A multi-exon deletion within *WWOX* is associated with a 46,XY Disorder of Sex Development.

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Running title: WWOX and Disorders of Sex Development

Abstract

Disorders of sex development (DSD) are congenital conditions where chromosomal, gonad or genital development is atypical. In a significant proportion of 46,XY DSD cases it is not possible to identify a causative mutation, making genetic counseling difficult and potentially hindering optimal treatment. We present here the analysis of a 46,XY DSD patient that presented at birth with ambiguous genitalia. Histological analysis of the surgically-removed gonads showed bilateral undifferentiated gonadal tissue and immature testis, both containing malignant germ cells. We screened genomic DNA from this patient for deletions and duplications using an Illumina whole genome SNP microarray. This analysis revealed a heterozygous deletion within the *WWOX* gene on chromosome 16, removing exons 6-8. Analysis of parental DNA showed that the deletion was inherited from the mother. cDNA analysis confirmed that the deletion maintained the reading frame, with exon 5 being spliced directly onto exon 9. This deletion is the first description of a germline rearrangement affecting the coding sequence of *WWOX* in humans. Previously described *Wwox* knockout mouse models showed gonadal abnormalities, supporting a role for WWOX in human gonad development.

Key words: Disorders of Sex Development, copy number, WWOX, gonad, microarrays.

Introduction

In the early stages of human embryogenesis the developing gonads are bipotent, being capable of forming either testes or ovaries. In males the expression of the Y chromosomal *SRY* gene initiates testis development, while ovarian development in

principle occurs only in the absence of *SRY* (reviewed in [1]). Following the establishment of sex-specific expression of key regulatory genes in the gonad, gonadal differentiation results in develoment of the external genitalia. As a result there are two main stages in gonad formation, and disruption of either can lead to disorders of sex development (DSD). DSD are surprisingly common, with ambiguous genitalia estimated to occur with an incidence of 1 in 4500 live births [2].

A number of genes important in the regulation of sex determination have been identified, yet in as many as 70% of 46,XY DSD cases no genetic cause has been identified. We have previously demonstrated the power of whole genome copy number analysis with high density microarrays to identify causative mutations in DSD [3, 4]. Here we describe the use of this approach to identify a multi-exon heterozygous deletion in the *WWOX* gene of a 46,XY DSD patient.

Materials and Methods

Array hybridization and analysis

Genomic DNA was hybridized onto an Illumina 610-Quad microarray at the Australian Genome Research Facility (Melbourne, Australia) following manufacturer's instructions. Data were analyzed using Genome Studio data analysis software (Illumina).

MLPA analysis

Deletion screening of the *WWOX* gene was performed with Multiplex Ligation-dependent Probe Amplification (MLPA). Probe design, the MLPA reaction and data analysis were performed as previously described [5].

RNA extraction, cDNA generation and breakpoint PCR

RNA was extracted from lymphocytes obtained from the index case using standard procedures, with cDNA generated using random hexamers and the Transcriptor High Fidelity cDNA synthesis kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The PCR amplification across the deletion used the following primers; F – 5' CGAAACCGCCAAGTCTTTT 3', R – 5' CGTCTCTTCGCTCTGAGCTT 3', and was run under the following conditions:

1 cycle: 60 seconds 95°C; 35 cycles: 30 seconds 95°C; 30 seconds 58°C; 60 seconds 72°C; 1 cycle: 20 min 72°C.

DNA Sequencing

Sanger sequencing was conducted at the Department of Pathology, University of Melbourne.

Histological and Immunohistochemical Analysis

Research on human tissue samples was performed according to the Code for Proper Secondary Use of Human Tissue in The Netherlands, as developed by the Dutch Federation of Medical Scientific Societies (FMWV) version 2002, and approved by an institutional review board (MEC 02.981). Immunohistochemical detection of formalin-fixed paraffin-embedded tissue was performed for SOX9 and FOXL2 [6], OCT3/4 [7], and KITL [8], as described previously.

Results

Physical examination of the index patient at the age of ten days revealed unfused labioscrotal folds, impalpable gonads, clitoral hypertrophy 20mm in size, and a perineal urogenital sinus. Genitography demonstrated the presence of a vagina and underdeveloped uterus. Chromosomal analysis showed a 46,XY karyotype with no

visible aberrations. Sequence analysis of the *SRY* and *NR5A1* genes did not reveal any variants, and MLPA analysis with a commercially available kit (MLPA P185-B1) containing probes targeted at the *WNT4, SRY, NR0B1, SOX9* and *NR5A1* genes did not show any deletions or duplications.

Small dysgenic gonads were present in the abdomen, and pathological analysis following complete removal at two years of age showed that they contained oedematous infantile testicular parenchyma (Figure 1). The epididymis was completely separated from the rete testis on both sides, and tubair epithelium was identified at the left side. The left gonad consisted of centrally located oedematous testicular tissue, positive for immunohistochemical detection of SOX9 (indicative for Sertoli cells) and negative for FOXL2 (a granulosa cell marker). A gradual transition towards undifferentiated gonadal tissue, containing both SOX9 and FOXL2 positive cells at the upper and lower poles were identified. Undifferentiated gonadal tissue is a gonadal pattern found specifically in patients with gonadal dysgenesis and is typically characterized by the combined expression of FOXL2 and SOX9 (usually with a preponderance of FOXL2), suggesting limited differentiation of the supportive cell lineage into pre-granulosa and pre-Sertoli cells[9]. Immature OCT3/4-positive germ cells were also found, either dispersed in ovarian-like stroma or organized together with Sertoli/granulosa cells in cord-like structures, reminiscent of sex cords. These structures have been recognized as the precursor lesion for gonadoblastoma[10].

In spite of the presence of ovarian-like stroma, no ovarian follicles were present, and therefore the histology did not allow the diagnosis of ovarian differentiation. The right gonad displayed a similar morphology, with predominant, SOX9 positive testicular differentiation, except for the upper pole, where a large area of FOXL2 positive

undifferentiated gonadal tissue was seen, also containing scarce SOX9 positive cells. Based on morphological criteria and immunohistochemical analysis for OCT3/4 and TSPY (showing co-expression of both markers), presence of pre-malignant germ cells was diagnosed. The co-expression is assumed to identify the earliest pathogenetic changes in the genesis of malignant germ cell tumors. That the germ cells are indeed transformed was confirmed by staining for KITL. A limited hormonal work-up was carried out in the neonatal period, with normal values for ACTH, cortisol and 17 hydroxy progesterone. At 28 weeks, serum values were obtained for LH and FSH and were 1.0 and 5.2 U/l respectively. Although FSH levels were not measured prior to this, the relatively high value at this age suggests a higher value (in the range of gonadal dysgenesis) during full mini-puberty. A HCG test performed at 18 months shows a moderate rise of testosterone (testosterone 231 ng/dl after HCG, 500U, 2x/week for 3 weeks).

Microarray analysis did not reveal deletions or duplications covering any genes already known to be involved in DSD. The most promising candidate rearrangement was a 767 kb deletion on chr 16 (chr16:76956767-77723905; hg18) (Figure 2a). This deletion was located within the *WWOX* gene, and removed exons 6-8. MLPA probes for each of the nine *WWOX* exons were designed, and MLPA analysis confirmed that the deletion was restricted to these three exons (data not shown). The deletion is predicted to generate an alternative, in-frame mRNA product, with exon 5 being spliced directly onto exon 9. This alternative transcript was verified by sequencing cDNA derived from matched lymphocytes (Figure 2b).

MLPA analysis of parental DNA samples revealed that the deletion was maternally inherited, with no evidence of mosaicism [11]. The clinical history of the mother included a relatively late menarche (16 yrs) with irregular menstruation up to the first pregnancy. Prior to this pregnancy she had received hormonal stimulation, but this was stopped prior to conception. Four subsequent pregnancies occurred naturally. DNA analysis of the children (two brothers and two sisters, all unaffected) showed that none had inherited the deletion. Analysis of the *WWOX* gene in the index case did not show any sequence variants.

Discussion

We report here the first germline rearrangement affecting the coding sequence of the *WWOX* gene. *WWOX* consists of 9 exons, and is >1 Mb in size. It is located on the long arm of chromosome 16 in a known fragile site frequently rearranged in a wide range of cancers [12], and is a suspected tumour suppressor gene [13].

WWOX encodes a 414 aa, 46 kDa protein [14]. At the amino terminus there are two WW domains, with a short chain oxidoreductase (SDR) domain within the central portion of the protein [15]. The WW domains are believed to be involved in protein-protein interactions, including a number of transcription factors [16, 17]. The SDR region is thought to play a role in steroid metabolism [18], with the exon 6-8 deletion identified in this study maintaining the reading frame but effectively ablating the SDR domain from the predicted protein (Figure 2c).

Two different *Wwox* knock-out mouse models have show gonadal abnormalities, including defects of Leydig cell function in the testis [18, 19]. There are various possible mechanisms for WWOX-mediated testicular dysfunction. WWOX expression inhibits

the Wnt/β-catenin pathway in a dose dependent manner, although this inhibition is reduced if the SDR domain is removed [20]. The Wnt/β-catenin pathway is involved in normal ovarian development, and increased Wnt/β-catenin activity in the developing gonad through compromised WWOX activity may interfere with normal testis development. Indeed, expression of a stabilized form of β-catenin in the somatic cells of XY mice leads to male to female sex reversal [21].

WWOX expression is high in endocrine tissues such as pituitary, testis and ovary [22], and it may have a role in gonadotrophin or sex-steroid biosynthesis. It has been suggested that a truncated WWOX protein consisting of both WW domains but without a functional SDR domain may act as a competitor of the full-length protein. The truncated WWOX could bind to WWOX-interacting proteins via the WW domains, but would not have any SDR activity [20].

Although the mother of the index case carried the same deletion there was a milder gonadal phenotype, with late menarche. An increase in activity of the Wnt/β-catenin pathway due to reduced WWOX activity would not necessarily be expected to have an effect on ovarian development, as this pathway is already active during this process. There may however be a disturbed ovarian function due to impaired steroidogenesis, as was observed in one of the mouse models [18].

In conclusion, we have identified a multi-exon deletion in *WWOX* in a 46,XY DSD patient with ambiguous genitalia. This finding is the first germline rearrangement affecting *WWOX* coding sequence described in humans, and implicates *WWOX* in normal gonadal development.

Acknowledgements

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Conflict of Interest Statement

The authors declare no conflict of interest.

Figure 1. Representative histological and immunohistochemical findings for the left gonad: A) total overview of the gonad histology (H & E); B) higher magnification (2.5x magnification, indicated by square in A); immunohistochemical detection of C) SOX9, positive in the Sertoli cells; D) FOXL2, positive in the granulosa cells; E) OCT3/4; F) TSPY; G) KITL, all positive in the transformed germ cells. All immunohistochemical images are at the magnification of 100x, except G, being 50x.

Figure 2. Molecular analysis of DNA from the 46,XY DSD patient. (A). The deletion identified by microarray analysis, removing 767 kb of genomic DNA on chromosome 16. Data are plotted along the chromosome, with each point representing the copy number estimate of an individual probe. The breakpoints of the deletion are shown by the broken vertical lines. (B) PCR analysis of the deletion. cDNA was derived from lymphocytes of the index patient, and primers were designed to amplify a PCR product across the predicted breakpoint. Sequence analysis shows that exon 5 is spliced directly onto exon 9. (C) Effect of the deletion on the WWOX protein. The full length WWOX protein has two WW domains at the N-terminal, and a short chain oxidoreductase (SDR) domain at the C-terminal. A deletion of exons 6-8 is predicted to result in an in-frame but shortened product, with the SDR domain largely missing.

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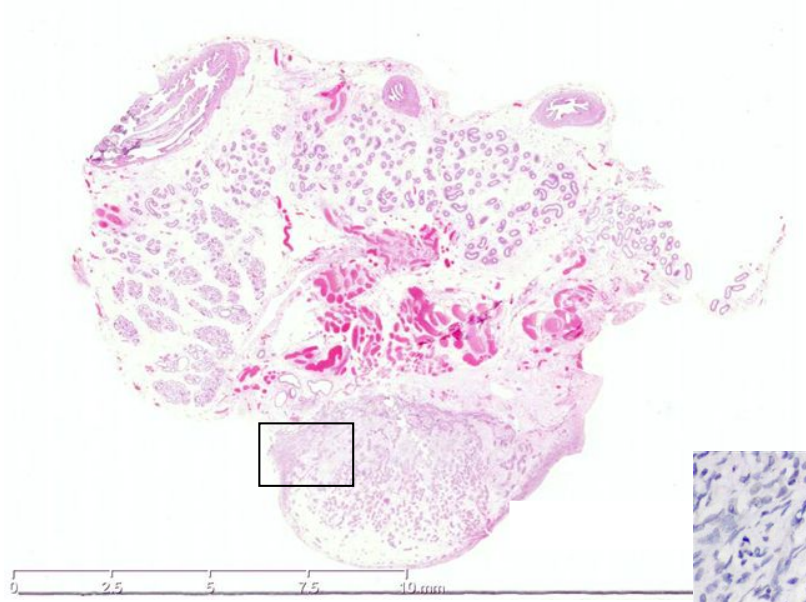

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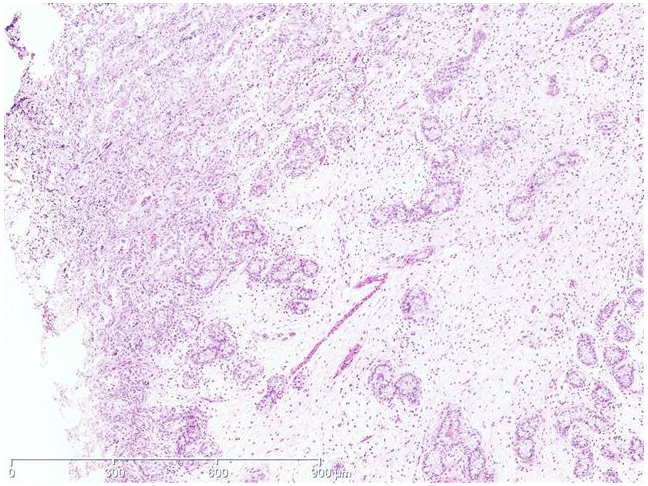

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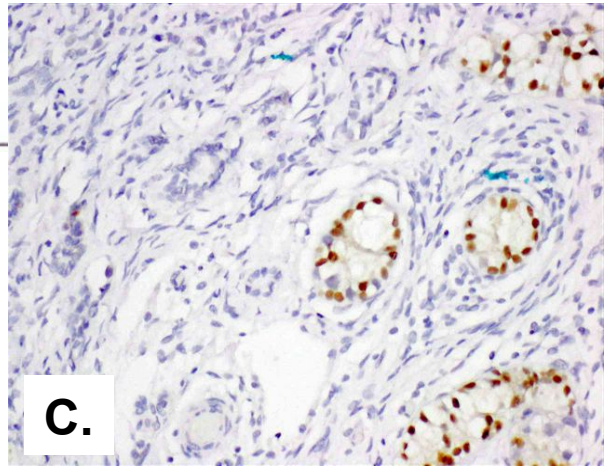

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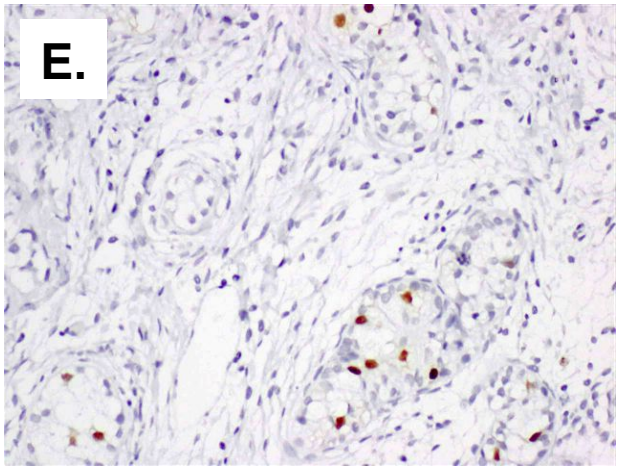

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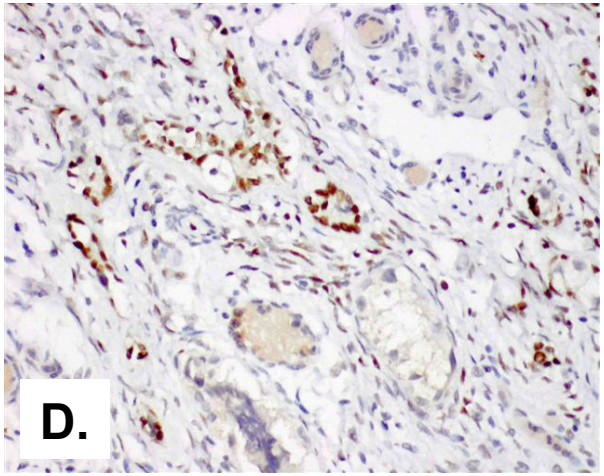

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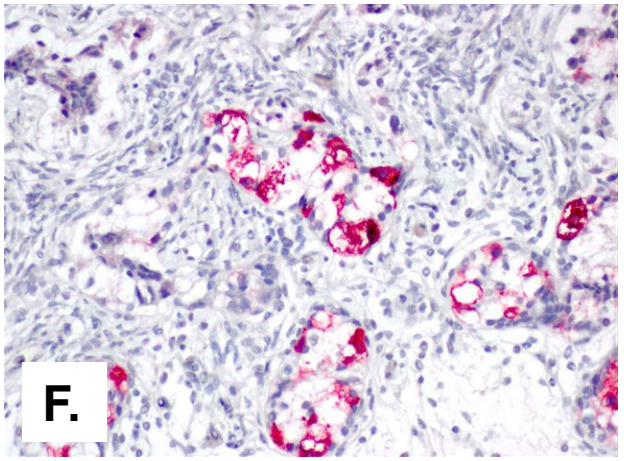

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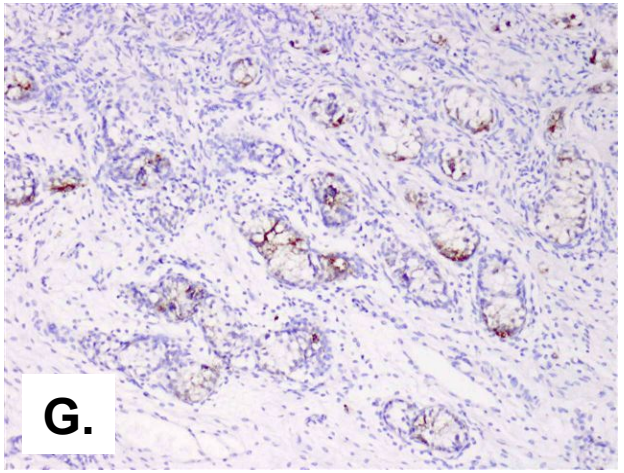

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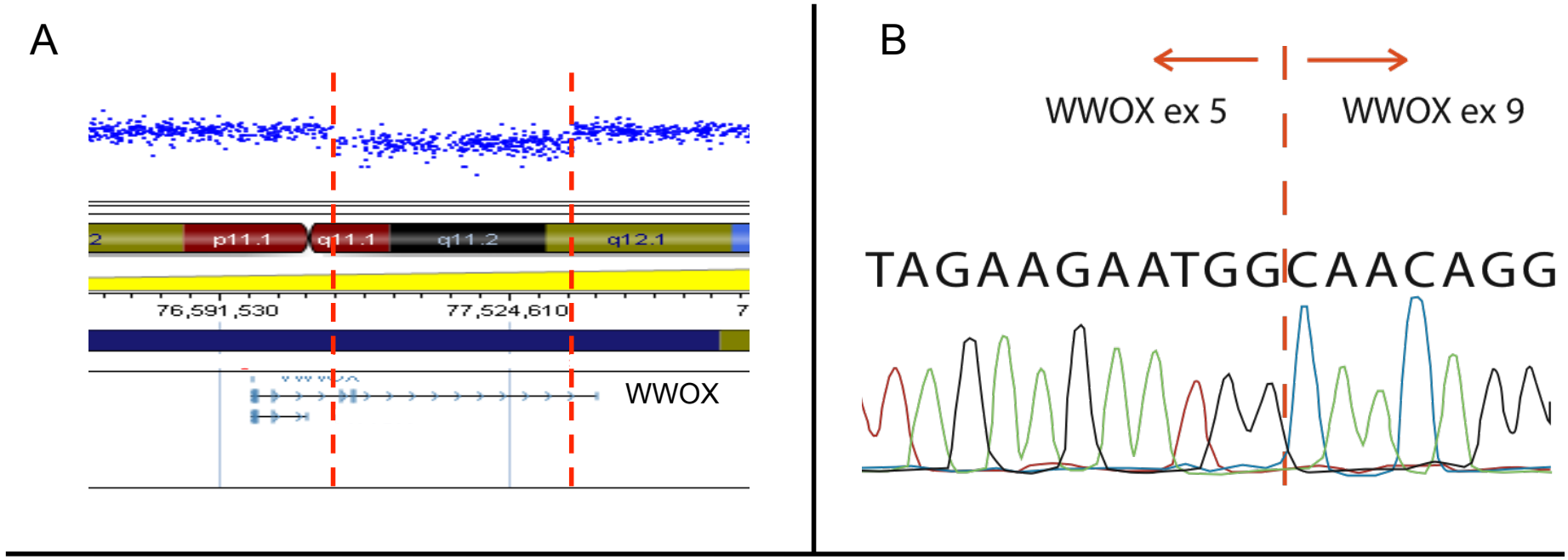
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WWOX
B
WWOX ex 5
WWOX ex 9
TAGAAGAATGGCAACAGG
C
WW domains
SDR domain
a)
Full-length WWOX
b)
WWOX lacking
exons 6-8

Figure 2