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Novel loss-of-function mutation in *MCM8* causes premature ovarian insufficiency

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Abstract

Background: Premature ovarian insufficiency (POI) is one major cause of female infertility, minichromosome maintenance complex component 8 (*MCM8*) has been reported to be responsible for POI.

Methods: Whole-exome sequencing was performed to identify the genetic variants of women with POI. Sanger sequencing was used to validate the variants in all the family members. Various bioinformatic software was used for the pathogenicity assessment. Reverse transcription polymerase chain reaction (RT-PCR), real-time quantitative PCR, and a chromosomal instability study induced by mitomycin C were performed to analyze the functional effects of the variant.

Results: A novel homozygous frameshift mutation (NM_032485.4:c.351_354 delAAAG) of *MCM8* gene was identified in the patients, segregated with POI in this family. This mutation is predicted to produce truncated MCM8 protein and to be pathogenic. Reverse transcription polymerase chain reaction revealed that the frameshift mutation led to a remarkably reduced level of *MCM8* transcript products, and chromosomal instability study showed that the ability of mutant MCM8 to repair DNA breaks was impaired.

Conclusion: We identified a novel homozygous frameshift mutation in the *MCM8* gene in two affected sisters with POI, and functional analysis revealed that this mutation is pathogenic. Our findings enrich the *MCM8* mutation spectrum and might help clinicians to make a precise diagnosis, thereby allowing better family planning and genetic counseling.

KEYWORDS

loss-of-function, *MCM8* gene, premature ovarian insufficiency, whole-exome sequencing

1 | INTRODUCTION

Premature ovarian insufficiency (POI) is one major cause of female infertility, which is characterized by loss of normal ovarian function before the age of 40 years. Patients with POI present with primary or secondary amenorrhea, low serum levels of estrogen, and elevated level of follicle-stimulating hormone (FSH > 25 mIU/ml; European Society for

Human Reproduction and Embryology (ESHRE) Guideline Group on POI; Webber et al., 2016). The etiology of POI is complex, including autoimmune factors, metabolic factors, iatrogenic factors, infectious factors, environmental and psychological factors, and genetic factors (Vegetti et al., 1998). The genetic factors for POI are heterogeneous and only a few of the genes have been identified as candidate genes, including X-linked genes (e.g., *BMP15* (Pasquale, Beck-Peccoz, & Persani, 2004) and *FMR1* (Conway, Payne, Webb, Murray, & Jacobs, 1998)) and autosomal genes (e.g., *FSHR* (Aittomäki et al., 1995), *STAG3* (He et al., 2017), *XRCC2*; (Zhang et al., 2019), and *MCM8* (AlAsiri et al., 2015; Tucker, Grover, Bachelot, Touraine, & Sinclair, 2016)).

MCM8, a member of the minichromosome maintenance complex component (MCM) family, plays an important role in homologous recombination and repair of double-strand DNA breaks (DSBs). *Mcm8* knockout mice had impaired homologous recombination-mediated DNA repair during gametogenesis, which led to POI and infertility (Lutzmann et al., 2012). However, mutations reported in *MCM8* related to POI are rare, and only nine mutations have been described (AlAsiri et al., 2015; Bouali et al., 2017; Desai et al., 2017; Dou et al., 2016; Yardena et al., 2015), including two frameshift variants (Table 1; Figure 1c).

In our study, a novel homozygous frameshift mutation in the *MCM8* gene was identified in a consanguineous Han Chinese family by whole-exome sequencing (WES), which segregates with POI in this family. Furthermore, functional characterization revealed that this frameshift mutation is pathogenic.

2 | MATERIALS AND METHODS

2.1 | Study subjects

Written informed consent was obtained from all participating individuals. This study was approved by the institutional ethics committees of the Reproductive and Genetic Hospital of CITIC Xiangya of Central South University.

A consanguineous Han Chinese family was recruited to identify the genetic etiology of infertility at the Reproductive and Genetic Hospital of CITIC Xiangya (Changsha, Hunan, China). The family includes normal parents (family members III-1 and III-2), a normal daughter (family member IV-2), and two daughters affected with POI (family members IV-3 and IV-1; Figure 1a). The healthy parents were first cousins, living in Hunan Province, with normal fertility. The proband (IV-3) and her elder sister (IV-1) had received a diagnosis of primary infertility after more than 3 years of marriage. Routine analyses of their husbands' semen revealed normal fertility.

2.2 | Genomic DNA extraction and WES

Genomic DNA from peripheral blood samples was extracted using a QIAamp® DNA blood midi kit (Qiagen) according to the manufacturer's protocol. The proband was subjected to WES. Sequences were captured by SureSelect v.4 (Agilent Technologies), and enriched libraries were sequenced using an Illumina HiSeq2000. After removing joints and low-quality raw data, the WES raw reads were aligned to NCBI GRCh37 (reference genome Hg19) using Burrows-Wheeler Aligner, and then Picard was used to remove and sort the copies of polymerase chain reaction (PCR; http://broadinstitute.gib.io/picard/). The GATK package was used for mutation identification, including base recalibration variant calling with Haplotype Caller and variant quality score recalibration, and the ANNOVAR software was used for mutation annotation to screen out possible pathogenic mutations. The pathogenicity of the mutation was predicted by bioinformatics software (SIFT [https://sift.bii.a-star.edu.sg/], MutationTaster [http://www.mutationtaster.org/], and Combined Annotation Dependent Depletion [CADD; cadd.gs.washington.edu/]). Structural analysis was performed using the SWISS-MODEL software (https://swissmodel.expasy.org).

We use the following inclusion criteria to identify the candidate pathogenic genes: (a) had a frequency of less than 1% in the 1,000 Genomes variant database, NHLBI-GO exome sequencing project, and Exome Aggregation Consortium; (b) bioinformatics software predicted the genes to be deleterious variants; (c) homozygous variants were considered with priority; and (d) the genes were relevant to the phenotype (including biological process associated with oogenesis or the existence of a mouse knockout model with a female sterility phenotype similar to our patients).

The *MCM8* mutation was validated by Sanger sequencing, and the specific primers used are listed in Table S1.

2.3 | Reverse transcription polymerase chain reaction

First, we performed reverse transcription PCR (RT-PCR) to analyze the functional effects of the variant. The RNA was extracted from the peripheral blood of the two POI patients and a normal female control according to the instructions of the RNA extraction kit (Qiagen). The primers used for RT-PCR are listed in Table S1. Then, the amplified PCR products were analyzed using 3.0% agarose gel electrophoresis to determine whether *MCM8* transcripts were degraded due to a nonsense-mediated mRNA decay (NMD) pathway.

TABLE 1 Clinical characteristics of earlierly reported cases of POI with MCM8 mutations

Variants	Menstrual history	FSH (mIU/ml)	LH (mIU/ml)	Estradiol (Pg/ml)	Ultrasonographic examination		Other clinical abnormalities	Age at diagnosis (years)	Reference
c.446C>G:p.P149R (Hom)	Primary amenorrhea	73.25	22.1	1.11	Right ovarian volume (cm^3)	Not visualized	Hypergonadotropic hypogonadism; hypothyroidism	NA	Saleh et al. (2015)
					Left ovarian volume (cm^3)	4.3			
		95.09	28.93	10.37	Right ovarian volume (cm^3)	Not visualized			
					Left ovarian volume (cm^3)	2.7			
		42.13	12.05	10.82	Right ovarian volume (cm^3)	0.7			
					Left ovarian volume (cm^3)	0.4			
c.1954-1G>A (Hom)	Primary amenorrhea	NA	NA	NA	No ovaries and no uterus (15, 17, 18 years) No ovary, 6-mm long uterus with 4-mm mucosa (20 years)		Agenesis of left kidney; middle conductive high-frequency hearing loss in left ear; temporal epilepsy; mild mental retardation	15	Yardena et al. (2015)
c.1469-1470insTA (Hom)	Primary amenorrhoea	NA	NA	NA	No ovaries , small prepubertal uterus length 5 cm (14.5 years)		Normal	14.5	
c.464G>A:p.C155Y (Het) c.548A>G:p.N183S (Het) c.1334G>A:p.R445Q (Het)	Primary amenorrhea	NA	NA	NA	NA		NA	NA	Desai et al. (2017)
c.950A>T:p.H317L (Het)	Primary amenorrhea	>40	NA	NA	NA		NA	NA	Dou et al. (2016)
c.1802A>G:p.H601R (Het)	Secondary amenorrhea	>40	NA	NA	NA		NA	NA	
c.482A>C:p.H161P (Hom)	Primary amenorrhea	86	20	45	Atrophic ovaries and uterus		Normal	14	Bouali et al. (2017)
C.351_354delAAAG:p.K118Efs*5 (Hom)	Primary amenorrhea	76.91	35.36	5	NA		Normal	28	This study
		77.02	26.26	6.78	Uterus: 32 × 13 × 26 mm Right ovary: 11 × 6 × 8 mm Left ovary: 10 × 6 × 7 mm		Normal	26	

Note: Normal value range: estradiol (pg/ml), female: 21-251pg/ml; FSH (mIU/ml), female: 3.03–8.08 mIU/ml; LH (mIU/ml), female: 1.80–11.78 mIU/ml.

Abbreviations: FSH, follicle-stimulating hormone; Het, heterozygote mutation; Hom, homozygote mutation; LH, luteinizing hormone; NA, data not available; POI, premature ovarian insufficiency.

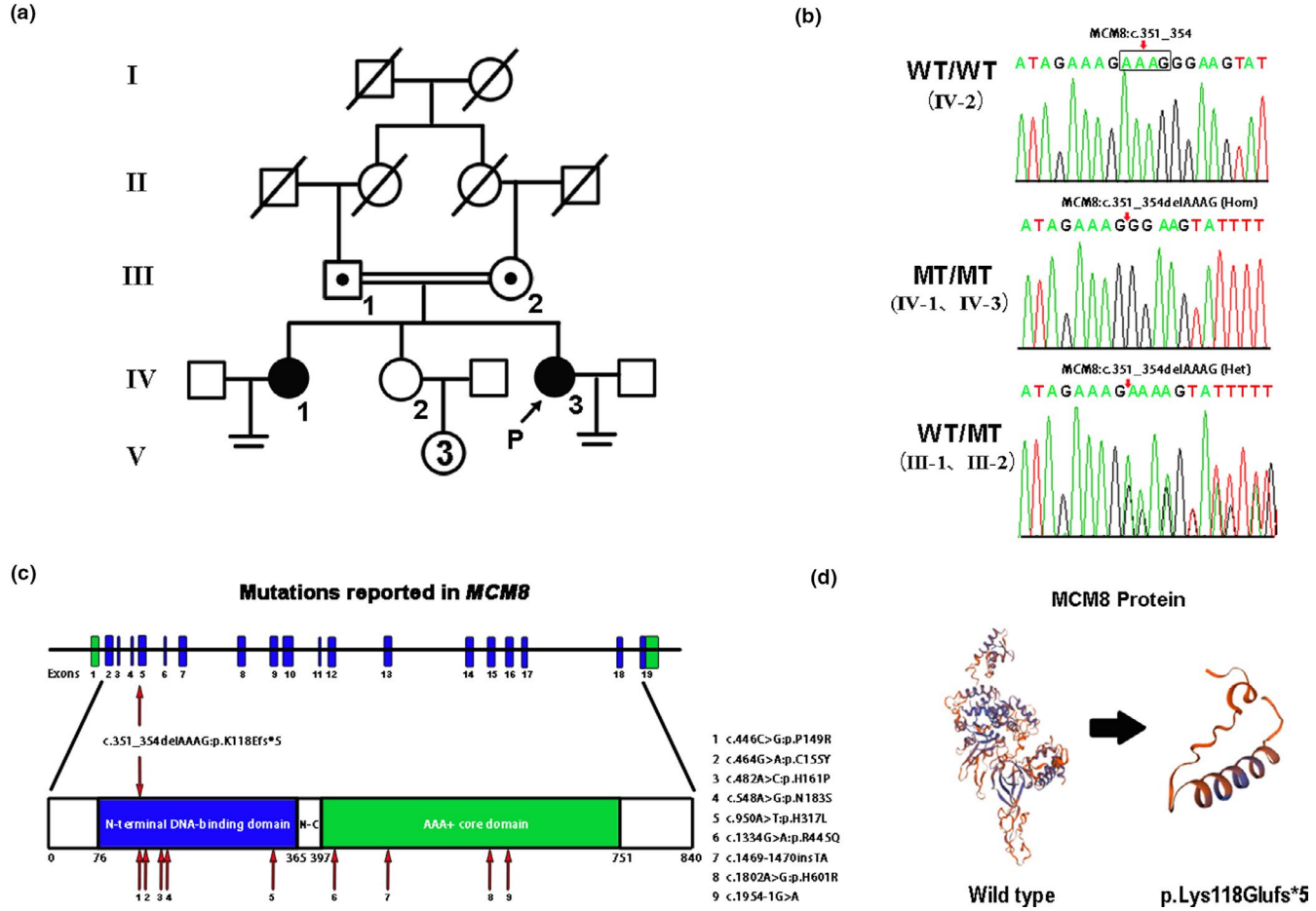

FIGURE 1 Pedigree of the consanguineous Chinese family in this study and Sanger sequencing of the c.351_354delAAAG mutation of *MCM8* in this family. (a) Two first cousins (III-1 and III-2) in generation 3 married to each other with two affected children (IV-1 with POI and IV-3 with POI). The proband is marked with a black arrow. Open symbols indicate the unaffected members. Heterozygous carriers are indicated with a dot in the middle of the symbol. Filled symbols indicate the affected members with POI. (b) Sanger sequencing of the c.351_354delAAAG (pointed by red arrows) mutation of *MCM8* in this family. Two affected sisters (IV-1 and IV-3) are homozygous for the mutation. The healthy parents are heterozygous for the mutation, while their healthy daughter (IV-2) is free of the mutation. MT, mutation-type allele; POI, premature ovarian insufficiency; WT, wild-type allele. (c) *MCM8* gene is located on chromosome 20, consisting of 19 exons and encoding 840 amino acids (the coding exons are present as blue full boxes, noncoding exons are show as green full boxes, and introns are present with black lines). It has two important domains: N-terminal DNA-binding domain and AAA+ core domain. The mutation (NM_032485.4:c.351_354delAAAG) reported in our study is located in exon 5 and lies in the N-terminal DNA-binding domain of MCM8 (Middle red arrows indicate this mutation). Up to now, the nine mutations in *MCM8* that have been reported to be related to POI are indicated by red arrows below the schematic of the MCM8 domains. (d) Structural analysis of MCM8 using the SWISS-MODEL software (https://swissmodel.expasy.org). The MCM8 (NP_001268449.1) variant was predicted to form a truncated MCM8 protein of 121 amino acids (p.Lys118Glufs*5)

2.4 | Real-time quantitative PCR

Real-time quantitative PCR was performed on LightCycler® 480 Instrument II (Roche) using the LightCycler® 480 SYBR® Green I Master (Roche) according to the manufacturer's instruction. The expression level of *MCM8* was normalized to the endogenous expression of *β-ACTIN*. Reactions were performed using two pairs of primers, one pair spanning exons 3 and 4 of *MCM8*, which are located upstream of the mutation we identified, and the other pair spanning exons 5 and 6, which includes the mutation site we identified. GraphPad Prism 5 software and a two-tailed *t* test assuming equal variance were used to compare the expression level of *MCM8* between homozygous (IV-1 and IV-3) and the wild-type control (IV-2). We considered the difference to be statistically significant when the calculated corresponding *p* value was <.05. The detailed primer sequences used in RT-qPCR are shown in Table S1. The assays were performed in independent triplicate.

2.5 | Chromosomal instability study

The peripheral blood of two patients and a normal female control were collected. Lymphocytes were cultured in peripheral blood

karyotyping medium with phytohemagglutinin in the presence of mitomycin C (MMC) from *Streptomyces caespitosus* at different concentrations (0, 300 nM). Cells were harvested after 72 hr of incubation at 37°C and placed onto microscope slides. Chromosomal breaks of metaphase cell spreads were counted under a microscope (the total number of metaphase cell divisions was more than 100 in each study slide). The corresponding percentages of chromosomal breaks were calculated (total number of chromosomal breaks/total number of metaphase cell spreads counted). Finally, GraphPad Prism 5 software and a two-tailed *t* test assuming equal variance were used to compare the percentages of chromosomal breaks between homozygous (IV-1 and IV-3) and a normal control (NC). We considered the difference to be statistically significant when the calculated corresponding *p* value was <.05.

3 | RESULTS

3.1 | Clinical findings

The proband (IV-3), a 26-year-old woman, was diagnosed with POI at age 20, with primary amenorrhea. Transvaginal ultrasound examination revealed that her uterus was significantly small (32 × 13 × 26 mm), and the adnexa on both sides were impalpable. After hormone replacement therapy, ovaries were observed but still small (left ovary: 10 × 6 × 7 mm; right ovary: 11 × 6 × 8 mm), and no visible follicle was identified. She had low estradiol levels (6.78 pg/ml; normal range 21–251 pg/ml), high levels of FSH (77.02 mIU/ml; normal range 3.03–8.08 mIU/ml) and LH (26.26 mIU/ml; normal range 1.80–11.78 mIU/ml), and normal levels of prolactin and thyroid-stimulating hormone. Additionally, her anti-mullerian hormone (AMH) and inhibin B levels were too low to be detected.

The sister of proband (IV-3) also received the diagnosis of POI with similar clinical symptoms, including primary amenorrhea, low estradiol levels (5 pg/ml; normal range 21–251 pg/ml), and high levels of FSH (76.91 mIU/ml; normal range 3.03–8.08 mIU/ml) and LH (35.36 mIU/ml; normal range 1.80–11.78 mIU/ml). The detailed clinical examination data of the affected individuals in the family are shown in Table S2.

Their mother (III-2) has normal pubertal development and regular menstrual period. Their unaffected sister (IV-2) was fertile and conceived spontaneously. The two patients showed normal 46, XX karyotypes and a normal range of FMR1 CGG repeats. No associated endocrinopathies or autoimmune disorders were found in this family.

3.2 | Identification of the *MCM8* mutation

Whole-exome sequencing was performed on the proband (IV-3) to identify the pathogenic variants. Raw data (average of 15.65 Gb) were generated with a mean depth of 141.21-fold for the target regions, indicating the high quality of sequencing (as shown in Table S3). After mapping to the reference genome sequence (Hg19), approximately 99.71% of the targeted bases were covered sufficiently to pass quality assessment for calling single-nucleotide polymorphisms and indels. According to the filtering strategy, only one novel homozygous variant in *MCM8* (NM_032485.4:c.351_354delAAAG;p.Lys118Glufs*5) fulfilled these criteria (detailed data are shown in Table S4), which was predicted to result in the formation of a truncated MCM8 protein of 121 amino acids (p.Lys118Glufs*5; Figure 1d). Sanger sequencing verified that the proband and her affected sister were homozygous, her mothered sister was free of this variant, and their healthy parents were heterozygous carriers (Figure 1b), co-segregated with POI in this family.

3.3 | The frameshift mutation led to the loss of *MCM8* transcript products

Based on the mechanism of NMD, it is predicted that being homozygous for this mutation will result in a loss of *MCM8* transcript products (Lejeune & Maquat, 2005). Firstly, we analyzed the level of *MCM8* transcript products in the patient's peripheral blood. Reverse transcription polymerase chain reaction showed that it was unknown whether the *MCM8* transcript products of the affected siblings (IV-1 and IV-3) were partly degraded or not (Figure 2a). Subsequently, we used real-time quantitative PCR to identify the actual levels of *MCM8* transcript products, which revealed a remarkably reduced level of *MCM8* transcript products in the affected subjects compared to their sister free of this mutation (IV-2; Figure 2b). Taken together, the frameshift mutation leads to the loss of MCM8 transcript products in patients with POI in this family, which may underlie the pathogenesis of this disease in women.

3.4 | The repair of chromosomal breaks in mutant MCM8 was impaired

Previous study has suggested that MCM8 participates in the DSBs repair and affects gametogenesis in mice (Lutzmann et al., 2012). Therefore, we examined the chromosomal breakage repair by culturing peripheral lymphocytes exposed to 300 nM MMC using peripheral blood cells from two patients (IV-1 and IV-3) and a NC. A total of more than 110 cells were assessed per sample to score broken chromosomes. Percentages of chromosomal breaks observed in metaphase spreads of NC, IV-1, and IV-3 revealed that the homozygous affected siblings (IV-1 and IV-3) had more chromosomal breaks than that of the NC at 300 nM MMC concentration (Figure 2c,d). Thus, we concluded that the ability to repair

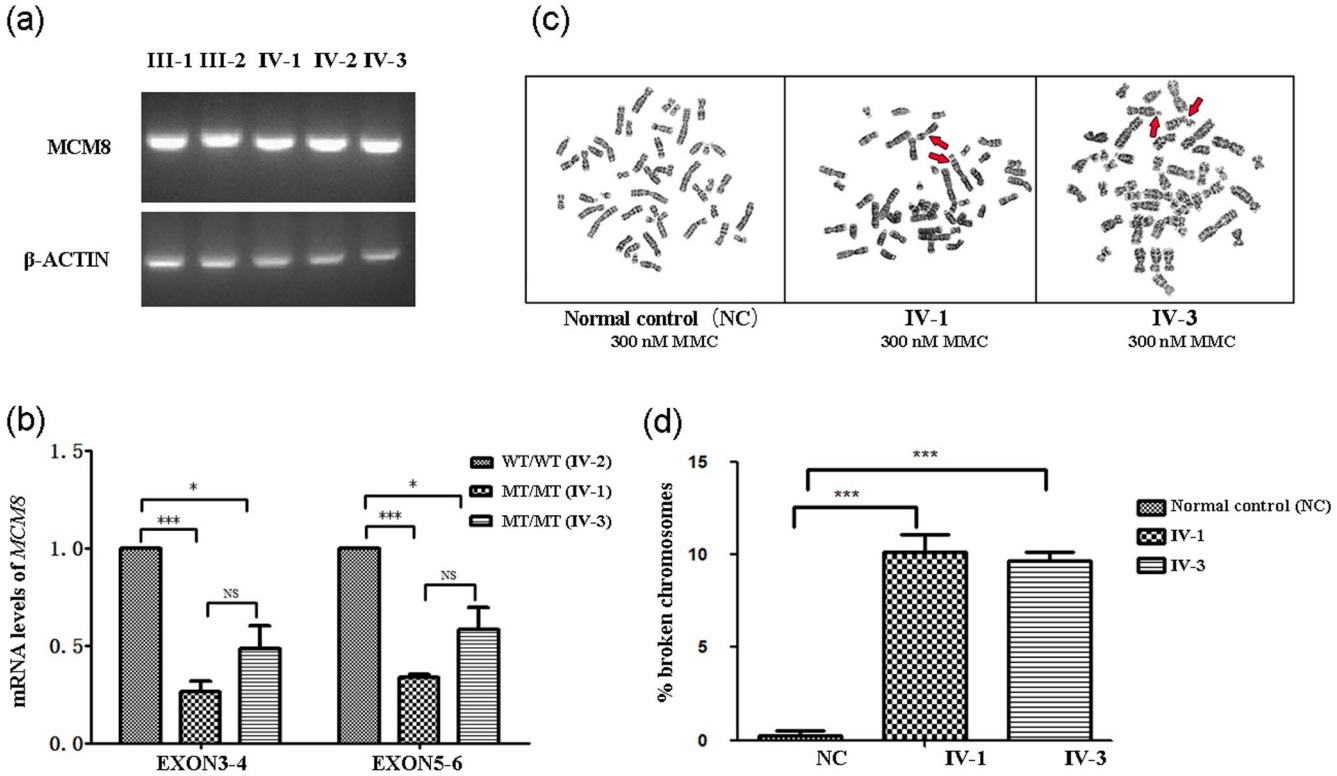

FIGURE 2 Functional analysis of the c.351_354delAAAG mutation of *MCM8*. (a) 3.0% agarose gel electrophoresis of the reverse transcription polymerase chain reaction (PCR) products from five members of the family. The brightness of five bands was similar, which reveals that the MCM8 transcript products of affected siblings (IV-1 and IV-3) were not partially or completely degraded. (b) Statistical bar chart of real-time quantitative PCR data. Real-time quantitative PCR was performed on two affected sisters (IV-1 and IV-3) and their sister free of this mutation (IV-2), which revealed a remarkably reduced level of MCM8 transcript products in the affected subjects compared to IV-2 (p values comparison between IV-1 and IV-2:0.0004 [exon3~4] and <.0001 [exon5~6]; p values comparison between IV-3 and IV-2:0.0168 [exon3~4] and 0.0312 [exon5~6]). WT, wild-type allele; MT, mutation-type allele; NS, no significant difference. *$p < .05$ were considered statistically significant. ***$p < .001$. (c) Metaphase spreads of peripheral blood lymphocytes (normal control, IV-1, and IV-3) exposed to 300 nM MMC. The red arrows mark the broken chromosomes. (d) Statistical bar chart of percentages of broken chromosomes (observed in metaphase spreads of normal control, IV-1, and IV-3 at 300 nM MMC), which revealed that the homozygous affected siblings (IV-1 and IV-3) had more chromosomal breaks than that of the normal control (p values comparison between IV-1 and normal control: .0005; p values comparison between IV-3 and normal control: <.0001). MMC, mitomycin C. *$p < .05$ were considered statistically significant. ***$p < .001$

DNA breaks with mutant MCM8 was impaired in affected patients.

4 | DISCUSSION

In the present study, we identified two affected sisters with POI in a consanguineous Han Chinese family. In order to find out the genetic cause of the disease in this family, we performed WES on the proband, which revealed a novel homozygous frameshift mutation (c.351_354delAAAG;p.Lys118Glufs*5) in the *MCM8* gene. Functional analysis revealed that this mutation leads to a decrease of *MCM8* transcript products in patients with POI in this family, which may be due to the mechanism of NMD; the decreased *MCM8* transcript products might result in the impairment of the repair of chromosomal breaks. These findings support that this mutation is the pathogenic cause of POI in this family.

MCM8 gene is located on chromosome 20, consisting of 19 exons and encoding 840 amino acids (Johnson, Yayoi, & Daniel, 2003). It has two important domains: the N-terminal DNA-binding domain and AAA+ core domain, which are highly conserved among primates. Mutations (c.1954-1G>A; c.1469-1470insTA) that resulted in truncated MCM8 with partial N-terminal DNA-binding domain have been reported to impair repair of chromosomal breaks and result in POI (Yardena et al., 2015). The mutation (c.351_354delAAAG; p.Lys118Glufs*5) identified in our patients is also predicted to result in the formation of a truncated MCM8 with partial N-terminal DNA-binding domain, indicating that it might be pathogenic. Furthermore, the peripheral lymphocytes of our patients showed impaired repair of chromosomal breaks with high sensitivity to MMC, compared to the normal individual.

Hence, we suggest that the identified frameshift mutation affects the function of MCM8 and is responsible for POI in this family.

MCM8 is a member of the minichromosome MCM family, which is involved in important physiological processes such as DNA replication, meiosis, and homologous recombination repair (Deegan & Diffley, 2016). Homologous recombination repair is a vital important process to ensure the integrity of DNA. Oocytes with repair defects of DSBs tend to undergo apoptosis, and thus ultimately cause POI (Ceccaldi, Rondinelli, & D'Andrea, 2015; Katari et al., 2018). Up until now, four homozygous *MCM8* mutations (c.446C>G:p.P149R; Saleh et al., 2015; c.482A>C:p.H161P; Bouali et al., 2017; c.1954-1G>A; Yardena et al., 2015; c.1470_1471insAT; Yardena et al., 2015) have been reported to be related to POI. In our study, the patients showed impaired repair of chromosomal breaks and were diagnosed with POI with no visible follicles. Therefore, we suggested that this homozygous mutation of *MCM8* impaired the repair of DSBs, hindered the meiotic processes of the oocytes, and resulted in POI.

In a previous study, substantial evidence revealed that patients with defective genes related to repair of DSBs (*MCM8*, *MCM9*, *BRCA1*, and *BRCA2*) show a series of clinical features. Women with defects of *MCM9* resulted in POI and short stature (Wood-Trageser et al., 2014). In addition, patients who carried mutations of *BRCA1* or *BRCA2* have the risk of cancer susceptibility, as well as POI (Kutluk, Volkan, Shiny, Robert, & Lin, 2015). Previous findings demonstrated that patients with *MCM8* mutations presented with POI and other disorders, including hypergonadotropic hypogonadism, hypothyroidism (Saleh et al., 2015), agenesis of kidney, hearing loss, epilepsy, and mild mental retardation (Yardena et al., 2015). Furthermore, *Mcm8*-null female mice tended to develop ovarian adenomas and sex cord-stromal tumors (Lutzmann et al., 2012). In our study, the affected patients had normal height, normal thyroid function, and we did not find other abnormalities at present. It could be explained by the fact that the late-onset diseases may have not yet occurred in the patients at this age, such as tumors, or that genetic background (individual variations) was able to modulate the severity of mutant phenotypes (Vu et al., 2015). Therefore, we suggest that regular medical examinations are required for the patients in our study and other infertile patients caused by DSBs repair-related genes, in order to identify other related diseases as soon as possible and treat them early.

In conclusion, we identified a novel *MCM8* loss-of-function mutation that contributes to POI, extending the mutation spectrum of the *MCM8* gene and having important significance for genetic counseling of POI families. Follow-up studies will be necessary to expand the phenotype spectrums in females with POI related to *MCM8* variants and the mechanisms of MCM8 involvement in gametogenesis still need to be explored further.

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CONFLICT OF INTEREST

The authors declare that they have no conflicting interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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