Original Article

Copy Number Variation Study of A Cohort of 46,XY DSD Patients

Mona K. Mekkawy[1], Ola M. Eid [1], Shereen A. Sayed[1], Azza E. Abd-Elnaby[1], Aya Elaidy[2], Alaa K. Kamel[1], Inas M. Mazen[2]

[1]Human Cytogenetics Department, [2]Clinical Genetics Department, Human Genetics and Genome Research Institute, National Research Centre, Cairo, Egypt

ABSTRACT

Background: Differences of sex development (DSD) is a group of heterogeneous conditions with a diverse pathophysiology. They are generally characterized by an abnormality of the chromosomal, gonadal or phenotypic features that typically define sex development.

Aim: The study aim was to introduce Multiplex ligation-dependent Probe Amplification (MLPA) technique in the diagnostic workup of 46,XY DSD patients and to correlate genotypic abnormalities with clinical phenotype for more understanding of etiologic background.

Methods: The study was carried out on thirty five 46,XY DSD patients selected from the Endocrinology clinic, Institute of human genetics and genome research, National Research Centre, Egypt. Patients underwent thorough clinical examination, hormonal assessment, pelvic ultrasonography and genitography. Laparoscopy with gonadal biopsy and histopathological evaluation were done when indicated. All patients were subjected to karyotype and MLPA analysis.

Results: Clinically significant Copy number variations (CNVs) were detected in three patients in the form of *SOX9* gene deletion in 2 patients, deletion of *DMRT1* in 1 patient, while heterozygous duplication (of unknown significance) in *HSD17B3* gene was detected in 1 patient, with an overall rate of 11.4%.

Conclusion: MLPA is a robust cost-effective technique for screening of 46,XY DSD patients as a complement to Sanger sequencing. CNV analysis should be added to the first-line diagnostics in 46,XY DSD, especially if the phenotype includes malformations in other systems.

Key Words: 46,XY DSD, CNV, MLPA, SOX9.

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Corresponding Author: Mona K. Mekkawy, Human Cytogenetics Department, Human Genetics and Genome Research Institute, National Research Centre, Cairo, Egypt. **Tel.:** 01227432579,
E-mail: Monamekkawy2005@yahoo.com

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INTRODUCTION

Differences of sex development (DSD) is a revised term referred to as disorders of Sex Development (**Johnson *et al.,* 2017**). It includes a group of heterogeneous conditions with diverse pathophysiology. They are generally characterized by an abnormality of the chromosomal, gonadal or phenotypic features that typically define sex development (**Hughes *et al.,* 2006**). Such conditions usually present with atypical genitalia in the newborn period or as delayed puberty or primary amenorrhea in an adolescent or present later in life as infertility (**Hughes, 2008**).

A number of genes contributes, both in the very early and later stages, in the process of sex determination and differentiation. The determination and maintenance of gonadal sex as either male or female occurs through alternate fate suppression. *SRY*, located on the Y short arm is the testis determining gene, which directly upregulates the autosomal gene, *SOX9*, to initiate a cascade of complex gene interactions ultimately leading to the formation of testes, while suppressing the female pathway (**Lucas-Herald and Bashamboo, 2014**). In the absence of *SRY*, the female fate proceeds and certain genes like *WNT4* and R-spondin1 that lead to β-catenin stabilization will suppress the male pathway (**Lucas-Herald and Bashamboo, 2014; Lundgaard *et al.,* 2024**). On the other hand, androgens produced by the testes derive the differentiation of fetal internal and external genitalia and the development of secondary sex characters at puberty (**Sinisi *et al.,* 2003**). Imbalances affecting genes contributing to the antagonistic gonadal genetic pathways or those that control androgen biosynthesis or action will result in abnormal prenatal and postnatal sexual development, resulting in differences of sex development (DSD).

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46,XY difference of sex development (46,XY DSD) is a congenital disorders characterized by inconsistency between gonadal and phenotypic sex. It is considered one of the major concerns for parents and clinicians regarding gender assignment, health problems, medical and surgical intervention, psychological health and reproductive life (**Mazen *et al.,* 2008; Hou *et al.,* 2021; Faradz *et al.,* 2023; Mary *et al.,* 2023**).

However, identification of a discrete genetic etiology in 46,XY DSD is available only in about 50% of patients (**Ahmed *et al.,* 2011**).

Many studies reported clinically significant CNV in 46,XY undiagnosed patients using chromosomal microarray (CMA) and Multiplex Ligation-dependent Probe Amplification (MLPA) techniques and suggested that rearrangements of certain coding and non-coding sequences can disturb gene regulation and account for a significant proportion of undiagnosed DSD cases (**Ledig *et al.,* 2010; White *et al.,* 2011; Harrison *et al.,* 2014; Baetens *et al.,* 2017**).

CMA is a genome-wide technique that represents a powerful tool to identify submicroscopic imbalances but their expense may restrict their wider application (**Parivesh *et al.,* 2019**). Therewith, MLPA technique is an easy and reliable method for detection of copy number variation in multiple genes throughout the genome. This technique is able to distinguish sequences differing in only one nucleotide and has a higher sensitivity in detecting atypical deletions and determining the deletion size and regulatory regions of some genes (**Schouten *et al.,* 2002**).

The aim of the present study was to introduce MLPA technique in the diagnostic workup of 46,XY DSD patients and to correlate genotypic abnormalities with clinical phenotypes for more understanding of etiologic background.

PATIENTS AND METHODS:

The study was carried out on thirty five 46,XY DSD patients referred from the Endocrinology Clinic, to the Human Cytogenetics Department, Institute of human genetics and genome research, National Research Centre (NRC), Cairo, Egypt. An informed written approval was obtained from patients or parents/guardians of patients by the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments involving human subjects and the approval of the NRC ethics committee. The selected cases included patients presenting with atypical genitalia, hypospadias, cryptorchidism, and micropenis or female phenotype with primary amenorrhea and gonadal dysgenesis. Age ranged from birth to adulthood. Exclusion criteria included; patients with androgen insensitivity syndrome, Karyotypes with sex chromosomal constitution other than 46,XY.

Thorough clinical examination was done with special emphasis on the phenotypic description of external genitalia, classified according to **Quigley *et al.,* (1995).** Pubertal staging was done following **Tanner and Davies, (1985).** Pelvic ultrasonography and genitography were performed for visualization of internal genitalia

Hormonal assessment in the form of basal serum FSH, LH, basal and post HCG stimulation of testosterone and its precursors, DHT and delta-4 androstenedione (Δ4) to testosterone (T) ratio. In addition to AMH and inhibin B to assess Sertoli cell function were performed.

Laparoscopy with gonadal biopsy taking and histopathological evaluation were done when indicated for proper diagnosis.

Peripheral blood sample collection (5ml) is carried out on K2-EDTA in vacutainer tube for DNA extraction and (2.5ml) on Lithium heparin vacutainer tube for blood lymphocytes culture.

Blood lymphocytes culture and chromosomes preparation were carried out for all patients according to **Verma and Babu, (1995)**. Karyotypes nomenclature was done according to the **ISCN, (2020)**. A total of 20 metaphases were analyzed for each patient. FISH was carried out for female reared patients when required for identification of *SRY* gene using LSI *SRY* probe (Yp11.31/Yq12), Cytocell FISH probes, Oxford Gene Technology, UK.

DNA extraction from the peripheral blood lymphocytes for the studied patients and the reference samples (one reference for 7 patients sample with minimal 3 references per test) was carried out using PAXgene kit (PreAnalytix, Hiden, Germany) according to the manufacturer's instruction. The quality and quantity of the DNA samples were determined using the NanoDrop spectrophotometer.

MLPA analysis was carried out using SALSA MLPA probemix P334-A3 Gonadal Development Disorder and SALSA MLPA probemix P185-C2 Intersex, according to the manufacturer's instruction (MRC-Holland). SALSA MLPA probemix P334-A3 Gonadal Development Disorder contains probes for four genes *DMRT1*, *CYP17A1, SRD5A2* and *HSD17B3*. While, P185-C2 Intersex probemix contains probes for *NR0B1* (*DAX1*) and *CXorf21* on Xp21.2, *SOX9* on 17q24.3, *SRY* and *ZFY* on Yp11.3, *WNT4* on 1p36.12 and *NR5A1* on 9q33. DNA denaturation and overnight hybridization of the MLPA probemix was done, followed by probe ligation and

amplification in the next day. Separation of amplified products was done using Genetic Analyzer ABI 3500 (USA). Interpretation of the results was performed using Coffalyser. Net software (MRC-Holland). Ratios less than 0.75 were considered as deletion, between 0.75 and 1.30 as normal and more than 1.30 as duplication.

RESULTS

Clinical evaluation and Hormonal analysis

The present study was conducted on thirty five patients with 46,XY DSD. The patients' presenting features included atypical genitalia (20 patients), Hypospadius (8 patients), primary amenorrhea (6 patients) and inguinal hernia (1 patient), table (1). Their ages ranged from 1 month to 21 years with a mean age of 7 years. Parental consanguinity was detected in 21 patient (60%). Twenty five patients (71%) were reared as males and 10 patients (29%) were reared as females. Six of the female-reared patients presented during early and late teens with primary amenorrhea and 5 of them had a hypoplastic uterus. While 3 presented in early childhood with atypical genitalia and 1 with an inguinal hernia.

The patients were classified according to the initial clinical diagnosis and hormonal assessment into five groups: 21 patients (60%) with gonadal dysgenesis, 5 patients (14%) with 17 beta hydroxysteroid dehydrogenase type 3 defficiency, 2 patients (5.7%) diagnosed with 5 alpha reductase deficiency, 2 patients (5.7%) with isolated hypospadius and 5 patients (14%) with DSD associated with other abnormalities The general characteristics of the studied patients and the summary of their data are presented in table (1).

Gonadal histopathological study

Histopathological examination revealed a dysgenetic gonad in 21 patients, out of 26 patients studied, and an early developing gonadoblastoma in the inguinal gonad in one female reared patient presented with primary amenorrhea.

Conventional Cytogenetic and FISH analysis:

Patients with 46,XY chromosomal constitution were selected, one patient showed 46,XY,del(9)(p22), while two patients, brother and sister, showed a pericentric inversion of the X chromosome: 46,XY,inv(X)(p11q13). FISH analysis for all female reared patients using LSI *SRY* probe (specific for the *SRY* gene Yp11.31/ Yq12) revealed positive signals.

MLPA analysis:

MLPA analysis was conducted using the Intersex probemix for 8 patients, while both Intersex and Gonadal Development Disorder probemixes were used for the rest of the patients. Clinically relevant CNVs were detected in three patients in the form of *SOX9* gene deletion in 2 patients, deletion in *DMRT1* in 1 patient, while heterozygous duplication (of unknown significance) in *HSD17B3* gene was detected in 1 patient with an overall rate of 11.4%.

Clinical report on MLPA positive patients

The first patient was a 1-year-old male presenting with atypical genitalia and skeletal anomalies. Genital examination showed anchored penis, left undescended testis, and Quigley score of 3. General examination revealed dysmorphic features in the form of flat face, depressed nasal bridge and hypertelorism, short stature (-5 SD), short lower limbs with bowing and bilateral talipus. Abdominal-pelvic ultrasound showed left gonad at the left inguinal canal with no uterine shadow. MLPA assay using intersex probemix showed complete *SOX9* deletion (affecting exons 1, 2 and 3) (Figure 1).

The second patient was a 3-year-old male presenting with delayed milestones and atypical genitalia with a bilaterally undescended testis and a Quigley score of 3. General examination revealed blepharophimosis with mild dysmorphic features in the form of bilateral epicanthic folds, hypertelorism and depressed nasal bridge, with short stature (-4 SD) and severe microcephaly (-4 SD). Abdominal- pelvic ultrasonography showed both testes at the corresponding inguinal canals. Conventional cytogenetic analysis showed 46,XY karyotype, while MLPA assay using intersex probemix showed partial *SOX9* deletion affecting exon 2 (Figure 2).

The third patient was a 5-months-old male presenting with undescended testes and delayed motor and mental development. Genital examination showed a bilaterally undescended testis. General examination revealed dysmorphic features in the form of abnormal skull shape, prominent metopic suture, arched sparse eye brows, bilateral epicanthic folds, upward slanting of the palpebral fissures, hypertelorism, depressed nasal bridge, long philtrum, micrognathia, elevated lower lip, low set ears, short neck, high arched palate, marked hypotonia and bilateral talipus in the lower limbs. Echocardiography revealed congenital heart defect in the form of patent ductus arteriosis. Brain MRI showed agenesis of the corpus callosum. Conventional cytogenetic analysis showed 9p deletion: 46,XY,del(9)(p22). MLPA assay using Gonadal Development Disorder probemix confirmed the complete *DMRT1* deletion (Figure 3).

The fourth patient was a 15-year-old male presenting with atypical genitalia. Genital examination revealed a bilaterally undescended testis and hypospadias with Quigley score 3. General examination showed no associated features. Abdominal-pelvic ultrasonography showed both testes at the corresponding inguinal canals. The hormonal profile showed low testosterone levels (T), high androstenedione (Δ4) levels and high ratio of serum Δ4/T in the basal levels and after HCG stimulation test, thus favoring the diagnosis of 17β-Hydroxysteroid dehydrogenase III deficiency. MLPA assay using

Gonadal Development Disorder probemix showed partial duplication of *HSD17B3* gene at exon 1.

Clinical report on patients with inversion X and no CNV

One of the patients was a 4-year-old male presenting with intellectual disability, Duchenne muscular dystrophy (*DMD*) and atypical genitalia. Genital examination showed two palpable gonads with Quigley score of 3. Gonadal biopsy revealed testicular tissue with immature sertoli cells.Conventional cytogenetic analysis showed a pericentric inversion of X chromosome 46,XY,inv(X)(p11q13). MLPA assay using intersex probemix showed no copy number changes in *DAX1* gene. Similar results were detected in his female reared sib, who was 7-year-old presenting with intellectual disability, *DMD* and atypical genitalia. Genital examination showed bilateral inguinal gonads and a Quigley score of 3. Conventional cytogenetic analysis showed a pericentric inversion of X chromosome 46,XY,inv(X)(p11q13). MLPA assay also revealed no copy number alteration in *DAX1* gene.

Table 1: General Characteristics of the studied patients:

Presentation Data		Cases	
		No	%
Sex	Male	25	71%
	Female	10	29%
Age at presentation	neonatal/ childhood	24	69%
	Pubertal	11	31%
Presenting Feature	Atypical genitalia	20	57%
	Hypospadias	8	23%
	Primary amenorrhea	6	17%
	Inguinal hernia	1	3%
Parental Consanguinity	Positive	21	60%
	Negative	14	40%
Associated findings	Isolated DSD	30	86%
	DSD with associated clinical abnormalities	5	14%

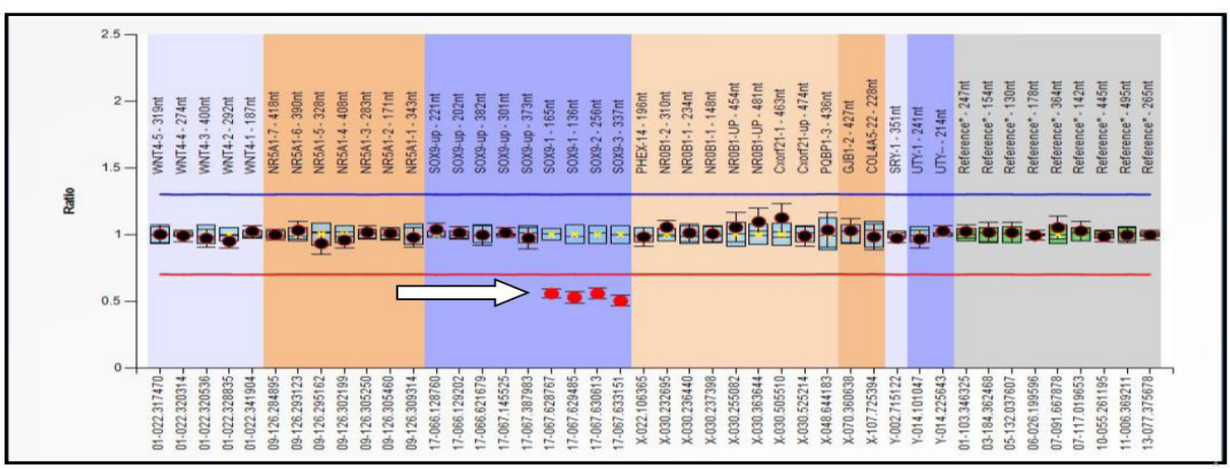

Figure 1: MLPA assay using intersex probemix showing complete *SOX9* deletion affecting exons 1, 2 and 3.

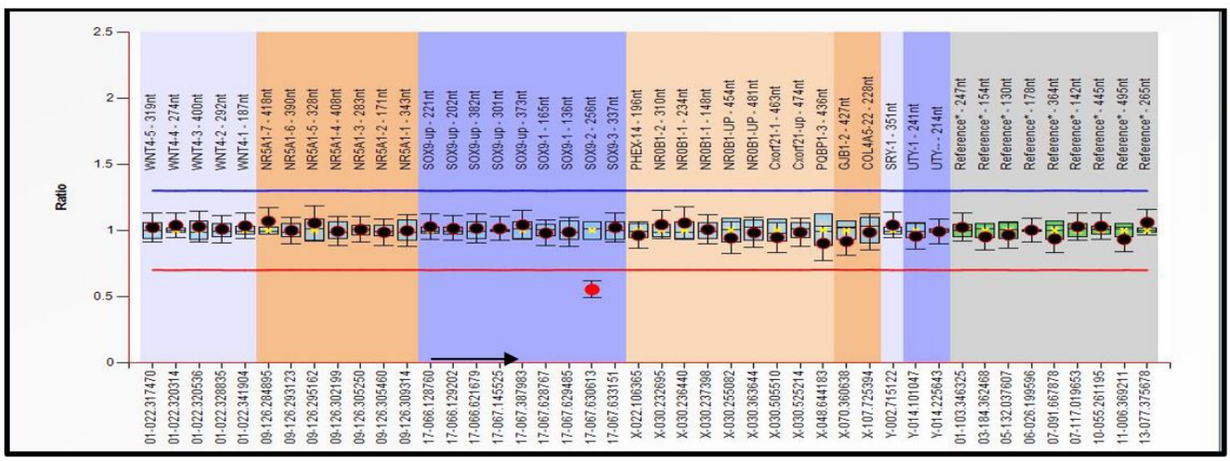

Figure 2: MLPA assay using intersex probemix showing partial *SOX9* deletion affecting exon 2.

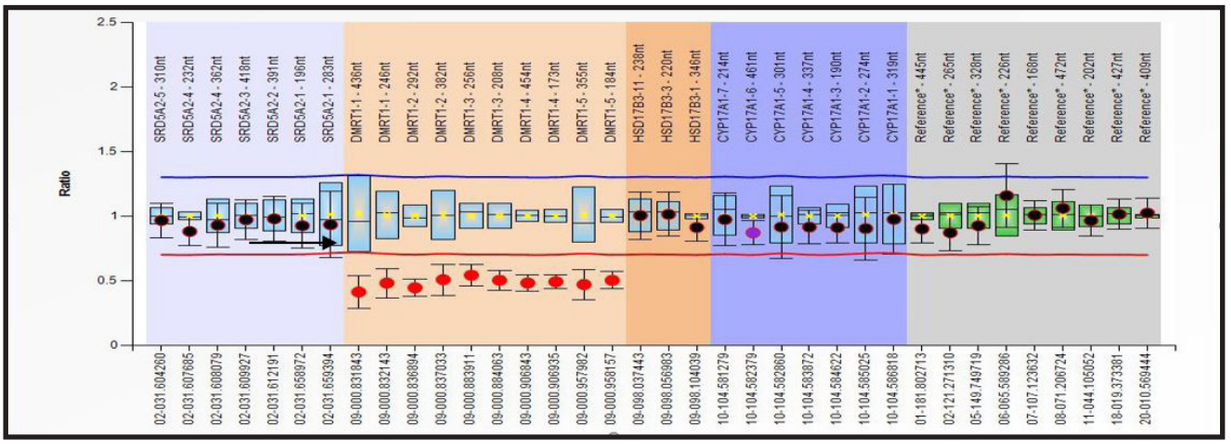

Figure 3: MLPA assay using Gonadal Development Disorder probemix showing complete *DMRT1* deletion.

DISCUSSION

The prevalence of genital abnormalities ranges from 0.3-1% and could be associated with life threatening conditions and other medical and psychological risk factors (**Mazen *et al.,* 2008; Mazen and Ismail, 2010**). This emphasizes the importance of careful examination and diagnosis of the neonates and children with DSD.

There is a gap in management and diagnosis of 46,XY DSD patients because only about 50% pursue their genetic and molecular etiology (**Ahmed *et al.,* 2013**). Recurrent CNVs of coding and regulatory non coding elements have been established as an important etiology in undiagnosed DSD patients. It could affect more than one gene resulting in variable syndromic DSD phenotypes (**Lee *et al.,* 2008; George *et al.,* 2010; Audi *et al.,* 2018; Ahmadifard *et al.,* 2019**). The precise molecular cytogenetic diagnosis is important as it gives explanation of the clinical picture and inform patient management in relation to possible gender development, risk factors and medical and surgical intervention (**Ahmadifard *et al.,* 2019; Reisch *et al.,* 2019; Bagas *et al.,* 2018**).

The current study included 35 46,XY DSD patients with the most frequent presenting feature being atypical genitalia (57% patients), This nearly comes in agreement with **Sema *et al.,* (2011)** who reported atypical genitalia as the main presenting complain among 95 DSD patients. While each of short stature, hypospadias and primary amenorrhea were the presenting feature in 10% of the patients. Parental consanguinity was detected among 60% of our patients, this is nearly similar to the rate reported by **Mazen and Ismail, (2010)** who mentioned high rate of parental consanguinity among DSD patients reaching 61–65% in Egypt. This high consanguinity rate could lead to the appearance of the autosomal recessive gene disorders such as 17β hydroxysteroid dehydrogenase 3 deficiency (17βHSD3) and 5 alpha reductase deficiency (5α-RD2).

71% of our patients were reared as males and 29% were reared as females with age ranging from 1 month to 20 years. Similarly, **Vasundhar *et al.,* (2016)** reported nearly the same rate. 74% of his 46,XY DSD patients were reared as males and 26% were reared as females, with age ranging from months to adulthood. While **Bastian et al., (2015)** and **Gangaher et al., (2017)** reported the gender assignment as 64.3% and 72% as females and 35.7% and 28% as males, respectively. Most of our 46,XY female patients presented in their teens with primary amenorrhea, while those having atypical genitalia presented in their early childhood. Most of 46,XY female phenotype may result from complete gonadal dysgenesis, complete androgen insensitivity syndrome (CAIS) or 17βHSD3 deficiency and usually present with primary amenorrhea (**Basri *et al.,* 2021**). On the other hand, female reared patients with atypical genitalia usually present early in life and may commonly result from partial gonadal dysgenesis, partial androgen insensitivity syndrome, 5-alpha reductase type 2 deficiency and 17βHSD3 deficiency (**Sinisi *et al,* 2003**). About 60% of our patients were diagnosed to have gonadal dysgenesis (GD), which is a rare disorder of sex development with a prevalence of 1:100,000 births. The diagnosis depends on the histology of the gonads that ranges from fibrous streak gonads to partial GD with testicular tissue (**Crone *et al.,* 2002; King and Conway, 2014**). In complete 46,XY GD, the anti-Mullerian hormone (AMH) is deficient resulting in a female phenotype with Mullerian structures (**Bashamboo and McElreavey, 2013**).

DSD associated with other congenital abnormalities was detected among 5 (14%) of our patients. Abnormal sexual development may occur as a part of several malformation syndromes in over a quarter of DSD cases (**Cox *et al.,* 2014**). Many previous studies reported 46,XY DSD cases associated with dysmorphic features, intellectual disability or congenital anomalies (**Swinkel *et al.,* 2008; Ledig *et al.,***

2010; Bastian *et al.,* 2015; Bruni *et al.,* 2019). This was ascribed to other somatic effects of the gene contributing to DSD pathway or due to involvement of other genes.

The rate of MLPA detection of clinically relevant CNVs among our patients reached 11.4% including deletion of *SOX9* gene in 2 patients, deletions of *DMRT1* gene in 1 patient, while duplication of *HSD17B3* gene (of unknown significance) was detected in 1 patient. Pathogenic CNVs were previously detected in 10%-13% of 46,XY DSD (**Garcia–Acero *et al.,* 2019; White *et al.,* 2011; Igarashi *et al.,* 2013,** respectively). Thereafter, CNVs were established as an important causative etiology for about one fifth of syndromic and non syndromic DSD cases. It can cause disruption of known DSD genes as *SRY*, *SOX9*, *WT1*, *DMRT1*, *SF1* (*NR5A1*), *DAX1* (*NR0B1*), *GATA4*, *ATRX*, *AR*, *HSD17B3*, and *SRD5A2* (**Barbaro *et al.,* 2008; Tannour *et al.,* 2010; White *et al.,* 2011; Igarashi *et al.,* 2013**) or detect novel CNV suspected to affect new genes as *ADCY2*, *EMX2*, *CAMK1D* and *HOXD* cluster (**Mary *et al.,* 2023**). Recurrent CNVs of regulatory non coding sequences upstream of *SOX3* or *SOX9* were also reported to affect gene expression and result in DSD phenotype (**Ledig *et al.,* 2010; Tannour *et al.,* 2010; White *et al.,* 2011**).

In the current study *SOX9* deletion was detected in two patients, affecting exons 1,2 and 3 in the first patient and exon 2 in the second patient. *SOX9* (*Sry*-Related HMG-Box Gene 9; OMIM#608160) is located on chromosome 17q24.3 and belongs to the highly conserved HMG family members. *SOX9* is one of the key genes that plays a critical role in development of testis and long bones. It functions immediately downstream of *SRY* and is critical for Sertoli cell differentiation (**Morais da Silva *et al.,* 1996**). It acts also as a chondrogenic transcription factor and is a target of signaling by the parathyroid hormone-related peptide in the growth plate of endochondral bones (**Huang *et al.,* 2001**). *SOX9* abnormalities have been reported in 46,XY DSD with or without Campomelic dysplasia (OMIM#114290), which is an autosomal dominant skeletal dysplasia characterized by congenital shortness and bowing of long bones, face flattening and club feet. It have been associated with 46,XY gonadal dysgenesis with a wide range of severity (**Matsushita *et al.,* 2013**). Our first patient had a deletion in the three coding exons of *SOX9* with typical features of campomelic dysplasia in addition to atypical genitalia with a unilateral undescended testis, compared to the second patient who had deletion of one exon, and exhibited short stature with delayed milestones, microcephaly, blepharophimosis and mild dysmorphic features and a bilaterally undescended testis. Acampomelic campomelic dysplasia is a rare form of autosomal dominant congenital short-limbed dwarfism and also results from mutations in *SOX9* gene family. It is frequently misdiagnosed due to the absence of campomelic features (**Ledig *et al.,* 2010; Chen *et al.,* 2012; Pasupathy *et al.,* 2016**). In the past years, several isolated 46,XY DSD were reported due CNV affecting *SOX9* upstream regulatory region without affecting the coding sequence. This region contains transcription factor binding sites essential to maintain *SOX9* expression during sex development and represents a crucial factor in normal male development (**White *et al.,* 2011; Bhagavath *et al.,* 2014; Baetens *et al.,* 2017; Croft *et al.,* 2018**).

DMRT1 deletion was detected in a patient with 46,XY,del(9)(p22), who was presenting with inguinal hernia associated with dysmorphic features as well as congenital heart disease and intellectual disability. Distal 9p deletions have been reported in patients with syndromic 46,XY DSD. Within this region the strongest candidate for the gonadal dysgenesis phenotype is *DMRT1* (Double sex and mab-3 related transcription factor gene; OMIM#602424). It is located in 9p24.3 at the terminal region of 9 short arm and is found in a cluster with two other members of the gene family (*DMRT1-3*), having in common a zinc finger-like DNA-binding motif (DM domain) (**Raymond *et al.,* 2000; Eser and Ayaz, 2018**). *DMRT1* activates testis-specific genes such as *SOX9* and *SOX8*, and represses ovary-specific genes encoding Foxl2, Wnt4 and R-spondin-1 signaling proteins, and estrogen receptors (**Kim *et al.,* 2015**). **Swinkel *et al.,* (2008)** had divided the patients with 9p deletions into two groups. One group includes patients with the phenotype of 9p deletion syndrome, whereas the other group included patients that do not meet all the criteria of the consensus phenotype. This depends on the involvement of other disease-associated loci at 9p22.3–23 resulting in various malformations (**Igarashi *et al.,* 2013; Mohamed *et al.,* 2021**). Our patient had the syndromic features of 9p deletion and DSD in the form of two inguinal testes, which could be ascribed to *DMRT1* deletion. More severe DSD phenotype associated with a delay in psychomotor development was reported by **Bastian *et al.,* (2015)** in three patients with 9p24 deletion, among a cohort of 46,XY gonadal dysgenesis studied by CMA. On the other hand, recurrent CNVs affecting *DMRT1* were reported as potential causes of idiopathic azoospermia (**Lopes *et al.,* 2013**). Small deletions affecting *DMRT1* regulatory region were also reported as a cause of 46,XY gonadal dysgenesis (**Calvari *et al.,* 2000**). Varying degrees of sexual developmental disorders associated with the same genetic defect may result due to involvement of other regulatory genes or epigenetic factors contributing to the complex cascade of gonadal development (**Matson *et al.,* 2011**).

One of our patients was a 15- year-old male who presented with atypical genitalia and bilateral inguinal gonads. His clinical and hormonal assessments denoted 17β-HSD-3 deficiency and MLPA analysis detected duplication in exon 1 of *HSD17B3* gene. Malfunction in the *HSD17B3* gene (OMIM#605573) affects androgen synthesis in testis and results in disorder in the conversion of androstenedione to testosterone (**Neocleous *et al.,* 2012**). 17-β-HSD3 deficiency (OMIM#264300) is a rare

autosomal recessive disorder of male sex differentiation that occurs in 1:147,000 newborns (**Boehmer *et al.,* 1999**). Phenotype ranges from atypical genitalia with undescended or inguinal gonads to female external genitalia. Mutations in the *HSD17B3* gene may be in the form of homozygous or compound heterozygous mutations affecting 46,XY individuals. Many individuals raised as females develop a male gender identity and then decide to be reassigned as males after puberty. Diagnosis can be established by elevated Δ4 and low T serum levels that result in a T/Δ4 ratio lower than 0.8. (**Sullivan *et al.,* 2017**). However, the genetic testing is crucial for diagnosis besides the hormonal.

As the17β-HSD-3 deficiency is an autosomal recessive disorder, the partial heterozygous duplication of this gene is considered of unknown significance as we have not examined all the gene exons by Sanger sequencing. It is noteworthy that our finding comes in agreement with **Neocleous *et al.,* (2012)**, who similarly reported a case of atypical genitalia with a bilaterally palpable gonads in the inguinal canal and no Müllerian structures. He showed duplication in *HSD17B3* gene in exons 3-10 by MLPA and a heterozygous mutation in p.R80Q region by sequencing. His parents' sequencing revealed the presence of duplication in the mother and a heterozygous mutation in the father. This may be the case in our patient, who should undergo re-evaluation and sequencing of all exons to explore the possibility of presence of a heterozygous mutation that could explain the hormonal and MLPA findings of this patient. None of other patients with suspected 17β-HSD-3 deficiency or 5-alpha-reductase type 2 (5α-RD2) deficiency obtained positive results by MLPA, indicating the importance of Sanger sequencing of genes commonly implicated in DSD phenotypes in complementing MLPA (**Barbaro *et al.,* 2008; Biesecker and Green, 2014**).

5α-RD2 deficiency is one of the common causes of atypical genitalia in children. The phenotype can vary from underdeveloped male genitalia to a complete female phenotype. It is diagnosed by estimating the ratio between testosterone and dihydrotestosterone (DHT) after human chorionic gonadotropin (hCG) stimulation, whereas there is an increase in the ratio of testosterone to DHT. However, this test is not totally diagnostic as the ratio may vary based on the severity of enzyme deficiency and the children's age. It is also not useful in partial enzyme deficiencies (**Kumar and Barboza-Meca, 2022**).

In the present study, two siblings had a pericentric inversion of the X chromosome and presented with atypical genitalia associated with partial gonadal dysgenesis, intellectual disability and Duchenne muscular dystrophy (*DMD*). The gene on X chromosome related to XY gonadal dysgenesis and sex reversal is *DAX1* (*NR0B1*; OMIM#300018) mapped to Xp21.2, while the gene causing *DMD* is the dystrophin (*DMD*) gene (OMIM#300376) residing also in the same region: Xp21.2-Xp21.1. This deviated the attention to the possibility of disruption or copy number alteration affecting this region as a result of X inversion. However, MLPA result was not informative. Several cases of pericentric inversion of X chromosome with different breakpoints have been reported with phenotypes ranging from normal to gonadal dysgenesis (**White *et al.,* 2011; Garcia-Acero *et al.,* 2019**). Other studies previously detected X chromosome rearrangements involving *DAX1* region by MLPA analysis in patients with gonadal dysgenesis, which was usually associated with mental subnormality (**Barbaro *et al.,* 2008; White *et al.,* 2011; Garcia-Acero *et al.,* 2019**). Nonetheless, not only gene regions should be investigated but also intergeneic sequences using different methods could be helpful. It was suggested that the X rearrangement in our two sibs had led to disruption of a regulatory region or disturbed an upstream regulatory gene, resulting in *DAX1* gene overexpression and abnormal expression of *DMD* gene that led to disturbance of the testicular developmental pathway and *DMD*, respectively. Other methods are further required to detect the expression level of *DAX1* and *DMD* genes as chromosomal microarray and quantitative real-time PCR. *DAX1* gene have an important role in gonadal sex determination and is expressed in the first stages of gonadal and adrenal differentiation and in the developing hypothalamus. In normal 46,XY males, the *DAX1* is held inactive by *SRY* and thus, a male gonadal differentiation pathway is followed. Duplications in XY individuals lead to *SOX9* inactivation and block of testicular formation and patients develop as females with a hypoplastic uterus (**Suntharalingham *et al.,* 2015; de Oliveira *et al.,* 2024**). Nevertheless, our two sibs exhibited atypical genitalia without Mullerian structures, suggesting that the *DAX1* is partially overexpressed to the extent that only led to partial testicular dysgenesis. *DMD* gene encodes dystrophin, a large muscle protein that its mutations lead to Duchenne (310200) and Becker (300376) muscular dystrophy (**Kilimann *et al.,* 1992**). *DMD* is defined as progressive deterioration of muscle tissue resulting in increasing muscular weakness. The association of ID in our two patients, could be hypothesized to be due to abnormal expression of *IL1RAPL1* gene (interleukin-1 receptor accessory protein-like, gene 1; OMIM#300206), mapped to the same region: Xp21.3-p21.2, just distal to *DAX1*. Deletions of this gene or telomeric from *DAX1* have been shown to be associated with X-linked intellectual developmental disorder (OMIM#300143). Moreover, *MRXS17* gene (Mental Retardation, X-Linked, Syndromic 17), residing in the vicinity Xp21.1-p11.23 may be contributed to the phenotype due to abnormal expression or disruption of its regulatory elements by the Xp breakpoint resulting in ID. This region has been mapped by **Marom *et al.,* (2011)** to be associated with X-linked Intellectual developmental disorder (OMIM#300858).

Lack of proper testicular development makes a favorable micro-environment for an increased risk for

malignant transformation (**García-Acero *et al.,* 2020**). The overall tumor risk is between 15-33%. DSD patients are considered at high risk of development of testicular carcinoma in situ, germ cell tumors, gonadoblastoma and dysgerminoma (**Cools, 2014; Grinspon *et al.,* 2020**). Females with intra-abdominal gonads carry the highest risk of tumor development. It is recommended to perform prophylactic gonadectomy at different timings depending on diagnosis. Pre-operative assessment is essential to locate the gonads precisely (**Basri *et al.,* 2021**).

CONCLUSION

In the present study, MLPA could detect CNV in 11.4% of 46,XY DSD patients. It is useful as a screening method that can detect copy number changes at the exon level of genes known to be involved in DSD, including small intragenic rearrangements and can analyze several samples at a time. However, it should be complemented by sequencing analysis of genes commonly implicated in DSD to increase the diagnostic yield and to identify gene mutations accompanied with the deletion or duplication. As the genetic testing technology is evolving, CNV analysis remains a powerful tool to solve undiagnosed DSD patients, especially the syndromic cases. More sophisticated techniques such as chromosomal microarray and Optical genome mapping can significantly augment solving undiagnosed cases.

CONFLICT OF INTEREST

There are no conflicts of interest.

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