Novel *PMM2* missense mutation in a Chinese family with non-syndromic premature ovarian insufficiency

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Abstract

Purpose This study sought to identify a disease-related gene in a consanguineous Chinese family in which there were two premature ovarian insufficiency (POI) sisters.

Method We used whole-exome sequencing and Sanger sequencing to identify the disease-causing gene. Results were verified using an assay of mutant protein and in silico analyses.

Result We identified a novel missense mutation (NM_000303: c.556G>A, p.Gly186Arg) in the *PMM2* gene. The two sisters suffer from premature ovarian insufficiency (POI) only and have no other symptoms of congenital disorder of glycosylation type-1a (CDG-Ia). We found that the enzymic activity of the mutant *PMM2* protein was reduced by 55.21% ($p < 0.05$) when compared with wild type, and many in silico tools suggested the mutation is disease-related.

Conclusion This particular gene modification results in changes in activity of phosphomannomutase modification, which could lead to *PMM2*-CDG-Ia with an uncommon phenotype.

Keywords Premature ovarian insufficiency (POI) · Congenital disorder of glycosylation type-1a (CDG-Ia) · Non-syndromic premature ovarian insufficiency · *PMM2*

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Introduction

Premature ovarian insufficiency (POI) is a clinical syndrome characterized by the depletion or reduction of normal ovarian function before the age of 40. It is characterized by menstrual disturbance, raised gonadotropins levels, and low estradiol levels [1]. Usually, POI is diagnosed when a woman under 40 presents with a 4–6-month period of amenorrhea or oligomenorrhea and two serial measurements of elevated levels of follicle-stimulating hormone (FSH) (> 40 mIU/ml taken > 4 weeks apart) [2]. POI is a heterogeneous disease that is often caused by genetic mutations [3]. With the development of sequencing technology, many POI-related genes have been identified: *MCM8* [4], *STAG3* [5], *CSB-PGBD3* [6], and *DMC1* [7].

Congenital disorder of glycosylation type-1a (CDG-Ia) often leads to POI; most POI female patients manifest elevated levels of gonadotropin and absence of puberty [8]. The *PMM2* gene encodes phosphomannomutase, which is an isomerase that converts the mannose-6-phosphate to mannose-1-phosphate during an early step in N-glycosylation. Deficiency of phosphomannomutase (*PMM2*, OMIM #601785) causes CDG-Ia [9]. CDG-Ia is an autosomal recessive disorder that

affects multiple organs. In most cases, the progress of CDG-Ia commonly undergoes the following four stages: the infantile multi-systemic stage, the childhood ataxia-mental retardation stage, the teenage leg atrophy stage, and the adult hypogonadal stage. However, not all patients go through all these stages. The severity can drastically vary from asymptomatic to fatal, even in the cases with exactly the same mutation [10].

In this study, we report two sisters that both have the same homozygous variant in the *PMM2* (NM_000303: c.556G>A, p.Gly186Arg). They are from a consanguineous family with POI. Their parents and the unaffected brother are heterozygous for the same variant. According to the patient's POI symptoms and genetic testing results, *PMM2*-CDG-Ia can be diagnosed. Unlike the multi-system CDG-Ia reported previously [11], both of our patients only suffer from POI, rather than multi-system diseases. To verify the pathogenicity of this variant, three types of *PMM2* proteins were obtained using heterologous expression in *E. coli*. We found reduced activity of the mutant protein, which suggests this novel variant is disease-related and likely causal in the disease etiology.

Methods

Pedigree and participants

We obtained the peripheral blood and collected relevant information from five members from a Han Chinese family for the following: two affected sisters (IV-1, IV-2), one unaffected brother (IV-3), and the two unaffected parents (III-1, III-2) (Fig. 1a). The parents of the proband were first cousins. We used the peripheral blood to extract Genomic DNA (gDNA) from leukocytes using a QIAamp DNA blood midi kit (Qiagen, Hilden, Germany) per the manufacturer's instructions.

Whole-exome sequencing and variant analysis

Familial POI is a Mendelian-inherited disease. Whole-exome sequencing (WES) is a widespread approach to discover potential Mendelian disease-related genes. We sent gDNA samples from the proband, her affected sister, and her parents to the WES program of BGI for WES analyses (BGI Genomics, B G I - S H E N Z H E N). The project used SureSelect_Human_All_Exon_V6 for exon capture and Xten (Illumina, Inc., San Diego, CA, USA) for sequencing. Sequence reads were aligned with the human reference genome (hg19) using the Burrows-Wheeler Aligner algorithm (Li and Durbin R, 2009). Using SAMtools (www.huslib.org), we excluded duplicate reads and reads that were mapped to multiple locations in the exome. Variants (single nucleotide variations and small insertions/deletions) were identified using certain inclusion criteria: (1) Variants with frequencies less than 5% according to three different public databases (1000

Genomes, <http://browser.1000genomes.org/index.html>; Exome Aggregation Consortium, ExAC, <http://exac.broadinstitute.org/>; and NHLBI GO Exome Sequencing Project, ESP, <http://evs.gs.washington.edu/EVS/>); (2) variants that were either a missense, frameshift, splice site, or nonsense; and (3) variant in a gene that has been reported to be related to POI. In addition, we placed priority on the homozygous variants found in the sisters because of the genetic pattern in this family.

Sanger sequencing

We used Sanger sequencing to confirm the mutation of the candidate gene, *PMM2*, identified via WES. We used specific PCR primers (*PMM2*-F: 5'-CCGCAAATATGCCGGATGC-3' and *PMM2*-R: 5'-TCAATTTCCCCCATCAAGCG-3') to amplify the target region. The amplified PCR products were run on 2.0% agarose gel electrophoresis. Then, we identified the band size, and the PCR products were sequenced on ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA).

In silico analyses and evolutionary conservation

To assess the disease-related potential of the identified mutation, we used various in silico tools that analyzed its evolutionary conservation and pathogenicity. We used the amino acid sequences of different species to analyze the evolutionary conservation of *PMM2*; sequences were obtained from the NCBI (<https://www.ncbi.nlm.nih.gov/>). We used seven tools to predict the potential pathogenicity: Polyphen2 (<http://genetics.bwh.harvard.edu/pph2>), MutationTaster (<http://www.mutationtaster.org/>), SIFT (<http://sift.bii.a-star.edu.sg/>), MutationAssessor (<http://mutationassessor.org/r3/>), FATHMM (<http://fathmm.biocompute.org.uk/inherited.html>), GERP_plus (<http://mendel.stanford.edu/SidowLab/downloads/gerp/index.html>), and PhastCons (<http://varianttools.sourceforge.net/Annotation/PhastCons>). The structure of the *PMM2* protein was modeled by Phyre 0.2 (<http://www.sbg.bio.ic.ac.uk/phyre>), and we used PyMol (<http://www.pymol.org>) for structure visualization.

Tissues

We obtained human fetal ovary tissue from a fetus after induced abortion at 25 weeks. Of note, the tissues were obtained from the Department of Obstetrics and Gynecology, Reproductive Medicine Center, Second Xiangya Hospital of Central South University with the approval of the Ethics Committee of the Central South University.

Immunohistochemistry

Ovary tissues were fixed in a 4% paraformaldehyde solution for 16–20 h. After dehydration, tissues were embedded in paraffin and sectioned at a thickness of 4 μ m and an interval of 100 μ m. The sections were dewaxed using xylene for 20 min and hydrated by absolute ethyl alcohol. Sections were covered by 3% H_2O_2 at 37 $^{\circ}C$ for 20 min to completely inactivate endogenous peroxidase. For antigen retrieval, sections were immersed in a 10 mmol/L citric acid buffer, following which the buffer was boiled to 100 $^{\circ}C$ for 10 min then exposed and cooled to room temperature. Goat serum was used to block for 2 h, then the sections were incubated with 1:200 anti-PMM2 antibody (Abcam, ab229996) at 4 $^{\circ}C$ overnight and incubated with biotinylated secondary antibody at 37 $^{\circ}C$ for 1 h. The sections were stained with diaminobenzidine (DAB) and hematoxylin, and were mounted with coverslips. PBS was used as the negative control.

Expression of recombinant wild type and mutant in *E. coli*

To produce the PMM2 proteins for phosphomannomutase assay, the expression vectors for PMM2-WT, PMM2-G186R, and PMM2-R141H were inserted into the expression vector plasmid pET 3a, which contained a His tag. The R141H variant was used as a positive control; it has been reported to reduce PMM2 enzymic activity [12]. We obtained the sequence for the Human PMM2 cDNA from NCBI (www.ncbi.nlm.nih.gov). The plasmid constructs were used to transform *E. coli* BI21 (DE3) cells, which were then cultured in LB medium at 37 $^{\circ}C$ until OD600 reached 0.2. Isopropylthiogalactoside (IPTG) was then added to the culture medium, and incubated for 4 h. The mixture was centrifuged at 13,000 rpm for 20 min, and the supernatant was discarded, leaving the bacterial cells. The cells were lysed by ultrasound for 20 min. We purified the protein from the lysate using Ni-chelating affinity chromatography. The protein was obtained at a concentration to 0.6 mg/ml and analyzed by the SDS-PAGE to confirm the protein is PMM2.

Fig. 1 Pedigree of the family and analyses of PMM2 in the family. **a** Two first cousins (III-1 and III-2) in the same generation married to each other and had two affected children IV-1 and IV-2, with premature ovarian insufficiency (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. Sequence chromatograms for PMM2 (NM_000303: c. G556A, p.G186R) in this family, the

unaffected parents and brother are heterozygous carriers of the variation of PMM2, c.G556A, whereas the two affected siblings are homozygous for the variation. **b** Alignment of PMM2 proteins from various species. The G186 site of human PMM2 is 100% conserved in the aligned sequences. **c** Modeling of the PMM2 wide type and mutant protein. The 186th amino acid change from glycine to arginine

Table 1 Hormone examinations of affected individuals in the family

	Item	IV-1	IV-2	Normal value range
Age	–	35	31	–
Basal sexual hormone	FSH	70.03	51.86	3.03–8.08 mIU/ml
	T	0.56	0.77	0.38–1.97 ng/ml
	LH	7.5	9.08	1.80–11.78 mIU/ml
	PRL	20.54	19.78	5.18–26.53 ng/ml
	E ₂	17.80	11.65	21–251 pg/ml

FSH, human follicle-stimulating hormone; T, testosterone; LH, human luteinizing hormone; PRL, prolactin; E₂, estradiol

Phosphomannomutase assay

We used the enzyme activity assay method established by Van Schaftingen and Jaeken [13]. The assay mixtures contained 50 mM Hepes (pH = 7.1); 5 mM MgCl₂; 0.25 mM NADP; 10 µg/ml yeast glucose 6-phosphate dehydrogenase; 0.1 mM mannose 1-phosphate; 1 µM glucose 1,6-diphosphate; 10 µg/ml phosphoglucose isomerase; 3.5 µg/ml phosphomannose isomerase; and 0.6 mg/ml protein extract. The final reaction was 250 µl and incubated for 30 min at 30 °C. The absorbance was measured using a fluorometer reader (Enspire 2300 multilabel reader; Perkin Elmer). The rate of NADPH formation was followed for 1 min. One unit is equal 1 nmol of NADPH/min being formed. The experiment was repeated three times.

Results

Clinical manifestation of the patients in the POI pedigree

The parents (III-1 and III-2) of the proband are cousins. We observed POI in the proband (IV-1, age = 35) and her sister (IV-2, age = 31; Fig. 1a). They both had primary amenorrhea and they displayed dysplastic secondary sex characteristics (mammary dysplasia and lack of pubic hair). Both

of their serum FSH concentrations exceeded 40 mIU/ml (Table 1). Ultrasonographic examination showed infantile uteri and atrophic ovaries. Other than POI, the two affected siblings had no other CDG-Ia symptoms. They had normal facial features, normal intelligence, and no neurologic disorders. The proband's mother had regular menstrual cycles and began menopause at a reasonable age, and the brother is healthy and fertile. All the family members have no known chromosomal abnormalities, ovarian surgery, chemotherapy or radiotherapy, or history of other diseases.

Identification of mutation by WES

The affected siblings (IV-1, IV-2) and their healthy parents (III-1, III-2) were chosen for WES. POI is a rare genetic disease; variants with frequencies greater than 5% frequency were filtered out in the 1000 Genomes, dbSNP, ESP6500, and ExAC databases (Supplementary Table SI). Because the proband and her sister were from a consanguineous family and their parents are healthy, we gave priority to homozygous variants—six variants were retained (Supplementary Table SII). We also focused on conservation, phenotype, and reported POI-related genes. We found a variant in *PMM2* (NM_000303: c.556G>A, p. Gly186Arg). *PMM2* is reported to cause POI and the mutation point of *PMM2* G186R is extremely conserved across species. Therefore, we speculate that the novel variant in *PMM2* is likely to be the disease-associated gene. Sanger sequencing and cosegregation analysis were then performed using DNA samples available from the family members. G186R was found in the affected patients (IV-1 and IV-2). The unaffected parents (III-1 and III-2) and the healthy brother (IV-3) were heterozygous carriers for this same *PMM2* variant (Fig. 1a).

In silico analysis of *PMM2* G186R mutations and evolutionary conservation

The potential pathogenicity of *PMM2* G186R was analyzed by different in silico tools. Seven different prediction software (SIFT, PolyPhen2_HVAR, Mutation Taster, Mutation Assessor, FATHMM, GERP+, and PhastCons) all predicted

Table 2 In silico analysis of *PMM2* variants

No.	Database	Score	Annotations
1	SIFT	0	Deleterious (< 0.05)
2	PolyPhen2_HVAR	1	Probably damaging (≥ 0.909), possibly damaging (0.447–0.909); benign (≤ 0.446)
3	MutationTaster	0.99999	The closer the value is to 1, the more probable the nucleotide is conserved.
4	MutationAssessor	3.575	Deleterious (> 1.938)
5	FATHMM	– 5.26	Deleterious (< – 1.5)
6	GERP_plus	4.8	Deleterious (> 3)
7	PhastCons	0.997	Deleterious (> 0.6)

Fig. 2 Expression of PMM2 in fetus ovary. Scale bars, 100 μ m and 50 μ m

this novel variant in *PMM2* to be deleterious and indicated it is disease-associated (Table 2). We compared the protein sequences of different species, which we downloaded from Genbank (human, chimpanzee, mouse, rat, *Castor Canadensis*, zebrafish, yeast, *Arabidopsis thaliana*, *Aspergillus bombycis*), and found the signal nucleotide polymorphism (SNP), G186R, in these species (from microorganism to mammal) was 100% conserved (Fig. 1b). This indicates that G186R is located in a relevant functional domain.

PMM2 expression in fetal ovary

In the tissue obtained, there were only primordial follicles in the ovary. We found that the PMM2 had high expression in the cytoplasm of the oocytes (Fig. 2), suggesting that PMM2 might be an essential factor in regulating follicle development at the early stage.

The impact of PMM2 G186R mutations

The G186 variant is located at exon seven in the *PMM2* gene. The SNP caused the 186th amino acid of the *PMM2* protein to change from glycine to arginine. Remarkably, the mutant site was highly conserved in almost all the species analyzed (Fig. 1b). Of note, arginine is an amino acid with a

side chain, whereas the original glycine has no side chain (Fig. 1c). The side chain of arginine can form hydrogen bonds with surrounding groups, which can cause protein misfolding [14]. To explore the effects of the mutant protein, we measured its enzymatic activity. Wild type and the two *PMM2* variants (G186R and R141H) were successfully produced using *E. coli*. After purification, SDS-PAGE analysis showed that the size of the three purified proteins was consistent with the expected molecular weight of PMM2, which suggested that the heterologous expression of these three PMM2 proteins was successful (Fig. 3a). Enzyme activity was measured as previously described. Enzyme activity of the variant (4.13 ± 0.29 nmol/min ml) and positive control (1.14 ± 0.33 nmol/min ml) was reduced when compared with wild type (7.48 ± 0.48 nmol/min ml; $p < 0.05$) (Table 3, Fig. 3b).

Table 3 Specific activities of recombinant wild type and mutant PMM2

Protein type	Production (nmol/min·ml)
PMM2-WT	7.48 ± 0.48
PMM2-G186R	4.13 ± 0.29
PMM2-R141H	1.41 ± 0.33

Fig. 3 Recombinant proteins analyzed by the SDS-PAGE and activity assay. **a** Three recombinant proteins were analyzed by SDS-PAGE and could identify as PMM2. **b** Activities of recombinant PMM2. Each bar represents the average protein activity. Comparing with the wild type PMM2, the mutation protein PMM2-G186R and PMM2-R141H have the significant reduction of activity. (* $p < 0.05$)

Discussion

Using whole-exome sequencing, we revealed an unreported missense mutation (NM_000303: c. 556 G>A, p. Gly186Arg) in *PMM2*. The proband and the proband's sister had both been diagnosed with POI. Unlike most patients, during their infancy and childhood, they did not develop typical symptoms such as axial hypotonia, hyporeflexia, esotropia, developmental delay, or multiple organ system involvement [15]. In China,

approximately 1–2% of the reproductive-age female is affected by POI, of which 25% cases are due to genetic factors [16]. As CDG-Ia is not common in China, only few cases have been reported to date. Hence, this novel *PMM2* mutation with unusual symptoms in the consanguineous family studies has a certain significance.

Using *E. coli* expression, we found that the *PMM2* variant had a decrease ($p < 0.05$) in enzymatic activity relative to the wild-type protein. The positive control, R141H, also had significantly reduced activity compared with the wild type ($p < 0.05$), which is consistent with results from previous reports (Kjaergaard et al., 1999); this result indicates that our assay worked. Decreased enzymatic activity of *PMM2* results in incomplete glycosylation of the protein, which may cause disease.

The *PMM2* protein is highly conserved among many species; there are similarities even between yeast and mammals. This indicates how important the amino acid sequence is for protein function. Additionally, the mutation site found in this family is highly conserved across species; therefore, it likely plays an important role in disease etiology.

Thousands of CDG cases caused by mutations in *PMM2* have been reported, most of which affect organs throughout the body. The severity of the disease ranges from subclinical to fatal. However, this study may be the first to report a mutation in *PMM2* that only affects the female reproductive system. The polyphenotypic mechanism of *PMM2*-CDG is elusive, which suggests that genetic modifiers are important in determining the specific phenotypic outcome [10]. In addition, another characteristic of *PMM2*-CDG is the strange relationship between genotype and phenotype—different phenotypes can appear with the same genotype. In our study, the two sisters, who are both homozygous for G186R, show similar symptoms. This may be related to the fact that the parents of the proband are consanguineous first cousins, so the *PMM2* gene has a high degree of genetic homology, and the two sisters have a large degree of common genomic background.

The uncommon phenotype shown in this family gives us a new perspective for studying ovarian function. Interestingly, almost all of the plausible candidate genes people identified from the non-syndromic POI pedigrees using WES are involved in meiosis and DNA repair [3]. There are also many syndromic POI genes, such as *FMRI*, *GALT*, *EIF2B*, *FOXL2*. For example, patients who have the mutation in the *FOXL2* often suffer from both POI and the blepharophimosis-ptosis-epicanthus inversus syndrome [17]. However, the novel *PMM2* variant, which we observed in these two sisters who had non-syndromic POI, and *PMM2* is highly expressed in the primordial follicles, suggests an important relationship between ovaries and glycosylation. One group studied this relationship using a conditional knockout mouse model. The authors knocked out the O-glycans (*C1gal1*) and N-glycan (*Mgat1*) genes in oocytes [18]. Follicles were absent in the mutated ovaries, and the mutated female mice presented with premature ovarian failure, which

included changes in cumulus cell signaling and extracellular matrix composition [19]. In another model, oocyte-specific deletion of complex and hybrid N-glycans (Mgat1) led to defects in preovulatory follicle and cumulus mass development [20]. Many important proteins in the ovary are glycosylated, including BMP15 [21] and GDF9 [22]. The mutation in *PMM2* may prevent glycosylation.

Our study gives us a new look at the relationship between glycosylation and ovarian function. When we find genetic risk factors for an idiopathic POI in patients without other symptoms, we should think about not only the classical non-syndromic genes but also the syndromic type. Of course, to investigate further, animal models should be established. Unfortunately, few useful knockout mouse models are available; mice null for genes that are mutated in CDGs usually die in early development [10]. Mouse models that simulate the most common *PMM2* variants in humans (*PMM2*^{R141H/F122L}) have been established [23, 24]. Mice with these two variants show multiple organ dysfunction, and the heterozygous parent mouse required oral mannose to rescue the offspring [24]. Due to the phenotypic mechanism of phenotype corresponding to *PMM2* point mutation, the single knock-in allele that mimics the mutation model may not be able to present the expected phenotype. Therefore, a conditional *PMM2* knockout mouse model may be a better choice to study the pathogenic mechanism of incomplete glycosylation in ovaries.

Electronic artwork

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Compliance with ethical standards Each participating member signed an informed consent form. This study was approved by the Ethics Committee of Central South University.

Conflict of interest The authors declare that they have no conflicts of interest.

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