

A Congenital Diaphragmatic Hernia and 46,XX Disorder of Sex Development Caused by a *WT1* Pathogenic Variant

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Abbreviations:

DSD: Disorder of Sex Development; CDH: Congenital Diaphragmatic Hernia; GCT: Germ Cell Tumour

1. Abstract

WT1 is an important gene in gonadal differentiation process, especially in the male differentiation. It is known in some syndromic and non-syndromic pathology. This gene is associated with under-virilization in 46,XY patient. Here we report a 46,XX case presenting with external genital virilization, diaphragmatic hernia and wilms tumor. A screening on a Next Generation Sequencing (NGS) panel of 11 genes involved in 46,XX disorder of sex development (DSD) revealed the heterozygous *de novo WT1* nonsense variant NM_024426.4 c.1468C>T p.(Gln490*). A gain-of-function effect

seems to be predicted for his variant affecting the fourth zinc finger region of the protein, as previously described. This case report confirms the implication of *WT1* gene in 46, XX DSD and expands its phenotype with the association of diaphragmatic hernia.

2. Introduction

Most of 46,XX disorder of Sex development (DSD) are due to androgen excess, with congenital adrenal hyperplasia as the main cause. Less frequently, 46,XX DSD are associated with disorder of gonadal development such as ovotesticular DSD or a genetic involvement caused by the translocation of *SRY* or a *SOX9* du-

plication [1, 2]

Concerning other determining genes, *WT1*, gene [MIM*607102] (Wilm's tumor suppressor gene 1), located at chromosome band 11p13, is involved in various embryological process stages such as in early gonadal differentiation [3], kidney formation [4] and diaphragm development formation [5]. Heterozygous *WT1* variants have either been reported in individuals with nonsyndromic Wilms tumor [MIM#194070] [4] or nephrotic syndrome type 4 [MIM#256370], and in association with syndromic forms in Frasier Syndrome [MIM#136680] or Denys-Drash Syndrome [MIM#194080]. Moreover, the contiguous gene deletion at 11p13 is well-known as WAGR Syndrome [MIM#194072], causing the association of Wilms tumor, aniridia, genitourinary anomalies and mental retardation syndrome (aniridia due to *PAX6*, other features probably due to *WT1*).

Interestingly, two descriptions of 46, XX DSD associated with *WT1* variants were recently reported. The first case identified a missense variant by whole exome sequencing in a 46, XX case presenting with a syndromic form with male external genitalia, dysgenic testis, microcephaly and small uterus [6]. Secondly, Gomes *et al* identified a frameshift variant by target massively parallel sequencing in a 46, XX girl with atypical genitalia characterized by clitoromegaly, single perineal opening, short blind-ending vagina, and bilateral testes with seminiferous tubules [7]. In both cases, *WT1* variants affected the fourth zinc-finger DNA-binding domain of the WT1 protein.

Here, we present an original case of a 46, XX DSD borned with Congenital diaphragmatic hernia (CDH) with genital virilization in which we identified a novel heterozygous *WT1* variant.

3. Materials & Methods

This study was designed in compliance with the tenets of Helsinki declaration and informed consent was obtained for all individuals included.

Molecular analysis consisted on a Massive Parallel Sequencing (MPS) panel of 11 genes involved in 46,XX DSD. Pathogenic and probably pathogenic variants were confirmed by Sanger analysis.

3.1. MPS Methods:

Custom capture probes were designed with SeqCap EZ Choice and NimbleDesign software (Roche, USA) targeting 11 genes involved in 46,XX DSD (more informations on demand). A library of all coding regions +/- 50 bp was prepared using the Kapa Nimblegen (Roche, USA) following the manufacturer's instructions. Paired-end 2X150-bp sequencing was performed on a NextSeq 500 (Illumina, San Diego, CA, USA). Sequence alignment to the human reference genome (hg19) and variants call and annotation were performed using an in-house bioinformatic pipeline Variant

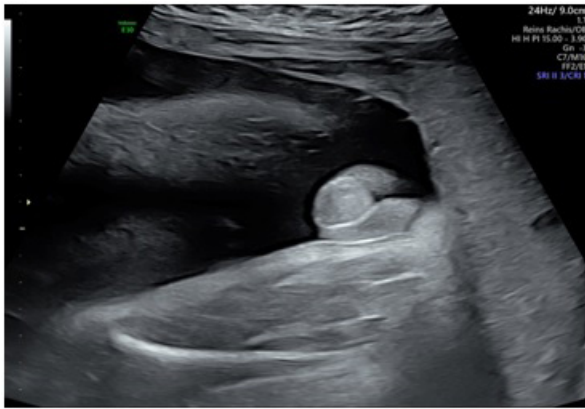
classification followed ACMG recommendations [8].

3.2. Sanger Methods:

A PCR amplification was performed, then PCR fragments were bidirectionally sequenced by capillary electrophoresis (3730xl sequencer, SeqScape software, Life Technologies). Sequence variations were numbered with the Adenine of the ATG initiation codon considered as the first nucleotide (NM_024426.4).

4. Results

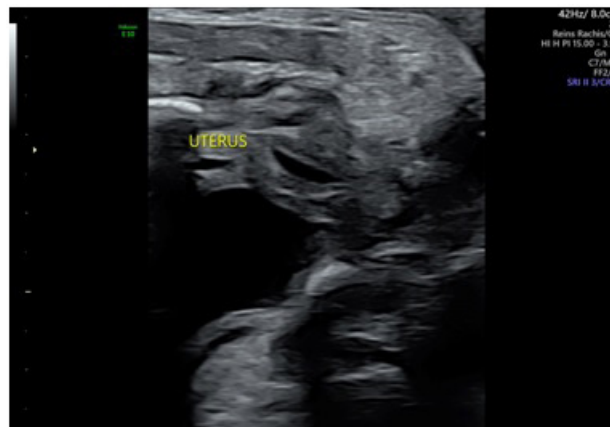
The case is a one-year-old girl, the second child of unrelated parents. During pregnancy, ultrasound sonography revealed a left diaphragmatic hernia at 22 weeks of amenorrhea, then associated with a clitoridomegaly and an uterus (Figure 1). An amniocentesis was performed and showed antenatal karyotype was 46, XX. She was born after at 39 weeks of gestation with a weight of 3020 gr. Rapid surgical treatment of her hernia was done on day two, with succinate hydrocortisone in order to avoid a potential adrenal insufficiency. Post-operative recovery was uncomplicated. On clinical examination, she presented with a clitoromegaly Prader 2 characterized by a 2.5 cm erectile genital bud, a misplacement of the urinary meatus too low implanted and a single short urogenital opening (Figure 2), with a 5 mm common channel. Ultrasonography and MRI revealed a right hemi-uterus (16 x 7,5mm) without any ovarian structure but composed with an endometrial line, and a renal asymmetry (44 mm for the right one vs 30 mm for the left one). Biological testing at day 1 showed elevated testosterone (105 ng/dL) and normal adrenal hormones without adrenal insufficiency. Mini-puberty occurred between the first and the fifth months of life. At one month of age, elevated testosterone (140 ng/dL) was detected, with elevated inhibin B (109 pg/mL) levels, low estradiol (11 pg/mL) and low anti-mullerian hormone (11 ng/mL). Testosterone level varied from 159 ng/dL at three months to 63 ng/dL at five months. A male hormonal profile was confirmed by testosterone and inhibin levels showing persistent Leydig cells and Sertoli cells secretion (Table 1). Renal function was unremarkable without proteinuria. Control postnatal karyotype was 46,XX, with no evidence of *SRY* translocation and no chromosomal imbalance detected on the array-CGH. A screening on a Next Generation Sequencing (NGS) panel of 11 genes involved in 46,XX DSD revealed the heterozygous *de novo WT1* nonsense variant c.1468C>T p.(Gln490*). A right-kidney nephroblastoma was discovered at the age of three months, and treated by chemotherapy and nephrectomy. After an expert national meeting consensus, two decisions were made: first to keep the female gender assignment accepted by the family, and secondly not to perform surgery on the gonads for the moment. A right streak gonads with fallopian tube and uterine hypoplasia was detected during the nephrectomy laparoscopy.



1a. Sagittal section centered on clitoral hypertrophy, 30 amenorrhea week



1b. 3D representation of the external genitalia, 30 amenorrhea week



1c. Sagittal section centered on the uterus, 32 amenorrhea week

Figure 1: Ultrasound sonography

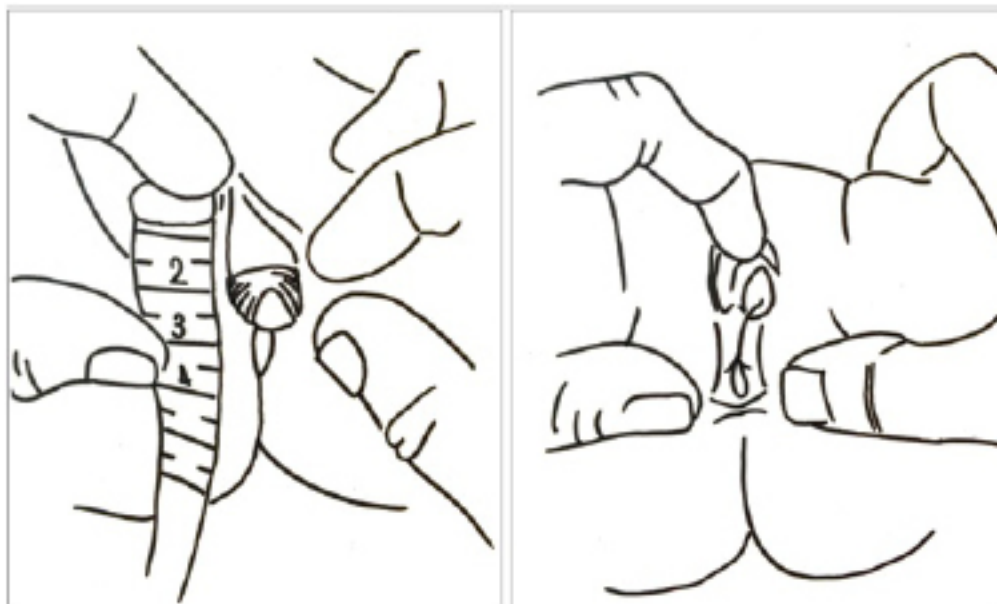


Figure 2 : Representation of the external genitalia

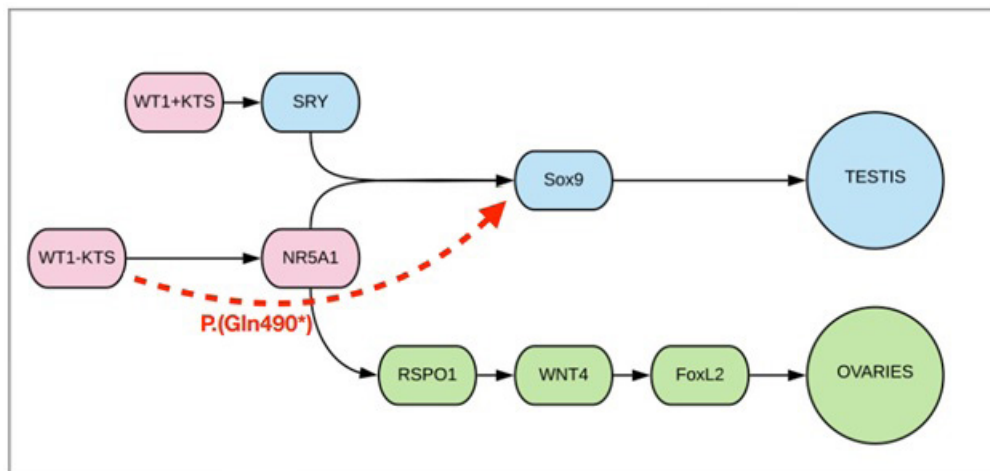


Figure 3 : Schematic overview of gonadal differentiation signalling pathway the red arrow represents the hypothetical mechanism of diversion from the normal pathway by the *WT1* nonsense variant c.1468C>T p.(Gln490*)

5. Discussion

Here we identified a novel heterozygous de novo variant in *WT1*: c.1468C>T p. (Gln490*) in a 46,XX DSD child with diaphragmatic hernia. This nonsense variant was never reported in the literature and was absent from GnomAD. According to the ACMG guidelines [8], this variant can be considered as pathogenic (PVS1, PM2, PP3). A gain-of-function effect seems to be predicted as proved by *in vitro* and *in silico* studies of protein interaction and stability [7, 9].

WT1 gene [MIM*607102] (Wilm's tumor suppressor gene 1), located at chromosome band 11p13, is involved in various embryological process stages such as in early gonadal differentiation [3], kidney formation [4] and diaphragm development [5].

WT1 is a 50 kb gene encoding for a four-zinc finger DNA-binding protein with 10 exons and various isoforms with two majors due to the insertion of three amino acids (KTS) between fingers three and four, named as -KTS or +KTS isoforms [10].

Hammes *et al.* generated mice lacking those specific isoforms of *WT1*. Heterozygous mice with reduced +KTS protein levels developed a glomerular syndrome and presented a model for Frasier syndrome. Each type of homozygous mice died early at birth due to impaired renal development. Interestingly, mice lacking the +KTS isoform showed complete XY reversion by reduced SRY expression. Lack of -KTS isoforms resulted in a more severe developmental phenotype than loss of +KTS isoforms [11].

WT1 has a key role in gonad differentiation, by the early enabling activation and maintenance of *NR5A1* through the *WT1*(-KTS) isoform [3, 12]. At the same time, *WT1*(+KTS) activates *SRY* expression [13]. SF1 and SRY act as cofactors to activate the transcription of *Sox9*[14]. Activating this pathway leads to male gonadal differentiation. Comparatively, *WNT4* and *RSPO1* play a

role in the activation of female pathway in XX gonads. [15, 16]. In the now three different cases of 46,XX DSD with *WT1* variant affected the fourth zinc finger, we describe the activation of male gonadal pathway. (Figure 3)

Heterozygous *WT1* variants have either been reported in individuals with non-syndromic Wilms tumor [MIM#194070] or nephrotic syndrome type 4 [MIM#256370], and in association with syndromic forms in Frasier Syndrome [MIM#136680] (gonadal dysgenesis and focal segmental glomerulosclerosis) [15], Denys-Drash Syndrome [MIM#194080] (gonadal dysgenesis and diffuse mesangial sclerosis)[16] or Meacham syndrome [MIM#608978] (gonadal dysgenesis, cardiac malformation, and diaphragmatic defect with pulmonary hypoplasia) [17]. Moreover, the contiguous gene deletion at 11p13 is well-known as WAGR Syndrome[MIM#194072], causing the association of Wilms tumor, aniridia, genitourinary anomalies such as hypospadias or cryptorchidism for boys and vaginal, uterine or ovarian abnormalities for girls and mental retardation syndrome (aniridia due to *PAX6*, other features probably due to *WT1*) [5, 18]. All of these syndromes are linked with *WT1* decreased expression and lead to a male virilization defects even to complete sex reversal comparatively to no effect on female gonadal development.

During mammalian embryonic development, *WT1* is expressed in both pleural and abdominal mesothelium which contribute to the diaphragmatic formation [5]. The link between diaphragmatic hernias and *WT1* variants was already described [19]. Homozygous *WT1* null-mice who died early at birth had diaphragmatic hernias and urogenital defects [20]. The association of a gonadal dysgenesis with diaphragmatic hernia must therefore lead to a screening of *WT1*.

At the moment, the child does not have gonadoblastoma but his

gonads need close monitoring because some DSDs are associated with a high risk of cancer. Rathered together, the prevalence of germ cell tumour (GCT) in DSDs is 12% [21]. Known risk factors for GCT cancer are: cryptorchidism (RR x 2.9), presence of streak gonads, familial predisposition, presence of the Gonadoblastoma-Y-locus region on the Y chromosome and more specifically the *TSPY* gene [22, 23]. On out of 292 DSD XY studied, 15% developed GCT, of which 51% were malignant. Average age at diagnosis is between 14 to 21 years [24]. Incomplete testicular differentiation with delayed maturation or blockage of germ cells is also associated with an excess risk of GCT. This can be assessed by immunohistochemistry with the presence of the OCT 3/4 protein [21, 22]. Frasier syndrome seems to have the highest risk of degeneration, with a 67% of GCT risk apparition. They appear around the age of 12[15]. In both 46,XX Denys-Drash or Frasier syndromes, no GCT was reported. In Gomes case, the presence of bilateral testes with seminiferous tubules containing predominantly Sertoli cells and rare germ cells was confirmed; no gonadoblastoma was encountered [7].

Two tools can be assessed for the risk of degeneration. On one hypothesis, testicular germ cell-derived tumours (TGCTs) in humans have a highly different gene expression profile and a specific epigenetics regulation from normal germ cells residing in adult testes [25]. Kristensen *et al* [26] proposed the exploration of DNA methylation profiles as a predictive risk of GCT. Voorhoeve *et al* [27] showed that some microRNAs were overexpressed in all Germ Cell Cancer including carcinoma *in situ*. The detection of these microRNAs, especially miR-371-3 and miR-302, could be used as biomarkers in the screening and monitoring of GCT [28-30], as serum sensibility detection is high, at 98%[30]. This analysis could be then interesting to perform in our family.

6. Conclusions

This case report confirms the implication of *WT1* gene with the third description of a predicted gain-of-function variant in a 46, XX DSD child and expands its phenotype with the association of diaphragmatic hernia.

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