Novel *DMRT1* 3'UTR+11insT mutation associated to XY partial gonadal dysgenesis

Nova mutação 3'UTR+11insT no gene *DMRT1* associada à disgenesia gonadal parcial XY

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SUMMARY

The Y-chromosome-located *SRY* gene encodes a small testis-specific protein containing a DNA--binding motif known as the HMG (high mobility group) box. However, mutations in *SRY* are not frequent especially in cases of 46,XY partial gonadal dysgenesis. Several sex-determining genes direct the fate of the bipotential gonad to either testis or ovary. In addition, heterozygous small deletions in 9p can cause complete and partial XY gonadal dysgenesis without other symptoms. Human *DMRT1* gene, which is located at 9p24.3, is expressed in testis and ovary and has been considered, among others, a candidate autosomal gene responsible for gonadal dysgenesis. In this report we describe a nucleotide insertion in *DMRT1* 3'UTR in a patient of XY partial gonadal dygenesis. The 3'UTR+11insT is located within a conserved motif important for mRNA stabilization. Arq Bras Endocrinol Metab. 2010;54(8):749-53

SUMÁRIO

O gene *SRY*, localizado no cromossomo Y, codifica uma proteína testículo-específica contendo um domínio HMG (grupo de alta mobilidade) de ligação ao DNA. No entanto, mutações no gene *SRY* não são frequentes, especialmente nos casos de disgenesia gonadal parcial em indivíduos 46,XY. São atualmente conhecidos vários genes que participam do processo de diferenciação gonadal, tanto para o desenvolvimento testicular quanto para o ovariano. Além disso, pequenas deleções heterozigotas em 9p podem causar disgenesia gonadal XY completa ou parcial, sem outros sintomas associados. O gene *DMRT1* humano, que está localizado em 9p24.3, é expresso no testículo e ovário no período fetal e tem sido considerado um dos genes autossômicos envolvido na etiologia das disgenesias gonadais. Neste trabalho, descrevemos a inserção de um nucleotídeo em 3'UTR do gene *DMRT1* em um paciente 46,XY com disgenesia gonadal parcial. A mutação 3'UTR+11insT está localizada dentro de um motivo conservado importante para a estabilização do mRNA. Arq Bras Endocrinol Metab. 2010;54(8):749-53

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INTRODUCTION

Disorders of sex development (DSD) are defined as congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical (1). XY gonadal dysgenesis (OMIM ID #400044) is a DSD in which the embryonic gonadal development is defective (2). Clinically, XY gonadal dysgenesis may manifest as complete or partial forms. Complete gonadal dysgenesis in 46,XY individuals is characterized by a female phenotype with full development of unambiguous female genitalia, normally developed Müllerian structures, and streak gonads. In general, these patients come for clinical assistance because of delayed puberty (3). Conversely, 46,XY partial gonadal dysgenesis is characterized by partial testicular differentiation therefore ambiguous genitalia is usually observed in the

newborn period. Gonadal histology is variable but frequently consists of hypoplastic testicular tubules intermixed with areas of ovarian stroma (4). Internal ducts typically consist of a combination of Wolffian and Mullerian ducts (4).

The failure in testis development may be a consequence of mutations in the *SRY* gene (5). The Y-chromosome-located *SRY* gene encodes a small testis-specific protein containing a DNA-binding motif known as the HMG (high mobility group) box. However, mutations in *SRY* are not frequent especially in cases of 46,XY partial gonadal dysgenesis (6,7).

Several sex-determining genes direct the fate of the bipotential gonad to either testis or ovary (8). It is well known that heterozygous small deletions in 9p can cause complete or partial XY gonadal dysgenesis (OMIM ID #154230) without other symptoms (9,10). A human gene located at 9p24.3 with sequence similarities to genes that regulate sexual development in insects and nematodes has been described as responsible for XY gonadal dysgenesis. This gene is called *DMRT1* (doublesex and mab-3 related transcription factor 1) and in adults it is expressed in the testis and ovary (11,12). The gene has five exons and is predicted to encode a protein of 373 amino acids with a DM domain near its N-terminal portion (13). There are four *DMRT1* mutations in codons 45, 221, 281, and 295, but their association to a characteristic 46,XY gonadal dysgenesis phenotype is not well defined (13).

We report here a *DMRT1* nucleotide insertion in 3'UTR found by sequence analysis in a case of XY partial gonadal dygenesis. The 3'UTR+11insT is located within a conserved motif important for directing alternative splicing and/or promoting mRNA stabilization.

SUBJECT AND METHODS

Blood specimens and clinical data of the patient and relatives were collected with approval by the appropriate Institutional Review Board; signed informed consent was obtained.

Clinical data

A 7-month-old male infant was referred to us due to sex ambiguity. The child was delivered by cesarean section after an uneventful 39-week pregnancy. Birth weight was 3,100 g and length 48 cm. He was the second child of young healthy unrelated parents. When first examined by us, weight was 6,820 g and length 65.1 cm. There was no dysmorphic picture, and genital examination revealed a 2.3 cm phallus with *chordee*, penoscrotal hypospadias, scrotum with rugae and pigmentation, and both gonads were palpable at inguinal region with 1.0 cm at the greatest diameter. Hormonal evaluation at the age of 7 months revealed normal gonadotropin levels (FSH = 3.1 IU/L, normal range (NR) = 1.5-12.4 IU/L; LH = 0.1 IU/L, NR = 1.7-8.6 IU/L), low levels of total testosterone (after 3 days of 1,500 IU of hCG per day) (0.02 ng/mL, NR > 1.5 ng/mL) and anti-müllerian hormone (114 pMol/L, NR = 265-679 pMol/L), but also normal values of ACTH (21 pg/mL, NR < 46 pg/mL), cortisol (12 µg/dL, NR = 5-25 µg/dL), progesterone (0.7 ng/mL, NR = 0.1-1.4 ng/mL), 17-OH progesterone (0.8 ng/mL, NR = 0.2-1.5 ng/mL), androstenedione (1.0 ng/mL, NR = 0.7-3.6 ng/mL) and DHEA (3.7 ng/mL, NR = 3.0-6.1 ng/mL). G-banding karyotype in 32 cells revealed a 46,XY karyotype. After three injections of testosterone enanthate (50 mg over successive months), phallus size increased 2 cm. The diagnosis of 46,XY DSD due to partial gonadal dysgenesis was confirmed by biopsy of both gonads, which revealed bilateral prepubertal testis with marked tubular hypoplasia, severe germinal hypoplasia and Sertoli cells hyperplasia. This patient is patient number 5 of the paper published by Ribeiro-Scolfaro and cols. (4).

Molecular analysis

Genomic DNA was extracted from peripheral blood leukocytes by the standard phenol/chloroform method.

For the microdeletion investigation, the following polymorphic markers located in the distal part of 9p: D9S143 (14); D9S1779, D9S1858, D9S1813, and D9S54 (http://www.ensembl.org/Homo_sapiens/index.html) were used. Each microsatellite was amplified in an independent radioactive PCR. PCR was performed in a final volume of 12.5 µL containing 50-100 ηg of genomic DNA, 20 ρmol of each primer, 200 M of each dNTPs (0.2 mM dATP + 0.2 mM dTTP + 0.2 mM dGTP + 0.1 mM dCTP + 1 µCi [α-32P] dCTP), 0.25 U Taq DNA polymerase (Invitrogen, CA, USA) reaction buffer for the enzyme, 1.0 mM to 1.5 mM $MgCl_2$. PCR cycles were: 94°C for 5 minutes, 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds (25 cycles) and a final step of 72°C for 5 min. Radioactive PCR products were mixed in a loading buffer containing 95% formamide, 0.05% bromophenol blue, 20 mM EDTA, and pH 8.0. Then the samples were

denatured for 5 minutes at 94°C and applied to denaturing polyacrylamide gel 6%. Electrophoresis was performed at 1500 V, 50 W, 50 mA, for 2-4 hours. Gels were placed in a gel dryer for an hour and then exposed to X-ray films (Hyperfilm MP – Amersham-Pharmacia Biotech) at -70°C for 2-8 hours.

DMRT1 gene was amplified by PCR amplification of the entire coding region including exon-intron junctions and both 5'UTR and 3'UTR regions using synthetic oligonucleotides (Invitrogen) as primers (Table 1) which were designed using Primer 3 open access software (http://primer3.sourceforge.net). The amplified fragments were directly sequenced using Big Dye TM Terminator Cycle Sequencing Kit V3.1 Ready Reaction (ABI PRISM/PE Biosystems, Foster City, CA, USA). The sequences obtained in an ABI3700 Automated Sequencer (ABI PRISM/PE Biosystems) were compared to the normal sequence of the gene (ENSG00000137090).

RESULTS

To investigate the possibility of a 9p deletion in the patient, a study using DNA microsatellite in the critical interval 9p24.3 was carried out. There was no evidence of microdeletion in the *DMRT1* region because the patient was heterozygous for all microsatellite tested, but D9S54.

DMRT1 sequence analysis on the patient's DNA revealed the heterozygous insertion of a thymine located 12 nucleotides beyond the stop codon in the 3'UTR (Figure 1). Sequencing also revealed heterozygosity for a nucleotide change T>A in exon 1 that caused p.S45T protein variation (data not shown), considered to play no biological effect on protein function, therefore a polymorphism (rs16925431-dbSNP125). Unfortunately, parents were not available to evaluate the segregation of both allelic variants.

Table 1. *Primers* for *DMRT1* gene amplification and sequencing

Primer	Sequence (5 → 3')	Tm (°C)
E1FA	CTC CGG AGC GTC GCT GTC CGT CGG	63
EIRA	GAG CCA AGA TCG CGC CAC TAC ACT GC	59
E1FS	TCC CTG GCA GCA GTC TCC AGG CGA G	61
E1RS	AGC CTG GCA ACC GAG CGA GAC TC	57
E2FS	GTG TTT TGG CAA AGC TGA TTC TGG	51
E2RS	TGC AAC CTT CGG CCT CCC TCA TGC	57
E3FS	AGA AGT AAA GTT TCG TGG ACT AAC	47
E3RS	TGC ACA TGC ATG TGG CTT TCA CAC	52
E4F	CAC TGT GCC CAG CCT GTT ACC TTG	56
E4R	AAG CCA TTA GAC ACA GCT AAT GAC	49
E5F	GAA TAA TGA ATG TAT AAA GAC CAG CCA C	50
CR64	CAG CCT CTG GAC TAA ACT CTA AGG	52
CR76	GGC AAA GCG TCT GGG GCG CTA GTG	59

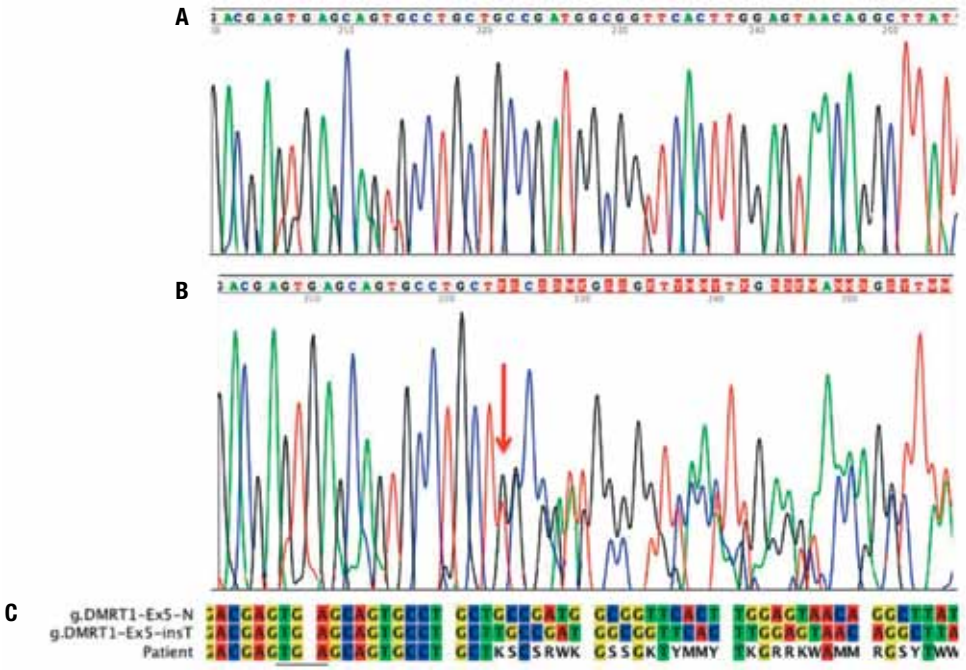

Figure 1. Eletropherograms showing the end of exon 5 and part of 3'UTR of *DMRT1* gene. **(A)** normal sequence. **(B)** Heterozygosis for a T insertion in the patient's sample; the red arrow indicates the insertion site. **(C)** Sequence alignment: sequence on the top shows the normal allele, in the middle, 3'UTR+11insT, and at the bottom sequencing reading of the heterozygous patient; the stop codon TGA is underlined. (K) = G or T; (M) = A or C; (S) = G or C; (Y) = C or T; (W) = A or T.

DISCUSSION

A novel insertion in *DMRT1* gene was identified in the patient described here with 46,XY partial gonadal dysgenesis. *SRY* and *WT1* genes were studied before but the patient did not carry mutations in either gene (7). Therefore, deletions and mutations on 9p and *DMRT1* gene, respectively, were investigated.

Recent studies on *DMRT1* gene have shown a very complex pattern of expression in male gonads involving the production of alternative transcripts in both mice and humans (15,16). Cheng and cols. (15) have characterized multiple transcript isoforms in human testis. These transcripts code for different putative proteins, two of them generated by alternative splicing in the 3'UTR. Other transcripts are results of the intronic exonization including Alu elements that are not randomly distributed in the non-coding regions. In mice, alternative splicing processes were detected in adult testis and in undifferentiated gonads. All forms are produced with a similar pattern of expression peaking at 13.5 days after fertilization and maintaining a baseline pattern of expression in male gonads (16). These experiments indicate an important role of this gene in the testicular determination and differentiation in mammals. The genomic region where the 3'UTR+11insT mutation occurred is recognized as an Exonic Splicing Enhancer by PSEX (Putative Exonic Splicing Enhancers/Silencers) algorithm (17,18). A preliminary in silico analysis using ESEfinder 3.0 to identify exonic splicing enhancers (19,20) indicates the abolishment of a SC35 protein recognition site in the presence of the 3'UTR+11insT mutation.

Herpin and cols. (21) studied mechanisms that regulate the expression of specific genes in the gonads during embryonic development. They found the protein-binding conserved motif CUGCUGCCGAU located in the *DMRT1* 3'UTR that participates in the gonad-specific stabilization of mRNA during development. The presence of this sequence indicates the importance of cis and trans elements forming mRNA-protein complexes in the control of specificity and selectivity of gene expression. The insertion described here is located exactly in the middle of the highly conserved region changing this sequence to CUGCU**U**GCCGAU.

In conclusion, the mutation identified in the patient with partial gonadal disgenesis may cause the phenotype either by modifications in the alternative splicing process or by preventing mRNA stabilization by protein binding and, consequently, leading to mRNA degradation.

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REFERENCES

1. Hughes IA. Disorders of sex development: a new definition and classification. Best Pract Res Clin Endocrinol Metab. 2008;22:119-34.
2. Andrade JG, Guerra-Júnior G, Maciel-Guerra AT. 46,XY and 45,X/46,XY testicular dysgenesis: similar gonadal and genital phenotype, different prognosis. Arq Bras Endocrinol Metabol. 2010;54:331-4.
3. Michala L, Goswami D, Creighton SM, Conway GS. Swyer syndrome: presentation and outcomes. BJOG. 2008;115:737-41.
4. Ribeiro-Scolfaro M, Cardinalli IA, Stuchi-Perez EG, de Mello MP, Assumpção JG, Baptista MTM, et al. Morphometry and histology of gonads from 13 children with dysgenetic male pseudohermaphroditism. Arch Pathol Lab Med. 2001;125 652-6.
5. Berta P, Hawkins JR, Sinclair AH, Taylor A, Griffiths BL, Goodfellow PN, et al. Genetic evidence equating SRY and the testis-determining factor. Nature. 1990;348:448-50.
6. Knower KC, Kelly S, Harley VR. Turning on the male-SRY, SOX9 and sex determination in mammals. Cytogenet Genome Res. 2003;101:185-98.
7. Tagliarini EB, Assumpção JG, Scolfaro MR, Mello MP, Maciel-Guerra AT, Guerra Jr G, et al. Mutations in SRY and WT1 genes required for gonadal development are not responsible for XY partial gonadal dysgenesis. Braz J Med Biol Res. 2005;38:17-25.
8. Biason-Lauber A. Control of sex development. Best Pract Res Clin Endocrinol Metab. 2010;24(2):163-86.
9. Veitia RA, Nunes M, Quintana-Murci L, Rappaport R, Thibaud E, Jaubert F, et al. Swyer syndrome and 46,XY partial gonadal dysgenesis associated with 9p deletions in the absence of monosomy-9p syndrome. Am J Hum Genet. 1998;63:901-5.
10. Calvari V, Bertini V, De Grandi A, Peverali G, Zuffardi O, Ferguson-Smith M, et al. A new submicroscopic deletion that refines the 9p region of sex reversal. Genomics. 2000; 65:203-12.
11. Raymond CS, Shamu CE, Shen MM, Seifert KJ, Hisch B, Hodgkin J, et al. Evidence for evolutionary conservation of sex-determining genes. Nature. 1998;391:691-4.
12. Pask AJ, Behringer RR, Renfree MB. Expression of DMRT1 in the mammalian ovary and testis--from marsupials to mice. Cytogenet Genome Res. 2003;101:229-36.
13. Raymond CS, Parker ED, Kettlewell JR, Brown LG, Page DC, Kusz K, et al. A region of human chromosome 9p required for testis development contains two genes related to known sexual regulators. Hum Mol Genet. 1999;8:989-96.
14. Furlong RA, Lyall JE, Lush MJ, Affara NA, Ferguson-Smith MA. Four dinucleotide repeat polymorphisms on chromosome 9 (D9S143-146). Hum Mol Genet. 1992;1:447.
15. Cheng HH, Ying M, Tian YH, Guo Y, McElreavey K, Zhou JR. Transcriptional diversity of DMRT1 (dsx- and mab3-related transcription factor 1) in human testis. Cell Res. 2006;16:389-93.

16. Lu H, Xiao H, Liao Z, Yiqing G, Hanhua C, Rongjia Z. Multiple alternative splicing of mouse Dmrt1 during gonadal differentiation. Biochem Biophys Res Commun. 2007;352: 630-4.
17. Smith PJ, Zhang C, Wang J, Chew SL, Zhang MQ, Krainer AR. An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers. Hum Mol Genet. 2006;15:2490-508.
18. Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR. ESEfinder: a web resource to identify exonic splicing enhancers. Nucleic Acid Res. 2003;31:3568-71.
19. Zhang XH, Chasin LA. Computational definition of sequence motifs governing constitutive exon splicing. Genes Dev. 2004;18:1241-50.
20. Zhang XH, Kangsamaksin T, Chao MS, Banerjee JK, Chasin LA. Exon inclusion is dependent on predictable exonic splicing enhancers. Mol Cell Biol. 2005;25:7323-32.
21. Herpin A, Nakamura S, Wagner TU, Tanaka M, Schartl M. A highly conserved cis-regulatory motif directs differential gonadal synexpression of Dmrt1 transcripts during gonad development. Nucleic Acids Res. 2009;37:1510-20.