ABSTRACT

Disorders of sex development (DSD) are a group of rare conditions characterized by discrepancy between chromosomal sex, gonads and external genitalia. Congenital abnormalities of the kidney and urinary tract are often associated with DSD, mostly in multiple malformation syndromes. We describe the case of an 11-year-old Caucasian boy, with right kidney hypoplasia and hypospadias. Genome-wide copy number variation (CNV) analysis revealed a unique duplication of about 550 kb on chromosome Xq27, and a 46,XX karyotype, consistent with a sex reversal phenotype. This region includes multiple genes, and, among these, SOX3 emerged as the main phenotypic driver. This is the fifth case reporting a genomic imbalance involving the SOX3 gene in a 46,XX SRY-negative male, and the first with associated renal malformations. Our data provide plausible links between SOX3 gene dosage and kidney malformations. It is noteworthy that the current and reported SOX3 gene duplications are below the detection threshold of standard karyotypes and were found only by analyzing CNVs using DNA microarrays. Therefore, all 46,XX SRY-negative males should be screened for SOX3 gene duplications with DNA microarrays.

Keywords: Congenital anomalies of kidneys and the urinary tract (CAKUT); Copy number variations (CNVs); Disorders of sex development (DSD).
SOX3 gene encodes for a transcription factor expressed in the central nervous system (CNS) of vertebrate embryos, which is essential for pituitary, craniofacial and neuronal development [15-22].

Prior human genetics studies implicated SOX3 in brain development and gender determination. Laumonnier et al. [17] described a pericentric inversion of the X chromosome involving the IL1RAPL at Xp21.3 and the polyalanine repeat of SOX3 at Xq26.3, in a 10-year-old girl affected by mild memory deficiency, strabism, speech impairment and hypotonia; because previous studies showed that female carriers of microdeletions involving IL1RAPL do not show intellectual disability [23,24], the phenotype was likely attributable to another gene in the duplicated region. Analysis of an independent family segregating X-linked intellectual disability demonstrated an in-frame duplication of 33 bp involving a polyalanine repeat of SOX3, thus pointing to SOX3 mutations as the cause of neurodevelopmental delay.

A sub-microscopic duplication of 685.6 kb at Xq27.1 involving SOX3, has been reported in two siblings affected by hypopituitarism and abnormalities of corpus callosum, while a duplication involving the SOX3 polyalanine repeat was identified in three male siblings from another family, segregating panhypopituitarism and abnormalities of the pituitary gland: all of these patients had absent infundibulum and did not present an intellectual disability [18]. A duplication of 3.9 Mb involving the Xq27 region containing SOX3, has been reported in males affected by X-linked hypopituitarism [25]. In a study of 16 SRY-negative 46,XX male patients, DNA microarray analysis showed genomic rearrangements of the SOX3 regulatory region in three patients, two duplications and one deletion: the CNVs involved genomic regions in close proximity of SOX3 in all three patients [26].

Interestingly, a recent report described a SRY-negative, 46,XX boy affected by ovotesticular DSD, with hypo-spadias and cryptorchidism with a de novo duplication of a 502 kb fragment of the long arm of chromosome X, involving SOX3, as well as RPS17P17, CDR and MIR 320D2. The role of the RPS17P17, CDR1 and MIR320D2 genes has not been investigated [27]. In summary, SOX3 genetic variants have been associated with X-linked intellectual disability with isolated growth hormone deficiency as well as X-linked panhypopituitarism and 46,XX sex reversal in males. Until now, no other developmental phenotypes have been associated to SOX3 gene dosage.

**CASE PRESENTATION**

We were consulted on a 11-year-old white Caucasian male for the findings of hypoplasia of the right kidney and coronal moderate hypospadias, after surgical correction of the urethra anomaly. He was the first child of a non-consanguineous couple. His parents and younger sister were healthy. His intelligence was normal (IQ 92) and he had no other anomalies. The behavior, growth and development were all normal. His testes volume was >4 mL and the penis length was 5 cm. Abdominal ultrasound and magnetic resonance imaging (MRI) did not show internal female genitalia, and confirmed right kidney hypoplasia (Figure 1, Table 1). The left kidney size was 80 x 32 mm, while the right kidney size was 57 x 23 mm.

The patient was investigated as part of a study approved by the institutional review board at our International Centre for genetic Engineering and Biotechnology in Skopje (Republic of Macedonia) and at the Department of Nephrology, Columbia University, New York, NY, USA. This patient was already reported as part of our prior study on copy number variations (CNVs) in kidney malformations [28].

An additional 23 patients were selected to perform targeted Sanger resequencing of SOX3. We selected 23 males affected by urinary tract developmental defects (10 renal hypoplasia; three vescicoureteral reflux; two posterior urethral valve; four obstructive uropathy; one bladder anomaly, one ectopic, one accessory kidney and one horseshoe kidney) and associated DSD (11 hypospasias, nine cryptorchidism, one epispadia and one congenital hidrocele).

**Endocrine Analysis.** Plasma concentrations of steroid hormones, comprising mineralocorticoids, glucocorticoids and androgens, were determined using UPLC Quattro Premier/Xe system (Waters, Milford, MA, USA) as previously described [29-31].

In brief, aliquots of plasma samples, calibrator and controls with a volume of 0.1 mL were combined with an internal standard mixture to monitor recovery. All samples were extracted using Oasis MAX SPE system Plates (Waters).
Table 1. Comparison of our patient characteristics with cases reported in the literature.

| Parameters | References | [26] | [26] | [26] | [27] | This Study |
|------------|------------|------|------|------|------|------------|
| Age (years) | Patient 1  | M-30 | M-19; M-26 (histology) | M-19 months | M-30 months | M-11 |
| Height     | Patient 2  | 165 cm | 167.5 cm | 75 cm | 87.8 cm (11.8 kg) | 148 cm (42 kg) |
| Penile size| Patient 3  | 10.2 cm long; 2.6 cm wide | 3.4 cm long | 32 mm long; 13 mm wide | 5 cm |
| Testicular size | Patient 4 | ~5 mL | ~6 mL | right testicle appear smaller than left testicle | 4 mL |
| Genitals and testes | Patient 5 | scrotal hypoplasia; retractile testes; histology; atrophic changes with loss of normal hypoplastic scrotum; spermatogenesis; thickening and hyalinization of the tubular basal lamina and diminished number of interstitial cells; normal spermatic cords | cryptorchidism; hypospadias | moderate coronal hypospadias |
| Secondary sexual characteristics | normal | Tanner stage 5 pubic hair and penile development with small testes; onset age 13 years | NA | NA |
| Developmental issues | gender dysphoria from 6 years; referred to behavioral therapist | microcephaly; developmental delay; growth retardation | none | crossdressing |
| CAKUT | – | – | – | – | hypospadias; kidney hypoplasia |
| Genetic alterations | two microduplications of ~123 and 85 kb, the former of which spanned the entire \( SOX3 \) gene | microdeletion; a single 343 kb immediately upstream of \( SOX3 \), suggesting that altered regulation of \( SOX3 \) is the cause of XX male sex reversal | a large ~6 Mb duplication that encompasses \( SOX3 \) and at least 18 additional distally located genes | de novo duplication (0.5 Mb) at Xq27.1 comprising \( SOX3 \), \( CDRI \) and \( MR320D2 \) | a unique 550 kb duplication involving \( SOX3 \), the non-coding RNA \( LINC00632 \), \( AK054921 \), \( CDRI \) and the miRNA \( MR320D2 \) |

NA: not available; CAKUT: congenital anomalies of the kidneys and urinary tract.

**Genetic Analyses.** After receiving informed consent, collected according to the Ethics Board of the Macedonian Academy of Sciences and Arts (Skopje, Republic of Macedonia), genomic DNA was obtained from peripheral blood samples using standard methods. Genome wide genotyping was conducted on patient MCD_13 using Illumina 610-Quad chip (Illumina Inc., San Diego, CA, USA) [32].

Copy number variation analysis was performed as previously described and data were compared to 21,575 multiethnic controls [28,33-35]. Briefly, genotype calls and quality-control analyses were conducted using GenomeStudio v.2010.3 (Illumina Inc.) and PLINK software [36]. Standardized genotyping methods implemented by the PennCNV program [37] were used for genome-wide CNV calls. The human reference genome hg18 (NCBI build 36.1, March 2006) was the reference assembly used to map the CNVs. The annotation of the CNVs was then performed using the UCSC RefGene and RefExon (CNVision program) [38].

Specific primers were designed to direct polymerase chain reaction (PCR) at the exon and exon-intron boundaries of \( SOX3 \), and bidirectional Sanger sequencing was performed by BigDye® terminator (Nimegen BV, Nijmegen, The Netherlands) reaction followed by a run on an automatic capillary DNA sequencer. Sequence and alignment was conducted using Sequencer 5.4 software (Gene Codes Corp., Ann Arbor, MI, USA).

An adreno corticotropic hormone (ACTH) test showed normal basal and stimulated 17OH-progesterone excluding a form of 46,XX DSD due to 21-hydroxylase deficiency. The 11-deoxycorticosterone (DOC) and 11-deoxycortisol were normal at both baseline and after ACTH stimulation, excluding 11-hydroxylase deficiency. Cortisol levels were in the mid-normal range at baseline and responded to stimulation, excluding primary adrenal insufficiency.
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SOX3 DUPLICATION IN CAKUT PATIENT

Figure 2. The 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 hCG (human chorionic gonadotropin) test found testosterone in the low-normal range for male sex and age at baseline. After stimulation, it raised up to 146.0 ng/mL indicating the presence of functional Leydig cells targeted by hCG. The stimulated ratio A:T was below 1, not supporting 17-β-hydroxysteroid dehydrogenase type 3 deficiency. The stimulated ratio T:DHT was 5.6, not supporting 5 α-reductase insufficiency. Microarray-based copy number analysis was previously performed in this patient as part of a larger study on congenital kidney defects [28].

In our 11-year-old male patient affected by renal dystrophy (RHD) and DSD (MCD_13), the microarray analysis showed an unique duplication of about 550 kb of the chromosome region Xq27, involving multiple genes and transcripts: SOX3, RP1-177G6 and CDR1, the non coding RNA LINC00632, and the miRNA MIR320D2 [28] (Figure 2). None of the genes within the duplication locus has previously been reported to be in association with kidney and urinary tract phenotypes [39,40]. The chromosomal microarray analysis confirmed the 46,XX female karyotype. Parental DNA material was not available to test segregation; therefore, we could not verify if the Xq27 duplication was a de novo or inherited genomic imbalance. No causal mutations were detected in the 23 male patients selected for targeted resequencing indicating that SOX3 coding variants might be a very rare cause of urinary tract malformations associated with DSDs.

DISCUSSION

There are four cases reported with SOX3 duplications in 46,XX SRY-negative males in the literature [26,27,41] (Table 1). Two of the 46,XX male patients, 30 and 26 years old, respectively, reported by Sutton et al. [26], had normal intelligence and growth; the third one had developmental delay, growth retardation and microcephaly. The patient described by Grinspon et al. [27] had normal growth and intelligence, but was affected by hypospadias and cryptorchidism, with ovotestis and hypoplastic testes. Histology analysis showed atrophic changes and loss of normal spermatogenesis. Our patient’s clinical phenotype was characterized by normal development and intelligence, DSD characterized by hypospadias and males genitalia with 46,XX karyotype, and, unique compared to all other reported patients in the literature, hypoplasia of the left kidney. Interestingly, our patient, as well as the patient described by Grinspon et al. [27], both with karyotype 46,XX SRY-negative, were characterized by duplications involving the Xq27, encompassing the same genes: SOX3, the non coding RNA LINC00632, AK054921, CDR1 and the miRNA MIR320D2.

The question is whether the kidney defect observed in our patient is biologically related to the duplication of SOX3 or the other genes in the CNV, or if it represents a coincidental finding. Analysis of publicly available expression data (www.gudmap.org) indicates high expression of Sox3 in the mouse developing bladder neck at embryonic day E13.5, thus suggesting a possible link to lower urinary tract malformations. The SOX3 gene is known to be regulated by PBX1 through direct interaction with its transcription binding site [42]. Interestingly, another patient with renal hypoplasia from our cohort, was found to carry a de novo 0.51 kb deletion affecting PBX1 [28]. Inactivation of Pbx1 in the mouse results in urinary malformations including renal agenesis and hypoplasia [43]. Finally, a recent report implicates haploinsufficiency of PBX1 in the pathogenesis of syndromic forms of congenital anomalies of the kidney and urinary tract [44].

These data provide plausible links between SOX3 gene dosage and kidney malformations. Formal proof of a causal link will require additional genetic and functional data. It is noteworthy that the current and reported SOX3 duplications are below the detection threshold of standard karyotype and were found only by analyzing CNVs using
DNA microarrays. Therefore, it is important to convey that all 46,XX SRY-negative males should be screened for SOX3 duplications with DNA microarrays.

We report a case of an 11-year-old male with a duplication of chromosome Xq27, involving SOX3, and leading to a male sex reversal and, possibly, kidney hypoplasia. This is the second case of 46,XX SRY-negative affected by DSD and characterized by CNV involving the SOX3 locus, described so far. We speculate that the genomic duplication involving SOX3 could be responsible not only for pituitary hormone deficiencies in humans and male sex reversal, but also for CAKUT. All 46,XX SRY-negative patients, should be screened for duplications affecting SOX3.

Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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