A duplication upstream of *SOX9* was not positively correlated with the *SRY*-negative 46,XX testicular disorder of sex development: A case report and literature review

XIN-YI XIA[1*], CUI ZHANG[1*], TIAN-FU LI[1], QIU-YUE WU[1], NA LI[1],
WEI-WEI LI[1], YING-XIA CUI[1], XIAO-JUN LI[1] and YI-CHAO SHI[2]

[1]Department of Reproduction and Genetics, Institute of Laboratory Medicine, Jinling Hospital,
Nanjing University School of Medicine, Nanjing, Jiangsu 210002; [2]Department of Reproduction and Genetics,
Suzhou Municipal Hospital, Nanjing Medical University Affiliated Suzhou Hospital, Suzhou, Jiangsu 215002, P.R. China

Abstract. The 46,XX male disorder of sex development (DSD) is rarely observed in humans. Patients with DSD are all male with testicular tissue differentiation. The mechanism of sex determination and differentiation remains to be elucidated. In the present case report, an 46,XX inv (9) infertile male negative for the sex-determining region of the Y chromosome (*SRY*) gene was examined. This infertile male was systemically assessed by semen analysis, serum hormone testing and gonadal biopsy. Formalin-fixed and paraffin-embedded gonad tissues were assessed histochemically. The *SRY* gene was analyzed by fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR). The other 23 specific loci, including the azoospermia factor region on the Y chromosome and the sequence-targeted sites of the *SRY*-box 9 (*SOX9*) gene were analyzed by PCR. The genes *RSPO1*, *DAX1*, *SOX3*, *ROCK*, *DMRT1*, *SPRY2* and *FGF9* were also assessed using sequencing analysis. Affymetrix Cytogenetics Whole Genome 2.7 M Arrays were used for detecting the genomic DNA from the patient and the parents. The patient with the 46,XX inv (9) (p11q13) karyotype exhibited male primary, however, not secondary sexual characteristics. However, the patient's mother with the 46, XX inv (9) karyotype was unaffected. The testicular tissue dysplasia of the patient was confirmed by tissue biopsy and absence of the *SRY* gene, and the other 23 loci on the Y chromosome were confirmed by FISH and/or PCR. The *RSPO1*, *DAX1*, *SOX3*, *ROCK*, *DMRT1*, *SPRY2* and *FGF9* genes were sequenced and no mutations were detected. A duplication on the 3 M site in the upstream region of *SOX9* was identified in the patient as well as in the mother. The patient with the 46,XX testicular DSD and *SRY*-negative status was found to be infertile. The duplication on the 3 M site in the upstream region of *SOX9* was a polymorphism, which indicated that the change was not a cause of 46,XX male SDS. These clinical, molecular and cytogenetic findings suggested that other unidentified genetic or environmental factors are significant in the regulation of SDS.

Introduction

The 46,XX male disorder of sex development (DSD) is a rare genetic condition (1). Patients with the 46,XX karyotype display various degrees of testicular tissue development. As far as the sexual phenotype is concerned, three clinical categories of sex-reversed 46,XX individuals have been identified: i) Classic XX males, infertility with normal male internal and external genitalia; ii) XX males with ambiguous genitalia, usually detected at birth by external genital ambiguities, including hypospadias, micropenis or hyperclitoridy; iii) XX true hermaphrodites, who exhibit internal or external genital ambiguities detected at birth (2-4).

At the molecular level, XX males can be classified by the sex-determining region Y gene (*SRY*) location on the Y chromosome, as *SRY*-positive or *SRY*-negative, which is important for encoding testis determining factor (*TDF*) (5). It is known that ~90% of these patients exhibit Y chromosomal material, including the *SRY* gene, which is usually translocated to the distal tip of the short arm of the X chromosome or autosomal chromosomes (6). The 46,XX males with *SRY*-positive status are clinically heterogeneous. The majority of the patients are normal prior to puberty and are diagnosed following puberty based on infertility. By contrast, 20% of 46,XX males positive for *SRY* exhibit an external genitalia abnormality at birth with the classical phenotype being hypospadias. In addition, certain patients are negative for *SRY*, and these patients always exhibit external genital ambiguities and contact the doctor for

Correspondence to: Dr Yi-Chao Shi, Department of Reproduction and Genetics, Suzhou Municipal Hospital, Nanjing Medical University Affiliated Suzhou Hospital, 26 Daoqian Street, Suzhou, Jiangsu 215002, P.R. China
E-mail: yichaoshi71@gmail.com

*Contributed equally

infertility and gynecomastia (7). The mechanism by which the development of testicular tissue in 46,XX males negative for *SRY* is regulated remains to be elucidated.

Of note, several genes have been identified to be associated with *SRY*-negative 46,XX male sex reversal patients. *SOX9* and *DAX1* have been suggested to function as early mediators downstream of the *SRY* gene in the male sex-determination pathway (8,9). *SOX3* has been demonstrated to upregulate the expression of *SOX9* via a similar mechanism to that of *SRY* and to be responsible for XX male sex reversal in humans through gain-of-function mutations mediated by genomic rearrangements around *SOX3*, possibly leading to the alteration of regulation (10). Disruption in the R-spondin1 (*RSPO1*) gene has been previously reported in a recessive syndrome characterized by XX sex reversal, palmoplantar hyperkeratosis and pre-disposition to squamous cell carcinoma (11). *SPRY2* and *FGF9* are also associated with 46,XX male sex reversal (12,13). Testes formation may be initiated by an alternative signaling pathway attributed to *ROCK1* activation in the XX testes. On the basis of the inhibitory assay *in vitro*, it has been suggested that *ROCK1* phosphorylates and activates *SOX9* in Sertoli cells (14). The haploid dose of *DMRT1* may lead to testicular dysplasia with XY male-to-female sex reversal (15,16). Seeherunvong *et al* (17) described a partial duplication of 22q in a case of 46,XX sex reversal, *SRY*-negative, and almost complete androphany of patient's exogenous genitals. Others have revealed that 46,XX *SRY*-negative males have a duplication in a regulatory region upstream of the *SOX9* gene, which was overexpressed in these individuals (18-23).

The present case report described the clinical, fluorescence *in situ* hybridization (FISH) and molecular analyses of a 46,XX male DSD patient negative for *SRY* and aimed to investigate the association between the clinical characteristics and the chromosomal karyotype. The possible mechanisms to explain the etiology of the 46,XX sex reversal male negative for the *SRY* gene was also investigated.

Materials and methods

Case presentation. A 29-year-old male visited the outpatient clinic of the Center for Reproduction and Genetics (Suzhou Municipal Hospital, Nanjing Medical University Affiliated Suzhou Hospital, Suzhou, China) with a complaint of infertility. The patient reported that he had a surgical history of correction of congenital hypospadias at the age of 5 and presented with the development of mammary glands at 19 years of age. The parents are in a non-consanguineous marriage and the family members exhibited no clinical manifestations. The patient was found to be short in stature and the physical examination revealed no prominentia laryngea, armpit hair or beard, pale skin, a marginal increase of breast bilaterally and a surgical scar on the abdomen. The testicular volumes were small and the texture was hard. Rectal touch revealed a detectable prostate; however, the volume was low. Endocrinological data were indicated as follows: No sperm and spermatogenic cells according to semen examination; fructose in the seminal plasma was normal; seminal plasma α-glucosidase levels of 22.0 U/ml (normal range, 35.1-87.7 U/ml); seminal plasma acid phosphatase levels of

31.2 U/ml (normal range, 48.8-208.6 U/ml); a Serum T levels of 6.02 nmol/l (normal range, 9.4-37 nmol/l); estradiol levels of 0.11 nmol/l (normal range, 0.129-0.239 nmol/l); follicle-stimulating hormone levels of 51 IU/l (normal range, 1.5-11.5 IU/l), luteinizing hormone levels of 35.5 IU/l (normal range, 1.1-8.2 IU/l) and prolactin levels of 332.6 IU/l (normal range, 95.4-400 IU/l). No abnormality was identified during brain and adrenal computerized tomography examination. No uterus or ovary was detected, and other clinical indicators were normal.

All procedures used in the present study were performed according to the Declaration of Helsinki. The Ethics Committee of Jinling Hospital (Nanjing, China) approved the present study. Written informed consent was obtained from all participants.

Histological analysis. Formalin-fixed and paraffin-embedded gonad tissue from the affected individual was obtained from the Department of Pathology, Faculty of Medicine, Jinling Hospital (Nanjing, China) by punch biopsy. A 5 µm-thick serial section was cut from the tissue block and stained with hematoxylin and eosin (Beyotime Institute of Biotechnology, Inc., Haimen, China).

Cytogenetic analysis and FISH analysis. G banding karyotype analysis was performed on the peripheral blood lymphocytes, including 100 metaphase cells, from the patient by conventional operating techniques. The identical analysis was performed on the blood samples from the parents and the sister. The karyotype analysis was performed using FISH with the following two probes: Dual color centromere probe, *DXZ1*, with Spectrum Green and *DYZ3* with Spectrum Orange (Vysis, Downers Grove, IL, USA; cat. no. 32-111051), and *SRY* with Orange (Vysis; cat. no. 30-190079). A total of 10 mitotic phases were analyzed, according to the manufacturer's instructions. Microscopic examination was performed using an Olympus BX51 microscope (Olympus, Tokyo, Japan), and analyzed by Cytovision 3.0 image analysis software (Leica Biosystems, Oberkochen, Germany). FISH analysis was also performed on samples from the patient's father.

Polymerase chain reaction (PCR) amplification and sequencing of the coding region of candidate genes. The genomic DNA was isolated from whole-blood leukocytes from the parents and paraffin-embedded tissue from the patient using phenol/chloroform extraction. The purpose of DNA extraction was to determine the presence or absence of *SRY* in the peripheral blood lymphocytes. Two primer pairs for *SRY* were designed using Primer 5.0 software (Premier Biosoft, Palo Alto, CA, USA) and the primers were synthesized by BGI (Beijing, China). Each PCR reaction (25 µl) contained 2 µl genomic DNA, 1 µl forward primer, 1 µl reward primer, 2.5 µl 10X buffer, 1.5 µl Mg^{2+}, 2 µl deoxynucleotide triphosphates, 0.25 µl Taq DNA polymerase and 15 µl double-distilled water. PCR was performed under the following conditions: 95˚C for 5 min followed by 35 cycles of 94˚C for 30 sec, 56˚C for 30 sec and 72˚C for 60 sec. PCR amplification products were assessed on 2% agarose gels. The products were then sequenced by BGI company. A total of 24 sequence-targeted sites (STS) were investigated to determine the presence or absence of
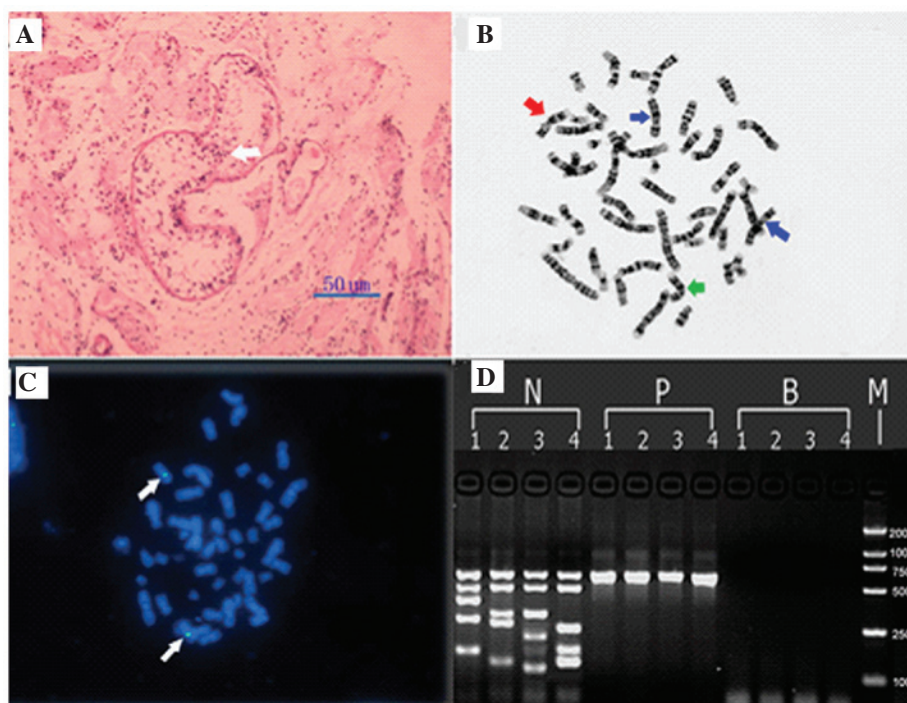
Figure 1. Patient results, including histological analysis, cytogenetic analysis, FISH analysis and PCR. (A) A percutaneous testicular biopsy demonstrating the convoluted seminiferous tubules were hypogenetic with interstitial fibrosis (hematoxylin and eosin staining; magnification, 50 μm). A few sertoli cells were observed in seminiferous tubules (white arrow). (B) Chromosome analysis demonstrating a pericentric inversion of the chromosome 9 (red arrow). The normal chromosome 9 is indicated by a green arrow and the normal chromosome X is indicated by a blue arrow. (C) FISH demonstrating that the orange signal of the *SRY* gene was absent in the patient, while two green signals were observed on the X chromosome (white arrows). (D) PCR analysis demonstrating the absence of azoospermia factor regions. Lanes: 1, ZFX/ZFY (690 bp), SRY (472 bp), sY254 (400 bp), sY127 (274 bp) and sY86 (155 bp); 2, ZFX/ZFY (690 bp), SRY (472 bp), sY134 (301 bp), sY84 (255 bp) and sY255 (126 bp); 3, ZFX/ZFY (690 bp), SRY (472 bp), sY157 (286 bp), sY239 (201 bp) and sY124 (109 bp); 4, ZFX/ZFY (690 bp), SRY (472 bp), sY242 (233 bp), sY132 (160 bp) and sY152 (126 bp). N, fertile male; P, patient; B, blank; M, DL2000 marker (2,000, 1,000, 750, 500, 250, 100 bp). FISH, fluorescent *in situ* hybridization; PCR, polymerase chain reaction.

Y chromosome material in the genome of the patient's blood. Firstly, sY14 (*SRY*), sY84, sY86, sY124, sY127, sY132, sY134, sY152, sY157, sY239, sY242, sY254 and sY255 were detected. Primers and amplification conditions were derived from the Genome Database (http://www.ncbi.nlm.nih.gov). To further confirm the presence or absence of the Y chromosome, 11 STS, including DYS19, DYS385, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438 and DYS439, were detected. At the same time, 15 autosomal sites, including D3S1358, D21S11, D18S51, D5S818, D13S317, D7S820, D16S539, D8S117P, TH01, CSFIPO, Penta E, Penta D, vWA, TPOX and FGA, were detected. The PCR products were analyzed on a DNA PowerPlex-Y sequencing system (Promega, Madison, WI, USA). The *RSPO1*, *DAX1*, *SOX9*, *SOX3*, *ROCK*, *DMRT1*, *SPRY2* and *FGF9* genes were amplified using custom-synthesized oligonucleotide primers.

The internal locus, including D17S794, D17S1350, D17S1351, D17S1352 and D17S1807 in *SOX9*, and D9S1858 in *DMRT1*, were detected. These loci were amplified, according to the information provided by the Genome Database, using fluorescently-labeled forward primers. The PCR products were analyzed on a DNA sequencer (ABI3500; Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and sequence analysis was performed by Genecore Company (Shanghai, China).

Single nucleotide polymorphism (SNP) array. The patient and the parents were analyzed using Affymetrix Cytogenetics 2.7 M

(Affymetrix, Inc., Santa Clara, CA, USA). The genomic DNA was extracted from whole-blood leukocytes from the patient and the parents. The array experiment was performed according to the manufacturer's instructions. Briefly, the genomic DNA was denatured and neutralized, and subsequently amplified by PCR. The PCR products were purified, fragmented and end-labeled with biotin. The fragmented, labeled PCR products were subsequently hybridized to the arrays overnight. The copy number variation was analyzed by Affymetrix Chromosome Analysis Suite software (ChAS) v1.2.2 (Affymetrix, Inc.). The GRCh37 (Hg19) was used to determine the chromosome positions. The abovementioned experiments were performed with the assistance of Capital Bio Corporation (Beijing, China).

Results

Histological analysis. Assessment of a testicular biopsy of the patient demonstrated that the number of testicular seminiferous tubules significantly decreased in a few sertoli cells (Fig. 1A); however, this was not observed at all levels of spermatogenic cell.

Cytogenetic analysis and FISH analysis. Karyotype analysis confirmed the 46,XX inv (9) (p11q13) karyotype (Fig. 1B) and FISH analysis demonstrated negative expression of the *SRY* gene (Fig. 1C). It was determined that inv (9) (p11q13) was inherited from the patient's mother. No other abnormality was observed in the family members of the patient.
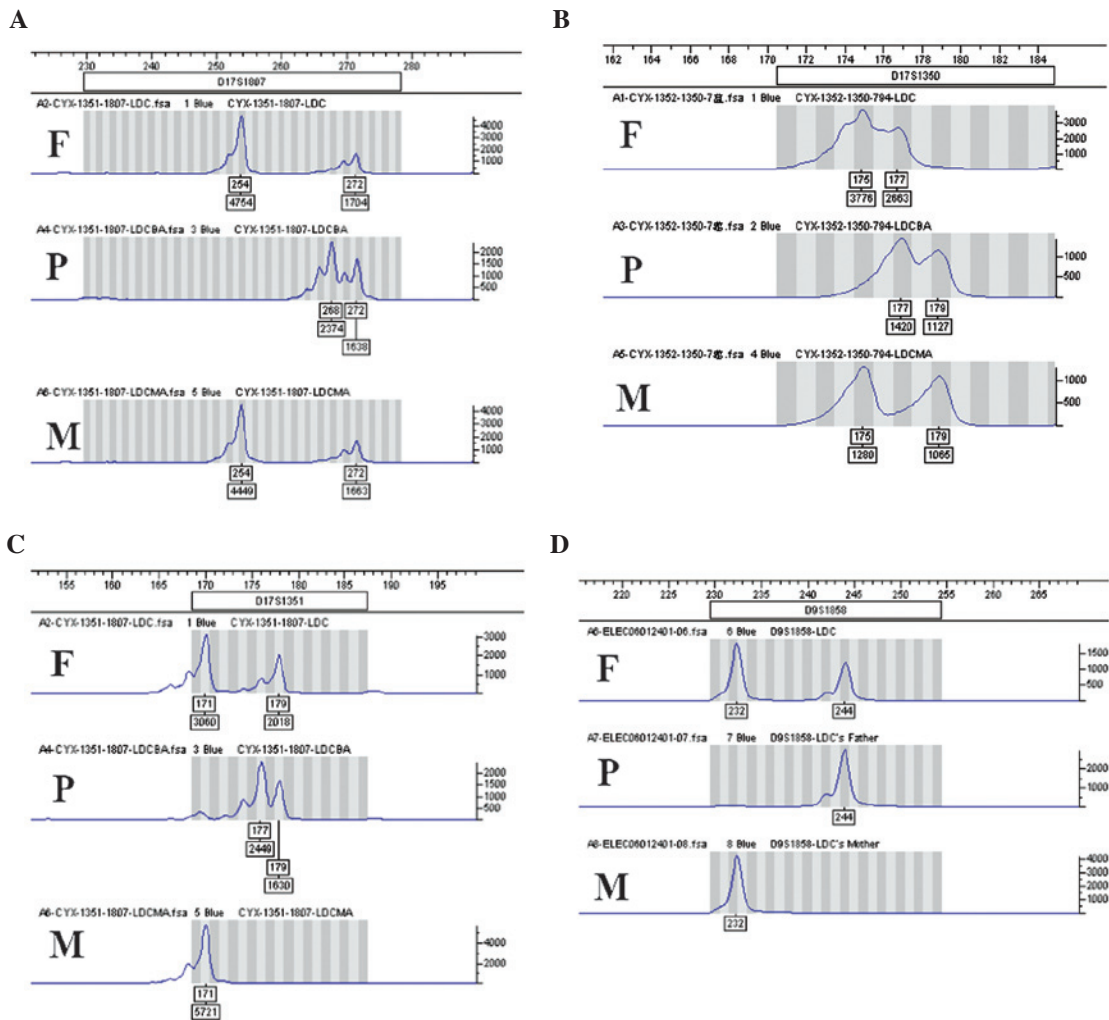
Figure 2. *SOX9* and *DMRT1* internal locus analysis. (A) Two kurtoses of 254 and 272 bp of D17S1807, (B) two kurtoses of 175 and 177 bp of D17S1350 and (C) two kurtoses of 171 and 179 bp of D17S1351 in the *SOX9* gene. There were (D) two kurtoses of 232 and 244 bp of D9S1858 in the *DMRT1* gene. No abnormal dose was detected in any STR site. P, patient; F, father; M, mother.

PCR amplification and sequencing of the coding region of candidate genes. The *SRY* gene was negative in peripheral blood leukocytes, as confirmed by FISH and PCR using two different primer pairs (Fig. 1C and D). STS detection revealed that the azoospermia factor (AZF)a, AZFb and AZFc regions were absent (Fig. 1D). Absence of PCR amplification products of the other 11 sites further confirmed the lack of Y chromosome sequences in the patient genomic DNA. The coding region and exon/intron boundaries of the *RSPO1*, *DAX1*, *SOX9*, *SOX3*, *ROCK*, *DMRT1*, *SPRY2* and *FGF9* genes were sequenced, and no mutation was detected.

The five STS markers D17S794, D17S1352, D17S1807 (Fig. 2A), D17S1350 (Fig. 2B) and D17S1351 (Fig. 2C) in the *SOX9* gene were normal. D9S1858 (Fig. 2D) was detected in the *DMRT1* gene. All markers revealed no abnormality of dosage, in comparison with the results of the patient's parents.

SNP array. Following comparison of the results of the patient with those of the parents, it was identified that the patient exhibited a ~88-kb duplication in a region upstream of *SOX9* at chromosome 17: 67,024,087-67,112,435 (Fig. 3). However, the duplicated region was also identified in the mother, which was a polymorphism (Fig. 3).

Discussion

The 46,XX male DSD syndrome is a rare genetic condition, which occurs with an incidence of ~1:20,000 in newborn males (24,25). The 46,XX male DSD syndrome was first reported in 1964 by de la Chapelle (24). The patients are all male with testicular tissue differentiation. Normal male sexual differentiation predominantly depends on the Y chromosome, which contains *TDF*. In general, sex determination and differentiation of mammals begins with the expression of the *SRY* gene, which is located in the short arm of the Y chromosome, and is the major sex determination gene (26-28). The undifferentiated gonad is differentiated into the testicle via the *SRY* gene, which secretes testosterone and mullerian duet inhibiting substance (MIS) to launch a series of male sexual differentiation mechanisms. At the molecular level, XX males can be classified as *SRY*-positive and *SRY*-negative. In the present study, a case of 46,XX *SRY*-negative males was identified. The mechanism by which the induction of testicular tissue is performed in *SRY*-negative patients remains to be elucidated.

Several hypotheses have been suggested regarding testicular development in *SRY*-negative patients. Hiddengonadal

Figure 3. Affymetrix cytogenetics whole genome 2.7 M arrays were used for detecting the genomic DNA from the patient and the parents. An 88-kb duplication was identified at the chromosome 17: 67,024,087-67,112,435 region in the patient and the mother (black arrows). This result indicated that the change was a polymorphism and not a cause of 46,XX male disorder of sex development.

mosaicism of *SRY* exists in patients. Domenice *et al* (29) indicated that no *SRY* was detected in the blood lymphocytes or skin tissue. Unfortunately, the present study was unable to detect the Y chromosome or the *SRY* gene in the blood samples. Certain autosomal or X-linked genes, which repress the male pathway (i.e. mutations in this pathway can result in de-repression of the male pathway in XX gonads) (1). For instance, a point mutation in the Wilm's tumor suppressor gene *WT1* on the autosome can lead to sex reversal, as previously described (30). Altered expression of other sex-determining genes, such as the *DAX1* gene, which are located downstream of *SRY* has been reported to be the cause of sex reversal (31). *SRY* is located in the short arm of the male Y chromosome, which is adjacent to the pseudoautosomal region. *SRY* negatively regulates the expression of the Z locus of the autosome, which may contain the male-determination gene. The reason that males with a deletion of *SRY* cannot express the male-determination gene is the degeneration of the Z locus. Therefore, a 46,XX male has normal male internal and external genitalia.

A previous study demonstrated that the overexpression of *SOX9*, which is located in 22q13, can trigger the development of testicles in the absence of *SRY* (17). The transcription factor *SOX9* is the most important gene in the sex-determining gene high-mobility group box family, which is associated with *SRY*. *SOX9* is the first gene expressed in precursor cells of Sertoli cells following *SRY*, and the normal expression of *SOX9* is associated with testicular differentiation (20). Ectopic expression of *SOX9* in the female gonad of XX mice, which lack *SRY*, caused complete female-to-male sex reversal (20,32). These data demonstrated that *SOX9* can support the formation of functional Sertoli cells from precursor cells. As the primary molecular marker of *SRY* differentiation, MIS can induce precursor cells of Leydig cells

in undifferentiated gonad to Leydig cells under the action of Sertoli cells secreting testosterone and promoting the normal development of the male reproductive tract (33). The genomic domain regulating the expression of *SOX9* was located 500-600 kb upstream of *SOX9* (1,21,23). It has been suggested that disruptions in certain regions upstream of *SOX9* can lead to various diseases, which result from the removal of variable long-range tissue-specific regulatory elements, which alter the expression of *SOX9* (34). Certain studies have suggested that duplication of the region containing *SOX9* is able to trigger sex reversal. Huang *et al* (18) have reported a case of 46,XX *SRY* negative male sex reversal caused by duplication of *SOX9*. Benko *et al* (23) demonstrated that the copy number variations of the region containing *SOX9* were the genetic basis for 46,XX DSDs of variable severity (ranging from mild to complete sex reversal).

In the present study, no change in the levels of *SOX9* was detected by *SOX9* internal locus analysis. In addition, deletion/duplication analysis was performed on the patient and a genomic-wide high-density Cyto2.7M™ Array kit (Affymetrix Inc.) was used to assess the parents. The patient exhibited a *de novo* partial duplication originating from the upstream of *SOX9*, involving ~88 kb in the chromosome 17: 67,024,087-67,112,435 region. An identical duplication was also detected in the mother. Therefore, it was suggested that the change was merely a polymorphism. The duplication located in the regulatory region upstream of *SOX9* was not a direct cause of the 46,XX testicular disorder of sex development (18). Another explanation may be that besides *SRY*, there are other genes associated with male gonad differentiation. The clinical, molecular and cytogenetic findings of the present study suggested that other unidentified genetic or environmental factors are significant in the regulation of SDS. Further studies will aim at identifying the exact pathogenesis of the disease.

Acknowledgements

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