# Identification of an *SRY*-Negative 46,XX Infertility Male with a Heterozygous Deletion Downstream of *SOX3* Gene

**Shengfang Qin** ( qinshengfang@126.com )
Chengdu Medical College https://orcid.org/0000-0001-9391-890X
**Xueyan Wang**
Chengdu Medical College
**Jin Wang**
Chengdu Medical College

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# Abstract

**Background:** A male individual with a non-chimeric karyotype of 46,XX is very rare. We explored the genetic aetiology of an infertility male with 46,XX and *SRY* negative.

**Methods:** The peripheral blood sample was collected from the patient and subjected to a range of genetic testing, including conventional chromosomal karyotyping, short tandem repeat (STR) analysis for chromosome 13, 18, 21, X, Y contained *SRY* gene, azoospermia factor (*AZF*) deletion analysis including *SRY* gene, fluorescence in situ hybridization (FISH) with specific probes for CSP X/CSP Y/SRY, chromosomal microarray analysis (CMA) for genomic copy number variations (CNVs), and whole-genome analysis(WGA) for SNV&InDel variants, and the X chromosome inactivation (XCI) analysis for *AR* gene.

**Results:** The patient was found to have a 46,XX karyotype. Neither *AZF*a+b+c nor *SRY* band was detected in the electrophoresis result. FISH results of both interphase cells with CSPX/CSPY probe and metaphase cells with CSPX/CSPY/SRY probe showed two green fluorescence signals at the centromeres of X chromosomes, but no Y chromosome and *SRY* fluorescence signal. QF-PCR results showed that the patient had only the *AMELX* fluorescence peak of the X chromosome but no *AMELY* and *SRY* fluorescence peak. All results of the Karyotype, FISH, and STR did not suggest limited Y chimerism. CMA showed he had a heterozygous deletion of about 867 kb in Xq27.1 (hg19: chrX: 138,612,879-139,480,163 bp), located at 104 kb downstream of *SOX3* gene, including *F9*, *CXorf66*, *MCF2* and *ATP11C*; Meanwhile, whole-genome sequencing also found no SNV&InDel mutation associated with abnormal sex development. 75% X chromosome inactivation was detected.

**Conclusions:** Although the pathogenicity of 46,XX male patients with *SRY* negative remains unclear, *SOX3* expression of the acquired function may be associated with partial testis differentiation. Therefore, copy number variation of *SOX3* gene and regulatory region should be performed routinely for these patients.


# Background

XX male sex Reversal (46,XX sex Reversal, SRXX) is a disease caused by the abnormalities of ovarian development, characterized by 46,XX karyotype and male phenotype, which is often referred to as 46,XX male[1]. The incidence rate in male newborns is about 1/20,000[2]. It has been reported that over 90% of 46,XX male genomes contain *SRY* gene that is 46,XX(*SRY*+) male, which is often caused by the translocation of Y chromosome fragment containing *SRY* gene to X chromosome[3-7]. Among the remaining 10%, a very few are 46,XX males without *SRY* gene, namely 46,XX(*SRY*-) males exclusive of the Y chromosome chimerism[8,9].

In previous reports, the causes of 46,XX(*SRY*-) male mainly include dose change or mutation of *SOX9* gene[9-12], loss of function mutation of ovarian stimulating gene *WNT4* or *RSPO1* gene[13,14], heterozygous mutation of *NR5A1* gene[15-17], recently there are also a few reports about the copy number variation(CNV) of *SOX3* (*SRY*-box transcription factor 3) gene[18]. Here, we will proceed with the

molecular genetic identification of a 46,XX(*SRY*-) male patient admitted to our clinic, and the research results will enrich the theoretical knowledge and guide the clinical treatment of this kind of patient.

## Methods

## Subject

A 31-years-old patient, heigh 166cm and weigh 52.5kg, went to our clinic due to primary infertility. Physical examination showed a male appearance, a thin beard, Adam's apple, two broad bean-size of testicles and an average size of the penis. No sperm was found in routine semen examination. Hormone test results were follow: Testosterone: 1.75ng/mL(reference value range(RVR): 2.80-8.00), progesterone: 0.11ng/mL(RVR: 0.20-1.40), prolactin: 208.44uIU/mL(RVR: 86.00-324.00), estradiol: 5.00pg/mL(RVR:27.10-52.20), luteinizing hormone: 29.32mIU/mL(RVR: 1.70-8.60), follicle-stimulating hormone 37.88mIU/mL(RVR: 1.50-12.40). Deny genetic history. The parents of the patient refused the karyotype analysis.

## Specimen preparation and DNA extraction

5mL of venous blood was collected from patients with heparin sodium and EDTA-$Na_2$ anticoagulant tubes, respectively, and ready for use. According to the manufacturer's protocols, genomic DNA was extracted from EDTA-$Na_2$ anticoagulated blood using QIAamp DNA Mini Kit (QIAGEN Company). DNA was qualified when the concentration was more than 30ng/uL, the OD260/280 value between 1.8 to 2.0 determined by ultraviolet spectrophotometer Nanodrop 1C (Thermo Fisher Scientific).

## Chromosome karyotype analysis

Lymphocytes of heparin sodium anticoagulated blood were cultured, harvested, and prepared for microscope slides before Giemsa staining according to conventional cell culture methods. Zeiss karyotype analysis system (Karl Zeiss,Germany) was adopted for chromosome count and karyotype analysis, the same as previous studies[19].

### AZF **detection**

Multiplex amplified was performed with *AZF* detection kit (Yaneng corp.), then 2.0% agarose electrophoresis and imaging, according to manufacturer's instructions.

## QF-PCR detection

Multiplex PCR amplification was performed with Devyser compact v3 kit (Devyser AB, Sweden). The amplification condition was 95℃ for 15 min; 94℃ 30 sec, 58℃ 1 min 30 sec, 72℃ 1 min 30 sec, 27 cycles;72 ℃ for 30 min. The amplified products were subjected to capillary electrophoresis with AB 3500Dx gene analyzer, and the electrophoresis data were analyzed by GeneMapper software. The fluorescence peak of AMELX in Xp22.2, AMELY in Yp11.2 and *SRY* in Yp11.31 were used to evaluate the patient's gender. The experimental method was referred to in the previous report[20].

# FISH analysis

Lymphocytes in EDTA-Na2 anticoagulant blood were isolated by lymphocyte separation solution and hybridized by CSP18/CSP X/CSPY probe (Jin Pujia corp.), as the same method as previously reported[21]. Meanwhile, the metaphase cells harvested from lymphocyte culture were co-hybridized with CSPX/CSPY probe (Jinpuga Company) and *SRY* probe (Abbott Company). The MIX-1 was prepared by *SRY* hybridization buffer and *SRY* probe at a ratio of 9:1, and the MIX-2 was made with CSP hybridization buffer and CSP X/CSP Y centromeric probe at a ratio of 4:1, and then added the MIX-1 and MIX-2 to the metaphase cells loaded on the glass slide. The Glass slide was denaturated at 78℃ for 10 minutes and hybridized at 42℃ for more than 16 hours. Refer to reagent instruction for the experimental operation. A fluorescence microscope observed the fluorescence signal of hybridization.

# CMA analysis

500-1000ng of patient DNA and the equivalent amount of reference DNA was taken for simultaneous experiments. After digestion, the labelled patient sample was mixed with the reference sample and co-hybridized to SurePrint G3 CGH+SNP (180K) chip. Agilent DNA Microarray Scanner was used to scan the fluorescence signals after the slides were washed. Agilent Feature Extraction Software extracted the data from the images(.tif) and converted it to log-ratios data. Agilent CytoGenomics software was used to analyze CNV. Agilent Technologies provide the reagents, chips, instruments and analytical software. Refer to the instructions for specific methods. CMA analysis mainly adopts some online databases such as OMIM (https://omim.org/), DGV (http://dgv.tcag.ca/dgv/), Decipher (https://decipher.sanger.ac.uk/), ClinGen (https://wwW.clinicalgenome.org/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/).

# WGA analysis

Illumina HiSeq PE150 high-throughput dual-terminal sequencing was performed after random interruption into tiny fragments of DNA, terminal repair, phosphorylation, a-tail addition, connector and library construction. Quality control was carried out on the raw sequencing data to obtain high-quality clean data; Then, fastp software[22] was used for comparative analysis of clean data and human reference genome sequence, and data such as sequencing depth and coverage of the target region were counted and obtained Bam files. Finally, SNP/InDel was detected and annotated based on Bam files to obtain all mutation information. The Haplotyper tool of Sentieo software[23] was used to detect SNP and InDel mutation, and ANNOVAR software[24] was used to annotate the mutation results accompanied by multiple databases (such as dbSNP, 1000G, ESP6500, HGMD, OMIM). Meanwhile, CNVkit software was used to analyze CNV[25].

# XCI detection

The sample DNA of undigested and digested by HpaII, which methylation-sensitive restriction enzyme, was amplified by androgen receptor (*AR*) gene-specific primers and capillary electrophoresis subsequently. *MIC2* was used as the reference gene, and the Hpa II enzyme was digested overnight in a 37℃ water bath. The samples were amplified by double PCR before and after enzyme digestion. As

reported in the literature[26], FAM fluorescein was added to the 5 'end of the forward primer[27]. PCR reaction conditions followed: 95℃ for 5min; 28 cycles of 95℃ for 45sec, 58℃ for 30sec, 72℃ for 30sec; 72℃ for 7min. PCR products were subjected to capillary electrophoresis. XCI ratio was calculated according to formula (d1/u1)/(d1/u1 + d2/u2), and >70% was determined as XCI bias[28,29].

# Results

### Cytogenetic analysis

The patient's karyotype was 46,XX, as shown in Figure.

## *AZF*

Electrophoresis results of the amplification products of patients showed no *SRY* bands nor corresponding bands of *AZF*a (sY84, sY86), *AZF*b (sY127, sY134), and *AZF*c (sY254, sY255) regions.

QF-PCR

The gender site of the patient was *AMELX* but not *AMELY*, and there was no fluorescence peak at the *SRY* site, as shown in Figure 2.

### FISH analysis

FISH results of interphase cells hybridized by the centromere probes of 18, X and Y, showed two 18 and two X fluorescence signals but no Y signal, as shown in Figure 3A. FISH results of metaphase cells hybridized with X, Y, and *SRY* probes showed two green signals of X chromosome centromere, but neither of *SRY* nor Y chromosome, as shown in Figure 3b.

### Results of Y chromosome chimerism

No Y chimerism was detected by karyotype analysis, CSPY analysis in FISH, and *AMELY* and *SRY* analysis in QF-PCR.

### CMA

Taking 46,XX normal females as a reference, the patient's Xq27.1 (hg19: chrX: 138,612,879-139,480,163 bp) had about 867Kb of heterozygotic deletion, and the deletion region was located at 104Kb downstream of the *SOX3* gene, including *F9*, *CXorf66*, *MCF2*, *ATP11C* four protein-coding genes, as shown in Figure 4a and 4b.

### WGA

The WGA results showed no pathogenic or likely pathogenic SNV&InDel variant related to sexual development, which can clearly explain the patient phenotype; meanwhile, the results also showed about 892kb heterozygous deletion (hg19: chrX: 138,609,392-139,501,392) in Xq27.1.

### XCI

The XCI ratio of patients was about 75%, which was non-random inactivation. See Figure 5.

### Comparison of the clinical phenotypes of 46,XX *SRY*-negative male patients with CNV of *SOX3*

Table 1 summarises the clinical features of 46,XX males with *SRY*-negative individuals involved in the CNV of *SOX3*. The patients all had the typical male appearance and showed common abnormal phenotypes, including spermatogenous testicular dysplasia because they were absent from the entire Y chromosome. Among the seven patients, five patients(case 1, 3, 4, 5, 6) had microduplications spanning the entire *SOX3* gene, another two patients, including the case 2 and our patient, had microdeletions near the *SOX3* gene, which were speculated to play a regulatory role for *SOX3* expression. Our patient showed a CNV near the *SOX3* gene in CMA, and WGA excluded no other SNV&InDel mutations associated with sex development. Meanwhile, he had a skewed X chromosome inactivation, which was not inconsistent with the case 1.

## Discussion

*SRY* gene is recognized as the best TDF candidate gene. As long as the *SRY* gene exists in the individual genome, male gonadal development will occur even without the Y chromosome, the primary mechanism of 46,XX(*SRY*+) male pathogenesis. Therefore, Y chromosome chimerism in 46,XX(*SRY*-) males should be excluded first. This study detected no Y chromosome chimerism in the patient's peripheral blood through several experimental analysis methods. Of course, it was impossible to rule out completely the gonad limited chimerism due to the inability to obtain the gonad tissue. Other gene mutations may cause the pathogenesis of 46,XX(*SRY*-) males in the testicular development pathway, which makes the gonads of individuals without the *SRY* gene develop towards the testis[4,5].

Some 46,XX(*SRY*-) male individuals were previously reported to be caused by abnormalities of the *SOX3* gene[9] and found the repetition of the *SOX3* regulatory region can up-regulate SOX9 gene expression[30]. In our patient, There is a heterozygous deletion near the *SOX3* gene in Xq27.1, which may be related to its pathogenesis.

*SOX3* gene is one of 20 SOX(*SRY*-related HMG-box) gene family members. Stevanovic et al. cloned the *SOX3* gene and identified its location at Xq27.1 in 1993[31]. *SOX3* gene consists of a single exon and contains an HMG box, encoding 446 amino acids of a transcription factor SOX-3 protein[32]. *SOX3* gene sequence is highly conserved among different species and has high homology with *SRY* and other SOX family genes. *SOX3* gene is the ancestral gene of the *SRY* gene[9,33]. *SOX3* encodes a transcription factor expressed in the central nervous system of vertebrate embryos[32], which plays a vital role in the pituitary, craniofacial and adrenal development. The variation of this gene is associated with X-linked mental retardation, growth hormone deficiency, X-linked hypothyroidism, 46,XX male sex reversal, and other diseases[9,34-37].

Loss-of-function of *SOX3* induced by mutation does not cause sex determination abnormality in mice and humans[38]. However, studies in transgenic mice have shown that in-situ expression of *SOX3* in bipotent gonads leads to up-regulation of Sox9 expression, leading to testicular induction and XX male sex reversal. The mechanism of *SOX3* rearrangement causes sex reversals, and the frequency of sex development disorders remain unknown. The study of Moalem S et al. provided evidence that the de novo duplication of *SOX3* in XX bipotent gonads causes the acquisition of *SOX3* function[39]. In XX mice lacking the *SRY* gene, it was found that the expression of the *SOX3* gene with the acquired function was related to partial differentiation of testis. Overexpression of *SOX3*, synergistically expression with SF1, up-regulated SOX9 stimulated gonad development into testis in XX mice[9,40]. *SRY* gene was derived from a new mutation in the regulatory region of the *SOX3* gene and expressed in the early gonad. The data of transgenic mice indicated that *SOX3* and *SRY* were interchangeable in sex determination function.

The type of variation in *SOX3* that has been associated with 46,XX male sex reversal disorders is copy number variation of *SOX3* (CNV) in previous reports. To date, 6 cases of *SOX3* CNV related 46,XX male patients have been reported. In some cases, CNV duplication, including the *SOX3* gene[39,41,42], resulted in the change of gene product dose. Some CNVS do not contain the *SOX3* gene but are adjacent to the *SOX3* gene region, or the breaking point of CNV falls in the regulatory region of *SOX3*, so it is speculated that CNV has a positional effect on *SOX3* gene expression[9,43]. The elemental clinical manifestations of the patient include sexual reversal and gonadal dysplasia, azoospermia and infertility(adult individual), which may be combined with other abnormal clinical phenotypes if other genes are present. CMA showed that our patient had a heterozygous deletion of about 867kb in Xq27.1 (hg19: chrX: 138,612,879-139,480,163 bp), which was located at 104kb downstream of the *SOX3* gene, including F9, CXorf66, MCF2 and ATP11C; Meanwhile, whole-genome sequencing also found an 892kb heterozygosity deletion in Xq27.1 (hg19: chrX: 138,609,392-139,501,392), but no SNV&InDel mutation associated with abnormal sex development. This patient is the first report of whole-genome sequencing in 46,XX male patients, and the use of WGA can entirely exclude pathogenic or likely pathogenic SNV&InDel variants in genes associated with the patient's abnormal sexual phenotype. About this deletion area, no similar report was found in the DGV database of the normal population, and no sexual reversal phenotype was reported in Decipher and ClinVar databases. Similar to previous reports[9,41], we speculated that the deletion region might involve the regulation region of the *SOX3* gene, leading to differentiation and development of male testis through weakening inhibition of *SOX3* and increasing expression *SOX3*[9,42].

Since the deletion of the patient in this study occurred on the X chromosome, the inactivation of the X chromosome is also an essential factor affecting the clinical significance. XCI is a dose-compensation mechanism, which usually occurs in early embryonic development when one of the two X chromosomes in a woman is inactivated, resulting in only one paternal or maternal chromosome being expressed in each cell of the female individual. In general, XCI is random, i.e., the ratio of the two X chromosomes inactivated in females is 50%: 50%[44]. However, if the inactivation is not random, it is called skewed inactivation. In 46,XX (*SRY*+) males, some studies[27,45] showed a high degree of XCI bias (greater than 90%), some were random XCI in other patients[46]. The phenotype of 46,XX males (*SRY*+) differed with the variable inactivation of the X chromosome carrying the *SRY* gene[47,48]. However, XCI has not been

reported in 46,XX male(*SRY*-)patients, so we conducted the XCI analysis in our patient. The XCI test found a moderate XCI bias; about 75% of X chromosomes in the patient's peripheral blood were inactivated. We speculate that the expression of positive selection of the deficient X chromosomes results in the development of male gonads of the patient, similar to previous reports[49]. Since the sample of the patient's mother could not be collected, it is unknown whether the patient's X chromosome deletions are de novo or inherited from an unphenotypic mother (the XCI bias inactivates the abnormal X chromosome). The XCI ratio of gonads tissues may differ from peripheral blood[28], but it cannot be accurately known because the sample is inaccessible.

Similar to the two adults patients reported by Sutton E et al.[9], the main features of our patient are azoospermia and infertility[50] because of a deletion of the entire Y chromosome, namely deletion of *AZF* region, contained *USP9Y, DBY, PRY, RBMY, DAZ, BPY2* and other genes related to sperm production, development and maturation. *AZF* region is recognized as the most common factor of male infertility[51-53], which account for 10%-15% of azoospermia and 5%-10% of severe oligozoospermia[54].

At present, the pathogenesis of *SRY* positive 46,XX male patients is relatively straightforward. However, the molecular mechanism, signalling pathway and genetic regulation of *SRY* negative 46,XX male are not understood enough, and the diagnosis and treatment of these patients are still relatively complex. Sutton et al.[9] identified three arrangements including or adjacent to the *SOX3* gene, accounting for 19% (3/16) in 16 *SRY*-negative 46,XX male patients. Subsequently, several case reports indicated that *SOX3* is a critical pathogenic factor in *SRY* negative 46,XX male patients. Therefore, it is crucial to conduct the CNV determination involved in the *SOX3* gene in all *SRY* negative 46,XX male patients. It is noteworthy that the current and reported *SOX3* duplications or deletions are below the detection threshold of conventional karyotype and were found only by analyzing CNVs using CMA. Therefore, CMA detection is routinely recommended to detect CNVs, and high-throughput sequencing can simultaneously rule out other SNV/INDEL mutations. In addition, the XCI test in XX diseases also should be considered.

# Declarations


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### Authors' contributions

Shengfang Qin conceived and designed the study, interpreted the data and drafted the manuscript. Xueyang Wang enrolled the patients, performed genetic counseling and provided clinical information. Jin Wang performed the laboratory detection and experimental data acquisition.


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### Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

### Ethics approval and consent to participate

The research was approved by the Institutional Committee for the Protection of Human Subjects (Institutional Review Board of Sichuan Provincial Maternity and Child Health Care Hospital, 20201113-98), and the patient signed the informed consent.

### Consent for publication

The patient had provided his consent for publication.

### Competing interests

The authors declare that they have no competing interests.

## References

1. Lee PA, Houk CP, Ahmed SF, Hughes IA. Consensus statement on management of intersex disorders. International Consensus Conference on Intersex. Pediatrics. 2006;118(2):e488-500. https://doi.org/10.1542/peds.2006-0738

2. DELACHAPELLE A, HORTLING H, NIEMI M, WENNSTROEM J. XX SEX CHROMOSOMES IN A HUMAN MALE. FIRST CASE. Acta Med Scand. 1964;175:SUPPL 412:25-8. https://doi.org/10.1111/j.0954-6820.1964.tb04630.x.

3. Andersson M, Page DC, de la Chapelle A. Chromosome Y-specific DNA is transferred to the short arm of X chromosome in human XX males. Science. 1986;233(4765):786-8. https://doi.org/10.1126/science.3738510.

4. Ferguson-Smith MA, Cooke A, Affara NA, Boyd E, Tolmie JL. Genotype-phenotype correlations in XX males and their bearing on current theories of sex determination. Hum Genet. 1990;84(2):198-202. https://doi.org/10.1007/BF00208942.

5. Donlon TA, Müller U. Deletion mapping of DNA segments from the Y chromosome long arm and their analysis in an XX male. Genomics. 1991;10(1):51-6. https://doi.org/10.1016/0888-7543(91)90483-u.

6. Vorona E, Zitzmann M, Gromoll J, Schüring AN, Nieschlag E. Clinical, endocrinological, and epigenetic features of the 46,XX male syndrome, compared with 47,XXY Klinefelter patients. J Clin Endocrinol

Metab. 2007;92(9):3458-65. https://doi.org/10.1210/jc.2007-0447.

7. Ahmad A, Siddiqui MA, Goyal A, Wangnoo SK. Is 46XX karyotype always a female. BMJ Case Rep. 2012;2012. https://doi.org/10.1136/bcr-2012-006223.

8. Inoue H, Nomura M, Yanase T, Ichino I,Goto K, Ikuyama S, et al. A rare case of 46,XX true hermaphroditism with hidden mosaicism with sex-determining region Y chromosome-bearing cells in the gonads. Intern Med. 1998;37(5):467-71. https://doi.org/10.2169/internalmedicine.37.467.

9. Sutton E, Hughes J, White S, Sekido R, Tan J, Arboleda V, et al. Identification of SOX3 as an XX male sex reversal gene in mice and humans. J Clin Invest. 2011;121(1):328-41. https://doi.org/10.1172/JCI42580.

10. Cox JJ, Willatt L, Homfray T, Woods CG. A SOX9 duplication and familial 46,XX developmental testicular disorder. N Engl J Med. 2011;364(1):91-3. https://doi.org/10.1056/NEJMc1010311.

11. Kim GJ, Sock E, Buchberger A, Just W, Denzer F, Hoepffner W, et al. Copy number variation of two separate regulatory regions upstream of SOX9 causes isolated 46,XY or 46,XX disorder of sex development. J Med Genet. 2015;52(4):240-7. https://doi.org/10.1136/jmedgenet-2014-102864

12. Mengen E, Kayhan G, Kocaay P, Uçaktürk SA. A Duplication Upstream of SOX9 Associated with SRY Negative 46,XX Ovotesticular Disorder of Sex Development: A Case Report. J Clin Res Pediatr Endocrinol. 2020;12(3):308-14. https://doi.org/10.4274/jcrpe.galenos.2019.2019.0101

13. Parma P, Radi O, Vidal V, Chaboissier MC, Dellambra E, Valentini S, et al. R-spondin1 is essential in sex determination, skin differentiation and malignancy. Nat Genet. 2006;38(11):1304-9. https://doi.org/10.1038/ng1907

14. Lee GM, Ko JM, Shin CH, Yang SW. A Korean boy with 46,XX testicular disorder of sex development caused by SOX9 duplication. Ann Pediatr Endocrinol Metab. 2014;19(2):108-12. https://doi.org/10.6065/apem.2014.19.2.108

15. Mandel H, Shemer R, Borochowitz ZU, Okopnik M, Knopf C, Indelman M, et al. SERKAL syndrome: an autosomal-recessive disorder caused by a loss-of-function mutation in WNT4. Am J Hum Genet. 2008;82(1):39-47. https://doi.org/10.1016/j.ajhg.2007.08.005

16. Baetens D, Stoop H, Peelman F, Todeschini AL, Rosseel T, Coppieters F, et al. NR5A1 is a novel disease gene for 46,XX testicular and ovotesticular disorders of sex development. Genet Med. 2017;19(4):367-76. https://doi.org/10.1038/gim.2016.118

17. Igarashi M, Takasawa K, Hakoda A, Kanno J, Takada S, Miyado M, et al. Identical NR5A1 Missense Mutations in Two Unrelated 46,XX Individuals with Testicular Tissues. Hum Mutat. 2017;38(1):39-42. https://doi.org/10.1002/humu.23116

18. Bashamboo A, Donohoue PA, Vilain E, Rojo S, Calvel P, Seneviratne SN, et al. A recurrent p.Arg92Trp variant in steroidogenic factor-1 (NR5A1) can act as a molecular switch in human sex development. Hum Mol Genet. 2016;25(16):3446-53. https://doi.org/10.1093/hmg/ddw186.

19. Liu Y, Kong XD, Wu QH, Li G, Song L, Sun YP. Karyotype analysis in large-sample infertile couples living in Central China: a study of 14965 couples. J Assist Reprod Genet. 2013;30(4):547-53. https://doi.org/10.1007/s10815-013-9964-6.

20. Masoudzadeh N, Teimourian S. Comparison of quantitative fluorescent polymerase chain reaction and karyotype analysis for prenatal screening of chromosomal aneuploidies in 270 amniotic fluid samples. J Perinat Med. 2019;47(6):631-6. https://doi.org/10.1515/jpm-2019-0069.

21. Song Y, Chen Q, Zhang Z, Hou H, Zhang D, Shi Q. Effects of age on segregation of the X and Y chromosomes in cultured lymphocytes from Chinese men. J Genet Genomics. 2009;36(8):467-74. https://doi.org/10.1016/S1673-8527(08)60136-8.

22. Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018;34(17):i884-884i890. https://doi.org/10.1093/bioinformatics/bty560.

23. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25(14):1754-60. https://doi.org/10.1093/bioinformatics/btp324.

24. García-Alcalde F, Okonechnikov K, Carbonell J, Cruz LM, Götz S, Tarazona S, et al. Qualimap: evaluating next-generation sequencing alignment data. Bioinformatics. 2012;28(20):2678-9. https://doi.org/10.1093/bioinformatics/bts503.

25. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Ling Lin L, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res. 2012;22(3):568-76. https://doi.org/10.1101/gr.129684.111.

26. Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont JW. Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. Am J Hum Genet. 1992;51(6):1229-39.

27. Bouayed Abdelmoula N, Portnoi MF, Keskes L, Recan D, Bahloul A, Boudawara T, et al. Skewed X-chromosome inactivation pattern in SRY positive XX maleness: a case report and review of literature. Ann Genet. 2003;46(1):11-8. https://doi.org/10.1016/s0003-3995(03)00011-x.

28. Sharp A, Robinson D, Jacobs P. Age- and tissue-specific variation of X chromosome inactivation ratios in normal women. Hum Genet. 2000;107(4):343-9. https://doi.org/10.1007/s004390000382.

29. Minks J, Robinson WP, Brown CJ. A skewed view of X chromosome inactivation. J Clin Invest. 2008;118(1):20-3. https://doi.org/10.1172/JCI34470.

30. Mizuno K, Kojima Y, Kamisawa H, Moritoki Y, Nishio H, Nakane A, et al. Elucidation of distinctive genomic DNA structures in patients with 46,XX testicular disorders of sex development using genome wide analyses. J Urol. 2014;192(2):535-41. https://doi.org/10.1016/j.juro.2014.02.044.

31. Stevanović M, Lovell-Badge R, Collignon J, Goodfellow PN. SOX3 is an X-linked gene related to SRY. Hum Mol Genet. 1993;2(12):2013-8. https://doi.org/10.1093/hmg/2.12.2013.

32. Collignon J, Sockanathan S, Hacker A, Cohen-Tannoudji M, Norris D, Rastan S, et al. A comparison of the properties of Sox-3 with Sry and two related genes, Sox-1 and Sox-2. Development. 1996;122(2):509-20.

33. Foster JW, Graves JA. An SRY-related sequence on the marsupial X chromosome: implications for the evolution of the mammalian testis-determining gene. Proc Natl Acad Sci U S A. 1994;91(5):1927-31. https://doi.org/10.1073/pnas.91.5.1927.

34. Lagerström-Fermér M, Sundvall M, Johnsen E, Warne GL, Forrest SM, Zajac J D, et al. X-linked recessive panhypopituitarism associated with a regional duplication in Xq25-q26. Am J Hum Genet. 1997;60(4):910-6.

35. Laumonnier F, Ronce N, Hamel BC, Thomas P, Lespinasse J, Raynaud M, et al. Transcription factor SOX3 is involved in X-linked mental retardation with growth hormone deficiency. Am J Hum Genet. 2002;71(6):1450-5. https://doi.org/10.1086/344661.

36. Rizzoti K, Brunelli S, Carmignac D, Thomas PQ, Robinson IC, Lovell-Badge R. SOX3 is required during the formation of the hypothalamo-pituitary axis. Nat Genet. 2004;36(3):247-55. https://doi.org/10.1038/ng1309.

37. Solomon NM, Ross SA, Forrest SM, Thomas PQ, Morgan T, Belsky JL, et al. Array comparative genomic hybridisation analysis of boys with X-linked hypopituitarism identifies a 3.9 Mb duplicated critical region at Xq27 containing SOX3. J Med Genet. 2007;44(4):e75. https://doi.org/10.1136/jmg.2007.049049.

38. Laronda MM, Jameson JL. Sox3 functions in a cell-autonomous manner to regulate spermatogonial differentiation in mice. Endocrinology. 2011;152(4):1606-15. https://doi.org/10.1210/en.2010-1249.

39. Moalem S, Babul-Hirji R, Stavropolous DJ, Wherrett D, Bägli DJ, Thomas P, et al. XX male sex reversal with genital abnormalities associated with a de novo SOX3 gene duplication. Am J Med Genet A. 2012;158A(7):1759-64. https://doi.org/10.1002/ajmg.a.35390.

40. Igarashi M, Mikami H, Katsumi M, Miyado M, Izumi Y, Ogata T, et al. SOX3 Overdosage Permits Normal Sex Development in Females with Random X Inactivation. Sex Dev. 2015;9(3):125-9. https://doi.org/10.1159/000377653.

41. Grinspon RP, Nevado J, Mori Alvarez Mde L, Rey GD, Castera R, Venara M, et al. 46,XX ovotesticular DSD associated with a SOX3 gene duplication in a SRY-negative boy. Clin Endocrinol (Oxf). 2016;85(4):673-5. https://doi.org/10.1111/cen.13126.

42. Tasic V, Mitrotti A, Riepe FG, Kulle AE, Laban N, Polenakovic M, et al. Duplication of The SOX3 Gene in an Sry-negative 46,XX Male with Associated Congenital Anomalies of Kidneys and the Urinary Tract: Case Report and Review of the Literature. Balkan J Med Genet. 2019;22(1):81-8. https://doi.org/10.2478/bjmg-2019-0006.

43. Bowl MR, Nesbit MA, Harding B, Levy E, Jefferson A, Volpi E, et al. An interstitial deletion-insertion involving chromosomes 2p25.3 and Xq27.1, near SOX3, causes X-linked recessive hypoparathyroidism. J Clin Invest. 2005;115(10):2822-31. https://doi.org/10.1172/JCI24156.

44. Amos-Landgraf JM, Cottle A, Plenge RM, Friez M, Schwartz CE, Longshore J, et al. X chromosome-inactivation patterns of 1,005 phenotypically unaffected females. Am J Hum Genet. 2006;79(3):493-9. https://doi.org/10.1086/507565.

45. Kusz K, Kotecki M, Wojda A, Szarras-Czapnik M, Latos-Bielenska A, Warenik-Szymankiewicz A, et al. Incomplete masculinisation of XX subjects carrying the SRY gene on an inactive X chromosome. J Med Genet. 1999;36(6):452-6.

46. Gunes S, Asci R, Okten G, Atac F, Onat OE, Ogur G, et al. Two males with SRY-positive 46,XX testicular disorder of sex development. Syst Biol Reprod Med. 2013;59(1):42-7. https://doi.org/10.3109/19396368.2012.731624.

47. McElreavey K, Rappaport R, Vilain E, Abbas N, Richaud F, Lortat-Jacob S, et al. A minority of 46,XX true hermaphrodites are positive for the Y-DNA sequence including SRY. Hum Genet. 1992;90(1-2):121-5. https://doi.org/10.1007/BF00210754.

48. McElreavey K, Vilain E, Abbas N, Herskowitz I, Fellous M. A regulatory cascade hypothesis for mammalian sex determination: SRY represses a negative regulator of male development. Proc Natl Acad Sci U S A. 1993;90(8):3368-72. https://doi.org/10.1073/pnas.90.8.3368.

49. Fechner PY, Rosenberg C, Stetten G, Cargile CB, Pearson PL, Smith KD, et al. Nonrandom inactivation of the Y-bearing X chromosome in a 46,XX individual: evidence for the etiology of 46,XX true hermaphroditism. Cytogenet Cell Genet. 1994;66(1):22-6. https://doi.org/10.1159/000133656.

50. Vetro A, Ciccone R, Giorda R, Patricelli MG, Mina ED, Forlino A, et al. XX males SRY negative: a confirmed cause of infertility. J Med Genet. 2011;48(10):710-2. https://doi.org/10.1136/jmedgenet-2011-100036.

51. Tiepolo L, Zuffardi O. Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm. Hum Genet. 1976;34(2):119-24.

https://doi.org/10.1007/BF00278879.

52. Motovali-Bashi M, Rezaei Z, Dehghanian F, Rezaei H. Multiplex PCR based screening for micro/partial deletions in the AZF region of Y-chromosome in severe oligozoospermic and azoospermic infertile men in Iran. Iran J Reprod Med. 2015;13(9):563-70.

53. Colaco S, Modi D. Genetics of the human Y chromosome and its association with male infertility. Reprod Biol Endocrinol. 2018;16(1):14. https://doi.org/10.1186/s12958-018-0330-5.

54. Yu XW, Wei ZT, Jiang YT, Zhang SL. Y chromosome azoospermia factor region microdeletions and transmission characteristics in azoospermic and severe oligozoospermic patients. Int J Clin Exp Med. 2015;8(9):14634-46.

# Tables

Table 1 Comparison of the clinical phenotypes of 46,XX SRY-negative male patients with CNV of SOX3.

| Case | Reference | CNV | Clinical phenotype | Detection method | X chromosome inactivation |
|---|---|---|---|---|---|
| 1 | Sutton E *et al*. 2011(9) | The patient contained two microduplications of approximately 123 kb and 85 kb, the former of which spanned the entire *SOX3* gene. | The patient was an infertility male of 30-year-old. His height was 165 cm, and he weighed 64 kg, with no abnormal symptoms. Infertility was indicated by two spermograms, which confirmed azoospermia. The patient presented with small testicles and normal secondary sexual characteristics. | CMA | X-inactivation studies showed no evidence for skewed inactivation in DNA derived from lymphocytes |
| 2 | Sutton E *et al*. 2011(9) | The patient contained a single 343-kb microdeletion immediately upstream of *SOX3*; the coding sequence of *SOX3* is not affected.<br>It is suggested that altered regulation (and not increased dosage) of *SOX3* is the cause of XX male sex reversal. | The patient was a 35-year-old with gender dysphoria. Height was 167.5 cm, weight 73.5 kg, with no medical problems apart from ongoing gender identity issues. The external genitalia was typical male, apart from small, soft 6-ml testes. There was little body hair. Primary hypogonadism, with FSH and LH elevated, and testosterone low. Histological examination showed atrophic changes in the testes, with loss of normal spermatogenesis, thickening and hyalinization of the tubular basal lamina, and diminished interstitial cells. The patient was *SRY*-negative in both peripheral blood and testicular tissue. The *SOX3* rearrangement was not present in his mother. | CMA | NA |
| 3 | Sutton E *et al*. 2011(9) | The patient has a large (approximately 6-Mb) duplication that encompasses *SOX3* and at least 18 additional distally located genes, overexpression of which probably contributes to the phenotypic complexity. Notably, the proximal breakpoint falls within the *SOX3* regulatory region. | The patient was a boy of 19 months who presented with a more complex phenotype that also includes scrotal hypoplasia, microcephaly, small testis on one side, developmental delay(height 75.2 cm [<5th percentile]), and growth retardation. There were no significant problems during pregnancy or the newborn period. No endocrine evaluation or parental DNA was available. | CMA | NA |
| 4 | Moalem S *et al*. 2012(30) | The patient has a de novo 494 kb copy number gain in region Xq27.1 (NCBI 36/hg18: 139,354,859 – 139,848,664), containing the *SOX3* RP1-177G6.2, CDR1 and MIR320D2 genes. | The patient was a 46,XX male newborn with hypospadias. Ultrasound examination revealed normal testicular size and structure. | CMA | NA |
| 5 | Grinspon RP *et al*. 2016(31) | The patient has a de novo gain at Xq27.1. The duplicated region was around 0.5 Mb, and encompassed the *SOX3* gene (arr[hg19] Xq27.1(139,541,737-140,043,863)x3). Other genes included were RPS17P17, CDR1, MIR320D2. | The patient was a two years six months boy. He had a trophic phallus 32 mm long and 13 mm wide with ambiguous genitalia and bilateral ovotesticular DSD. | CMA | NA |
| 6 | Tasic V *et al*. 2019(32) | The patient has a 550 kb duplication at Xq27 (ChrX: 139,360,520-139,908,320), involving *SOX3*, the non-coding RNA LINC00632, AK054921, CDR1 and the miRNA MIR320D2. | The patient was an 11-year-old boy with right kidney hypoplasia and moderate coronal hypospadias. His testes volume was >4 mL, and the penis length was 5 cm. | CMA | NA |
| 7 | Present study | CMA found the patient contained a heterozygous deletion of about 867kb in Xq27.1 (138612879-139480163 bp), located at 104kb downstream of *SOX3* gene, including F9, CXorf66, MCF2 and ATP11C. Whole-genome sequencing also found an 892kb heterozygosity deletion in Xq27.1 (GRCH37: ChrX:138609392-139501392), but no | The patient was a 31-years-old primary infertility patient with two small testicular, his height was 166cm, and he weighed 52.5kg. Physical examination showed a male appearance, a little beard, Adam's apple, the testicles are the size of broad bean, the penis is average size. No sperm was found in routine semen examination. Hormone test results showed testosterone and estradiol were low, FSH and LH were high. The parental sample was unavailable. | CMA, WGA | The X chromosome inactivation ratio of patients was about 75%, which was non-random inactivation. |

SNV&InDel mutation associated with abnormal sex development.

# Figures

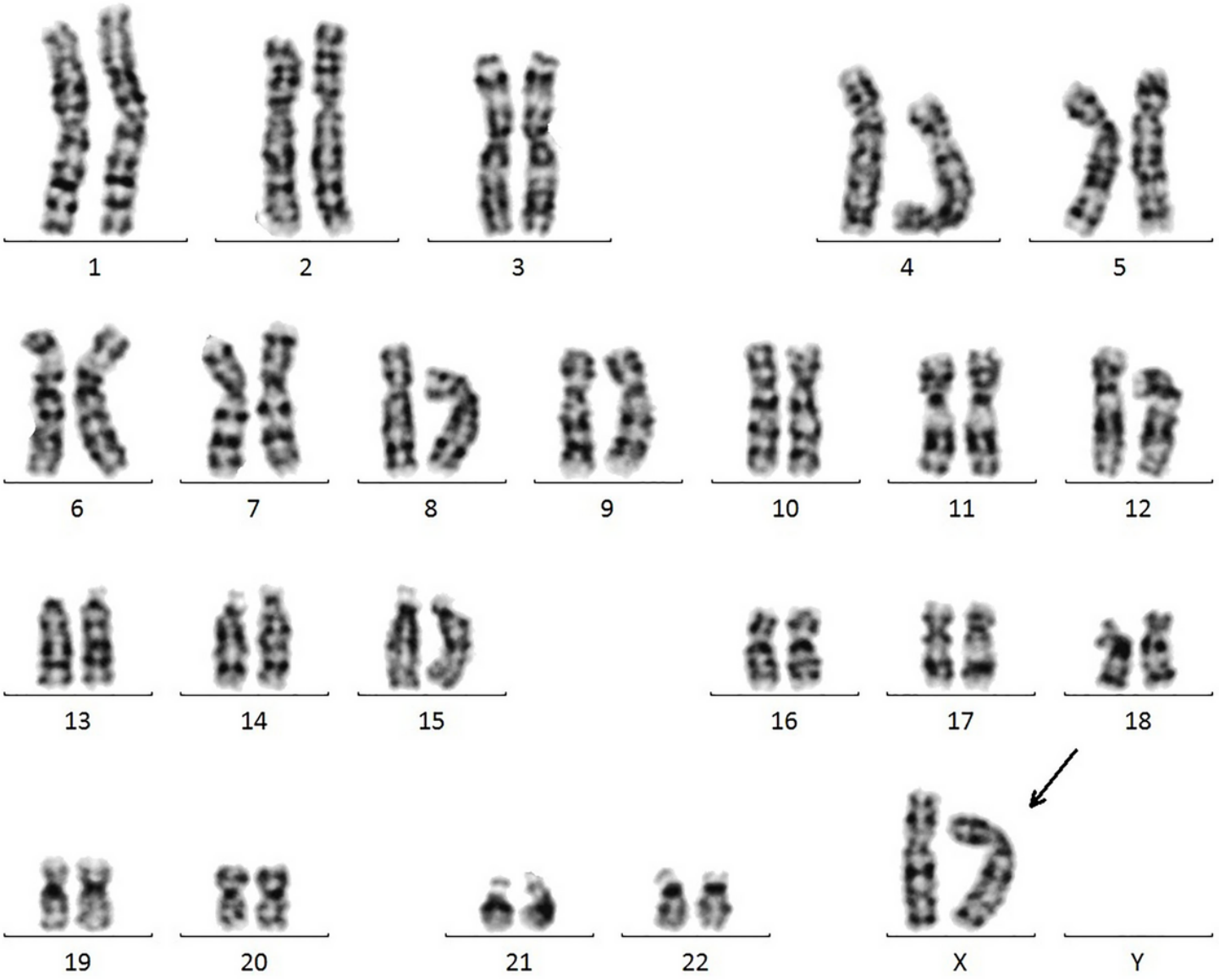
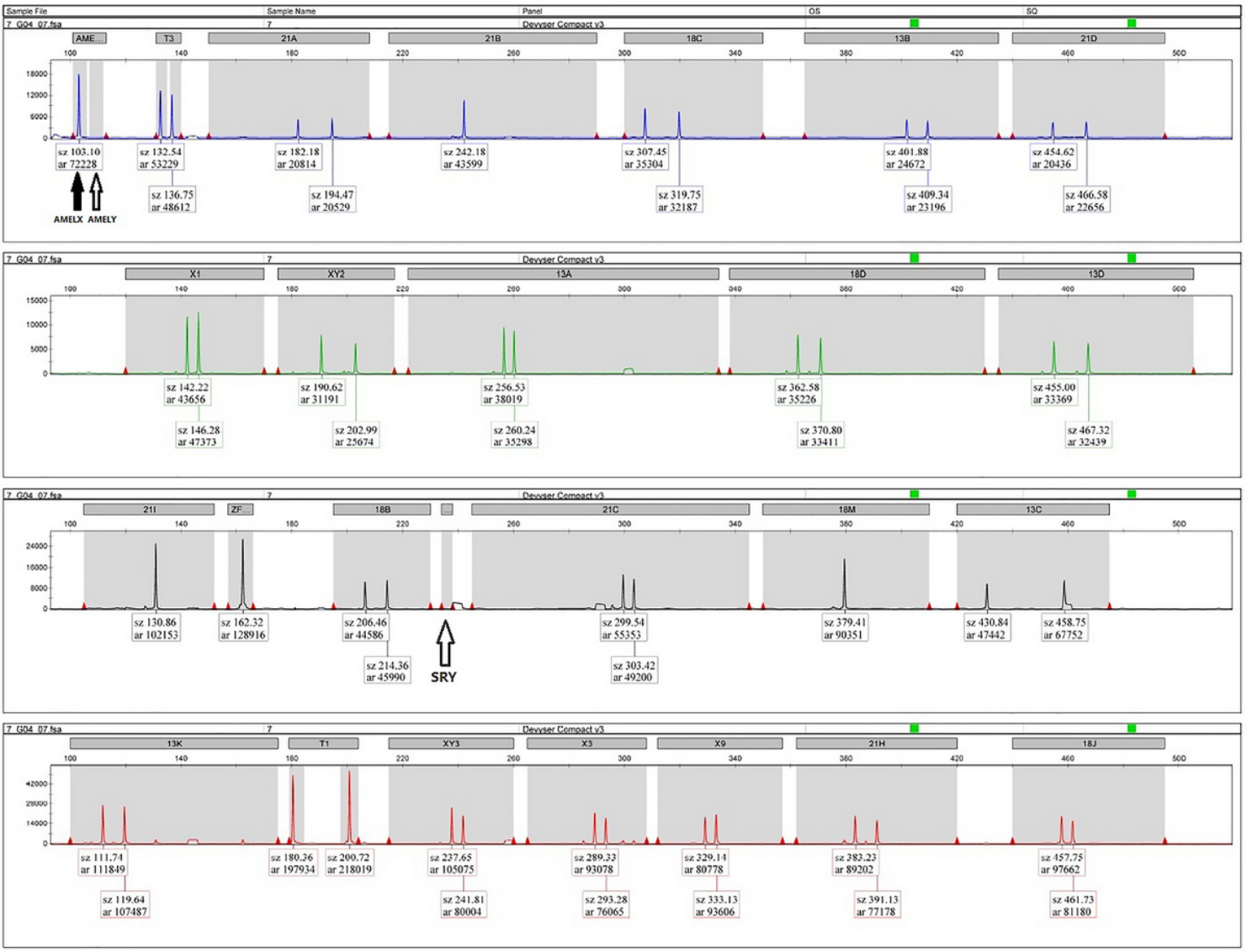


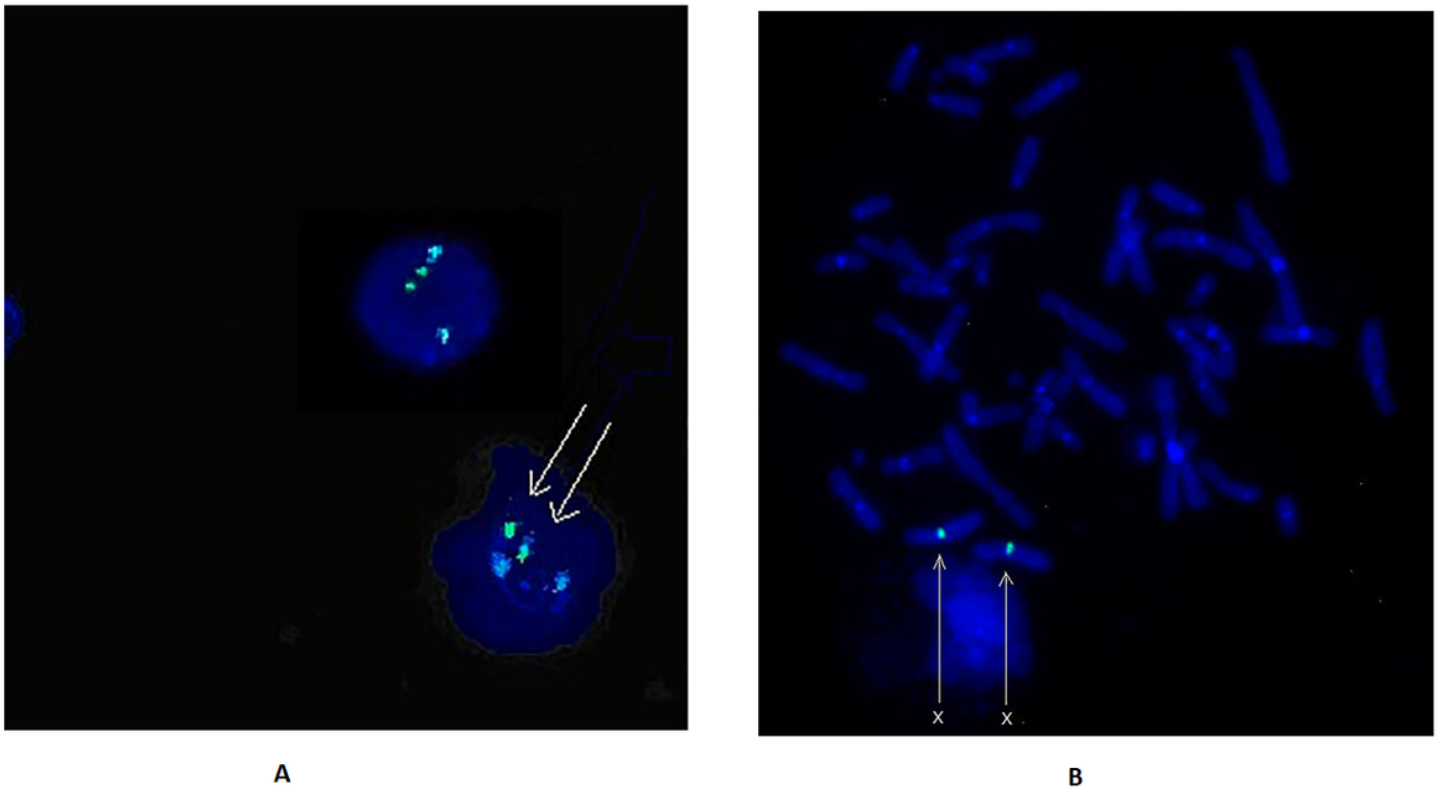

**Figure 1**

Chromosome karyotype of the patient. The sex chromosome of 46,XX male sex reversal patient is XX, as the arrow indicated, but no Y chromosome.



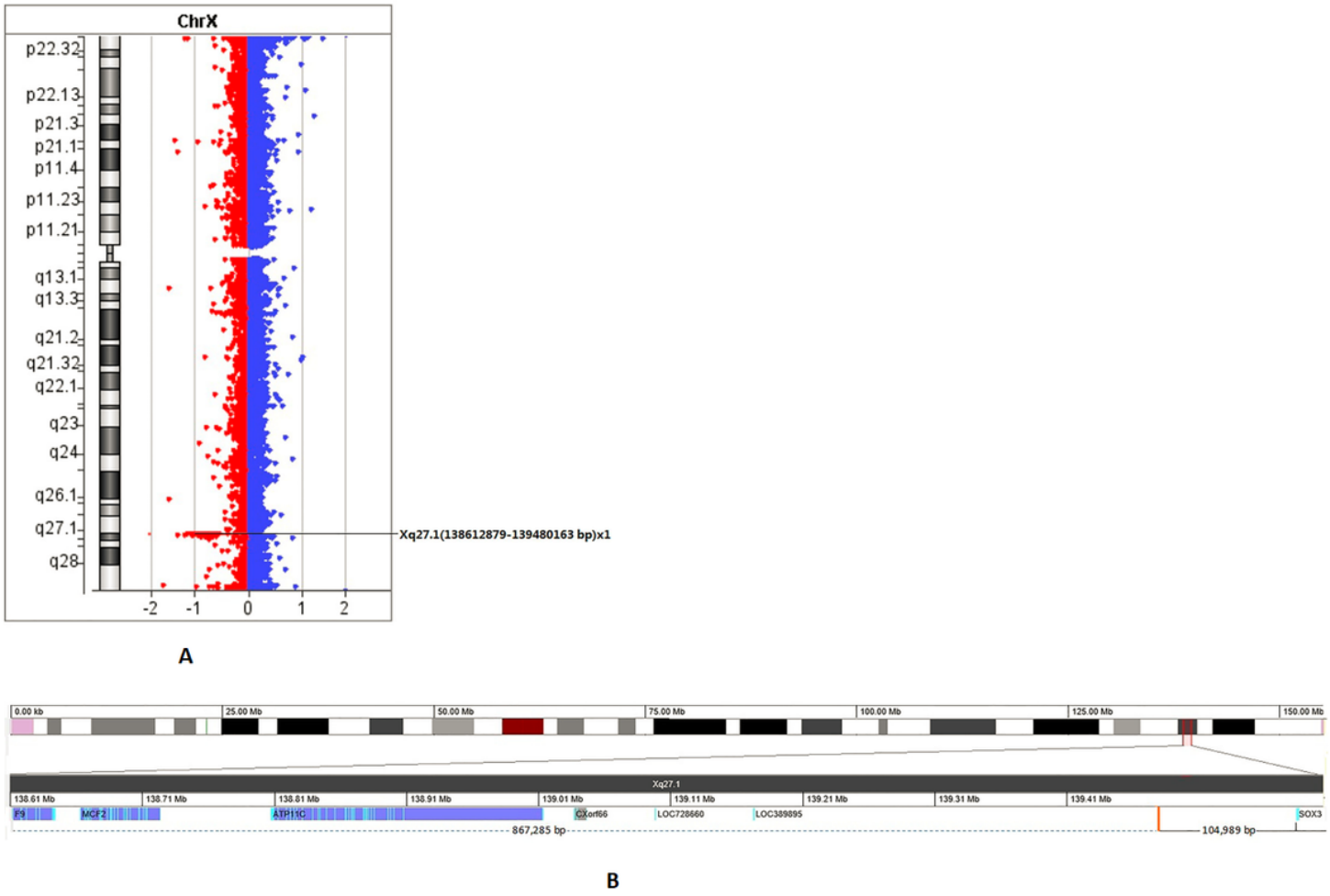

## Figure 2

QF-PCR electrophoresis diagram of the 46,XX (SRY-) male patient. QF-PCR results of the patient showed a fluorescence peak of AMELX but no AMELXY and SRY. AMELX, AMELXY and SRY represent the loci of Xp22.2, Yp11.2 and Yp11.31, respectively. The solid arrow indicated a specific amplification peak, while the hollow arrow indicated no amplification peak.



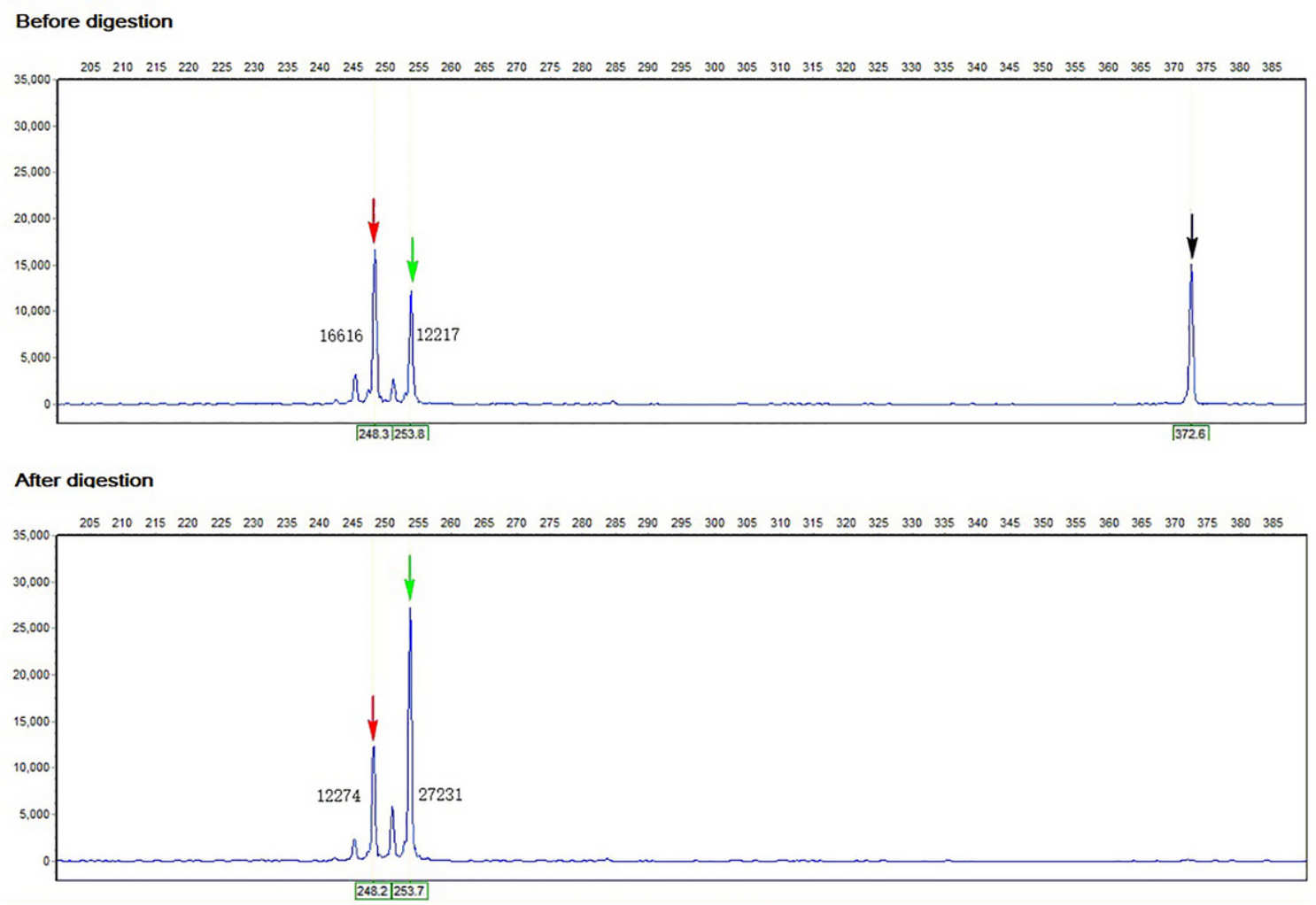

## Figure 3

a. Interphase cells FISH of the 46,XX (SRY-) male patient using 18, X, and Y centromeric probes. The blue signal represents the centromeres on chromosome 18, while the green signal represents the centromeres on chromosome X, as indicated by the arrow b. Metaphase cells FISH of the 46,XX (SRY-) male patient with X, Y and SRY probes. The green signal represents the centromeres of the X chromosome, as indicated by the arrow.




## Figure 4

a. CMA results of the 46,XX (SRY-) male patient. There was about 867kb heterozygous loss in Xq27.1 (hg19: chrX: 138,612,879-139,480,163bp), as the line indicated. b. Schematic diagram of gene chip deletion region of the 46,XX (SRY-) male patient. The deletion region (hg19: chrX: 138,612,879-139,480,163 bp) is located at 104kb downstream of the SOX3 gene in Xq27.1. The dotted line is the deletion region with a size of about 867kb.




## Figure 5

X chromosome inactivation results of the 46,XX (SRY-) male patient. The figure above and below show the fluorescence peaks of the X chromosome before and after HpaII digestion, respectively. The ordinate and abscissa represent fluorescence intensity and fragment length. The black arrow indicates the amplified products of the reference gene. After complete digestion, there is no amplified products peak (as shown below). The red and green arrows indicate the two alleles of the AR gene in the X chromosomes. X chromosome inactivation was calculated according to the formula (d1/u1)/(d1/u1 +d2/u2). The X chromosome inactivation rate in this patient was about 75%, which was non-random. d1: the height of the higher peak after enzyme digestion, u1: the height of the undigested peak, which corresponding to d1; d2: the height of the shorter peak after digestion; u2: the height of the undigested peak, which corresponding to d2.