The Mutational Spectrum of *WT1* in Male Infertility

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Purpose: We evaluated the impact of *WT1* mutations in isolated severe spermatogenic impairment in a population of European ancestry. *WT1* was first identified as the gene responsible for Wilms tumor. It was later associated with a plethora of clinical phenotypes often accompanied by urogenital defects and male infertility. The recent finding of *WT1* missense mutations in Chinese azoospermic males without major gonadal malformations broadened the phenotypic spectrum of *WT1* defects and motivated this study.

Materials and Methods: We analyzed the *WT1* coding region in a cohort of 194 Portuguese patients with nonobstructive azoospermia and in 188 with severe oligozoospermia with increased depth for the exons encoding the regulatory region of the protein. We also analyzed a group of 31 infertile males with a clinical history of unilateral or bilateral cryptorchidism and 1 patient with anorchia.

Results: We found 2 *WT1* missense substitutions at higher frequency in patients than in controls. 1) A novel variant in exon 1 (p.Pro130Leu) that disrupted a mammalian specific polyproline stretch in the self-association domain was more frequent in azoospermia cases (0.27% vs 0.13%, $p = 0.549$). 2) A rare variant in a conserved residue in close proximity to the first zinc finger (pCys350Arg) was more frequent in severe oligozoospermia cases (0.80% vs 0.13%, $p = 0.113$).

Conclusions: Results suggest a role for rare *WT1* damaging variants in severe spermatogenic failure in populations of European ancestry. Large multicenter studies are needed to fully assess the contribution of *WT1* genetic alterations to male infertility in the absence of other disease phenotypes.

Key Words: testis; infertility, male; genes, Wilms tumor; mutation; European continental ancestry group

Abbreviations and Acronyms

DDS = Denys-Drash

NOA = nonobstructive azoospermia

NR5A1 = nuclear receptor steroidogenic factor 1

WT1 = Wilms tumor 1

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THE *WT1* gene located at 11p13 encodes a protein with a C-terminal zinc finger domain that is involved in DNA and RNA binding. WT1 acts as a transcription factor through the interaction of WT1 activation and repression domains with its targets.[1,2] This activity is thought to be modified by dimerization of WT1 with other proteins (heterodimerization) or with itself (homodimerization) at the N-terminal self-association domain.

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Genetic defects in *WT1* typically result in 1 of 3 congenital syndromes, including WAGR (Wilms tumor, aniridia, genitourinary alteration and mental retardation), DDS or Frasier syndrome, characterized by malformation of the gonadal ridge (gonadal dysgenesis, hypospadias and cryptorchidism) and kidneys (horseshoe kidney and renal hypoplasia).[3] This association is in accord with the preponderant role of *WT1* in urogenital system development and differentiation.

WT1 is a crucial factor in male sex determination with an essential role in the male gonadal differentiation pathway. This is supported by the fact that gonads fail to develop in $Wt1^{-/-}$ mice.[4] Moreover, several lines of evidence demonstrate its importance in different stages of testicular and germ cell development. Chang et al reported that WT1 dependent suppression of WNT/β-catenin signaling in Sertoli cells is essential for the normal development of primordial germ cells.[5] Studies in which *Wt1* was specifically inactivated in Sertoli cells at different developmental stages revealed its importance in testicular differentiation[6] and in the maintenance of Sertoli cell polarity and spermatogenesis in adulthood.[7] Interestingly the phenotype of germ cell loss observed in *Wt1* conditional knockout mice resembles that observed in Sertoli-cell only syndrome and in accord several missense mutations in the *WT1* gene were recently described in a cohort of Chinese patients with NOA.[7]

Phenotypic expression of *WT1* defects varies and depends on the affected protein domains. Patients with C-terminal missense or nonsense mutations typically show severe gonadal dysgenesis and/or nephropathy resulting from a dominant negative action of heterozygous *WT1* missense mutations or from haploinsufficiency.[3,8,9] Most mutations reported in the first exons of this gene result in truncated proteins and were found in patients with renal tumor and genitourinary abnormalities. In fact, missense mutations affecting only the WT1 protein N-terminus are expected to have a milder impact on its physiological function and result in milder gonadal malformations since the DNA binding domain should remain intact.[10] Accordingly most mutations found to date in infertile patients without major disturbances of testicular development are located in the WT1 protein N-terminus.[7]

We resequenced the *WT1* coding region in a cohort of Portuguese patients, focusing on the first 6 exons, which encode the regulatory domain. To determine the impact of the 2 identified missense *WT1* variants we 1) reassessed the protein conservation across vertebrates and 2) reviewed the spectrum of *WT1* mutations affecting male fertility and compared them to those in control populations from large genome sequencing projects.

MATERIALS AND METHODS

Patients and Controls

DNA samples from peripheral blood leukocytes of 194 men with NOA and 188 with severe oligozoospermia (fewer than 1 million sperm per ml) who had idiopathic spermatogenic failure were collected at the Human Genetics Department, National Institute of Health Dr. Ricardo Jorge, and the Genetics Department, Faculty of Medicine, University of Porto, where routine molecular diagnosis was done for male infertility. A group of 31 infertile males with a clinical history of unilateral or bilateral cryptorchidism and 1 patient with anorchia were also selected after physical examination, hormonal testing (follicle-stimulating hormone and testosterone) and standard clinical genetic screening for karyotypic anomalies and Y chromosome microdeletions. Patients with a known cause of infertility were excluded from study. Molecular studies were performed in coded DNA samples after receiving informed consent.

As controls we obtained DNA from peripheral blood from 373 Portuguese men, including 72 with normozoospermia (normal sperm parameters) and 301 who had fathered at least 1 child. This study was included in the project, Copy Number Variation in Infertile Men Genomic Regions: Screening in the Portuguese Population (PTDC/SAU-GMG/101229/2008), which was approved by the National Institute of Health Dr. Ricardo Jorge ethics committee.

Analysis

WT1 coding sequence. Supplementary table 1 (http://jurology.com/) lists the *WT1* primers used in this study. To analyze the *NR5A1* sequence we used the primers described by Bashamboo et al.[11] DNA fragments were amplified and sequenced, and all putative variants were individually confirmed.

Restriction fragment length polymorphism. This technique was applied to screen Portuguese controls for the c.1048T>C variant in *WT1* exon 6. The restriction endonuclease *Cfr42I* was used to cleave a 1,020 bp amplicon into 2 fragments of 832 and 188 bp, respectively, which were resolved by electrophoresis in polyacrylamide gel.

In silico. WT1 protein sequences of several species of mammals and from chickens were retrieved from the Ensembl database (http://www.ensemble.org/) and manually curated and aligned with the ClustalW algorithm in Geneious v.5.5.8 (http://www.geneious.com/). PolyPhen-2 (Polymorphism Phenotyping, version 2)[12] (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/) were used to predict the impact of nonsynonymous substitutions in *WT1* patients and controls. As a reference we used the Ensembl WT1 protein ENSP00000331327 (transcript sequence ENST00000332351). This transcript comprises all 10 *WT1* exons (3,122 bp) and initiates with the upstream CUG codon. We retrieved variant data from 1000 Genomes (http://www.1000-genomes.org/),[13] NHLBI (National Heart, Lung and Blood Institute) GO (Grand Opportunity) ESP (Exome Sequencing Project)

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