Novel Variants in *HFM1* Lead to Male Infertility Due to Non-obstructive Azoospermia

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Research

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Abstract

Background Non-obstructive azoospermia (NOA) is the most severe form of male infertility. More than half of the NOA patients were idiopathic for their etiology, in whom it's difficult to retrieve sperm despite the application of microsurgical testicular sperm extraction (microTESE). Therefore, we conducted to this study to identify the potential genetic factors responsible for NOA, and investigate the sperm retrieval rate of microTESE for the genetic defected NOA.

Methods One NOA patient from a consanguineous family (F1-II-1) and fifty NOA patients from non-consanguineous families were included in the study. Semen analyses, chromosome karyotypes, screening of Y chromosome microdeletions, sex hormone testing, and subsequent testicular biopsy were performed to categorize NOA or obstructive azoospermia. Potentialgenetic variants were identified by whole exome sequencing (WES),and confirmed by Sanger sequencing in F1 II-1. The candidate genes were screened in the other fifty NOA patients. Further experiments including quantitative real time-polymerase chain reaction and western blotting were performed to verify the effects of gene variation on gene expression.

Results Normal somatic karyotypes and Y chromosome microdeletions were examined in all patients. Hematoxylin and eosin staining (H&E) of the testicular tissues suggested meiotic arrest, and a novel homozygous *HFM1* variant (c.3490C>T: p.Q1164X) was identified in F1 II-1. Furthermore, another homozygous *HFM1* variant (c.3470G>A: p.C1157Y) was also verified in F2 II-1 from the fifty NOA patients. Significantly decreased expression levels of HFM1 mRNA and protein were observed in the testicular tissues of these two mutants compared with controls. MicroTESE was performed in these two patients, while no sperm were retrieved.

Conclusions Our study identified two novel homozygous variants of *HFM1* that are responsible for spermatogenic failure and NOA, even microTESE can not contribute to retrieve sperm in these patients.

Introduction

Azoospermia is a medical condition characterized by an absence spermatozoa in the ejaculated semen. It is the most serious form of male infertility, and approximately 1% of the male population and 10% of the infertile males are affected by this condition worldwide [1, 2]. According to spermatogenic function, azoospermia is classified into obstructive azoospermia (OA) and non-obstructive azoospermia (NOA). The development of assisted reproductive technology (ART), such as intracytoplasmic sperm injection (ICSI), has allowed most patients with OA to conceive their own offspring [3, 4]. However, sperm retrieval in NOA patients has always been difficult even by microsurgical testicular sperm extraction (microTESE) [5, 6]. Therefore, it is important to investigate the etiology and pathogenesis of NOA, which will facilitate the development of a targeted therapy.

It has been reported that some factors, such as abnormal chromosomes, Y-chromosomal microdeletion, and cryptorchidism, etc can cause NOA. However, a majority of the NOA cases remain idiopathic and no

medical etiologies have been identified [4]. Recent studies have revealed several NOA-associated genes, such as *TEX11* (OMIM: 300311), *TEX14* (OMIM: 605792), *FANCM* (OMIM: 609644), *SPINK2* (OMIM: 605753), *MEIOB* (OMIM: 617670), and *STAG3* (OMIM: 608489) [7–13]. Although these identified genes can only account for a small proportion of NOA cases, they really deepen our understanding of the causes of NOA.

Helicase family member 1 (HFM1) plays an important role in crossover formation and complete synapsis during meiosis [14]. Previous studies have reported that variants in *HFM1* were related to female primary ovarian failure (POF) and male NOA or severe oligozoospermia in human [15, 16]. And phenotypes of POF and NOA were also found in *HFM1*$^{-/-}$ female and male mice, respectively [17]. In the present study, we reported two novel homozygous variants in *HFM1* leading to NOA, which upgrade the clinical significance of *HFM1* as a pathogenic gene for NOA. Additionally, we also investigated results of microTESE in cases with *HFM1* variants for the first time, which may provide a clinical reference that microTESE is not beneficial for these patients.

Methods

Subjects

An azoospermic patient from a Chinese consanguineous family (F1 II-1), who was admitted to the First Affiliated Hospital of Anhui Medical University for further diagnosis and treatment, was included in the study. Additionally, other 50 NOA patients were also recruited in the study. The ejaculated semen and urine were centrifuged, and some other analyses, including the determination of somatic karyotypes, screening of Y chromosome microdeletions, sex hormone testing, subsequent testicular biopsy, and testicular pathological analysis, were performed to identify the NOA phenotype in patients.

Ethical approval

All participants, including patients, a control individual with normal fertility, and two controls with OA, signed written informed consent to participate in the study. The study was approved by the review board committee of the First Affiliated Hospital of Anhui Medical University, and it was conducted in accordance with the Declaration of Helsinki.

Whole exome sequencing (WES), Sanger sequencing, and bioinformatics analysis

DNA was extracted from the whole peripheral blood. WES, Sanger sequencing, and bioinformatics analysis were performed according to previous studies [18, 19]. Sanger sequencing was performed to verify the identified mutations and parental origins of F1-II-1. The primers used are listed in Supplementary table 1.

Hematoxylin and eosin (H&E) staining and immunofluorescence (IF)

Fluorescence staining was performed as previous described. The following antibodies were used: mouse monoclonal antibody γH2AX (1:1000, Millipore #05-636, to detect meiotic DSBs and the XY body in pachytene nuclei, and apoptotic metaphases in case of pan-chromosomal signal). Histopathological assessment of spermatogenesis was performed by H&E staining. The location for candidate gene expression in seminiferous tubules was determined by IF. H&E staining and IF assay were performed as previously described [18, 19]. The Probable ATP-dependent DNA helicase HFM1 antibody was used as the primary anti-HFM1 antibody (Abbexa, 1:50).

Quantitative real-time PCR (RT-PCR) and western blotting

The mRNA and protein expression levels of the candidate gene were detected by RT-PCR and western blotting, respectively, according to previously described procedures [19, 20]. The same antibody used for IF was applied for western blotting (1:1000), and the PCR primers used are listed in Supplementary table 2.

MicroTESE

As no sperm was detected in routine testicular biopsy, microTESE was performed to retrieve testicular sperms, according to a previous study [21].

Results

Homozygous mutations in HFM1 were identified in two of the selected patients

Normal somatic karyotypes and Y chromosome microdeletions were observed in all 51 patients. H&E staining of the testicular tissue verified NOA in all selected patients. A rare homozygous *HFM1* mutation (c.3490C>T: p.Q1164X) was observed and verified by WES and Sanger sequencing in F1 II-1, and his parents were classified as heterozygous carriers. Subsequently, sequencing of the coding region of *HFM1* was performed in the 50 idiopathic NOA patients to detect *HFM1* mutations. Another novel mutation of the *HFM1* gene (c.3470G>A: p.C1157Y) was observed in four of the fifty NOA patients. Three of the four cases were heterozygous and one was homozygous from a non-consanguineous family (F2 II-1). The detailed clinical features and genetic information of the mutants are presented in Table 1 and Figure 1.

Expression of HFM1 was significantly decreased in the testicular tissues of F1 II-1 and F2 II-1

Testicular tissues of F1 II-1 and F2 II-1 were collected and examined to determine the potential effects of *HFM1* mutations. IF of the testicular tissues of OA patients revealed that *HFM1* was concentrated in cytoplasm of spermatogonia and spermatocytes in seminiferous tubules. The IF signal of HFM1 were significantly decreased in both F1 II-1 and F2 II-1. Detailed information is present in Figure 2. Similarly, the expression levels of HFM1 mRNA and protein in the testicular tissues of F1 II-1 and F2 II-1 were significantly low compared with those of the control group (Figure 3).

Outcome of microTESE in F1-II-1

After an exhaustive understanding of microTESE, both of F1 II-1 and F2 II-1 provided consent to undergo the procedure. However, no sperm were found in these two cases, although many spermatocytes were observed.

Discussion

In this present study, two homozygous variants of *HFM1* were identified as genetic causes of spermatogenesis impairment in two patients with NOA. Additionally, the expression levels of HFM1 mRNA and HFM1 protein were significantly decreased in the two patients with homozygous *HFM1* mutations. Moreover, no sperm were found in mutants despite the application of microTESE. These results indicate that *HFM1* variants are novel causative mutations of NOA in humans.

The NOA phenotype in *HFM1*$^{-/-}$ adult male mice has been reported previously [17]. *HFM1* variants related to NOA in human was first reported in a study conducted in 2017, and compound heterozygous variants of *HFM1* were observed in only one patient with NOA [16]. *HFM1*, comprising 39 exons mapped to the human chromosome 1q22, is a meiosis-specific gene that plays important roles during synapsis and meiotic recombination [14-17]. *HFM1* variants are not only associated with male NOA, but also female POF [15, 16, 22]. Apart from *HFM1*, a series of meiosis-related genes, such as *STAG3* (OMIM: 608489) and *DMC1* (OMIM: 602721), are also associated with fertility, and their variants can lead to male spermatogenic failure or female POI due to germ cell defects [12, 23, 24]. *STAG3* encodes a meiosis-specific protein, which plays an important role in chromosomal axis formation and sister chromatid recombination. Additionally, *DMC1* is also a meiosis-specific gene that plays an essential role in homologous recombination during meiosis and DNA double-strand break repair [22]. In our study, we observed two novel homozygous *HFM1* variants in two unrelated NOA patients. F1 II-1 carried a homozygous stop-gain variant in *HFM1* (c.3490C>T: p.Q1164X), which led to a truncated protein. This variant was classified "likely pathogenic" according to the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines [25]. The functions of the truncated protein may be different from those of the normal one, and it can cause defects in chromosome synapsis and recombination, leading to spermatogenesis failure. F2 II-1 carried a homozygous missense variant in *HFM1* (c.3470G>A), which changed the amino acid at position 1157 from cysteine to tyrosine. The replacement of a highly conserved hydrophilic amino acid with a hydrophobic amino acid may lead to a change in the secondary structure of the *HFM1* protein and influence its function. Potential high pathogenicity of the variant was predicted from Sorting Intolerant from Tolerant (SIFT), PolyPhen-2, and MutationTaster. The RT-PCR and western blot analyses also reported that the expression levels of HFM1 mRNA and protein were significantly decreased in the two patients with homozygous *HFM1* mutations compared with the normal individuals.

Routine testicular biopsy of these two patients revealed no sperm. Considering the high sperm retrieval rate by microTESE in NOA patients [26, 27], F1 II-1 and F2 II-1consented to undergo the procedure. However, no sperms were retrieved, either. It is the first time to report outcomes of microTESE in NOA

patients with variants in *HFM1*. Although only two patients were enrolled, it may provide a clinical reference that microTESE is not beneficial for these patients.

Some limitations should be taken into consideration in this study. Firstly, since sequencing of *HFM1* was not performed in healthy fertile males, we cannot comment on the variant frequency of the gene in normal fertile males. Secondly, the chromosome spread experiments, which can detect possible defects during synapsis and recombination, was not performed as no enough testicular samples. Thirdly, only two cases with homozygous variants in *HFM1* underwent microTESE, further research is necessary to verify the probability of successful sperm retrieval by microTESE in NOA patients with bialletic *HFM1* mutations.

Conclusions

Our results provide further evidences for *HFM1* is a candidate gene of NOA in human, and suggest that homozygous variants in *HFM1* can cause autosomal recessive male infertility due to NOA. In addition, it is likely that microTESE cannot contribute to retrieve sperm in these patients.

Abbreviations

NOA: non-obstructive azoospermia

microTESE: microsurgical testicular sperm extraction

WES: Whole exome sequencing

H&E: Hematoxylin and eosin staining

OA: obstructive azoospermia

ART: assisted reproductive technology

ICSI: Intracytoplasmic sperm injection

HFM1: Helicase family member 1

POF: primary ovarian failure

IF: immunofluorescence

RT-PCR: Quantitative real-time PCR

ACMG: American College of Medical Genetics and Genomics

AMP: Association for Molecular Patholog

SIFT: Sorting Intolerant From Tolerant

Declarations

Ethics approval and consent to participate

This study was approved and consented by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University.

Consent for publication

All the individuals and their family members, as well as two controls signed written informed consents after having received complete information about the research.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YC, XH, DT and ML designed the study. DT, CW, CX and HG collected the data. YG, and KL analyzed the data of WES and Sanger sequencing. DT, HC, GL and QS performed the experiments of RT-PCR and immunofluorescence staining. DT, ML and YG wrote the paper. All authors have read and approved the final manuscript.

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Table

Table 1
Clinical features and genetic information of HFM1 mutations in F1 II-1 and F2 II-1.

Individuals	**F1 II-1**	**F2 II-1**
Clinical features		
Age	28	31
Secondary sexual characteristics	Normal	Normal
testicular volume(Left/Right, ml)	12/12	10/10
Somatic karyotype	46,XY	46,XY
Y Chromosome microdeletions	No	No
Sex hormone		
Follicle-stimulating hormone(IU/L)	14.75	25.79
Luteinizing hormone(IU/L)	7.53	15.10
Testosterone(nmol/L)	11.04	12.86
Estradiol(pmol/L)	209.00	109.00
Prolactin(ng/ml)	13.92	6.34
Information of HFM1 **mutations**		
cDNA mutation	c.3490C > T	c.3470G > A
Mutation type	Stopgain	Missense
Protein alteration	p.Q1164X	p.C1157Y
Allele frequency in human population		
1KGP	0	0.005
ExAc_all	0	0.002
gnomAD	0	0.002
Functional prediction		
SIFT	N/A	Damaging
PolyPhen-2	N/A	Probably Damaging
MutationTaster	Disease_causing Automatic	Disease_causing
RefSeq accession number of *HFM1* is NM_001017975.5		
Abbreviations: 1KGP, 1000 Genomes Project; ExAc_all, all the data of Exome Aggregation Consortium; gnomAD, the Genome Aggregation Database; N/A: Not applicable.		

Figures

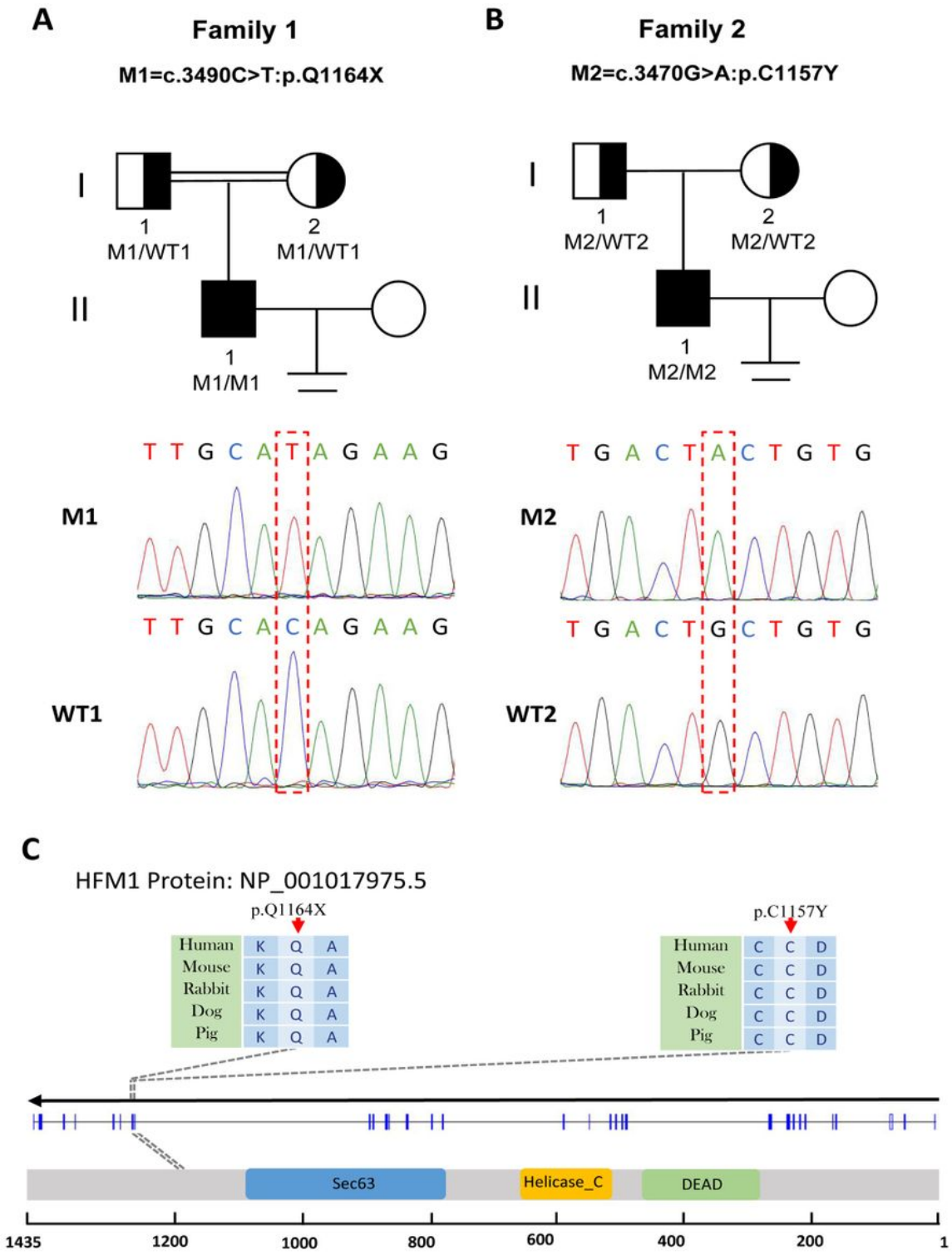

Figure 1

Variants of HFM1 in the two cases. (A-B) The two families affected by the variants in HFM1. The red frames indicate mutated positions in the Sanger sequencing results. (C) The mutated positions of HFM1

are conserved among species. And the red arrows indicate the locations of HFM1 variants occurred in the domains of HFM1 protein. M, HFM1 mutation; WT, wild type.

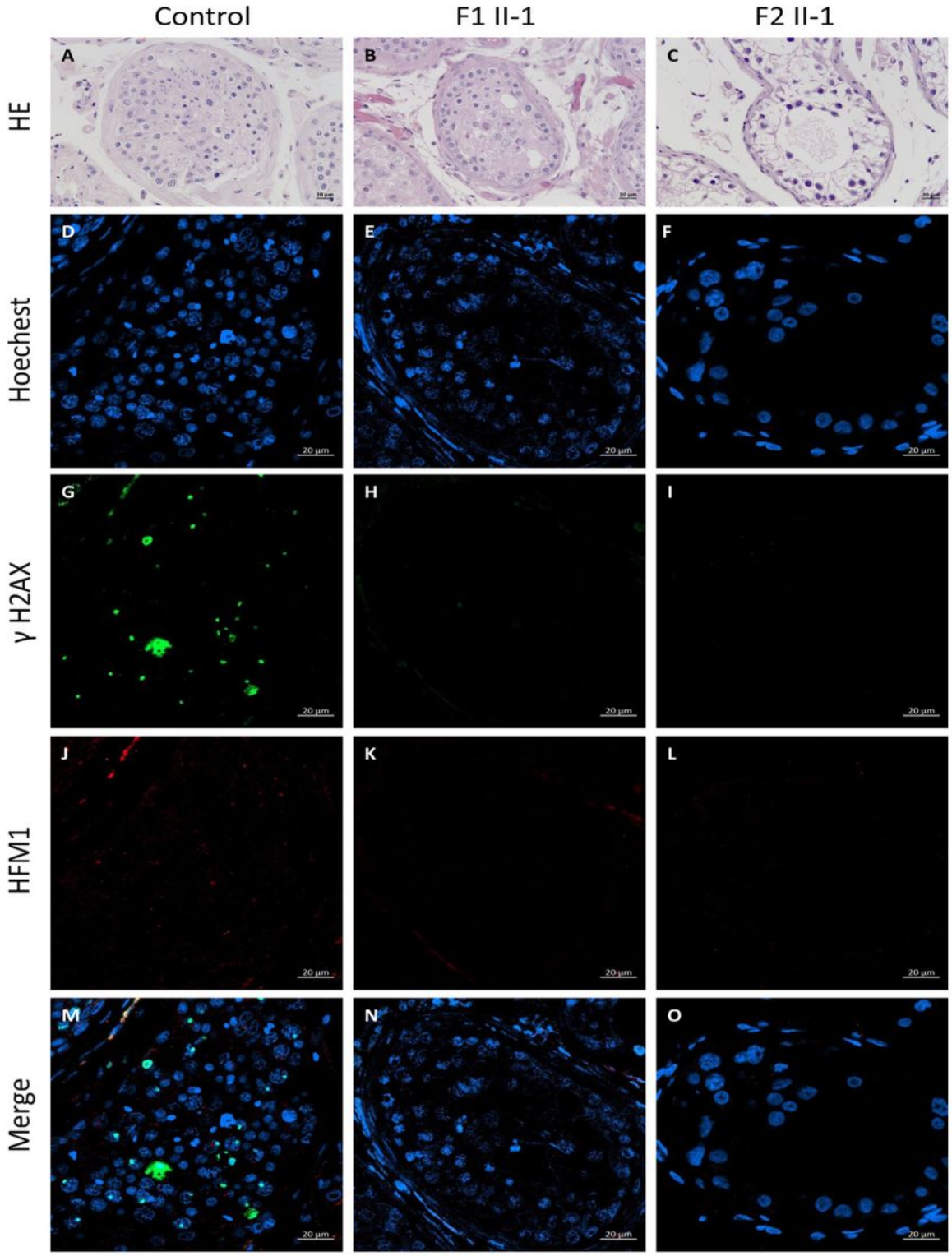

Figure 2

Testicular histopathology of H&E staining and the expressive location of the HFM1 of IF assay. (A) Normal testicular histopathology from a OA patient. (B) Testicular histopathology of meiotic arrest from F1 II-1 and F2 II-1. (D, G, J, M) HFM1 immunostaining (red) was primarily concentrated in cytoplasm of

spermatogonia and spermatocytes in seminiferous tubules in normal control. (E, F, H , I, K, L, N, O) The immunostaining was decreased in the testicular tissues of F1 II-1 and F2 II-1. Hoechst (blue) was stained as a nuclear marker. The γH2AX (green) was stained as a marker of spermatocyte.

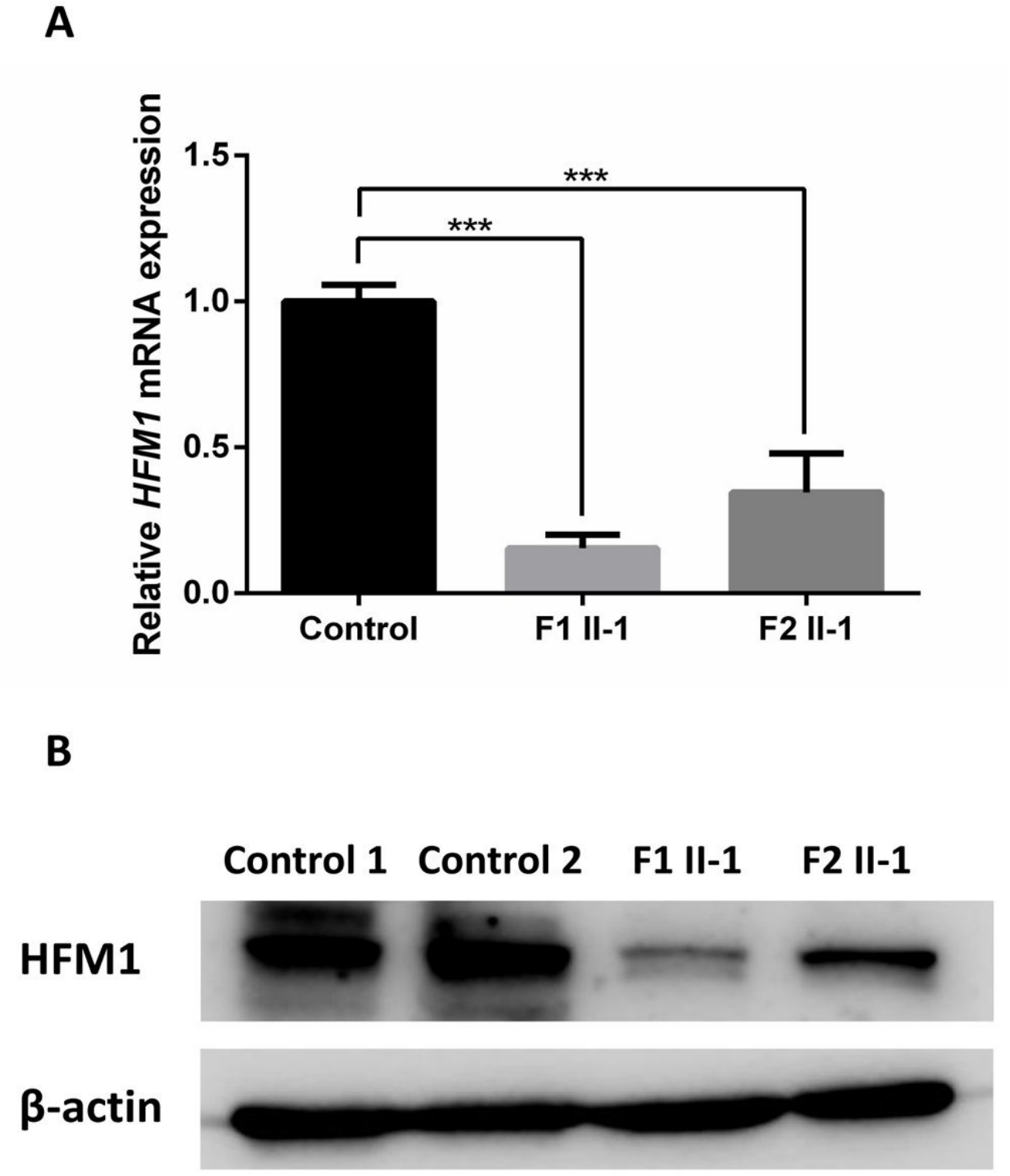

Figure 3

(A) The expressions of HFM1-mRNA are significantly lower in F1 II-1 and F2 II-1 compared with normal control by RT-PCR. (B) The expressions of HFM1-protein are significantly lower in F1 II-1 and F2 II-1

compared with normal controls by western blot.

Supplementary Files

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