Case Report

Intragenic duplication of *DMRT1* in a *SRY*-negative boy with 46,XX disorder of sex development

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Abstract: Background. 46,XX disorders of sex development are rare. Approximately, 90% of XX males are *SRY*-positive, while testicular development in the absence of *SRY* takes place in a minority. **Methods**: A boy with 46,XX karyotype (*SRY*-negative; absence of *SOX9* duplications) was investigated by targeted Next Generation Sequencing (NGS), Multiplex ligation-dependent probe amplification (MLPA), and Comparative Genomic Hybridization array (CGH-array). **Results**: The boy had normal male phenotype and normal prepubertal values of testicular hormones. He presented a heterozygous duplication of 49.626 bp, encompassing exons 2 and 3 of *DMRT1*. The result was arr[GRCh37] 9p24.3(845893_895518)x3. Since both breakpoints are harbored in the intronic regions, the duplication does not stop or shift the coding frame. Additional known pathogenic or uncertain variants in pro-testis gene cascade were not identified. **Conclusions**: This study report a boy with 46,XX testicular disorder of sex differentiation, showing a de novo partial intragenic duplication of *DMRT1*. This intragenic duplication may result in a gain of function, acting as primary pro-testis gene (or anti-ovary gene) in a 46,XX human foetus and permitting normal pre-pubertal endocrine testis function.

Keywords: 46,XX disorder of sex development, *SRY*-negative male, Testis determination, *DMRT1*, *DMRT1* duplication.

1. Introduction

In mammals, sex determination is the outcome of a battle between mutually antagonistic male and female regulatory networks, that canalize the development of primordial bipotential gonad towards testis or ovary pathway while actively repress the other one [1]. In 46,XY embryos, the transient expression of *SRY* (sex determing region of the Y chromosome) triggers a cascade of gene interactions ultimately leading to the formation of a testis from the indifferent gonadal ridge. The SRY-box gene *SOX9* is the earliest up-regulated gene in the testis pathway downstream of *SRY* [1-3]. After testis determination, the testicular anti-müllerian hormone (AMH) and androgens guide the differentiation of male internal and external genitalia. In the XX foetuses, due to the absence of *SRY*, *SOX9* expression remains low and is overcome by *DAX1*, *FOXL2*, *WNT4* and *RSPO1*, which up-regulate β-catenin; a feed-forward loop between these pro-ovarian factors is established, resulting in the differentiation of the female gonad and the pro-testis

gene down-regulation. Since the ovary does not secrete AMH and androgens, female internal and external genitalia develop [1-3].

Disorders (or Differences) of Sex Development (DSD) can result from the disruption of the delicate balance among these opposing molecular networks [1,2]. Among DSD, males with 46,XX karyotype are rare (~1:20.000 male births) [3]. These individuals may have gonads completely differentiate into testes, a condition known as 46,XX testicular DSD, or the coexistence of ovarian and testicular tissue, a condition known as 46,XX ovo-testicular DSD [3]. About 90% of males with 46,XX testicular DSD show a translocation of *SRY* gene onto one of the X chromosomes or onto an autosome, whereas the other ones (~10%) are *SRY*-negative [3]. In the last years, an increasing number of genetic variants associated with *SRY*-negative 46,XX testicular DSD have been described, including both gain-of-function of pro-testis genes (as *SOX9*) and loss-of-function of members of the pro-ovarian pathway (as *WNT4*/*RSPO1* signaling pathway) (reviewed in 3). Anyway, the genetic causes of *SRY*-negative 46,XX testicular DSD remain unknown in some patients.

Human *DMRT1* (located on 9p24.3) is a gene involved in testis differentiation and in maintenance of male somatic cell fate after differentiation [4, 5]. It encodes a protein with a DM domain, a zinc-finger-like DNA-binding motif, first identified in the sex regulators Doublesex (Dsx) of Drosophila and Male abnormal-3 (MAB-3) of C. elegans [6]. Genes encoding for a DNA-binding DM domain transcription factor are deeply conserved in a broad range of metazoa and act as triggers for primary sex determination in multiple phyla [5,7-9]. In mammals, this role is played by *SRY*, a more recently evolved gene, and *DMRT1* is thought to be less relevant for switch-on male sex determination [4]. Anyway, experimental data in a transgenic mouse model demonstrated that the ectopic overexpression of *Dmrt1* in XX foetal gonads is sufficient to drive testicular differentiation in the embryo and male secondary sex development after birth [4, 10]. Studies in mice also indicate that this gene is essential to maintain testis differentiation long after its determination, showing that mammalian gonads may have some degrees of plasticity throughout life. In adult mice, the loss of *Dmrt1* function causes a sex-cell-fate reprogramming, leading Sertoli cells to trans-differentiate into granulosa cells and testicular tissue to reorganize toward an ovarian morphology [5,11]. Moreover, *Dmrt1* overexpression in the mouse ovary can cause male sex determination or female-to-male cell fate transdifferentiation [12].

In humans, heterozygous deletions in 9p24.3, involving *DMRT1* of this gene have been reported in 46,XY patients with gonadal dysgenesis or ovotestes, indicating that its haploinsufficiency is associated with defective testicular development and male-to-female sex reversal [reviewed in 5]. Its critical role in male sex determination has been confirmed by the evidence of missense point mutations in females with 46,XY karyotype and gonadal dysgenesis [reviewed in 5]. *SRY*-negative 46,XX testicular DSD attributable to *DMRT1* upregulation has not been described in humans [3].

Here, a boy with 46,XX testicular DSD, *SRY*-negative, is reported. He does not show any alteration in *SOX9* or other pro-testis genes, but a *de novo* heterozygous intragenic duplication of *DMRT1* involving the second and third exons was detected.

2. Results

Clinical findings

The boy showed normal male phenotype. Height was about one standard deviation score (SDS) below the mean for both male (phenotype) and female (karyotype) reference standards (Table 1), but adequate for his target height (mid-parental height -0,7 SDS). Both testes were located into the scrotum with low normal volume for prepubertal age (Table 1).

Sonography demonstrated normal testicular structure without any additional findings. Ultrasound scanning of the pelvis showed the absence of Müllerian derivatives.

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Endocrine evaluation demonstrated normal values of gonadotropin and androgens for prepubertal age (data not shown). Both antimüllerian hormone (AMH; 756,8 pmol/L; reference range 321 – 1,218 pmol/L) and inhibin B (108,0 pg/ml; reference range 23 – 252 pg/ml) serum levels resulted in their normal prepubertal range.

Table 1. Boy with 46,XX testicular DSD (*SRY* negative): clinical findings at the age 3,2 years.

Clinical parameters	Data according to reference values for	
	males	females
Height, SDS	-1,2	-1,0
Weight for age, SDS	0,1	0,3
Weight for height, SDS	1,1	1,2
Body mass index, SDS	1,3	1,3
Mean testicular volume, ml	1,0	—
Mean testicular volume, SDS	-1,1	—

SDS = standard deviation score

Genetics

No pathogenic or uncertain variants were identified in the patient by NGS analysis. SALSA MLPA P185-C2 Intersex kit detected a normal copy number for *NR0B1*, *CXorf21*, *SOX9*, *WNT4*, and *NR5A1* genes and showed the absence of *SRY*. MLPA analysis using SALSA MLPA P334-A3 Gonadal kit resulted in a ratio value for the probe hybridizing to *DMRT1* exon 2 (13074-L14293 and 13078-L14297 probes) and exon 3 (13072-L14291 and 13069-L14288 probes) consistent with a heterozygous duplication (Fig. 1).

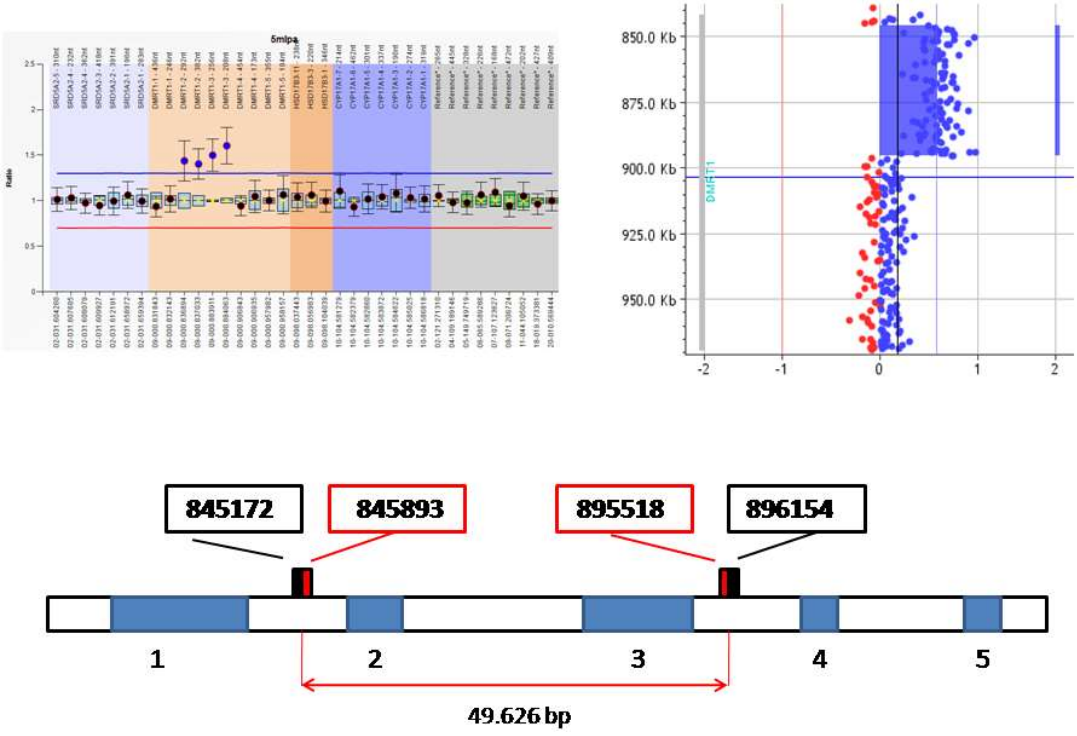

Figure 1. Results of MLPA test (top, left panel) and array CGH (top, right panel), showing the duplication of *DMRT1*. At the bottom, schematic representation of *DMRT1* gene with the position of probes delimitating the duplication breakpoints is depicted (blue boxes: exons 1 to 5).

The probes targeting the other exons of the *DMRT1* showed normal copy number (Fig.1). The *DMRT1* intragenic duplication was confirmed by a customized CGH-array platform, that allowed to better define the distal and proximal intervals of breakpoints. The duplication extent was of 49,626 bp. The proximal breakpoint lay between the oligo in position 845,172 pb (not duplicated) and the oligo in position 845,893 pb (duplicated); the distal one was between the oligo in position 895,518 pb (duplicated) and that in position 896,154 pb (not duplicated). The result was arr[GRCh37] 9p24.3(845893_895518)x3. Since both breakpoints are in the intronic regions, the duplication does not stop or shift the coding frame, but gives rise to a transcript with a duplication of the second and third exons (Fig. 1).

Family study demonstrated the *de novo* origin of the *DMRT1* duplication in this boy with 46,XX testicular DSD.

3. Discussion

Here, a *SRY*-negative boy with 46,XX testicular DSD and normal male phenotype is reported. Albeit no histological gonadal data were available, prepubertal "functional" testicular tissue can be inferred by normal male values of AMH and inhibin B, indicating normal Sertoli cell function. This findings largely avoid the possibility of streak gonads or postnatal ovarian trasdifferentiation. On the other hand, the normal masculinization of genitalia and the absence of Müllerian derivatives demonstrate that optimal levels and function of fetal AMH and androgens were operative during prenatal sex differentiation [1-3].

This boy showed a *de novo* isolated heterozygous duplication of 49.626 bp, encompassing exons 2 and 3 of *DMRT1* (Fig. 1). Extensive molecular investigation of genes and genomic regions related to sex determination and differentiation did not reveal other pathogenetic variants.

DMRT1 acts as master sex-determining gene in a broad range of metazoa and prevents female reprogramming in the postnatal mammalian testis [4,5,7,9,11]. In mammals, sex determination is primarily driven by *SRY*. However, experimental data showed that ectopic overexpression of *Dmrt1* in XX fetal gonads of a mouse model is sufficient to drive testicular differentiation at the fetal stage and maintenance of male secondary sex development postnatally [10]. In mouse, *Dmrt1* appears to be a bifunctional transcription factor, that represses female sex-determining genes (i.e. *Wnt4* and *Rspo1*) whilst promotes the continuous expression of male sex-determining genes (i.e. *Sox9*) [5,10,12].

In humans, the molecular mechanism of *DMRT1* action during the fetal period remains largely unknown (Database GTEx, www.gtexportal.org/home). Anyway, *DMRT1* is involved in testis determination, since its haploinsufficiency due to either distal 9p monosomy or heterozygous point mutations is able to derange the normal male pathway, leading to dysgenetic gonads or ovotestis [13-21]. These findings are confirmed by experimental data in 46,XY Dmrt1+/− and Dmrt1−/− murine knock-out models (7).

The present heterozygous *DMRT1* duplication encompassing exons 2 and 3 of *DMRT1* does not interrupt the reading frame, because both duplication breakpoints fall in intronic regions and the mutated protein is expected to be bigger with a duplicated internal "core" of aminoacids corresponding to the second and third exon. Thus, this duplication unlikely cause an impaired DMRT1 function, but it may cause a *DMRT1* gain of function, determining testis determination in a 46,XX human foetus. Experimental goat studies suggested that *DMRT1* up-regulation in a XX contest might be able to promote *SOX9* activation independently from *SRY* and then testis

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determination [22]. By contrast, it could be speculated that this duplication of *DMRT1* exons 2 and 3 may prevent ovary determination. These mechanisms can be hypothized, but not proven, in the present boy.

The complete *DMRT1* three-dimensional structure is not yet available and the lack of sequence similarity to other templates makes impossible to build a trustable 3D model of this mutant protein based on homology. However, interaction between the *DMRT1* DM domain and DNA in mouse and human has been analyzed by X-ray crystallography [9] (PDB code 4YJ0). DM domain is a unique intertwined double zinc-binding module followed by a C-terminal alpha helix, which binds to DNA at a pseudopalindromic consensus element. The alpha helix inserts into the DNA major groove, involving the recognition of specific bases, while the zinc-binding module spans the minor groove primarily through phosphate backbone contacts. *DMRT1*, in vivo, can bind DNA using different stoichiometries, it interacts as tetramer, trimer or dimer with specific DNA binding target sides [5]. A particularly noteworthy feature of *DMRT1*-DNA binding is that two adjacent alpha helices of two *DMRT1* proteins lie antiparallel together in the major groove of the consensus element (Fig. 2). No other protein interacts with DNA through a pair of alpha helices so close to be both inserted the same section of a major groove. The DM domain is encoded by the first and second exon, so the intragenic duplication here presented likely produces a protein with a partial duplication of this functional domain that may mimic the effect of a *DMRT1* dimer.

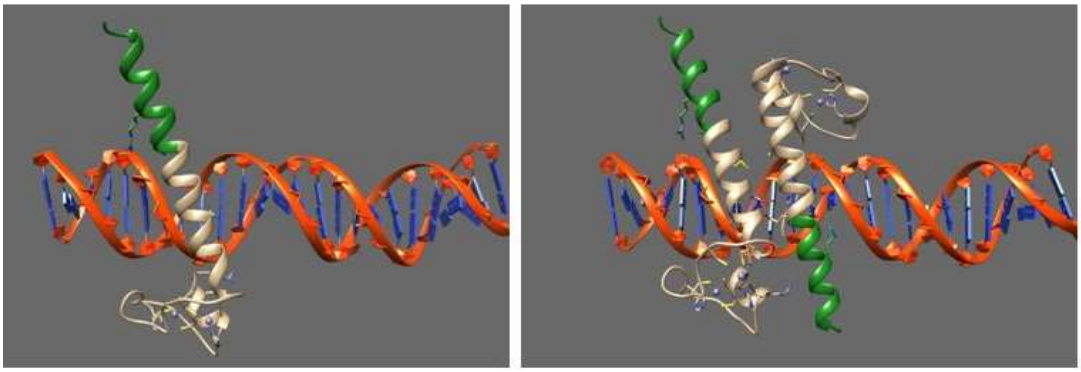

Figure 2: Three dimensional structure of the DM domain (fragment Ser70-Leu131) bound to a 25-mer DNA. Left panel: a single helix (ribbons) bind the major groove of the DNA molecule, the interacting aminoacids are drawn as sticks, a Zinc ion is reported as a cyan sphere. The partial product of exon 2 is colored in green. Right panel: two adjacent helices, are binding the major groove of the same 25-mer, as determined by X-ray crystallography.

This paper shows some limitations. Assuming that the present duplication of *DMRT1* may increase the DMRT1 protein function or stability, an *in-vitro* assay should be performed as well as a whole exome sequencing could allow to better define the mechanisms underlying the phenotype of this boy with 46,XX testicular DSD. Finally, a loss of function by changes in the three-dimensional conformation of the protein was not excluded by specific investigations. However, it is high unlikely that loss of function of a pro-testis gene switch-on testicular development in a 46,XX context. At this regard, distal 9p hemizygosity does not preclude female phenotype, ovarian differentiation and oogenesis [5].

In conclusion, a *de novo DMRT1* intragenic duplication possibly acting as primary pro-testis gene during foetal sex determination period has been found in in a *SRY*-negative boy with 46,XX testicular DSD. Pathogenetic or uncertain genetic variants of other genes

involved in testis determination were not found, but some additional investigations should be performed. At now, biomarkers of testicular function are in their normal prepubertal range indicating male gonadal function in early childhood, but long-term follow-up is needed to elucidate his long-life testicular fate.

4. Materials and Methods

Clinical methods

Height and weight were expressed as SDS according to WHO standards (http://www.who.int/childgrowth/software/en/). Correct mid-parental height was calculated using measured parental heights adjusted for male or female sex: father's height + mother's height/2 plus 6.5 cm.

Baseline blood samples were obtained in the fasting state between 8: 00 and 9: 00 a.m. to assess LH, FSH, testosterone anti-müllerian hormone (AMH), inhibin B. Normative levels were from values reported in a recent review [23]. Testicular volume was evaluated by Prader orchidometer and expressed according the normative values of Goede et al. [24].

Next Generation Sequencing (NGS)

A 20-genes NGS custom panel for DSD (Supplementary Table S1) was performed using SureSelect XT target enrichment (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions and the products were sequenced using MiSeq Illumina platform (Illumina, San Diego, CA, United States) . Annotation and filtering of variants were performed with Variant Interpreter platform; the NGS coverage was analyzed in detail using Integrative Genome Viewer version 2.3.

Multiplex ligation-dependent probe amplification (MLPA)

MLPA was performed using SALSA MLPA P334-A3 Gonadal kit including probes for CYP17A1, DMRT1, HSD17B3 and SRD5A2 genes, and SALSA MLPA P185-C2 Intersex kit containing probemix of the following genes: NR0B1, CXorf21, SOX9, SRY and ZFY (Yp11.3), WNT4, NR5A1 (MRC-Holland, Amsterdam, The Netherlands). Amplified products were separated by size using capillary electrophoresis system on an ABI-3500 Genetic Analyzer (Applera, Italy) and data were analyzed by the Coffalyser.Net Software (MRC-Holland, Amsterdam, The Netherlands). A peak was considered abnormal when the ratio was <0.65 (deletion, copy number change from two to one allele) or >1.30 (duplication, copy number change from two to three or more alleles) compared to the peaks of the reference probes. Information on probe sequences can be freely accessed on the MRC Holland website (www.mlpa.com).

CGH-array

DNA from a 46,XX healthy subject was used as control (Agilent Technologies, Santa Clara, California, U.S.A.). Five-hundred ng of genomic DNA both from the patient (test sample) and the control (reference sample) were differentially labeled with Cy5-dCTP or with Cy3-dCTP using random primer labeling according to manufacturer's protocol (Agilent). The labeling reactions were applied to the oligo-arrays and incubated for 24 h at 67 °C in an oven. Slides were washed and scanned using the Agilent scanner, and the identification of individual spots on scanned arrays and quality slide evaluation was performed with the Agilent dedicated software (Feature Extraction, Agilent). The array CGH was performed on a customized 180K SurePrint G3 Human CGH Microarray (Agilent), enriched in the genes correlated to DSD (Supplementary Table S1). For these

genes the overall median probe spacing is about 1.8 KB. Copy number variants (CNVs) were identified with Cytogenomics 4.0.3.12 (Agilent), using the ADM-2 (Aberration Detection Method-2) algorithm. We analyzed all the CNVs, independently of their absolute size; they were compared to those reported in the http://dgv.tcag.ca/variation.

5. Patient

The boy was referred to us at the age 3 years, 3/12. He was the third child of a non-consanguineous Caucasian parents, who had one previous miscarriage. He was born at term (40 weeks); pregnancy and perinatal period were uneventful; prenatal amniocentesis revealed a 46,XX karyotype, while *SRY* was not detected by FISH and PCR analysis. Prenatal ultrasound and perinatal evaluation demonstrated normal male genitalia without internal female genitalia. At birth, absence of *SRY* was confirmed and *SOX9* duplications was ruled out. The boy presented mild speech and motor delay in his first two years of life.

Supplementary Materials: Table S1 are available online at www.mdpi.com/xxx/s1,

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Informed Consent Statement: The parents had given their informed written consent before any clinical and genetic investigation. The study was conducted according to the Declaration of Helsinki and the standard protocol of investigation of a child with DSD in our Department.

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Conflicts of Interest: The authors declare no conflict of interest.

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