The Natural History of a Man With Ovotesticular 46,XX DSD Caused by a Novel 3-Mb 15q26.2 Deletion Containing NR2F2 Gene

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Gonadal sex determination is a complex genetic process by which an embryonic primordium is driven to form an ovary or a testis, which requires a delicate dosage balance involving many genes. Disruption in this molecular pathway can lead to differences of sex development (DSD). Although some genetic mechanisms leading to 46,XY DSD have been elucidated, little is known about copy-number variation (CNV) causing testicular or ovotesticular 46,XX DSD. We describe a 20-year natural history of a man with SRY-negative 46,XX who was born with atypical male external genitalia, aortic coarctation, and bilateral blepharophimosis-ptosis. The molecular study identified a de novo heterozygous 3-Mb 15q26.2 deletion, a gene-poor locus containing NR2F2, which encodes the nuclear receptor COUP-TFII that is highly expressed in ovary and cardiac arteries. Immunohistochemistry confirmed the low COUP-TFII expression on his ovotestis tissue. Monosomy of 15q26.2, encompassing the NR2F2 gene, may act as a Z-factor regulating the male sex determination negatively. This finding supports a novel type of CNV resulting in DSD in an individual who developed male puberty spontaneously.

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46,XX ovotesticular differences/disorders of sex development (DSD), formerly known as true hermaphroditism [1], comprise a spectrum of sex anatomy promoted by rare variants of sexually dimorphic gonadal genes [2]. The sex determination in humans is a complex

Abbreviations: CNV, copy-number variation; DSD, differences of sex development; TFB, transcription factor binding.
1. Case Report

A. Clinical History

The reported patient is a 20-year-old man diagnosed with ovotestis. He was born from nonconsanguineous parents after an uneventful pregnancy, the third youngest boy in a healthy family. His lymphocyte culture revealed a 46,XX karyotype. At birth, a 3-cm penis with midshaft hypospadia, palpated inguinal gonads, and bilateral blepharophimosis-ptosis were detected. Low-set ears, webbed neck, pectus excavatum, and sinus bradycardia were noted (Fig. 1A and 1B). He presented low weight (5th percentile) but standard stature (75th percentile) for his age and surpassed parental target height (Fig. 1C). Asymmetric blepharophimosis-ptosis was documented by his parents (Fig. 1D). Urethrography showed a small prostatic urethra and a failure to fill the contrast in the posterior urethra (Fig. 1E). Cystoscopy identified a prominent bulging corresponding to verumontanum (Fig. 1F). Corneal topography diagnosed keratoconus when he was 11 years old (Fig. 1G). He underwent zetaplasty when he was 12 years old for correcting penile curvature that developed late after hypospadia repairs (Fig. 1H). At ~14 years of age, he began spontaneous puberty, even though he presented with bilateral microorchidism (1 and 1.8 mL). He attained pubertal spurt and male secondary characteristics and no gynecomastia was evidenced. He developed an average penile length, male muscular distribution, and pubic hair, although scarce on the face (Fig. 1A). When he was 16 years old he underwent left gonadal biopsy and right gonadectomy because of a firm nodule in the right gonad diagnosed on histopathology as fibrotic tissue. Revised gonadal biopsies confirmed ovotesticular 46,XX DSD, comprising follicular epithelium mainly with granulosa cells in the ovarian part and Leydig cell hyperplasia and Sertoli cell-only tubules in the testicular part (Fig. 2A–2D). Gonadal axis tests are summarized in Table 1. He was assigned and raised as a boy, identified himself as male during childhood and adolescence, and has been living as heterosexual.
Figure 1. Clinical and surgical investigation of a 46,XX man carrying heterozygous deletion at 15q26.2 encompassing NR2F2 gene. (A) Low-set posteriorly rotated ears, webbing of the neck, and mild pectus carinatum inferiorly with pectus excavatum superiorly. (B) Holter monitor indicating sinus bradycardia with the average measurement of 48 bpm. (C) Growth chart during clinical and surgical follow-up indicating his height (in/cm) and weight (lb/kg). (D) Asymmetric blepharophimosis-ptosis when he was 2 y old. (E) Urethrography showing the presence of a small prostatic utricle (*) and a failure to fill the contrast in the posterior urethra (**). (F) Cystoscopy identified a prominent lesion in verumontanum (vm) corresponding to the same position as the contrast-fill failure in urethrography. (G) Corneal topography indicating keratoconus when he was 11 y old during an investigation for low visual acuity. (H) Intracorporeal macroscopic aspect of the right gonad obtained during zetaplasty performed for the treatment of penile curvature when he was 12 y old, which emerged in late postoperative of hypospadias repairs at 4 and 6 y old, showing

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B. Genetic and Immunohistochemistry Analyses

Upon detailing his 46,XX karyotype, single nucleotide polymorphism-array analysis was performed and revealed a de novo 3-Mb deletion on 15q26: arr[GRCh37]15q26.2(95127653_98146649)

This region is an evolutionarily conserved locus among mammals and contains two genes (NR2F2 and SPATA8), three noncoding genes (NR2F2-AS1, SPATA8-AS1, and miR-1469), and three pseudogenes (PGAM1P12, RPL31P5, and FAM149B1P1) (Fig. 1I). The locus 15q26.2 also encompasses regulatory regions, containing more than 15,000 transcription factor binding (TFB) sites, among them, 282 to SRY, 147 to SOX5 and 103 to SOX9 (data not shown). PCR and Sanger sequencing from peripheral blood DNA were negative for common 46,XX DSD variants in SRY, RSPO1, SOX9, as well as NR2F2. MLPA revealed no CNV for WNT4, NR5A1, and SOX9 (Fig. 1J). Additionally, whole-exome sequencing performed in the proband and his parents resulted in no pathogenic DSD variants.

Once we ruled out SOX9, SOX3, WNT4, FOXL2, RSPO1, and NR2F2 missense mutations and that NR2F2 is missing in the 3-Mb 15q26 deletion, we further performed immunohistochemistry in the resected gonadal tissue and found lower COUP-TFII expression in granulosa-type cells and Sertoli cells compared with sex-cord stromal cells (Fig. 2E–2F).

2. Discussion

A de novo 3-Mb deletion on 15q26, encompassing NR2F2 gene and hundreds of sex-associated TFB sites, constitutes a novel genetic rearrangement in a 46,XX subject born with atypical male genitalia who developed all secondary male sexual characteristics. Examining his histopathology gonad thoroughly, we hypothesized that the ovotestis might have evolved to progressive gonadal fibrosis. Likely it may have begun slightly during the fetal period and then gradually increased up to the time of the midfinal period of puberty. The gradual testis atrophy leading to micro-orchidism must have resulted in Sertoli cells death, therefore, a decrease of inhibin B secretion and FSH increase as observed at the end of his adolescence. By compromising Leydig cells subsequently, the ongoing atrophy might have resulted in decreased testosterone synthesis and elevated LH level by a negative feedback loop.

This new 15q26.2 CNV implies NR2F2/COUP-TFII haploinsufficiency and is consistent with its diminished expression observed in ovotestis gonad, indicating its role in controlling DSD. Many recognized chromosomal rearrangements had been associated with urogenital and gonadal phenotypes [8]. In 2017, we reported the 15q26.2 CNV in association with 46,XX ovo-testicular DSD, and cardiac phenotype [10]. Most CNVs are related to a loss-of-function mechanism, and few are related to upregulation of putative protestis factors in the developing 46,XX gonads as reported in SOX9 and SOX3 [8]. Other 46,XX testicular or ovotesticular DSDs result from mutations in pro-ovary/antitestis genes of the WNT4/RSPO1 signaling pathway, including loss-of-function mutations in WNT4 and homozygous RSPO1 mutations. Also, mutations in the FOXL2 gene are linked to ovary insufficiency and blepharophimosis-ptosis phenotype [8].
The verified NR2F2 heterozygous deletion can explain the genital ridge mesenchyme switch from an ovary to testis caused by the disruption of ovary-specific signaling, which would oppose testis differentiation. Zhao et al. (2017) recently showed that Nr2f2 (2/2)46,XX embryos developed mixed ductal mesenchyme, leading to both female and male reproductive tracts [11]. Our report is in agreement with Zhao’s active pro-ovarian mechanism and validates in human the

**Figure 2.** Immunohistochemical characterization of steroid hormone nuclear receptor COUP-TFII expression on the ovotestis gonad from 46,XX subject carrying a 3-Mb 15q26.2 heterozygous deletion. (A) Low-power scanning field showing left to right ovotesticular dysgenetic gonad containing testicular seminiferous tubules (st), merged rete testis/rete ovarii (rt/ro), and ovarian follicular epithelium (fe) with hematoxylin and eosin (H&E) staining. (B) Dysgenetic ovarian follicular epithelium showing granulosa (*) and theca (**) cell layers, surrounded by ovarian stroma (***) on H&E (200×). (C) Granulosa cells lining in a theca lutein cyst where no primary oocytes were found (H&E). (D) Seminiferous tubules are devoid from germinative cells and contain only Sertoli cells surrounded by some luteinized stromal cells (H&E). Weak nuclear immunostaining for COUP-TFII immunoperoxidase in granulosa (E) and Sertoli (F) cells fulfilling seminiferous tubules of ovotesticular tissue in comparison with ovary (from young woman/200×) and testis (elderly man/400×) external control tissues used in the same immunostaining bath, which are zoomed in the upper-right-hand corner of E and F.
hypothesis that the regression of Wolffian ducts in 46,XX embryos is actively driven by COUP-TFI
by suppressing mesenchyme-epithelium crosstalk responsible for Wolffian maintenance [11]. It has
been described that NR2F2 is expressed at the same time of WT1 in early gonadal embryogenesis.
Therefore, our data are consistent with previous observations on the role of NF2R2 [7, 11]. We
hypothesized that the dosage-sensitive loss of NR2F2 could release WT1 to trigger the expression
of SOX9 through NR5A1 in the absence of SRY (Fig. 2K).

Moreover, the 3-Mb 15q26.2 deletion also containing putative protestis (SOX9, SOX3, and
SOX10) TFB sites may impair sex dimorphic regulation of target genes in the bipotential
gonad. This regulatory haploinsufficiency can lead to incomplete testicular development, as observed in our patient, once SOX9 partial activation is not sufficient to completely block
alleged pro-ovary gene expression (Fig. 2K). Bashamboo et al. [7], applying exome sequencing
on 46,XX SRY-negative individuals with unexplained virilization or testicular/ovotesticular
DSD, identified heterozygous mutations in NR2F2 in three young children. They presented
congenital heart disease, without palpable gonads, and two of them having blepharophimosis-
epicanthus inversus syndrome [7]. For that reason, the 15q26.2 region encompassing
NR2F2 and hundreds of putative protestis TFB sites may function as a Z-factor region, being
crucial for ovary development, as hypothesized in 1993 by McElreavey et al. [9].

Therefore, our data show that the locus 15q26.2 can be considered one of the Z-factor
regions for the sex development in XX embryos because both the monosomy of this chromosomal region and the haploinsufficiency of NR2F2 are not capable of defining the full
differentiation of either female or male gonads.

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Table 1. Gonadal Axis Hormone Measurements During 20-Year Follow-Up of a Man with Ovotesticular
46,XX Due to 15q26.2 Haploinsufficiency

| Period                          | Age  (yr) | Total T ng/dL (male NR) | LH IU/L (NR) | FSH IU/L (NR) |
|--------------------------------|-----------|------------------------|--------------|---------------|
| Newborn                        | 66 d      | 120 (<30)              | NA           | NA            |
| During childhood               | 2         | 30 (<30)               | 0.52 (<0.10) | 3.0 (<0.3)    |
|                                | 3.2       | 22 (<30)               | 0.90 (< 0.10)| 2.2 (<0.3)    |
| Very early to late adolescence | 11.7      | 127 (30–150)           | NA           | NA            |
|                                | 12.5      | 188 (30–150)           | 1.86 (<2.28) | 4.6 (0.30–4.00) |
|                                | 13        | 202 (30–150)           | 5.1 (0.31–5.29) | 6.9 (0.30–4.00) |
|                                | 14.6      | 340 (241–827)          | 4.8 (0.31–5.29) | 19.1 (0.40–7.40) |
|                                | 16        | 407 (241–827)          | 32.4 (1.50–9.30) | 63.8 (0.40–7.40) |
|                                | 17.8      | 177 (241–827)          | 39 (1.50–9.30) | 91 (0.40–7.40) |
|                                | 19.7      | 122 (241–827)          | 43 (1.50–9.30) | 97.7 (0.40–7.40) |

Normal range refers to the interval of basal levels in control subjects matched according to age and male sex. The conversion factor used: testosterone ng/dL × 0.034 for nmol/L.
Abbreviations: NA, not available; NR, normal reference; T, testosterone.
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Data Availability: Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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