MCM8 and *MCM9* Nucleotide Variants in Women With Primary Ovarian Insufficiency

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Objective: To assess the frequency of variants, including biallelic pathogenic variants, in *minichromosome maintenance 8* (*MCM8*) and *minichromosome maintenance 9* (*MCM9*), other genes related to MCM8–MCM9, and DNA damage repair (DDR) pathway in participants with primary ovarian insufficiency (POI).

Design: *MCM8*, *MCM9*, and genes encoding DDR proteins that have been implicated in reproductive aging were sequenced among POI participants.

Setting: Academic research institution.

Participants: All were diagnosed with POI prior to age 40 years and presented with elevated follicle-stimulating hormone levels.

Interventions: None.

Main Outcome Measures: We identified nucleotide variants in *MCM8*, *MCM9*, and genes thought to be involved in the DNA damage response pathway and/or implicated in reproductive aging.

Results: *MCM8* was sequenced in 155 POI participants, whereas *MCM9* was sequenced in 151 participants. Three of 155 (2%) participants carried possibly damaging heterozygous variants in *MCM8*, whereas 7 of 151 (5%) individuals carried possibly damaging heterozygous variants in *MCM9*. One participant carried a novel homozygous variant, c.1651C>T, p.Gln551*, in *MCM9*, which is predicted to introduce a premature stop codon in exon 9. Biallelic damaging heterozygous variants in both *MCM8* and *MCM9* were identified in 1 participant. Of a total of 10 participants carrying damaging heterozygous variants in either *MCM8* or *MCM9*, 2 individuals carried heterozygous damaging variants in genes associated with either MCM8 or MCM9 or the DDR pathway.

Conclusions: We identified a significant number of potentially damaging and novel variants in *MCM8* and *MCM9* among participants with POI and examined multiallelic association with variants in DDR and MCM8–MCM9 interactome genes. **(*J Clin Endocrinol Metab* 102: 576–582, 2017)**

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Abbreviations: DDR, DNA damage repair; ExAC, Exome Variant Server, GWAS, genome-wide association study; MCM, minichromosome maintenance; POI, primary ovarian insufficiency; SNP, single nucleotide polymorphism; WES, whole-exome sequencing; VUS, variant of unknown significance.

Primary ovarian insufficiency (POI) is genetically heterogeneous and presents as hypergonadotropic hypogonadism, syndromic or nonsyndromic. In POI (MIM 311360), ovaries become devoid of oocytes prior to age 40 years (1, 2). The condition is characterized by amenorrhea, infertility, hypoestrogenism, and elevated follicle-stimulating hormone serum levels. Women with POI are also susceptible to the development of cancer, osteoporosis, and heart diseases (1).

Minichromosome maintenance 8 (*MCM8*) and *minichromosome maintenance 9* (*MCM9*), members of minichromosome maintenance (MCM) family of genes (MCM2–9), are autosomal genes that have recently been implicated in causing hypergonadotropic hypogonadism when inherited in an autosomal recessive fashion in rare consanguineous families (3–7). In mice lacking either *Mcm8* or *Mcm9*, females are infertile, with early loss of germ cells, and were predisposed to cancer, including hepatocellular carcinoma and ovarian tumors/hyperplasias (8, 9). Moreover, genome-wide association studies (GWASs) on reproductive aging have identified a nonsynonymous single-nucleotide polymorphism (SNP) within the *MCM8* locus (10, 11) that strongly associates with the age of menopause. These recent studies suggest that *MCM8* and *MCM9* are essential regulators of gonads from their genesis to senescence.

The central role of *MCM8* and *MCM9* in reproductive aging prompted us to hypothesize that *MCM8* and *MCM9* variants are present in a significant subset of individuals with POI. We screened a cohort of 173 participants for variants in *MCM8* and *MCM9*, genes interacting with MCM8–MCM9, and DNA damage repair (DDR) genes implicated in reproduction to assess the presence of homozygous, heterozygous, and biallelic variants in individuals with POI.

Materials and Methods

Participants

We investigated *MCM8* and *MCM9* variants in a cohort of 173 participants diagnosed with POI. The study was approved by the Institutional Review Board of the University of Pittsburgh (PRO09080427). Among this cohort, 141 women self-identified as white, 4 as Hispanic, 3 as of Indian descent, 1 as African American, and 1 as mixed white and African American, with 23 participants not self-identifying with a race. This cohort contained 75 women diagnosed with primary amenorrhea, 44 women diagnosed prior to age 25 years with secondary amenorrhea, 33 women diagnosed after age 25 years with secondary amenorrhea, and 21 for whom the type of POI was not classified. Of a total of 173 participants, 133 individuals were sequenced for both *MCM8* and *MCM9* genes, whereas 155 participants were sequenced only for *MCM8* and 151 participants were sequenced only for *MCM9*. In addition, in the 2 sequencing methods used as described here, 19 participants were analyzed by both Sanger sequencing and whole-exome sequencing (WES). Specifics for each sample can be found in Supplemental Table 1.

Sanger sequencing

Of a total of 173 participants' blood samples, 89 were analyzed by Sanger sequencing. Polymerase chain reaction amplification was conducted with the KAPA HiFi Hotstart PCR Kit (Kapa Biosystems, Inc. Wilmington, MA) with deoxynucleotides of every exon, including the exon-intron boundaries of *MCM8* and *MCM9* using primers provided in Supplemental Table 2. Polymerase chain reaction products were then purified and sequenced at Beckman Coulter Genomics (Danvers, MA). Results were analyzed using Sequencher (Gene Codes Corporation, Ann Arbor, MI). Specifics for each sample can be found in Supplemental Table 1.

Exome sequencing

In total, 103 women were subjected to WES. All variants present in *MCM8* and *MCM9* were queried for analysis. We used the Agilent SureSelect V5 capture kit (Agilent Technologies, Santa Clara, CA) to sequence these samples, and this capture covers the intron-exon boundaries. The WES coverage on average was between 150 and 250 reads per base pair, and error rate of calling the variant was $<1\%$ (3, 4). Specifics for each sample can be found in Supplemental Table 1. Exons and splice sites were captured using a specified capture kit (Supplemental Table 3), followed by 2×100–base pair paired-end WES performed on an Illumina HiSEquation 2500 (Illumina, Inc, San Diego, CA). Reads were prepared for analysis using Cutadapt to remove the adapters and the Fastx Toolkit to trim the first 5 base pairs at the 5′ end of reads (Cold Spring Harbor Lab, Cold Spring Harbor, NY). Data were aligned to NCBI37/hg19 using Burrows-Wheeler Aligner (12, 13). Local realignment around insertions and deletions, reads base quality recalibration, and variant calling were conducted using GATK Tool Kits (Broad Institute, Cambridge, MA) (99% accuracy in read determination and read-depth coverage of ≥ 30). GATK Haplotype Caller was used to call variants. Versions of all software used are provided in Supplemental Table 3 by sample. Variants not present in the 1000 Genomes Project, Exome Variant Server data sets, Exome Aggregation Consortium (ExAC, Cambridge, MA), or the Single Nucleotide Polymorphism database (dbSNP) were considered novel variants and were corroborated by Sanger sequencing (14).

Variant classification

We used the most recent guidelines from the American College of Medical Genetics and Genomics to classify variants based on pathogenicity (15). The pathogenicity of the variants was established using *in silico* analyses. Literature quotes these tools as predicting pathogenicity in 70% to 80% of variants (16, 17). In brief, we used the following *in silico* criteria to assign variants: variants with minor allelic frequency of $<1\%$ according to the ExAC and which resulted in loss of function due to nonsense, frameshift, canonical ± 1 or 2 splice sites, initiation codon, and single or multiexon deletion considered damaging. Common variants with frequency $>1\%$ were not considered pathogenic and were excluded from the analysis. Variants $<1\%$ in the population were considered benign if prediction algorithms (Sorting Tolerant from Intolerant, Polyphen-2, PhyloP) did not suggest a significant effect on a gene or gene product

(18, 19). If *in silico* tools did not unanimously identify a variant as damaging or benign, it was assigned as a variant of unknown significance (VUS). Since our population was overwhelmingly white, we used white population variant frequencies for comparison.

Statistical analysis

Association of the variants identified in *MCM8* and *MCM9* with a POI phenotype was assessed by comparing the frequency of the variants present within our POI cohort with the frequency of variants in the European (non-Finnish) population present in the following publicly available databases: ExAC or Exome Variant Server, NHLBI Exome Sequencing Project (Seattle, WA) (20). Allele frequencies were compared using a Fisher exact test. The odds ratio with the 95% confidence interval, *P* value of the Fisher exact test, and the adjusted *P* value using Benjamini and Hochberg's method to control the false discovery rate were calculated. *P* value <0.05 was considered significant. The statistical analysis was performed using the statistical computing software R (R Core Team, Vienna, Austria).

Results

MCM8 variants

We sequenced all exons of the *MCM8* gene in 155 participants with POI. A total of 10 nonsynonymous variants in *MCM8* were identified in 18 women (Table 1). Four of these 10 variants were considered benign (rs145133959, rs116926921, rs16991591, and rs41282946). The variant rs16991591 was significantly associated with POI ($P = 0.0071$) compared with the reference value reported in the ExAC database. Three variants were predicted to be damaging (rs140044814, rs377142760, and rs368499896) and were not statistically associated with the POI phenotype. The 2 previously reported variants (rs58487183 and rs61754763) and 1 novel variant, c.1334G>A (p.Arg445Gln), were of unknown significance (VUS). Of these 3 variants with VUS, variant rs58487183 was significantly associated with POI phenotype ($P = 0.0450$). Moreover, variant c.1334G>A was novel, not present in current databases, and predicted to cause a missense mutation, p.Arg445Gln, in 1 participant. All of the *MCM8* variants identified through sequencing were heterozygous. We did not identify *MCM8* homozygous or possible compound heterozygous variants. The locations of the damaging variants in *MCM8* identified in the studied cohort are depicted in Supplemental Fig. 1A.

MCM9 variants

We analyzed a total of 15 individuals for the presence of *MCM9* variants either by WES or Sanger sequencing and identified a total of 10 nonsynonymous variants in 13 individuals (Table 2). Two variants were predicted to be benign (rs34213490 and rs61742362). The variant at rs34213490 was significantly associated with POI ($P = 0.005397$). Six variants were predicted to be damaging. Four of these 6 variants were known and heterozygous in the affected individuals (rs200078427, rs78231991, rs149099524, and rs78791427). Two other damaging variants were novel (c.1651C>T; p.Gln551* and c.2011C>A; p.Glu670*) and predicted to insert a premature stop codon, resulting in a truncated form of MCM9 protein. Of these 2 stop gain variants, the c.1651C>T variant was homozygous in 1 participant with primary amenorrhea. This patient was the offspring of a consanguineous marriage whose family was not available for additional testing, whereas the other variant (c.2011C>A) was present in heterozygous form in 2 participants with secondary amenorrhea.

We also identified 2 novel missense heterozygous variants c.1784C>G; p.Thr595Arg and c.2422G>A; p.Val808Ile, in our POI cohort. However, the pathogenicity of these 2 variants was classified as VUS. The locations of the damaging variants in *MCM9* identified in the studied cohort are depicted in Supplemental Fig. 1B.

Table 1. *MCM8* Variants in 155 Individuals With Idiopathic POI

No. of Individuals								Minor Allele Frequency				
Heterozygous	Homozygous	Exon	Genomic Coordinate (GRCh37)	dbSNP	cDNA Notation	Protein Notation	Variant Classification	Reported by ExAC	POI Cohort (No. Alleles/No. Total Alleles)	Odds Ratio (95% Confidence Interval)	Fisher's Exact Test (*P* Value)	Adjusted *P* Value
1	0	5	20:5935825	rs145133959	c.414A>G	p.Ile138Met	Benign	0.004497 (300/66,716)	0.0032 (1/312)	0.711 (0.036–3.418)	1	1
2	0	5	20:5935832	rs116926921	c.421A>G	p.Ile141Val	Benign	0.001319 (88/66,716)	0.0064 (2/312)	4.883 (0.851–15.861)	0.0662	0.1277
3	0	5	20:5935875	rs58487183	c.464G>A	p.Cys155Tyr	VUS	0.001394 (93/66,694)	0.0096[a] (3/312)	**6.950 (1.853–18.569)**	0.0103	**0.0450**
1	0	5	20:5935893	rs140044814	c.482A>G	p.His161Arg	Damaging	0.001411 (94/66,638)	0.0032 (1/312)	2.276 (0.115–11.080)	0.3585	0.5179
5	0	6	20:5937833	rs16991591	c.548A>G	p.Asn183Ser	Benign	0.002044 (135/66,044)	0.016[a] (5/312)	**7.950 (3.067–17.211)**	0.0005	**0.0071**
1	0	12	20:5953779	Novel	c.1334G>A	p.Arg445Gln	VUS	NA	0.0032 (1/312)	—	—	—
1	0	14	20:5963639	rs377142760	c.1561G>A	p.Asp521Asn	Damaging	0.0001049 (7/66,730)	0.0032 (1/312)	30.641 (1.375–190.79)	0.0366	0.0952
1	0	14	20:5963655	rs368499896	c.1577A>G	p.Gln526Arg	Damaging	0.000077[b] (1/8600)	0.0032 (1/312)	27.606 (0.716–1060.9)	0.0687	0.1277
2	0	16	20:5966763	rs61754763	c.2149A>G	p.Ile717Val	VUS	0.01196 (798/66,714)	0.0064 (2/312)	0.532 (0.094–1.693)	0.5959	0.7747
1	0	18	20:5974291	rs41282946	c.2380A>G	p.Ile794Val	Benign	0.001147 (76/66,288)	0.0032 (1/312)	2.801 (0.141–13.701)	0.3035	0.4932

Abbreviations: cDNA, complementary DNA; NA, not available.

[a]Fisher exact test was used to compare the frequencies of the variants in the POI cohort as in the ExAC database. The odds ratio with the 95% confidence interval, *P* value of the Fisher exact test, and the adjusted *P* value using Benjamini and Hochberg's method to control the false discovery rate were calculated. Significant *P* values ($P < 0.05$) are presented in bold.

[b]Value from Exome Variant Server, minor allele frequency in European American population. Value not available in ExAC.

Table 2. *MCM9* Variants in 151 Individuals With Idiopathic POI

No. of Individuals								Minor Allele Frequency				
Heterozygous	Homozygous	Exon	Genomic Coordinate	dbSNP	cDNA Notation	Protein Notation	Variant Classification	Reported by ExAC	POI Cohort (No. of Alleles/No. of Total Alleles)	Odds Ratio (95% Confidence Interval)	Fisher's Exact Test (*P* Value)	Adjusted *p* Value
2	0	2	6:119245200	rs34213490	c.397T>C	p.Thr139Ala	Benign	0.0001798 (12/66,740)	0.0065[a] (2/304)	**36.806 (5.872–139.48)**	0.001799	**0.005397**
1	0	3	6:119243187	rs200078427	c.686T>G	p.Val229Gly	Damaging	0.0002549 (17/66,686)	0.0032 (1/304)	12.93 (0.625–69.039)	0.078618	0.134773
2	0	5	6:119234579	rs78231991	c.911A>G	p.Asn304Ser	Damaging	0.00508 (337/66,338)	0.0065 (2/304)	1.297 (0.228–4.138)	0.669875	0.803851
1	0	6	6:119234586	rs149099524	c.905-1G>T	Splice variant	Damaging	0.000116[b] (1/8600)	0.0032 (1/304)	28.342 (0.734–1088.89)	0.067122	0.134244
0	1	9	6: 119149171	Novel	c.1651C>T	p.Gln551[b]	Damaging	NA	0.0065 (2/304)	—	—	—
1	0	10	6:119147976	Novel	c.1784C>G	p.Thr595Arg	VUS	NA	0.0032 (1/304)	—	—	—
1	0	12	6:119137445	rs78791427	c.1974G>T	p.Gln658His	Damaging	0.01172 (50/4268)	0.0032 (1/304)	0.278 (0.013–1.38)	0.257221	0.342962
2	0	12	6:119137408	Novel	c.2011G>T	p.Glu670[b]	Damaging	NA	0.0065 (2/304)	—	—	—
1	0	12	6:119136997	Novel	c.2422G>A	p.Val808Ile	VUS	NA	0.0032 (1/304)	—	—	—
1	0	12	6: 119136133	rs61742362	c.3286A>G	p.Met1096Val	Benign	0.003522 (19/5394)	0.0032 (1/304)	0.933 (0.045–4.918)	1	1

Abbreviations: cDNA, complementary DNA; NA, not available.

[a]Fisher exact test was used to compare the frequencies of the variants in the POI cohort as in the ExAC database. The odds ratio with the 95% confidence interval, *P* value of the Fisher exact test, and the adjusted *P* value using Benjamini and Hochberg's method to control the false discovery rate were calculated. Significant *P* values ($P < 0.05$) are presented in bold.

[b]Value from Exome Variant Server, minor allele frequency in European American population. Value not available in ExAC.

Compound and biallelic *MCM8* and *MCM9* variants

We could not determine the presence of compound heterozygous variants in all of our participants because parents' DNA samples for all participants were not available for genotype analysis. One participant (POI-63) with primary amenorrhea carried 2 damaging heterozygous variants at the c.905-1G>T and c.1784C>G (p.Thr595Arg) position in the *MCM9* gene, but we could not determine if in *cis* or *trans*. In our cohort of 133 participants who were sequenced for both *MCM8* and *MCM9*, 1 individual (POI-44) with primary amenorrhea carried damaging heterozygous variants in both *MCM8* (c.1561G>A, p.Asp521Asn) and *MCM9* (c.911A>G, p.Asn304Ser) alleles (Supplemental Table 1).

DDR gene variants

We analyzed DDR genes associated with reproductive aging (10) and genes involved in the MCM8–MCM9 interactome (21–23) for the presence of rare (frequency <0.01%, known or novel) heterozygous variants predicted to be damaging or with unclear significance in participants carrying at least 1 heterozygous damaging variant in either the *MCM8* or *MCM9* gene. We identified 2 participants who carried heterozygous variants that were predicted to be damaging in genes associated with either the MCM8–MCM9 pathway or DDR (Supplemental Table 4). In participant POI-63, 2 heterozygous variants were identified in *MCM9* c.1784C>G, p.Thr595Arg (VUS) and a splice variant at c.905-1G>T position (damaging), and 1 nonsynonymous substitution variant with unknown significance was observed in the *BRCA1* gene (c.2521C>T, p.Arg841Trp). Similarly, 1 participant (PPOI-173), carrier of a pathogenic heterozygous variant in *MCM8* (p.His161Arg), carried additional heterozygous variants in *RAD54L* (p.Arg202Cys), predicted to be damaging, and a variant with uncertain significance in the *HELB* (p.Gln523Pro) gene.

Discussion

DDR is essential for cells to survive when faced with metabolic and environmental damage and is also a key process in meiosis to resolve DNA damage incurred during homologous recombination (10, 11). Meiotic arrest and loss of oocytes are caused by deficiencies in genes that are critical in DNA damage repair. For example, a number of mouse mutants in DDR genes, such as *Dmc1*, *Msh5*, *Stag3*, and *Syce1*, lose oocytes rapidly in part because of their inability to process double strand breaks (24–27). Mutations in *DMC1*, *MSH5*, *STAG3*, and *SYCE1* have been identified in women with POI (26, 28, 29). DDR genes, including a single-nucleotide polymorphism in *MCM8* (rs16991615), have been implicated in the onset of menopause by GWASs performed in the population related to white, Hispanic, and African American ethnicity (10, 11).

We previously reported that *MCM8* and *MCM9* mutations, when inherited in an autosomal recessive fashion, are associated with POI and chromosomal instability (3, 4). Subsequent studies in additional families corroborated the importance of MCM8 and MCM9 in human gonadal development (5–7). Mouse knockout studies on *Mcm8* and *Mcm9* also validate their significance in gonadal development (8, 9). In the current study, we evaluated a total of 173 participants with idiopathic POI to identify the prevalence of *MCM8* and *MCM9* variants.

We sequenced the *MCM8* gene in 155 participants who presented with POI. Of a total of 10 *MCM8* variants identified among 18 participants, we identified 3 damaging variants in 3 participants; all 3 presented with primary amenorrhea. No participant with secondary amenorrhea

had damaging *MCM8* variants. We identified a novel variant in *MCM8* at position c.1334G>A (p.Arg445Gln) in 1 participant, but since this variant p.Arg445Gln exists in a heterozygous state, it is unlikely to be a single cause of the POI phenotype. It was interesting to note that the 2 *MCM8* variants rs58487183 (VUS) and rs16991591 (benign) were statistically associated with the POI phenotype. Apart from these 2 variants, the other known benign or damaging variants of *MCM8* identified in our POI cohort were not significantly associated with the POI phenotype compared with the control population in public databases such as ExAC/Exome Variant Server. Interestingly, the *MCM8* menopause-associated variant rs16991615 was not statistically associated with our POI cohort.

MCM9 was not identified in GWASs on reproductive aging (10, 11), highlighting an inherent limitation of GWASs to identify all genetic contributions to a phenotypic condition. We know from mouse studies that MCM8 and MCM9 proteins physically interact and are codependent in their actions (8, 9). Moreover, we and others have shown that several families with loss-of-function *MCM9* gene variants have members that present with hypergonadotropic hypogonadism and chromosomal instability (4, 6, 7). In the current study, we screened 151 participants for the presence of *MCM9* variants. Of a total of 10 variants identified, in a total of 13 participants, 12 individuals were heterozygous for the variants and 1 individual carried a homozygous variant, whereas 1 participant (POI-63) carried 2 heterozygous variants [c.905-1G>T (damaging) and c.1784C>G, p.Thr595Arg (VUS)] in *MCM9*. We do not know whether these 2 variants are present in a compound heterozygous condition, as DNA from the parents was not available for evaluation. Eight participants carried 6 variants that were predicted to be pathogenic, including variants predicted to disrupt splicing and introduce a stop codon. Of these 8 participants with POI, 3 were diagnosed with primary amenorrhea, whereas the remaining 5 participants were classified as having secondary amenorrhea.

In *MCM9*, 1 benign variant, rs34213490, was significantly associated with POI, as it was more prevalent in our POI cohort compared with a control population. However, none of the other known variants that were previously reported in public databases were statistically associated with the POI phenotype. We have identified 4 novel variants in *MCM9* among 5 participants. Of these 4 variants, 1 variant (c.1651C>T) is present in a homozygous condition in an individual (POI-49) with primary amenorrhea. This variant creates a stop codon (p.Gln551*) in the AAA^+ core MCM domain, predicted to cause a nonfunctional truncated protein (Supplemental Fig. 1B). Likewise, the previous report also documented the presence of the novel homozygous mutation in the MCM domain at p.E495* of MCM9 in women with primary amenorrhea (7). Therefore, this homozygous variant with the loss of function likely accounts for the participant's POI.

Previous studies have shown that the stability of MCM8 and MCM9 proteins depends on the expression of binding partner (9). The epistatic relationship between *MCM8* and *MCM9* in POI has not been explored. We explored biallelic variants in individuals with POI. We observed that 1 participant (POI-44) carried both the damaging heterozygous variants in *MCM8* and *MCM9*.

We also explored multiallelic inheritance of *MCM8* and *MCM9* genes with genes that are part of the MCM8–MCM9 pathway or DDR genes implicated in reproductive aging. *In vitro* studies have explored the MCM8–MCM9 pathway in DDR and have identified few proteins that interact with MCM8 and MCM9 during homologous recombination (21–23). Moreover, 1 GWAS identified 34 genes involved in the DDR pathway that are in the vicinity of a total of 44 loci and highly associated with early age of menopause (10). Of 10 participants carrying either *MCM8* or *MCM9* heterozygous damaging variants, we identified triallelic inheritance of variants (either damaging or with VUS) in genes such as *MCM9* and *BRCA1* in 1 participant and in the *MCM8*, *RAD54L*, and *HELB* genes in another participant.

In conclusion, we identified novel and potentially damaging variants in *MCM8* and *MCM9* genes as well as a possibly multiallelic association in DDR or MCM8–MCM9 interactome genes. The overall incidence of mutations in both alleles for *MCM8* (0/155) and *MCM9* (1/151) genes was low. We identified potential pathogenic variants in 3 of 155 participants in *MCM8* and 7 of 151 participants in *MCM9*. However, further functional analysis is required to decipher the role of heterozygous variants in disease causation. We have also identified 1 participant with biallelic damaging variants in *MCM8* and *MCM9* genes and 2 participants with multiallelic variants in DDR and MCM8–MCM9 interactome genes. The limitations of our study include the following: (1) small and mixed cohort of POI patients who have a varied phenotype ranging from primary to secondary amenorrhea; (2) lack of availability of parental DNA samples for testing, limiting segregation analyses; (3) incomplete knowledge of the MCM8–MCM9 interactome, thus limiting a comprehensive analysis; (4) variants classified based on the *in silico* tools and thus require following functional analysis; and (5) this study only exploring the coding region and exon-intron boundaries of the *MCM8*, *MCM9*, and DDR related genes and would have missed the noncoding regulatory regions. Future *in vitro* and *in vivo* functional studies are necessary to

understand the functional significance of *MCM8* and *MCM9* gene variants identified in this study as well as the multiallelic interactions.

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