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Case report

A duplication upstream of *SOX9* associated with *SRY* negative 46,XX ovotesticular disorder of sex development: A case report

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What is already known on this topic?
The 46,XX ovotesticular disorder of sex development (DSD) is rarely observed in humans. It is known that the excessive expression of SRY-related high mobility group box 9 (*SOX9*) is the cause of *SRY*-negative 46,XX ovotesticular DSD in the absence of *SRY*.

What this study adds?
This report presents the first case announced in Turkey, exhibiting *SOX9* duplication in *SRY*-negative 46,XX ovotesticular DSD.

Abstract
The 46,XX ovotesticular disorder of sex development (DSD) is rarely observed in humans. This disorder is generally described as ambiguous genitalia, the presence of ovarian and testicular tissues in different gonads or in the same gonad. Almost no subjects with 46,XX ovotesticular DSD have sex-determining region of the Y chromosome (*SRY*) gene. It is known that the excessive expression of SRY-related high mobility group box 9 (*SOX9*) is the cause of *SRY*-negative 46,XX ovotesticular DSD in the absence of *SRY*. Here, we analyzed our *SRY*-negative case with 46,XX ovotesticular DSD. In the array comparative genomic hybridization (CGH) study conducted on the peripheral blood sample of the patient, we detected a duplication of 1114 kb (Hg19 coordinates: chr17:69006280-70120619) in the region of 17q24.3 containing *SOX9*. This report presents the first case announced in Turkey, exhibiting *SOX9* duplication in *SRY*-negative 46,XX ovotesticular DSD.
Key words: 46,XX ovotesticular disorder of sex development, *SRY-negative, SOX9*.

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Introduction

Normal gonadal differentiation and sex development depend on the synchronization of the pathways reflecting the effects and interactions of certain genes, transcription factors, and hormones in the genetic sex presence determined by the chromosomal structure. First, ovarian or testicular development from a primitive gonad, then differentiation of internal and external genital structures take place. Modifications of these complex gene regulatory networks or deterioration of the gene expression regulating the fetal gonadal development lead to disorders of sex development (DSD) (1, 2).
These disorders include a heterogeneous abnormality spectrum in which the chromosomal, genetic, gonadal, hormonal or phenotypical aspects of sex are atypical. The gap in terminology provides a frame for approaching differential diagnosis in a patient. The DSD categories include sex chromosome DSDs such as 45,X/46,XY; ovotesticular DSD; 46,XY DSDs such as disorders of testicular development, disorders of androgen synthesis and action, and XY sex reversal; and 46,XX DSDs such as masculinization of the XX individual and XX sex reversal. Approximately 1 in 4000 infants is born with a DSD (1).
Ovarian differentiation is the normal way in the 46,XX fetus (1). However, in rare cases, 46,XX gonads can either differentiate in testes, which is known as 46,XX testicular DSD (named as XX male before), or turn into a condition causing ovarian and testicular tissue to couple in the same individual, known as 46,XX ovotesticular DSD (real hermaphroditism before) (3). Ovotesticular DSD is a rare DSD in which an individual generally has ambiguous genitalia, ovarian and testicular tissues are present in separate gonads or in the same gonad; two third of these have the 46,XX karyotype. Molecular studies revealed that almost 90 % of 46,XX with ovotesticular DSD were sex-determining region of the Y chromosome (*SRY*) gene negative (4). In these *SRY* negative individuals, testicular development may depend on the presence of an additional dose or excessive expression of an autosomal gene which influences male sexual differentiation such as SRY-related high mobility group box 9 (*SOX9*) (5).
So far, the definite mechanism of testicular differentiation in 46,XX ovotesticular DSD has not been explained; three mechanisms were suggested to explain the testicular determination: (i) Hidden mosaicism with a cell line carrying Y; (ii) translocation of Y-material from paternal Y to the X chromosome including the *SRY* gene; (iii) in a gene which is autosomal or connected to X, defects allowing testicular determination in the absence of *SRY*, *SOX9* is one of these genes (4).
SOX9 duplication is not a common cause of the testicular development in cases with *SRY*-negative 46,XX testicular or ovotesticular DSD (6). Only 3 studies have been published exhibiting SOX9 duplicationin *SRY*-negative 46,XX ovotesticular

DSD (4, 5, 7). This report presents the first case announced in Turkey, exhibiting *SOX9* duplication in *SRY*-negative 46,XX ovotesticular DSD. In addition, we present a literature review of SRY-negative 46,XX ovotesticular DSD and discuss the role of *SRY* and *SOX9* in testicular development.

Case presentation

A 3-year and 4-month-old male patient was brought due to uncertainty in the genital region. The patient was born through normal vaginal delivery with a weight of 3450 g [0.28 standard deviation score (SDS)] and a height of 50 cm (-0.05 SDS) in the 39th gestational week, and this was the 4th pregnancy of the 32-year-old healthy mother. The patient, whose atypical genitalia was discovered after birth, was examined due to the disorder of sexual differentiation in the external center. The parents are in a non-consanguineous marriage and the family members exhibited no clinical manifestations. The family is of Turkish origin. Peripheral blood chromosome analysis was assessed as 46,XX. Patient's available examinations, when he was 36 days old: serum testosterone (T): 86.69 ng/dl (normal range, 75–400), dihydrotestosterone (DHT): 7.09 ng/dl (normal range, DHT decreases rapidly the first week, then increases to 12–85 ng/dL between 30–60 days. Levels then decrease gradually to prepubertal values by seven months. Prepubertal children< 3 ng/dl). When he was 4 months old, basal 17-Hydroxyprogesterone (17OHP) level of 1.4 ng/ml (normal range, 2 ng/mL), peak cortisol response level of 22.3 µg/dl and peak 17OHP level of 4.9 ng/ml were observed in his ACTH stimulation test, and adrenal insufficiency was excluded. His hormonal evaluation when he was 8 months old: Serum T level of 12.9 ng/dl (normal range, < 3–10); estradiol level of < 1 ng/dl (normal range, < 1.5 ng/dL); follicle-stimulating hormone (FSH) level of 0.28 mIU/mL (normal range, 0.16 – 4.1), luteinizing hormone (LH) level of 0.03 mIU/mL (normal range, 0.02 – 7.0) and prolactin level of 10.16 ng/ml (normal range, 3–18). After the human chorionic gonadotrophin (hCG) stimulation performed to assess testicular functions at the age of 1, the serum T value was 129.7 ng/dl (normal value, 65-250 ng/dl), and it was considered as a sufficient response. Afterward, the patient discontinued his follow-up in the external center. The patient is currently 3 years 4 months. In the physical examination of the patient, his body weight was 12.6 (-1.69 SDS), his height was 89.5 cm (-2.4 SDS) and his head circumference was 50 cm (-0.35 SDS). There was no dysmorphism, scoliosis or skeletal dysplasia. Asymmetry was observed in his genital examination and phallus was 3.9 cm, cordis was observed, there was a single narrow urethral orifice opening to the phallus base. A 1 ml gonad was palpated in the labioscrotal fold on the right, a 1 ml gonad was palpated in the inguinal canal proximal on the left. In Figure 1, the external genital structure is observed. Hormonal assessment of the patient; Serum T level of < 1 ng/dl (normal range, < 3–10); estradiol level of < 1.2 ng/dl (normal range, < 1.5 ng/dL); FSH level of 0.87 mIU/mL (normal range, 0.26–3.0), LH level of 0.53 mIU/mL (normal range, 0.02–0.3) and serum anti-Mullerian hormone level of (AMH) 19 ng/ml (normal range, 48.0–83.2). The hCG test was executed again, 1500 units of hCG were applied for 3 consecutive days. After the test, the serum T level was 40 ng/dl (normal value, 65-250 ng/dl).

In the pelvic ultrasonographic examination, there was an appearance of ovarian tissue with the dimensions of approximately 14x10x5 mm containing millimetric cystic areas in the left inguinal canal,there was a testicular tissue with the dimensions of 5x7x10 mm at the level of hemiscrotum-labium majus on the right and a homogeneous internal structure. In the left rectovesical area, there was an appearance that might belong to a rudimentary structure with a front-back diameter of 5 mm. In the abdominal and pelvic MRI examination, the right testis was scrotal located and its dimensions were measured as approximately 13x8x11 mm. At the level of the inguinal canal proximal on the left, a structure was observed complying with ovarian tissue with T2 hyperintense cystic areas that belong to follicle cysts with the dimensions of 15x10 mm (Figure 2, 3) . There is a structure with the dimensions of 7x5x17 mm complying with the rudimentary uterus in the left posterior-inferior section of the (Figure 4). The biopsy was taken from the wedge and central sections of the bilateral gonads. The gonadal tissues stabilized with formalin and buried in paraffin were assessed histochemically. The right gonad was found compliant with testis parenchyma containing a seminiferous tubule structure, the left gonad was found compliant with ovarian tissue containing follicle structures at a different maturation stage (Figure 5). The *SRY* gene was shown to be negative in the peripheral blood leucocytes through the methods of Fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR). Moreover, the *SRY* gene was found to be negative in the analysis of the gonad biopsy material. In the array comparative genomic hybridization (CGH) study conducted on the peripheral blood sample of the patient (Agilent 8x60K array, Santa Clara, Ca, USA), a duplication of 1114 kb (Hg19 coordinates: chr17:69006280-70120619) was detected in the region of 17q24.3 (Figure 6). This duplication covers the part from the 1.1 Mb upstream region of the SOX9 gene (including the promoter region) until the 3' UTR region. Furthermore, it was found out as a result of the array CHG that the X chromosome had two copies and the Y chromosome was not available.

The patient was evaluated in the sex research commission. As a result of the psychiatric evaluation of the patient, it was discovered that the patient's selection of games and toys complied with the male sex and he selected playmates and clothes in compliance with the male sex according to the information given by the family, and as per the clinical conclusion reached, the patient's development of sexual identity was male-oriented. As a result of the patient's upbringing as a boy so far, the willingness of the family for a male-oriented corrective operation and the medical and psychiatric evaluations, a decision was made on the implementation of male-oriented corrective operations.

Result

We diagnosed 46,XX Ovotesticular DSD in our patient with clinical findings and molecular studies. The *SRY* was negative with the FISH and PCR methods. In the CGH study conducted on the peripheral blood sample of the patient, a duplication of 1114 kb (Hg19 coordinates: chr17:69006280-70120619) was detected in the region of 17q24.3. This duplication covers the part from the 1.1 Mb upstream region of the SOX9 gene (including the promoter region) until the 3' UTR region. The blood samples taken from the parents were found insufficient, and it could not be confirmed whether this copy was *de novo* as blood samples could not be taken again.

Discussion

Ovotesticular DSD is a rare form of DSD with variable prevalence and karyotypes in different parts of the world. Ovotesticular DSD is defined as the presence of ovarian tissue with follicles and testicular tissue with seminiferous tubules in the same individual. Although an ovotestis is the most commonly identified gonad in ovotesticular DSD, there can be an ovary on one side and a testis on the other (1). All the studies agree that the 46,XX karyotype is the most common karyotype

observed in blood samples of patients with ovotesticular DSD. Its frequency varies between 65% and 90%. In remaining cases, there is a Y chromosome (46,XY, 46,XX/46,XY or other mosaics) that explains the development of the testicular tissue (3).

When the testicular tissue differs in a 46,XX *SRY*-negative gonad, 2 different mechanisms can be foreseen: increased expression of the pro-testis genes or insufficient expression of the provarian/anti-testis genes (3).

The binary switch responsible for testicular development is the *SRY* gene located on the short arm of the Y chromosome. The *SRY* protein contains a high-mobility group (HMG) domain and is encoded by a single exon gene. The *SRY* protein is expressed in pre-Sertoli cells, where it triggers a molecular switch to induce Sertoli cell differentiation, thus, initiating the process of male sexual differentiation. A threshold *SRY* level must be achieved at a critical time during gestation to establish male sexual differentiation. Otherwise, the ovarian differentiation pathway is activated. Available data suggest that steroidogenic factor-1 (*NR5A1*) promotes *SRY* expression (1).

SRY expression is independent of the presence of germ cells. *SRY* increases the expression of the SRY-related HMG box-containing-9 (*SOX9*) gene. *SOX9* is a member of the SRY-related HMG domain gene family located at chromosome 17q24.3-17q25 (1). *SOX9* is expressed in various tissues including chondrocytes and testes, furthermore, it is found in the bile duct, central nervous system, hair follicles, heart, lungs, pancreas, and retina (3). *SOX9* is highly expressed in Sertoli cells where it functions to promote Sertoli cell differentiation. Phenotype-genotype studies of humans and mice demonstrate that *SOX9* expression is a crucial step, downstream of *SRY*, in testis development. Upstream from the *SOX9* transcription start site, there appears to be a testis-specific enhancer element (hTES). *SOX9* then re-regulates Fibroblast Growth Factor 9 (FGF9) and Prostaglandin D2 (PGD2), and a positive feedback cycle is established to regulate *SOX9*, which gradually becomes independent of *SRY*, further. *SOX9* is responsible for the Sertoli cell specification, thus, testicular differentiation starts and the AMH production is triggered (1, 3).

Most of the *SOX9* duplications were identified in 46,XX testicular DSD patients (4). Firstly, Huang et al. (1999) reported an individual who had *SRY*-negative 46,XX testicular DSD and the duplication of the 17q chromosome (8). So far, there have been only 3 studies on individuals with *SRY*-negative 46,XX ovotesticular DSD and duplication upstream of *SOX9*. Firstly, Benko et al. (2011) identified the upstream duplications of *SOX9* in three cases with *SRY*-negative 46,XX ovotesticular DSD. These patients exhibited genital virilization at various levels; one of them had gonads as bilateral ovotestis, another one had ovarian remnant on the left and testis on the right, the third patient had a streak gonad with partial ovarian differentiation on the right and ovotestis on the left. Molecular studies showed a different level of duplication in each patient. The region about 500 kb upstream *SOX9* and covering 78 kb is accepted as the sex determination region (RevSex region). These authors asserted that the upstream region duplication of *SOX9* observed in 46,XX DSD patients had one or more regulating element, which is critical for gonadal development (5). Secondly, Kim et al. (2015) defined two people with 46,XX ovotesticular DSD. An upstream duplication of *SOX9* was found in both patients (including RevSex region) (7). Lastly, López-Hernández et al. (2018) conducted a molecular study on 10 patients with *SRY*-negative ovotesticular DSD independently from each other. Only in 1 patient, they found a heterozygosis duplication around 581 kb in 5' upstream region including almost all the coding region of *SOX9*. This patient was 6 years and 12 months old and brought up as a girl; one of the gonads was an ovary, the other one was an ovotestis (4). Differently, *SOX9* duplication was also detected in 46,XX DSD studies. Vetro et al. (2015) analyzed 19 individuals with 46,XX DSD. In a patient with ambiguous external genitalia and 46,XX ovotesticular DSD, they detected a copy containing the gene-desert region upstream *SOX9*, including the RevSex region (9). Recently, Ohnesorg et al. (2017) have reported an individual 46,XX with ovotesticular DSD with a heterozygous duplication upstream of *SOX9* encompassing a minimal region of 248 kb at 17q24.3 (10).

Consequently, these studies set forth the importance of *SOX9* copies in male sexual differentiation with breasts and show that this is a key gene in testicular differentiation. We analyzed our *SRY* negative case with 46,XX ovotesticular DSD, the Y chromosomal sequence was not shown in our patient. Therefore, testicular differentiation occurred in our patient in the absence of *SRY*. In the array CGH study conducted on the peripheral blood sample of the patient, a duplication of 1114 kb (Hg19 coordinates: chr17:69006280-70120619) was detected in the region of 17q24.3. This duplication covers the part from the 1.1 Mb upstream region of the *SOX9* gene (including the promoter region) until the 3' UTR region.

Duplication in the region of 17q that contains *SOX9* is not a common cause of testis development in subjects with SRY-negative 46,XX ovotesticular DSD. Seeherunvong et al. (2012) analyzed 30 *SRY*-negative people including 23 with 46,XX testicular DSD and 7 with 46,XX ovotesticular DSD. They researched the possible copies of *SOX9* and duplication of the *SOX9* region in 17q was not detected in any subject (6). Rajender et al. (8) used the PCR and the microsatellite analysis in order to examine a person with *SRY*-negative 46,XX testicular DSD, however, they could not detect the microduplication of *SOX9* (11). In a similar study conducted on twins, one of whom had 46,XX testicular DSD and the other one had 46,XX ovotesticular DSD, none of them had *SOX9* duplication (12). Lastly, Temel et al. referred to a large family with 9 members who had 46,XX testicular or 46,XX ovotesticular DSD. None of the individuals affected in the family group had *SOX9* duplication (13).

Ovotesticular DSD requires the presence of seminiferous cords and ovarian follicles together with oocytes. The clinical picture is not different from the other types of DSD, there is a range extending from a male phenotype with mild hypospadias and cryptorchidism to a female phenotype with clitoromegaly and minor labial fusion. Internal genital is usually associated with external virilization and Mullerian derivatives are observed in less virilized patients. Moreover, the ultrasonographic evaluation may be deceptive as a diagnostic tool in neonates or infants. Histological analysis of the gonads is mandatory for diagnosis (3). The phenotype of our patient was male-dominant and he had a gonad as the testis and another gonad as ovarian tissue together with the rudimentary Mullerian structure.

Testosterone and AMH levels are generally medium between the normal male and female ranges (14, 15). Estradiol levels can reflect the amount of functional ovarian tissues in neonates and teenagers. Gonadotropins may be increased or normal (12, 14), the ovarian estrogen effect is reflected in these cases as the testicular tissue is dysgenetic in most conditions. Although the serum testosterone level in mini-puberty and the serum testosterone level in the hCG test, which was conducted at the age of 1, were adequate, the serum testosterone level decreased substantially in the hCG test repeated afterward. In

addition, the AMH level was found to be under the age-compliant reference range. This showed us clearly that the function of the testicular tissue decreased as grown older.

Sex assignment is a problem in these patients and opinions resemble those in DSD (16). In patients with ovotesticular DSD, the ovarian tissue can be normal enough to produce oocyte. For this reason, attention can be paid to the preservation of the ovarian tissue and female assignment can be preferred. However, male sex was assigned to our patient. It was concluded that this selection resulted from the patient's development of male sexual identity as a result of his upbringing as a boy so far, the family's willingness for male sex and psychiatric evaluation.

Partial gonadectomy requires specific interpretation: for children raised male, the ovarian part must be removed before the pubertal age in order to prevent estrogen increase resulting in gynecomastia or other heterosexual pubertal development characteristics and also the cystic follicle complications that might emerge as a response to high FSH. In rare cases, it was explained that male patients with the medical history of hypospadias and cryptorchidism applied with cyclic haematuria in puberty (17). In patients raised female, the testicular tissue must be removed to prevent virilization in puberty. Regarding the risk of tumor growth in the testicular part, it was reported to be low even if the tissue is dysgenetic possibly because the Y chromosomal sequences are not available (18). For our case, a male-oriented corrective operation was planned due to the male sexual assignment.

Conclusion

The *SOX9* gene is considered as the target of *SRY* and thus induces a gene expression resulting in the testicular assignment. Studies revealed the importance of SOX9 copies in male sexual differentiation with breasts and show that this is a key gene in testicular differentiation. Duplication in the region of 17q that contains *SOX9* is a rare cause of testis development in subjects with *SRY*-negative 46,XX ovotesticular DSD. Such DSDs are very rare and require a careful systematic, sensitive approach to diagnosis and management of the diagnostic and ethical challenges. This report presents the first case announced in Turkey, exhibiting *SOX9* duplication in *SRY*-negative 46,XX ovotesticular DSD.

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Ethics

Informed Consent: A written informed consent was obtained from the patient's family

Authorship Contributions

Surgical and Medical Practices: Eda Mengen, Seyit Ahmet Uçaktürk, Gulsum Kayhan, Pınar Kocaay

Concept: Eda Mengen, Seyit Ahmet Uçaktürk,

Design: Eda Mengen, Seyit Ahmet Uçaktürk

Data Collection or Processing: Eda Mengen, Seyit Ahmet Uçaktürk, Gulsum Kayhan, Pınar Kocaay

Analysis or Interpretation: Eda Mengen, Seyit Ahmet Uçaktürk, Gulsum Kayhan, Pınar Kocaay

Literature Search: Eda Mengen, Seyit Ahmet Uçaktürk,

Writing: Eda Mengen

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Figure 1. The genital photograph of the patient was taken at the age of 3 years and 4 months.

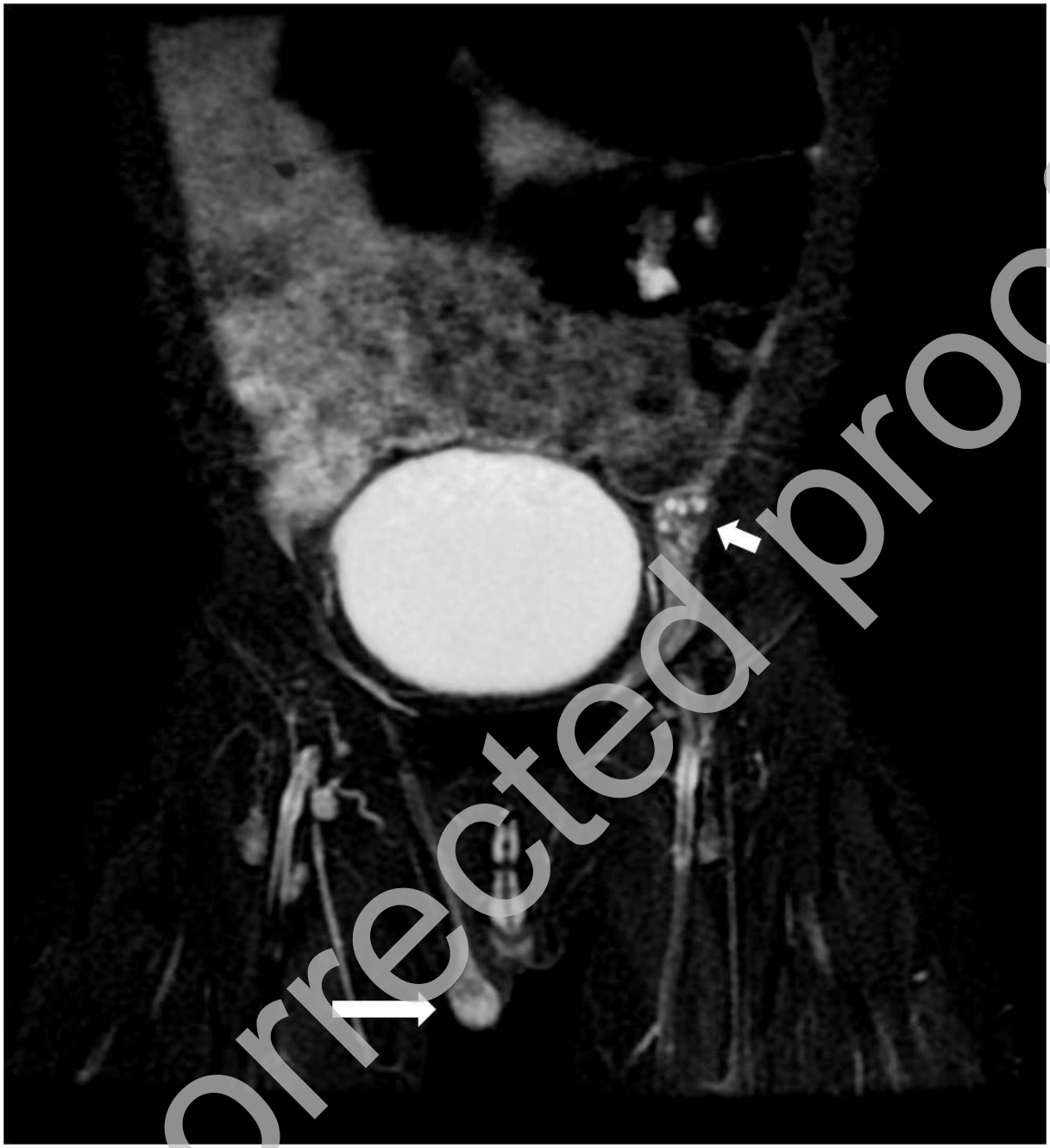

Figure 2. Coronal fat-suppressed T2-weighted image of the MR sequence shows testicular tissue in the right scrotum. There is no testicular tissue in the scrotal area on the left. Ovarian tissue with millimetric follicle cysts is view in the left inguinal region.

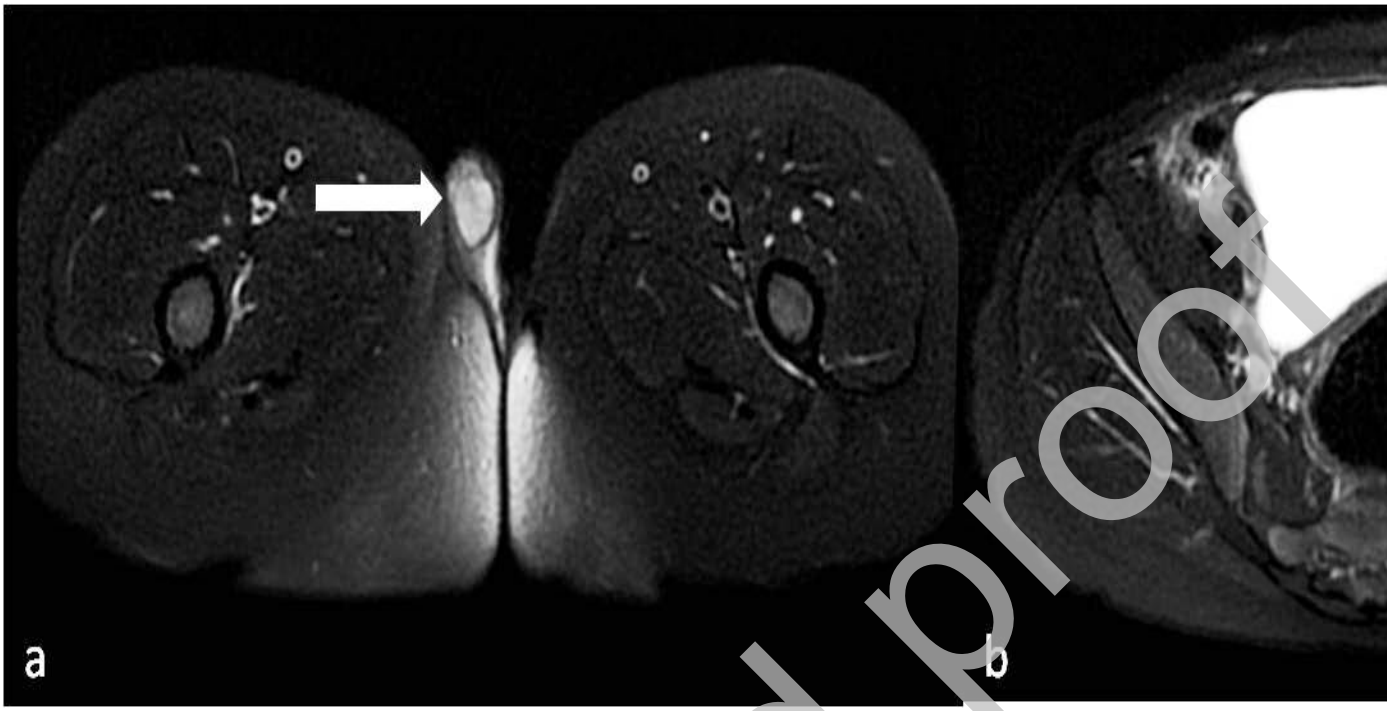

Figure 3. Axial fat-suppressed T2-weighted image of the MR sequence shows testicular tissue in the right scrotum. On the testis side, normal epididymis and spermatic cord are observed (a). Ovarian tissue with millimetric follicle cysts is shown in the left inguinal region (b).

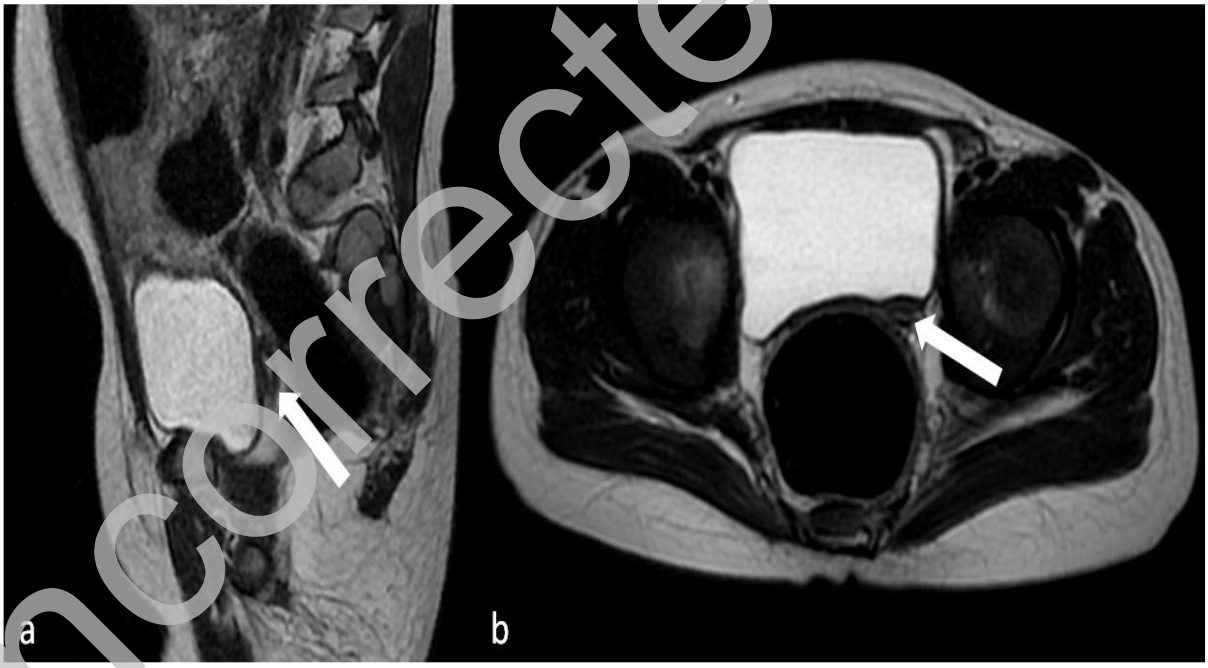

Figure 4. Rudimentaty uterus is shown on the sagittal T2 (a) and axial (b) T2-weighted magnetic resonance images.

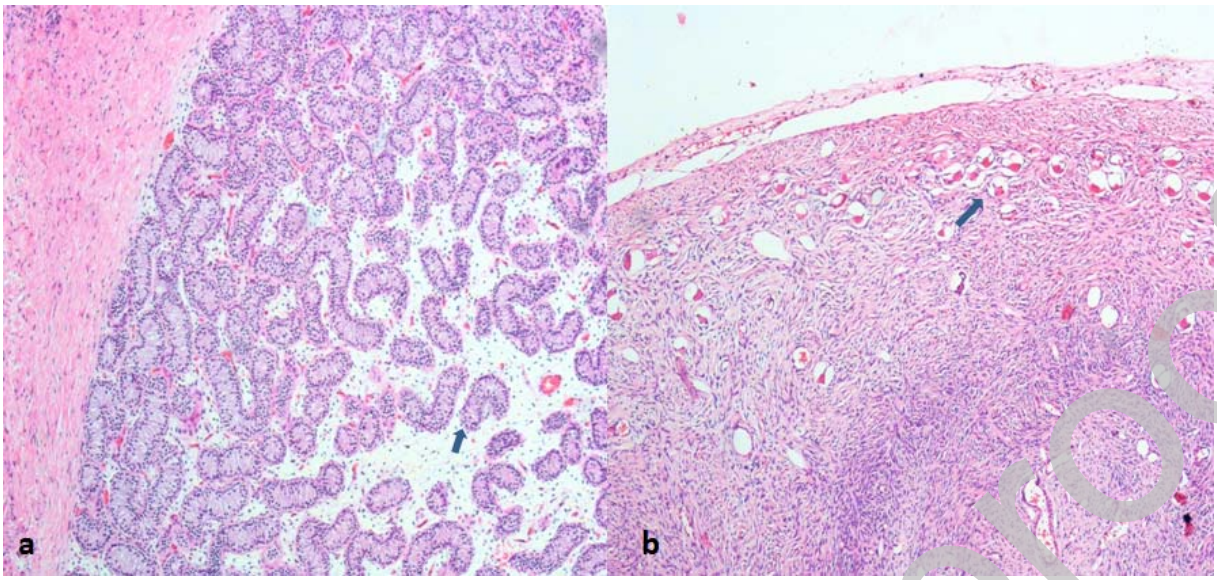

Figure 5. Testicular tissue with seminiferous tubules structure (a) and ovarian tissue containing follicle structures at a different maturation stage (b).

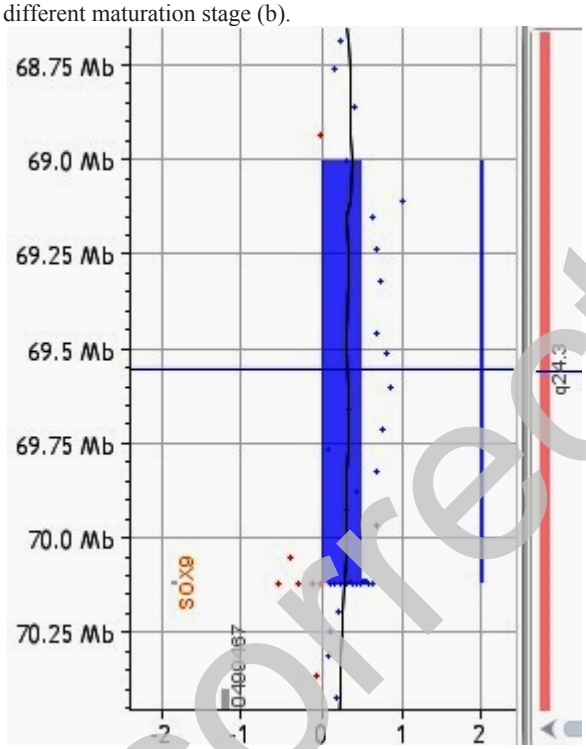

Figure 6. Patient resut, in the CGH study conducted on the peripheral blood sample of the patient, a duplication of 1114 kb (Hg19 coordinates: chr17:69006280-70120619) was detected in the region of 17q24.3. This duplication covers the part from the 1.1 Mb upstream region of the SOX9 gene (including the promoter region) until the 3' UTR region.