

# Genetic Screening of Iranian Patients with 46,XY Disorders of Sex Development

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## Abstract

**Background:** Disorders of sex development (DSDs) belong to uncommon pathologies and result from abnormalities during gonadal determination and differentiation. Various gene mutations involved in gonadal determination and differentiation have been associated with gonadal dysgenesis. Despite advances in exploration of genes and mechanisms involved in sex disorders, most children with severe 46,XY DSDs have no definitive etiological diagnoses; therefore, the possibility that other genes or loci might play important roles in these disorders needs to be explored.

**Methods:** Patients (37) clinically suspicious for 46,XY gonadal dysgenesis (46,XY GD) of unknown etiology were studied. *SRY*, encoding the sex-determining region Y protein, *NR5A1*, encoding a transcription factor called steroidogenic factor 1, and *DHH*, encoding the desert hedgehog protein, were directly sequenced. Multiplex ligation-dependent probe amplification (MLPA) was used to detect deletions in *NR0B1*, encoding the DAX1 protein, and *WNT4*, encoding the WNT4 protein, and real-time PCR (qPCR) confirmed the MLPA data. Other potential loci have been investigated in the complete genome using Array-Comparative Genomic Hybridization, (Array CGH).

**Results:** The *SRY* deletion was found in five patients. One each of previously described *NR5A1*, *DHH*, and *AR* (androgen receptor) allelic variants were identified. A pathogenic c.2522G>A *AR* mutation was found in two affected brothers. A heterozygous partial deletion was found in *NR5A1* and heterozygous partial duplications were found in *WNT4*. These deletions and duplications (del/dup) were confirmed by qPCR. The Array CGH result demonstrated one partial deletion in *SOX2-OT*, which encodes a member of the SOX family of transcription factors, and the exact region of the rearrangements.

**Conclusions:** According to our study, del/dup mutations could be checked prior to point mutations, *SOX2-OT* has a potential role in gonadal dysgenesis, and Array CGH has a prominent role in gonadal dysgenesis diagnosis.

**Keywords:** Array-Comparative Genomic Hybridization, (Array-CGH), Disorders of sex development (DSDs), Mutation

## Introduction

Disorders of sex development (DSDs) are a heterogeneous group of inherited and congenital disorders related to sex determination and

differentiation (1). The frequency of DSDs is estimated to be about 1 in 4500 live births (1) and DSDs account for about 7.5% of all birth defects.

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Genetic alterations including sex chromosomal deletions, translocations, and gene mutations have been reported in DSD patients (2-6). Disorders of Sex Development are classified in three main groups: 1) sex chromosome DSDs, 2) 46,XY DSDs, and 3) 46,XX DSDs (1). However, genetically and clinically, DSDs are extremely heterogeneous and difficult to categorize in distinct classes. 46,XY DSDs are mainly suspected in females with primary amenorrhea and/or insufficient breast development during puberty (7). The main features of patients with 46,XY karyotype are ambiguity in external sex organs and/or presence of female organs (7). Based on the etiology of the disease, 46,XY DSDs are further divided into three groups, including 1) deficiency in hormonal biosynthesis (8, 9), 2) androgen receptor defects (9), and 3) gonadal development abnormalities. 46,XY gonadal dysgenesis (46,XY GD) belongs to the latest group of this classification in which gonads develop unusually during embryonic stages. Genetic abnormalities, including chromosomal aberrations and gene mutations, have been identified in the pathobiology of the disease (4). Abnormal translocation between the X and Y chromosomes could result in *SRY* deletion (10). Deletions in chromosomes 9p, 2q, and 10q (11), and duplication in chromosome 1p (12) have also been reported in 46,XY GD patients. Molecular analyses have identified several genes responsible for gonadal development, including *SRY* (13-16), *DHH*, *NR5A1*, *NR0B1*, and *WNT4* (4). Mutations in *SRY* (Yp chromosome) (17), *NR5A1* (9p33 chromosome) (18-22), and *DHH* (12q13.1 chromosome) (23-25) have been reported in affected patients. Conversely, duplication of *NR0B1* (26, 27) and *WNT4*, which play pivotal functions in female sex determination, leads to increased protein activity and their targets in some 46,XY DSD individuals (4, 12, 28). Therefore, genetic analyses following clinical examinations seems essential for detection and management of affected patients with different DSDs, especially 46,XY DSDs. Although several genetic abnormalities have been discovered through genetic analyses, the genetic causes in about 50% of affected patients remain unknown. In this study we aimed to identify genetic defects in patients with 46,XY DSDs. The investigations comprised both

low (karyotyping) and high (array-based techniques and sequencing) -resolution approaches to identify genomic abnormalities. Molecular techniques identified mutations in *SRY*, *NR5A1*, *DHH*, and *AR*, which are involved in sex development. A second aim was to better understand the genetic mechanisms of DSDs and improve management of DSD patients.

## Materials and Methods

### Patient screening and selection

Thirty-seven patients, with a mean age of 14 years, and clinical and biochemical inclusion criteria of 46,XY GD, defined as complete female external genitalia and/or ambiguous genitalia associated with penoscrotal hypospadias, poor gonadogenesis, absence and/or presence of female internal genitalia including uterus and fallopian tubes, considerable increase in FSH and LH levels, decreased testosterone levels in serum, absence or very low levels of testosterone after muscular injection of human chorionic gonadotropin (hCG), or absence or very low rates of spermatogenesis, and 46,XY karyotype who were referred to pediatric endocrinology centers of the Tehran University of Medical Sciences were selected for this study. Pedigrees for all patients were determined after obtaining clinical information, including ultrasounds and pathological findings. The study was approved by the Ethics Committee of the Tehran University of Medical Sciences and all participants or their legal guardians signed informed consent forms.

### DNA extraction and PCR for mutation analysis

DNA was extracted from white blood cells using a standard salting-out protocol. *SRY*, *NR5A1*, *DHH*, and *AR* were analyzed for mutations using PCR and sequencing. Multiplex PCR was used to investigate the *SRY* deletion. The dysbindin protein homolog gene (*DSBN*) was used as an internal control. Primers were designed using Primer 3 and Gene Runner software. Each PCR contained AMS buffer (10X), MgCl<sub>2</sub> (50 mM), forward and reverse primers (100 pmol), DMSO (5-10%), dNTPs (10 mM), 1-2 µg of sample DNA, and up to 25 µl of ddH<sub>2</sub>O. The PCR primers and conditions of related genes are available upon request.

### Multiplex Ligation-Dependent Probe Amplification (MLPA) analysis

Multiplex Ligation-Dependent Probe Amplification was performed using kit P185-B1 according to the manufacturer's protocol (MRC-Holland, Amsterdam, the Netherlands). Fragment analysis was performed on an ABI 3100 capillary sequencer and subsequent data was analyzed using GeneMarker software version 1.9 (SoftGenetics, www.softgenetics.com). Results with relative peaks less than 0.75 and greater than 1.35 were considered as abnormal. Multiplex Ligation-Dependent Probe results that indicated abnormalities were confirmed by real-time PCR (qPCR).

### Array-Comparative Genomic Hybridization (Array-CGH)

To perform array-CGH, extracted DNA samples were analyzed using a  $2 \times 400\text{K}$  microarray kit (Agilent Technology), which could analyze 411,000 regions in the complete genome. Sample DNAs were digested with *Alu* I and *Rsa* I restriction enzymes and labeled with *Cy5* and *Cy3* using a Human Genome CGH Microarray kit 400A. Treated DNAs were purified (Microcon YM-30; Millipore) and hybridized with human Cot-1 DNA (1mg/ml; Roche). The slides were scanned by InnoScan 710 (Arrayit Corporation,

CA, USA) after post-hybridization washes. Data analyses, image processing, and quality controls were all performed using BlueFuse Multi 2.3 analyzing software.

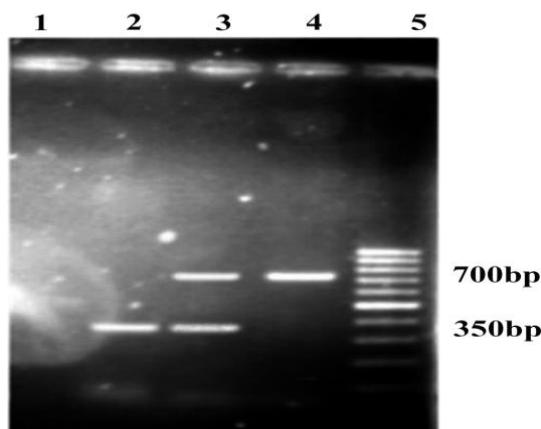
## Results

### Patients

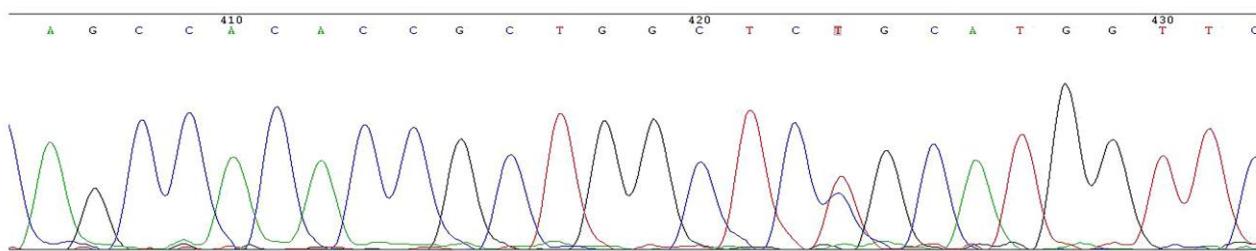
Thirty-seven patients (phenotypically 13 males and 24 females) between 1 month and 30 years of age were selected. Pedigree analysis showed that 21% of the patients were born into families with relative consanguinity. Three of these cases were accompanied with syndromic features, which included congenital abnormalities with mental retardation, and facial and skeletal anomalies.

### PCR Sequencing of *SRY*, *NR5A1*, and *DHH*

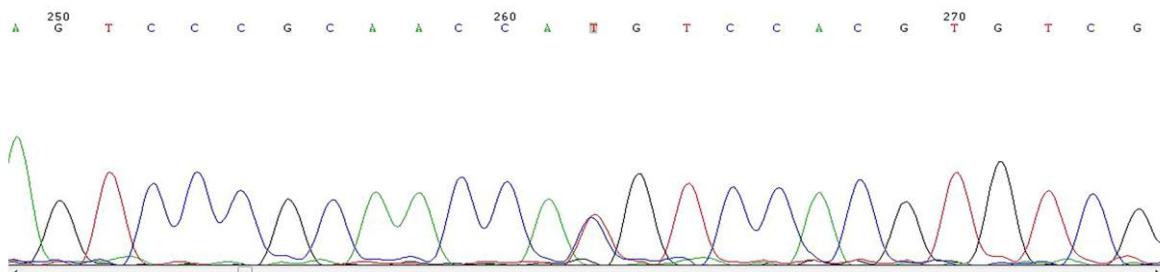
The patients with normal cytogenetic 46,XY karyotypes were analyzed for molecular mutations. The genomes of five patients (13.5% of all samples) contained deletions of *SRY* (Fig. 1). A heterozygote nucleotide change (c.82C>T) in the 3' UTR of exon 7 of *NR5A1* was identified in three patients (Fig. 2), and a heterozygote polymorphism (rs: 117527954, c.543C>TDHH) in exon 2 of *DHH* was detected in one patient (Fig. 3). The clinical significance of these mutations is not yet known.



**Fig. 1.** Agarose gel electrophoresis of the *SRY* deletion after multiplex PCR. Lane 1: Negative control to check for PCR contamination. The 350 bp bands in lanes 2 and 3 were internal controls amplified from *DSBN*. The 700 bp bands in lanes 3 and 4 were amplified from *SRY*. The internal control was not included in lane 4. No *SRY* product was amplified in lane 2. Lane 5: molecular weight marker.



**Fig. 2.** Heterozygous nucleotide change at nucleotide 423 in the 3' UTR of *NR5A1*.



**Fig. 3.** Heterozygous nucleotide change at nucleotide 262 in exon 2 of *DHH*.

### MPLA and qPCR

The samples with no nucleotide changes in the analyzed genes and from the patients with normal 46,XY karyotypes were further analyzed for other genetic abnormalities using MLPA. Our MLPA result confirmed the deletions found in *SRY* by PCR. One deletion was identified in *NR5A1* in one male patient. Moreover, duplications were identified in the first exon of *WNT4* in three patients. The deletions and duplications were consequently confirmed using qPCR.

### Array CGH analysis

Array CGH analysis confirmed the structural anomalies found in the patients with abnormalities in chromosomes 7 and 13 (29). Furthermore, one deletion was found in a patient in the 15q11.2-q13.1 region, which indicates the syndromic features of Prader-Willi with ambiguous genitalia. Another patient had a deletion in 3q26.33. This deletion included *SOX2-OT* (not shown).

### Discussion

Disorders of sex development could result from genetic abnormalities ranging from chromosomal abnormalities to molecular defects in genes involved in normal sex development (2, 4-6, 12). Sex chromosome aberrations are among the first defects that can be identified in DSD patients (2, 3). In this study karyotype analysis identified a patient with partial monosomy for 13q and partial trisomy for 7q (29). These findings suggest that any chromosomal change, even in autosomal chromosomes, could cause DSDs. Various mutations in *SRY*, *NR5A1*, *DHH*, and *WNT4*, all of which are involved in sex organ differentiation, have been reported in previous studies (12). Deletions and mutations in *SRY* have been shown to be involved in sex developmental syndromes and infertility (14, 30, 31). Point mutations in this gene are

usually inherited de novo and result in complete female phenotype (4, 32). However, in some cases, mutations in *SRY* could also be inherited from heterozygote mosaic fathers (10). In the present study, *SRY* was completely deleted in five patients (13.5% of all samples). Two of these patients had ambiguous external genitalia and were reared as males. The other three patients showed complete female phenotypes. The *SRY* deletion frequency found in our study is similar to that reported in other studies (4). The relatively high *SRY* deletion percentage in our and other similar studies emphasizes the important role of *SRY* in sex development. We emphasize that the only mutation we found of this gene was its complete deletion; no point mutations were identified. Therefore, analysis for the presence of this gene should be a first step in the evaluation of DSD patients, followed by karyotype. The steroidogenic factor 1 (*NR5A1*) gene product is a nuclear receptor that controls regulation of gene complexes involved in gonadal development, fertilization, and adrenal axis (33). Mutations in *NR5A1* have been reported in families and affected patients with gonadal dysgenesis (19-21, 34, 35). We detected one nucleotide change in the 3' UTR of *NR5A1* in three patients with incomplete gonadal dysgenesis. The clinical significance of this change is not yet understood. Multiplex Ligation-Dependent Probe Amplification results showed deletion of the third exon of *NR5A1* in one patient. Our data confirmed the role of *NR5A1* mutation in gonadal dysgenesis. The effect of the *DHH* mutation is not yet known.

To investigate the role of *WNT4* expression in inducing ectopic gonadal development, deletion and duplication analyses were performed using MLPA. Our data showed duplication of *WNT4* in one patient and indicated a dose-dependent effect of this gene in gonadal dysgenesis (28, 36). Regarding the existence of deleted rather than missense mutations in our candidate genes, we suggest that del/dup mutations are

likely to provide more information than full-coding sequence analysis. One of our patients was diagnosed with Prader-Willi syndrome via array CGH analysis. It seems this technique could help to diagnose of syndromic cases (37). Furthermore, through array CGH analysis, deletion 228,572 bp for SOX2-OT was detected in one patient.

Fantes et al. (2003) introduced *SOX2-OT* as a non-coding gene with 5 exons. *SOX2* is a single-exon gene that exists inside the intron region of *SOX2-OT*. Williamson et al. (2006) reported an inactivating mutation in *SOX2* that causes anophthalmia-esophageal-genital (AEG) syndrome. Pedace et al. (2009) reported a 6-month old child with micropenis and a two base-pair insertion in *SOX2*. As the patient's hypothalamus-pituitary axis was intact, it was suggested that this gene has a direct role in the development of external genitalia. Our finding in a DSD patient increases the possibility of involvement of this gene in sex developmental disorders; however, the role of *SOX2* in the etiology of DSDs needs further investigation.

In the present study, some important factors involved in sex determination during embryogenesis were analyzed; nevertheless, it seems that few of the selected factors are involved in the etiology of DSDs in our patients. We therefore plan to explore other genes for their potential roles in DSDs.

Regarding the heterogenic clinical manifestations and various causes of DSDs, it is difficult to predict the exact incidence of this disease. On the one hand, some clinical diagnosis criteria are being challenged; for example, plasma testosterone levels do not likely indicate *NR5A1* or *MAMLD1* mutations, as these mutations have been detected in patients with normal testosterone. On the other hand, to date, no relationship has been found between in vitro functional studies and clinical manifestations or biochemical findings. Combined with these data, diagnosis and confirmation of 46,XY DSDs using genetic methods is strongly recommended.

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In addition to known etiologic causes of DSDs, another new candidate gene for this disease is *MAMLD1*. It has been shown that this gene is deleted in patients with myopathy and external genital abnormalities (38-40). *MAMLD1* is co-expressed with *NR5A1* and appears to have a role in the regulation of sex developmental gene expression. Moreover, it also appears to have a role in testosterone production. Therefore, we believe *MAMLD1* should be investigated for a potential role in 46,XY DSDs (41).

Although these disorders display extreme heterogeneity in terms of both clinical and genetic aspects, they may be categorized in distinct classes by genetic screening and diagnostic tests. Therefore, genetic analyses following clinical examinations seems essential to identify and manage patients affected with different of DSDs, especially 46,XY DSDs. In this regard, genetic counseling is critical to aid parents and future parents in decision making.

Our study showed a high prevalence of *SRY* deletions in DSD patients and verified the role of *NR5A1* and *WNT4* in the etiology of 46,XY DSDs. *SOX2* could also be added to the panel of DSD patients' gene analyses. However, because the etiologies of DSDs are known in only 50% of cases, and regarding the various genetic heterogeneity, a single gene-by-gene approach is inefficient, and using high throughput techniques, such as array and/or next generation sequencing, are strongly recommended. Array CGH would be the first choice for syndromic cases of 46,XY DSDs.

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