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Anomalies in Human Sex Determination: Usefulness of a Combined Cytogenetic Approach to Characterize an Additional Case with Xp Functional Disomy Associated with 46,XY Gonadal Dysgenesis

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What is already known on this topic?

Xp-Yp translocation, t(X;Y)(p21;p11.3), is a rarely occurring rearrangement resulting in pure functional disomy of Xp, including the dosage sensitive sex (DSS) reversal region and is associated with 46,XY gonadal dysgenesis (GD).

What this study adds?

We report the fourth case of Xp;Yp translocation with Xp21.2-pter duplication associated with XY GD. Molecular cytogenetic methods are still relevant for the characterization of the exact chromosomal mechanism responsible for severe clinical features including DSD at an early age. This may contribute to understanding the possible genetic cause of syndromic 46,XY DSD cases and provide special and personalized support for these cases.

Abstract

Objective: Disorders of sexual development (DSD) are a heterogeneous group of genital defects affecting chromosomal, gonadal and anatomical sex. 46,XY DSD is a subset of DSD which covers a wide range of phenotypes in which 46,XY gonadal dysgenesis (GD) is the most severe form. In this study, we report on the clinical and molecular cytogenetic findings of a study on a Tunisian girl with the syndromic form of 46,XY DSD.
Methods: This case was a phenotypic female patient having several congenital anomalies including growth retardation. Karyotype, fluorescence in situ hybridization and array Comparative Genome Hybridization (array CGH) were performed.
Results: The proband exhibited a de-novo 46,X,der(Y) karyotype. Array CGH revealed a pathogenic 27.5Mb gain of an Xp21.2 chromosome segment leading to Xp functional disomy. No deletion was observed in the Y-chromosome. The duplicated region encompassed the NR0B1 (DAX1) and MAGEB genes, located within the dosage sensitive sex (DSS) reversal locus, known as promote genes responsible

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for human sex reversal and testis repression. The extra-dosage and interactions of these genes with different specific genes could result in the impairment of the male sex pathway. Over-dosage of KAL1 and IL1RAPL1 genes fall within the somatic features observed in the patient.

Conclusion: To the best of our knowledge, we report on the fourth case of Xp21.2-pter duplication within Xp;Yp translocation associated with XY GD. Our findings suggest that when duplicated, the NR0B1 and MAGEB genes could be a major cause of XY GD. Therefore, we emphasize the usefulness of a combined cytogenetic approach in order to provide an accurate genetic diagnosis for those patients having syndromic XY DSD in a clinical setting.

Keywords: Disorders of sexual development, dosage sensitive sex reversal locus, functional disomy Xp, 46,XY gonadal dysgenesis

Introduction

The type of the gonad of an individual is usually a testis or ovary governed by sex chromosomes. In 46,XY individuals, the presence of the Y chromosome testis-determining gene *SRY* (OMIM#601947) initiates the formation of the testis and inhibits the formation of the ovary. DSD are a set of rare congenital conditions in which the chromosomal, phenotypic, and anatomical sex are discordant and they can occur in isolated or syndromic forms (1).

The subset 46,XY DSD, which includes gonadal dysgenesis (GD), disorders of androgens synthesis or action, or disorders of anti-Müllerian hormone (AMH) synthesis or action in XY GD, is the most prevalent etiology of this condition. It is characterized by an abnormal formation of the testis due to chromosomal imbalances or mutations involving key genes implicated in the formation of the gonad (2).

The emergence of next-generation sequencing technology has allowed for the identification of the genetic etiologies in 50% of DSD cases (3,4). Yet, we cannot ignore the role of banding and molecular cytogenetic techniques in the diagnosis of DSD in which the etiology has been determined in 20% of cases (5).

Remarkably, a large proportion of DSD is caused by copy number variation (CNV) involving critical dosage-sensitive genes with a large spectrum of gonadal phenotypes.

Duplications of chromosomal regions containing Xp21, also termed Xp functional disomy, are known to cause syndromic 46,XY DSD and all reported patients presented with sex reversal as part of a complex phenotype which includes dysmorphic features and/or mental retardation (6,7). Notably, der(Y)t(X;Y)(p21.1;p11.3) is a rarely occurring rearrangement in which the translocation of the duplicated Xp segment to the Y chromosome results in a pure functional disomy of the Xp encompassing the dosage sensitive sex (DSS) locus.

Only three cases with der(Y)t(X;Y)(p21.2;p11.3) have been reported as being raised as females even with the presence of an intact Y chromosome and *SRY* gene (6,7,8).

The duplicated DSS locus contains the melanoma antigen, Family B (*MAGEB)* genes and the nuclear receptor subfamily 0, Group B *NR0B1* gene, the most probable causes of XY GD, if overexpressed (6,7,8,9).

In this paper, we report on an additional case with syndromic 46,XY GD due to Xp functional disomy within Xp;Yp translocation. We underline the complementarity between the different cytogenetic techniques to characterize the duplication and translocation events and their contribution to the management of XY GD cases. Therefore, the Comparative Genome Hybridization (CGH) array can be considered as an efficient tool for the diagnosis of chromosomal aberrations, when investigating syndromic forms of DSD.

Methods

Clinical Presentation of the Patient

The patient was a seven-month-old girl with dysmorphic features and profound failure to thrive.

She was referred to our department for genetic diagnosis. Written approval was obtained from the patient's parents in order to perform genetic analyses and complementary studies, as well as to publish this data.

The Local Ethics Board of the University Teaching Hospital Farhat Hached approved the present study (no: IRB00008931, date: 15.03.2022), written consent was taken from the parents for photo publication and consent for the genetic analysis and publication of the case were obtained from the parents.

Peripheral Blood Karyotype

Reverse Heat Giemsa banded karyotype was performed on the metaphase chromosome preparations obtained from peripheral blood lymphocytes of both the patient and her parents according to standard protocols (450-550 band level). Metaphase chromosome spreads were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes. Cell cultures were incubated for 72 hours. A minimum of 20 R-banded metaphase chromosomes were

analyzed using Cytovision® Karyotyping software version 4.0. Karyotypes were classified according to the International System of Human Cytogenomic Nomenclature (2020) (10).

FISH Analysis

Fluorescence *in situ* hybridization (FISH) was carried out on metaphase chromosomes of the patient according to the standard protocol, using commercial probes (Kreatech Diagnostics): Whole chromosomes painting (WCPX,WCPY), centromeric probe for chromosome X (CENX), SHOX (Xp22.33 and Yp11.2), SRY (Yp11.3), XIST probes (Xq13.2) and STS (Xp22.31) were used as telomeric probes. Bac clone RP11-89I17 (*NR0B1* gene), RP11-147O4 (*KAL1* gene) and RP11-639O (primary Pseudoautosomal region) were also used. Probes were applied to metaphase slides and co-denaturized for 7 mins at 75 °C. After 24 hours of hybridization at 37 °C and washing, the chromosomes were counterstained with a 4.6 diamino-2-phenylindole and observed using an Axioskop Zeiss® fluorescent microscope. Images were captured with a CCD camera (Cytovision, Applied Imaging®).

Array CGH

CGH 4x44K micro-array was performed using the agilent platform as previously described (11,12). Agilent® oligonucleotide array was performed according to the manufacturer's instructions (Agilent Human Genome CGH Microarray kit 44K®).

Statistical Analysis

Percentile study rank level was used to generate the following: baby girl growth chart, infant boy growth chart, height, weight, body mass index and cranial perimeter (https://www.childgrowthcalculator.com/#grafica_longitud).

Percentiles are given according to the World Health Organization data by comparing the growth chart of our patient with most of the children at her age.

Results

Clinical Report

The investigated case was the first child of an apparently healthy consanguineous Tunisian couple (second-degree relatives). Her birth weight was 2.350 kg (percentile = 1.8). Her height was 49 cm (percentile = 46), and her head circumference was 33 cm (percentile = 21.2). At the age of seven months, she was referred to our department for exploration of dysmorphic features associated with profound failure to thrive. The anthropometric measurements were below the 3rd percentile. The child's length percentile was 1.4; and her weight and cranial perimeter percentiles were 0.3. She had craniofacial dysmorphic features including long face, exophthalmos, hypertelorism, ogival palate, a relatively short and flat philtrum and strabismus (Figure 1). Moreover, she had exhibited a remarkable weight stagnation since the age of 2 months with marked hypotonia. The family history was unremarkable.

At the age of 11 months, she continued to have mild generalized hypotonia, and was still unable to hold her head up. She had severely retarded psychomotor development. Her anthropometric measurements continued to be below the 3rd percentile. An abdominal ultrasound study was performed and revealed small kidneys, invisible uterine or ovarian structure. Supplementary investigations with pelvic magnetic resonance imaging (MRI) showed an absence of internal female organs.

An echocardiogram showed an inter-atrial communication (IAC) heart defect. When last assessed at the age of 16 months, she was still unable to sit independently with a marked axial hypotonia and nystagmus. Anthropometric measurements continued to be below the 3rd percentile, and the IAC diameter increased (Figure 2).

At the age of eight years, the patient still had severe growth delay and was operated on for a percutaneous closure of IAC. At this age, her hormonal profile was as follows:

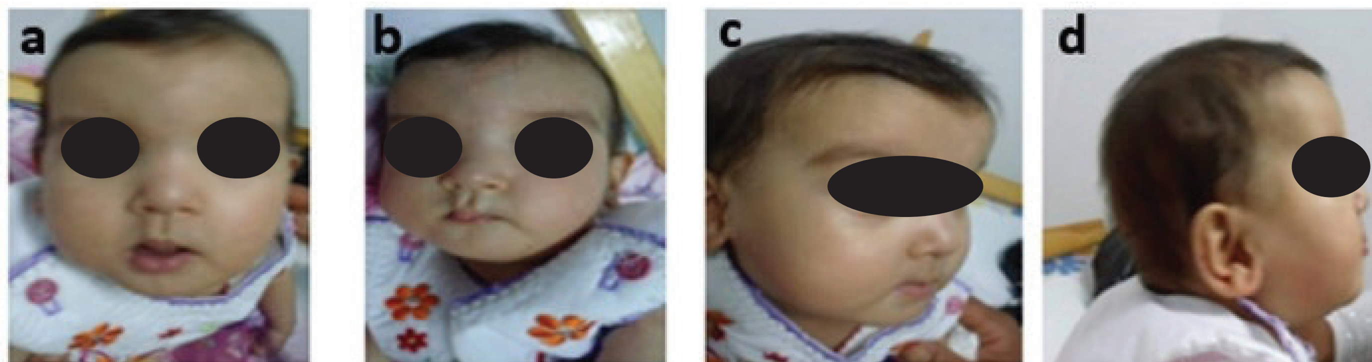

Figure 1. Photographs of face (a, b) and profile picture (c, d) of the patient at 7 months

Figure 2. Photographs of face of the patient at 16 months

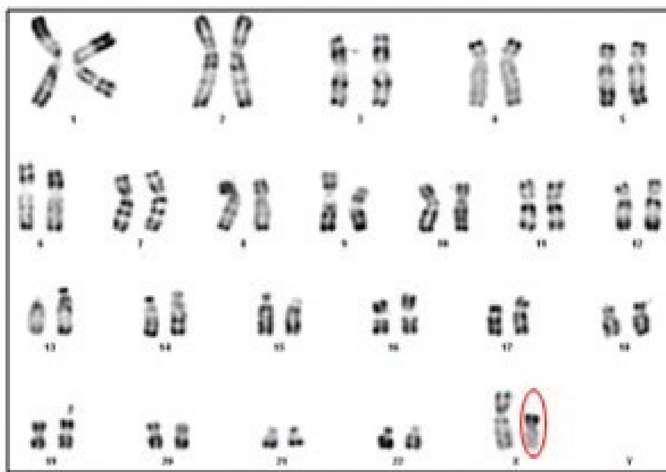

Figure 3. RHG banded-karyotype showing a marker chromosome (red circle)

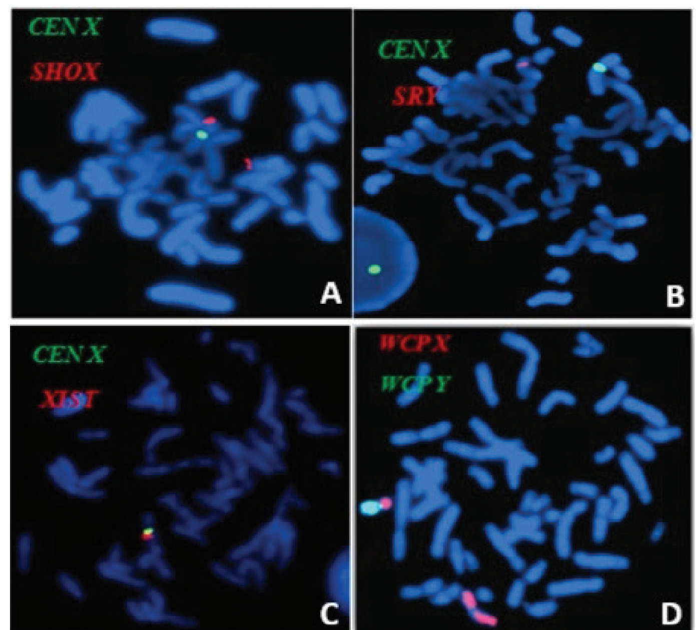

Figure 4. FISH analysis. A) FISH results using SHOX probe, two red spots were detected. B) FISH results using SRY probe, one red spot was detected. C) FISH results using XIST probe, one red spot was detected. D) FISH analysis using WCPX/WCPY showed the presence of a part from chromosome X on the Y chromosome

FISH: fluorescence in situ hybridization

luteinizing hormone (0.1 IU/L; reference range: 1.1-10), follicle-stimulating hormone (1.2 IU/L; reference range: 1.3-11), AMH (0.67 ng/µL; reference range: 1.43-11.6) and estradiol (10 pg/mL; reference range: <30).

Genetic Results

Cytogenetic analysis revealed a 46,X,der(Y) karyotype in all metaphase cells from the proband (Figure 3). The parent's karyotypes were normal (data not shown).

The extra material was a *de novo* rearrangement and was identified as a der(Y). FISH using whole-chromosome X and Y painting probes showed labeling along the entire length of the normal X chromosome and on the terminal segment of the short arm of the der(Y) chromosome (Figure 4D).

FISH analysis with specific loci probes showed the presence of the *SRY* gene on the short arm of the Y chromosome and the presence of the *SHOX* gene on each of the sex chromosomes. The XIST probe was present on the long arm of the X chromosome (Figure 4A, 4B, 4C). Specific locus probes of the X chromosome, *NR0B1* (Xp21.2) and *KAL1* (Xp22.31), were present in double copies on the normal X chromosome and the other on the der(Y) respectively (Figure 5A, 5B). Using the *STS* gene probe, one signal was detected on the X and der(Y) chromosomes telomeres (Figure 5B). Array CGH displayed a gain of genetic material on the short arm of chromosome X encompassing approximately 27.5Mb mapping from 2,710,316 to 30,248,793 according to the Genome reference Consortium Human build 36 assembly (hg18/NCBI36) (Figure 6).

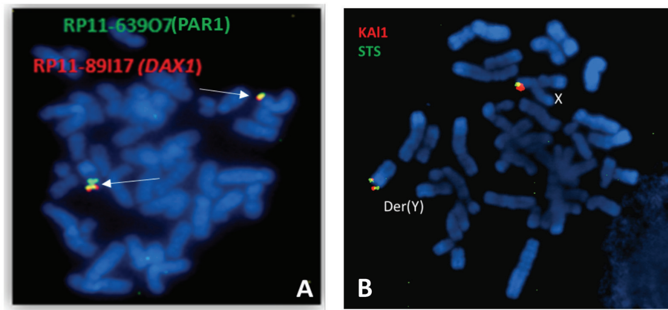

Figure 5. FISH analysis using specific probes: A) NR0B1 probe showed its presence on both sex chromosomes (white arrows); B) KAL1 and STS probes showed their presence on both sex chromosomes

FISH: fluorescence in situ hybridization

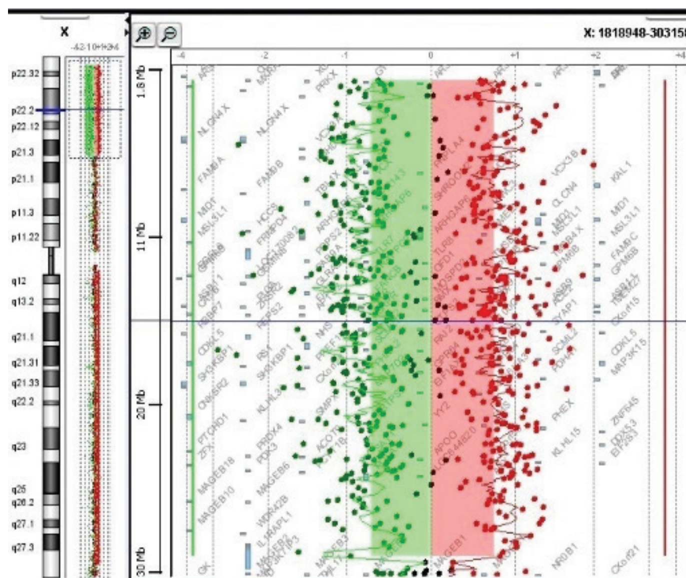

Figure 6. 4×44K Agilent Technologies oligonucleotides array profile of our patient showing Xp21.1 duplication of approximately 27.5 Mb

No other chromosomal rearrangement was detected, particularly within the Y chromosome.

Based on these results, the final karyotype of our patient was designated as:

46,X,der(Y)t(X;Y)(p21.2;p11.3).arr[NCBI36]Xp21.2-p22.3 (2,710,316_30,248,793)x2 dn

Discussion

Sex determination is a complex process that implicates specific genes required for the progressive development of the undifferentiated gonad. An adequate dosage of these genes is required for proper gonadal development. CNV consistent with deletions or duplications of the genomic area including these genes may lead to DSD (13).

In this study, the patient investigated represents one of a small number of reported Xp;Yp translocation cases and the first reported Xp functional disomy case from Tunisia due to a large duplication on the Xp chromosome (Table 1).

The proband presented a 46,X,der(Y) male karyotype. The rearranged Y chromosome was the product of a translocation between sex chromosomes resulting from a non-allelic homologous recombination (NAHR) during paternal meiosis or in the early stages of embryogenesis. The parental karyotypes were normal, indicating a *de novo* origin of the unbalanced chromosome translocation. Array CGH was performed and it showed a gain of nearly 27 Mb on the Xp21 chromosome with a log-ratio equal to 0.58.

Based on the clinical and phenotypical criteria, biochemical assays, and genetic investigations, we can confirm that our patient presents with the syndromic form of 46,XY GD (OMIM≠300018) including a range of extra-gonadal abnormalities (growth delay, mental retardation, hypotonia, dysmorphic features and IAC). Remarkably, our patient had a complete Y-chromosome within an intact *SRY* gene and is a female. This reveals that the male sex development process is relatively complex and further factors are necessary for early testis formation with adequate dosage.

Thus, the duplicated region covers several genes, namely the *NR0B1, MAGEB, KAL1* and *IL1RAPL1* genes, resulting in ectopic expression and causing a disturbance in several developmental systems.

NR0B1, also called dosage-sensitive sex reversal gene (*DAX1*;OMIM#300018), is located in the DSS region at Xp21.2. When duplicated, *NR0B1* is considered to be the most likely factor for 46,XY GD (19,20,21,22).

NR0B1 is an orphan nuclear receptor which acts in a mutually antagonist pathway to ensure testis determination. Its expression has been shown in different tissues (adrenal cortex, gonad, anterior pituitary, and hypothalamus, and also in adult adrenal cortex, Sertoli and Leydig cells in the testis, theca, granulosa, and interstitial cells in the ovary), highlighting its pleiotropic function (20,21,22). The link between *NR0B1* and the sex development process has been established by several studies. Previously, the *NR0B1* gene was known to be a dosage-sensitive ovarian determining gene. It is in fact down-regulated in the developing testis and persists in the ovary in mice (16,21). Conversely, in the last five years, numerous studies have shown that *NR0B1* plays an important role in male gonadogenesis and acts as an anti-testis factor within a critical window of sex development (23,24).

Table 1. Comparison of clinical and genetic findings in cases with partial disomy Xp21. 1-Xpter and a Y chromosome to Xp disomy with Xp;Yp translocation

References	Ogata et al. (6) 1992	Sanlaville et al. (7) 2004	Ashton et al. (8) 2013	Barbaro et al. (14) 2007	White et al. (15), 2011; case 13	Bardoni et al. (16) 1994; case 711	Bardoni et al. (16) 1994; case BG	Ledig et al. (17) 2010; case 9	Ledig et al. (17) 2010; case 52	Bernstein et al. (18), 1980	Present study
Karyotype	46,X,der(Y) t(X;Y) (p21;p11.3)	46,X,der(Y) t(X;Y) (p21.2;p11.3)	46,X,der(Y) t(X;Y) (p21.1;p11.3)	46,XY	46,XY	46,Y,dup(X) (p21.2-p22.3)	46,Y,dup(X) (p21.2-p22.2)	46,XY	46,XY	46,Y,dup(X) (p21-pter)	46,X,der(Y)t(X;Y) (p21.2;p11.3)
Mechanism	t(Xp;Yp)	t(Xp;Yp)	t(Xp;Yp)	Duplication	Duplication	Duplication	Duplication	Duplication	Duplication	Duplication	t(Xp;Yp)
Size of the CNV	NA	NA	NA	637 Kb	771 Kb	NA	NA	16.23 Mb	729 Kb	NA	27.5 Mb
Gender	F	F	F	F	F	F	F	F	F	F	F
Age at diagnosis, years	2 years and 3 months	Postnatal	4 months	15 years old	NA	NA	NA	NA	NA	3 year old	7 months
Cranio-facial dimorphism	- Frontal bossing - Antimongoloid slant - Large, low set ears with thick auricular folds - Cleft palate	- Bitemporal narrowness - Short nose - Prominent forehead - Scarce hair - Low-set ears - Cleft palate	- A bulbous nasal tip - Pinched nares with protuberant columella - Prominence of the philtral pillars - Small mouth	NA	-	NA	NA	+	NA	- Cleft palate - Prominent forehead - Mild hypertelorism - Short nose - Large ears	- Long face exophthalmos - Hypertelorism - Ogival palate - Short and flat philtrum - Strabismus
Growth retardation	+	+	+	NA	-	NA	NA	+	-	+	+
Hypotonia	+	+	+	NA	-		NA	+	-	+	+
Delayed mental development	+	+	+	NA	-	NA	NA	+	-	+	+
Delayed motor development	+	+	+	NA	-	NA	NA	+	-	+	+
External genitalia	F	F	F	F	F	Hypoplastic female external genitalia	F	F	Clitoris hypertrophy	F	F
Internal genitalia				Normal Mullerian derivatives	Normal Mullerian derivatives	Normal vagina	Normal Mullerian derivatives	Uterus	NA	Normal vagina and cervix, hypoplastic uterus, and fallopian tubes	No uterus and vagina

Table 1. Continued

References	Ogata et al. (6) 1992	Sanlaville et al. (7) 2004	Ashton et al. (8) 2013	Barbaro et al. (14) 2007	White et al. (15), 2011; case 13	Bardoni et al. (16) 1994; case 711	Bardoni et al. (16) 1994; case BG	Ledig et al. (17) 2010; case 9	Ledig et al. (17) 2010; case 52	Bernstein et al. (18), 1980	Present study
Gonads	Streak gonads	-	-	Streak gonads	-	-	Absent left gonad and right streak gonad with primordial sex cords	-	Testicular residues	-	-
Other features	Autoimmune disease	Partial agenesis of the corpus callosum	Partial agenesis of the corpus callosum	NA	-	NA	NA	NA	-	A ventricular septal defect	Inter-atrial communication heart defect
Clinical diagnosis	Syndromic XY GD	Syndromic XY GD	Syndromic XY GD	Isolated XY GD	Isolated XY CGD	NA	Isolated XY GD	Syndromic XY GD	Isolated XY GD	Syndromic XY GD	Syndromic XY GD

F: female, +: present, -absent; NA: not available; GD: gonadal dysgenesis; CGD: complete gonadal dysgenesis, CNV: copy number variation

In normal XY males, a single copy of *NR0B1* is required for normal testis cord formation and testicular hormone synthesis (25,26,27). When *NR0B1* is overexpressed in 46,XY individuals, as in Xp duplication, it inhibits *SOX9* gene expression and antagonizes the synergy between *SF1* and *SOX9* by inactivating the *AMH* gene promoter. The inactivation of the AMH promoter gene blocks the regression of the Müllerian ducts. Thus, testicular formation is disrupted and a female pathway is followed (23,24,28). However, no uterus or gonad were observed in this case. Non-visualized uterus and gonad on MRI do not exclude the possibility of a GD diagnosis, which was confirmed by our genetic finding, since the structures may be too small to be detected at the time of examination. Small-sized structures could lead to suboptimal signal or resolution of the MRIs or suboptimal visualization and subsequently misinterpretation (29).

In addition to the *NR0B1* gene, the duplicated region contained testis expressed genes, called *MAGEB* genes located within the DSS locus (20). Recently, it has been speculated that overexpression of these genes could be involved in male to female sex reversal and may have a role in maintaining fetal testicular identity (14,28). Also, the *MAGEB* (1,2,3) genes seem to be functionally required for X chromosome inactivation mediated by XIST (30). Interestingly, the deletion of the same region containing *NR0B1* and MAGEB has been reported to be responsible for the opposite phenotype in a 46,XX SRY-negative ovotesticular DSD (28). So far, an adequate dosage of both *NR0B1* and *MAGEB* genes is needed for both male and female sex development and most likely these genes belong to overlapping complex molecular cascades in the testicular/ovarian tissue (i.e. from sex determination to sex differentiation).

Hence, a breakage within the DSS region may interfere with the spatiotemporal expression pattern resulting in ectopic expression, and incomplete stimulation/repression of male or female sex development, leading to different stages of sexual ambiguity. All these disorders can be responsible for infertility in adulthood.

Additionally, the duplicated region, in addition to the *NR0B1* gene, included several other genes which may be responsible for the patient's phenotype.

The *KAL1* gene (OMIM#308700) encodes a secreted heparin-binding protein (KAL or anosmin-1) which plays an important role in the embryonic development of the kidneys and human central nervous system. *KAL1* stimulates the signaling activity of the fibroblast growth factor receptor (FGFR1), which is involved in a variety of developmental processes including the formation, growth and shaping of

different tissues and organs (31). The overexpression of *KAL1* may interfere with FGFR1 signaling activity, which may be indirectly responsible for developmental and speech delay, intellectual disability and genital abnormalities (32,33).

The duplicated region also encompassed the gene encoding the IL1 receptor accessory protein-like1 gene (*IL1RAPL1,* #OMIM;300143*)*, a protein with high levels of expression in hippocampal neurons known to be involved in the memory system. Deletions and mutations in this gene were found in patients with mental retardation, which suggests a specific role in the physiological processes underlying memory and learning abilities (14). Within large Xp21 duplications, disruption of this gene could explain mental retardation.

To summarize, cytogenetic techniques are still as important as ever in the detection of chromosomal rearrangements, especially when the clinical manifestations are highly evocative of a known syndrome.

In fact, in a review of 116 patients with idiopathic DSD, array CGH was able to detect clinically relevant CNV in 21.5% of the patients (34). In another study of a cohort of 87 patients, array CGH identified CNV in 31.25% of syndromic DSD cases and in 29.57% of non-syndromic DSD cases (17). This justifies its relative contribution to the identification of CNV related to different DSD phenotypes by characterizing the exact size, and breakpoints as well as the expansion of the pool of candidate genes in disease pathogenesis in a single step. The discovery of new genomic analysis tools such as Hi-C technology may provide new insights into the physical genomic interactions and support the hypothesis that a common genomic region can be bound by both pro-testis and pro-ovarian transcription factors and genes (35,36).

Study Limitations

A possible limitation of the present study may be that we reported on a single case which may not be that conclusive. The number of patients presenting with GD resulting from Xp;Yp translocation is also limited due to the rarity of this rearrangement.

Conclusion

The results presented in this study illustrate the first Tunisian case having 46,XY GD due to a large duplication within the Xp21.2 DSS locus and associated with an X;Y translocation event.

Such DSD cases are very rare and require a careful, systematic, and sensitive approach to diagnose. Together, the karyotype, FISH and array CGH can prove useful in delivering a conclusive genetic diagnosis for those patients with the syndromic form of DSD by identifying chromosome abnormalities associated with dosage changes in genes, such as *NR0B1*, which play a pivotal role in human sex development.

Acknowledgments

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Ethics

Ethics Committee Approval: The Local Ethics Board of the University Teaching Hospital Farhat Hached approved the present study (no: IRB00008931, date: 15.03.2022).

Informed Consent: Written consent was taken from the parents for photo publication and consent for the genetic analysis and publication of the case were obtained from the parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ikbel Hadj Hassine, Afef Jelloul, Ali Saad, Soumaya Mougou-Zerelli, Concept: Khouloud Rjiba, Fethi El Amri, Monia Zaouali, Kenneth Mcelreavey, Design: Khouloud Rjiba, Kenneth Mcelreavey, Data Collection or Processing: Wafa Slimani, Meriem Gaddas, Hela Ben Khelifa, Analysis or Interpretation: Khouloud Rjiba, Wafa Slimani, Literature Search: Khouloud Rjiba, Writing: Khouloud Rjiba.

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