Tumorigenesis and Neoplastic Progression

CTNNB1 Mutations and Overexpression of Wnt/ β -Catenin Target Genes in WT1-Mutant Wilms' Tumors

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Gain-of-function mutations in exon 3 of β -catenin (CTNNB1) are specific for Wilms' tumors that have lost WT1, but 50% of WT1-mutant cases lack such "hot spot" mutations. To ask whether stabilization of β -catenin might be essential after WT1 loss, and to identify downstream target genes, we compared expression profiles in WT1-mutant versus WT1 wild-type Wilms' tumors. Supervised and unsupervised hierarchical clustering of the expression data separated these two classes of Wilms' tumor. The WT1-mutant tumors overexpressed genes encoding myogenic and other transcription factors (MOX2, LBX1, SIM2), signaling molecules (TGFB2, FST, BMP2A), extracellular Wnt inhibitors (WIF1, SFRP4), and known β -catenin/TCF targets (FST, CSPG2, CMYC). β -Catenin/TCF target genes were overexpressed in the WT1-mutant tumors even in the absence of CTNNB1 exon 3 mutations, and complete sequencing revealed gain-of-function mutations elsewhere in the CTNNB1 gene in some of these tumors, increasing the overall mutation frequency to 75%. Lastly, we identified and validated a novel direct β -catenin target gene, GAD1, among the WT1-mutant signature genes. These data highlight two molecular classes of Wilms' tumor, and indicate strong selection for stabilization of β -catenin in the WT1-mutant class. β -Catenin stabilization can

initiate tumorigenesis in other systems, and this mechanism is likely critical in tumor formation after loss of WT1. (Am J Pathol 2004, 165:1943–1953)

Mutation of the WT1 tumor suppressor gene occurs in ~10% of Wilms' tumors, and this subset is enriched in tumors that occur in two heritable syndromes, Denys-Drash and Wilms' tumor aniridia genitourinary anomaly retardation syndrome (WAGR).^{1,2} Both syndromes involve abnormalities of urogenital development, and consistent with a key role for WT1 in urogenital development, mice lacking this gene fail to form kidneys.³ WT1 encodes a transcription factor, and several candidate WT1 target genes are expressed in metanephric structures.^{4–6} But although WT1 is a prototypical tumor suppressor,⁷ it is not yet known how its loss predisposes to Wilms' tumorigenesis.⁸

β -Catenin, encoded by CTNNB1, is a nascent transcription factor that transmits signals from Wnt ligands to the cell nucleus. β -Catenin mutations, occurring selectively at amino acid residues that are normal sites of phosphorylation, create a nondegradable form of this protein.⁹ Such mutations have been documented in Wilms' tumors,^{10,11} and a previous study suggested that they might occur almost exclusively in cases with WT1 mutations.¹² But ~50% of WT1-mutant Wilms' tumors lacked such mutations, leaving open the question of an essential link between loss of WT1 and activation of the β -catenin/TCF pathway. We have now answered this question positively, using mutational analysis, gene expression profiling, and functional assays. In addition, our data uncover a novel β -catenin/TCF target gene, GAD1, and also show relative overexpression of specific myogenic transcription factors, signaling molecules, and signaling inhibitors in WT1-mutant compared to WT1 wild-type Wilms' tumors.

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Materials and Methods

Tumors and Control Tissues

Sporadic Wilms' tumors were newly diagnosed and archival cases resected at Babies Hospital of Columbia University and at Hackensack University Hospital. The newly diagnosed cases were studied with informed consent, whereas the archival cases were studied blind to patient identifiers, under an exempt protocol approved by the Columbia University Institutional Review Board. Cryopreserved biopsies of syndromic Wilms' tumors were obtained anonymously from the National Wilms' Tumor Study Group.

Oligonucleotide Microarray Analysis

HG-U95A GeneChips (Affymetrix), which query 10,000 genes (12,627 probe sets), were used for analyzing Wilms' tumors. The cRNA probes were synthesized as described.¹³ After scanning, mRNA expression values were determined using Affymetrix GeneChip software version 5.0. Expression values for each probe set were first filtered for a minimal level of variation (1.5-fold variation from the experiment mean in at least six tumors) and for reliability (Affymetrix presence calls in at least five tumors). Genes that passed these criteria were subjected to non-supervised clustering using the five-cluster *K*-means algorithm. For supervised analysis we labeled the cases as WT1 wild type or WT1-mutant. Starting with the probe sets that passed the initial criteria of variability and reliability, we asked for the genes that differed in their expression most strongly between these two groups of tumors, using the nonparametric Mann-Whitney *U*-test. Identical lists of the genes with the strongest differential expression were obtained when the genes were ranked using parametric tests. We used GeneSpring (Silicon Genetics, Redwood City, CA) for data analysis and for creating dendrograms.

Northern Blotting

RNA from Wilms' tumor and fetal kidney tissues, pulverized under liquid nitrogen, and RNA from cell lines, was prepared using Trizol (Invitrogen, Carlsbad, CA) and were resolved on formaldehyde-containing agarose gels and transferred to Nytran membranes (Schleicher and Schull, Keene, NH). Probes were partial cDNAs prepared by reverse transcriptase-polymerase chain reaction (PCR) using gene-specific primers (sequences available on request). Hybridization was in ULTRAhyb (Ambion, Austin, TX) at 42°C overnight; washing was at 64°C in 0.1% sodium dodecyl sulfate/0.1× standard saline citrate. Band intensities on the autoradiograms were measured with a Storm Phosphorimager (model 840; Molecular Dynamics/Amersham Biosciences Corp., Piscataway, NJ).

Wilms' Tumor Explants and Transforming Growth Factor (TGF)- β 2 Treatment

Wilms' tumor explants were dispersed by mincing, and maintained and passaged in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 10 mmol/L penicillin-streptomycin, and 10 mmol/L L-glutamine. TGF- β 2 peptide (Sigma, St. Louis, MO) was added to an epithelial-appearing explant from a sporadic Wilms' tumor (passage 4) at concentrations of from 0 to 50 ng/ml when the cells were 30% confluent. Plates were photographed after 4 days, cells were harvested, and total RNA was isolated for Northern blot analysis.

Sequencing of CTNNB1 and WT1

PCR primers for amplifying and sequencing exon 3 of *CTNNB1* from genomic DNA, and for amplifying the entire *CTNNB1* coding region from cDNAs, were based on sequences in GenBank. All *CTNNB1* mutations found at the cDNA level were confirmed by exon-specific PCR of genomic DNA. *WT1* was sequenced in each case both from genomic DNA (primers spanning individual exons 1 to 10) and from partial cDNAs, using primers located in exons 4 and 10. Primer sequences are available on request.

Tissue Microdissection

Paraffin-embedded tissue sections were stained with toluidine blue, and the epithelial and stromal components microdissected using a PixCell Ile LCM System (Arcturus, Mountain View, CA). For documentation, microscopic images were digitally captured before and after the microdissection procedure. Genomic DNA was purified from the tissue fragments using proteinase K/sodium dodecyl sulfate digestion followed by phenol/chloroform extractions. Pellet-paint co-precipitant (Novagen) was then added; the DNA was precipitated with 70% ethanol, dissolved in distilled water, and used for PCR followed by direct sequencing. Because relatively small amounts of DNA were obtained from the microdissected blocks, special precautions were taken to avoid PCR contamination; in addition to running standard minus-DNA controls, which were negative, the *CTNNB1* PCR primers for the microdissection experiments were repositioned in more distal upstream and downstream intronic sequences flanking exon 3, outside of the sequences used in previous analyses of whole tissue DNA.

Promoter-Reporter Assays

Human 293 transformed kidney cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 10 mmol/L penicillin-streptomycin. The *GAD1* (*GAD67*) promoter, nucleotides 73,250 to 72,781 of GenBank accession AC007504 (−400 to +60 relative to

the transcription initiation site), was cloned upstream of LUC in the pGL3 vector. A site-directed mutagenesis kit (QuikChange; Stratagene, La Jolla, CA) was used to mutate the TCF site (sequence CTTTGAT → CTTTGGG). The dominant-negative TCF4 construct (pPGS-CITE-CMV-Tcf4dN, dnTCF4) and S37A β -catenin construct, used to ascertain TCF-dependent promoter activity, have been previously described.¹⁴ To measure β -catenin/TCF responses, cells in 35-mm plates were transfected in triplicate with 1.5 μ g of plasmid DNA containing 0.4 μ g of *GAD1* promoter-reporter plasmids, with or without 0.6 μ g of S37A β -catenin plasmid and with or without 0.2 μ g of dnTCF4 plasmid, and 0.1 μ g of pCMV- β -galactosidase (β -gal). Carrier pCMV (pPGS-CITE-CMV-Neo) was added to make the total plasmid DNA concentration equal in each experimental condition. As positive and negative controls, pTopflash (containing four concatenated TCF sites) and pFopflash (containing mutated versions of these sites) were transfected with 0.1 μ g of the S37A β -catenin plasmid. Luciferase and β -galactosidase assays were performed according to the manufacturer's protocol (Luciferase Assay System; Promega, Madison, WI).

Co-Transfection Assays for Activity of Mutant β -Catenins

To assess the functions of the non-hotspot *CTNNB1* mutations, the complete protein-coding regions of the mutant alleles were amplified by reverse-transcription (Superscript, Invitrogen) followed by PCR using *CTNNB1* primers, and cloned into the pEGFP-N1 vector (Clontech, Palo Alto, CA), producing GFP-fusion proteins with expression driven from the CMV promoter. These plasmids, 0.5 μ g, were co-transfected with the pTopflash or pFopflash luciferase reporter plasmids, 0.4 μ g, together with 0.1 μ g of the Renilla luciferase control plasmid, into 293 cells. Expression values were determined from the ratio of firefly luciferase to Renilla luciferase activity using the Dual Luciferase Assay System (Promega).

Immunohistochemistry

Procedures were essentially as previously described.¹³ Antigen retrieval for detection of β -catenin was done by boiling the deparaffinized sections on slides for 10 minutes in 1 mmol/L ethylenediaminetetraacetic acid, pH 8.0, in a microwave oven. The anti- β -catenin (β -catenin; BD Transduction Laboratories, Lexington, KY) was used at a dilution of 1:400, and the anti-SIM2 antibody (C-17; Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 1:500. For detecting β -catenin, the secondary antibody (biotinylated horse anti-mouse; Vector Laboratories, Burlingame, CA) was used at a dilution of 1:400. For detecting SIM2, the secondary antibody (biotinylated

rabbit anti-goat, Vector Laboratories) was used at a dilution of 1:400.

Results

CTNNB1 Stabilizing Mutations Are Restricted to *WT1*-Mutant Wilms' Tumors

We first sequenced *WT1* in 19 sporadic and 17 syndromic Wilms' tumors. Only a single sporadic tumor (WT564) showed a *WT1* mutation, whereas each of six Denys-Drash-associated tumors and five of nine WAGR-associated tumors sequenced as positive controls showed the expected point mutations in the zinc finger region (Table 1). We next sequenced the mutational hotspot region of *CTNNB1*. This region, in exon 3, encodes phosphorylation sites (serine and threonine residues) that regulate β -catenin stability. Among 17 *WT1*-mutant syndromic cases, 9 carried missense mutations or deletions affecting this exon of *CTNNB1* (Table 1). In contrast, only 1 of the 19 sporadic Wilms' tumors carried an exon 3 mutation and, strikingly, this was the single tumor with a *WT1* mutation. Combining our analysis with the previous study,¹² it is clear that *CTNNB1* hotspot mutations occur in 50% of cases *WT1*-mutant Wilms' tumors, but are rare or absent in their more common *WT1* wild-type counterparts.

The major effect of *CTNNB1* hotspot mutations is thought to be stabilization of β -catenin and consequent accumulation of this nascent transcription factor in the nucleus. To verify this prediction, we assessed the subcellular distribution of β -catenin by immunohistochemistry in genetically characterized primary Wilms' tumors. This revealed clusters of tumor cells with nuclear β -catenin in both of two cases with *CTNNB1* mutations, whereas no such nuclear accumulation was seen in multiple sections of five Wilms' tumors lacking *CTNNB1* mutations (Figure 1 and data not shown). This analysis does not exclude that there could be a minor subset of Wilms' tumors with nuclear β -catenin even in the absence of *CTNNB1* mutations, but it does indicate a good correlation between the presence of such mutations and the nuclear accumulation of β -catenin.

Interestingly, in both of the mutation-bearing cases, the tumor cells with nuclear β -catenin were only observed in the malignant stromal component (Figure 1, C and D). When we performed tissue microdissection and isolated DNA from the epithelial and stromal components of one of these cases, the heterozygous (gain-of-function) *CTNNB1* mutation was clearly identified in both components (Figure 2). As expected, the *WT1* homozygous mutation was also detected in both components (data not shown). Thus, mutation of the *CTNNB1* gene occurred early enough in tumorigenesis that the mutation was present in all cells of the surgically resected tumor. These combined immunohistochemical and genetic data suggest that, despite its constitutive stabilization, a cytoplasmic or membrane component anchors the mutant β -catenin in the cytoplasm in the epithelial tumor cells, while this mutant protein is free to translocate to the nucleus in the malignant stromal cells.

Table 1. Mutational Analysis of *CTNNB1* and *WT1* in Wilms' Tumors

Tissue bank no.	Syndrome	Study no.	<i>CTNNB1</i> exon 3 hotspot		<i>CTNNB1</i> non-hotspot	<i>WT1</i>
			Nucleotide	AA		
WT2621B	Sporadic	1	nc	nc	—	nc
WT2110B	Sporadic	2	nc	nc	—	nc
WT2113	Sporadic	3	nc	nc	—	nc
WT2122A	Sporadic	4	nc	nc	—	nc
WT2345A	Sporadic	5	nc	nc	—	nc
WT2409A	Sporadic	6	nc	nc	—	nc
WT2562	Sporadic	7	nc	nc	nc	nc
WT2596B	Sporadic	8	nc	nc	—	nc
WT2071A	Sporadic	9	nc	nc	nc	nc
WT2767A	Sporadic	10	nc	nc	nc	nc
WT2769B	Sporadic	11	nc	nc	nc	nc
WT616B	Sporadic	12	nc	nc	nc	nc
WT899A	Sporadic	13	nc	nc	nc	nc
WT914	Sporadic	14	nc	nc	nc	nc
WT2509D	Sporadic	15	nc	nc	nc	nc
WT3531	Sporadic	16	nc	nc	nc	nc
WT3663	Sporadic	17	nc	nc	nc	nc
WT3665	Sporadic	18	nc	nc	nc	nc
WT564	Sporadic	19	TCT → CCT	S45P	—	R301Opal*
TB3309	WAGR	20	nc	nc	K335I	R394W
TB3306	WAGR	21	TCT → TTT	S33F	nc	nc
TB3494	WAGR	22	TCT → TGT	S45C	—	nc
TB3498	WAGR	23	347ΔTCT	ΔS45	—	ins(1nt), ex10*
TB3499	WAGR	24	nc	nc	W383R	Δ4bp/exon 7*
TB3497	WAGR	25	nc	nc	W383R	ins4bp/exon 7*
TB3308	WAGR	26	347ΔTCT	ΔS45	—	nc
TB3495	WAGR	27	347ΔTCT	ΔS45	—	ins(1nt), ex7*
TB3496	WAGR	28	nc	nc	nc	nc
TB3500	WAGR	29	nc	nc	(ΔE632-S681) [†]	—
TB3502	D-D	30	nc	nc	nc	C388S
TB3311	D-D	31	nc	nc	—	R394Y
TB3501	D-D	32	del 24bp	ΔP44-N51	—	C388Y
TB3310	D-D	33	nc	nc	nc	R394L
TB3307	D-D	34	347ΔTCT	ΔS45	—	D396N
TB3503	D-D	35	TCT → TAT	S45Y	—	R394Y
TB3965	D-D	36	ACC → GCC	T41A	—	R3890Opal*

* Chain-terminating homozygous mutations.

[†] A 150-bp deletion, with an insertion of 6 bp; replacing 50 amino acids with two amino acids, SV (see Figure 7).
nc, No change; (—), not done.

Microarray Analysis Distinguishes *WT1*-Mutant from *WT1*-Replete Tumors

For convenience in the following discussion we refer to two Wilms' tumor classes: class 1 tumors are *WT1* wild-type whereas class 2 tumors are *WT1*-mutant. Our hypothesis in the next series of experiments was that constitutive activation of the Wnt/ β -catenin pathway, either by *CTNNB1* mutations or by lesions in other components of this pathway, might occur in all *WT1*-mutant (class 2) Wilms' tumors. Thus, sets of genes differentially expressed between class 1 and class 2 Wilms' tumors might be enriched in β -catenin/TCF targets. We performed expression profiling of 17 sporadic Wilms' tumors and a comparison group of 11 syndromic Wilms' tumors (seven WAGR syndrome and four Denys-Drash syndrome), with cases selected only on the basis of RNA availability. Both supervised and unsupervised, hierarchical clustering of the microarray data readily distinguished the (primarily syndromic) *WT1*-mutant cases from the sporadic *WT1* wild-type cases (Figure 3). Significantly, the single sporadic tumor with a *WT1* deletion (WT564) showed a pattern of gene expression that

closely matched the consensus of the syndromic *WT1*-mutant cases (Figure 3).

Although the unsupervised and supervised analyses both produced clear class 1 and class 2 consensus patterns, there was variation among the cases, with two of the sporadic cases showing a pattern of gene expression that appeared intermediate between the two consensus patterns (Figure 3, arrowheads). Both of these outlier cases were classical triphasic Wilms' tumors, but analysis of additional tumor samples will be necessary to determine whether a specific histology, perhaps stromal abundance analogous to that seen in many of the syndromic tumors, and/or an as yet unidentified genetic lesion, correlates with this intermediate pattern of gene expression.

For validation of the GeneChip data, we hybridized Northern blots with probes for 10 of the differentially expressed genes, selected based on their interesting potential biological functions. For each gene the pattern of expression closely matched that predicted from the microarray data (examples in Figure 4A). Measuring the band intensities on these blots by Phosphorimaging con-

Figure 1. Subcellular localization of β -catenin in genetically characterized Wilms' tumors. Formalin-fixed, paraffin-embedded sections of two *WT1* wild-type/*CTNNB1* wild-type (class 1) Wilms' tumors (A, B) and two *WT1*-mutant/*CTNNB1*-mutant (class 2) Wilms' tumors (C, D) were immunostained with anti- β -catenin. Immunoreactive β -catenin is prominent in all of the tumors, but nuclear β -catenin is restricted to the class 2 cases, in which it is seen in clusters of cells (asterisks) restricted to the neoplastic stromal component (Str). Neoplastic epithelial cells (Epi) show cytoplasmic and membrane staining for β -catenin and neoplastic blastema (Bl) shows cytoplasmic staining. Several spindle-shaped endothelial cells are also densely stained (arrow).

firmed that the GeneChips were giving a valid readout of differential mRNA expression (Figure 4B).

Moreover, for one of the differentially expressed genes, the transcription factor encoded by *SIM2*, we confirmed differential expression at the protein level by immunohistochemistry. This nuclear protein was seen fo-

cally in the epithelial component of both of two class 2 tumors, and was strongly expressed by neoplastic stromal cells adjacent to the epithelial areas in both of these cases (Figure 5). Among five class 1 Wilms' tumors examined, four showed no detectable SIM2 protein, while one case showed detectable SIM2 protein focally in the epithelial component, but not in the stromal component.

Class 2 Wilms' Tumor Signature Genes Include β -Catenin/TCF Targets, Extracellular Wnt Inhibitors, Myogenic Transcription Factors, and Signaling Molecules

The genes with the greatest differential expression between the two Wilms' tumor classes are described and ranked by significance in Tables 2 and 3. Although the differentially expressed genes clearly distinguished the class 1 from class 2 tumors, additional analysis showed these two classes otherwise share a common expression profile. Specifically, of the 28 genes identified in our previous analysis as overexpressed in sporadic Wilms' tumors compared to normal fetal kidney and several heterologous tumors and tissues,¹³ only 3 (*GPR39*, *PAX2*, *PRAME*) were differentially expressed in the comparison of class 1 versus class 2 Wilms' tumors. This finding would be predicted based on general similarities in histogenesis. Although there are histological differences, notably a tendency for more mesenchymal and myogenic differentiation in the syndromic tumors,^{15,16} the pathological criteria for diagnosis are identical in these tumors and their sporadic counterparts.

Among the signature genes overexpressed in the class 2 tumors are examples encoding important transcriptional activators and repressors, signaling agonists and antagonists, extracellular matrix proteins, and enzymes. Notable is the consistent overexpression of two genes encoding extracellular inhibitors of Wnt signaling, *WIF1* and *SFRP4* (fold differences and *P* values in Table 3). A gene encoding a third extracellular Wnt inhibitor, *DKK1*, was also differentially expressed, although at a less stringent *P* value (fold difference, 3.6; *P* value, 2.2×10^{-4}) that was below the cutoff for the list in Table 3. Consistent with the known tendency of syndromic Wilms' tumors to undergo myogenic differentiation, two genes encoding myogenic transcription factors, *MEOX2* (also known as *MOX2*) and *LBX1*, appeared in the class 2 signature set (Table 3; see also Figure 4 for Northern blot confirmation of *MEOX2* overexpression).

Undifferentiated mesenchyme is also prominent in syndromic Wilms' tumors, and one factor enforcing this phenotype may be TGF- β 2, which has a well known ability to induce epithelial-mesenchymal transitions, and which is encoded by the class 2 signature gene, *TGFB2* (Table 3). In fact, when we exposed a class 1 Wilms' tumor explant to exogenous TGF- β 2, the cells underwent a morphological epithelial-mesenchymal transition, and showed changes in the expression of several signature genes (upregulation of *CSPG2* and down-regulation of *IFI16* and *PAX8*) suggesting a partial transition to a class 2 pheno-

Figure 2. *CTNNB1* mutation in both the stromal and epithelial component of a class 2 Wilms' tumor. Tissue microdissection with capture of an epithelial area (Epi), illustrated by premicrodissection (A) and postmicrodissection (B) photomicrographs. Stromal areas (Str) were captured using the same procedure (not shown). C: Sequence chromatograms of genomic PCR products from DNA isolated from the epithelial and stromal components of the tumor, showing a heterozygous gain-of-function mutation in exon 3 of *CTNNB1* in both components. As expected, the mutation is absent from nonneoplastic kidney DNA of the same patient. T, gross tissue sample from the tumor; N, sample of normal kidney adjacent to the tumor.

Figure 3. Microarray analysis of gene expression in sporadic and syndromic Wilms' tumors. Results of the nonsupervised five-cluster *K*-means analysis are on the **left**, with two of the five clusters (**bottom**) crudely distinguishing WT1 wild-type from WT1-mutant tumors. The most significant probe sets identified by supervised analysis and clearly distinguishing these genetic classes of Wilms' tumor are shown at the **right**. Genes are on the y axis and tumors on the x axis. Red indicates high expression and blue low expression, with the color scale at 0 to 6 relative to the experiment mean. The **double line** under the **right** panel marks data from two independent cRNA probes from a single tumor, illustrating reproducibility. The **asterisk** indicates the single sporadic tumor with a *WT1* mutation. The **arrowheads** indicate two *WT1* wild-type (class 1) tumors that show an expression profile intermediate between the class 1 and class 2 consensus patterns.

type (Figure 6). As discussed below, the versican gene (*CSPG2*) is likely to be a convergent target of activation by TGF- β 2 signaling and by the nuclear activity of β -catenin/TCF in class 2 Wilms' tumors. Additional signaling molecules in TGF- β -related pathways, the agonist BMP2A and the antagonist follistatin (*FST* gene), were also found among the class 2 signature genes (Table 3).

Although we have chosen to focus on the class 2 signature genes in the remainder of this report, the list of class 1 signature genes will be useful for future studies of growth-related genes, stage of differentiation, and cell-of-origin in sporadic Wilms' tumors. The class 1 tumors evidently use a distinct array of signaling proteins, with the genes related to calcium signaling (*S100A13*, *ITPR1*, *GPR39*) highly represented, and a distinct array of transcription factor genes, notably *PAX8* and *PAX2*, relatively overexpressed.

Known and Novel β -Catenin/TCF Targets Are Overexpressed in WT1-Mutant Wilms' Tumors with and without CTNNB1 Hotspot Mutations

Three known β -catenin target genes, encoding c-Myc, cyclin-D, and Fra-1¹⁷⁻¹⁹ did not appear among the class 2 signature genes. Nevertheless, examination of the primary data showed that *CMYC* was in fact modestly but consistently overexpressed in the class 2 Wilms' tumors, with a mean difference of 2.3-fold in class 2 compared to class 1 tumors. In addition, two recently reported β -catenin target genes,²⁰ encoding follistatin (*FST*) and versican (*CSPG2*), showed strongly differential expression and did appear in the class 2 signature set (Table 3).

The *GAD1* gene, encoding glutamic acid decarboxylase, is necessary for production of γ -aminobutyric acid in

Figure 4. Validation of the differentially expressed genes by Northern blotting and Phosphorimaging. **A:** Probes for the indicated genes were hybridized sequentially to two duplicate Northern blots (**left** and **right**), which were stripped between hybridizations. Class 1 tumors are WT1 wild-type and class 2 tumors are WT1-mutant. Positions of 28S and 18S ribosomal RNAs are indicated as markers. FKi, normal human fetal kidney. Several of the signature genes are not only differentially expressed between class 1 and class 2 tumors, but are also absolutely overexpressed relative to the control fetal kidney. **B:** Band intensities from Northern blotting were measured by Phosphorimaging. Expression levels are graphed in arbitrary units, with the values normalized to the signal obtained in the same lane after rehybridization with a glyceraldehyde 3-phosphate dehydrogenase probe (similar results were obtained after normalizing to β -actin). Each data point (**diamonds**) is the value for one tumor (1, class 1; 2, class 2) or normal fetal kidney (FKi). *PAX2* and *GPR39* are class 1 signature genes; *WIF1* and *GAD1* are class 2 signature genes.

neurons. But cleft palate observed in mice lacking *Gad1* suggests that this gene also plays an essential role in epithelial-mesenchymal interactions.^{21,22} *GAD1* has not been reported as a β -catenin/TCF target, but it is consistently overexpressed in class 2 Wilms' tumors (Figures 3 and 4). Analysis of sequences in GenBank (accession AC007504) showed that the proximal promoter of this gene contains a consensus TCF-binding site, located at

Figure 5. Validation of *SIM2* as a class 2 signature gene by immunohistochemistry. The *SIM2* transcription factor is visualized as nuclear staining in a class 2 Wilms' tumor containing a *CTNNB1* mutation (**A**), and is not detected above background in a class 1 Wilms' tumor (**B**). Both of two class 2 tumors were strongly positive for nuclear *SIM2* in neoplastic stromal cells adjacent to epithelial areas, and focally in the epithelial component, whereas four of five class 1 tumors showed no *SIM2* positivity. The fifth class 1 tumor examined showed focal epithelial but no stromal *SIM2* positivity.

298 to 292 bp upstream of the transcriptional start site, and reporter assays in conjunction with site-directed mutagenesis of this site revealed that the *GAD1* promoter is β -catenin/TCF responsive (Figure 7). Thus, *GAD1* is a novel direct target of β -catenin/TCF uncovered by our screen.

Additional candidate β -catenin/TCF target genes are also present in our dataset. Notably, *SIM2*, a gene that is overexpressed both at the mRNA and protein level in class 2 compared to class 1 Wilms' tumors, contains at least one consensus TCF site in its promoter region. Additional studies will be necessary to validate *SIM2* as a β -catenin/TCF-responsive gene, but it is intriguing that a major phenotype in *Sim2*-knockout mice is cleft palate, similar to the phenotype caused by mutation of *Gad1*.^{23,24}

Some WT1-Mutant Wilms' Tumors Carry Non-Hotspot CTNNB1 Mutations

As predicted from the hypothesis that β -catenin stabilization is an obligate second event in WT1-mutant tumor precursor cells, a statistical analysis showed that bona fide β -catenin/TCF target genes were relatively overexpressed both in the class 2 tumors with *CTNNB1* hotspot mutations and in the remaining class 2 cases that lacked such mutations, in comparison with the class 1 Wilms' tumors (Figure 8). To explain this general overexpression of β -catenin/TCF target genes in class 2 Wilms' tumors, we searched for mutations that might stabilize β -catenin in those cases that lacked *CTNNB1* hotspot mutations. Complete sequencing of *CTNNB1* showed unusual heterozygous truncating and missense mutations, outside of exon 3, in several tumors in this category, with some of these mutations occurring in the APC/Axin-binding region (Table 1 and Figure 9A). Other genes in the Wnt/ β -catenin pathway need to be examined, but based on these data hotspot plus non-hotspot *CTNNB1* mutations already account for constitutive β -catenin stabilization in 75% of the WT1-mutant tumors in our series.

To directly assess the functional consequences of the non-hotspot β -catenin mutations, we cloned these mutant alleles as GFP-fusion constructs and compared their activities with that of two common hotspot mutant alleles (Δ S45, S37A), and with wild-type β -catenin, in co-transfections with pTopflash and pPopflash reporters in 293 cells. As shown in Figure 9B, both of the non-hotspot mutations tested proved to be gain-of-function alleles, reproducibly activating pTopflash transcription at levels twofold higher than wild-type β -catenin. As might be expected from their less frequent biological selection during tumor formation, these rare alleles were somewhat less potent than the hotspot Δ S45 and S37A mutant β -catenins, which produced fourfold to sixfold increases in pTopflash transcription in this assay (Figure 9B).

Discussion

Our data show that Wilms' tumors with loss-of-function mutations in the WT1 tumor suppressor (class 2) can be

Table 2. Genes Highly Expressed in WT1-Wild-Type Compared to WT1-Mutant Wilms' Tumors

Probe set	Gene description	GenBank	Gene category	Fold Difference	P value
37554_at	Kallikrein 6 (KLK6)	U62801	Protease	9.2	5.50E-09
35704_at	HRAS-like suppressor 3 (HRASLS3)	X92814	Intracellular signaling	6.2	2.30E-08
39712_at	S100A13	A1541308	Ca++ binding protein	6.6	4.41E-08
755_at	Type 1 inositol 1,4,5-trisphosphate receptor (ITPR1)	D26070	Ca++ signaling receptor	6.0	4.41E-08
32779_s_at	Type 1 inositol 1,4,5-trisphosphate receptor (ITPR1)	U23850	Ca++ signaling receptor	4.4	4.41E-08
38749_at	GPR39	A1936826	Signaling receptor	4.3	1.46E-07
35810_at	ARPC3 actin related protein 2/3 complex	A1525393	Cytoskeletal regulation	2.2	1.46E-07
32778_at	Type 1 inositol 1,4,5-trisphosphate receptor (ITPR1)	D26070	Ca++ signaling receptor	3.8	2.54E-07
32174_at	Solute carrier (SLC9A3R1) (EBP50)	AF015926	Transporter	4.2	4.30E-07
41536_at	Helix-loop-helix protein ID4	AL022726	Transcription antagonist	3.7	4.30E-07
41271_at	SLC7A8 solute carrier family 7	Y18483	Transporter	3.6	4.30E-07
40302_at	ORF P02454	AL031186	Unknown	2.9	4.30E-07
1456_s_at	Interferon-gamma induced protein (IFI-16)	M63838	Transcriptional modulator	7.7	1.14E-06
40556_at	Reticulocalbin (RCN1)	D42073	Ca++ binding protein	2.4	1.14E-06
1970_s_at	FGFR2	Z71929	Signaling receptor	6.1	2.79E-06
41723_s_at	MHC class II HLA-DR beta-1 (DR2.3)	M32578	MHC antigen	4.1	2.79E-06
31488_s_at	Phosphoglycerate kinase (PGK1)	S81916	Metabolic enzyme	3.2	2.79E-06
34354_at	FGFR2	M80634	Signaling receptor	2.9	2.79E-06
994_at	hR-PTPu PTPRM	X58288	Receptor tyrosine phosphatase	2.5	2.79E-06
35396_at	Hyaluronic acid synthase 2 (HAS2)	U54804	Extracellular matrix synthesis	4.2	4.24E-06
39133_at	GCN5L1	A1525379	Transcriptional activator	2.5	6.33E-06
36869_at	PAX8	X69699	Transcription factor	7.6	1.35E-05
1984_s_at	Rho GDP-dissociation Inhibitor 2 ARHGDI B	G69549	Intracellular signaling	7.6	1.35E-05
39009_at	LSM3 homolog, U6 small nuclear RNA associated	N98670	RNA metabolism	2.2	1.35E-05
36155_at	Testican (SPOCK2)	D87465	Extracellular matrix protein	3.9	1.93E-05
36812_at	BCAR3 contains SH2 and RAS-GEF domains	U92715	Intracellular signaling	2.5	1.93E-05
37383_f_at	HLA class I locus C heavy chain	X58536	MHC antigen	2.5	1.93E-05
121_at	PAX8	X69699	Transcription factor	3.1	2.72E-05
34642_at	YWHAZ 14-3-3 protein	U28964	Intracellular signaling	2.5	2.72E-05
723_s_at	HLA DR1B	M32578	Histocompatibility antigen	2.1	2.72E-05
34194_at	cDNA DKFZp564B076 LOC340210	AL049313	Unknown	3.8	3.79E-05
1363_at	Fibroblast growth factor receptor (K-sam)	M87770	Signaling receptor	2.6	3.79E-05
651_at	Replication protein A 14kDa subunit (RPA3)	L07493	DNA replication	2.1	3.79E-05
33130_at	HOXD1 homeo box D1	AW001001	Transcription factor	9.0	5.22E-05
41352_at	Beta-galactoside alpha-2,6-sialyltransferase	X62822	Sialyltransferase	2.5	5.22E-05
38186_g_at	PAX2	U45255	Transcription factor	3.7	7.11E-05
1143_s_at	FGFR2	M80634	Signaling receptor	3.0	9.59E-05
37225_at	KANK kidney ankyrin repeat-containing protein	D79994	Cytoplasmic signaling	2.8	9.59E-05
995_g_at	hR-PTPu PTPRM	X58288	Receptor tyrosine phosphatase	2.6	9.59E-05
35259_s_at	SFRS2IP	Y11251	Splicing factor	2.0	9.59E-05

distinguished from WT1-replete Wilms' tumors (class 1) by differential expression of discrete sets of genes, and that these sets include known and novel targets of β -catenin. Based on the expression of these genes, together with our sequencing data for *CTNNB1* showing frequent gain-of-function mutations, both in the exon 3 hotspot and in other regions of the gene, restricted to the class 2 tumors, the β -catenin/TCF pathway is evidently hyperactivated in nearly all WT1-mutant tumors. This dichotomy between class 1 and class 2 Wilms' tumors had a practical benefit in that it allowed us to validate several β -catenin/TCF targets, that were originally found in cell line experiments, in the physiological setting of a well-defined class of primary human cancers, and also to identify a novel direct β -catenin/TCF target gene, *GAD1*. This technical approach—gene expression profiling in genetically defined classes of primary tumors—should continue to be useful for identifying downstream targets of oncogenic transcription factors.²⁵

The strong expression of genes encoding extracellular inhibitors of Wnt signaling (*WIF1*, *SFRP4*) that we have shown is characteristic of the class 2 tumors is entirely

consistent with the genetic data, because cells with constitutive activation of the intracellular arm of the Wnt pathway (via somatic mutations of *CTNNB1*) are expected to be at least partly resistant to growth inhibition by these extracellular molecules. One of these inhibitors, Dickkopf-1 (*DKK1* gene) was previously reported as overexpressed in some Wilms' tumors, although *WT1* and *CTNNB1* status was not determined.²⁶

In principle, WT1 transcriptional targets might also be expected among the sets of differentially expressed genes reported here. Several targets of WT1 identified in cell line systems and in normal development were not differentially expressed between class 1 and class 2 Wilms' tumors in our study, whereas other candidate WT1 target genes were found differentially expressed in the predicted direction, but did not pass our statistical criteria. Specifically, p21, podocalyxin, and amphiregulin do not appear among the class 1 signature genes. For podocalyxin, this is trivially accounted for by its lack of its representation on the GeneChip; for amphiregulin, the mRNA levels were not statistically different in the two classes of Wilms' tumors, being low but detectable in

Table 3. Genes Highly Expressed in WT1-mutant Compared to WT1-Wild-Type Wilms' Tumors

Probe set	Gene description	GenBank	Gene category	Fold difference	P value
32872_at	cDNA DKFZp564I083	AL049279	Intronic ITF2 transcript	6.5	5.50E-09
40380_at	RNA binding protein ELAVL4 HuD antigen	M62843	RNA binding protein	2.6	2.30E-08
37183_at	GAD1 67-kDa isoenzyme	M81883	Metabolic enzyme	12.0	4.41E-08
38356_at	Follistatin precursor (FST)	M19481	Signaling ligand	7.1	8.17E-08
40398_s_at	Mesenchyme homeo box 2 (MEOX2)	AI743406	Myogenic transcription factor	7.1	1.45E-07
40399_r_at	Mesenchyme homeo box 2 (MEOX2)	AI743406	Myogenic transcription factor	7.4	2.54E-07
1262_s_at	Transforming growth factor-beta-2 precursor (TGFB2)	M19154	Signaling ligand	4.9	7.09E-07
38112_g_at	Versican (CSPG2)	X15998	Extracellular matrix protein	3.9	7.09E-07
35178_at	Wnt inhibitory factor-1 (WIF1)	W27944	Extracellular Wnt inhibitor	9.4	1.14E-06
39842_at	Cytokine receptor family (CLF1)	AF059293	Signaling receptor	6.8	1.14E-06
39665_at	Glycine receptor beta subunit (GLRB)	U33267	Signaling receptor	3.6	1.14E-06
33235_at	KIAA0938 (NAV3)	AB023155	Cytoskeletal regulation?	4.9	1.80E-06
35226_at	Eyes absent homolog 2 (EYA2)	U71207	Transcription factor	4.4	2.79E-06
38111_at	Versican (CSPG2)	X15998	Extracellular matrix protein	3.2	2.79E-06
35961_at	cDNA DKFZp586O1318	AL049390	Unknown function	4.3	4.24E-06
38396_at	mRNA for 3' UTR of unknown protein	Y09836	Unknown function	3.5	4.24E-06
38972_at	LOC115207 hypothetical protein BC013764	AF052169	Unknown function	3.2	4.24E-06
214_at	Homeobox protein HOX7	M97676	Transcription factor	2.9	4.24E-06
40856_at	PEDF	U29953	Neurotrophic serpin	2.6	9.31E-06
40199_at	Homeobox protein HOX7	M97676	Transcription factor	4.7	9.31E-06
41544_at	Serum-inducible kinase (SNK)	AF059617	Signaling kinase	3.3	9.31E-06
33240_at	SEMACP3	AB029018	Semaphorin associated protein	2.6	9.31E-06
39608_at	Transcription factor SIM2 long form mRNA	U80456	Transcription factor	4.3	1.35E-05
37154_at	Protocadherin 68 (PCH68) mRNA, complete cds.	AF029343	Adhesion molecule	3.9	1.35E-05
31609_s_at	Procollagen C-endopeptidase enhancer (PCOLCE)	L33799	Extracellular matrix synthesis	2.8	1.35E-05
41348_at	Iroquois-class homeodomain protein IRX-2a	U90304	Transcription factor	4.8	1.93E-05
36455_at	Collagen alpha 3 type IX (COL9A3)	L41162	Extracellular matrix protein	3.3	1.93E-05
1731_at	Platelet-derived growth factor receptor alpha (PDGFRA)	M21574	Signaling receptor	3.3	1.93E-05
37829_at	GAS2	U95032	Cytoskeletal protein	3.9	5.22E-05
41405_at	SFRP4	AF026692	Extracellular Wnt inhibitor	3.8	7.11E-05
971_s_at	Transforming growth factor-beta-2 precursor (TGFB2)	Y00083	Signaling ligand	2.9	7.11E-05
31513_at	LBX1	X90828	Myogenic transcription factor	5.9	9.59E-05
41073_at	G protein-coupled receptor 49 (GPR49)	AI743745	Signaling receptor	4.8	9.59E-05
34631_at	Eyes-absent homologue 4 (EYA4)	Y17114	Transcription factor	3.6	9.59E-05
1113_at	Bone morphogenetic protein 2A (BMP2A)	M22489	Signaling ligand	3.0	9.59E-05

both types of tumors, while p21 mRNA showed substantial case-to-case variability. Another candidate WT1 target, the cyclin E gene, was also expressed robustly in all of the Wilms' tumors, regardless of WT1 status. The E-cadherin gene, which is WT1-responsive in some cell types, was variably expressed, although at levels much reduced from normal fetal kidney controls, without a significant difference between WT1-mutant and WT1-replete tumors. The connective tissue growth factor gene (*CTGF/IGFBP8*), previously identified as induced (3.1-fold) by a dominant-negative mutant form of WT1 in a cell line from an aggressive Wilms' tumor²⁷ did not pass our statistical and absolute expression value cutoffs, but examination of our primary expression data showed that it was in fact modestly (2.3-fold) overexpressed in class 2 (WT1-mutant) compared to class 1 (WT1-replete) tumors, consistent with its candidacy as a target gene for repression by wild-type WT1. Similarly, *TGFB2* mRNA was identified in that previous screen (induced 2.9-fold by the dominant-negative WT1 construct), and this gene also appears in our class 2 signature set, consistent with its candidacy as a target for repression by wild-type WT1.

In agreement with our data, *CMYC* was recently independently reported as differentially expressed in WT1-mutant Wilms' tumors,²⁸ and from this evidence, as well

Figure 6. Exogenous TGF- β 2 induces an epithelial-mesenchymal transition and a partial shift to a class 2 expression pattern in a class 1 Wilms' tumor explant. **A:** Cells explanted from a primary class 1 Wilms' tumor were exposed to TGF- β 2 in complete medium, or to complete medium alone (control), and photographed under phase-contrast after 4 days. **B:** The same explant cells were exposed to the indicated concentrations of TGF- β 2 for 4 days and then harvested for RNA extraction. The Northern blot was hybridized with the indicated cDNA probes. Ethidium bromide (EtBr) staining of 28S and 18S RNA is shown as a loading control. The class 2 signature gene *CSPG2* is induced, whereas the class 1 signature genes *IFI16* and *PAX8* are suppressed.

Figure 7. β -Catenin/TCF responsiveness of the *GAD1* promoter. The *GAD1* promoter is activated by co-transfection of oncogenic β -catenin (S37A), and dominant-negative TCF4 (TCF4-dn) abrogates this effect. Site-directed mutagenesis of the single TCF binding site (pGAD1- Δ TCF) abrogates inducible promoter activity. The pTopFlash construct (pTop), containing four consensus TCF sites, was used as a positive control and a version of this construct with the TCF sites mutated (pFop) was used as the negative control: pTop luciferase signal was elevated 6 $\times$ over the signal obtained with pFop (not shown).

as a previous study using cell transfections,²⁹ this gene was proposed as a candidate WT1 target. Based on the uniform overexpression of β -catenin/TCF targets in class 2 Wilms' tumors, and the fact that *CMYC* is a β -catenin/TCF target in other cell types, it seems likely that overex-

Figure 8. Similarities in the expression of β -catenin/TCF targets in class 2 Wilms' tumors with and without *CTNNB1* hotspot mutations. Gene expression is shown relative to the mean value for the entire experiment, including Wilms' tumors and control fetal kidneys. *CMYC*, *FST*, and *CSPG2* are class 2 signature genes that β -catenin/TCF targets base on published evidence^{17,20} and *GAD1* is a class 2 signature gene that is a direct β -catenin/TCF target based on the current work. *WIF1* is a strong class 2 signature gene that has not yet been evaluated as a direct β -catenin/TCF target but whose overexpression may be related to the constitutive stabilization of β -catenin in the class 2 tumor cells (see text). The class 2 cases are subdivided into those with *CTNNB1* exon 3 mutations (labeled 2) and those lacking such mutations (labeled 2'), and the class 1 cases are labeled 1. *t*-Tests for nonequivalence of the mRNA expression distributions indicate similar patterns of gene expression in class 2 tumors with and without *CTNNB1* hotspot mutations. The *t*-test *P* values are: *CSPG2* 2 versus 2' (0.57), 2 versus 1 (2.41E-05), 2' versus 1 (2.01E-04); *FST* 2 versus 2' (0.76), 2 versus 1 (3.00E-05), 2' versus 1 (4.27E-04); *CMYC* 2 versus 2' (0.83), 2 versus 1 (0.014), 2' versus 1 (0.027); *GAD1* 2 versus 2' (0.084), 2 versus 1 (2.09E-05), 2' versus 1 (1.01E-06); *WIF1* 2 versus 2' (0.45), 2 versus 1 (1.25E-06), 2' versus 1 (3.42E-04). *HAS2* is shown as an example of a class 1 signature gene.

Figure 9. Functional assessment of non-hotspot *CTNNB1* mutant alleles. **A:** Map of *CTNNB1* mutations in the class 2 Wilms' tumors. The exon 3 mutational hotspot, containing serine and threonine phosphorylation sites, and several regions of important protein-protein interactions are indicated. The shaded rectangles indicate the armadillo-repeats.³¹ **B:** Co-transfection assays comparing the ability of wild-type (wt) and mutant alleles in GFP-fusion constructs to activate transcription from pTopflash (pTop) or the negative control plasmid pFopflash (pFop). The luciferase values were sequentially normalized, first to the Renilla luciferase internal control and then to the mean value for the wild-type constructs. Each bar represents the results obtained with a single plasmid preparation transfected in triplicate. The non-hotspot mutant alleles activate pTop more strongly than the matched wild-type allele, but less strongly than the hotspot mutant alleles. GAL, β -galactosidase control plasmid.

pression of *CMYC* in these tumors is at least in part because of hyperactivation of the β -catenin/TCF pathway. Loss of WT1 may play a synergistic role in activating c-Myc in class 2 Wilms' tumors.

Our data also have implications for the histopathology of syndromic Wilms' tumors. These WT1-mutant tumors are known to have, in general, a more extensive stromal (mesenchymal) component than their sporadic, WT1-replete, counterparts.¹⁶ The TGF- β 2 protein, which is a class 2 Wilms' tumor signature gene, is a potent mesenchyme-inducer in many cell systems, and we have shown here that it can have this effect on Wilms' tumor cells. Relative overexpression of *TGFB2* may therefore contribute to the abundance of the neoplastic stromal component in the class 2 tumors. Interestingly, we observed nuclear β -catenin only in the malignant stromal component, suggesting that the stromal component may be important for the malignant behavior of these tumors. Similarly, the strong overexpression of the myogenic transcription factor genes *MEOX2* and *LBX1*, also identified as class 2 signature genes in this study, may account for the focal myogenic (skeletal muscle) differentiation that is another consistent feature of WT1-mutant Wilms' tumors.^{15,30}

Overall, our findings, together with the previous observation that homozygous mutation of *Wt1* is cell-lethal in a mouse knockout³ suggests that β -catenin stabilization in *WT1*-mutant tumor progenitor cells may be necessary for survival and proliferation of these cells. Our data in no way exclude that *WT1* transcriptional targets may play a direct role in tumor suppression by inhibiting cell proliferation, but our positive findings support an alternative model in which the tumor suppressor role of the *WT1* gene is explained by a less direct biological mechanism. In this scenario, loss of function of *WT1* in primitive metanephric cells predisposes these cells to apoptosis, thereby providing an ongoing selection, during kidney growth, for cell rescue and clonal expansion via activation of the Wnt/ β -catenin pathway. Alternatively, absence of *WT1* and stabilization of β -catenin may be synergistic and continuously necessary for growth of class 2 Wilms' tumors. These hypotheses make different predictions, which may be testable in mouse genetic models.

Lastly, we have pointed out a minor subset of class 1 Wilms' tumors that, despite lacking *CTNNB1* mutations, show an expression profile intermediate between the class 1 and class 2 consensus patterns. In addition, 25% of class 2 Wilms' tumors lack *CTNNB1* mutations, but the tumor cells in these cases nonetheless overexpress β -catenin/TCF target genes, adhering to the consensus class 2 signature. Whether these tumors contain alternative genetic lesions that activate the Wnt/ β -catenin signaling pathway will be an interesting topic for future investigation.

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