The importance of the multiplex ligation-dependent probe amplification in the identification of a novel two-exon deletion of the *NR5A1* gene in a patient with 46,XY differences of sex development

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Abstract

Gonadal dysgenesis (GD) is a rare cause of differences of sex development (DSD) with highly variable clinical and genetic conditions. Although identification of the causative genetic alterations can offer a clearer prognosis and personalized management to patients, more than 50% of the DSD cases still do not have an accurate genetic diagnosis. *NR5A1* (previously known as *SF-1*), is a transcriptional regulator of genes required for normal development and functional maintenance of the gonads and the adrenal glands. Nucleotide sequence variants of the *NR5A1* gene have been reported in numerous patients with GD with or without adrenal failure, however, microdeletion or partial deletion in the *NR5A1* gene have been described only in a few GD cases. In this case study, we present a subject with female phenotype, mild clitoromegaly, partial GD and normal adrenal function. Cytogenetic analysis revealed a 46,XY *SRY*+ karyotype. Microarray analysis did not identify pathogenic copy number variations, nor did panel sequencing of the most common DSD genes. Subsequently, multiplex ligation-dependent probe amplification (MLPA) was performed to test for small deletion/duplication of the most frequently affected genes associated with GD. Using this method, we have identified a novel heterozygous deletion involving exons 5 and 6 of the *NR5A1* gene as the cause of abnormal sexual development of the patient. This report expands our knowledge about the range and pathogenetic role of *NR5A1* mutations associated with partial gonadal dysgenesis in 46,XY DSD. Furthermore, our data emphasises the indispensable role of MLPA in the diagnosis of DSD with unclear etiology.

Keywords Gonadal dysgenesis · *NR5A1* · MLPA · Novel partial deletion · 46,XY DSD

Introduction

Differences of sex development (DSD) are congenital conditions in which the development of chromosomal, gonadal and anatomical sex is atypical. The frequency of DSD is about 1 in 4500 live births [1]. The etiological background of DSD is extremely heterogeneous as it can

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be induced by numerical or structural sex chromosome aberrations, variations of genes involved in the gonadal and/or genital development or steroidogenesis, maternal factors, hormonal disturbances and epigenetic modifications [2–4].

Based on sex chromosomal content DSD are classified into three groups: sex chromosome DSD, 46,XX DSD and 46,XY DSD [5]. In clinical practice, less than 50% of the DSDs with gonadal dysgenesis (GD) have specific molecular genetic diagnosis resulting from highly variable phenotype, heterogeneous genetic background and low genotype–phenotype correlation. Due to the high proportion of DSD cases with unknown genetic background, this field is still under intensive research. Heterozygous mutations of *NR5A1* is the most prevalent genetic cause in 46,XY DSD individuals, with a frequency of about 15–20% [6]. *NR5A1* (previously known as steroidogenic factor-1, *SF-1*) is a transcription factor that belongs to the nuclear receptor superfamily and regulates a number of genes involved in early development of bipotential gonads, in subsequent differentiation of the testes, and in steroidogenesis. Human *NR5A1* mutations were first reported in association with 46,XY DSD and adrenal insufficiency [7], but later studies revealed that mutations are more frequent in 46,XY DSD patients without adrenal failure [8, 9]. Most commonly DSD is associated with ambiguous external genitalia, with or without Mullerian structures, due to variable degrees of testicular dysgenesis with compromised androgen and anti-Mullerian hormone (AMH) production [6]. The *NR5A1* gene, located on chromosome 9q33.3, is composed of 7 exons including the non-coding first exon [10]. To date, the identified variants are mainly missense, nonsense, splicing mutations, small deletions and insertions [11–13]. Large scale mutations, such as microdeletion of 9q33 chromosomal region or partial gene deletions involving *NR5A1* have been reported in only 5 cases with 46,XY DSD (Table 1) [14–18].

In this study, we present a novel mutation of the *NR5A1* gene in a female patient with 46,XY karyotype and GD. The genetic alteration, a small partial deletion including exon 5 and 6, was identified by multiplex ligation-dependent probe amplification (MLPA). This case is the first report of a de novo exon 5 and 6 deletion of the *NR5A1* gene identified in 46,XY DSD. Our finding shows that although intragenic copy number alterations are a rare cause of GD, testing for these genetic variations should be considered in DSD patients with unknown etiology.

Materials

The proband is the first child of non-consanguineous Caucasian healthy parents. Family history was negative for DSD, premature ovarian failure (POF), fertility problems or any genetic disease. The patient was born after an uneventful pregnancy at 36 weeks of gestation with a birth weight of 3200 g. At birth clinodactyly of the 5th fingers and 2–3 toe cutaneous syndactyly were noticed on both sides. Both of these are common isolated malformations in the general population. External genitalia showed female appearance with slight clitoromegaly (Prader stage I). At 9 months of age, the child was admitted to a hospital because of abdominal pain and vomiting. Bilateral inguinal hernias were found with gonads, suspected testes, during transabdominal ultrasonography (US). Upon laparoscopy mixed internal genitalia with a very small uterus, fallopian tubes and epididymis were detected. Biopsy of the gonads identified testicular tissue with no spermatogonia and a small number of Leydig cells. The gonads were placed into the abdomen. At that time G-banded chromosome analysis was performed and a *SRY* positive 46,XY karyotype was revealed. Complete androgen insensitivity syndrome was excluded based on low testosterone level (<0.15 nmol/L).

Table 1 46,XY DSD cases with deletions containing *NR5A1* gene

Size of deletion	Clinical and gonadal phenotype	Other symptoms	Genetic testing	Inheritance	References
3.07 Mb	Clitoromegaly, ovotestis, absence of uterus	Genitopatellar syndrome	Array CGH	Not done	Schlaubitz et al. [17]
1.54 Mb	Female external genitalia	Mild mental retardation, minor dysmorphism	Array CGH	De novo	Brandt et al. [15]
0.97 Mb	Clitoromegaly, absence of uterus or Mullerian structure, fusion of the labia majora, shallow vaginal entrance, gonad in the left labium	Left-sided ptosis	Array CGH	De novo	van Silfhout et al. [18]
0.24 Mb	Ambiguous genitalia with perineal hypospadias, bilateral gonads palpable in the labioscrotal folds	–	Array CGH	Maternal	Harrison et al. [16]
3.1–4.8 kb	Leydig cell hyperplasia, scarce germ cells, carcinoma in situ	–	Custom MLPA	Not done	Barbaro et al. [14]
Max. 7.642 kb	Clitoromegaly, absence of uterus, blind-ending vagina, dysgenetic gonads	–	MLPA	De novo	This study

Gonadotropin hormone levels were the following: follicle-stimulating hormone (FSH): 6.4 mIU/L (ref.: 0.1–6 mIU/L), luteinizing hormone (LH): 1.5 mIU/L (ref.: 0.1–4 mIU/L). The patient had normal serum cortisol and adrenocorticotropin hormone values and there was no evidence of adrenal insufficiency. The parents raised their baby as a female and the child was referred to an endocrinologist only at the age of 5 years when the transabdominal US examination failed to show Mullerian structures and magnetic resonance imaging (MRI) could not identify an uterus. At that time the AMH level was 87.3 pmol/L, above the normal female (2–32 pmol/L) and below the normal male reference value (400–1300 pmol/L), this suggested clinical diagnosis of partial gonadal dysgenesis, a form of 46,XY DSD, consistent with the results of the earlier histology examination of the gonads. At 5.5 years of age, laparoscopy and a urethra-cystovaginostomy was done and gonadectomy was performed to clarify the diagnosis and prevent tumorous transformation of the dysgenic testes. A blinded end vagina was identified, but uterus could not be found. Histological examination showed testicular parenchyma, epididymis and a small part of ductus deferens with a piece of tissue resembling the fallopian tube in the right side gonad (10×5×7 mm), while in the left side gonad (16×80×70 mm) testicular and epididymidis tissues were found. The seminiferous tubules showed only Sertoli cells without spermatogonia on both sides (Fig. 1). These examinations confirmed the diagnosis of 46,XY with a highly suspected genetic background.

Methods

G-banded chromosome analysis was carried out on cultured lymphocytes using standard protocols. Fluorescence in situ hybridization (FISH) was performed according to the manufacturer's instructions using locus probe for *SRY* gene and the centromeric region of chromosome X (Cytocell, Rainbow Scientific Inc., Windsor, CT). Genomic DNA for molecular genetic studies was extracted from peripheral blood leukocytes using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Array comparative genomic hybridization (CGH) was performed using CytoScan 750 K Array as specified by the manufacturer with overall average probe spacing 4.125 bp per probe (Affymetrix, Thermo Fisher Scientific, Waltham, Massachusetts). Affymetrix Chromosome Analysis Suite (ChAS) Software was used for analysis. Exome sequencing was performed using Complete Genomics platform by the Beijing Genomics Institute. Targeted analysis of known genes involved in DSD was performed. MLPA was performed using SALSA MLPA P185-C2 Intersex probe-mix containing selected exons of the following genes: *NR0B1* (*DAX1*), *CXorf21* (Xp21.2), *SOX9* (17q24.3), *SRY* and *ZFY* (Yp11.3), *WNT4* (1p36.12), *NR5A1* (9q33) (MRC-Holland, Amsterdam, The Netherlands). Amplified products were separated by size on ABI-3130 (Applied Biosystems, Foster City, CA) and data were analyzed by the Coffalyser.Net Software (MRC-Holland, Amsterdam,

Fig. 1 **a** Histology of the right gonad (Hematoxylin Eosin (HE) staining, 20× magnification); **b** Histology of the left gonad (HE staining, 20× magnification); **c** Seminiferous tubules with only Sertoli cells present (HE staining, approx. 400× magnification); **d** Seminiferous tubules with only Sertoli cells present (inhibin alpha immunohistochemistry, approx. 400× magnification)

Fig. 2 **a** Result of MLPA analysis. Sample was normalized using the average of 2 normal controls. Red dots show a heterozygous deletion of exons 5 and 6 of the *NR5A1* gene. Chromosomal locations are displayed on the x-axis and the y-axis represents dosage quotient (Ratio). Normal copy number is between 0.65 to 1.30. **b** Electropherograms of the patient with heterozygous deletion (in red), superimposed on the electropherograms of a control subject (in blue). Whereas control regions (*HSD17B3* and *USH2A*) show a similar peak height in the patient and control, heterozygous deletion reduces the peak height (*NR5A1* exon 5 and 6) by half. **c** Array CGH result of the patient shows that exons 5 and 6 of *NR5A1* gene is not covered by copy number probes (exon numeration from left to right, exon 1–7). The genomic position of presenting probes before exon 5 is 127,259,618 within intron 4 and after exon 6 is 127,251,976 within intron 6 (GRCh37). (Color figure online)

The Netherlands). A peak was considered abnormal when the ratio was < 0.65 (deletion) or > 1.30 (duplication) compared to the peaks of the reference probes. QMPSF (Quantitative Multiplex PCR of Short Fluorescent Fragments) conditions and primer sequences are detailed in Supplementary Material.

Peripheral blood samples were collected from the proband after obtaining informed consent according to national regulations including permission of the parents for publication of the medical details. The MLPA study was approved by the Ethics Committee of the Ministry of Human Capacities (# RITK-MULTI-001).

Results

Routine cytogenetic studies revealed *SRY* positive 46,XY karyotype. Targeted gene panel analysis showed no pathogenic variants in DSD related genes. After excluding nucleotide sequence mutations, array CGH was performed to identify copy number alterations. Pathogenic copy number variation (CNV) was not detected by the oligonucleotide array. Assuming that intragenic deletions/duplications may be responsible for GD, the most commonly affected DSD genes were screened by MLPA. A heterozygous partial deletion encompassing exons 5 and 6 of the *NR5A1* gene was identified (Fig. 2a). Probes targeting all other exons of the *NR5A1* gene showed normal copy number. QMPSF assay confirmed the two-exon deletion (Fig. 2b). This small intragenic deletion could not be detected by the formerly used oligonucleotide array as exons 5 and 6 were not covered by the CNV probes. The two CNV probes that showed normal copy numbers proximal and distal to the deletion were located in introns 4 and 6 suggesting intronic breakpoints of the deletion (Fig. 2c). Family studies confirmed the de novo origin of the mutation that was undetectable in the genomic DNA of the parents. Diagnosis of 46,XY DSD with partial gonad dysgenesis was indicated.

Discussion

We report a case study of a patient with female external genitalia, clitoromegaly, absent uterus, blind-ending vagina and dysgenetic gonads with mixed tissues consisting of testis, epididymis, ductus deferens and tuba uterina. The proband also manifested mild dysmorphic features. Chromosome analysis revealed *SRY* positive 46,XY DSD. Clarification of the genetic background started with targeted DSD gene panel analysis and whole genome array CGH, both of these tests detected no pathogenic variants. MLPA analysis of DSD related genes identified a new partial heterozygous deletion within the *NR5A1* gene including exon 5 and 6. The genetic alteration was confirmed by QMPSF method. To our knowledge, the patient is the first reported case carrying mutation affecting exons 5 and 6 of the *NR5A1* gene.

46,XY DSD may include complete or partial gonadal dysgenesis due to disturbances in testis differentiation or undervirilization/undermasculinization as a result of aberrant androgen synthesis or action. The most common gene mutations associated with 46,XY DSD include *ARX*, *ATRX*, *CBX2*, *DAX1*, *DHH*, *DMRT1*, *EMX2*, *ESR2*, *FGFR2*, *GATA4*, *HHAT*, *MAP3K1*, *NR5A1*, *SOX8*, *SOX9*, *SRY*, *TSPYL1*, *WNT4*, *WT1*, *ZFPM2*, and *ZNRF3* [2, 19].

The *NR5A1* protein is a transcription factor necessary for the expression of key genes involved in male sex differentiation (e.g. *SOX9*, *SRY*) that along with the product of the *WT1* gene, regulates expression of AMH by Sertoli cells leading to regression of the Mullerian structures. In testicular Leydig cells, it stimulates the expression of enzymes required for testosterone biosynthesis that are essential for Wolffian duct development and formation of external genitalia [12]. Targeted deletion of *Nr5a1* in XY mice cause adrenal and GD, sex reversal with female external genitalia and persistence of Mullerian structures [20].

In humans, loss of function mutations of the *NR5A1* gene are associated with highly variable clinical conditions including male factor infertility, hypospadias, undescended testes, bilateral anorchia, primary ovarian insufficiency (in 46,XX female), GD and in rare cases adrenal insufficiency. To date, more than 40 *NR5A1* heterozygous mutations have been described in 46,XY GD [12] with no clear genotype–phenotype relationships established. In contrast to nucleotide sequence mutations, CNVs are extremely rarely detected in 46,XY DSD individuals [21, 22]. Significant numbers of urogenital defects are associated with major congenital malformations or minor abnormalities, which is highly suggestive of chromosomal alterations as etiological factors [23]. While conventional karyotyping due to its limited resolution (5–10 Mb) is not

suitable to identify most of these aberrations, array CGH and MLPA have permitted the detection of submicroscopic CNVs throughout the genome and at gene level in DSD. A review by Kon and Fukami [24] in summarizing the results of 15 studies in which array CGH or MLPA were used to detect CNVs, emphasizes the significance of CNVs as one of the genetic causes of DSD. So far, all reported deletions or duplications were detected in heterozygous form leading to haploinsufficiency of the affected genes which may disturb sex developmental processes. The CNVs were mainly de novo and, in rare cases, POF has been described in carrier mothers. Only a few cases with CNVs involving the *NR5A1* gene have been published [14–18]. The reported CNVs are heterogeneous in regards to their sizes and clinical phenotypes. The clinical and genetic details of these cases and modes of inheritance are listed in Table 1.

The *NR5A1* protein consists of a DNA-binding domain (DBD), two zinc finger domains, a hinge region, a ligand-binding domain (LBD) with 12-helix structure typical in nuclear receptors, and two activation function domains [25]. The LBD is composed of exon 4 (partial), exon 5, 6 and 7. In our case, the partial deletion affecting exons 5 and 6 results in a truncated *NR5A1* gene. The mutation is out-of frame leading to a stop codon right after the coding part of exon 4, thus no functional *NR5A1* is synthesized from this allele. The other allele did not appear affected as exome sequencing did not reveal any sequence mutation in this allele. Based on these results haploinsufficiency of *NR5A1* can cause abnormal sexual development as observed in the proband.

In conclusion, a new partial deletion including exons 5 and 6 of the *NR5A1* gene was identified by MLPA in a female patient with 46,XY partial gonadal dysgenesis that represents a novel genetic cause of 46,XY DSD. Our results also emphasize the importance of MLPA suitable for the detection of small size CNV and intragenic deletions/duplications that can improve the diagnostic yield in routine practice. Molecular diagnosis is highly beneficial for DSD patients as it can help in the assessment of adrenal and gonadal functions, determining the risk of malignancy of the gonads, improving the accuracy of genetic counseling and developing personalized management. CNVs are extremely rarely detected in individuals with gonadal dysgenesis, investigation of these type of mutations is not part of the genetic diagnostic procedure. Based on our data targeted CNV analysis of DSD related genes including *NR5A1* by MLPA in routine genetic screening of patients with 46,XY DSD with unknown etiology should be considered.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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