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Mutations in *SRY* and *WT1* genes required for gonadal development are not responsible for XY partial gonadal dysgenesis

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Abstract

The *WT1* transcription factor regulates *SRY* expression during the initial steps of the sex determination process in humans, activating a gene cascade leading to testis differentiation. In addition to causing Wilms' tumor, mutations in *WT1* are often responsible for urogenital defects in men, while *SRY* mutations are mainly related to 46,XY pure gonadal dysgenesis. In order to evaluate their role in abnormal testicular organogenesis, we screened for *SRY* and *WT1* gene mutations in 10 children with XY partial gonadal dysgenesis, 2 of whom with a history of Wilms' tumor. The open reading frame and 360 bp of the 5' flanking sequence of the *SRY* gene, and the ten exons and intron boundaries of the *WT1* gene were amplified by PCR of genomic DNA. Single-strand conformation polymorphism was initially used for *WT1* mutation screening. Since shifts in fragment migration were only observed for intron/exon 4, the ten *WT1* exons from all patients were sequenced manually. No mutations were detected in the *SRY* 5' untranslated region or within *SRY* open-reading frame sequences. *WT1* sequencing revealed one missense mutation (D396N) in the ninth exon of a patient who also had Wilms' tumor. In addition, two silent point mutations were found in the first exon including one described here for the first time. Some non-coding sequence variations were detected, representing one new (IVS4+85A>G) and two already described (-7ATG T>G, IVS9-49 T>C) single nucleotide polymorphisms. Therefore, mutations in two major genes required for gonadal development, *SRY* and *WT1*, are not responsible for XY partial gonadal dysgenesis.

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Key words
- XY partial gonadal dysgenesis
- *SRY* open reading frame
- *SRY* 5' untranslated region
- *WT1* exons
- Denys-Drash syndrome

Introduction

XY gonadal dysgenesis is a disorder of sexual determination and differentiation that includes a complete (pure) and a partial form. Patients with a 46,XY karyotype and pure gonadal dysgenesis have a female phenotype with full development of unambiguous female genitalia, well-developed Müllerian structures and streak gonads. In contrast, patients with 46,XY partial gonadal dysgenesis are characterized by partial testicular differentiation, low levels of testosterone, ambiguous genitalia, and persistence of Müllerian structures. Patients with both forms are at increased risk for gonadal neoplastic transformation (1). Depending on the severity of testicular dysgenesis, anti-Müllerian hormone levels may be low or undetectable in patients with abnormal testicular determination (2,3). Gonadal histology is typically characterized by poorly developed seminiferous tubules surrounded by wavy ovarian stroma, but may range from apparently normally differentiated testes to streak gonads. According to Berkovitz et al. (4), patients with 46,XY partial gonadal dysgenesis may present a wide range of characteristics depending on the extent of testicular development and they may have either bilateral dysgenetic testes or one dysgenetic testis and one streak gonad. Scolfaro et al. (5) described this histological variability in a series of 13 children with partial gonadal dysgenesis.

Sex-determining genes direct the fate of the bipotential gonad to either testis or ovary. They can be categorized into transcription factors involved throughout gonadal morphogenesis (e.g., *SF1*, *WT1*), inducers of testicular development (*SRY*, *SOX9*), and "anti-testis" genes and potential promoters of ovarian development (*DAX1*, *WNT4*). All of these genes are expressed in the developing genital ridges, and their products interact with each other as part of a complex genetic pathway leading to gonadal differentiation into one sex or the other (6).

Mutations in or abnormal expression of any of such genes will impair the formation of testes in an XY background or the formation of ovaries in an XX background (6). Indeed, mutations in the *SRY* (sex determining region on the Y chromosome), the major gene required for male sex determination, have been reported to account for 20 to 67% of cases of 46,XY pure gonadal dysgenesis (7). In most cases the mutations are located within the high mobility group box, which is a DNA-binding domain essential for SRY function as a transcriptional regulator. Only seven *SRY* mutations of the 44 described so far are found outside this domain (8). Nucleotide changes in the 5' untranslated region (UTR) flanking sequence of the *SRY* gene seem not to be a major cause of disturbance in the male differentiation pathway but a few mutations that might affect *SRY* expression have been reported in XY females (9).

WT1 (Wilms' tumor gene 1) encodes a transcription factor containing four "zinc-finger" motifs which binds to and acts synergistically with SRY to activate transcription from promoters containing SRY-binding sites (10), being also required for the normal development and function of the urogenital tract (11,12). The complexity of WT1 action is reflected at the molecular level, since up to 24 different isoforms may result from a combination of alternative translational sites, alternative RNA splicing and RNA editing (13). Constitutional mutations are associated with familial Wilms' tumor and syndromes such as Denys-Drash syndrome characterized by nephropathy, genital anomalies and often a predisposition to Wilms' tumor (12). Transcripts of the *WT1* gene are detected in the gonadal ridge as early as 6 weeks postconception in human embryos, before testis determination (14). According to Fuqua et al. (15), mutations in the *WT1* gene may result in abnormalities of gonadal ridge formation that delay testis determination. Since the *WT1* gene is also expressed in

the gonadal ridge after testis determination (14), defects in *WT1* may also delay the differentiation after SRY expression.

In this report, we present the data obtained for 10 patients with a diagnosis of partial gonadal dysgenesis who were investigated for mutations in the *SRY* and *WT1* coding and non-coding sequences.

Patients and Methods

The series consisted of 10 patients with a diagnosis of partial gonadal dysgenesis and a 46,XY karyotype. They were examined by the members of the Grupo Interdisciplinar de Estudos da Determinação e Diferenciação do Sexo at Hospital das Clínicas, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil. The protocol was approved by the Ethics Committee of Faculdade de Ciências Médicas, UNICAMP. Informed consent was obtained from the parents of the children included in the study.

The diagnosis of XY partial gonadal dysgenesis was based on findings of ambiguous genitalia and bilateral cryptorchidism, low levels of testosterone and anti-Müllerian hormone (Table 1). All patients showed evidence of Müllerian duct derivatives, which was confirmed by laparoscopy.

Patients 9 and 10 had a personal history of unilateral Wilms' tumor. Patient 10 also had progressive renal failure and died at 13 months of age; at autopsy, kidney evaluation revealed diffuse mesangial sclerosis. This patient had a Denys-Drash syndrome phenotype. No patient had a family history of intersex.

The diagnosis of gonadal dysgenesis was confirmed by a careful histologic and morphometric evaluation of the gonads based on the following criteria: gonadal position, mean tubular diameter, number of germ cells, number of Sertoli cells, and presence or absence of Müllerian and Wolffian duct derivatives. These results were published in a report by Scolfaro et al. (5), in which the present patients 1 to 9 were patients 2, 3, 4, 6, 7, 8, 9, 13, and 12, respectively. At autopsy, bilateral streak gonads were found in patient 10.

Genomic DNA was extracted from peripheral blood leukocytes by standard techniques (17). The *SRY* open reading frame (ORF) was amplified with primers XES10

Table 1. Genital phenotype and hormonal data of 10 children with 46,XY partial gonadal dysgenesis who participated in the study.

Patient	Age (months)	External genitalia[1]	Gonadal position (right/left)	AMH (normal values for age) (pmol/l)	T (nmol/l)[2]	FSH (IU/l)[3]	LH (IU/l)[4]
1	108	2	A/A (left = streak)	114 (234-438)	0.7	5.4	0.9
2	30	2	I/I	52 (360-638)	<0.3	1.2	0.6
3	0.5	3	I/I	98 (251-679)	1.7[5]	3.9	0.8
4	92	2	I/A (left = streak)	113 (234-438)	<0.3	5.8	0.8
5	78	2	I/-	73 (309-566)	1.0	6.3	1.0
6	13	2	I/I	107 (360-638)	<0.3	1.0	0.5
7	20	4	I/A (left = streak)	11 (360-638)	<0.3	1.5	1.1
8	44	3	I/A (left = streak)	71 (360-638)	<0.3	0.9	0.9
9#	29	3	A/A	116 (360-638)	3.1	0.7	0.3
10#	12	2	I/A	NP	<0.3	7.8	3.4

AMH = anti-Müllerian hormone (2); T = total testosterone; FSH = follicle-stimulating hormone; LH = luteinizing hormone; A = abdominal; I = inguinal canal; –, not found; NP = not performed.
[1]According to the classification of Quigley et al. (16). [2]Total testosterone level after the human chorionic gonadotropin stimulation test (normal value: above 1.4 nmol/l). [3]Normal values of prepubertal FSH: 0.1-1.4 IU/l. [4]Normal values of prepubertal LH: 0.1-1.0 IU/l. [5]Basal total testosterone level. #Patient with a past history of Wilms' tumor.

and XES11 described by Hawkins et al. (18). PCR amplifications were performed by the method of Assumpção et al. (8). For the amplification of the 360-bp *SRY* 5' flanking sequence, encompassing the *SRY* putative core promoter, we used the primers and PCR conditions described by Schmitt-Ney et al. (19). Five microliters of PCR amplification products were submitted to direct sequencing with the Thermo-sequenase radiolabeled terminator cycle sequencing kit (Amersham-Pharmacia Biotech, Uppsala, Sweden). Sequencing was carried out twice with products from different PCR procedures and with both sense and antisense primers to confirm the results.

The 10 exons and their flanking regions of the *WT1* gene were amplified by PCR from genomic DNA with primers described in Table 2. Due to its long size and some difficulties in fragment amplification, 3 different primer pairs were used for exon 1. The PCR amplifications were performed as described before for *SRY* (8), except that DMSO was omitted. After a first denaturation step (10 min, 95ºC), the cycling profile was: 95ºC, 1 min; 54-64ºC, 1 min; 72ºC, 1 min (30 cycles), followed by 10 min at 72ºC (final extension). The PCR products were analyzed in 1% ethidium bromide-stained agarose gels to determine the size of the fragments. Amplified products were screened for sequence variations by non-radioactive single-strand conformation polymorphism (SSCP) analysis. Electrophoresis was performed with the PhastSystem™ (Pharmacia Biotech) using 12.5 and 20% non-denaturing polyacrylamide homogeneous Phastgel with PhastGel SDS and native buffer strips and the gels were silver stained. Direct sequencing of PCR products was carried out in two separate reactions using sense and anti-

Table 2. Primers designed for *WT1* amplification.

Exon	Sequences 5′-3′	(nt positions)	Annealing temperature (ºC)	Size (bp)	GenBank accession number
1	1S - AGCCAGAGCAGCAGGGAGT	(665-683)	64	278	X61631
	1AS - ACGACCCGTAAGCCGAAGC	(922-940)			
	1BS - ATGGGCTCCGACGTGC	(790-805)	54	215	
	1BAS - ATGAAGGAGTGAGGCGG	(988-1004)			
	1CS - TTCGGCTTACGGGTCGTTGG	(924-943)	62	397	
	1CAS - CAAAAGGGGTAGGAGAGGGG	(1301-1320)			
2	2S - CCGTCTTGCGAGAGCACC	(3-20)	58	262	M80218
	2AS - CTAATTTGCTGTGGGTTAGG	(245-264)			
3	3S - GCTCAGGATCTCGTGTCTCC	(5-24)	64	318	M80219
	3AS - GCCTCCAAGACCCAGCAT	(305-322)			
4	4S - CAGTTGTGTATTATTTTGTGG	(235-255)	56	285	X61633
	4AS - AACTAGGGGAAGGAGGAAA	(502-520)			
5	5S - CCACTCCCCACCTCTTC	(190-206)	56	115	X61634
	5AS - CGCCATTTGCTTTGCC	(290-305)			
6	6S - CCTTTTTCCCTTCTTTG	(159-175)	52	179	X61635
	6AS - TAAGTAGGAAGAGGCAGT	(320-337)			
7	7S - GCTTAAAGCCTCCCTTC	(157-173)	54	231	X61636
	7AS - CTTGAACCATGTTTGCCC	(370-387)			
8	8S - GAGATCCCCTTTTCCAG	(178-194)	56	177	X61637
	8AS - CACAGCTGCCAGCAATG	(338-354)			
9	9S - CTCACTGTGCCCACATTG	(868-885)	58	210	X61637
	9AS - CAATTTCATTCCACAATAG	(1059-1077)			
10	10S - ACTTCACTCGGGCCTTGATAG	(149-169)	62	276	X61638
	10AS - AGTGGAGAGTCAGACTTGAAAG	(403-424)			

nt = nucleotide; bp = base pairs.

sense primers as described above.

Results

The patients with 46,XY partial gonadal dysgenesis did not present any mutation within the *SRY* 5' UTR or the *SRY* ORF sequence, whereas SSCP analysis of the *WT1* gene revealed shifts only for the fragments obtained with primers 4S and 4AS. After sequencing, it was concluded that the shifts observed in the SSCP gels arose as a result of a non-described nucleotide variation in the 3' flanking region of the fourth exon, located within intron 4 (IVS4+85A>G). Since the SSCP technique was unable to detect sequence variations in the majority of amplified fragments, sequencing of the whole *WT1* coding region was performed. This procedure revealed three single nucleotide polymorphisms (SNPs) already described in normal populations (20) (rs22344582, rs1799925 and rs1799937 according to the Single Nucleotide Polymorphism Database) and a new 163G>A silent point mutation at codon 54 (Pro54Pro; patients 3, 5 and 8). Table 3 shows the patients' genotypes for these polymorphic sites. The only pathogenic mutation in this study was a nucleotide change (1186G>A), which was found in heterozygous condition in patient No. 10. This transition is located within the ninth exon and causes a missense mutation which changes the predicted aspartic acid in codon 396 to asparagine (D396N).

Discussion

The absence of *SRY* mutations observed in the present study reinforces previous literature data which suggest that *SRY* mutations in patients with partial gonadal dysgenesis are much less frequent than in XY pure gonadal dysgenesis (22). Among the 44 different *SRY* ORF mutations reported to date, only three were associated with XY partial gonadal dysgenesis (reviewed by Assumpção et al., 8). One of them was a variant sequence (S18N) also found in normal relatives of the patient (23). The second (R30I) was found in affected and non-affected members of a family, including the father, two siblings with partial gonadal dysgenesis, a phenotypic female with pure gonadal dysgenesis, and three

Table 3. *WT1* single nucleotide polymorphism genotypes in 10 children with partial gonadal dysgenesis.

Patient	Exon 1			Intron 4 +85 23840228[2] A>G	Intron 9 -49 rs1799937[1] T>C
	rs22344582[1] G>T (-7 from first ATG)	rs1799925[1] C>T (Pro42Pro)	23857703[2] G>A (Pro54Pro)		
1	T/T	C/C	A/A	A/A	C/C
2	T/T	C/C	G/G	A/G	T/T
3	G/G	T/T	G/G	G/G	C/C
4	T/T	T/T	G/G	A/G	C/C
5	T/T	C/C	G/G	A/G	C/C
6	T/T	T/T	G/G	A/A	T/T
7	T/T	C/C	G/G	G/G	T/T
8	T/T	C/C	A/A	G/G	T/T
9	T/T	C/C	A/A	A/A	T/T
10	G/G	T/T	G/G	A/A	T/T
Normal male control	T/T	C/C	G/G	A/G	T/T

[1]rs = reference sequence number according to http://www.ncbi.nlm.nih.gov/SNP (20). [2]Numbering according to *H. sapiens* chromosome 11 genomic contig (gi|29807454|ref|NT_009237.15|Hs11_9394[29807454] – http://www.ncbi.nlm.nih.gov/ (21).

non-affected male siblings (8). Both mutations are located in the non-high motility group box, while the third one (Y129N) is located at C-terminal of the high motility group box sequence (24). *In vitro* studies revealed that R30I mutant protein was poorly phosphorylated and showed reduced DNA-binding capacity (7), while the Y129N mutant exhibited altered protein-protein interactions (24). In addition, a case of XY partial gonadal dysgenesis with a *de novo* interstitial deletion at the 3' end of the *SRY* ORF was reported by McElreavey et al. (25), who proposed that this deletion could be responsible for the abnormal gonadal development by diminishing *SRY* expression.

To date, no nucleotide changes in the 5' UTR flanking sequence of the *SRY* gene have been reported in XY partial gonadal dysgenesis. A paternally inherited 3-bp deletion in the *SRY* 5'UTR Sp1 binding site has been demonstrated in a 46,XY female with pure gonadal dysgenesis (Assumpção JG, Maciel-Guerra AT, Marques-de-Faria AP, Guerra Jr G, Scolfaro MR and De Mello MP, unpublished data). The patient's father had surgically corrected hypospadias and penoscrotal fusion as a child and other affected relatives with genital ambiguity have been reported in this family. These findings suggest that, although rare, *SRY* mutations should be screened in cases of partial gonadal dysgenesis, especially if familial recurrence of genital ambiguity is reported.

In the present study, analysis of the *WT1* gene revealed the D396N deleterious mutation in one of the patients with unilateral Wilms' tumor (patient No. 10). This mutation had already been described in patients with Denys-Drash syndrome (26,27), being the third most frequent mutation reported in the Universal *WT1* mutation database (28). According to Little and Wells (29), the Denys-Drash phenotype often arises from the alteration of one allele by a missense point mutation, usually in the zinc finger binding domain of *WT1*. The second and third zinc finger regions, encoded by exons 8 and 9, are considered to be hot spots for mutations in patients with typical Denys-Drash nephropathy (30). Based on experimental evidence, some investigators have claimed that the Denys-Drash syndrome mutations behave in a dominant-negative fashion (27,31,32), while others have proposed that heterozygous point mutations cause functional loss of one *WT1* allele; therefore, haploinsufficiency may be responsible for the phenotype (33).

The constitutional heterozygous missense D396N, identified in patient 10 described here, affects the third zinc finger region of the protein. It has been shown that D396N has a slightly reduced binding affinity for selected DNA sequences when compared with the wild-type WT1 (34). Thus, the clinical phenotype of Denys-Drash syndrome in our patient may be associated with a modest reduction in the DNA binding affinity of WT1, either in a near-haploinsufficiency condition or in a dominant-negative fashion. Moreover, the persistence of the Müllerian ducts in this patient may indicate a *MIS* gene deregulation, since WT1 is an essential factor for the transactivation of *MIS* promoter (35). Indeed, co-transfection experiments with several zinc finger mutant *WT1*s (D396N included) with either *SOX9* or *SF1* failed to activate synergistically reporter constructs containing the *MIS* promoter (36). WT1 is also considered a key regulator of the visceral epithelial cells (podocytes) of the mature glomerulus function, by repressing or activating *PAX2* expression (37,38) and binding to conserved elements within the Podocalyxin gene promoter (39). Reduced expression levels leading to impairment of the podocytes cause crescentic glomerulonephritis and mesangial sclerosis (40) and could explain the early manifestations of nephrotic syndrome in our patient, which began after surgical excision of a left-sided Wilms' tumor at the age of 12 months. In a retrospective study of 12 patients treated for

WT1 gene-related disorders, Auber et al. (30) reported that in Denys-Drash syndrome patients, proteinuria occurred very early and diffuse mesangial sclerosis or mesangial hyperplasia was the cause of end-stage renal disease, in the presence of missense point mutations affecting the third zinc finger.

We were unable to demonstrate any pathogenic mutation after sequencing the 10 exons and the corresponding flanking regions of the *WT1* gene of patient 9 described in this paper, who also had a Wilms' tumor at age four but remained free of renal disease after 5 years of follow-up. However, neither a *WT1* intragenic deletion nor mutations in the promoter region that could significantly affect the transcription rate were excluded in the present case. Baird et al. (27) reported a similar case of an atypical XY Denys-Drash syndrome female patient with Wilms' tumor and ambiguous genitalia, but no nephropathy or *WT1* gene mutations, arguing that such cases raise the possibility of the existence of other genes which may also predispose to this clinical condition.

No *WT1* mutations were detected in patients with partial gonadal dysgenesis without renal abnormalities or Wilms' tumor manifestations (patients 1 to 8). Among the five SNP identified in some of these patients, three can be found in the SNP database, with allelic frequencies obtained from samples of 90 individuals (20). For the *WT1* SNP located at -7 from the first ATG (G>T), the "G" and "T" allele frequencies are of 0.722 and 0.278, respectively. An inverted rate was found for our patients, with the "T" allele being the most frequent one (0.8) but the small size of our sample precludes any conclusion. The "C" and "T" allelic frequencies reported for the *WT1* SNP located at exon 1 are 0.711 and 0.289, respectively. In our sample, the frequencies were of 0.6 and 0.4 for the "C" and "T" alleles, respectively. The third SNP (IVS9-49 T>C) has frequencies of 0.55 and 0.45 reported for the "T" and "C" alleles, respectively. The "T" and "C" allelic frequencies in our patients were of 0.6 and 0.4, respectively. Two not yet described sequence variations were identified in the present study, possibly representing new polymorphisms if we take into account the genotypic variation observed in the patient sample. Although the pathogenic significance of *WT1* SNPs is largely unknown, it is possible to propose that some haplotypes could play a role in the efficiency of mRNA processing or translation due to secondary structures.

Our results suggest that *WT1* gene mutations are not a common cause of 46,XY partial gonadal dysgenesis in the absence of renal abnormalities or Wilms' tumor manifestations. In conclusion, although mutations in the two major genes required for male gonadal development account for a minority of cases of XY partial gonadal dysgenesis, genetic analysis of the *WT1* gene should be performed in these patients looking for Wilms' tumor or nephropathy risk factor. This clearly indicates that mutations at other chromosomal loci should be investigated in order to elucidate these complex clinical situations.

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