Biallelic Mutations in WDR12 is Associated With Male Infertility With Tapered-Head Sperm

juan hua (✉ hjhuajuan@163.com)

Anhui Medical University

Lan Guo

Anhui Medical University

Yao Yao

Anhui Medical University

Yangyang Wan

The First Affiliated Hospital of USTC: Anhui Provincial Hospital

Wen Hu

Hefei xingzhike biotechnology

Hui Jiang

Third Hospital of Beijing University: Peking University Third Hospital

Xiansheng Zhang

Anhui Medical University

Bo Xu

The First Affiliated Hospital of USTC: Anhui Provincial Hospital

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Abstract

Teratozoospermia is a rare disease associated with male infertility. Unfortunately, approximately 30% of the genetic causes associated with teratozoospermia remain unknown. Several recurrent genetic mutations have been reported to be associated with globozoospermia, macrozoospermia and acephalic spermatozoa, whereas the genetic basis of tapered-head sperm is relatively less well-understood. In this study, whole-exome sequencing (WES) identified a homozygous [WD repeat domain 12](#) (*WDR12*) (p.Ser162Ala/c.484T>G) variant in an infertile patient with tapered-head sperm from a consanguineous Chinese family. Bioinformatic analysis predicted this mutation to be a pathogenic variant. To further verify the effect of this variant, we analyzed *WDR12* protein expression in the patient's spermatozoa by western blot and found *WDR12* to be significantly down-regulated. Also, we found that *WDR12* expression is increased in pachytene spermatocytes, and intense staining was visible throughout the round spermatids in mouse testis. Based on our results, we concluded that a rare biallelic pathogenic missense variant (p.Ser162Ala/c.484T>G) in the *WDR12* gene causes teratozoospermia. These results will provide novel insights into understanding the molecular mechanisms of male infertility and will help clinicians provide accurate diagnoses.

Introduction

According to the WHO (World Health Organization) report, more than 15% of couples (approximately 50 million couples) are infertile and seek clinical help for fertility. Approximately half of these infertility cases are caused by male-factor with abnormal semen quality (Mascarenhas, et al., 2012). Teratozoospermia is defined by the presence of spermatozoa with abnormal morphology in over 96% of sperms (WHO, 2010). The malformations of sperm morphology include abnormalities in the head, neck, and tail. Malformations of the sperm head are a major cause of male infertility and have been classified based on several specific phenotypes, such as round-head, small-head, tapered-head, large-head, and acephalic spermatozoa (Dehghanpour, et al., 2017). Up to now, several recurrent mutations have been identified in three specific phenotypes, which are macrozoospermia (*AURKC*) (Ben Khelifa, et al., 2011; Ma, et al., 2018), globozoospermia (*SPATA16*, *DPY19L2*, *PICK1*, *ZBP1*, and *CCDC62*) (Dam, et al., 2007; Koscinski, et al., 2011; Liu, et al., 2010; Oud, et al., 2020; Yatsenko, et al., 2012) and acephalic spermatozoa (*SUN5*, *TSGA10*, *PMFBP1*, *DNAH6*, *BRDT*, and *CEP112*) (Elkhatib, et al., 2017; Li, et al., 2017; Li, et al., 2018; Sha, et al., 2020; Sha, et al., 2018; Zhu, et al., 2018; Zhu, et al., 2016). In addition, morphological defects described in sperm head also include tapering, small, amorphous, and pyriform head, whereas the genetic basis of them is rarely reported. Recent studies suggested that tapered-head spermatozoa are related to Sertoli cell ectoplasmic specialization deficiency, sperm protamine deficiency and acroplaxome deficiency, but the genetic basis of tapered-head sperm is still lacking (Dehghanpour, et al., 2017; Tang, et al., 2010). In the present study, we performed WES to identify the genetic reason of male infertility in a man with tapered-head sperm from a consanguineous Chinese family.

WDR12 is a member of PeBow complex and plays significant roles in numerous biological processes, such as ribosome biogenesis (Holzel, et al., 2005). High expression levels of *WDR12* mRNA have been

reported in the testis of adult mice. However, little is known about the role of WDR12 during human spermatogenesis and no WDR12 mutations have been reported to be associated with human male infertility. The outcomes of this research study provide the first viewpoint of a physiological role for WDR12 in human male infertility.

PATIENTS AND METHODS

Patients And Methods

Patient

The patient (30 years old, married in 2017) was referred to the Center for Reproductive Medicine at the First Affiliated Hospital of University of Science and Technology of China for infertility. The parents of the proband had a consanguineous marriage in their three closest generations. The proband exhibited a normal erection and ejaculation, and reported having sexual intercourse two to three times/week; but his wife has been unable to get pregnant. The proband had no history of contacts with toxic or other adverse chemicals. Physical examination revealed that the patient had normal bilateral testicular size, normally developed male external genitalia, and no abnormality in the bilateral spermatic veins upon palpation. The proband did not have primary microcephaly or respiratory disease. The patient had a normal chromosomal karyotype (46; XY), and no Y chromosome deletions were found. The hormone levels of the proband were normal. Semen analyses were carried out during routine examination of the individuals according to the WHO guideline (the fifth Edition). Sperm morphology was assessed by Papanicolaou staining. At least 200 spermatozoa were examined. The percentages of morphologically abnormal spermatozoa were evaluated according to the WHO guidelines. The brother of the proband exhibiting normal spermatozoa. Samples used as controls were obtained from fertile patients exhibiting normal spermatozoa. Aniline blue staining indicated no difference between the patient and the control group (figure. S1). The control subjects were obtained from fertile patients exhibiting normal spermatozoa.

Animal studies

All animal experiments were performed in C57BL/6 mice (*Mus musculus*). Experimental methods for all animal experiments were in accordance with guidelines of the Institutional Animal Care and Use Committee of Anhui Medical University. Animals were housed in a specific-pathogen-free facility and maintained at 22°C with a 12-hour light/dark cycle.

Genomic DNA preparation and WES

The patient and his parents' genomic DNA were extracted as previously described ([Hua and Wan, 2019](#)).

Genomic DNA samples were subjected to WES. WES analysis was performed by BGI in Shenzhen on a HiSeq2000 sequencing platform (Illumina, San Diego, California, USA). WES raw reads analysis was performed as previously described ([Hua and Wan, 2019](#)). Variants meeting the following criteria were

considered as candidate genes: (i) non-silent variants (nonsense, missense, frame-shift and splice site mutations); (ii) variants that were absent or rare (MAF<0.01) in the 1000G, ESP6500, and ExAC databases. (iii) variants that were homozygous in the patient, and heterozygous in his parents. (IV) variants in genes that have no function in spermatogenesis based on literature are excluded. (V) variants predicted to be non-deleterious by >30% software covering them were excluded (SIFT, Polyphen-2, CADD, Mutation assessor, Mutation taster, REVEL, MetaLR LRT, MetaSVM, and FATHMM).

Sanger sequencing

The mutation in *WDR12* was validated by Sanger sequencing of DNA samples from the patient and his parents. PCR products of the third exon of the *WDR12* were amplified using specific primes (forward primer: 5'-CTGAGCAAGTGACTATCTCC-3', reverse primer: 5'-CTTTGGTTTCAGAACATGATG-3').

Reverse transcription PCR

RNA was extracted from whole blood using TRI REAGENT®BD (Molecular Research Center) according to the manufacturer's protocol, as previously described ([Hua and Wan, 2019](#)).

Quantitative PCR (Q-PCR)

Q-PCR was performed according to the manufacturer's protocol (*WDR12* forward primer: 5'-CAAGAACACCCTTGGTGACC-3', *WDR12* reverse primer: 5'-GACCGCCAGACTCAACATCC-3'). The fold change in gene expression was quantified by the relative quantification method ($2^{-\Delta\Delta CT}$) using *GAPDH* as the internal control (*GAPDH* forward primer: 5'-GTCAAGGCTGAGAACGGGAA-3', *GAPDH* reverse primer: 5'-AAATGAGCCCCAGCCTTCTC-3'). Data are shown as the average fold increase $\pm$ standard error of the mean. Statistical significance was determined using the Student's *t*-test (***p*<0.01).

Histological analysis

Fresh testicular tissue was fixed in 10% formaldehyde, dehydrated, and embedded in paraffin, and 5 μ m sections were attached to microscope slides. After de-paraffinization and re-hydration, the sections were incubated with anti-WDR12 polyclonal antibody (Invitrogen, PA5-57635) at 4°C overnight. After washing with TBS three times, the sections were incubated with biotinylated and streptomycin-labeled goat anti-mouse antibodies (Maixin Bio, KIT-5010,) at 37 °C for 10 minutes. Nuclei were counterstained with hematoxylin. Morphology images were acquired using a fluorescence microscope (Olympus BX61).

Immunoblot analysis

Protein lysates were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. Blots were then probed with primary antibodies. After washing with TBST three times, blots were incubated with alkaline phosphatase (AP)-conjugated anti-mouse/rabbit IgG antibody (Promega, S372B, WI, USA). AP activity was detected using a Lumi-Phos kit (Pierce Biotechnology, KJ1243353).

Construction of wild-type and mutant expression plasmids

DNA from HepG2 cells was used as a template for constructing wild-type (WT) and mutant (MT) constructs by using RT-PCR. Primers used to generate WT and MT expression plasmids are listed in Table. S2. Briefly, the WT fragment was amplified by PCR. pcDNA3.1 vector was linearized by *XhoI* and *KpnI* digestion and, 100 ng of linear vector was mixed with 40 ng WT amplicon in 10 μ L to construct the WDR12 WT expression plasmid. Next, the MT fragment was amplified by PCR by using specific primers. The reaction products were digested with DpnI, and the mixture was transfected into DH5a strain. Sequences of wild-type and mutated expression plasmids were confirmed by Sanger sequencing. WDR12 wild-type and mutant cDNAs were cloned in frame with an N-terminal FLAG.

Results

Characterization of the morphological defects in the sperm of an infertile patient

In this study, we examined an infertile man from a consanguineous family (Fig. 1A). Routine sperm analysis indicated that the patient suffered from teratozoospermia. In particular, the Papanicolaou staining revealed that 83.8% of the spermatozoa presented a tapered-head sperm phenotype (Table 1 & Fig. 1B). To further characterize the spermatozoa defects of the patient, transmission electron microscopy (TEM) was used to analyze ultrastructure in sperm cells of the patient as well as the control. Compared with normal control, most sperm cells from the patient exhibited smaller and less condensed nuclei, while acrosomes of the spermatozoa of patient were either absent or morphologically defective (Fig. 1C). Thus, we speculate that the tapered-headed sperm might be the cause of the infertility for this patient.

Identification of a mutation in *WDR12* in a patient with tapered-head sperm by WES analysis

To identify a possible genetic cause of the infertility in this patient, we analyzed the peripheral blood genomic DNA obtained from the patient and his parents using WES. WES data was analyzed and described in Figure 2. Briefly, variants with MAF >0.01 in human genetic variation databases (1000 Genomes, ESP6500, and ExAC) were excluded. Because of consanguinity loop in the family, we hypothesized that homozygous mutations in the patient may be responsible for the infertility. Further, we assumed a recessive mode of inheritance, which resulted in 13 variants in 12 genes (Fig. 2A). To investigate whether any of these 12 genes may be related to male infertility, we first excluded genes with no function in spermatogenesis based on data mining of the related literatures and genes which have no expression level in testis. Next, we excluded variants which predicted to be non-deleterious by >30% prediction tools (SIFT, Polyphen-2, CADD, Mutation assessor, Mutation taster, REVEL, MetaIR, LRT, MetaSVM, FATHMM) (Fig. 2A & Table S2). These filters resulted in only two mutations (NM_005245.3, p.Asp1113Asn/c.3337G>A of *FAT1* and NM_018256.3, p.Ser162Ala/c.484T>G of *WDR12*). Given that the patient did not display facial dysmorphism, colobomatous microphthalmia, ptosis, or syndactyly defects, which are associated with mutations in *FAT1*. we focused on the homozygous variant (p.Ser162Ala/c.484T>G) of *WDR12*.

Validation by Sanger sequencing

Sanger sequencing revealed that a patient affected by teratozoospermia was homozygous for this *WDR12* variant, whereas his parents carried the variant in a heterozygous state (Fig. 2B).

Detrimental effects of the identified *WDR12* variant

To investigate the effect of the homozygous *WDR12* (p.Ser162Ala/c.484T>G) variant at the molecular level, we analyzed WDR12 protein expression levels. Western blot analysis on spermatozoa protein extracts revealed WDR12 protein expression to be significantly decreased in the patient's spermatozoa (Fig. 3A&B). Furthermore, we assessed WDR12 expression from WT and MT *WDR12* expression plasmids in 293T cells respectively by western blot. The result showed that WDR12 protein level was highly expressed from the WT plasmid than the MT plasmid (Fig. 3C), suggesting that this missense variant affects WDR12 expression.

In silico analysis of the *WDR12* mutation

Bioinformatic analysis utilizing online pathogenicity prediction tools (Polyphen-2, Sift, Mutation Assessor, CADD, Mutation taster) predicted that the c.484T>G mutation in *WDR12* is probably a damaging mutation, suggesting that *WDR12* (p.Ser162Ala/c.484T>G) should be a disease-causing mutation (Table. 2). The conservation of this mutation site was predicted by computational analysis and the result showed that Ser residue at the 162 site is highly conserved (Fig.3D). These results suggest the Ser residue at the 162 site had an important role.

WDR12 expression and localization in mouse testis

To explore the biological function of *Wdr12* in the mouse, we detected tissue- and developmental stage-specific expression of WDR12. First, high expression of *Wdr12* mRNA was observed in the adult mouse testis (Fig. 4 A). Further, Q-PCR and western blot analysis revealed that the expression of WDR12 was very weak in 7dpp mouse testis, but increased over time, peaking at 21dpp, which is coincident with the appearance of round spermatids. Then, WDR12 expression level was reduced in testes of adult mice (Fig. 4B, C). Immunohistochemical staining further showed that WDR12 is predominantly expressed in late pachytene spermatocytes and round spermatids (Fig. 4D). Collectively, the expression and localization patterns of WDR12 during spermatogenesis suggests its potential role in round spermatid development.

ICSI using the patient's sperm and pregnancy outcome

After centrifugation on density gradients and careful examination, a few 'normal-looking' spermatozoa that fit into an ICSI micropipette were selected. ICSI cycle was attempted for the patient at the first affiliated hospital of USTC. Nine eggs were used for the ICSI cycle, and 4 eggs form viable embryos, then two embryos were transferred, and his wife became pregnant. These results indicate that the infertility of *WDR12* homozygous mutation could be overcome by ICSI.

Discussion

Sperm morphology is a crucial semen parameter, and previous researches have proved a relationship between sperm morphological defects and fertility potential. In recent years, several human genes, such as *SUN5*, *AURKC*, *DPY19L2*, *TSGA10*, *PMFBP1*, and *RNF220* have been reported as causative agents for macrozoospermia, acephalic spermatozoa, small-headed sperms and globozoospermia through studies on the familial cases of infertility or groups of infertile patients (Ben Khelifa, et al., 2011; Jiang, et al., 2019; Kosciński, et al., 2011; Sha, et al., 2018; Zhu, et al., 2018; Zhu, et al., 2016). However, the genetic basis of tapered-head sperm is rarely reported. In this study, we analyzed a consanguineous Chinese family wherein we identified a homozygous single-base mutation (p.Ser162Ala/c.484T > G) in the *WDR12* gene in a patient with tapered-head sperm by WES.

WDR12 (WD repeat protein 12) is a member of the WD repeats family and contains seven WD repeats at its carboxyl terminus (Nal, et al., 2002). In mammals, *WDR12* forms a stable nucleolar PeBoW complex with Pes1 (Pescadillo 1) and Bop1 (Block of proliferation 1), and plays significant roles in numerous biological processes, such as ribosome biogenesis (Holzel, et al., 2005). As a member of PeBoW, endogenous *WDR12* is a crucial factor for the processing of 32S precursor rRNA (ribosomal RNA), cell division, signal transduction, cell cycle progression, apoptosis, and cell proliferation (Rohrmoser, et al., 2007). Previous studies have suggested a role of *WDR12* in myocardial infarction, coronary artery disease and Hepatocellular carcinoma (Yin, et al., 2018). High expression levels of *WDR12* mRNA have been reported in the testis of adult mice. However, little is known about the role of *WDR12* during human spermatogenesis and no *WDR12* mutations have been reported to be associated with human male infertility. In our present study, *WDR12* expression in the postnatal testis was found to peak at 21-28dpp, which coincides with the appearance of round spermatids and elongated spermatids. Immunostaining of testis sections revealed that *WDR12* is expressed in the nuclei of late pachytene spermatocytes in seminiferous tubules at 21dpp. *WDR12* expression progressively increased in pachytene spermatocytes, and intense staining was visible throughout the nucleus of round spermatids in 28dpp. This distinct expression pattern of *WDR12* indicates that it may function during round spermatid development. However, mutations in *WDR12* have not been reported to be associated with human male infertility. In this study, we identified a homozygous missense mutation (p.Ser162Ala/c.484T > G) in the *WDR12* gene associated with tapered-head sperm phenotype. The T-to-G transition at position 484 in the *WDR12* gene resulted in the substitution of an Ala for Ser at position 162. Interspecies comparison revealed significant conservation of this Ser residue. Investigating the effect of the mutation on *WDR12* expression showed that *WDR12* protein expression was reduced in the patient sperms, suggested that this missense mutation (c.484T > G) probably has influence on *WDR12* expression and function. Endogenous *WDR12* is functional in several processes in the cell, such as cell division and proliferation, cell cycle control, and ribosome biogenesis. *WDR12* dysfunction disrupt rRNA processing and blocked ribosome biogenesis. Previous studies have proposed the linkage between ribosome function and male infertility. Zhang *et al* (Zhang, et al., 2018) systematically analyzed the gene expression profiles of patients with teratozoospermia and various ribosomal genes, including *RPS3*, *RPS5*, *RPS6*, *RPS16* and *RPS23*, were observed to be downregulated in abnormal sperm. Another similarly studies also reported that various

ribosome genes, such as RPS25, RPS11, RPS13, RPL30, RPL34, RPL27, RPS were down-regulated in the asthenozoospermic group in comparison to the control group. Additionally, *RPS6* has been reported to regulate the viability of sertoli cells in blood-testis barrier dynamics in rats (Mok, et al., 2014). In addition, *Rpl10l*, is a ribosomal component in mouse spermatogenic cells and *Rpl10l*-deficient male mice are sterile and exhibit disrupted ribosome biogenesis in late-prophase spermatocytes (Jiang, et al., 2017). Furthermore, a homozygous *RPL 10L* mutation was recently identified to cause male infertility with SO by WES (Tu, et al., 2020). Collectively, the consistency between previous studies and the results of the present study suggest that *WDR12* mutant may disrupt ribosome biogenesis that leads to male infertility.

In summary, our study, to the best of our knowledge, is the first one to report a homozygous missense mutation (p.Ser162Ala/c.484T > G) in the *WDR12* gene associated with tapered-head sperm. This work provides researchers novel insights into understanding the molecular mechanisms of male infertility and will improve clinicians to make an accurate diagnose.

Declarations

Ethics approval and consent to participate

This work was consented by the ethics committee of Anhui Medical University (Approve ID:20190346 and 20190329). Written informed consent was signed by the patient.

Consent for publication

Not applicable for that section.

Data Availability Statement

The datasets of variants for this study can be found in the NCBI dbVar database (SUB7725323).

Authors' contributions

LG, YYW, YY and WH performed most of the experiments. JH analyzed the WES data and carried out the bioinformatic analysis. BX and JH wrote the manuscript. YYW collected peripheral blood samples from the consanguineous Chinese family. All authors reviewed the manuscript.

Conflict of interest

The authors declare no potential conflict of interest.

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Not applicable

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Tables

Due to technical limitations, table 1 and 2 is only available as a download in the Supplemental Files section.

Figures

Figure 1

A patient with tapered-head sperms from a consanguineous family. (A) Family tree of the patient. (B) Morphological abnormalities of the spermatozoa were observed by light microscopy. (C) Ultrastructure of the sperm heads from control and patient with WDR12 mutation. Control spermatozoa presents with a normal sperm head and highly condensed chromatin. sperm from patient display less chromatin condensation (green arrow). Moreover, the acrosome was quite thin and defective with barely recognizable inner and outer membranes (blue arrow)

Figure 2

Exome sequencing revealed a biallelic variant in WDR12. (A) Gene filter strategies used in this study. (B) Sanger sequencing validated the biallelic mutation in the patient and his parents. The arrow shows the mutated base.

Figure 3

Protein expression levels of WDR12 as detected by western blot analysis in the patient and a normal control. (A) WDR12 protein levels were determined by western blot. (B) The density of each band was quantified with ImageJ. B-actin was used as the loading control. Data are expressed as the mean $\pm$ SD of three independent experiments. Data were analyzed with spss 18.0 software. *** $p < 0.001$. (C) Western blot showed WDR12 expression is decreased in the MT plasmid compared to the WT plasmid in 293T cells. (D) Results of ConSurf32 analysis of the Ser 162. Color intensity denotes conservation, “e” and “b” denote predicted exposed and buried residues respectively, and “f” and “s” denote predicted functional and structural residues respectively.

Figure 4

WDR12 is highly enriched in mouse testes. (A) Q-PCR analysis of WDR12 mRNA in adult mouse tissue lysates (B, C) Western blot and Q-PCR analyses of WDR12 expression level in mouse testis tissue collected at different time points during postnatal development. (D) Localization patterns of WDR12 in mouse testis tissue collected at different time points during postnatal development. Primary spermatocyte (Spc), pachytene spermatocytes (Pac), round spermatids (RS), and elongating spermatids (ES). β-actin was used as an internal loading control.

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