Original Article

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Three cases of rare *SRY*-negative 46,XX testicular disorder of sexual development with complete masculinization and a review of the literature

Bom Yi Lee[1], Shin Young Lee[1], Yeon Woo Lee[1], Shin Young Kim[1], Jin Woo Kim[1], Hyun Mee Ryu[1,2], Joong Shik Lee[3], So Yeon Park[1,*], and Ju Tae Seo[3,*]

[1]Laboratory of Medical Genetics, Cheil General Hospital and Women's Healthcare Center, Seoul, Korea
[2]Department of Obstetrics and Gynecology, [3]Department of Urology, Cheil General Hospital and Women's Healthcare Center, Dankook University College of Medicine, Seoul, Korea

Purpose: To identify the clinical characteristics of *SRY*-negative male patients and genes related to male sex reversal, we performed a retrospective study using cases of 46,XX testicular disorders of sex development with a review of the literature.
Materials and Methods: *SRY*-negative cases of 46,XX testicular disorders of sex development referred for cytogenetic analysis from 1983 to 2013 were examined using clinical findings, seminal analyses, basal hormone profiles, conventional cytogenetic analysis and polymerase chain reaction.
Results: Chromosome analysis of cultured peripheral blood cells of 8,386 individuals found 19 cases (0.23%) with 46,XX testicular disorders of sex development. The *SRY* gene was confirmed to be absent in three of these 19 cases (15.8%).
Conclusion: We report three rare cases of *SRY*-negative 46,XX testicular disorders of sex development. Genes on autosomes and the X chromosome that may have a role in sex determination were deduced through a literature review. These genes, through differences in gene dosage variation, may have a role in sex reversal in the absence of *SRY*.

Key words: Azoospermia, Infertility, *SRY* genes, 46,XX testicular disorder of sex development.

Introduction

The critical gene for male sex determination, *SRY* (sex-determining region Y), which is located on chromosome Yp11.3, initiates gonads to differentiate into testes, induces Leydig cells to secrete testosterone, develops Wolffian ducts, and forms male external genitalia. At the same time, Sertoli cells secrete Müllerian inhibiting factor that induces regression of Müllerian ducts that in females would differentiate into the uterus. These processes for sex determination do not occur in females in the absence of *SRY*. Sex differentiation related genes such as *SOX9*, *FGF9*, *DAX1*, *WT1*, *RSPO1*, and *SOX10*, which are located on either autosomes or the X chromosome, may have a role in gonad development and function. These genes were studied in

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*Co-corresponding author: Ju Tae Seo, M.D., Ph.D.
Department of Urology, Cheil General Hospital and Women's Healthcare Center, Dankook University College of Medicine, 17 Seoae-ro 1-gil, Jung-gu, Seoul 04619, Korea.
Tel: +82-2-2000-7682, Fax: +82-2-2278-4574, E-mail: jtscmc@naver.com
So Yeon Park, Ph.D.
Laboratory of Medical Genetics, Cheil General Hospital and Women's Healthcare Center, 17 Seoae-ro 1-gil, Jung-gu, Seoul 04619, Korea.
Tel: +82-2-2000-7680, Fax: +82-2-2278-4574, E-mail: paranip5@hanmail.net
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XX testicular/ovotesticular disorders of sex development (DSDs) in the absence of the *SRY* gene.

46,XX male sex reversal (also known as testicular DSD) is reported in 1:20,000 to 1:25,000 of newborn males [1], and is categorized using clinical phenotypes or molecular genetic analysis depending on the presence or absence of the *SRY* gene. Clinical sexual phenotypes of individuals with 46,XX testicular DSD range from complete masculinization to true hermaphrodites (also known as ovotesticular DSD) and are comprised of three groups: 1) XX testicular DSD with normal genitalia of which 85% of cases are normal males with internal and external genitalia, and are usually diagnosed after puberty because of hypogonadism, gynecomastia, and/or infertility [2]; 2) patients with XX testicular DSD with ambiguous genitalia are identified at birth by external genital ambiguities such as micropenises, cryptorchidism, or hypospadias; and 3) patients with XX ovotesticular DSD have internal and external genital ambiguities that are detected at birth or histologically—these patients have either an XX karyotype (60%) or a 46,XX/46,XY or 46,XY karyotypes.

46,XX testicular DSD is also classified genetically based on the presence or absence of the *SRY* gene [3]. In 90% of cases in which the *SRY* gene is present, the disorder is the result of an aberrant Y to X chromosomal interchange during meiosis I in paternal gametogenesis. Affected males show a similar phenotype compared to males with Klinefelter syndrome, which is characterized by a 47,XXY karyotype and consists of an active X chromosome, an inactive X chromosome, and the *SRY* gene. Rarely, the terminal region of the Y chromosome, that includes the *SRY* gene, is cytogenetically detectable on the X chromosome. For the majority of cases in which the *SRY* gene is absent, patients with 46,XX testicular DSD have genital ambiguities such as a micropenis, hypospadias, and cryptorchidism. However, completely virilized 46,XX testicular DSD with a *SRY* deficiency has rarely been reported in the literature. Therefore, other autosomal or X-linked genes may have regulatory roles with *SRY* in the sex-determination process.

We present three rare cases of *SRY*-negative 46,XX testicular DSD found in the past 31 years at our center, and our review of the literature on previous cases of 46,XX testicular DSD with a focus on recently reported related genes since the characterization of *SRY*.

Materials and Methods

1. Clinical findings and cytogenetic analysis

We retrospectively investigated the clinical records of 8,386 males who were referred for cytogenetic analysis from 1983 to 2013 at the Cheil General Hospital, Seoul, Korea. We examined hormonal profiles, histological findings, and semen analysis. For the hormonal profile, we evaluated the levels of serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), 17β-estradiol, testosterone (T), prolactin, and sex hormone-binding globulin.

Conventional cytogenetic analyses were performed according to standard techniques. High-resolution chromosomes of 700-band levels by GTL-banding were prepared using cultured peripheral blood cells. We analyzed 100 metaphases of each patient to exclude potential sex chromosome mosaicism.

Detection of the *SRY* gene in some patients was confirmed using fluorescence *in-situ* hybridization (FISH) analysis with the Y-specific probes: CEP Y Sat III Spectrum Green/CEP Y Alpha Spectrum Orange (Vysis Inc., Downers Grove, IL, USA) and the LSI SRY Spectrum Orange/CEP X Spectrum Green (Vysis Inc.) (data not shown).

2. Molecular genetic analysis

Three individuals with 46,XX testicular DSD were further investigated using azoospermia factor (*AZF*) microdeletion analysis with multiplex polymerase chain reaction (PCR). The oligonucleotides used for multiplex PCR are described in our previous study [4]. Briefly, PCR was performed using the following cycle conditions: an initial denaturation at 95°C for 10 minutes, followed by 35 cycles at 95°C for 10 minutes, 62°C for 90 seconds, and 65°C for 90 seconds with a final extension at 65°C for 10 minutes. Each round of PCR that was performed using patient's DNA included normal male DNA and normal female DNA as a positive and a negative control, respectively. Amplified products were separated on 3% agarose gel by electrophoresis and visualized using ultraviolet illumination.

3. Ethical considerations

This study was approved by the Ethics Committee of Cheil General Hospital and Women's Health Care Center (#CGH-IRB-2014-47) and consent was obtained from all patients.

Table 1. Detailed data from 19 cases of 46,XX testicular DSD in 8,386 cases of male individuals referred to cytogenetic analysis from 1983 to 2013

Case	Age (yr)	Indication	Marriage (yr)	SA	*SRY*	T (ng/mL)	LH (mIU/mL)	FSH (mIU/mL)	E_2 (pg/mL)	PRL (ng/mL)	SHBG (nmol/L)	Free T (pg/mL)	Testis R, L (mL)	H (cm)/ W (kg)	Urological findings
1	37	XXY	6	Azo	NR								5, 5		Hypoplasia
2	35 days	Ambiguous genitalia			NR	0.1 (0-15)	0.8 (2-20)	5.6 (2-10)						49.5/3.4	Bilateral cryptorchidism, hypospadia, pencile type penis, penoplasty; female E, VD, V; (–)R, L
3	37	Infertility	4	Azo	NR	3.4 (0-15)	9.7 (2-20)	22 (2-10)	25 (30-120)				3, 3		Penis (5 cm) E, VD, V; (–)R, L
4	36	Infertility	3	Azo	NR	1.9 (0.2-0.8)	18 (1.8-13.4)	42 (2-12)	13 (30-120)				8, 8	165/59	penis (8 cm) E, VD, V; (–)R, L
5	28	Infertility	3	Azo	(+)	3.6 (0.2-0.8)	22 (8.0-13.4)	64 (2-12)		7.8 (0-15)			4, 4	165/55	Penis (7 cm) E, VD, V; (–)R, L
6	31	Infertility	9	Azo	NR	2.2 (0.2-0.8)	14 (1.8-13.4)	42 (2-12)					3, 3		Penis (4 cm), tubular sclerosis & hyalinization, focal increased number of Leydig cells
7	30	Infertility			NR	5.1 (0.2-0.8)	4.5 (1.8-13.4)	16 (2-12)	12 (30-120)				10, 10		E, VD, V; (–)R, L
8	33		5	Azo	NR								4, 4	/65	Germ cell aplasia
9	37	Infertility	4	Azo	NR	4.7 (0.2-0.8)	17 (1.8-13.4)	48 (2-12)			19 (10-73)	18 (8.8-27)	2, 2	162/65	Penis (6 cm), E, VD, V; (–)R, L
10	33	Infertility	3	Azo	NR	1.95 (2.5-8.8)	17.2 (0-12)	22.3 (0-15)					3, 3	174/73	E, VD, V; (–)R, L
11	38	Infertility	4	Azo	(+)	3.5 (0.2-0.8)	18 (1.8-13.4)	45 (2-12)		4.3 (0-15)			3, 3	160/62	Leydig cell hyperplasia, *AZF* gene microdeletion E, VD, V; (–)R, L
12	28	Infertility	3	Azo	NR	1.8 (0.2-0.8)	13 (1.8-13.4)	29 (2-12)					12, 12		Germ cell aplasia, R. testicular atrophy, bilateral retractile testis, bilateral orchiopexy (undescended) E, VD, V; (–)R, L
13	36	Infertility	7	Azo	(+)	2.1 (0.2-0.8)	13 (1.8-13.4)	35 (2-12)			35 (10-73)	13 (8.8-27)	3, 3	173/76	Penis (9 cm), *AZF* gene microdeletion
14	29	Infertility	1	Azo	(+)	2.78 (1.3-8.1)	9.2 (1.5-9.2)	44 (1-14)	11 (15-80)	3.8 (0-15)	25 (10-73)		5, 5	173/80	Germ cell aplasia (60-70%) atrophy (30-40%) E, VD, V; (–)R, L
15	29	Infertility		Azo	(+)	1.3 (1.3-8.1)	5.9 (1.5-9.2)	34 (1-14)	8 (15-80)	8.8 (2.7-19.7)	11 (10-73)		2, 2	163/72	E, VD, V; (–)R, L
16	41	Azo	4.11	Azo	(+)	1.27 (1.3-8.1)	30.7 (1.7-8.6)	48.8 (1.5-12.4)	9.9 (7.4-42.6)	7.6 (4.0-15.2)	39.8 (10-73)	2.09 (8.8-27)	2, 2	163/60	E, VD, V; (–)R, L
17	37	Infertility	6.11	Azo	(–)	0.58 (1.3-8.1)	8.4 (1.7-8.6)	5.3 (1.5-12.4)	144.7 (7.4-42.6)	14.4 (4.0-15.2)	79 (10-73)		2, -	165/56	Male hormonal treatment E, VD, V; (–)R, L
18	42	Known XX	5	Azo	(–)	1.62 (1.3-8.1)	13.3 (1.7-8.6)	45.1 (1.5-12.4)		8.1 (4.0-15.2)	38.3 (10-73)	3.2 (8.8-27)	2, 2	156/45	Male hormonal treatment E, VD, V; (–)R, L
19	36	Azo	6	Azo	(–)	0.47 (1.3-8.1)	19.1 (1.7-8.6)	27 (1.5-12.4)	17 (7.4-42.6)	2.9 (4.0-15.2)	25.4 (10-73)	0.8 (8.8-27)	2, 2	172/78	Male hormonal treatment E, VD, V; (–)R, L

DSD, disorders of sex development; SA, semen analysis; LH, luteinizing hormone; FSH, follicle-stimulating hormone; E_2, 17β-estradiol; PRL, prolactin; SHBG, sex hormone-binding globulin; T, testosterone; R, right; L, left; H, height; W, weight; Azo, azoospermia; NR, not reported; E, epididymis; VD, vas deferens; V, varicocele; (+), present; (–), absent; *AZF*, azoospermic factor.

Results

1. Clinical findings and cytogenetic analysis

Of 8,386 male individuals, we identified 1,172 cases with an abnormal karyotype, and 19 cases with 46,XX testicular DSDs. Of these 19 cases, three *SRY*-negative 46,XX testicular DSDs were confirmed experimentally, and all three cases showed azoospermia with small testes volumes. We found that these 19 patients with 46,XX testicular DSDs have serum concentrations that were partially out of range of normal hormonal profiles. Of those cases of XX testicular DSDs with the *SRY* gene, we found increased levels of LH, FSH, and T, whereas in cases of XX testicular DSDs without the *SRY* gene, we found increased levels of LH and FSH with or without decreased levels of T. Patient height ranged from 156 to 172 cm, and we found from our urological assessment that there were no cases of ovotesticular DSD or ambiguous genitalia. With regard to the three cases with 46,XX testicular DSDs without the *SRY* gene, all three patients showed masculinized external genitalia and small testes with azoospermia. Detailed clinical data is summarized in Table 1.

We analyzed 100 metaphase karyotypes of cultured lymphocytes from peripheral blood of the 19 cases with 46,XX testicular DSDs. All individuals showed a complete 46,XX karyotype. Regardless of the presence or absence of the *SRY* gene, we found that 0.23% (19/8,386 cases) of the male participants referred for cytogenetic analysis in our study had XX testicular DSDs. Furthermore, we found that 15.8% (3/19 cases) of cases with 46,XX testicular DSDs were *SRY*-negative or, alternatively, 0.26% (3/1,172 cases) of males with an abnormal karyotype in our cohort of 8,386 participants.

2. Molecular genetic analysis

Of the 16 sequence-tagged site (STS) markers tested, amplification products of 15 STS loci including the *SRY* gene (sY14) on the Y chromosome were not detected with the exception of *ZFX* (zinc finger protein, X-linked) located on chromosome Xp21.3, which indicates the complete absence of the Y chromosome (Fig. 1).

Discussion

Through our study of 8,386 cases and a literature review (summarized in Table 2 [2,5-29]), we found that the major characteristics of male sex reversal for DSD were showed the clinical phenotypes of short stature without a T-dependent pubertal growth spurt and the Y-specific growth gene, azoospermia, internal or external genital abnormalities, gynecomastia, and high levels of FSH and LH, and a low level of T. However, a limitation of our retrospective study was that no gonadal biopsy samples were available for analysis and thus, gonadal mosaicism cannot be ruled out. Reports of familial cases of DSDs in the literature demonstrate that the various clinical phenotypes from ovotesticular DSD to masculinization may be because of incomplete penetrance of one or more genes.

The *SRY* gene has a critical role in the process of sex differentiation. However, the *SOX9*, *FGF9*, and *MAP3K1*

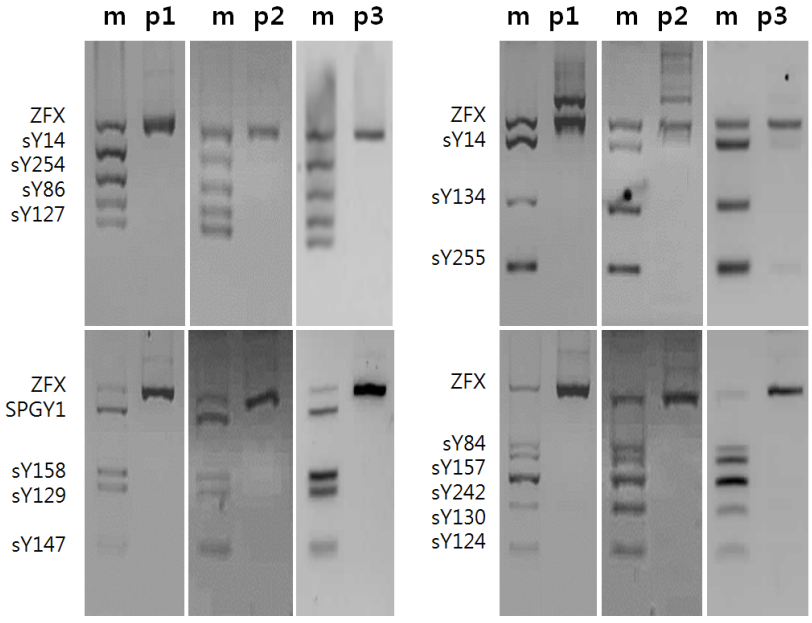

Fig. 1. Multiplex-polymerase chain reaction analysis using short tandem repeats for detection of *SRY* and microdeletion of the azoospermia factor (*AZF*) region. Samples (p1, p2, and p3) of *SRY*-negative 46,XX testicular disorders of sex development show complete deletion of the *AZF*b and *AZF*c regions. Lanes for the DNA ladder, blank, and female negative controls were loaded and electrophoresed (data not shown).
m, male positive control; p, patient; *ZFX*, zinc finger protein, X-linked.

Table 2. Summary of previously reported *SRY*-negative males with 46,XX testicular DSD or ovotesticular DSD

Case	Year [ref]	Age (yr)	Phenotype	Hormone	Diagnosis	Inheritance	Tested/proposed candidate gene(s)
1	1993 [5]		Normal male without ambiguity		XX TD	fam, sib	/*Z* gene theory, *Z*-/*Z*-, *Z*+/*Z*-, *Z*+/*Z*+; recessive mutations, wild type *Z* product, a negative regulator of male sex determination that is functional in wild-type female
2			Hypospadias, micropenis, hyperclitoridy		XX TD	fam, sib	
3			Internal & external genital ambiguity		OTD	fam, sib	
4	1993 [6]	>24	Normal penis, small testes, sister & mat cousin sister with OTD	FSH↑, LH↑	XX TD	fam, mat	*PABY, SRY, ZFY*/ autosomal dominant mutation or X-linked dominant gene downstream from *SRY*, incomplete penetrance
5	1997 [2]	28	6-cm penis, both gonads (3.78, 4.13 mL), sparse facial, body, & axillary hair, female pubic hair (Tanner stage 4), gynecomastia, hypergonadotropic hypogonadism, height 156 cm		XX TD	fam, sib	*PABY, SRY, ZFY*/ a homozygous loss-of-function mutation in a recessive, autosomal or X-linked gene, resulting in activation of the male sex-determining pathway
6		26	7-cm penis, both gonads (3.3, 4.12 mL), abundant facial, body, & axillary hair, female pubic hair (Tanner stage 4), height 162 cm		XX TD	fam, sib	
7	1998 [7]	4 wk	Small penis, hypospadias, a bifid scrotum, nonpalpable testes, intra-abdominal gonad (R), gonadal remnant (L) small midline uterus, vagina	T↓	OTD	fam, mat	/Autosomal or X-linked mutation, the different phenotypic effects arise because of variable penetrance
8		12	No pubertal signs, height in the 10th centile		XX TD	fam, mat	
9	1999 [8]	Infant	1.2-cm penis, scrotal hypospadias, the opening urethral meatus, bifid scrotum, palpable gonads, uterus (–), 46,XX,dup(17)(q23.1q24.3)/46,XX,dn		XX TD		/An extra dose of *SOX9* is sufficient to initiate testis differentiation in *SRY*(–)
10	1999 [24]	Infant	Small penis, scrotal sack (R), pigmented scrotal tissue (L), small vagina, uterus, & ovary, streak gonad (L), a small testis-like structure with VD & E (R), 46,XX,rec(22)dup(22q) inv(22)(p13q13.1)mat	T↑	OTD		Partial dup of chr 22/ genes on chr 22 that are involved in sex determination
11	2000 [9]	42	Sparse facial, body, axillary hair & male pubic hair, hypospadias, cryptorchidism, normal height	FSH↑, LH↑ T↓	XX TD		/Autosomal or X-linked sex-determining genes, both carriers for a recessive mutated allele in the *SOX9* locus, Huriez syndrome, sclerotylosis
12-17	2004 [26]		6 Cases		XX TD		Over expression of the *SOX10* gene at 22q13 in a sex reversal case; no evidence in 13 additional subjects with *SRY*(–) 46,XX sex reversal for microduplication of 22q *(SOX10)* /a gene on 22q that can trigger testis determination in the absence of *SRY*
18-24			7 Cases		OTD		
25	2005 [10]		Hypospadias & hypogenitalism, gynecomastia, PPK/SSC		XX TD	fam, sib	Linkage analysis of 15 loci for PPK and 9 loci for sex determination /differentiation a single mutation/ possibly affecting contiguous genes may underlie both sex reversal & PPK/SCC. The *SOX9* gene is very close to the locus for "tylosis".
26			Hypospadias & hypogenitalism, PPK		XX TD		
27			Hypospadias & hypogenitalism, two epididymal cysts (4.5 mm), gynecomastia, nodular hyperplasia of Leydig cells. PPK/SCC		XX TD		

Table 2. Continued

Case	Year [ref]	Age (yr)	Phenotype	Hormone	Diagnosis	Inheritance	Tested/proposed candidate gene(s)
28	2006 [12]	34	Testes (4.8, 5.1 mL), normal axillary & pubic hair, infertility, height 156 cm	FSH↑, LH↑	XX TD		*SOX9, DAX1*, 22q; no mutation, no dup in *SOX9*- 17q (6 STRs), 22q (3 STRs), no deletion in *DAX1*-X (53 STRs)/ a loss of function mutation in a 'gene' downstream to SRY in the male determining pathway
29	2007 [13]	15	4-5-cm penis, severe chordee, penoscrotal hypospadias, female pubic hair, blind ending vagina, palpable gonads		XX TD	fam	a mutation at a sex-determining locus other than *SRY* & *SOX9* as the cause for the XX sex reversal trait in this family, no a common *SOX9* haplotype identified among family members
30		13	4-5-cm penis, labiascrotal fusion, scrotal hypospadias, hypoplastic hyposcrotum		OTD		
31		5	3-4-cm penis, severe chordee, bifid scrotum, nonpalpable R. gonad, penoscrotal hypospadias. vaginal orifice (3 cm), penoscrotal transposition		OTD		
32		15	3-4-cm penis, severe chordee, perineal hypospadias, nonpalpable gonads, female pubic hair		OTD		
33		33	Normal penis, infertility, proximal glandular hypospadias, palpable gonads	FSH↑, LH↑	XX TD		
34		10	Normal penis & meatus, palpable scrotum, female pubic hair	Normal FSH, LH, T	XX TD		
35		26	Normal penis, megameatus, infertility, palpable gonads	FSH↑, LH↑	XX TD		
36		24	Normal penis & meatus, palpable gonads	FSH↑, LH↑	XX TD		
37		19	Normal penis size, distal glandular hypospadias, palpable gonads	FSH↑, LH↑	XX TD		
38	2008 [14]	Infant	1-cm penis, chordee, penoscrotal hypospadias, palpable gonads	Normal FSH, LH, T	XX TD	fam, twin	*SOX9, DAX1*; no mutation
39		Infant	0.5-cm penis, perineal hypospadias, gonad (R, nonpalpable; L, small)	FSH↑, LH↑	OTD	fam, twin	
40	2008 [11]	23	5-cm penis, hypospadias, testes (5.1, 0 mL), height 157 cm	FSH↑, LH↑, T↓	OTD		*SOX9, DAX1, Ad4BP/SF-1, WT1, GATA4, MIS. SOX9* expression↑ and expression↓ of *Ad4BP/SF-1, DAX1* & MIS /lesions affecting *SOX9* expression are the key factor in sex determination in *SRY*(–) XX males, the decreased expression of *Ad4BP/SF-1, DAX-1* & *MIS* contribute to their clinical features
41		28	3.6-cm penis, hypospadias, testes (3.3, 3.2 mL), height 150 cm	FSH↑, LH↑, T↓	OTD		
42		21	3.6-cm penis, hypospadias, testes (4.2, 3.8 mL), height 156 cm	FSH↑, LH↑, T↓	OTD		
43		5	Chordee, hypospadias (–)		OTD		
44		20	3.2-cm penis, hypospadias, testes (4.2, 3.1 mL)		OTD		
45	2010 [27]	29	Azo, testes (3, 5 mL), Leydig cell hyperplasia, height 165 cm	FSH↑, LH↑, T↓	XX TD		/25 cases review, a number of unknown genes downstream participate in sex determination
46	2010 [25]	30	Azo, small testes & scrotum, normal axillary & pubic hair, gynecomastia, hypo-thyroidism, height 170 cm	FSH↑, LH↑, TSH↑, T↓	XX TD		/Chronic autoimmune thyroiditis
47	2011 [16]	Adult	Azo, normal virilization, Leydig & Sertoli cells↓		XX TD	fam, pat	178-kb dup. 600-kb upstream of *SOX9*, gene desert region/ autosomal dominant sex-limited inheritance
48		Adult			XX TD	fam, pat	
49		Adult	Azo, normal virilization		XX TD	fam, pat	

Table 2. Continued

Case	Year [ref]	Age (yr)	Phenotype	Hormone	Diagnosis	Inheritance	Tested/proposed candidate gene(s)
50	2011 [17]	47	Azo, hypotrophic testes, germinal cell aplasia, mild gynecomastia	FSH↑, LH↑, T↓	XX TD	fam, pat	96-kb triplication 500-kb upstream of *SOX9* / *cis*-acting regulatory elements located within the smaller XX-sex reversal critical region; dup. increase *SOX9* expression driving testicular differentiation in *SRY*(–)
51		46	Azo, hypotrophic testes, germinal cell aplasia, mild gynecomastia	FSH↑, LH↑, T↓	XX TD	fam, pat	
52	2011 [18]	Infant	1.3-cm penis, hypospadias, bifid scrotum, male external genitalia, palpable gonads, bilateral fallopian tubes	FSH↑, LH↑, T↑	OTD		*SOX9*; minimum 78-kb dup located in gene desert region 517-595-kb upstream of the *SOX9* promoter/gonad specific *SOX9* transcriptional enhancer(s), the gain or loss of this region may act as a sex-determination switch in a tissue specific manner
53		Infant	Perineal hypospadias, asymmetric scrotum, L. scrotal with an ovarian remnant & fallopian tubes, surgery of a vagina & uterus		OTD	fam, mat	
54		Infant	Perineal hypospadias, 2.5-cm curved penis, hypoplastic & asymmetric scrotum, R. palpable gonad, vaginal pouch & uterus	FSH↑	OTD	fam, pat	
55-77	2012 [15]		The 23 subjects had range in the extent range of masculinization of the external genitalia		XX TD		*SOX9*-17q23.1-q24.3; no dup/ dup of *SOX9* is not a common cause, microduplication or rearrangement of the *SOX3* locus is a more common cause of 46,XX testicular & 46,XX ovotesticular DSD
78-84			Small penis, penoscrotal/perineal hypospadias, bilateral ovotestes		OTD		
85	2013 [21]	52	Azo, small testes, glandular hypospadia, virilization, height 160.3 cm	FSH↑, T↓	XX TD		Among *FGF9, WT1, NR5A1, SPRY2*, dup of *FGF9* & increase of *SPRY2* gene copy number/ may hinder *WNT4* expression and delaying of ovarian development in XX testicular DSD
86	2013 [28]	40	3.6-cm penis, Azo, testes (2, 2.5 mL), male pubic hair (Tanner stage 4), hypergonadotropic hypogonadism	Normal T, E_2, FSH↓,	XX TD		/Review, management guidance
87	2013 [19]	27	Azo, small testes, correction of congenital hypospadias	FSH↑, LH↑, T↓	XX TD		*DAX1, SOX9, RSPO1*; ~74 kb dup upstream of *SOX9* without mutation/ ~67 kb critical region of may lead to *SOX9* overexpression, causing female-to-male sex reversal
	2013 [22]		Genital ambiguity, hypospadias, bilateral cryptorchidism (two cases of Kojima et al. [11])				11 ↑expressed genes (*ROCK1, PQBP1, UCP2, OR13G1, ZNFX1, MPHOSPH8, GNRHR2, YIPF6, HSP90AB2P, KIF27*, and *YIF1B*) & 7 ↓expression genes (*EEF1A1, FTH1, UBB, RPS25, RPL7a, RPL6*, and *RPL32*); ↑expression of *ROCK1* in XX male, *ROCK1* phosphorylates & activates *SOX9* in Sertoli cells/ Testes formation by an alternative signaling pathway & *ROCK1*
	2014 [23]		Four cases of Kojima et al. [11] and Mizuno et al. [22]				High *SOX3* gene expression (Xq27.1) leads to testicular differentiation despite *SRY*(–)
88	2014 [20]	4	No gross anomalies, small testes without ambiguity, height 98.2 cm (10th-25th)	n LH, FSH, E_2, 17-OHP, hCG,	XX TD		Copy number dup of *SOX9* (1.44-1.45:1)
89	2014 [29]	14	Congenital scrotal type hypospadias, gynecomastia IA, Azo, small penis and testes, height 155 cm (age mean 165.9±7.21 yr)	nT, E_2, PRL, FSH↑, LH↑,	XX TD		No mutation of *DAX1, SOX9, SOX3, SOX10, ROCK1*, and *DMRT*, and no copy number variation in whole genome

Table 2. Continued

Case	Year [ref]	Age (yr)	Phenotype	Hormone	Diagnosis	Inheritance	Tested/proposed candidate gene(s)
90	2015 [current study]	37	Azo, testes (2,– mL), [E, VD, V R(–)], height 165 cm	E_2↑, SHBG, T↓	XX TD		The current study
91		42	Azo, testes (2, 2 mL), [E, VD, V R(–), L(–)], height 156 cm	FSH↑, LH↑, T↓	XX TD		
92		36	Azo, testes (2, 2 mL), [E, VD, V R(–), L(–)], height 172 cm	FSH↑, LH↑, T↓, PRL↓	XX TD		

DSD, disorders of sex development; ref, reference number; TD, testicular DSD; OTD, ovotesticular DSD; fam, familial; sib, sibling; FSH, follicle stimulating hormone; LH, luteinizing hormone; R, right; L, left; T, testosterone; chr, chromosome; STRs, short tandem repeats; PPK, palmoplantar keratoderma; TSH, thyroid-stimulating hormone; mat, maternal; SCC, squamous cell carcinoma; Azo, azoospermia; dup, duplication; hCG, human chorionic gonadotropin; E_2, 17β-estradiol; 17-OHP, 17-hydroxyprogesterone; pat, paternal; PRL, prolactin; SHBG, sex hormone-binding globulin; E, epididymis; V, varicocele; VD, vas deferens; (+), present; (–), absent; (↓), decreased; (↑), increased.

genes also have roles in the development of testis, the testis-androgen-genital tract to masculinization, anti-Müllerian duct regression, and the duct systems of external genitalia. In addition, *RSPO1* and *WNT4* function in gonads and have roles in ovary development, the duct system including fallopian tubes, the uterus, and external genitalia without the *SRY* gene [30,31]. These findings strongly indicate that DSDs with sex reversal involve the balanced regulation of genes in addition to the critical *SRY* gene, in the cascade of events that lead to development and sex differentiation.

McElreavey et al. [5] proposed the concept of a *Z* gene product that is a negative regulator of male sex determination. Therefore, homozygosity for loss-of-function and heterozygous mutants of *Z* alleles induce male sex reversal from *SRY*-negative complete masculinization to ovotesticular DSD.

Several familial studies have demonstrated that the varying clinical presentation of sexual development such as ovotesticular DSD or XX testicular DSD, may be a result of incomplete penetrance of paternal and maternal inheritance, while dosage-sensitive sex reversal may be a result of autosomal or X-linked genes in the sex-determining cascade [6,7,32].

DAX1 (also known as *NROB1*), an 'anti-testis' factor located on chromosome Xp21 that when duplicated was found to cause XY sex reversal. It has a role in ovarian development and/or in testis formation dependent on dosage-sensitivity during sex determination or sex differentiation pathways in mammals [32,33]. It was found that *Dax1* deficiency caused varying degrees of sex reversal in mice [34,35]. In humans, it was found that duplication of the Xp21.3 region containing the *DAX1* gene causes testicular regression in the presence of *SRY*, while the deletion of this region had no effect on male sex determination [36].

Haploinsufficiency of *SOX9* mutations have been proposed to cause sex reversal in patients with XY sex reversal, skeletal malformation syndrome, and campomelic dysplasia [37,38]. Huang et al. [8] reported that XX sex reversal was with campomelic dysplasia is caused by duplication of the *SOX9* gene, which is located on chromosome 17q24.3. Vernole et al. [9] reported XX testicular DSD with Huriez syndrome and palmoplantar keratoderma, and suggested that a gene related to sex determination that cause Huriez syndrome or tylosis, may be located at the *SOX9* locus. Radi et al. [10] reported that male sex reversal with palmoplantar keratoderma/squamous cell carcinoma that was paternally inherited in the same family, would be homozygous for a single gene mutation or contiguous genes. Moreover, increased expression of *SOX9* and reduced expression of *Ad4BP/SF-1* (also known as *NR5A1*), *DAX1* and anti-Müllerian hormone (AMH, also known as Müllerian inhibiting substance) in *SRY*-negative XX males, indicates that *SOX9* has a key role in sex determination in *SRY*-negative XX males, and that *Ad4BP/SF-1*, *DAX1* and AMH may contribute to their clinical features [11].

In contrast, Rajender et al. [12] found that there was no evidence of a relationship between *SOX9*, duplication of 22q, and *DAX1* in complete XX testicular DSD without *SRY*. Temel et al. [13] reported that *SOX9* was not duplicated, and no common *SOX9* haplotype was shared in nine familial cases with the absence of *SRY*, and a mutation at the *SOX9* locus; the authors proposed that *SRY*-negative XX testicular DSD may be caused by a monogenic impairment. Maciel-Guerra et al. [14] found no mutations in the *SOX9* and *DAX1* genes in case with *SRY*-negative XX maleness (testicular DSD) and monozygotic twins with XX ovotesticular DSD, but showed varying expression of incomplete penetrance of either an autosomal or a X-linked mutation. Seeherunvong et al. [15] investigated 30 subjects

without *SRY*-negative XX testicular or ovotesticular DSD and did not detect any mutations or duplications in the region of chromosome 17q that contains the *SOX9* gene using three short tandem repeat (STR) markers.

However, specific critical regions within the *SOX9* gene have been recently identified using microarray techniques. Cox et al. [16] found that three family members with 46,XX testicular DSD have a 178-kb microduplication of a gene desert region located 600-kb upstream of *SOX9* using SNP-microarray. Vetro et al. [17] found that two brothers who had the same paternal haplotype at the *SOX9* region had a 96-kb triplication of a region 500-kb upstream of *SOX9* at chromosome 17q24.3 using oligonucleotide array-comparative genomic hybridization. Benko et al. [18] proposed that a 78-kb minimal non-coding region found in a gene desert 517- to 595-kb upstream of the *SOX9* promoter included regions suggested by Cox et al. [16] and Vetro et al. [17], and that this region may have one or more gonad-specific *SOX9* transcriptional enhancers to induce activation or inactivation of *SOX9* gonadal expression in a tissue-specific manner. Xiao et al. [19] narrowed the candidate region to a 74-kb duplication in a 510- to 584-kb region upstream of *SOX9* in *SRY*-negative 46,XX testicular DSD, and proposed based on previous studies, that the candidate region related to gonadal development is a 67-kb region located 584- to 517-kb upstream of *SOX9*. Recently, there was a report of a *SOX9* duplication (~1.45 times) found in a Korean boy with XX testicular DSD [20].

Chiang et al. [21] investigated the *FGF9*, *WT1*, *NR5A1*, and *SPRY2* genes in cases with *SRY*-negative 46,XX testicular DSD and found that *FGF9* (located at chromosome 13q12.11) copy number was duplicated compared to that found in normal female controls and was significantly lower that of the normal male controls. Mizuno et al. [22] found that Leydig and Sertoli germ cells have increased expression levels of the Rho-associated, coiled-coil protein kinase 1 (ROCK1) protein, and proposed that testis formation may be regulated by an alternative ROCK1 signaling pathway.

Similar to *SOX9*, gain of function of the *SOX3* (SRY-box 3) gene showed it may be regulated by *SRY* in sex determination of transgenic mice, and in patients with XX male sex reversal [39]. More recently, Mizuno et al. [23] found copy number gain in the upstream region of the *SOX3* gene at chromosome Xq27.1.

A mutation in the *RSPO1* (R-spondin 1) gene in patients with 46,XX male sex reversal in an Italian family has demonstrated that sex reversal and palmoplantar hyperkeratosis/squamous cell carcinoma are regulated under via RSPO1 stimulation of keratinocytes and a reduction of β-catenin in the affected keratinocytes [40].

At other autosomal loci, deletion of the short arm of chromosome 11 led to the suggestion that the *WT1* (Wilms tumor 1) gene has a role in testis determination [41], while the *SF-1* gene has roles in primary adrenal failure and 46,XY gonadal dysgenesis [42]. Aleck et al. [24] reported a partial duplication of 22q13.1 (*SOX10*) in ovotesticular DSD without *SRY*. Jiménez et al. [43] proposed 'vanishing mosaicism' with 46,XX ovotesticular DSD without *SRY*; partial deletion of the *SRY* gene forms a testicular structure, and that an inactivated X chromosome carrying the *SRY* gene results in the development of ovarian tissue. Mustafa and Mehmet [25] reported an *SRY*-negative man with complete masculinization and autoimmune thyroiditis, but did not test for any specific genes.

In summary, we report three rare *SRY*-negative 46,XX testicular DSD cases with complete masculinization and provide a literature review on XX testicular DSD. Although *SRY* has a master role in sex determination and differentiation, there is a tightly coordinated expression between related genes under *SRY* for sex development from bipotential gonads. Therefore, the development of maleness in the absence of the *SRY* gene may be a result of a disruption of this balanced gene expression and gene mutation. Over the past two decades, several genes such as *SOX9*, *SOX3*, *DAX1*, and *RSPO1*, and chromosomal regions have been reported as potentially critical candidate genes for sex reversal. However, a more comprehensive understanding of other genes that are involved in the underlying networking mechanisms of the sex-determination cascade is necessary.

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