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

LEILA C.A. CARDOSO1,2, KELLY R.L. DE SOUZA2, ADRIANA HELENA DE O. REIS2, RAISSA COELHO ANDRADE1,2, ALBERTO C. BRITTO Jr2, MARIA A.F.D. DE LIMA2, ANNA C.E. DOS SANTOS1,2, PAULO S. DE FARIA3, SIMA FERMAN4, HÉCTOR N. SEUÁNEZ1,2 and FERNANDO R. VARGAS1,2,5

1Department of Genetics, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, 21944-970; 2Genetics Program, National Institute of Cancer, Rio de Janeiro, RJ, 20231-050; 3Pathology Division, National Institute of Cancer, Rio de Janeiro, RJ, 20220-400; 4Department of Pediatric Oncology, National Institute of Cancer, Rio de Janeiro, RJ, 20230-130; 5Department of Genetics and Molecular Biology, Federal University of the State of Rio de Janeiro, Rio de Janeiro, RJ, 22011-040, Brazil

Received July 9, 2012; Accepted August 28, 2012

DOI: 10.3892/or.2012.2096

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.

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

Correspondence to: Professor Fernando Regla Vargas, Genetics Program, National Institute of Cancer, 37 André Cavalcanti Street, Rio de Janeiro, RJ, 20231-050, Brazil
E-mail: fvargas@inca.gov.br

Key words: sporadic Wilms' tumor, constitutional, somatic, WT1, WTX and CTNNB1 mutations, overall survival
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 WTX 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 neo-adjuvant 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 (Prodinol), 1 μM dNTPs (Life Technologies), 0.9 mM MgCl₂, 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 assembled 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 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 blastematus 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>
<thead>
<tr>
<th>Patients</th>
<th>Gender</th>
<th>Laterality</th>
<th>Histopathology</th>
<th>Dx age</th>
<th>WTI exons 8, 9, 10</th>
<th>WTX exon 2</th>
<th>CTNNB1 exon 3</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>F</td>
<td>U</td>
<td>Tri</td>
<td>44</td>
<td>rs16754 (B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>B</td>
<td>ILNR</td>
<td>6</td>
<td>rs16754 (B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>U</td>
<td>Tri</td>
<td>48</td>
<td>rs16754 (B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>U</td>
<td>Tri</td>
<td>10</td>
<td>rs2234593 (B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>U</td>
<td>Bl</td>
<td>136</td>
<td>rs16754</td>
<td>rs34677493 + rs61730681</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>U</td>
<td>Ep</td>
<td>62</td>
<td>rs2234593</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>U</td>
<td>Ep</td>
<td>59</td>
<td>rs16754</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>B</td>
<td>DA</td>
<td>29</td>
<td>rs16754</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>U</td>
<td>FA</td>
<td>36</td>
<td></td>
<td>R560W</td>
<td></td>
<td>Macrosomnia</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>U</td>
<td>Tri</td>
<td>48</td>
<td></td>
<td>rs61730681 (LOH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>U</td>
<td>Bl</td>
<td>4</td>
<td></td>
<td>rs61730681 (T, het)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>F</td>
<td>U</td>
<td>Tri</td>
<td>28</td>
<td></td>
<td>T41A (T)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td>U</td>
<td>Ep</td>
<td>57</td>
<td>rs16754</td>
<td>rs61730681</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>F</td>
<td>U</td>
<td>Bl</td>
<td>7</td>
<td>rs16754 (T)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>F</td>
<td>U</td>
<td>Tri</td>
<td>35</td>
<td></td>
<td>rs61730681 (LOH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>M</td>
<td>U</td>
<td>Tri</td>
<td>47</td>
<td>rs16754 + rs2234593</td>
<td></td>
<td></td>
<td>Beckwith-Wiedemann syndrome</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>U</td>
<td>Bl, DA</td>
<td>32</td>
<td>rs16754 (T)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>M</td>
<td>U</td>
<td>Tri</td>
<td>47</td>
<td></td>
<td>S45C (T)</td>
<td></td>
<td>Macrosemia</td>
</tr>
<tr>
<td>25</td>
<td>F</td>
<td>U</td>
<td>Bl/Ep, PLNR</td>
<td>19</td>
<td>rs16754 (T)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>M</td>
<td>U</td>
<td>Tri</td>
<td>28</td>
<td>rs16754 (B)</td>
<td>g.17896insT, c.439insT p.157X (T)</td>
<td>Proteinuria</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>M</td>
<td>U</td>
<td>Bl</td>
<td>25</td>
<td>rs16754 + R458X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>M</td>
<td>U</td>
<td>St</td>
<td>56</td>
<td>rs16754 (B) + rs2234593 (B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>F</td>
<td>U</td>
<td>Tri</td>
<td>12</td>
<td>rs16754 + rs2234593</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>F</td>
<td>B</td>
<td>NA</td>
<td>12</td>
<td>C428Y (B)</td>
<td></td>
<td></td>
<td>Denys-Drash syndrome</td>
</tr>
</tbody>
</table>

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 nephrogenic rests; ILNR, intralobar nephrogenic rests; (B), blood sample only; (T), tumor sample only; (LOH), loss of heterozygosis; (het), heterozygosis.
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.
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 WTI, 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.
Acknowledgements

This study was supported by Conselho Nacional de Desenvolvimento Científico (CNPq) grants 401966/2010-0, 476808/2010-3, 573806/2008-0 and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) E26/170.026/2008.

References