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Association analysis between *HFM1* variations and idiopathic azoospermia or severe oligozoospermia in Chinese Men

Wenxiang Zhang[1,2], Xiaomin Song[2], Feng Ni[2], Jinbao Cheng[2], Bai-Lin Wu[3,4*] & Hong Jiang[1,2*]

[1]*Clinical College of PLA Affiliated Anhui Medical University, Hefei 230031, China;*
[2]*Reproductive Medicine Center, the 105th hospital of PLA, Hefei 230031, China;*
[3]*Children's Hospital of Fudan University; Institute of Biomedical Science, Shanghai Medical College of Fudan University, Shanghai, China 200032;*
[4]*Boston Children's Hospital and Harvard Medical School, Boston, MA 02115, USA*

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Dear Editor,

Infertility affects 10%−15% of couples trying to conceive, with male factors contributing to as many as 50% of infertile cases (Meng et al., 2015). Azoospermia or severe oligozoospermia (oligo/azoospermia), clinically characterized by a complete or partial absence of sperm in the ejaculate, account for 10%−15% of male infertility (Wosnitzer et al., 2014). Approximately 20%−30% of men with oligo/azoospermia are attributed to the known genetic factors, including chromosomal and single gene alterations, while up to 80% of idiopathic oligo/azoospermia remain unknown and further research is required to identify candidate gene(s) (Stahl et al., 2012).

A number of genes involved in spermatogenesis have been identified being related to idiopathic oligo/azoospermia, such as *NPAS2* (Ramasamy et al., 2015). *HFM1* (Helicase Family Member 1), an ATP-dependent DNA helicase homolog, encodes a meiosis-specific protein necessary for homologous recombination of chromosomes, exhibited dimorphism in gametogenesis (Hawley et al., 2013). The *Hfm1* null mice were infertile for both sexes, of which males showed meiotic arrest at the end of prophase I. The phenotypes of *Hfm1*$^{-/-}$ males were similar to the clinical characteristics of men with oligo/azoospermia (Hawley et al., 2013). Compound heterozygous mutations in *HFM1* gene (c.1686-1G>C and c.2651T>G/p.I884S, c.2206G>A/p.G736S and c.3929_3930 delinsG/p.P1310R fs*41) were identified in women with familial or sporadic primary ovarian insufficiency (POI) in our previous study, suggesting that biallelic mutations in *HFM1* can result in recessive POI in humans (Wang et al., 2014). However, whether *HFM1* is involved in male infertility is still unknown. The aim of this study is to investigate the association of *HFM1* gene variants with the idiopathic oligo/azoospermia in Chinese men.

Mutational analysis was performed for 80 Chinese patients with idiopathic oligo/azoospermia and 160 healthy controls (matched for ethnic backgrounds and ages) by direct sequencing of the coding regions in *HFM1* gene. Seven variations (six were singleton and one was detected twice, summarized in Table 1) were found in five unrelated patients. Among of them, four novel variations (c.437C>A/p.Thr146Lys, c.1328A>G/p.Lys443Arg, c.1481T>C/p.Val494Ala and c.3689C>G/p.Ser1230Cys) were absent in 160 matched controls and population data from the public available databases (the dbSNP, the 1000 Genomes Project, the NHLBI-ESP, and the ExAC, summarized in Table 1). The variation rate of *HFM1* in oligo/azoospermia group was significantly higher than matched controls and database

*Corresponding author (email: bai-lin.wu@childrens.harvard.edu; jiangh105@sina.com)

Table 1 Genomic and bioinformatic analysis for *HFM1* variants that may associate with idiopathic azoospermia or severe oligozoospermia in Chinese men[a)]

Exon	Genome position	Nucleotide Change	Protein Change	Type	Function Prediction by Bioinformatics tools	MAF in Patients	MAF in Matched Controls	Allele Frequency		Patient information
								1000 Genome	ExAc	
4	Chr1: 91859707	c.437C>A	p.T146K	Missense	Moderately conserved nucleotide (phyloP: 2.63) SIFT: Deleterious (score: 0.03, median: 3.43) PolyPhen-2: benign with a score of 0.047 MutationTaster: polymorphism (*P*-value: 0.988)	0.00625	0	0	0	S28 Azoospermia
11	Chr1: 91843649	c.1328A>G	p.K443R	Missense	Weakly conserved nucleotide (phyloP: 1.50) SIFT: Tolerated (score: 0.32, median: 3.62) PolyPhen-2: benign with a score of 0.000 MutationTaster: polymorphism (*P*-value: 1) This variant is in the protein DEXDc domain	0.00625	0	0	0	S42 Oligozoospermia
12	Chr1: 91841199	c.1481T>C	p.V494A	Missense	Highly conserved nucleotide (phyloP: 4.97) SIFT: Tolerated (score: 0.06, median: 3.42) PolyPhen-2: probably damaging (score: 0.962) MutationTaster: disease causing (*P*-value: 0.972) This variant is in the protein P-loop	0.00625	0	0	0	S78 Azoospermia
27	Chr1: 91781740	c.2900T>C	p.I967T	Missense Splicing	Highly conserved nucleotide (phyloP: 4.81) SIFT: Deleterious (score: 0.03, median: 3.36) PolyPhen-2: probably damaging (score: 0.994) MutationTaster: disease causing (*P*-value: 1) This variant is in the protein Sec63 domain Splicing predictions: Predicted change at acceptor site 2 bps upstream (MaxEnt: −25.1%; NNSPLICE: −58.8%; HSF: −1.1%)	0.00625	0	0	0.00000-8527	S50 Oligozoospermia
31	Chr1: 91742541	c.3470G>A	p.C1157Y	Missense Splicing	Moderately conserved nucleotide (phyloP: 3.68) SIFT: Deleterious (score: 0, median: 3.36) PolyPhen-2: probably damaging (score: 1.000) Mutation Taster: disease causing (*P*-value: 1) This variant is in the Zinc finger motif Splicing predictions: Predicted change at donor site 3 bps downstream (MaxEnt: +7.4%; NNSPLICE: +25.5%; HSF: +1.0%)	0.0125	0.00625	0	0.00183-1	S12 oligozoo-spermia S28 Azoospermia
34	Chr1: 91739352	c.3689C>G	p.S1230C	Missense	Weakly conserved nucleotide (phyloP: 0.61) SIFT: Tolerated (score: 0.09, median: 3.36) PolyPhen-2: probably damaging (score: 0.973) Mutation Taster: polymorphism (*P*-value: 1)	0.00625	0	0	0	S50 Oligozoospermia

a) The *HFM1* protein includes helicase ATP-binding domain, helicase C-Terminal domain, and SEC63 domain. P-loop: A phosphate-binding loop is a common motif in ATP- and GTP-binding proteins. Zinc finger motif: All cysteine residues in the zinc finger motif were completely conserved. DEXDc: The DEAH/DEAD box helicase domain is in the Helicase ATP-Binding domain. PhyloP: the evolutionary nucleotide conservation score in different species is a reliable method for predicting possible pathogenicity of a missense variant (score$\geqslant$2). SIFT: Amino acid substitutions are predicted to be damaging if $\leqslant$0.05 and tolerated if >0.05. PolyPhen-2: Scores range from 0 to 1 with high scores being assigned to damaging variants. MutationTaster: The *P* value is the probability of the prediction, a value close to 1 indicates a high 'security' of the prediction. MaxEnt: A method based on the Maximum Entropy principle, developed by the Burge Laboratory at Massachusetts Institute of Technology. NNSPLICE: A prediction method based on neural networks. HSF: Human Splicing Finder based on position weight matrices with some position-dependent logic.

control group (P<0.008 and P<0.00001, respectively). Two novel variants were identified locating in protein domains (p.Val494Ala in P-loop and p.Lys443Arg in the DEAH/DEAD box helicase domain (DEXDc)). The former was predicted deleterious to *HFM1* protein function and the later was predicted benign by SIFT, Polyphen-2 and Mutation Taster. These two compound heterozygous variants were identified in two unrelated patients. Of them, one patient had a novel missense variant c.437C>A/p.Thr146Lys (predicted to be deleterious by SIFT) and a known missense variant c.3470G>A/p.Cys1157Tyr in zinc finger motif (predicted to be disease causing by SIFT, Polyphen-2 and MutationTaster, and affecting splicing sites by MaxEnt, NNSPLICE and HSF). The other patient had a novel missense variant c.3689C>G/p.Ser1230Cys (predicted to be probably damaging on the structure and/or function of the *HFM1* protein by PolyPhen-2) and a rare known variant c.2900T>C/p.Ile967Thr in SEC63 domain (predicted to be disease causing by SIFT, Polyphen-2 and Mutation Taster, and affecting splicing sites by MaxEnt, NNSPLICE and HSF). These results indicate that *HFM1* gene variants may associate with idiopathic oligo/azoospermia in Chinese male individuals.

HFM1 (the human homologue of yeast Mer3), a meiotic gene comprised of 39 exons mapped to human chromosome 1p22.2 and specifically expressed in germ-line tissues such as testis and ovary, is necessary for normal progression of homologous recombination and proper synapsis between homologous chromosomes during meiosis process in many organisms (Tanaka et al., 2006; Wang et al., 2009). Hawley et al. (2013) had demonstrated that the absence of *HFM1* protein would result in deficient recombination and incomplete meiosis-specific modification of chromosome structure of *Hfm1*$^{-/-}$ mouse model. *Hfm1*$^{-/-}$ adult mice presented no overt somatic phenotype apart from the lack of reproductive function. The phenotypes of *Hfm1* knockout female mice include a significant reduction in ovary size, reduced number of follicles and corpora lutea, and an increase in stromal cells, which were similar to the phenotypes of POI patients (Hawley et al., 2013; Wang et al., 2014). As demonstrated in our previous study, mutations in *HFM1* gene could result in recessive POI (Wang et al., 2014).

Since the phenotype of meiotic mouse mutants commonly show sexual dimorphism (Hawley et al., 2013; Li et al., 2002), the aim of this study is to validate *HFM1* as a candidate gene for idiopathic oligo/azoospermia. Here for the first time we demonstrated that *HFM1* gene variants may associate with idiopathic oligo/azoospermia. The *HFM1* protein mainly includes helicase ATP-binding domain, helicase C-Terminal domain, and SEC63 domain (Figure S1 in Supporting Information). Four out of six variants found in this study located within the functionally important domains: DEXDc, one of the super family of helicases could unwind DNA and/or RNA, located in the Helicase ATP-Binding domain; the phosphate-binding loop (p-loop), a common motif in ATP- and GTP-binding proteins, containing nucleoside triphosphate hydrolase is involved in the general folding of protein molecules; the SEC63 domain is required for assembly of functional endoplasmic reticulum translocons; the Zinc finger motif, in which all cysteine residues in the zinc finger motif were completely conserved (Saraste et al., 1990; Tanaka et al., 2006). In addition, the p.Ile967Thr located in SEC63 domain might result in an alteration at splice acceptor site 2 bps upstream in exon 27, while the p.Cys1157Tyr located in zinc finger motif might lead to a change at splice donor site 3 bps downstream in exon 31. These two variants involved in splicing sites probably lead to *HFM1* protein dysfunction.

In conclusion, oligo/azoospermia as a complex disease is characterized by genetic heterogeneity with ethnics (Stahl et al., 2012). Our study suggested that *HFM1* gene variants may be associated with idiopathic azoospermia or severe oligozoospermia in Chinese men. Additional study with more patients and functional assays would be valuable for not only confirming the association but also further exploring the biological mechanisms of *HFM1* variations in the spermatogenesis impairment.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 Information of HFM1 protein and sites of variants.

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