WT1, *WTX* and *CTNNB1* mutation analysis in 43 patients with sporadic Wilms' tumor

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Abstract. Wilms' tumor (WT) is a heterogeneous neoplasia characterized by a number of genetic abnormalities, involving tumor suppressor genes, oncogenes and genes related to the Wnt signaling pathway. Somatic biallelic inactivation of *WT1* is observed in 5-10% of sporadic WT. Somatic mutations in exon 3 of *CTNNB1*, which encodes β-catenin, were initially observed in 15% of WT. *WTX* encodes a protein that negatively regulates the Wnt/β-catenin signaling pathway and mediates the binding of WT1. In this study, we screened germline and somatic mutations in selected regions of *WT1*, *WTX* and *CTNNB1* in 43 WT patients. Mutation analysis of *WT1* identified two single-nucleotide polymorphisms, one recurrent nonsense mutation (p.R458X) in a patient with proteinuria but without genitourinary findings of Denys-Drash syndrome (DDS) and one novel missense mutation, p.C428Y, in a patient with Denys-Drash syndrome phenotype. *WT1* SNP rs16754A>G (R369R) was observed in 17/43 patients, and was not associated with significant difference in age at diagnosis distribution, or with 60-month overall survival rate. *WTX* mutation analysis identified five sequence variations, two synonymous substitutions (p.Q1019Q and p.D379D), a non-synonymous mutation (p.F159L), one frameshift mutation (p.157X) and a novel missense mutation, p.R560W. Two sequence variations in *CTNNB1* were identified, p.T41A and p.S45C. Overall survival of bilateral cases was significantly lower (P=0.005). No difference was observed when survival was analyzed among patients with *WT1* or with *WTX* mutations. On the other hand, the survival of two patients with the *CTNNB1* p.T41A mutation was significantly lower (P=0.000517) than the average.

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Introduction

Wilms' tumor (WT) is a heterogeneous neoplasia characterized by several genetic and epigenetic abnormalities, involving tumor suppressor genes, oncogenes and genes related to the Wnt signaling pathway. The incidence of WT is 1/10,000 and bilateral presentation is observed in 10% of affected individuals. In approximately 1-2% of WT, recurrence occurs in the family (1).

The *WT1* gene is an essential regulator of kidney development, critical to the survival and subsequent differentiation of kidney cells (2). The *WT1* somatic, biallelic inactivation is seen in 5-10% of sporadic WT (1). The WT1 protein contains an amino-terminal transactivator and a carboxyl-terminal DNA-binding domain consisting of four zinc fingers. Alternative *WT1* splicing results in four different isoforms of the protein, and the most abundant isoform (+KTS) is generated by insertion of amino acids lysine, threonine and serine (KTS), coded by exons 9 and 10 (3-6). Exon 9 represents an important target for germline mutations associated with Denys-Drash syndrome (DDS), and specific constitutional point mutations affect the properties of WT1 to bind with EGR1 (early growth response 1) consensus sequence (7).

Mutations in exon 3 of the *CTNNB1* gene, which encodes β-catenin, were initially observed in 15% of tumor samples from WT patients (8). The β-catenin N-terminal region contains consensus phosphorylation sites for the serine/threonine kinase GSK-3β (glycogen synthase kinase 3β) protein, whose function is to phosphorylate β-catenin at multiple sites (Ser33, Ser37, Thr41 and Ser45). In the absence of signs of

growth and differentiation, this phosphorylation results in β-catenin degradation mediated by ubiquitin (9,10). β-catenin stabilization in the nucleus activates the Wnt signaling pathway mediated by β-catenin/TCF (11), leading to the deregulation of β-catenin signaling, which is critical for the development of various malignancies, including WT (8).

Rivera *et al* (2) identified another gene, *WTX*, found inactivated in one third of the studied WT samples. The WTX protein forms a complex with β-catenin, APC, and other proteins to negatively regulate the Wnt/β-catenin signaling pathway, leading to the degradation of β-catenin (12). Rivera *et al* (13) showed that WTX shuttles between the cytoplasm and the nucleus, and mediates the binding of WT1, modulating its activity. Mutations in *WTX* and *WT1* were initially thought to be mutually exclusive, while most mutations observed in *CTNNB1* coincided with *WT1* mutations (2).

In the present study, we screened germline and somatic mutations in the *WT1* exons 8, 9 and 10, the *WTX* coding region and the *CTNNB1* exon 3 in 43 WT patients.

Materials and methods

Patients. This study involved 43 patients with documented WT. All tumor samples were collected following neoadjuvant chemotherapy. This study was approved by the local ethics committee and the parents or tutors of all participant patients signed an informed consent.

DNA extraction. DNA extraction from peripheral blood and fresh tumor samples followed procedures established by Miller *et al* (14) and Sambrook *et al* (15).

Sequencing of WT1, WTX and CTNNB1. Blood and fresh tumor DNA samples were screened for *WT1*, *WTX* and *CTNNB1* mutations with previously reported primers (2). PCR reactions contained 5 pmol of forward and reverse primers (Prodimol), 1 μm dNTPs (Life Technologies), 0.9 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.0), 25 μM KCl, 1 U Taq DNA polymerase (Life Technologies) and 100 ng of DNA in a 25 μl final volume. PCR conditions for *WT1* exons 8, 9 and 10 assay consisted of 94˚C for 5 min, 35 cycles at 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 30 sec and 72˚C for 7 min. PCR conditions for *WTX* exon 2 assay consisted of 94˚C for 5 min, 35 'step down' cycles at 94˚C for 30 sec, 66˚C (decreasing 0.3˚C per cycle) for 30 sec and 72˚C for 30 sec and 72˚C for 7 min. Finally, *CTNNB1* exon 3 amplification conditions were 94˚C for 5 min, 35 'step down' cycles at 94˚C for 30 sec, 60˚C (decreasing 0.2˚C per cycle) for 30 sec, 72˚C for 30 sec and 72˚C for 7 min. PCR products were purified using the GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare) and were subjected to nucleotide sequencing using BigDye v3.1 (Life Technologies). DNA samples and reference sequences (NG_009272 for *WT1*; NG_021345 for *WTX*; and NG_013302.1 for *CTNNB1*) (16) were aligned and compared to identify homozygous and heterozygous nucleotide positions using ChromasPro v. 1.41 and MEGA 5 software.

Statistical analysis. Kaplan-Meier curves were used to estimate 60-month survival rates and overall survival. The Mann-Whitney U test was used to compare age at diagnosis.

Results

Our sample consisted of 43 unrelated patients, 18 females and 25 males, diagnosed with WT. Bilateral disease was diagnosed in nine patients. WT mean age at diagnosis was 43 months for the whole sample (ranging from 4 to 137 months) and 32 months for bilateral cases only. Five patients also presented major phenotypic abnormalities: one with Beckwith-Wiedemann syndrome, one with hemihypertrophy, two with non-syndromic macrosomia and one with Denys-Drash syndrome. Table I shows clinical, histopathological and molecular data of all patients.

Mutation analysis of *WT1* exons 8, 9 and 10 identified four sequence variants, namely, two single-nucleotide polymorphisms (SNPs), one novel missense mutation and one nonsense mutation. The synonymous sequence variant, SNP rs16754 (p.R369R), located at exon 8, was the most frequent mutation, having been observed in 17 patients. With the exception of case 7, all blood samples were heterozygous for this SNP, and no loss of heterozygosity (LOH) was observed in the available tumors. The only possible case of LOH was patient 38, whose tumor sample showed this variation in a homozygous (or hemizygous) state, but no blood sample was available from this patient. The second most frequent sequence variant, located at intron 9, was SNP rs2234593, observed in five patients. In three of these patients (patients 13, 35 and 43) blood and tumor samples were studied and did not show LOH. The novel missense mutation, p.C428Y (g.47820G>A; c.1283G>A) (Fig. 1A), was observed in heterozygosis in patient 44, a female patient with bilateral WT diagnosed at 12 months and clinical findings of Denys-Drash syndrome. Finally, patient 41 presented a nonsense g.48510C>T (c.1372C>T) transition, resulting in the replacement of an arginine for a stop codon (p.R458X) (Fig. 1B). This mutation was observed in heterozygosis in both blood and tumor samples. This male patient developed unilateral blastematous WT diagnosed at 25 months and proteinuria without other clinical findings of Denys-Drash syndrome.

Analysis of *WTX* exon 2 identified five sequence variants, two synonymous substitutions (rs61730681 and rs150075206), a non-synonymous mutation (rs34677493), a novel missense mutation and one frameshift mutation. SNP rs61730681 (p.Q1019Q) was observed in four female and two male patients, and LOH was observed in two of the four female carriers. SNP rs150075206 (p.D379D) and the non-synonymous mutation rs34677493 (p.F159L) were observed, respectively, in one female and one male patient, in both cases in association with SNP rs61730681. The novel missense mutation p.R560W, resulting from a C>T transition at position g.19136 (c.1678C>T) (Fig. 1C), was identified in hemizygosis in both blood and tumor samples of one male patient (patient 18), whose unilateral tumor showed focal anaplasia. Mutation g.17896insT (c.439insT), resulting in a frameshift and subsequent stop codon in the protein (p.157X) (Fig. 1D) was observed in the tumor sample of one male patient.

Two sequence variants in the *CTNNB1* gene were identified in three patients, in all cases in a heterozygous state in the tumor samples. Sequence variation rs121913409 predicts the frequently described missense mutation p.S45C and was observed in one patient (patient 37). Variation rs121913412,

Table I. *WT1*, *WTX* and *CTNNB1* mutations, histopathology of the tumors, and patient clinical data.

Patients	Gender	Laterality	Histopathology	Dx age	*WT1* exons 8, 9, 10	*WTX* exon 2	*CTNNB1* exon 3	Phenotype
7	F	U	Tri	44	rs16754 (B)			Hemihypertrophy
8	M	B	ILNR	6	rs16754 (B)			
9	F	U	Tri	48	rs16754 (B)			
10	F	U	Tri	10	rs2234593 (B)			
11	M	U	Bl	136	rs16754	rs34677493 + rs61730681		
13	F	U	Ep	62	rs2234593			
14	M	U	Ep	59	rs16754			
16	M	B	DA	29	rs16754			
18	M	U	FA	36		R560W		
19	F	U	Tri	48		rs61730681 (LOH)		
21	F	U	Bl	4		rs61730681 (T, het)		
22	F	U	Tri	28			T41A (T)	Macrosomia
23	M	U	FA	57	rs16754	rs61730681		
24	F	U	Tri	13		rs150075206 + rs61730681 (het)		
25	M	U	Tri.	42	rs16754			
27	M	B	Tri, PLNR	61			T41A (T)	
28	M	U	Tri	67	rs16754 (B)			
32	F	U	Bl	7	rs16754 (T)			
34	F	U	Tri	35		rs61730681 (LOH)		
35	M	U	Tri	47	rs16754 + rs2234593			Beckwith-Wiedemann syndrome
36	M	U	Bl, DA	32	rs16754 (T)			
37	M	U	Tri	47			S45C (T)	
38	F	U	Bl/Ep, PLNR	19	rs16754 (T)			Macrosomia
39	M	U	Tri	28	rs16754 (B)			
40	M	U	Tri	28		g.17896insT, c.439insT p.157X (T)		
41	M	U	Bl	25	rs16754 + R458X			Proteinuria
42	M	U	St	56	rs16754 (B) + rs2234593 (B)			
43	F	U	Tri	12	rs16754 + rs2234593			
44	F	B	NA	12	C428Y (B)			Denys-Drash syndrome

NA, not available; M, male; F, female; Dx age, age at diagnosis (months); Tri, triphasic; Bl, blastemal; Ep, epithelial; St, stromal; DA, diffuse anaplasia; FA, focal anaplasia; PLNR, perilobar nefrogenic rests; ILNR, intralobar nephrogenic rests; (B), blood sample only; (T), tumor sample only; (LOH), loss of heterozygosis; (het), heterozygosis.

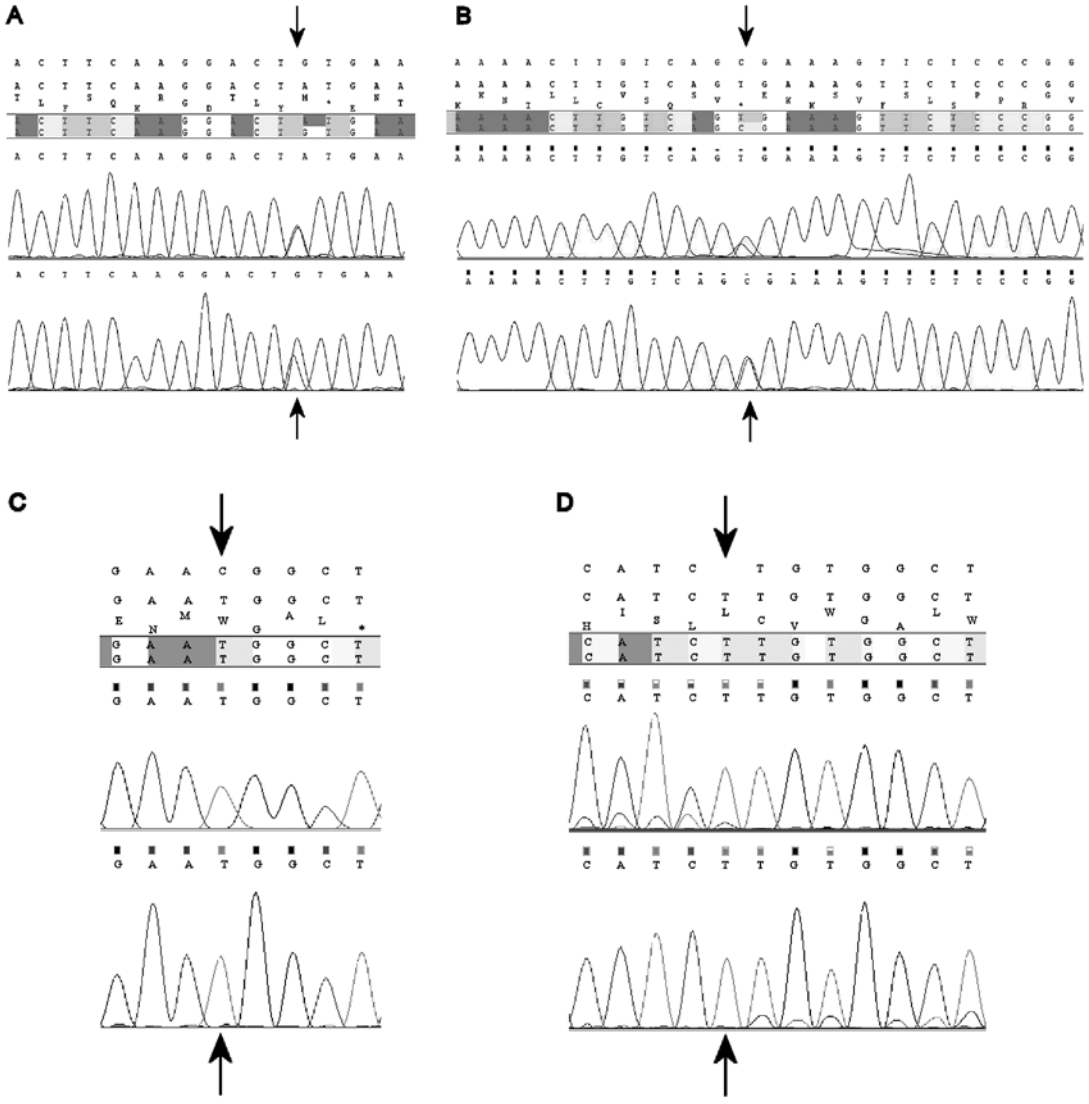

Figure 1. Electropherograms of *WT1* and *WTX* mutations observed in this study. (A) Electropherogram representing the novel *WT1* exon 9 missense mutation p.C428Y (g.47820 G>A) in heterozygosis; (B) electropherogram demonstrating *WT1* exon 10 nonsense transition (c.1372C>T), g.48510C>T, mutation p.R458X in heterozygosis; (C) the novel missense mutation p.R560W, resulting from a C>T transition at position g.19136 (c.1678C>T) in hemizygosis; (D) mutation g.17896insT (c.439insT), resulting in a frameshift and subsequent stop codon in the protein (p.157X) in hemizygosis. Black arrows indicate the mutations.

predictive of missense mutation p.T41A, was observed in two patients (patients 22 and 27).

The overall 60-month survival rate for the whole sample was approximately 80%. Overall survival of bilateral cases was significantly lower (P=0.005) (Fig. 2A). No difference was observed when survival was analyzed among patients with *WT1* mutations (P=0.778) (Fig. 2B), or in patients with *WTX* mutations (P=0.594) (Fig. 2C). On the other hand, survival of patients 22 and 27, carriers of the p.T41A mutation in *CTNNB1*, was significantly lower (P=0.000517) than the average (Fig. 2D). Overall survival of WT1 rs16754 carriers did not differ from the rest of the sample (P=0.561) and age at diagnosis did not differ between carriers and non-carriers of this sequence variant (P=0.817; data not shown).

Discussion

In the present study we screened for mutations in selected regions of *WT1*, *WTX* and *CTNNB1* in 43 WT patients.

In *WT1*, rs16754A>G, predictive of the synonymous mutation p.R369R, was the most frequently observed sequence variant (17/43 patients). No LOH was observed in 8/17 patients whose blood and tumor samples were analyzed. According to Milani *et al* (17), rs16754 corresponds to a cis-acting genetic variation regulating WT1 expression levels. This SNP has been associated with better overall survival in pediatric acute myeloid leukemia patients, and among rs16754 carriers, an increased expression of WT1 mRNA was observed (18). In our sample, allele rs16754G was not associated with differential age at diagnosis (P=0.817), or overall survival (P=0.561) among carriers.

Sequence variation *WT1* rs2234593 was present in five patients and in three of these no LOH was observed. This intronic variant, apparently, does not alter WT1 splicing sites (19).

The nonsense mutation p.R458X (p.R390X) was observed in one male patient with unilateral WT. This patient also had proteinuria and did not present genitourinary anomalies.

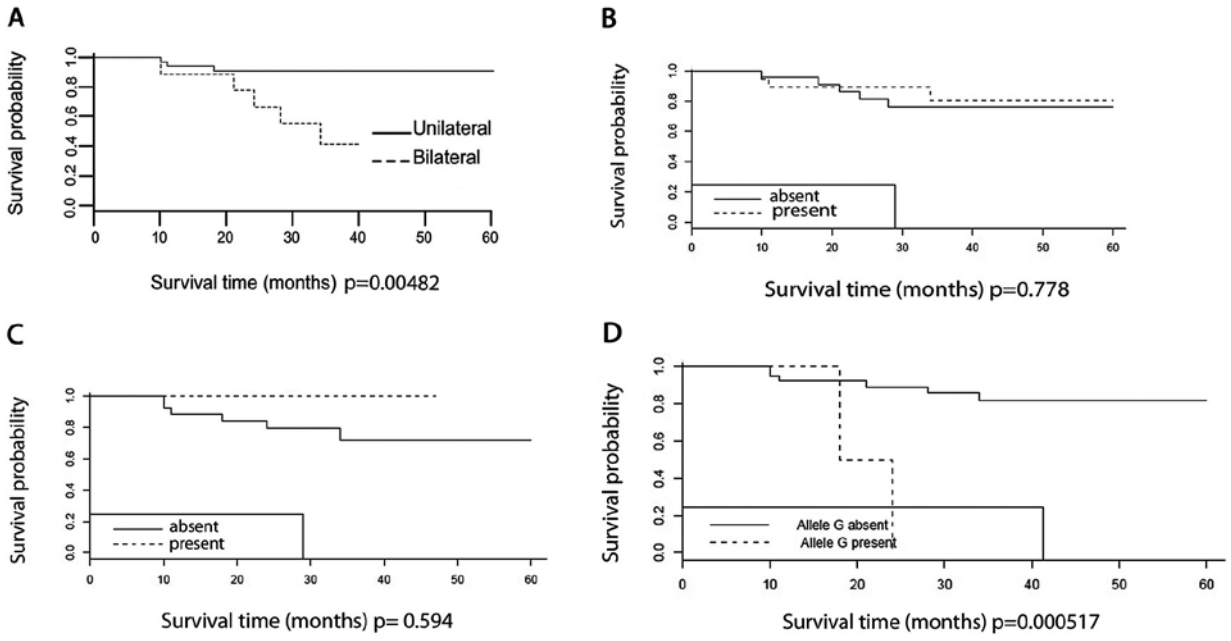

Figure 2. Estimated probability of overall survival. (A) Overall survival of bilateral cases was significantly lower (P=0.005); (B) no difference was observed when survival was analyzed among patients with *WT1* mutations (P=0.778); (C) no difference was observed when survival was analyzed among patients with *WTX* mutations (P=0.594), (D) survival of patients 22 and 27, carriers of the T41A mutation in *CTNNB1*, was significantly lower (P=0.000517) than the average.

This mutation has been frequently described in patients with DDS, and in at least one case of Frasier syndrome (20,21). Royer-Pokora *et al* (20) described three new cases of the *WT1* p.R390X nonsense mutation, and reviewed eight other cases from the literature (22-25), and observed that genitourinary anomalies were not present in 3/10 patients with p.R390X (20). Little *et al* (26) also described two female WT patients with p.R390X and without genitourinary anomalies. Shibata *et al* (25) studied seven cases of WT with rhabdomyogenic components (fetal rhabdomyogenic nephroblastoma), and found five cases with the p.R390X mutation. Corbin *et al* (27) observed the p.R390X mutation in a homozygous state in a tumor sample of one DDS patient who also presented the *CTNNB1* p.T41A mutation in a heterozygous state.

The missense WT1 p.C428Y (g.47820G>A; c.1283G>A;) mutation described in this study was observed in heterozygosis in a female patient with bilateral WT diagnosed at age 12 months. This patient had genitourinary anomalies, and developed early-onset proteinuria and end-stage renal disease. Another missense mutation in the same protein residue, p.C428G, has previously been reported. This mutation changes a cysteine residue important for the coordination of the zinc atom in the zinc finger domain (28).

Sixty-month overall survival among carriers of all WT1 mutations in our sample did not differ from global overall survival, a finding that was also observed by Royer-Pokora *et al* (29).

Five sequence variants were detected in *WTX*, two synonymous mutations (rs61730681; p.Q1019Q and rs150075206; p.D379D), two non-synonymous mutations (rs34677493; p.F159L and p.R560W) and one frameshift mutation. With the exception of p.R560W, all other *WTX* mutations observed in our patients had been previously identified by Rivera *et al* (2).

Transition c.1678C>T, predictive of undescribed missense mutation p.R560W, was observed in one male patient with unilateral WT and focal anaplasia. Corbin *et al* (27) observed another WTX missense mutation, p.T429I, in a WT patient who also presented anaplasia. Germline mutations in *WTX* were described in X-linked dominant osteopathia striata with cranial sclerosis (OSCS) (30), a disease not associated with WT risk. To the best of our knowledge, the *WTX* p.R560W mutation has not been previously described among WT (27,31-37) or OSCS (30,38) patients.

In our sample, 60-month overall survival among carriers of all *WTX* mutations did not differ from global overall survival (P=0.594), as observed by Wegert *et al* (32).

CTNNB1 exon 3 sequencing showed two previously well-known missense mutations (8,39,40) in three patients of our sample, all in heterozygosis: p.T41A in two patients and p.S45C in one patient. These somatic mutations remove a major phosphorylation site for GSK-3β, leading to the stabilization of β-catenin, and they exert a dominant effect at the level of the β-catenin/TCF-mediated transcription; therefore, these mutations may be associated with the development and/or survival of WT (8,39). Notably, the two carries of the p.T41A mutation showed a significantly lower overall survival rate (P= 0.000517) than the rest of the sample. In spite of the small number of p.T41A carriers in our group of patients (two individuals), we could not find an association of this somatic mutation with poorer survival rate among WT patients in the literature.

Additional studies of the impact of *WTX* mutations are essential to better understand the reasons why only somatic, and not germline mutations in this gene result in WT. Also, the interaction of the *WT1*, *WTX* and *CTNNB1* genes within the context of the Wnt signaling pathway, seems to be critical for the development and survival of various malignancies, including WT.

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