Clinical and Genetic Findings of Five Patients with WT1-Related Disorders

ABSTRACT

Aim: To present phenotypic variability of WT1-related disorders. Methods: Description of clinical and genetic features of five 46,XY patients with WT1 anomalies. Results: Patient 1: newborn with genital ambiguity; he developed Wilms tumor (WT) and chronic renal disease and died at the age of 10 months; the heterozygous 1186G>A mutation compatible with Denys-Drash syndrome was detected in this child. Patients 2 and 3: adolescents with chronic renal disease, primary amenorrhea and hypergonadotrophic hypogonadism; patient 2 had a gonadoblastoma. The heterozygous IVS9+4, C>T mutation, compatible with Frasier syndrome was detected. Patient 4: 9-year-old boy with aniridia, genital ambiguity, dysmorphisms and mental deficiency; a heterozygous 11p deletion, compatible with WAGR syndrome was detected. Patient 5: 2 months old, same diagnosis of patient 4; he developed WT at the age of 8 months. Conclusions: Constitutional abnormalities of WT1 cause gonadal and renal anomalies and predisposition to neoplasia and must be investigated in patients with ambiguous genitalia, chronic renal disease and/or Wilms tumors; primary amenorrhea with chronic renal disease; and aniridia, genital ambiguity and dysmorphisms. (Arq Bras Endocrinol Metab 2008; 52/8:1236-1243)

Keywords: Sex differentiation; WT1 gene; Denys-Drash syndrome; Frasier syndrome; WAGR syndrome

RESUMO

Achados Clínicos e Genéticos de Cinco Pacientes com Anomalias Relacionadas ao Gene WT1.


Descritores: Diferenciação sexual; Gene WT1; Síndrome de Denys-Drash; Síndrome de Frasier; Síndrome WAGR
INTRODUCTION

Sex determination is a complex and yet not fully elucidated process which depends on a complex network of interrelated genes. Gonadal development starts by the end of the 5th week of gestation with the migration of primordial germ cells from the yolk sac to the gonadal anlage. Formation of the primordial gonads, which have no apparent sexual differences up to 8 weeks, depend on the expression of many genes, including SF-1 (steroidogenic factor 1) (1), DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) (2) and WT1 (Wilms tumour 1) (3).

WT1 (OMIM 607102) is located at 11p13 and encodes a zinc finger motif-containing transcription factor involved in regulation of growth and differentiation (4). Beyond its role in the genesis of Wilms tumour (5-6), it regulates early gonad and kidney development (7).

Alternative splicing generates four major WT1 isoforms: an alternative splice site in intron 9 allows the addition of three amino acids (KTS) between zinc fingers 3 and 4 and the fifth exon, encoding 17 amino acids, may or may not be present. These isoforms are highly conserved among different species and play a crucial role in normal gene function. Gene action depends on the predominant isoform: WT1 (-KTS) variants act as transcriptional regulators, while WT1 (+KTS) participates on the regulation of certain genes at the post-transcriptional level (8-10).

WT1 (-KTS) isoforms act in association with the product of SF-1 to promote expression of anti-mullerian hormone (AMH), responsible for regression of the mullerian ducts in male embryos. The product of DAX1 can repress the synergistic action of WT1 and SF-1, resulting in down-regulation of AMH (11). In vitro experiments suggested that WT1 (-KTS) variants are also responsible for transcriptional activation of SRY, which activates the male differentiation pathway (10).

Mutations in WT1 are found in a variety of syndromes, including Denys-Drash (DDS, OMIM 194080), Frasier (FS, OMIM 136680) and WAGR (OMIM 194072) syndromes. Both DDS and FS are characterized by gonadal and renal anomalies and predisposition to neoplasia associated with “de novo” constitutional WT1 point mutations with a negative dominant effect (12).

In DDS, mutations often occur in the zinc finger region abolishing DNA binding capacity and leading to sex ambiguity as a result of dysgenetic testis, diffuse mesangial sclerosis with chronic renal disease and high incidence of Wilms tumour (WT) (13-14). The clinical picture of FS includes dysgenetic gonads with male-to-female sex reversal in 46,XY subjects and pubertal delay in both sexes, nephrotic syndrome and focal segmental glomerulosclerosis leading to chronic renal disease, and high incidence of gonadoblastoma but not of WT. Mutations in FS affect the splice site in intron 9, with WT1(+KTS) isoforms (15-16) losses.

WAGR (WT, aniridia, genitourinary malformations, mental retardation) is a contiguous gene syndrome arising from deletions of chromosome 11p13 which encompass at least both the PAX6 and WT1 genes (17-19).

We report five patients followed in a reference service for disorders of sex development which illustrate the broad spectrum of presentation of WT1-associated disorders.

SUBJECTS AND METHODS

Patients

The five patients were evaluated by the Interdisciplinary Study Group of Disorders of Sex Development (GIEDDS) of the State University of Campinas, São Paulo. The protocol was approved by the local Ethics Committee (N. 434/06) and informed consent was obtained from the parents of the children included in the study.

Laboratory assays

LH, FSH, testosterone were measured by electrochemiluminescence (BM/Hitachi Elecsys 2010, Roche Diagnostics, Boehringer, Mannheim, Germany).

Karyotype

Chromosome analysis of peripheral blood lymphocytes was performed by G-banding at 500-600 bands resolution using standard procedures.

Genomic DNA extraction, amplification and sequencing

Genomic DNA from peripheral blood leukocytes was purified by Proteinase K digestion and phenol/chloroform extraction followed by ethanol precipitation using standard techniques (20).
The 10 exons and the exon-intron junction regions of the WT1 gene were PCR amplified from genomic DNA with primers described in Table 1. Genomic sequence of the WT1 gene was obtained in the published sequence ENSG00000184937 (www.ensembl.org).

The final volume of all reactions was 50 μL and contained 10X Taq DNA polymerase buffer (Invitrogen, CA, USA), 1.0-1.5 mM MgCl2, 2 mM of each dNTP, 20 pmol of each primer, 300-500 ng genomic DNA templates 2 units recombinant Taq DNA polymerase (Invitrogen), 5% DMSO used only for exon 1. After the first denaturation step (5 min, 94°C), the cycling profile was: 94°C, 1 min; 53.5°C – 63.5°C, 1 min; 72°C 1-6 min (30 cycles), followed by 5 min at 72°C (final extension). The size of the PCR products was verified in 1% agarose gel electrophoresis stained with ethidium bromide. Before sequencing, purification of PCR products was performed using the Wizard® SV Gel and PCR clean-up system (Promega, Madison, WI, USA). Further direct sequencing using ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit (ABI PRISM/PE Biosystems, Foster City, CA, USA) was carried out in two separate reactions for each exon, except for exon 1 which required four reactions, using sense and antisense primers (Table 1). The sequences were obtained in an automatic sequencer ABI PRISM 3700 DNA Analyzer (ABI PRISM/PE Biosystems). Free softwares Chromas Lite and CLC Sequence Viewer v.5.0.1 were used to analyze and compare sequences with the published WT1 sequence at Ensembl database.

**RESULTS**

**Case 1**

A 1-month-old child was referred to us due to sex ambiguity. He was born at term after an uneventful preg-

**Table 1. Primers designed for WT1 coding sequence amplification.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Direction</th>
<th>Sequences 5’-3’</th>
<th>nt²</th>
<th>Ta (°C)³</th>
<th>Size (bp)⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>Forward</td>
<td>TGAGTGAATGGAGCGGCCGAG</td>
<td>512-532</td>
<td>60.5</td>
<td>1049</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGGGAAACGCTGGTAAAGAG</td>
<td>1539-1560</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intron 1</td>
<td>Forward</td>
<td>TTCATCAAACAGGAGCGCAG</td>
<td>1211-1230</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intron 1</td>
<td>Reverse</td>
<td>AAAGTGACAGTAAGGAGGC</td>
<td>1269-1288</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 2-3</td>
<td>Forward</td>
<td>CTGTCGAAAAGGTGACATCCAG</td>
<td>7324-7344</td>
<td>57.5</td>
<td>1015</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AATGATAGAGTGAGGACGAC</td>
<td>8313-8338</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intron 2</td>
<td>Forward</td>
<td>TAGATCTTGTGGTGTACATTC</td>
<td>7710-7731</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intron 2</td>
<td>Reverse</td>
<td>AAAGTTGCAATCCTCACTATG</td>
<td>13851-13873</td>
<td>53.4</td>
<td>1406</td>
</tr>
<tr>
<td>Exon 4-5</td>
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<td>GATTGACATATCTGCACTATG</td>
<td>19737-19757</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intron 3</td>
<td>Reverse</td>
<td>ATGCTACCTGATACCCACAG</td>
<td>18678-18700</td>
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<td></td>
</tr>
<tr>
<td>Intron 4</td>
<td>Forward</td>
<td>GGCCTCGTCTCTGCACTCTG</td>
<td>19475-19498</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intron 4</td>
<td>Reverse</td>
<td>AAATGACATCTGACGATGATG</td>
<td>35945-35965</td>
<td>57.5</td>
<td>375</td>
</tr>
<tr>
<td>Exon 6</td>
<td>Forward</td>
<td>GCACCTCAGTACCTGATGATG</td>
<td>36298-36319</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intron 5</td>
<td>Reverse</td>
<td>TGGGGGCTGGGAGGTGGTAATG</td>
<td>13958-13960</td>
<td>56.6</td>
<td>442</td>
</tr>
<tr>
<td>Intron 5</td>
<td>Forward</td>
<td>TGCATCTACAGTGGGCCCCTAG</td>
<td>40004-40027</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 7</td>
<td>Forward</td>
<td>TGGGGACTCAGTGGAGTGTAATG</td>
<td>43258-43277</td>
<td>55.1</td>
<td>1037</td>
</tr>
<tr>
<td>Intron 6</td>
<td>Reverse</td>
<td>TCTCTAACAACCTGAGTCAAGC</td>
<td>44271-44295</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intron 6</td>
<td>Forward</td>
<td>TGAGCGACATGTCAGACATG</td>
<td>43942-43968</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 8-9</td>
<td>Forward</td>
<td>CGGCTTCTCTGCTCAGCAG</td>
<td>46892-46913</td>
<td>56.1</td>
<td>890</td>
</tr>
<tr>
<td>Intron 8</td>
<td>Reverse</td>
<td>GAGCTCTAGTACATTCAACCT</td>
<td>47759-47781</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Internal primers used only for sequencing; nucleotide position in the sequence ENSG00000184937; °Anealing temperature used in PCR;Size of amplified fragments.*
nancy with a birth weight of 3,655 g and length 50 cm. He was the second child of unrelated parents and family history was unremarkable. He had a 2-cm phallus with chordee, penoscrotal hypospadias, shawl scrotum, bilateral cryptorchidism, and there was no dysmorphic picture associated to sex ambiguity. Sonography revealed no mullerian derivatives while genitography showed a urogenital sinus.

His karyotype was 46,XY, and there were normal basal levels of LH (5.4 U/L), FSH (3.8 U/L), free (5.4 pg/mL) and total (157 ng/dL) testosterone (T). When the child was 6 months old, a hCG stimulation test was performed (three intramuscular injections of Chorionic Gonadotropin (Profasi®, 1,000 IU, on successive days), and testosterone levels increased from 157 to 395 ng/dL. He developed unilateral WT and chronic renal disease at 8 months, and died 2 months later as a result of septicemia.

An 1186G>A heterozygous mutation was detected in exon 9 and confirmed the diagnosis of DDS; this case was first reported by Tagliarini and cols. (21).

**Case 2**

A 17.3-year-old girl was evaluated for primary amenorrhea and hypergonadotrophic hypogonadism. She was born at term after an uneventful pregnancy, with a birth weight of 3,560g and length 46cm. She was the second child of unrelated parents, and family history was unremarkable. She was subject to renal transplantation at 11 years as a consequence of chronic renal disease; at the same age, bilateral inguinal hernia repair was performed.

She referred spontaneous pubertal onset. On physical examination, she had normal external genitalia and pubertal development was on Tanner stage B3P2. There was no dysmorphic picture. Ultrasound revealed a 2.8cm³ uterus, and gonads could not be found.

Her karyotype was 46,XY, and there were high levels of FSH (188 U/L) and LH (46 U/L) and low estradiol (11pg/mL). Bilateral gonadectomy was performed, and histology revealed a right dysgenetic gonad with mesonephric remnants and a gonadoblastoma on the left. Female hormonal replacement therapy was initiated later on. Molecular analysis revealed an IVS9+4, C>T heterozygous mutation in intron 9 (Figure 1), thus confirming the diagnosis of Frasier syndrome.

**Case 3**

A 18-year-old girl was referred with a suspected diagnosis of FS to molecular analysis. She was the second child of unrelated parents, and family history was unremarkable. The girl had chronic renal disease treated with peritoneal dialysis, and primary amenorrhea was investigated when she was 15 years old – cytogenetic investigation revealed a 46,XY karyotype, bilateral gonadectomy was performed and histology revealed dysgenetic gonads. She has been on HRT since then.

On physical examination, she had no dysmorphic picture, external genitalia were normal, and breast development was incomplete.

Molecular analysis revealed the same mutation of case 2, thus confirming the diagnosis of FS.

**Case 4**

A 9-year-old boy presented with a history of sex ambiguity, aniridia, mental and motor retardation and dysmorphic features. He was born at term by cesarian section for breech presentation, after an uneventful pregnancy, with a birth weight of 2,550g. He was the
first child in a sibship of three; his parents were not related, and family history was unremarkable.

Physical examination revealed flat occiput, small and dysmorphic ears, short upslanted palpebral fissures, aniridia, nystagmus, short nose with high nasal bridge, clinodactyly of the V fingers, bilateral single transverse palmar crease, predominance of arches on the fingertips, fusiform fingers, nail hypoplasia, increased intermamillary distance and diastasis recti. He had a 4.5cm-phallus, bifid scrotum, penoscrotal hypospadia and nonpalpable gonads.

Ophthalmologic evaluation revealed macular hypoplasia. The testes were not seen on pelvic ultrasound and genitography did not reveal a urogenital sinus.

He had prepubertal levels of LH (<0.2 U/L), FSH (0.4 U/L), total (<20 ng/dL) and free (1 pg/mL) testosterone. An hCG stimulation test was performed, and testosterone levels increased from <20 to 396 ng/dL. Cytogenetic investigation revealed a \textit{de novo} 46,XY,del(11p) karyotype, thus leading to the diagnosis of WAGR syndrome.

Case 5
A 3-month-old boy was evaluated for aniridia and dysmorphic features. He was born in the 38\textsuperscript{th} week of gestation by cesarian section, and intrauterine growth retardation was noted at the 7\textsuperscript{th} month. Birth weight was 2,650g and length 44cm. He was the only child of unrelated parents, and family history was unremarkable.

On physical examination, he presented high forehead, low anterior hairline, dysmorphic ears, anteverted nostrils, notched \textit{alae nasi}, long and flat philtrum, thin upper lip, retrognathism, short neck, single transverse palmar crease and hypoplastic nails. He had a 3.5-cm phallus, bilateral cryptorchidism and hypoplastic scrotum. Ophthalmologic evaluation revealed photophobia, nystagmus, remnants of pupillary membrane and peripheral iris and mottled retinal pigment epithelium.

He had normal levels of LH (9.5 U/L), FSH (8.8 U/L), and total testosterone (669 ng/dL) for age. His karyotype was 46,XY,del (11p) \textit{de novo} (Figure 2), leading to the diagnosis of WAGR syndrome. When he was 8 months old, a unilateral Wilms tumour was detected by sonography. He was subject to nephrectomy, chemotherapy and radiotherapy. There was no tumor relapse until the age of 4 years, and renal function remained normal. Data on these five cases are summarized in Table 2.
Table 2. Description of five 46,XY patients with WT1-related disorders.

<table>
<thead>
<tr>
<th>N</th>
<th>Age at diagnosis</th>
<th>Sex assignment</th>
<th>Clinical picture</th>
<th>Neoplasia</th>
<th>WT1</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 mo</td>
<td>M</td>
<td>penoscrotal hypospadias, BL cryptorchidism, ESRD*</td>
<td>Wilms tumour</td>
<td>1186G&gt;A</td>
<td>Denys-Drash syndrome</td>
</tr>
<tr>
<td>2</td>
<td>17 y</td>
<td>F</td>
<td>Primary amenorrhea, hypergonadotrophic hypogonadism, ESRD</td>
<td>L gonado-blastoma</td>
<td>IVS9+4, C&gt;T</td>
<td>Frasier syndrome</td>
</tr>
<tr>
<td>3</td>
<td>18 y</td>
<td>F</td>
<td>Primary amenorrhea, hypergonadotrophic hypogonadism, ESRD</td>
<td>–</td>
<td>IVS9+4, C&gt;T</td>
<td>Frasier syndrome</td>
</tr>
<tr>
<td>4</td>
<td>9 y</td>
<td>M</td>
<td>Aniridia, perineal hypospadias, BL cryptorchidism, dysmorphic picture, motor and speech delay, mental deficiency</td>
<td>–</td>
<td>11p13 deletion</td>
<td>WAGR</td>
</tr>
<tr>
<td>5</td>
<td>2 mo</td>
<td>M</td>
<td>Aniridia, BL cryptorchidism, dysmorphic picture, motor and speech delay</td>
<td>Wilms tumour</td>
<td>11p13 deletion</td>
<td>WAGR</td>
</tr>
</tbody>
</table>

Bl = bilateral; ESRD = end stage renal disease; F = female; L = left; M = male; NB = newborn; *deceased (10 months)

DISCUSSION

Disorders of gonadal development (DGD) are a highly heterogeneous group of disorders of sex development (DSD) and include individuals with dysgenetic gonads (streaks), dysgenetic or rudimentary testes and true hermaphroditism or ovotesticular DSD. Some 46,XY individuals with DGD are born with sex ambiguity, and thus may be evaluated in infancy. However, those with female internal and external genitalia (male-to-female sex reversal) may be diagnosed only in adolescence because of pubertal delay. The latter are of great concern because of the risk of neoplastic transformation of dysgenetic gonads, which is significantly elevated after adolescence (22). Hormonal activity of gonadoblastoma may be found in some patients (23); in case 2, for instance, there was spontaneous breast development which may be due to an estrogen-producing gonadoblastoma.

Among DGD, WT1-related disorders are characterized by the association of gonadal and renal anomalies. As a consequence, screening for mutations in WT1 should be considered in 46,XY patients with ambiguous genitalia associated with chronic renal disease and (or) WT and in 46,XY females with hypergonadotrophic hypogonadism and history of chronic renal disease, thus allowing the diagnosis of DDS and FS.

In addition, all newborns with aniridia who do not have a family history of this ocular anomaly must be subject to high-resolution cytogenetic testing, which detects deletions involving 11p13 in up to 20% of individuals (24). FISH testing with probes spanning PAX6, WT1, the regions flanking PAX6, and the intervening sequence between PAX6 and WT1 can also be used to detect cryptic deletions in individuals with other clinical features of WAGR and normal cytogenetic studies (24).

Genotype-phenotype correlations in WT1-related disorders are well established. Mutations in DDS patients inactivate DNA binding by the zinc fingers, leading to early and severe impairment of renal function, dysgenetic testes and high incidence of WT, while in FS mutations in the donor splice site of intron 9 of the WT1 gene lead typically to dysgenetic gonads, end-stage renal failure in the second decade and gonadoblastoma. In turn, the reduced haploinsufficiency of WT1 in 11p13 deletion has a less pronounced effect on development, especially on that of the renal system.

However, there are some reports of atypical presentations, including a 46,XY child with sex ambiguity, nephrotic syndrome, gonadal tumour and normal testosterone production despite high levels of gonadotropins, who had a mutation associated with FS (25). In another study, a 46,XY child with sex ambiguity, normal testosterone production, aortic coarctation and no renal disease was found to have a P181S mutation in WT1 inherited from the mother (26).

Diagnosis of WT1-related disorders is more difficult in 46,XX subjects, who lack features of sex ambiguity and sex reversal. However, clinicians must have in mind that WT1 mutations may be found in up to 5% of
cases of steroid-resistant nephrotic syndrome (SRNS) (27-28). Routine evaluation of patients with this syndrome would allow early diagnosis of both DDS and FS in both sexes.

The heterozygous 1186G>A (D396N) mutation in exon 9 of patient 1 leads to an aspartic acid-asparagine substitution changing the structural organization of the third zinc finger of the WT1 protein. It was described in 1991 (13) and is a frequent finding in DDS; the most frequent mutation is 1180C>T (R394W) (29). The apparent dominant-negative nature of DDS mutations results from the action of altered WT1 in blocking the normal activity of the wildtype protein (12).

The heterozygous mutation in intron 9 found in both patients with FS (IVS9+4, C>T) is the most frequent mutation identified in these patients (52%) (15,25). This and the other four different mutations described in intron 9 of WT1 in patients with FS lead to reversal of the (+KTS)/(-KTS) ratio from 2:1 to 1:2 (15-16). Most of the patients with FS show the +4 C>T and +5G>A mutations; this hotspot is probably a consequence of the potential to deaminate 5-methylcytosine at the +4/+5 CpG dinucleotide (16).

Recurrence risk of WT1-related disorders varies according to each specific situation. DDS and FS usually arise as a consequence of de novo mutations, while 11p deletion in WAGR syndrome may be de novo or may result from transmission by a parent with a balanced chromosome rearrangement (24).

In conclusion, constitutional abnormalities of WT1 should be considered in patients with ambiguous genitalia and renal disease (chronic renal disease or Wilms tumors), primary amenorrhea with chronic renal disease (chronic renal disease or Wilms tumors), and those with aniridia, genital ambiguity and dysmorphic features (24). The apparent dominant-negative nature of DDS mutations results from the action of altered WT1 in blocking the normal activity of the wildtype protein (12).

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REFERENCES


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