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Wilms Tumor Genetics: Mutations in *WT1*, *WTX*, and *CTNNB1* Account for Only About One-Third of Tumors

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Abstract

Wilms tumor is genetically heterogeneous, and until recently only one Wilms tumor gene was known, *WT1* at 11p13. However, *WT1* is altered in only ~20% of Wilms tumors. Recently a novel gene, *WTX* at Xq11.1, was reported to be mutated in Wilms tumors. No overlap between tumors with mutations in *WTX* and *WT1* was noted, suggesting that *WT1* and *WTX* mutations could account for the genetic basis of roughly half of Wilms tumors. To assess the frequency of *WTX* mutations and their relationship to *WT1* mutations in a larger (n = 125) panel of Wilms tumors which had been thoroughly assessed for mutations in *WT1*, we conducted a complete mutational analysis of *WTX* that included sequencing of the entire coding region and quantitative PCR to identify deletions of the *WTX* gene. Twenty-three (18.4%) tumors carried a total of 24 *WTX* mutations, a lower *WTX* mutation frequency than that previously observed. Surprisingly, we observed an equivalent frequency of *WTX* mutations in tumors with mutations in either or both *WT1* and *CTNNB1* (20.0%) and tumors with no mutation in either *WT1* or *CTNNB1* (17.5%). *WTX* has been reported to play a role in the WNT/β-catenin signaling pathway, and, interestingly, *WTX* deletion/truncation mutations appeared to be rare in tumors carrying exon 3 mutations of *CTNNB1*, encoding β-catenin. Our findings indicate that *WT1* and *WTX* mutations occur with similar frequency, that they partially overlap in Wilms tumors, and that mutations in *WT1*, *WTX*, and *CTNNB1* underlie the genetic basis of about one-third of Wilms tumors.

INTRODUCTION

Wilms tumor is a childhood embryonal tumor of the kidney that arises from embryonal nephric mesenchyme and typically has a triphasic histology with blastemal, epithelial, and stromal elements. Until recently, the only known Wilms tumor gene was the *WT1* gene at 11p13, which was identified by virtue of being deleted (germline) in patients with WAGR (*W*ilms-*A*niridia-*G*enitourinary anomalies-mental *R*etardation) syndrome and which is mutated in ~20% of sporadic, nonsyndromic Wilms tumors (Huff, 1998). Wilms tumor occurs in both sporadic (98–99%) and familial (1–2%) forms, and the *WT1* gene is only rarely altered in familial Wilms tumors (FWT) (Huff, 1998). Genetic linkage analysis studies of several large Wilms tumor families led to the localization of two FWT genes,

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FWT1 at 17q12-21 (Rahman et al., 1996) and *FWT2* at 19q13.33-13.41 (McDonald et al., 1998), but neither gene has yet been identified. The existence of Wilms tumor families for which linkage to both *FWT1* and *FWT2* has been excluded indicates the existence of at least one additional FWT gene (Ruteshouser and Huff, 2004). Other loci, including 11p15, 1p, 2q, 7p, 9q, 14q, 16q, and 22, have also been implicated in the etiology of Wilms tumor through studies of loss of heterozygosity, loss of imprinting, and constitutional chromosomal defects (Mannens et al., 1990; Kaneko et al., 1991; Maw et al., 1992; Olson et al., 1995; Grundy et al., 1998; Ruteshouser et al., 2005). *CTNNB1*, the gene encoding β-catenin, is also mutated in about 15% of Wilms tumors (Koesters et al., 1999). Interestingly, most tumors with mutations in *WT1* also carry mutations in *CTNNB1*, and *CTNNB1* mutations rarely occur in the absence of *WT1* mutation (Maiti et al., 2000). The etiology of Wilms tumor is thus clearly both complex and heterogeneous.

WT1 encodes a zinc finger transcription factor for which many putative target genes have been identified; however, it is still not clear what the biologically relevant target genes are. β-catenin, encoded by the *CTNNB1* gene at 3p22.1, is involved in both cell adhesion and the WNT signaling pathway. Protein-stabilizing mutations in exon 3 of β-catenin have been observed in a variety of cancers as well as in Wilms tumors (Fukuzawa et al., 2007; Polakis, 2007). In some Wilms tumors with mutations in *WT1*, mutations in exons 7 and 8 of *CTNNB1* are also seen (Li et al., 2004; Fukuzawa et al., 2007), but the functional significance of these non-exon 3 mutations is not clear.

Recently, a third gene, *WTX*, was shown to be mutated in Wilms tumors (Rivera et al., 2007). *WTX* is located at Xq11.1, very close to the centromere, and encodes an 1135 amino acid protein with no conserved functional domains except for a predicted nuclear localization signal. Although *WTX* orthologs are found in other species, including zebrafish, the protein shows no substantial similarity to other proteins of known or unknown function. *WTX* appears to participate in the WNT signaling pathway and to promote the ubiquitination and degradation of β-catenin (Major et al., 2007). Previously *WTX* mutations, both deletions and point mutations, were observed in 15/51 (29.4%) of Wilms tumors, and the mutation frequency was approximately equal in males and females (Rivera et al., 2007). This study also noted that *WTX* alterations were never seen in tumors carrying mutations in either *WT1* or *CTNNB1*. The implication of these data was that the etiology of approximately half of Wilms tumors involved mutations in either *WT1* or *WTX*.

We have now assessed the frequency and type of *WTX* mutations in a larger group of Wilms tumors which had been completely analyzed for mutations in all 10 *WT1* coding exons, including deletions of all or part of the gene, and for mutations in exons 3, 7, and 8 of *CTNNB1*. We have assessed 125 tumors (80 with no *WT1* or *CTNNB1* mutations, 45 with mutations in either or both) for both point mutations and deletions in *WTX*, and an additional 52 tumors for *WTX* deletions only. We show that *WTX* alterations were approximately equally frequent in Wilms tumors with mutations in *WT1* and/or *CTNNB1* and in tumors with no mutation in either *WT1* or *CTNNB1*, and that *WTX* mutations occurred with about the same frequency as *WT1* mutations. Thus, about one-third of tumors carry mutations at *WT1*, *CTNNB1*, and/or *WTX*; gene mutations have yet to be identified for the remaining two-thirds of tumors.

MATERIALS AND METHODS

Tumor Samples

Wilms tumor samples were obtained from patients following informed consent. All tumor DNAs had been analyzed for mutations in *WT1* (all 10 exons) and *CTNNB1* (exons 3, 7, and 8), as previously described (Huff et al., 1995; Maiti et al., 2000). Briefly, tumor DNAs were assessed for *WT1* point mutations by analysis of PCR products from all 10 exons and for partial or complete deletion of *WT1* by Southern blot analysis and, in most cases, quantitative PCR analysis. Of 45 tumors with mutations in *WT1* and/or *CTNNB1*, 24 tumors carried mutations in both genes and 17 carried mutations in *WT1* only. Tumor staging ranged from Stages I to V (D'Angio et al., 1989). The age at diagnosis of these children ranged from 4 months to 17 years. Because we were interested in determining whether mutations in *WTX* and *WT1* were in fact mutually exclusive (as reported), the panel of tumors we assessed in this study was enriched for those with *WT1* mutations.

WTX Mutational Analysis

The entire coding region of *WTX* was amplified as a single PCR product from DNA samples from 125 Wilms tumors, including 80 *WT1*/*CTNNB1* wild-type tumors and 45 tumors with mutations in either or both *WT1* and *CTNNB1*. This PCR product was generated using primers F4 5′-AGCAAGCCAAGCATATCGAG-3′ and R1 5′-CTGGGCAGATGCACTTGAGT-3′, then sequenced by the University of Texas M. D. Anderson DNA Sequencing & Analysis Facility using an ABI 3730 DNA Analyzer with the F4 and R1 primers as well as F2 (5′-ACCGGAAGAGCAAGGTC-3′), R2 (5′CCAAGCAGGCCAATCATAG-3′), R3 (5′-GACGGAAGTCCCTCCAGTCT-3′), and R4 (5′-ATGGTCACTAGCCGTTCTTC-3′). Sequence data were generated with ABI Sequencing Analysis software v5.2 and chromatograms were produced using Chromas v1.45. If mutations were observed in the *WTX* DNA sequence, the analysis was repeated with both tumor and normal tissue DNA from the same patient to confirm the presence of the mutation and to determine if the mutation was present in the patient's germline.

Quantitative PCR for Genomic DNA

Quantitative PCR (qPCR) was performed using an ABI 7900HT Sequence Detection System thermal cycler (Applied Biosystems, Foster City, California) with SYBR Green. DNA (5 ng) from Wilms tumors was amplified in duplicate with primer sets from the 5′ and 3′ ends of *WTX* (Rivera et al., 2007) and from two reference amplicons; first, the intron 13 – exon 14 region of *NDST1* (*N*-deacetylase/*N*-sulfotransferase, located at 5q33.1; intron 13 5′-TCTGAGCTTTCCTTCCCGTTA-3′, exon 14 5′GGAAGTGTCTGGCCCATCTTATA-3′); and second, the intron 4 – exon 5 region of *FAH* (fumarylacetoacetate hydrolase, located at 15q25.1; intron 4 5′-GGTTGCTGATGGGATCTGTTG-3′, exon 5 5′-TTCTCCTTGTCCCTGAACATGAT-3′). Since both reference amplicons include intronic sequences, these primers would amplify only DNA. Analysis of the dissociation curves for all amplicons demonstrated that only the specific PCR product was generated. These chromosome 5 and 15 genes were used as reference amplicons because chromosomes 5 and 15 are two of the chromosomes most rarely amplified or deleted in Wilms tumors (Wang-Wuu et al., 1990; Kaneko et al., 1991) and both are unique, single-copy genes.

DNA samples were amplified in duplicate using SYBR Green PCR Master Mix (Applied Biosystems). Threshold cycle (Ct) values for duplicate samples were subtracted from the average Ct for each of the reference amplicons to calculate ΔCt. The SD of duplicate measurements of ΔCt was determined and used to generate error bars (Fig. 2). Normal control values for *WTX* 5′ and 3′ qPCR reactions were determined using the average of five unaffected male or five unaffected female DNA samples, and each experimental sample was normalized using these control values to produce data on relative genomic DNA content. If *WTX* deletions were observed in tumor samples, the analysis was repeated with both tumor and normal tissue DNA from the same patient to determine if the deletions were present in the patient's germline. qPCR was also performed on these samples with additional amplicons from the p and q arms of the X chromosome to determine if the deletions involved the entire X chromosome or the entire q arm of the X chromosome. These additional chromosome X amplicons were from the *PHEX* (phosphate regulating endopeptidase, X-linked) gene at Xp22.11 (intron 19 5′-TCTTCTTCTCTCACCAGGCTTACAG-3′, exon 20 5′-AAGAAGAGCTGGTTGTTGGTGAA-3′) and the *OCRL* (oculocerebrorenal syndrome of Lowe, phosphatidylinositol polyphosphate 5-phosphatase) gene at Xq25 (intron 19 5′-GACTTCTTTGGTAGGAGGACCTGTTC-3′, exon 20 5′-CGCAAAGGATACGGATTGT-CTC-3′). These amplicons include intronic sequences and hence these primers would amplify only DNA. Analysis of the dissociation curves for these amplicons demonstrated that only the specific PCR product was generated.

Quantitative PCR for RNA

cDNAs were prepared from Wilms tumor RNA by reverse transcribing 400 ng of total RNA from tumors using TaqMan Reverse Transcription reagents with random hexamers (Applied Biosystems). cDNAs were then amplified in duplicate in an ABI 7900HT Sequence Detection System thermal cycler using the TaqMan Gene Expression assay for *WTX* (aka *FAM123b*; Applied Biosystems), which spans the boundary between exons 1 and 2 of *WTX*/*FAM123b* in the region of *WTX* which is identical to the corresponding region of *FAM123b*. Expression of glyceraldehyde phosphate dehydrogenase (GAPDH) was determined as an endogenous gene expression control. The SD was determined for duplicate measurements of gene expression and used to generate error bars (Fig. 4). Expression of *WTX* in Wilms tumors was normalized to the expression in human fetal kidney (Clontech, Mountain View, California; total RNA from pooled fetal kidneys at 12–31 weeks of gestation).

RESULTS

Deletions and Mutations of *WTX*

A large panel of 125 Wilms tumors, including 45 tumors with mutations of *WT1*/*CTNNB1* (mutations in *WT1* or *CTNNB1* or both) and 80 tumors with no detectable mutations in either of these genes, was analyzed for point mutations by sequencing the coding region of the gene and for large deletions of the *WTX* gene (Fig. 1) using the technique of quantitative real-time PCR with two reference genes, an approach commonly and successfully used to accurately discriminate between zero, one, and two gene copies (Hoebeeck et al., 2005; Howald et al., 2006). *WTX* was mutated in 23 of these 125 tumors (18.4%); 16 tumors

carried deletions of the entire gene and seven tumors carried a total of eight point mutations (Tables 1 and 2, Figs. 2 and 3A). Of the eight point mutations, four were missense alterations (831G>T, D233Y, in one tumor; 1010G>C, K292N, in two tumors; and 2773C>T, P880L, in one tumor), three were nonsense mutations (all 763C>G, encoding S210X), and one was a single nucleotide deletion (367del(+)368C>T) at amino acid 78 that would result in a frameshift and a truncated protein terminating after amino acid 98 (Figs. 1D and 3A). Both deletions and point mutations in *WTX* were observed in Wilms tumors from both female and male patients (Table 1; Figs. 2A and Fig. 3A). For 20 of the 23 tumors, matched normal tissue from the same patient was available and normal tissue was assessed for the presence of the *WTX* mutation in the tumor. For all tumors with deletions and nonsense or frameshift mutations, the matched normal tissue showed no alteration in *WTX*, consistent with a somatic origin for the *WTX* alteration. For the four *WTX* missense mutants (one found in a tumor that also carried a nonsense mutation), the alteration was seen also in normal tissue (T67, T93, T140, and T170; see Table 1 and Fig. 3A).

On the basis of the previous study, we expected to find no *WTX* alterations in the group of *WT1*/*CTNNB1* mutant tumors, but when we partitioned the panel of 125 tumors by *WT1*/*CTNNB1* mutation status we were surprised to observe that *WTX* mutation was equally frequent in the two groups (Table 2). We identified *WTX* mutations in nine of 45 (20.0%) tumors with mutations in *WT1*/*CTNNB1* (see Fig. 3B) and in 14 of 80 (17.5%) tumors with no *WT1*/*CTNNB1* mutation. Four deletions of the entire *WTX* gene (Fig. 2B) and five point mutations (Table 1) were found in *WT1*/*CTNNB1* mutant tumors, versus 12 deletions and three point mutations in tumors with no mutation in *WT1* or *CTNNB1*.

The overall frequency of *WTX* mutations was approximately equivalent in Wilms tumors from males and females (20.9% vs. 15.5%, see Table 2), but the frequencies diverged when the tumors were partitioned into groups with and without *WT1* mutations. In the *WT1* wild-type tumors, *WTX* mutations occurred at a significantly higher rate in males than in females (27.3% vs. 5.6%; $P = 0.01$). In contrast, in *WT1*-mutant tumors, *WTX* mutations occurred more frequently in tumors from females (27.3% versus 13.0%), but this difference was not significant ($P > 0.05$).

DNAs from an additional group of 52 tumors, 49 with no mutation in either *WT1* or *CTNNB1*, two with mutations in both *WT1* and *CTNNB1*, and one with a *WT1* mutation only, were analyzed by qPCR only to assess *WTX* deletion status. We identified *WTX* deletions (9.6%) in five tumors with no *WT1* or *CTNNB1* mutation (data not shown), a frequency similar to that seen for the larger group of 125 tumors.

In each case in which we observed a single copy of *WTX* in a tumor from a female patient, we also carried out qPCR analysis with additional X chromosome amplicons (see Materials and Methods) to determine if the deletion was specific for *WTX* or encompassed the entire X chromosome or the entire q arm. In four cases of Wilms tumors from female patients we noted the presence of only one copy of X at the *WTX*, *PHEX* (Xp22.11), and *OCRL* (Xq25) genes (data not shown), indicating that one entire X chromosome was lost in these tumors, as has been previously noted in some Wilms tumors (Solis et al., 1988; Wang-Wuu et al., 1990). For all other cases the observed *WTX* deletion was confined to the *WTX* amplicons.

Tumors from female patients with loss of one entire X chromosome expressed *WTX* from the remaining, active X chromosome (see below) and so were not included in Tables 1-4 and in Figure 2.

In one patient with bilateral Wilms tumor, a *WTX* mutation was observed in addition to a germ-line mutation in *WT1* in one tumor (T162). Both tumors were reduced to homozygosity for the *WT1* mutation (Fig. 3B and data not shown) but were discordant for alterations in *WTX* and also *CTNNB1*; tumor T162 carried a deletion of the entire *WTX* gene but no *CTNNB1* exon 3 mutations, and the contralateral tumor from the same patient carried a *CTNNB1* exon 3 mutation but no *WTX* alteration. In another case (T41), a *WTX* deletion was observed in a tumor from one member of a family with familial predisposition to Wilms tumor linked to FWT2 at 19q13.4 (McDonald et al., 1998), and a tumor from another member of the same family carried no *WTX* alteration.

WTX Expression Analysis

WTX expression in fetal kidney and in 22 tumors without *WTX* deletions was approximately equal (Fig. 4, FK and WT). Five Wilms tumors from which RNA was available had been determined to carry deletions of *WTX*, and, as expected, expression of *WTX* in four of these tumors (three from females, T25, T137, and T157; one from a male, T17) was correspondingly low (<10% of *WTX* expression in fetal kidney; see Fig. 4). One tumor (T162) with a *WTX* deletion from a female patient had moderately low expression (41% of *WTX* expression in fetal kidney; Fig. 4). This tumor carried a homozygous *WT1* missense mutation (Table 1), and sequencing the tumor DNA showed the complete absence of the wild-type nucleotide at the position of the mutation (Fig. 3B), indicating that the tumor preparation was not contaminated with nontumor tissue. The most plausible explanation for the moderately low expression of *WTX* in T162 is that the inactive (nondeleted) X chromosome had undergone mosaic reactivation in some but not all cells within this tumor, although this was not tested.

Tumor T146, from a female patient, carried a heterozygous *WTX* frameshift mutation at codon 78 and showed normal expression of *WTX*. Sequencing of cDNA generated from the T146 *WTX* mRNA revealed that the expressed allele of *WTX* carried the frameshift mutation (data not shown). Two additional tumors, T54 and T62, showed very low (<10% of fetal kidney) expression of *WTX*, possibly indicating that these tumors carried an undetected alteration affecting *WTX* expression.

Correlation of *WTX* Status with Clinical Data

Age at diagnosis and assigned staging of tumor for Wilms tumors with alterations of *WTX* are given in Table 1. The tested panel of Wilms tumors was obtained from a group of patients with an average age at diagnosis of 39.6 months. The average age at diagnosis was 40 months for patients with *WTX*-mutant tumors and 39.5 months for patients with no detectable *WTX* mutation, indicating that mutation of *WTX* was not associated with a change in age at diagnosis. There was a slight preponderance of Stage III tumors in the group of tumors with *WTX* mutations when compared to the tumors with no mutation in *WTX* (Table 3), but statistical analysis of this distribution showed no significant difference ($P = 0.7$ by

Fisher's exact test). Likewise, there was no apparent correlation between *WTX* expression and age at diagnosis or tumor stage/histology (data not shown).

Mutation Frequency in Sporadic Versus Familial or Syndromic Wilms Tumors

We have previously presented data on the frequency of *WT1* mutations in sporadic versus familial and syndromic Wilms tumors (Huff, 1998). In Table 4, we show a similar analysis of the frequency of mutation of *WT1*, *CTNNB1*, and *WTX*, alone or in combination, in our panel of 125 Wilms tumors grouped into categories according to whether the tumors occurred sporadically or within a familial or syndromic context. Sporadic tumors were further grouped by laterality; unilateral, bilateral, or of unknown laterality. The frequency of *WTX* mutations, alone or in combination with *WT1* and/or *CTNNB1* mutations, was similar in sporadic unilateral, sporadic bilateral, and sporadic tumors of unknown laterality (15/68, or 22.1%; 2/12, or 16.7%; and 2/13, or 15.4%, respectively, Table 4). *WTX* mutations were less common in familial tumors (1/11, or 8.3%), but this difference was not significant.

DISCUSSION

We have conducted a thorough mutational analysis of the recently identified Wilms tumor gene, *WTX*, in a large panel of Wilms tumors which had previously been subjected to a complete mutational analysis for both the *WT1* gene and the exon 3 mutational hotspot in the *CTNNB1* gene as well as *CTNNB1* exons 7 and 8. We identified *WTX* mutations in 23 of 125 tumors (18.4%) which were subjected to both sequencing and qPCR analyses. We were able to assess matched normal tissue for the presence of *WTX* germline mutations in 20 of 23 of these cases. Matched normal tissue for all tumors with *WTX* deletions or nonsense/truncation mutations showed that the *WTX* alterations were somatic in origin. However, all four patients whose tumors (T67 and T140, from females; T93 and T170, from males) carried *WTX* missense alterations (831G>T, D233Y; 1010G>C, K292N; 2773C>T, P880L) also showed the *WTX* alteration in normal tissue, indicating that the variants were present in the germline. The aspartic acid at amino acid 233, the lysine at amino acid 292, and the proline at amino acid 880 are all conserved in most mammals but not in lower vertebrates. The K292N alteration was also previously observed as a somatic alteration (Rivera et al., 2007). None of the missense alterations were present in the National Center for Biotechnology Information (NCBI) SNP database (dbSNP) as known polymorphisms (Sherry et al., 1999). The functional significance of these missense alterations is not clear.

In strong contrast to previous data (Rivera et al., 2007), we identified *WTX* mutations at the same frequency in tumors with and without mutations in *WT1*. This result suggests that Wilms tumors with *WTX* mutations do not comprise a biologically distinct category of tumors. In agreement with this finding is our observation that Wilms tumors with *WTX* mutations were diagnosed at the same average age and stage as tumors with no mutation in *WTX*. The discrepancy between our data and the previous findings may be due to the presence of undetected *WT1* mutations since the incidence of *WT1* mutations in the previous study (<5%) is low relative to that observed when *WT1* is assessed for both intragenic mutations in the complete codingregion of the gene and also for exonic deletions. Analysis of the complete coding region of the *WT1* gene for both point mutations and also exonic

deletions previously demonstrated that *WT1* is mutated in ~20% of sporadic, nonsyndromic Wilms tumors (Huff, 1998).

The observed frequency of *WTX* mutations (18.4%) is lower than the 30% previously reported (Rivera et al., 2007). Although in the present study we enriched our Wilms tumor panel for tumors with mutations in *WT1* and/or *CTNNB1*, this is not expected to introduce a bias since *WTX* mutations were found to occur with equal frequency in *WT1*-mutant and *WT1*-wild-type tumors.

WTX expression analysis revealed, as expected, no or very low *WTX* expression in the one tested tumor with a *WTX* deletion from a male patient. *WTX* expression was also very low in three of four tested tumors with *WTX* deletions from female patients, indicating that the *WTX* deletion affected the active X chromosome. One tumor with a *WTX* deletion from a female patient expressed a moderate level of *WTX*. In this case, we were able to rule out contamination of the tumor DNA preparation with DNA from nontumor tissue (see Results), which suggests that the *WTX* expression in this tumor may be due to mosaic reactivation of the inactive (*WTX*-wild-type) X chromosome. Of 22 tumors with no *WTX* deletion, two (9%) showed little or no *WTX* expression, suggesting that in these tumors *WTX* has been silenced by promoter mutation or epigenetic alteration.

One *WT1*/*CTNNB1* wild-type tumor with a *WTX* deletion (T41) was from a patient from a Wilms tumor family which showed genetic linkage to 19q13.4. The *WTX* deletion was not present in the germline, and a tumor obtained from a second affected individual from this same family carried no alteration in *WTX*. This finding, together with the observations of *WTX* mutation in one but not both tumors in one bilateral case (T162), no evidence of linkage to the X chromosome in familial Wilms tumor, and the absence of germline *WTX* alterations that delete the gene or truncate the *WTX* protein, suggest that *WTX* mutation is unlikely to be an event predisposing to Wilms tumorigenesis, but may nevertheless play a role in tumorigenesis in predisposed individuals.

The molecular pathogenesis of Wilms tumor is still not well understood, but clearly is genetically heterogeneous. Our previous finding that mutations in *CTNNB1* show a highly significant association with mutations in *WT1* suggests that the mutation of two different cellular pathways is required for the development of a Wilms tumor (Maiti et al., 2000). The WNT signaling pathway is regulated in large part by modulation of β-catenin protein stability. β-catenin also plays an important role in cell adhesion. Therefore, the observation, in a subset of Wilms tumors, of *CTNNB1* mutations that act to stabilize the β-catenin protein implies that dysregulation of the WNT signaling pathway and/or altered cell adhesion is an important step in tumorigenesis in at least some tumors. This notion is further supported by the recent demonstration that the *WTX* protein forms a complex with members of the WNT pathway and promotes the ubiquitination and degradation of β-catenin (Major et al., 2007). These data also suggest that *WTX* mutation may have a similar “activating” effect on the WNT pathway as do *CTNNB1* mutations. It is therefore of interest to note that five of our Wilms tumors with both *WT1* and *WTX* mutations also carried somatic mutations of *CTNNB1*. These data would imply that *WTX* and *CTNNB1* mutations are not redundant. However, in three of the tumors the *CTNNB1* mutation is an exon 8 missense mutation

(1357T>G or 1358G>C) of unknown functional significance. The *WTX* variant detected in another tumor (T170) was also present in the germline and thus may represent a previously unreported, functionally neutral SNP. Only one tumor (T146) carried a *CTNNB1* mutation of known functional significance (343_345del) and also a *WTX* mutation predicted to be functionally significant (367del(+) 368C>T). With the exception of this single tumor, we found no overlap between functional, expressed *WTX* mutations and protein-stabilizing mutations in *CTNNB1*. These data provide further support for our hypothesis that the process of Wilms tumorigenesis requires inactivation of the WNT signaling pathway, via mutation of *CTNNB1* or *WTX* or another WNT pathway protein, as well as a cellular pathway involving *WT1*, and suggests that those tumors with *WT1* mutations but no *CTNNB1* or *WTX* mutation are likely to harbor mutation of another WNT pathway protein. The strong association of *CTNNB1* mutations and *WT1* mutations suggests that the effect of *WT1* ablation on tumor development is either enhanced by β-catenin stabilization and/or is necessary for the viability of *CTNNB1*-mutant (or, conversely, *WT1*-mutant) cells.

In contrast to *WT1*/*CTNNB1* mutation, our results indicate that there is no association between *WTX* and *WT1* mutations. The apparently random occurrence of *WTX* mutation in either a *WT1*-mutant or *WT1*-wild-type background sug-gests that *WT1* and *WTX* mutations are not functionally redundant and that there are not distinct mechanisms of Wilms tumorigenesis involving either an initial *WT1* mutation or an initial *WTX* mutation. In fact, our finding of a single case of bilateral Wilms tumors concordant for a germline *WT1* mutation but discordant for *WTX* mutation shows that, in this case, *WTX* mutation was not an initiating event in tumorigenesis.

Given that the respective mutation frequencies of *WT1* and *WTX* in Wilms tumors are roughly equal at about 20%, that *CTNNB1* mutations are found in about 15% of Wilms tumors but are rarely observed in the absence of a *WT1* mutation, and that *WTX* mutations appear to partition randomly between tumors with and without *WT1* mutations, alterations in all three of these genes together can underlie the genetic basis of only about one-third of Wilms tumors. Clearly, then, the alteration of additional genes or of their expression must be involved in the process of Wilms tumorigenesis for the remaining two-thirds of tumors. Interestingly, genetic and epigenetic events at chromosome 11p15.5, including loss of heterozygosity at *IGF2* and loss of imprinting in the *IGF2/H19* region, have been observed in ~70% of Wilms tumors (Ogawa et al., 1993; Rainier et al., 1993; Satoh et al., 2006). However, it is not clear whether these events at 11p15.5 are involved in tumorigenesis or tumor progression, or how alterations at 11p15.5, *WT1*, *WTX*, and/or *CTNNB1* may interact in the process of forming a Wilms tumor.

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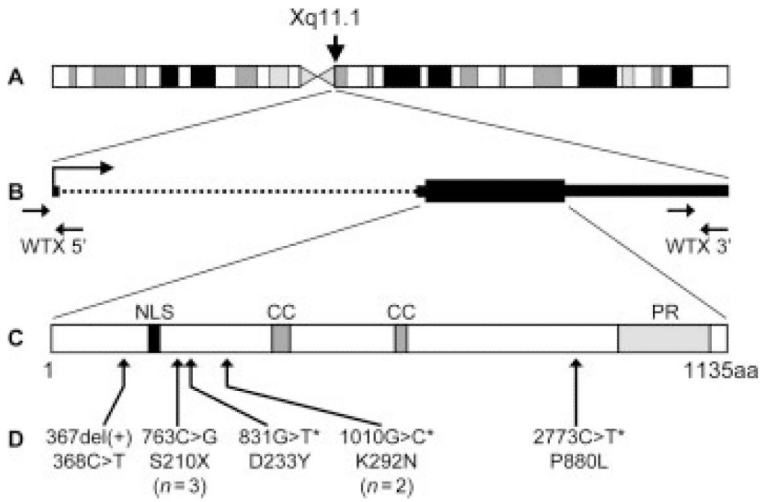

Figure 1.

The X chromosome gene *WTX* and its protein product. A: the location of *WTX* near the centromere at Xq11.1. B: the *WTX* gene. The large arrow indicates the direction of transcription. Small arrows mark the position of 5′ and 3′ primer sets used for quantitative PCR. The *WTX* coding region is indicated by a thick solid line, the 5′ and 3′ untranslated regions by thin solid lines, and the single intron by a dotted line. The orientation of the gene with respect to the X chromosome has been reversed. C: the 1135 amino acid *WTX* protein. NLS, nuclear localization signal. CC, coiled-coil domain; PR, proline-rich region. D: the location of point mutations in the *WTX* protein. Mutations observed more than once in our panel of Wilms tumors are indicated with the number of observations. Mutations present in the germ-line are indicated by an asterisk (*).

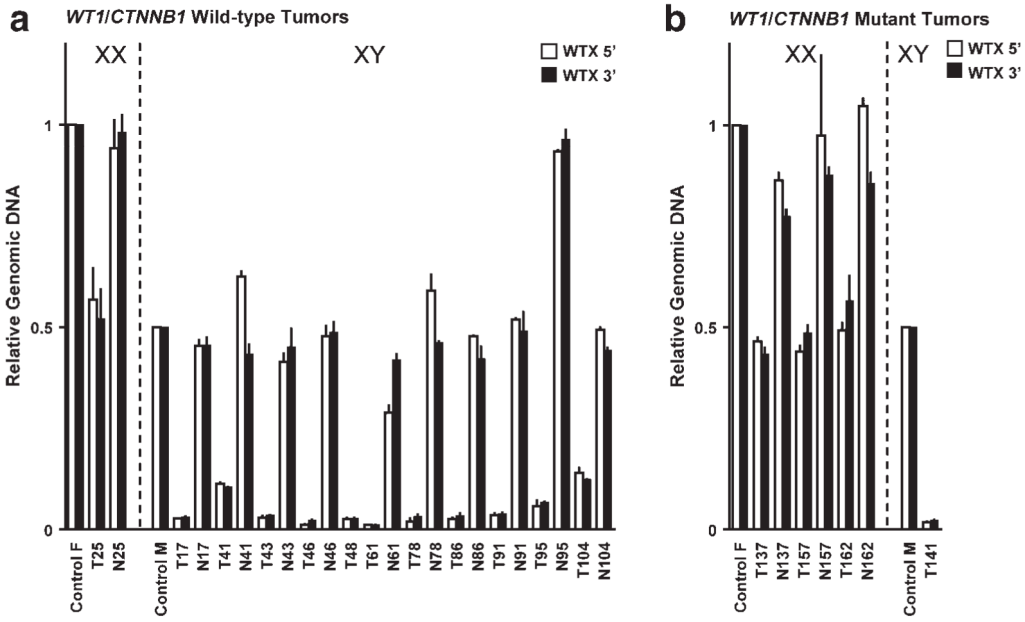

Figure 2.

Quantitative PCR (qPCR) of the 5′ and 3′ ends of the *WTX* gene. Control samples represent the average of DNA from five normal females or five normal males, set equal to 1.0 or 0.5, respectively. A: comparison of relative *WTX* genomic DNA in *WT1*/*CTNNB1*-wild-type tumors and matched normal tissues from one female and 11 males. For sample T48, no matched normal tissue DNA was available. B: comparison of relative *WTX* genomic DNA in *WT1*-mutant tumors and matched normal tissues from three females and one male. For sample T141, no matched normal tissue DNA was available. White bars, amplicon at 5′ end of *WTX* gene (see Fig. 1B). Black bars, amplicon at 3′ end of *WTX* gene. Data bars represent the average of duplicate assays normalized for both the chromosome 5 and 15 reference genes for tumor samples from females and the chromosome 5 reference gene alone for males. Error bars indicate SD from the mean. Note: Patient 95 was male but analysis of two normal tissues (N95) yielded results consistent with the presence of two X chromosomes. Both copies of *WTX* were lost in the tumor (T95); the tumor qPCR results are consistent with the loss of one entire X and the deletion of *WTX* from the second X.

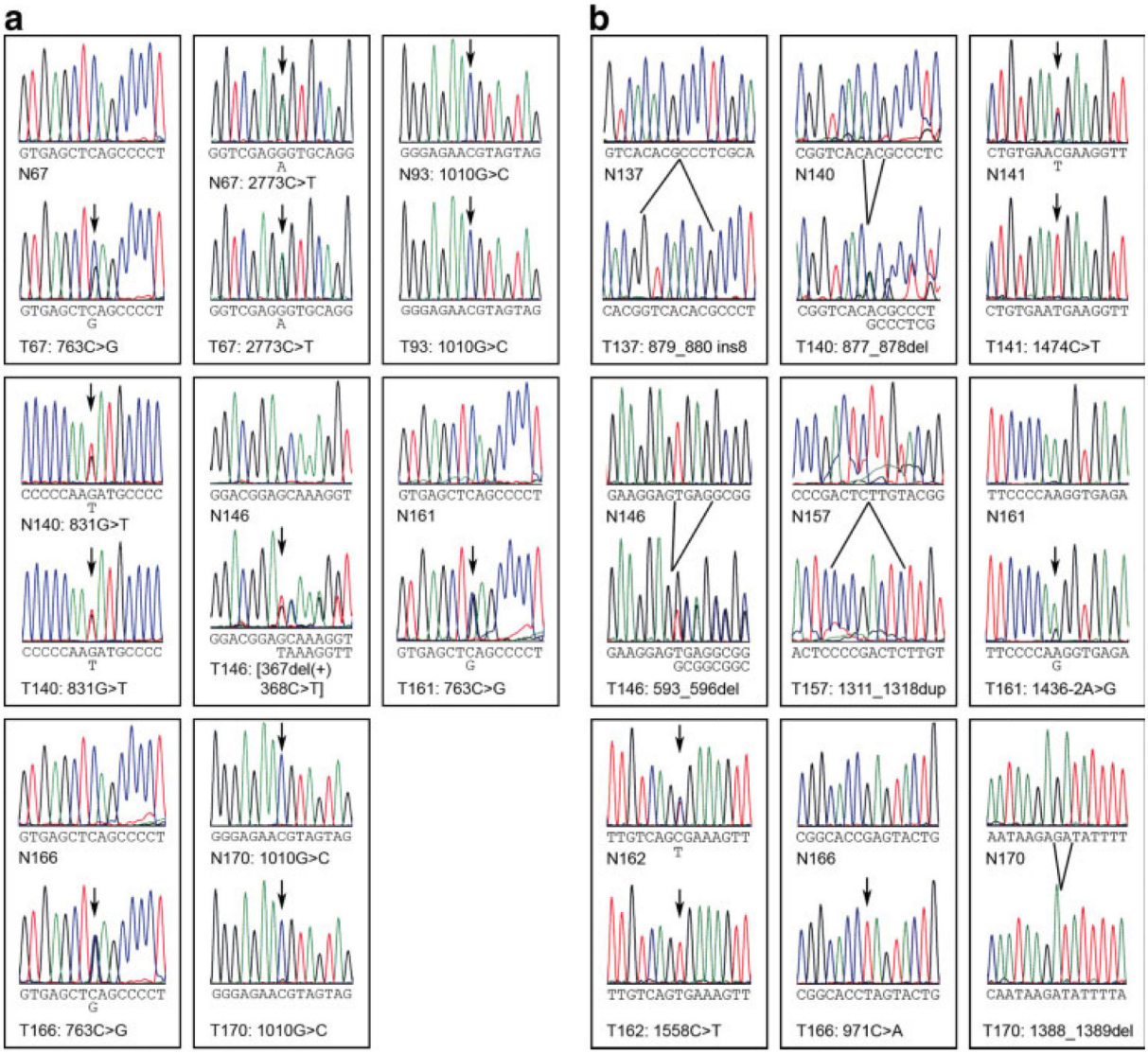

Figure 3.

Sequence chromatograms for *WTX* and *WT1* mutations. The sequence data are shown for the point mutations and small insertions/deletions listed in Table 1. A: *WTX* mutations. N67/T67 2773C>T sequences are from the non-coding DNA strand. B: *WT1* mutations. N166/T166 sequences are from the non-coding DNA strand. N, normal tissue; T, tumor; ins, insertion; del, deletion. Arrows indicate heterozygous or homozygous single-base alterations from the wild-type sequence. Note that patients 93 and 170 were male, with only one X chromosome, and hence were homozygous for germline missense mutations in *WTX*. Patient 161 was also male, but *WTX* and *WT1* sequencing results both indicate that tumor T161 was likely contaminated with normal tissue. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

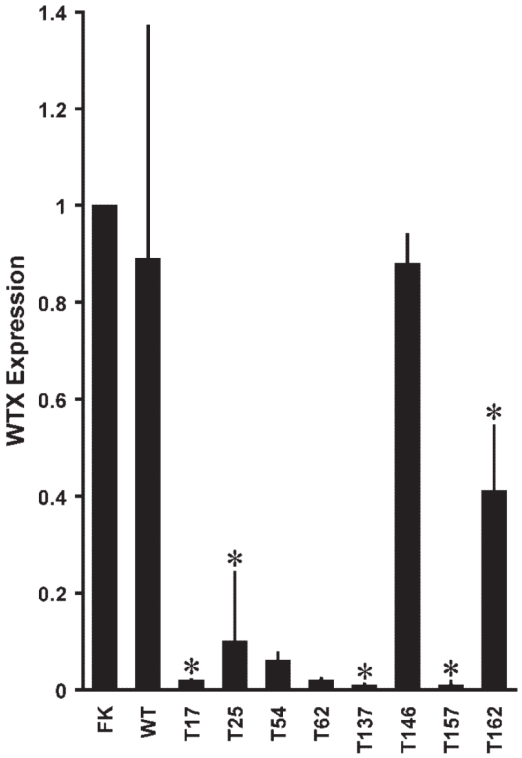

Figure 4.

Quantitative RT-PCR of the *WTX* gene. Expression of GAPDH, as the endogenous control, was measured in the same cDNA samples. FK, *WTX* expression in human fetal kidney. WT, the average of *WTX* expression in 22 Wilms tumors with no identified *WTX* deletions. Tumors with *WTX* deletions are indicated by an asterisk (*). Error bars indicate SD from the mean.

TABLE 1

Wilms Tumors with Alterations of WTX

				Mutations			
Tumor	**Sex Age**	**(m)[a]**	**Stage[b]**	***WTX*[c]**	***WT1*[c]**	***CTNNB1*[c]**	**WTX RNA[d]**
T17	M	50	-	Δ[e]	-	-	0.02
T25	F	36	-	Δ	-	-	0.10
T41	M	9	St V	Δ	-	-	-
T43	M	32	St I	Δ	-	-	-
T46	M	16	-	Δ	-	-	-
T48	M	35	St III	Δ	-	-	-
T61	M	55	St IV	Δ	-	-	-
T67	F	83	St IV	763C>G, S210X; 2773C>T, P880L[f]	-	-	-
T78	M	34	-	Δ	-	-	-
T86	M	26	St III	Δ	-	-	-
T91	M	5	St III	Δ	-	-	-
T93	M	54	St IV	1010G>C, K292N[f]	-	-	-
T95	M	30	-	Δ	-	-	-
T104	M	86	St II	Δ	-	-	-
T137	F	26	-	Δ	879_880ins8[g]	-	0.01
T140	F	25	St II	831G>T, D233Y[g]	877_878del[e]	-	-
T141	M	25	St III	Δ	1474C>T, R362X	-	-
T146	F	64	St III	[367del(1)368C>T]	593_596del	343_345del, DS45	0.88[h]
T157	F	35	St II	Δ	1311_1318dup[i]	-	0.01
T161	M	124	St III	763C>G, S210X	1436-2A>G	1358G>C, W383S	-
T162	F	14	St V	Δ	1558C>T, R390X	1357T>G, W383G	0.41
T166	F	36	St II	763C>G, S210X	971C>A, S194X	1358G>C, W383S	-
T170	M	9	-	1010G>C, K292N[f]	1388_1389del	1340C>G, P44A	-

[a] Age in months at diagnosis.

[b] Per the NationalWilms Tumor Study Group staging system (D'Angio et al., 1989).

[c] *WTX* gene version GI:121556877, *WT1* gene version GI:22027472, *CTNNB1* gene version GI:40254459.

[d] *WTX* expression level relative to level (1.00) in fetal kidney.

[e] Deletion.

[f] *WTX* mutation present in germline.

[g] Insertion.

[h] Mutant *WTX* allele expressed.

[i] Duplication.

TABLE 2

WTX Mutation Frequency in Genetically Defined Subsets of Wilms Tumors

	Tumors	Missense	Truncation[a]	Deletion	All mutations
Wilms tumors	125	4 (3.2%)	4 (3.2%)	16 (12.8%)	23[b] (18.4%)
Male	67	1 (1.5%)	1 (1.5%)	12 (17.9%)	14 (20.9%)
Female	58	3 (5.2%)	3 (5.2%)	4 (6.9%)	9[b] (15.5%)
Grouped by *WT1/CTNNB1* status:					
Wild-type[c]	80	2 (2.5%)	1 (1.3%)	12 (15.0%)	14[b] (17.5%)
Male	44	1 (2.3%)	0	11[d] (25.0%)	12[d] (27.3%)
Female	36	1 (2.8%)	1 (2.8%)	1[d] (2.8%)	2[b,d] (5.6%)
Mutant[e]	45	2 (4.4%)	3 (6.7%)	4 (8.9%)	9 (20.0%)
Male	23	1 (4.3%)	1 (4.3%)	1 (4.3%)	3 (13.0%)
Female	22	1 (4.5%)	2 (9.1%)	3 (13.6%)	6 (27.3%)

[a] Nonsense or frameshift mutation resulting in a truncated protein.

[b] Includes one tumor with both a missense and a truncation mutation.

[c] No mutations in either *WT1* or *CTNNB1*.

[d] Statistically significant ($P \leq 0.01$) difference between frequency of *WTX* mutations in males and females.

[e] Either or both *WT1* and *CTNNB1* mutated.

TABLE 3

Staging of Genetically Defined Subsets of Wilms Tumors

	Stage I	Stage II	Stage III	Stage IV	Stage V	Total
All tumors	24 (24.5%)	23 (23.5%)	27 (27.6%)	11 (11.2%)	13 (13.3%)	98
WTX wild-type	21 (26.6%)	19 (24.1%)	19 (24.1%)	8 (10.1%)	11 (13.9%)	79
WTX mutant	3 (15.8%)	4 (21.1%)	7 (36.8%)	3 (15.8%)	2 (10.5%)	19
NWTS[a]	40%	23%	23%	10%	5%	

[a](D'Angio et al., 1989).

TABLE 4

Frequency of Mutation of *WT1*, *CTNNB1*, and/or *WTX* in Sporadic, Familial, and Syndromic Wilms Tumors

	Sporadic unilateral	Sporadic bilateral	Sporadic unknown laterality	Familial	Syndromic[a]	All
Total tumors	68	12	13	11	21	125
No mutations	39 (57.4%)	6 (50.0%)	9 (69.2%)	7 (58.3%)	5 (23.8%)	66 (52.8%)
WT1 only	4 (5.9%)	1 (8.3%)	1 (7.7%)	0	7 (33.3%)	13 (10.4%)
CTNNB1 only	1 (1.5%)	0	0	2 (16.7%)	1 (4.8%)	4 (3.2%)
WTX only	9 (13.2%)	1 (8.3%)	2 (15.4%)	1 (8.3%)	1 (4.8%)	14 (11.2%)
WT1 + *CTNNB1*	9 (13.2%)	3 (25.0%)	1 (7.7%)	1 (8.3%)	5 (23.8%)	19 (15.2%)
WT1 + *WTX*	3 (4.4%)	0	0	0	1 (4.8%)	4 (3.2%)
All 3 genes	3 (4.4%)	1 (8.3%)	0	0	1 (4.8%)	5 (4.0%)

[a]Wilms tumors associated with genitourinary anomalies (n = 8), Denys-Drash syndrome (n = 5), WAGR syndrome (n = 4), Beckwith-Wiedemann syndrome (n = 3), or Perlman syndrome (n = 1).