Next-generation DNA sequencing in an appropriate sex assignment: case report of two phenotypically similar patients with 46, XY disorder of sex development

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ABSTRACT

Background: Disorders of sex development (DSD) are known as the inborn atypical development of chromosomal, gonadal, or anatomic sex. New opportunities in counseling DSD patients have emerged with advent of the next generation DNA sequencing (NGS) techniques.

Case Presentation: Two clinical 46, XY DSD cases having similar phenotypical features, including ambiguous genitalia, are presented in this paper. In the first patient, no causative variant was found, meanwhile, a heterozygous variant in the *CHD 7* gene considered as likely-benign was identified (chr8: 61693942, rs377139749, NM_017780.3:c.2053_2058dupGCAAAA p.Lys686_Thr687insAlaLys). Neither gonadal ability to produce androgens, nor tissue androgen sensitivity was impaired, therefore leading to a decision to maintain the initially assigned male sex in this patient. In the other patient, the study revealed previously reported heterozygous missense variant in the *SEMA3A* gene (chr7: 83636785, rs769957117, NM_006080.2:c.A1024G:p. Met342Val) responsible for HH type 16 (OMIM 614897). As well, a novel hemizygous variant in the *AR* gene (chrX: 66942818, AR: NM_000044:c.G2599C:p.Val867Leu) was identified. In conjunction with the features of HH, this leads to a decision to reassign the sex of rearing to a female.

Conclusion: NGS technique may be helpful in optimal sex assignment in DSD cases.

Keywords: Case report, disorders of sex development, next-generation sequencing, genes, genital ambiguity, gene variants.

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Background

Disorders of sex development (DSD) are known as inborn atypical developments of chromosomal, gonadal, or anatomic sex with a reported incidence rate as high as 1 per 100 live births. DSD is associated with an increased risk of malignancy, developmental disorders in puberty and infertility in adulthood, as well as with gender dysphoria and psychological distress, which may require in-depth professional counseling [1]. Three major clinical groups of DSD have been defined: chromosomal DSD, DSD with karyotype 46, XX, and DSD with karyotype 46, XY. The discrepancies between genetic, gonadal, and anatomical sex (when Y chromosome is present in a karyotype) may result from gonadal dysgenesis, biosynthesis disorders, or impaired steroid effect at the level of a specific receptor, as well as may represent the ovotesticular form of DSD.

In cases of female karyotype, ambiguous genitalia are usually associated with hyperandrogenemia in the first months of fetal development, which is commonly caused by congenital adrenal hyperplasia.

Importantly, patients with different forms of DSD may present with similar phenotypic features. The findings from physical examination and routine laboratory tests often fail to avoid diagnostic mistakes in the early stages of clinical investigation. This may lead to a wrong conclusion about the patient's sex of rearing and to inappropriate therapeutic approaches that may mismatch the patient's core gender identity later in life.

It has been acknowledged that a number of phenotypically similar diseases may have a number of different genetic causes [2]. Numerous genes have been reported that contribute in early or late phases to the process of sex determination and differentiation. It is more than likely that many others will be reported in the future, attesting to the biological complexity underlying these processes [1,3]. The wide range of genetic technologies, starting from the basic karyotyping and evolving into targeted genotyping and Sanger sequencing, became available for genetic diagnosis. However, it was with the advent of the next generation DNA sequencing (NGS) technique

that genetic heterogeneity could have been addressed by simply sequencing all the genes related to specific phenotypes. If clinical suspicion remains without any clear biochemical diagnosis, NGS panel testing should be pursued [4,5].

Case Presentation

Two phenotypically similar although genetically different 46, XY DSD cases, in whom the performance of NGS was critical in sex assignment and in opting for best therapeutic approaches, are presented. Parental informed consents on the performance of diagnostic procedures, clinical interventions, as well as on publication of the findings on a condition of anonymity have been obtained. The study was approved by the local Ethics Committee of the V.A. Almazov National Medical Research Centre (Protocol No. 24 of 12.10.2017).

Genetic investigation

Sequencing libraries were prepared using HaloPlex Custom Panel target enrichment kit (Agilent Technologies, Santa Clara, CA) with Illumina MiSeq instrument and MiSeq reagent kit v3 600 cycles PE chemistry (Illumina, San Diego) to screen for 80 DSD-associated genes (Table 1). Alignment (BWA-MEM-0.7.1), data processing (Picard 2.9), and variant calling (GATK4.0.11.0) were performed according to GATK Best Practice recommendation (Broad Institute), using hg19 human genome reference, within script described earlier [6]. Variant annotation was performed using Annovar [7]. Average target region coverage was ~×150, 95% of all target regions were covered at least ×20. Next, identified variants with a maximum frequency of 0.01% in several normal population variant databases (1000G, ESP, ExAC0.2, and gnomAD) and deep intronic variants were filtered out. Finally, variants in genes with a known expression in disorders of sex development were considered clinically relevant and confirmed by Sanger sequencing. The paternity was proved using short tandem repeat (STR) analysis (Loci D2S1360, D7S1517, D8S1132, D12S1064, SE-33) according to the previously published protocol [8].

A bioinformatics approach to predict the damaging effect of the missense mutation and variant classification

All identified genetic variants were classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines [9].

Patient 1

The child was born of third pregnancy unfolded under sustained threats of miscarriage and eclampsia. Based on the findings of the prenatal ultrasound examination, the fetus has been initially considered as a female. The baby was born pre-term at the 27 weeks of gestation with multiple congenital malformations, including bilateral inguinal hernia, scrotal hypospadia, cryptorchidism, congenital heart, and central nervous system (CNS) malformations. Cytogenetic examination performed at the third week postnatally revealed a 46, XY sex chromosome complement. The laparoscopic examination could not identify Müller ducts. These findings required reconsideration of the baby's sex, and the child was further treated as a male. At the age of 2 years, the child was admitted to the hospital for a further clinical workout. At admission, the patient presented with the signs of growth retardation and developmental delay. The external genitalia were of the ambiguous type. Although hypoplastic, the scrotum was split and contained testicles, each in its own separate pouch. The penis was underdeveloped and consisted of hypoplastic spongy bodies. Measurements of the serum hormonal levels yielded the following results: baseline testosterone <0.09 nmol/l, dihydrotestosterone (DHT) 67 pg/ml, prolactin 13.50 ng/ml, cortisol 237.80 nmol/l, estradiol < 5 pg/ml, luteinizing hormone (LH) < 0.1 mE/ml, follicle stimulating hormone (FSH) 1.1 mE/ml. Müllerian ducts were missing, and the serum level of anti-Müllerian hormone (AMH) was within normal range. hCG stimulation test consisting of three intramuscular injections of hCG on successive days (a total dose equal to 5,000 IU/m^2) has been performed. This raised the levels of serum testosterone up to 4.46 nmol/l, and of DHT up to 166.06 pg/ml. The differential diagnosis was between the disorders of adrenal hormone synthesis (OMIM 201910, 202010,

Table 1. Genes in panel related to DSD (Illumina MiSeq).

AKR1C2, AKR1C4, AMH, AMHR2, AR, ARL6, ARMC5, ARX, AVP, BBS1, BBS10
BBS12, BBS2, BBS4, BBS5, BBS7, BBS9, CBX2, CD96, CDKN1C, CEP41, CHD7
CYB5A, CYP11A1, CYP11B1, CYP11B2, CYP17A1, CYP19A1, CYP21A2, DHCR7
DHH, DMRT1, DMRT2, DUSP6, DVL1, ESR1, ESR2, FEZF1, FGF17, FGF8
FGFR1, FGFR2, FLRT3, FOXL2, FREM2, FSHB, GATA4, GNRH1, GNRHR
HS6ST1, HSD17B3, HSD3B2, IGF2, IL17RD, KAL1, KISS1, KISS1R, LHB
LHCGR, MAMLD1, MAP3K1, NR0B1, NR5A1, NSMF, PROK2, PROKR2
RSPO1, SEMA3A, SOX3, SOX9, SPRY4, SRD5A2, SRY, STAR, TAC3, TACR3
WDR11, WNT4, ZFPM2, ZNRF3

201710, 201810, etc), 5αR deficiency (OMIM 264600), partial androgen insensitivity syndrome (OMIM 312300), and central hypogonadism (OMIM 308700, 147950, 146110, 614837, 614841, 612702, 228300, etc.). Topical DHT gel applications on the penile tissues have been performed with some positive effect by the end of the third month of treatment. Meanwhile, taking into account largely reduced masculinization, the chances of this patient to functionally develop as a male subject looked most questionable. Therefore, a genetic screening test was undertaken. A heterozygous variant in the *CHD 7* gene (chr 8:61693942, rs 377139749, NM_017780.3:c.2053_2058dupGCAAAA p.Lys686_Thr687insAlaLys) considered as likely benign has been identified (Figure 1). No causative variants were found.

Since gonadal androgen biosynthesis, as well as peripheral androgens sensitivity largely remained intact, the decision was made to maintain the assigned male sex, to perform plastic surgery on the external genitalia and to suggest a replacement androgen steroid therapy at the age of spontaneous puberty.

Patient 2

The child was born of third pregnancy complicated by light maternal anemia and mycoplasmosis. The baby was born with ambiguous external genitalia, and the sex could not have been defined. The baby had a partially split scrotum, urogenital sinus, and shapeless penis. Right gonad was palpable in the scrotum. A tissue having the texture and the size similar to gonad was palpable in the left inguinal canal. Thorough workout of the DSD has been initiated at the age of 3 weeks postnatally. Routine cytogenetic investigation identified karyotype 46, XY. MRI failed to find Müller ducts. Measurements of the serum hormonal levels yielded the following results: estradiol 48 pg/ml, testosterone 3.54 mmol/l, DHT 301.64 pg/ml, LH 1.1 mlU/ml, and FSH 0.9 mlU/ml. The serum AMH level was within the normal range. hCG stimulation test consisting of three intramuscular injections of hCG on successive days has been performed with a positive response: this raised serum testosterone level to 20.19 nmol/l and serum DHT level to 385 pg/ml. Clinical test for androgen sensitivity was performed in that DHT gel has been topically applied on the penile surface in the course over 5 months. The effect of that therapy was very limited. The differential diagnosis was between 5αR deficiency (OMIM 264600) and partial androgen insensitivity syndrome (OMIM 312300). To complete diagnostic workout, genetic investigation using NGS was performed. This study revealed previously reported heterozygous missense variant in the *SEMA3A* gene (chr 7:83636785, rs769957117, NM_006080.2:c.A1024G:p.Met342Val) with low reported population frequency, which can be responsible for HH type 16 (OMIM 614897) (Figure 2). As well, a novel hemizygous variant in the *AR* gene (chrX: 66942818, AR:NM_000044:c.G2599C:p.Val867Leu) was identified (Figure 3). According to the ACMG release as of 2015, this variant has not been previously described and may be considered as likely pathogenic. Certain variants in the *AR* gene are known to be associated with partial androgen resistance syndrome (OMIM 312300). In conjunction with the features of HH, the identification of the above variant raised a question to the reassignment of the sex of rearing to a female with consideration of the appropriate surgical management of the ambiguous external genitalia in the future, and possible initiation of the estrogen replacement therapy at the age of spontaneous puberty.

Discussion

Genetic studies, including NGS, are becoming increasingly popular in clinical practice. Being the specific forms of congenital abnormalities, DSD are also the subject to extensive the genetic research. It was in the 1990s when the first genes influencing sex differentiation (such as *SRY*) have been identified. Our knowledge on the genetics of sex differentiation has largely expanded since then, and new panels of targeted genes potentially involved in the DSD now include 64 known and 967 candidate genes [10]. Despite the growing number of the variants identified

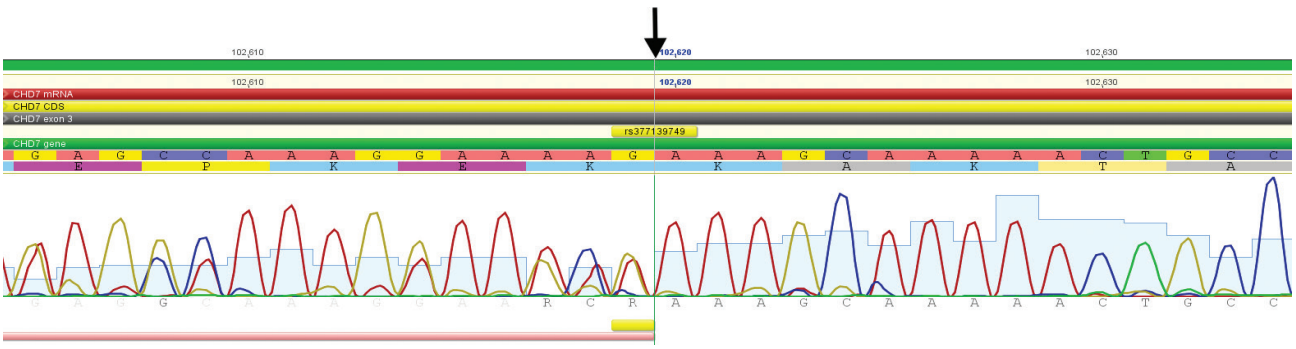

Figure 1. *DNA sequencing in Patient 1 (NGS, IlluminaMiSeq), confirmed by Sanger sequencing. Arrow: heterozygous variation in the CHD 7 gene (chr8:61693942, rs377139749, NM_017780.3:c.2053_2058dupGCAAAA p.Lys686_Thr687insAlaLys).*

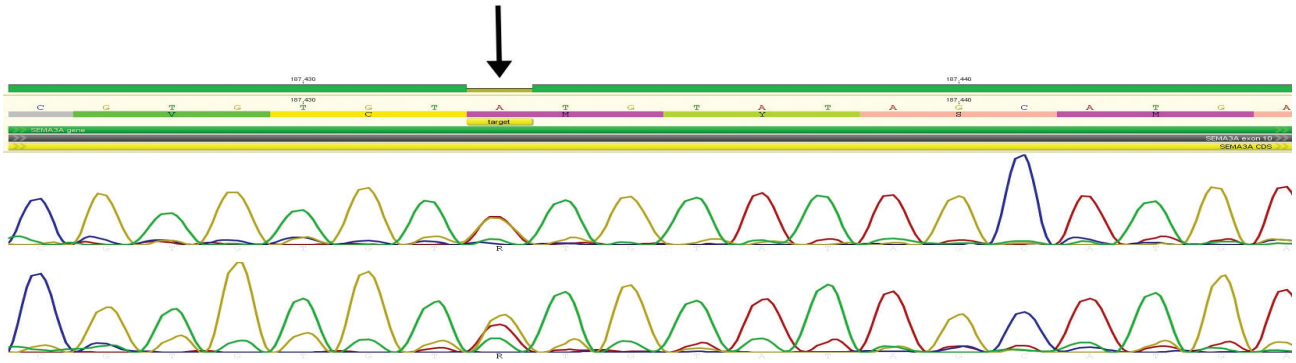

Figure 2. *DNA sequencing in Patient 2 (NGS, IlluminaMiSeq), confirmed by Sanger sequencing. Arrow: heterozygous mutation in the SEMA3A gene (chr7: 83636785, rs769957117, NM_006080.2:c.A1024G:p.Met342Val). Variation with low allele frequency (GnomAD 0,000008).*

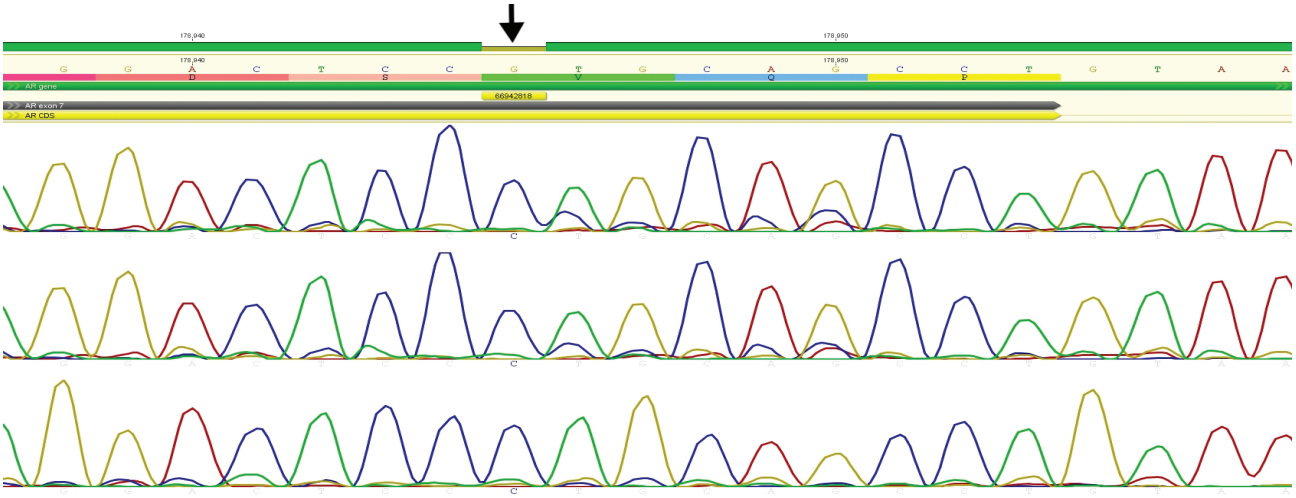

Figure 3. *DNA sequencing in Patient 2 (NGS, IlluminaMiSeq), confirmed by Sanger sequencing. Arrow: hemizygous mutation in the AR gene (chrX: 66942818, AR:NM_000044:c.G2599C:p.Val867Leu). According to ACMG 2015 criteria, considered as likely-pathogenic.*

with the help of high-resolution sequencing techniques, their clinical importance often remains unclear, and their attribution to either pathogenic or likely-pathogenic forms is a rather complicated issue.

The karyotypic group with the greatest diversity in the clinical spectrum of DSD is in individuals who have a 46, XY sex chromosome complement. Atypical sex development can occur through interruption of any part of testicular development, the hormonal hypothalamic–pituitary–gonadal axis, or morphogenesis of the urogenital region. Certain variants in the *CHD 7* gene (like the one found in Patient 1) have been previously described as a likely cause of HH type 5, either with or without anosmia (OMIM 612370) and of the CHARGE syndrome (OMIM 214800). Meanwhile, the estimated frequency of the allele rs377139749 in the affected *CHD 7* gene, which was found in Patient 1, ranges between 0.006814 and 0.03 and, therefore, is regarded as benign or likely benign [11]. Moreover, this variant has never been previously described in cases of DSD, its clinical significance remains unclear, and genetic re-evaluations may be required to further shed the light.

A large number of variants in the androgen receptor gene (*AR*) have been reported, with coding region variants found in nearly all complete androgen insensitivity syndrome patients but only in 20% of partial androgen insensitivity syndrome patients [12]. The *AR* gene variants are inherited in an X-linked manner. A novel hemizygous variant in the *AR* gene, considered as likely pathogenic, was identified in Patient 2 presented in this paper.

More than a half of the 46, XY DSD cases may remain genetically unexplained so far [13]. The same is true for the 46, XX DSD cases [14]. In a study covering more than 300 DSD patients, the authors could identify genetic causes in only 43% of cases [10]. The relatively low rate of the targeted genes identified in the DSD cases presumably may be due to the influence of epigenetic and environmental factors, the involvement of the somatic genes at the early stages of gonadal development [15].

In only 13 of 300 studied DSD patients, the cause could have been ascribed to the effects of one specific gene variant; this enabled the authors to consider certain DSD cases as having “oligogenic” rather than monogenic origin [10]. The findings from our study are in accord with

this suggestion: Patient 2 looks like a vignette of such an oligogenic form of 46, XY DSD.

Some authors reported gene variants known for their associations with certain types of HH in DSD patients. Similarly to the findings from our study, such patients may present with abnormal external genitalia [10]. It was also supposed that the causal genes responsible for DSD may express at the level of the hypothalamic–pituitary–gonadal axis affecting its function at different time points [10,11].

The limitation of the study was our inability to judge whether the diagnostic options would finely conform with the patients self-identity in the future. To answer this question, a follow-up study is required.

Conclusion

Modern approaches to genetic evaluation may be critical in an appropriate decision on the assignment of the sex of rearing in DSD patients.

Acknowledgments:

None

List of abbreviations:

ACMG	American College of Medical Genetics and Genomics
AMH	Anti-Müllerian hormone
CNS	Central nervous system
DHT	Dihydrotestosterone
DSD	Disorders of sex development
FSH	Follicle stimulating hormone
HH	Hypogonadotropic hypogonadism
LH	Luteinizing hormone
MRI	Magnetic resonance imaging
NGS	Next generation DNA sequencing
STR	Short tandem repeat

Conflicts of interest

None.

Consent for publication:

Parental informed consent was obtained.

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Summary of the case

Patient (gender, age)	1	2 years, undefined gender; 3 weeks, undefined gender
Final diagnosis	2	DSD
Symptoms	3	Ambiguous genitalia
Medications	4	NA
Clinical procedure	5	Next-generation DNA sequencing
Specialty	6	Pediatrics, endocrinology, genetics