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Gene variants identified by whole-exome sequencing in 33 French women with premature ovarian insufficiency

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Abstract

Purpose To investigate the potential genetic etiology of premature ovarian insufficiency (POI).

Methods Whole-exome sequencing (WES) was done on DNA samples from women diagnosed with POI. Mutations identified were analyzed by in silico tools and were annotated according to the guidelines of the American College of Medical Genetics and Genomics. Plausible variants were confirmed by Sanger sequencing.

Results Four of the 33 individuals (12%) carried pathogenic or likely pathogenic variants, and 6 individuals carried variants of unknown significance. The genes identified with pathogenic or likely pathogenic variants included *PMM2*, *MCM9*, and *PSMC3IP*.

Conclusions WES is an efficient tool for identifying gene variants in POI women; however, interpretation of variants is hampered by few exome studies involving ovarian disorders and the need for trio sequencing to determine inheritance and to detect de novo variants.

Keywords Premature ovarian insufficiency · Whole-exome sequencing · Gene variants

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Introduction

Premature ovarian insufficiency (POI) is a heterogeneous condition occurring when a woman experiences loss of ovarian activity before the age of 40. This disorder affects approximately 1 in 100 women and is clinically characterized by amenorrhea (primary or secondary), hypergonadotropic hypogonadism (syndromic or nonsyndromic), and infertility [1, 2].

Aside from external pathogenic factors such as chemotherapy, radiotherapy, and ovarian surgery, POI can be a developmental disorder caused by genetic abnormalities. Structural and numerical changes in X chromosomes, including Turner's syndrome (45, X) and X-linked mental retardation, make up significant subgroups of POI [3]. Also, over 50 genes [4] have been found to cause ovarian dysfunction in POI by modulating immune function, metabolism, gonadal development, hormonal signaling, meiosis, and DNA repair [2]. Currently, only karyotyping and fragile X mental retardation 1 screening [5, 6] are recommended in clinical genetic testing for POI. No specific recommendations exist to test individual or panel of genes. In this pilot study, we performed whole-exome sequencing (WES) on 33 French women diagnosed with POI.

Materials and methods

Participants

Participants were recruited at the Center for Endocrine Rare Disorders of Growth and Development and Center for Gynecological Disorders in France. The study was approved by the Institutional Review Board of University of Pittsburgh (PRO09080427). Informed consent was obtained from all individual participants in the study.

Thirty-three French Caucasian women presented with primary amenorrhea and elevated follicle-stimulating hormone (FSH) level (> 25 mIU/ml on two occasions 4 weeks apart) were diagnosed with POI and were enrolled in this retrospective cohort study for further evaluation. Hormone levels, including luteinizing hormone, estradiol, anti-Mullerian hormone (AMH), and testosterone, were determined in order to evaluate pituitary and ovarian function. Pelvic ultrasound was done to assess ovarian anatomy. Bone mineral density was examined to check for osteoporosis. Prolactin, thyroid-stimulating hormone, and antibodies against ovary, adrenal gland, and thyroid were measured to exclude endocrine and immune system disorders that could lead to amenorrhea. Karyotype analysis and fragile X screening were performed as part of the standard genetic screening. Consanguinity and family history of disease, including POI, autoimmune diseases, and other genetic defects, were recorded.

Whole-exome sequencing

Peripheral blood samples were collected at the Department of Endocrinology and Reproductive Medicine, Centre des Maladies Endocriniennes Rares de la Croissance et du Développement, Centre des Pathologies Gynécologiques Rares in France. DNA was extracted from peripheral blood samples. WES was performed on a HiSeq 2500 (Illumina, San Diego, CA) at Magee Clinical Genomics Laboratory, Pittsburgh, PA. Agilent SureSelect V5 Capture Kit (Agilent Technologies, Santa Clara, CA) was used for exome capture. The average coverage of WES was from 150× to 250× reads on the target regions of the capture kit, with an error rate of variant calling < 1% [7, 8]. The FastX Toolkit (Cold Spring Harbor Lab, Cold Spring Harbor, NY) was used to trim the first five base pairs at the 5′ end of reads, and Cutadapt was applied to remove the adapters for preparation. Data was aligned to NCBI37/hg19 using Burrows-Wheeler Aligner [9]. Local realignment around indels and quality recalibration of variants were conducted using genome analysis tool kit (Broad Institute, Cambridge, MA); for variant calling, we used genome analysis tool kit Haplotype Caller. We also filtered variants based on their location in genes known to be previously associated with female infertility (listed in Supplemental data 2). IGV alignment was used to visually inspect variant calls.

Sanger sequencing

FPOI41 DNA sample was analyzed by Sanger sequencing to determine if the two variants (c.496_497delCT, p.R166Afs; c.430_431insGA, p.L144*) in *PSMC3IP* were in cis or trans. Polymerase chain reaction (PCR) amplification was conducted with the LongAmp™ Taq2X Master Mix (New England Biolabs, NEB, MA) using the forward primer: 5′-AGTA CACTGTCCCCACGTTTC-3′ and reverse primer: 5′-GTGT AGCGGCTGATCTGGTG-3′. PCR products were then purified using NucleoSpin Gel and PCR Clean-up kit (Macherey nagel, Duren, Germany). The products were ligated into vectors using TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) and were transformed into NEB 5-alpha competent *Escherichia coli* cells (New England Biolabs, NEB, MA). Plasmids were extracted from individual *E. coli* colonies using PureLink Quick Plasmid Miniprep kit (Invitrogen, Carlsbad, CA) and sequenced at Genomics Research Core, University of Pittsburgh, Pennsylvania, PA.

Variant classification

Each gene variant was evaluated using the guidelines of the American College of Medical Genetics and Genomics (ACMG), published in 2015 [10]. These guidelines recognize five classes of variants: benign, likely benign, uncertain

significance, likely pathogenic, and pathogenic. We considered variants in classes of pathogenic or likely pathogenic as causative. Variants with minor allelic frequency in the Exome Aggregation Consortium (ExAC) database > 1% were excluded. Variants not present in the 1000 Genomes Project [11], Exome Variant Server data sets, Exome Aggregation Consortium (ExAC, Cambridge, MA) [12], or the Single Nucleotide Polymorphism database (dbSNP) [13] were considered novel variants. Novel variants and those with very low frequency found in population are considered as moderate evidence for pathogenicity (PM2). Nonsense and frameshift mutations, canonical ± 1 or 2 splice sites, initiation codon irregularities, and single- or multi-exon deletion variants in a gene where loss of function are known mechanisms of disease are strongly recognized as evidence for pathogenicity (PSV1). For nonsynonymous variants, prediction algorithms were applied. PhyloP, scale-invariant feature transform (SIFT), and PolyPhen-2 were applied to determine conservation, tolerance, and impact of amino acid substitution on protein function, respectively. These prediction tools have 70–80% predictive value in assigning pathogenicity of variants [14]. Variants that were conserved, deleterious, damaging, and previously published as disease causing were assigned as pathogenic (PP3).

Results

DNA samples from 33 individuals were sequenced and we identified plausibly causative variants in 10 individuals. The clinical characteristics for these 10 individuals are given in Table 1; complete clinical information can be found in Supplemental Data 1. They were labeled according to order of recruitment. The age of diagnosis ranged from 14 to 28. Among them, six patients presented with delayed pubertal development; the other patients presented with normal puberty. They all presented with ultrasound findings consistent with atrophic or undetectable ovaries and absent follicles, except FPOI16 for whom ultrasound was not performed.

Among 10 individuals with candidate variants, four individuals were identified with pathogenic variants. FPOI16 had pathogenic variants in *phosphomannomutase 2* (*PMM2*), FPOI24 and FPOI38 individuals had pathogenic variants in *mini-chromosome maintenance complex component 9* (*MCM9*), and FPOI41 had pathogenic variant in *proteasome 26s subunit interacting protein* (*PSMC3IP*). Six individuals were identified with variants of unknown significance (VUS). The variant information and the representative phenotypes are summarized in Table 2. Detailed variant information can be found in Supplemental data 3.

Discussions

Pathogenic variants

FPOI16 was previously diagnosed with congenital disorder of glycosylation (CDG), based on clinical presentation and biochemical studies, at the age of 15. CDG is a genetically heterogeneous syndrome, with a wide range of phenotypes. Autosomal recessive mutation in *PMM2* is the most common genetic cause for CDG and presents with central nervous system disorder including hypotonia, intellectual disability, cerebellar syndrome, and lack of secondary sexual development in adult female [15, 16]. *PMM2* gene has eight exons encoding the protein necessary for synthesis of guanosine diphosphate (GDP) mannose [17]. Two heterozygous variants in *PMM2*, missense variant on exon 6 (c.484C>T, p.R162W) and donor splicing site variant on exon 3 (c.255+1G>A), were identified in this woman. Both variants were identified as pathogenic in previous ClinVar submissions, (SCV000329703.5 and SCV000536849.1). c.484C>T was previously reported in CDG patients [18, 19] and functional studies suggest the damaging effect on *PMM2* protein stability [20]. Another French family with CDG carrying the same two heterozygous variants was reported in 2005 [21]. Karyotype analysis found that FPOI16 had fragile chromosome 17 on the short arm (46 XX+fra17p12); however, this is a well-known fragile site with no known functional consequence. We assumed that these two variants in FPOI16 were in trans, given the phenotype, biochemical studies, and known pathogenicity of variants. Parents were not available.

FPOI24 and FPOI38 both had pathogenic variants in *MCM9*, a gene that was recently implicated in POI [7]. FPOI24 was born to a consanguineous family and was diagnosed with POI when she was 18. FPOI38 was diagnosed with POI at the age of 20. FPOI24 and FPOI38 both had absent ovaries on the ultrasound. Homozygous variants and two heterozygous variants in *MCM9* were identified in FPOI24 (c.1651C>T, p.Q551X) and FPOI38 (c.1784C>G, p.T595R; c.905-1G>T), respectively. *MCM8* and *MCM9* are required in homologous recombination and the repair of double-stranded DNA breaks [4, 22]. In mice, deficiency of these two proteins causes infertility due to hypogonadism and oocyte depletion [22]. *MCM9* variants were previously associated with primary amenorrhea in unrelated consanguineous families [7, 23], and unlike previous report of short stature [7], FPOI24 and FPOI38 were of normal height. *MCM9* gene contains 13 exons [24], and the stop-gain novel variant of c.1651C>T in exon 9 is predicted to cause truncated protein, p.Q551X, and loss of *MCM9* function. Both of the variants in FPOI38, c.1784C>G and c.905-1G>T, that affects the acceptor splicing site on exon 6 were assigned to be pathogenic. The *MCM9* variants identified in this study were novel and were previously published by our group [25].

Table 1 Clinical profiles of 10 POI women identified with plausible variants

Patient ID	Puberty	FSH* (mIU/ml)	Estradiol* (pg/ml)	AMH* (ng/ml)	Karyotype	Pelvic ultrasonography			Variants of interest
						Ovary size* (mm)		Follicle	
						Right	Left		
FPOI8	Normal	104	19	0	Normal	16 × 10	15 × 6	Absent	NR5A1, c.1093C>T
FPOI10	Normal	100	56	ND	Normal	17 × 3	26 × 5	Absent	GDF9, c.1157G>T
FPOI11	Delayed	60	5	ND	Normal	Not viewable		Absent	MARF1, c.1684C>T
FPOI16	Delayed	50	34	ND	46, XX+ fra17p12	ND		ND	PMM2, c.484C>T, c.255+1G>A
FPOI22	Delayed	96	5	ND	46, X,t(X;14) (q22;q24)	16 × 6	13 × 6	Absent	LMNA, c.454C>A
FPOI24	Delayed	110	5	1	46, XX(t2:7)	Not viewable		Absent	MCM9, c.1651C>T
FPOI28	Normal	73	17	1.27	Normal	26 × 16	21 × 15	Present	FSHR, c.1274C>T, c.1209C>A
FPOI32	Normal	73	10	0.05	Normal	Not viewable		Absent	HARS2, c.1010A>G
FPOI38	Delayed	114	12	0.05	Normal	Not viewable		Absent	MCM9, c.1784C>G, c.905-1G>T
FPOI41	Delayed	140.3	12	0.05	Normal	Not viewable		Absent	PSMC3IP, c.496_497delCT, c.430_431insGA

*Normal ranges: FSH 3.5–12.5 mIU/ml, estradiol 13–166 pg/ml, AMH 0.5–3.8 ng/ml. Ovary size: 3–5 cm × 1.5–3 cm, referred from Pathology Associates of Lexington, P.A.

***ND* not done

FPOI41 was diagnosed with primary amenorrhea at 28 years of age and presented with elevated FSH level and ovaries were not visualized on ultrasound examination. Two heterozygous variants in *PSMC3IP* (c.496_497delCT, p.R166Afs; c.430_431insGA, p.L144*) were identified in this individual. *PSMC3IP* gene has nine exons and encodes a protein that functions in meiotic recombination and works as a coactivator of ligand-dependent transcription mediated by nuclear hormone receptors [26]. Autosomal recessive mutations in *PSMC3IP* lead to ovarian dysgenesis characterized by undetectable ovary and a hypoplastic uterus [26]. Both the exon 6 variant, p.R166Afs, and the exon 5 variant, p.L144*, identified in our study are novel and were predicted to cause frameshift mutation in *PSMC3IP*. We confirmed by Sanger sequencing that these two closely located variants were inherited in trans.

Variants of unknown significance

Aside from the pathogenic variants in *PMM2*, *MCM9*, and *PSMC3IP* mentioned above, six individuals, FPOI8, FPOI10, FPOI11, FPOI22, FPOI28, and FPOI32, were identified with variants of unknown significance in *nuclear receptor subfamily 5*, *group A* (*NR5A1*), *growth differentiation factor 9* (*GDF9*), *meiosis regulator and mRNA stability factor 1* (*MARF1*), *lamin A/C* (*LMNA*), *follicle-stimulating hormone receptor* (*FSHR*), and *histidyl-tRNA synthetase 2* (*HARS2*) (Supplemental data 3).

FPOI8 was diagnosed at 14 years of age with primary amenorrhea and experienced normal puberty. She presented with low estradiol and small ovaries. Heterozygous variant in *NR5A1* (c.1093C>T, p.R365W) was identified. *NR5A1* encodes a transcription factor that regulates the development of the adrenal and reproductive system [27]. Heterozygous *NR5A1* variants cause 46 and XX sex reversal and associate with POI [28]. This variant was predicted as potentially damaging by computational analyses. No further information was found in gene variant databases.

FPOI10 was diagnosed at 17 years of age with primary amenorrhea and experienced normal puberty. Ultrasound examination showed small ovaries and absent follicles. She had elevated FSH level and normal estradiol level. Homozygous variant (c.1157G>T, p.C386F) in *GDF9* was identified. *GDF9* is specifically expressed in oocytes and is an attractive candidate gene for POI. Mouse model deficient in *GDF9* is infertile and primary follicles do not grow [29]. *GDF9* variants in POI women have been previously reported in Indian [30], Chinese [31], and Caucasian [32] populations. All the previous variants reported in *GDF9* were heterozygous and unlikely to be pathogenic. One homozygous variant was identified in a Brazilian woman with primary amenorrhea [33]. FPOI10 variant was predicted to be conserved and damaging by computational analysis. However, we could not assign it as pathogenic due to the lack of functional studies and previous reports on this variant.

FPOI11 was diagnosed at 15 years of age with primary amenorrhea. She did not have identifiable ovaries on ultrasound examination. She was identified with homozygous variant (c.1684C>T, p.R562C) in *MARF1*. *MARF1* was found to be important in regulating oogenesis and genomic stability in mice [34]. We are not aware of previous reports associating *MARF1* variants and POI. c.1684C>T was predicted to be

Table 2 Variants identified in 10 POI women

Patient ID	Gene	Mutation type*		Gene MIM number [40]	Transcript ID	Nucleotide change	AA change	Reported dbSNP	ACMG evidence	Pathogenicity
FPOI8	NR5A1	Het	Missense	184757	NM_004959	c.1093C>T	p.R365W	Novel	PM2, PP3	VUS
FPOI10	GDF9	Hom	Missense	601918	NM_005260	c.1157G>T	p.C386F	Novel	PM2, PP3	VUS
FPOI11	MARF1	Hom	Missense	614593	NM_001184998	c.1684C>T	p.R562C	Novel	PM2, PP3	VUS
FPOI16	PMM2	Two het	Missense Splicing	PMM2-CDG,601785	NM_000303	c.484C>T c.255+1G>A	p.R162W	rs104894526 rs1060499598	PS4, PS3, PM2, PP3 PSV1, PS4, PM2, PP5	Pathogenic
FPOI22	LMNA	Het	Missense	Malouf syndrome, 150330	NM_170707	c.454C>A	p.L152I	Novel	PM1, PM2, PP2	VUS
FPOI24	MCM9	Hom	Stop-gain	610098	NM_017696	c.1651C>T	p.Q551X	Novel**	PSV1, PM2	Pathogenic
FPOI28	FSHR	Two het	Missense Missense	136435	NM_000145	c.1274C>T c.1209C>A	p.T425I p.N403 K	Novel Novel	PM2, PP3 PM2, PP3	VUS
FPOI32	HARS2	Hom	Missense	PRLTS2, 600783	NM_012208	c.1010A>G	p.Y337C	rs537198287	PM2, PP3	VUS
FPOI38	MCM9	Two het	Missense Splicing	610098	NM_017696	c.1784C>G c.905-1G>T	p.T595R	Novel** rs149099524	PM2, PP3 PSV1, PM2	Pathogenic
FPOI41	PSMC3IP	Two het	Deletion Insertion	608665	NM_016556	c.496_497delCT c.430_431insGA	p.R166Afs p.L144*	Novel Novel	PSV1, PM2 PSV1, PM2	Likely pathogenic

Hom homozygous, *Het* heterozygous dominant, *Two het* two heterozygous

**Same participant results have already been published in [25]

conserved and damaging by computational analysis; however, the lack of functional studies makes this a variant of unknown significance.

FPOI22 was diagnosed at 16 years of age with primary amenorrhea. She presented with elevated FSH and estradiol levels and had small ovaries and absent follicles on ultrasound examination. She carried a heterozygous variant (c.454C>A, p.L152I) in the *LMNA* gene. *LMNA* is a structural protein component of the nuclear lamina. *LMNA* variants have been found in individuals with POI and cardiomyopathy [35] (Malouf syndrome). Variants in this gene have not been reported in women with isolated POI. It is noteworthy that translocation between X chromosome and chromosome 14 was also detected. This may lead to loss of function of Xq22, which has been implicated in ovarian function [36]. Thus, for the FPOI22 case, we considered X chromosome translocation to be more plausible cause of POI phenotype than *LMNA* variant.

FPOI28 was diagnosed at 18 years of age with primary amenorrhea. She experienced normal puberty with elevated FSH and low AMH levels. She was identified with two heterozygous variants (c.1274C>T, p.T425I; c.1209C>A, p.N403K) in *FSHR*. FSH/FSHR interaction plays a significant part in regulating ovarian function by regulating follicle growth and estrogen production. *FSHR* mutations first reported in Finnish women were considered the first autosomal mechanism causing POI [37]. Variants of *FHSR* lead to oocyte dysfunction, characterized by a small ovary phenotype. Consistent with ovarian dysgenesis, small ovaries and small follicles were noted in FPOI28. These variants have not been previously reported as pathogenic, and functional studies are lacking.

FPOI32 was diagnosed at 27 years of age with primary amenorrhea and presented with absent ovaries on ultrasound examination. She was identified with homozygous variant (c.1010A>G, p.Y337C) in *HARS2*. The *HARS2* gene encodes histidyl-tRNA synthetase, a highly conserved protein that functions in mitochondria. Damage to this enzyme leads to Perrault syndrome (*PRLTS2*), characterized by primary amenorrhea and progressive sensorineural deafness in both males and females [38]. Hearing loss was not documented in FPOI32. This variant was damaging by computational analysis; however, it has not been previously reported as pathogenic and functional studies are lacking.

Conclusion

In this pilot study, we identified pathogenic and likely pathogenic variants in 4 out of 33 individuals (12%) with primary amenorrhea and hypergonadotropic hypogonadism. These results suggest a strong genetic etiology in these women and justify utilization of whole-exome sequencing for diagnostic

purposes. A small number of studies with a small number of participants among women with hypergonadotropic hypogonadism have hampered identification of variants associated with reproductive pathologies. It is difficult to assign pathogenicity when variants are novel and missense and functional studies are lacking. We were unable to get parental DNA samples; thus, whether different heterozygous variants in the same gene were in cis or trans could not be confirmed for all participants. Getting other family members from affected adults can be difficult, but it is very important to determine whether variants are in cis or trans, as well as whether variant is de novo. This is especially important since de novo variants contribute pathology to 40% of individuals with Mendelian disorders [39]. Although WES is an efficient tool for identifying gene variants in POI women, interpretation of variants is hampered by few exome studies involving ovarian disorders and the need for trio sequencing.

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Compliance with ethical standards

The study was approved by the Institutional Review Board of University of Pittsburgh (PRO09080427). Informed consent was obtained from all individual participants in the study.

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

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