

Case report

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A Korean boy with 46,XX testicular disorder of sex development caused by SOX9 duplication

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The 46,XX testicular disorder of sex development (DSD), also known as 46,XX male syndrome, is a rare form of DSD and clinical phenotype shows complete sex reversal from female to male. The sex-determining region Y (*SRY*) gene can be identified in most 46,XX testicular DSD patients; however, approximately 20% of patients with 46,XX testicular DSD are *SRY*-negative. The *SRY*-box 9 (*SOX9*) gene has several important functions during testis development and differentiation in males, and overexpression of *SOX9* leads to the male development of 46,XX gonads in the absence of *SRY*. In addition, *SOX9* duplication has been found to be a rare cause of 46,XX testicular DSD in humans. Here, we report a 4.2-year-old *SRY*-negative 46,XX boy with complete sex reversal caused by *SOX9* duplication for the first time in Korea. He showed normal external and internal male genitalia except for small testes. Fluorescence *in situ* hybridization and polymerase chain reaction (PCR) analyses failed to detect the presence of *SRY*, and *SOX9* intragenic mutation was not identified by direct sequencing analysis. Therefore, we performed real-time PCR analyses with specific primer pairs, and duplication of the *SOX9* gene was revealed. Although *SRY*-negative 46,XX testicular DSD is a rare condition, an effort to make an accurate diagnosis is important for the provision of proper genetic counseling and for guiding patients in their long-term management.

Keywords: XX male syndrome, Sex determining region Y-box 9, Sex-determining region gene on Y, Disorders of sex development

Introduction

The 46,XX testicular disorder of sex development (DSD), also known as 46,XX male syndrome, is a rare condition with an estimated prevalence of one in 20,000 males¹⁾. It can be classified into two subgroups, *SRY*-positive or *SRY*-negative, according to the presence or absence of the sex-determining region Y (*SRY*) gene. Approximately 80% of patients with 46,XX testicular DSD have *SRY* on one of two X chromosomes, which results from abnormal chromosomal translocation during gametogenesis. However, the remaining 20% of individuals with 46,XX testicular DSD are *SRY*-negative, and the cause of this condition is largely unknown¹⁾. Most individuals with 46,XX testicular DSD with *SRY* have normal genitalia and are usually not diagnosed until puberty does not proceed normally. However, *SRY*-negative individuals are more likely to have ambiguous genitalia than *SRY*-positive individuals. Therefore, 46,XX *SRY*-negative individuals showing complete male genitalia are quite rare²⁾.

The development of testes from bipotential gonadal primordia is controlled by complex molecular networks of expressions of multiple genes. Among the genes, *SRY* on the Y chromosome is a typical male-specific gene, which initiates testis development and directly upregulates the *SRY*-related HMG box-containing gene 9 (*SOX9*) gene expression. *SOX9* is a transcription factor with a DNA-binding site very similar to *SRY*, and plays a crucial role in the cascade of gene interactions for differentiation and development of the testis³⁾. In 1999, *SOX9*

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overexpression in a 46,XX gonad was identified as leading to an induction of male genitalia in the absence of *SRY*⁴⁾. Although there have been a few reports to date of 46,XX *SRY*-negative patients having duplication of *SOX9*⁴⁻⁷⁾, *SOX9* duplication is not a common cause of 46,XX testicular DSD⁸⁾. In Korea, less than 10 *SRY*-positive 46,XX patients have been reported, and there are no reports of 46,XX testicular DSD resulting from *SOX9* duplication⁹⁾.

In this study, we present an *SRY*-negative 46,XX Korean boy who presented with small testes and without any genital ambiguity. Molecular genetic studies confirmed *SOX9* duplication as the cause of 46,XX testicular DSD for the first time in Korea.

Case report

1. Subject

A 4.2-year-old boy was referred to the outpatient clinic at Seoul National University Children's Hospital for evaluation of an abnormal karyotype, 46,XX. Four months before the visit, his mother noticed that his testes were smaller than those of his newborn brother. He visited the Pediatric Urology Clinic at a regional hospital, and karyotype analysis using his peripheral blood resulted in a diagnosis of 46,XX. He was born at a gestational age of 40 weeks by vaginal delivery, with a birth weight of 3.2 kg (25th–50th percentile), without any perinatal problems. After birth, a small atrial septal defect was detected, which was closed spontaneously on follow-up.

On physical examination, his body weight and height were 14.9 kg (10th–25th percentile) and 98.2 cm (10th–25th percentile), respectively. His head circumference was 47.5 cm (5th percentile), and he showed normal developmental milestones. No gross anomalies were observed, and his external genitalia showed complete male phenotype without ambiguity (Fig. 1A). The phallus was 3.0 cm long, and the opening of the

urethral meatus was located at the tip of the glans penis. The perineal surface between the urethral meatus and the anal opening was closed. The scrotum was bifid with small palpable gonads. The longest testicle was less than 1 cm. No skeletal abnormalities were identified. His bone age was 4 years, and magnetic resonance imaging revealed that both testes were located in the scrotal sac; no structure like the uterus, ovary, or vagina was found (Fig. 1B).

Baseline serum concentrations of luteinizing hormone and follicle-stimulating hormone were <0.5 mIU/mL (prepubertal normal range, 0.02–1.03 mIU/mL) and 0.9 mIU/mL (prepubertal normal range, 0.25–1.92 mIU/mL), respectively. The estradiol level was 4 pg/mL (normal range for boys, 3–10 pg/mL), and the anti-Müllerian hormone level was 44.0 ng/mL (normal range for boys, 7–240 ng/mL). Levels of 17-hydroxyl progesterone and dehydroepiandrosterone sulfate were within normal ranges. The results of a human chorionic gonadotropin (hCG) stimulation test to assess testicular function showed a normal response: the baseline testosterone level was <0.1 ng/dL (normal range, <0.12 ng/dL), and the follow-up testosterone level after intramuscular injections for 3 consecutive days with 1,000 IU of hCG elevated to 2.10 ng/dL.

2. Genetic analyses

The parents provided formal informed consent to permit peripheral blood sampling for genetic testing, and the Institutional Review Board of Seoul National University Hospital approved this study. We repeated karyotype analysis using the patient's blood lymphocytes. Twenty cells were analyzed, and the karyotype was identified as 46,XX¹⁰⁾.

Fluorescence *in situ* hybridization (FISH) analysis to identify the presence of the *SRY* gene on an X chromosome was conducted; *SRY* was not revealed. Polymerase chain reaction (PCR) and electrophoresis were performed using specific primers for *SRY*; however, we could not find any band of amplified *SRY* materials.

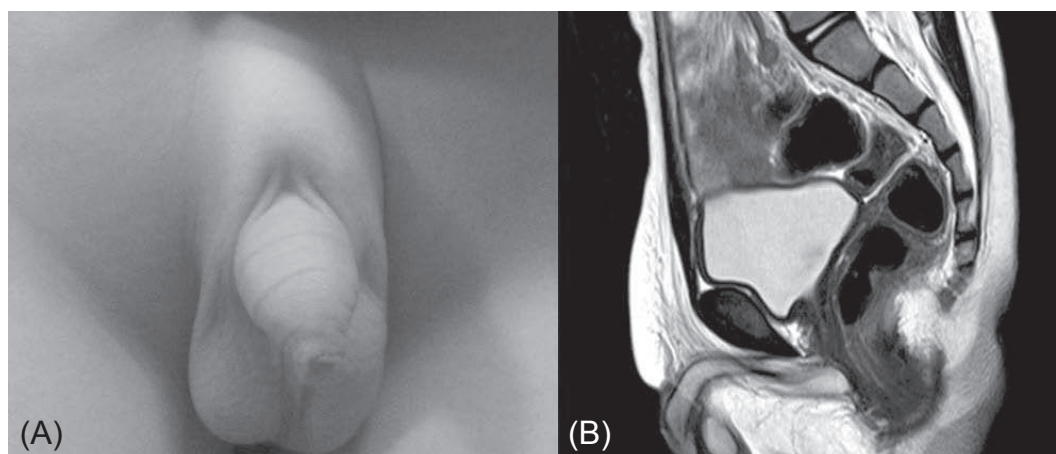


Fig. 1. (A) The genitalia photograph of the patient was taken at 4.2 years of age. His external genitalia show complete male phenotypes without ambiguity. (B) Normal internal genital organs are visible, and there is no evidence of female genital organs on the T2-weighted magnetic resonance image.

We performed additional molecular genetic analyses for assessing the *SOX9* gene. Direct sequencing analysis for all coding exons and exon-intron boundaries of *SOX9* did not identify any pathogenic mutations. Therefore, a real-time PCR assay was subsequently performed to identify the copy number of *SOX9*, using two sets of primer pairs. Each primer pair was designed to be located in the region approximately 5,000 bp upstream and downstream from the *SRY* gene. We used genomic DNA from a normal fertile 46,XY male as a control, and real-time PCR analyses with each primer pair were repeated three times. The results of real-time PCR reactions showed a duplicated copy number of *SOX9* in the patient: the ratio of the patient to the control male was 1.45:1 (using an upstream primer pair) and 1.44:1 (using a downstream primer pair) (Fig. 2).

Discussion

46,XX male syndrome was first described in 1964¹¹⁾, and the Chicago consensus renamed of this syndrome as 46,XX testicular DSD in 2005¹²⁾. Most patients have normal male phenotypes at birth, and are usually diagnosed in late adolescence because of delayed puberty, gynecomastia, or infertility²⁾. Approximately 15% of XX males have hypospadias, cryptorchidism, or more severe genital ambiguity¹³⁾. All 46,XX males are infertile owing to the absence of the azoospermia factor region in the long arm of the Y chromosome^{14,15)}. Although testosterone levels normally evolve during puberty, deficient testosterone production after a β -hCG stimulation test becomes evident and hypergonadotropic hypogonadism with severe testicular atrophy and azoospermia in biopsied testes is clearly manifested in adulthood^{15,16)}. In this case, there was normal response in the plasma testosterone level after β -HCG

stimulation performed at the age of 4.2 years, and this result only suggested that testis tissue was present. As testicular atrophy progresses with age, follow-up stimulation test performed during adolescence might show abnormal response as testicular atrophy progresses. Testosterone replacement therapy may be required at puberty.

Female-to-male sex reversal in individuals with 46,XX is most frequently caused by the presence of *SRY* on one of the X chromosome resulting from a translocation between the X and Y chromosomes¹⁷⁾. This translocation event occurs randomly during spermatogenesis in the affected individual's father. The *SRY* gene plays a critical role in the initiation of testicular differentiation in males¹⁸⁾. In XY embryos, *SRY*, which is normally located on the Y chromosome, induces the gonadal primordia to develop into testes. If a fetus is conceived from a sperm with an *SRY*-bearing X chromosome, it will develop as a male despite not having a Y chromosome.

In about 80% of individuals with 46,XX testicular DSD, the presence of *SRY* can be documented by FISH or PCR analyses and classified as *SRY*-positive; the remaining 20% without *SRY* are classified as *SRY*-negative. Some genotype-phenotype correlations have been suggested, and *SRY*-negative individuals have a higher incidence of genital ambiguity, which is not common in *SRY*-positive individuals^{2,17)}.

Because testicular differentiation occurs without *SRY* in approximately 20% of individuals with 46,XX testicular DSD, there might be other important genes that cause 46,XX testicular DSD¹⁹⁾. *SOX9* on chromosome 17q24.3 is one of the genes that plays an important role in the development of the skeleton and genital organs⁴⁾. During embryogenesis, *SOX9* is expressed immediately downstream of *SRY* and functions as a critical Sertoli cell differentiation factor. In addition, differentiated Ser-

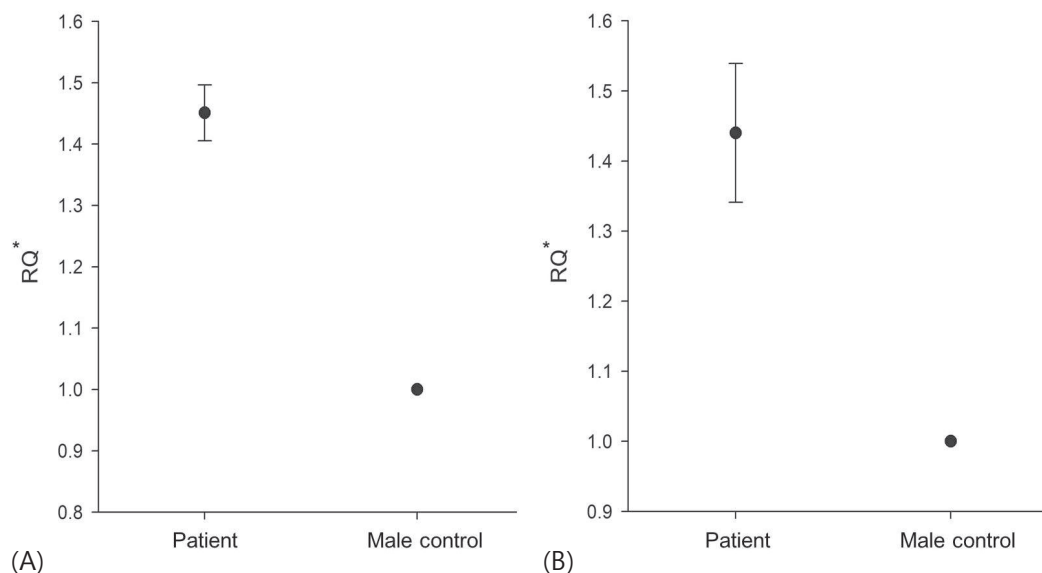


Fig. 2. Results of real-time polymerase chain reaction analysis using two sets of specific primer pairs (A, proximal primer pair; B, distal primer pair) show the increased copy number of *SOX9* in the patient (approximately 1.5 times) compared with a control male. RQ, relative quantification.

toli cells express anti-Müllerian hormone, which is required for Müllerian duct regression and differentiation of male genitalia¹⁴.

A family study recently reported that 46,XY persons with a 178 kb duplication in a region about 500 kb upstream of *SOX9* were completely normal fertile males, whereas 46,XX *SRY*-negative persons in the same family with duplications were clinically infertile males⁶. In this report, two of the three paternal cousins of their probands are known as infertile and the two probands share the same paternal haplotype for the *SOX9* region, confirming the possibility that the father was indeed the carrier of the same triplication. Therefore, we should investigate family members of 46,XX male patients caused by *SOX9* duplication.

Male sex differentiation is known to require a delicate dosage balance among male determining genes, including *SOX9*. Haploinsufficiency of *SOX9* from the loss of function mutations or deletions results in campomelic dysplasia (Online Mendelian Inheritance in Man #114290), which is a well-known skeletal dysplasia showing congenital bowing of long bones with male-to-female sex reversal in two-thirds of affected individuals with 46,XY²⁰. On the other hand, duplication of *SOX9* has been found in individuals with *SRY*-negative 46,XX testicular DSD. Previous studies have suggested that overexpression of *SOX9* could induce testis determination in the absence of *SRY*⁴. In the present study, we also revealed *SOX9* duplication as the cause of *SRY*-negative 46,XX testicular DSD by real-time PCR in a boy who presented with small testes and without any genital ambiguity. To our knowledge, this is the first report in Korea of 46,XX testicular DSD caused by *SOX9* duplication.

However, the results of a recent study suggested that duplication of *SOX9* is not a common cause of 46,XX testicular or 46,XX ovotesticular DSD⁸. In that study, the authors amplified microsatellite markers in the region of *SOX9* from a cohort of 30 patients with either 46,XX testicular or 46,XX ovotesticular DSD to detect *SOX9* duplications, but they could not find any duplication of the *SOX9* region in 17q. They implied that there might be another important cause of 46,XX testicular DSD⁸. Another gene, *SOX3*, has recently been identified as upregulating the expression of *SOX9* via a similar mechanism to *SRY* and as being responsible for XX male sex reversal in humans through gain-of-function mutations mediated by genomic rearrangements around *SOX3*, possibly leading to its altered regulation¹⁰. Furthermore, duplications of the *SOX3* locus were detected in three cases of 46,XX testicular DSD¹⁰. *SOX3* analysis can be considered the next step for the diagnosis of 46,XX *SRY*-negative testicular DSD with normal copies of *SOX9*.

Human sex determination and differentiation are complex processes associated with multiple genes including *SRY*, *SOX9*, *SOX3*, *DAX-1*, *SF-1*, and *WT-1*⁴. However, the exact mechanism underlying male sex determination is not completely understood yet, and multiple genes associated with testicular differentiation are not routinely or fully investigated in most cases of 46,XX testicular DSD. Although *SRY*-negative

46,XX testicular DSD is a rare condition, an effort to make an accurate diagnosis is important for the provision of proper genetic counseling and for guiding patients in their long-term management.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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