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SHORT REPORT

A 46,XX testicular disorder of sex development caused by a Wilms' tumour Factor-1 (*WT1*) pathogenic variant

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Molecular diagnosis is rarely established in 46,XX testicular (T) disorder of sex development (DSD) individuals with atypical genitalia. The Wilms' tumour factor-1 (WT1) gene is involved in early gonadal development in both sexes. Classically, *WT1* deleterious variants are associated with 46,XY disorders of sex development (DSD) because of gonadal dysgenesis. We report a novel frameshift *WT1* variant identified in an SRY-negative 46,XX testicular DSD girl born with atypical genitalia. Target massively parallel sequencing involving DSD-related genes identified a novel heterozygous *WT1* c.1453_1456del; p.Arg485Glyfs*14 variant located in the fourth zinc finger of the protein which is absent in the population databases. Segregation analysis and microsatellite analysis confirmed the *de novo* status of the variant that is predicted to be deleterious by *in silico* tools and to increase WT1 target activation in crystallographic model. This novel and predicted activating frameshift *WT1* variant leading to the 46,XX testicular DSD phenotype includes the fourth zinc-finger DNA-binding domain defects in the genetic aetiology of 46,XX DSD.

KEYWORDS
atypical genitalia, disorder of sex development; 46,XX testicular, WT1

1 | INTRODUCTION

In 46,XX individuals, there is a subset of patients born with male external genitalia or atypical genitalia because of the presence of testes or ovotestes, named as 46,XX testicular (T) and ovotesticular (OT) disorder of sex development (DSD), respectively. Most 46,XX T-DSD are caused by a gain-of-function in key testicular pathway genes.[1,2] Approximately, 90% of cases are caused by translocation of

SRY onto the X chromosome, particularly in patients with male external genitalia. In contrast, molecular diagnosis is rarely established in 46,XX OT-DSD and 46,XX T patients with atypical genitalia.[1] The genetic cause of SRY-negative 46,XX T-DSDs is related to *SOX9* upregulation, most commonly resulting from its duplication.[1]

WT1 encodes a DNA-binding protein containing four zinc fingers,[3] which is essential for normal mammalian urogenital development.[4] Classically, its pathogenic variants are associated with anomalies of testis development, leading to 46,XY DSD.[5,6] Here, we report a novel heterozygous *WT1* variant in a girl with 46,XX T-DSD.

2 | SUBJECTS AND METHODS

2.1 | Case report

A 1-year-old Brazilian girl was born after an uneventful pregnancy with atypical genitalia characterised by clitoromegaly, a single perineal opening, and a short blind-ending vagina. She was the second child of healthy and nonconsanguineous parents. A G-banded karyotyping analysis of 100 peripheral blood lymphocytes revealed a 46,XX karyotype. She underwent feminising genitoplasty and laparoscopy, which confirmed the presence of a hemiuterus. Biopsy of both abdominal gonads revealed testicular tissue with seminiferous tubules containing Sertoli cells but not germ cells. At 10 years of age, elevated basal follicle-stimulating hormone levels were detected (16 IU/L) with low basal estradiol (<1.83 PMol/L) and testosterone (2.08 nmol/L) levels. Gonadotropin-releasing hormone depot stimulation (aGnRH) test showed elevation of testosterone levels up to 5.65 nmol/L without an increase in estradiol levels (26.7 PMol/L) at 24 hours after injection.

She underwent bilateral gonadectomy, which confirmed the presence of bilateral testes with seminiferous tubules containing predominantly Sertoli cells with rare germ cells. An immature right uterine tube was also identified. Fluorescence in situ hybridization with *SRY*-specific probes excluded *SRY* translocation. The karyotypes of the gonads were also 46,XX.

2.2 | Genetic analysis

All research procedures followed the tenets of the Declaration of Helsinki, were approved by the local Ethics Committee with informed consent prior to genetic testing.

We designed an amplicon-based capture panel against exonic regions of 63 genes known to be associated with human DSDs and also candidate genes for DSD etiology[7] (see Table S1, Supporting Information).

Target sequences were captured using a custom Sure Select Target Enrichment System Kit (Agilent Technologies, Santa Clara, California). Sequencing was performed on the Illumina MiSeq platform (San Diego, California) (see Supporting Information).

Sanger sequencing confirmed the potentially pathogenic variant identified by targeted massively parallel sequencing and for segregation analysis. The identified variant was classified according to the American College of Medical Genetics (ACMG) criteria.[8]

Microsatellite analysis with 5 markers (deCODE, Généthon, and Marshfield) was performed to assess disease haplotypes in the

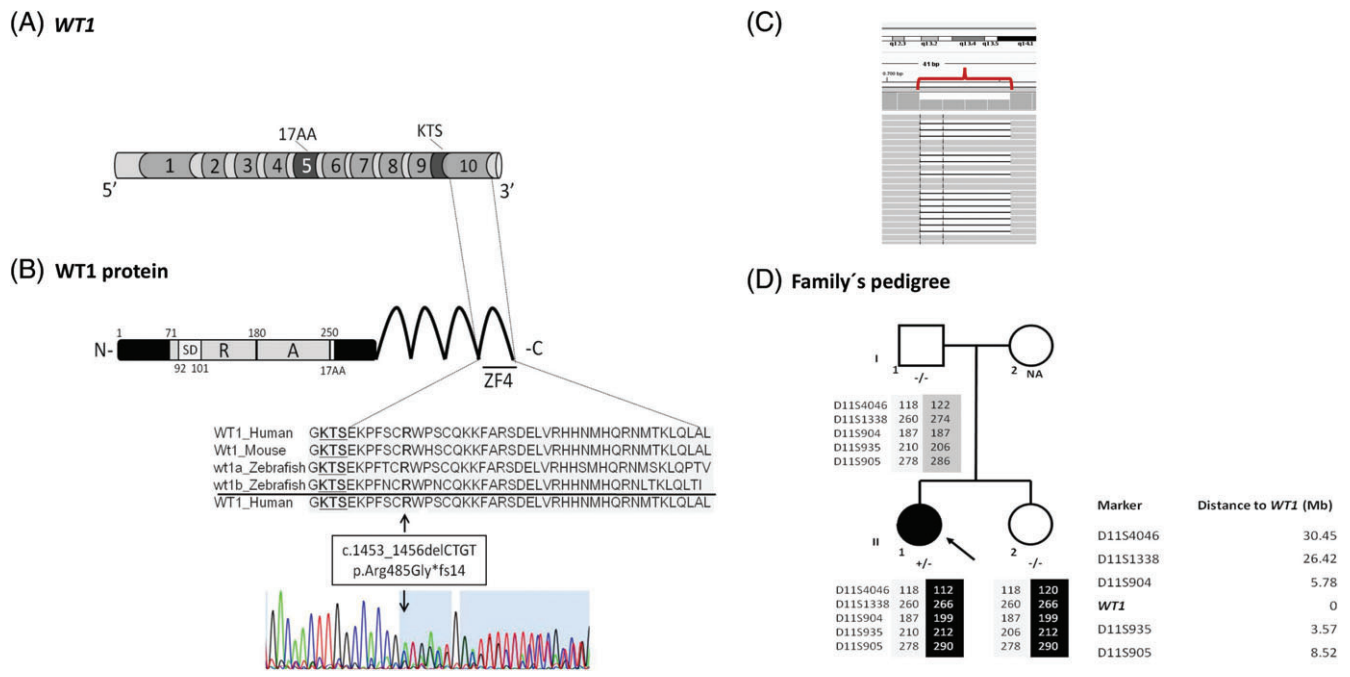

FIGURE 1 Schematic representation of the *WT1* variant, location on the protein, and amino acid conservation. A, the *WT1* is composed of 10 exons and has two alternative splice sites (KTS and 17AA). B, WT1 protein is shown indicating the suppression domain (SD), repression domain (R), and activation domain (a) with solid bars and zinc fingers (Zn) as waves. The c.1453_1456delCTGT variant is in exon 10, which encodes the fourth zinc-finger (ZF4) region. The affected amino acid is conserved among species. The electropherogram is depicted below. C, *WT1* variant visualisation using the integrative genomics viewer software: IGV 2.3—Broad institute/MIT/Harvard. D, pedigree and haplotype of the 46,XX T-DSD girl. The affected individual, heterozygous for the *WT1* c.1453_1456delCTGT variant, is indicated with a filled symbol. DNA from the patient's mother was not available (NA). The de novo status of the variant was confirmed by microsatellite analysis. The common haplotype inherited from the patient's mother is highlighted in a black box and by the father in a light grey [Colour figure can be viewed at wileyonlinelibrary.com]

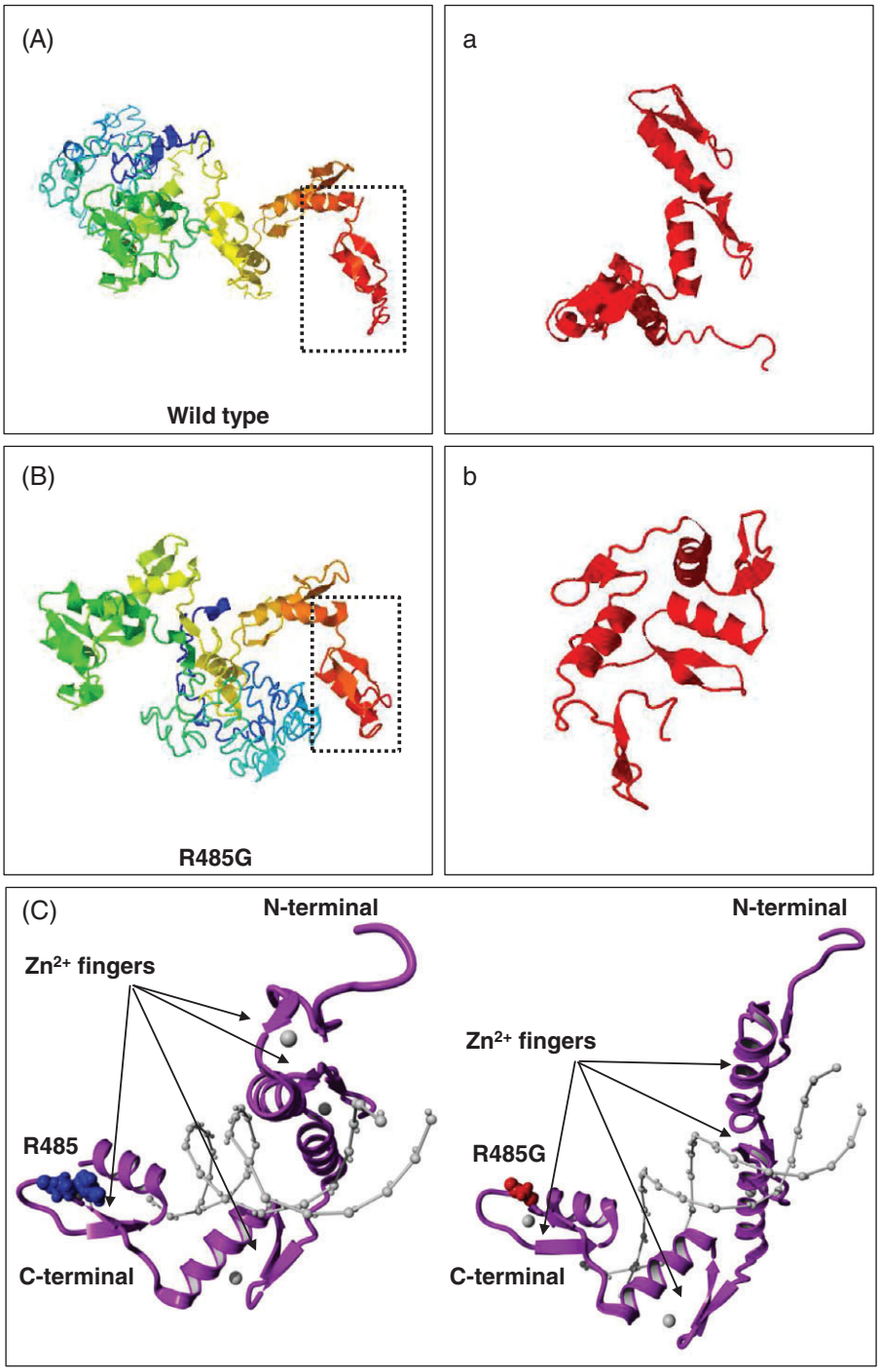

FIGURE 2 Structural analysis of R485G and wild-type WT proteins. A, WT1 wild-type protein prediction; (a) zoom of the wild-type WT1 fourth zinc finger; B, R485G protein variant prediction; (b) zoom of the R485G WT1 mutant region; (C) R485G functional prediction showing changes in the WT1 zinc finger domain (purple) bound to DNA (grey). The amino acid change promotes structural and functional disruption of WT1 [Colour figure can be viewed at wileyonlinelibrary.com]

propositus and in her mutation-negative father and sister. Data analysis was performed with GeneMapper v3.7 software (Applied Biosystems, Foster City, California).

2.3 | *In silico* modelling of the p.Arg485Glyfs*14 variant

The effect of the mutation on protein stability and function was calculated using the COFACTOR server (I-TASSER),[9] FoldX RepairPDB,[10] and BuildModel commands (YASARA).[11] The *in silico* interaction energies between the p.Arg485Gly WT1 region and DNA fragments for *NR5A1*, *FOXL2*, and *WNT4* were calculated using the FoldX interaction energy.

3 | RESULTS

Target sequencing analysis identified a novel heterozygous frameshift *WT1* c.1453_1456del variant (see Figure S1), which is located in exon

10 and encodes the fourth zinc finger of the protein (Figure 1A,B). No exonic variants were identified in the other DSD genes. This variant is absent in all population databases, including the Brazilian databases. This frameshift is predicted to be deleterious by Mutalyzer software[12] leading to a premature stop codon p.Arg485Glyfs*14. No copy number variation was identified.

This variant was not identified in the patients' father and unaffected sister, who share the same maternal haplotype with the patient, confirming the de novo status of the variant (Figure 1D). The variant is classified as pathogenic according to ACMG criteria.

3.1 | Protein structure and functional modelling of WT1 p.Arg485Glyfs*14

I-TASSER calculations predicted that the p.R485G variant affects the stability of the WT1 fourth zinc-finger domain to binding DNA. In the mutant model (Figure 2B), the glycine chain changes the protein conformation (WT1 C-score: 0.42, WT1 R485G C-score: −1.55) compared to the wild-type (Figure 2A). These protein structural and functional findings suggest that the p.R485G variant affects the DNA binding activity of WT1.

The estimated binding enthalpy was 9 kJ/mol for the wild-type complex and 17 kJ/mol for the R485G complex, representing a 102% increase in DNA-binding affinity of R485 WT1 (Figure 2C). This increased binding enthalpy in the mutant complex suggests that p.R485G is a gain-of-function variant that increases WT1 target activation.

4 | DISCUSSION

We described an *SRY*-negative 46,XX T-DSD girl with atypical genitalia harbouring a novel and de novo frameshift *WT1* variant. This presentation is very unusual for 46,XX T-DSD patients. Classically, these patients are characterised by a 46,XX karyotype, male external genitalia, small testis, and the absence of Mullerian structures.[13] Approximately, 85% of the patients are diagnosed after puberty because of their small testes, gynecomastia, and infertility. The other 15% of patients are diagnosed at birth based on atypical genitalia.[13]

In 46,XY individuals, pathogenic *WT1* allelic variants are responsible for Denys-Drash and Frasier syndromes, which are characterised by gonadal dysgenesis, early childhood cortico-resistant nephrotic proteinuria followed by renal failure, and Wilms' tumour or gonadoblastoma.[5,6] Denys-Drash variants are generally located in exons 8 and 9 and Frasier variants are located in intron 9.

In 46,XX individuals, two deleterious heterozygous *WT1* variants located outside the zinc-finger domains were previously described in two patients with premature ovarian insufficiency (POI).[14] Another two 46,XX gonadal dysgenesis patients with steroid-resistant nephropathy harboured variants located in exon 9 of *WT1*.[15,16] There is no description of atypical genitalia in any of these affected female patients.

The first description of a *WT1* variant causing 46,XX T-DSD was reported in 2017.[17] The missense *WT1* p.Arg495Gly variant was identified in a syndromic boy with microcephaly, normal kidney function, male external genitalia, dysgenic testis, and a small uterus.[17]

These findings resemble those reported for the *NR5A1* p.Arg92Trp pathogenic variant. This variant were also first associated with 46,XY DSD and POI[18] in 46,XX patients. Later, the *NR5A1* p.Arg92Trp variant was identified in familial and sporadic patients with 46,XX T and OT-DSD.[18–20]

Interestingly, the previously and presently described WT1 alterations associated with 46,XX T-DSD are located in the fourth zinc-finger DNA-binding domain of the WT1 protein.

There is one reported pathogenic variant, the p.X518Trp located in exon 10 of *WT1* in two female affected individuals.[21] In contrast to our frameshift variant, this variant leads to WT1 protein elongation of 22 amino acids but preserves fourth zinc-finger DNA-binding domain.

The 46,XX T-DSD phenotype can be explained based on the *WT1* role of in the ovarian determination pathway.[22] A previous study showed that the number of FOXL2-positive cells was dramatically reduced and SOX9-positive cells were observed in *Wt1*-deficient XX mice gonads.[23] In addition, the expression of ovary-specific genes

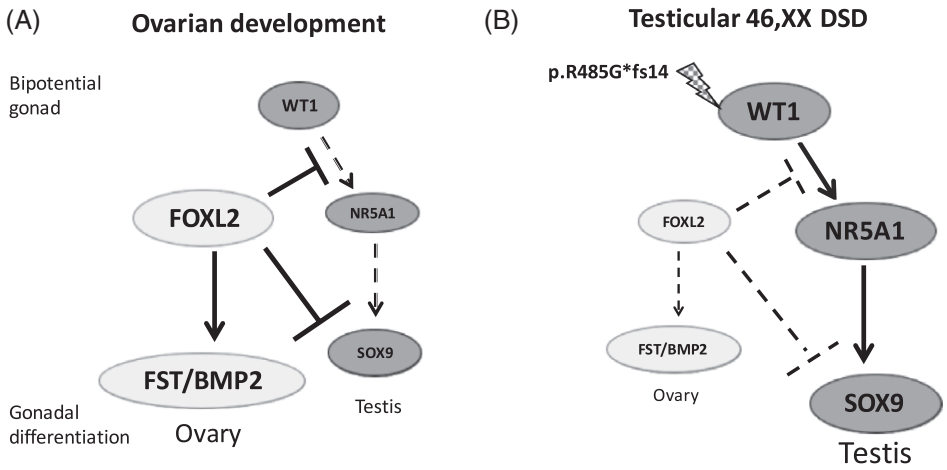

FIGURE 3 Schematic overview of the signalling pathway of ovarian development (A) and proposed mechanism of 46,XX DSD testicular in patients with heterozygous WT1 variant in exon 10 (B) in 46,XX individuals, FOXL2 transcriptionally represses *NR5A1* expression by antagonising WT1 during gonadal development, leading to suppression of the male pathway and activation of the ovarian determination cascade. We hypothesised that the WT1 p.R485G*fs14 variant, leads to NR5A1 expression, resulting in SOX9 up-regulation and testicular development in this 46,XX T-DSD girl

(*Wnt4*, *Rspo1*, *Foxl2*, *Bmp2*, and *Fst*) was also significantly reduced, whereas testis-specific genes expression was increased in *Wt1*-deficient XX gonads at E13.5.[23]

The mutant p.Arg485Glyfs*14 WT1 protein probably leads to an imbalance between the female and male gonadal determination pathway. The increased binding enthalpy of WT1 p.Arg485Glyfs*14, observed in the in silico model, probably leads to *NR5A1* overexpression followed by *SOX9* up-regulation and testis differentiation (Figure 3). Further in vitro studies are necessary to confirm this hypothesis.

The intriguing absence of renal manifestations of our patient might be because of her young age. The only reported variant in exon 10 (*WT1* p.X518Trp) led to progressive proteinuria in the female index case and her mother, reaching end-stage renal disease at 15 and 22 years old, respectively.[21] The propositus also presented with Wilms' tumour at 9 years old. These data indicate the need of follow-up of these individuals.

5 | CONCLUSION

The identification of a novel and predicted activating frameshift *WT1* variant leading to the 46,XX testicular DSD phenotype includes the fourth zinc-finger DNA-binding domain defects in the genetic aetiology of 46,XX DSD.

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CONFLICTS OF INTEREST

The author reports no conflict of interest in this work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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