of CDKN1C.

These findings add new elements to a growing understanding of the biological basis of WT. If extended to a larger number of samples, our findings might have important implications for the diagnosis and therapy of WT.

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