

## Utilization of Whole Exome Sequencing in Non-Syndromic Premature Ovarian Failure: *Ficolin-3* Gene Mutation in an Iranian Family

Soophia Mehrjooy<sup>1</sup>, Roshan Nikbakht<sup>2</sup>, Javad Mohammadi Asl<sup>3</sup>, Ataallah Ghadiri<sup>4</sup> and Pegah Ghandil<sup>1,5\*</sup>

<sup>1</sup>Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Jundishapur University of Medical Sciences, Iran; <sup>2</sup>Department of Gynecology, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; <sup>3</sup>Department of Medical Genetics Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; <sup>4</sup>Department of Immunology, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; <sup>5</sup>Diabetes Research Center, Health Research Institute Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

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### ABSTRACT

**Background:** Premature ovarian failure is a heterogeneous disorder, leading to early menopause. Several genes have been identified as the cause of non-syndromic POF. Our aim was to explore the genetic defects in Iranian patients with POF. **Methods:** We studied a family with three females exhibiting non-syndromic POF. WES was performed for one of the affected individuals after ruling out the presence of CGG repeat expansion at FMR1 gene in the family. Sanger sequencing was used to confirm the candidate sequence variants in the proband, and screening of the detected mutation was performed for the other affected and unaffected members of the family. **Results:** A homozygous frameshift mutation, c.349delC, was identified in *FCN3* gene in the proband and two other patients. The parents and two healthy brothers were heterozygous for the mutation, and an unaffected sister was homozygous for wild type. **Conclusion:** This is the first report of a mutation in *FCN3* gene in a family with POF. Our findings can lead to the enhancement of genetic databases of patients with POF, specifically for families with high-risk background. **DOI:** 10.52547/ibj.25.6.441

**Keywords:** Ficolin-3, Premature ovarian failure, Whole exome sequencing

**Corresponding Author:** Pegah Ghandil  
Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran;  
Tel./Fax: (+98-916) 5171534; E-mail: pghandil@yahoo.com or pghandil@yahoo.com

### INTRODUCTION

Premature ovarian failure refers to the depletion of follicular reserve and the permanent stop of menstrual cycles before the 4<sup>th</sup> decade of life<sup>[1]</sup>. Mechanisms that are involved in the pathogenesis of POF can be classified as genetic, autoimmune disorders, infections, metabolic disease, chemotherapy,

radiations, and idiopathic. Some POF cases are syndromic, in which the POF phenotype is associated with a specific disease, such as Turner syndrome. Moreover, FMR1 carriers are among the cases with syndromic POF<sup>[2]</sup>.

Non-syndromic POF cases, categorized as idiopathic, are thought to have a genetic basis<sup>[3]</sup>. The first gene identified for the molecular etiology of POF was

#### List of Abbreviations:

**BWA**, Burrows-Wheeler Aligner; **ExAC**, the Exome Aggregation Consortium; **FCN3**, ficolin-3; **FMR1**, fragile X mental retardation 1; **GATK**, Genome Analysis Toolkit ; **gmonAD**, Genome Aggregation Database; **GWAS**, Genome-wide association study; **HGMD**, Human Gene Mutation Database; **OMIM**, Online Mendelian Inheritance in Man; **POF**, premature ovarian failure; **SwissVar**, Swiss-Prot Variant; **WES**, Whole Exome Sequencing

*FSHR*, recognized by the candidate gene approach and gene linkage analysis in 1995<sup>[4]</sup>. In 1996, the first study was carried out using the gene sequencing method to identify non-syndromic POF causative genes, which led to the detection of *LHGR* gene<sup>[5]</sup>.

To date, several genes, such as *MCM9*, *MCM8*, *STAGE3*, and *SYCE1*, have been confirmed by the WES technique in relation to the etiology of the disease<sup>[6]</sup>. The *SYCE1* gene was identified based on the WES method in an Arab family with two daughters with non-syndromic POF from consanguineous marriage<sup>[7]</sup>. The *STAGE3* gene has also been identified by utilizing WES in a Palestinian family with several POF affected members and consanguineous parents<sup>[8]</sup>. Indeed, WES has been a quite effective method and is considered as a golden test in the study of non-syndromic POF<sup>[9]</sup>.

The increase of high prevalence and the lack of effective medication have made this disorder an attractive area of study<sup>[10]</sup>. Consanguineous marriages are an important factor in the prevalence of genetic disorders. In Iran, 38% of all marriages are reported to be consanguineous<sup>[11]</sup>; hence, a dramatic growth in the prevalence of autosomal recessive disorders, such as POF, is predicted. On the other hand, the psychological and physical complications of early menopause and the shortage of pregnancy opportunities highlight the necessity of molecular examination for these patients.

Altogether, the aim of this study was to identify genetic mutations associated with non-syndromic POF in an Iranian family using WES. Identification of molecular defects will be undoubtedly useful for accurate diagnosis and therapeutic targets in future for the patients and their families.

## MATERIALS AND METHODS

### Subjects

We selected the patients with POF, all from one family, referred to specialized clinics of gynecology in the southwest of Iran. The main criteria used for choosing the patients were based on positive family history of POF and secondary amenorrhea in at least three cases with a possible autosomal recessive inheritance pattern. The cases of the studied family were selected based on the reduction of follicular reserve, elevated levels of serum luteinizing hormone and serum follicle-stimulating hormone (normal, 1.0–14.7 IU/L for both), decreased estradiol levels and experience of menopause before the age of 30. We reviewed the patients' medical records to eliminate the POF cases caused by cancer, infection, and immunological diseases. To exclude the syndromic

cases, chromosomal analysis (G-Banding) using whole blood was carried out to confirm a 46, XX, normal female karyotype. Also, the possible expansion of CGG repeats at 5'UTR of *FMR1* gene in the proband was examined by PCR, demonstrating no expansion of the repeats<sup>[1]</sup>.

### WES

Peripheral blood samples were obtained from affected and unaffected family members. Genomic DNA was extracted using QIAamp DNA Blood Mini-Kit (QIAGEN, Hilden, Germany). WES was performed for the proband. Targeted gene capture was accomplished using a custom capture kit. On the Illumina sequencing platform, the libraries were sequenced with the average coverage of 80-100×. For recognizing variants relevant to the clinical indication, the obtained sequences were aligned to the human reference genome, GRCh37/hg19 using the BWA program<sup>[12,13]</sup> and analyzed by applying Picard and GATK version 3.6<sup>[14,15]</sup>. The GATK's best practices framework was employed for the identification of variants in the sample. Gene annotation of the variants was carried out using Veterans Entrepreneurship Program against the Ensembl release 87 of human genome. To be specific, clinically relevant mutations were annotated using published variants and a set of disease databases—ClinVar, OMIM, GWAS, HGMD, and SwissVar<sup>[16-20]</sup>. Variants with a minor allele frequency of >1% in population databases were filtered. The ExAC, gnomAD, dbSNP, and the 1000 Genomes Project human polymorphism database, and the National Heart, Lung, and Blood Institute databases were also employed. In the next step, only protein-coding, non-synonymous, stop gain, frameshift or splice variants, as well as intronic splice site variants were collected. Based on the information of the studied family, we suspected to have an autosomal recessive inheritance pattern in the family. For this reason, we selected homozygous variants, and the remaining variants were carefully evaluated for gene function and possible association with the occurrence of POF. Finally, the effect of non-synonymous variants was determined using several algorithms, including PolyPhen2 (<http://genetics.bwh.harvard.edu/pph2>), SIFT (<https://sift.bii.a-star.edu.sg>), MutationTaster2 (<http://www.mutationtaster.org>), MutationAssessor (<http://mutationassessor.org/r3>), and LRT ([http://www.genetics.wustl.edu/jflab/lrt\\_query.html](http://www.genetics.wustl.edu/jflab/lrt_query.html)).

### Mutation confirmation

We confirmed the detected mutation in the proband. PCR was accomplished with conditions described previously<sup>[21]</sup>. Gene-specific primers were designed by

Allele ID primer design software version 7 (Premier Biosoft, Palo Alto, USA). Sanger sequencing and data analyzing were performed for the PCR fragment of the proband using an ABI Prism 3700 apparatus (Big Dye Terminator sequencing kit; Applied Biosystems, Foster City, CA, USA). Afterwards, co-segregation analysis was conducted for other patients and healthy individuals in the family through utilizing Sanger sequencing.

### Ethical statement

The above-mentioned sampling protocols were approved by the Local Ethics Committee of the Ahvaz University of Medical Sciences, Ahvaz, Iran (ethical code: IR.AJUMS.REC.1396.100). Written informed consents were obtained from all individuals.

## RESULTS

Three females with POF exhibiting in the studied family were Iranian Lur ethnicity. The results of demographic, karyotype, and hormonal studies of all three patients are presented in Table 1. The first patient (III-2; Fig. 1A) was 25 years old and married four years ago. She referred to a physician since two years ago due to oligomenorrhea and infertility. Breasts were Tanner stage 5, and pubic and axillary hair were Tanner stage 4. Serum gonadotropin concentrations in two consecutive tests and pelvic ultrasonography results of this patient are presented in Table 1, which approves secondary amenorrhea. The second patient (III-1; Fig. 1A) was 29 years old and was married eight years ago. Her breast development was normal and referred to a physician since two years ago due to oligomenorrhea and infertility. The examination results of this patient are mentioned in Table 1. Another sister (III-3; Fig. 1A), 32 years old, has been married for

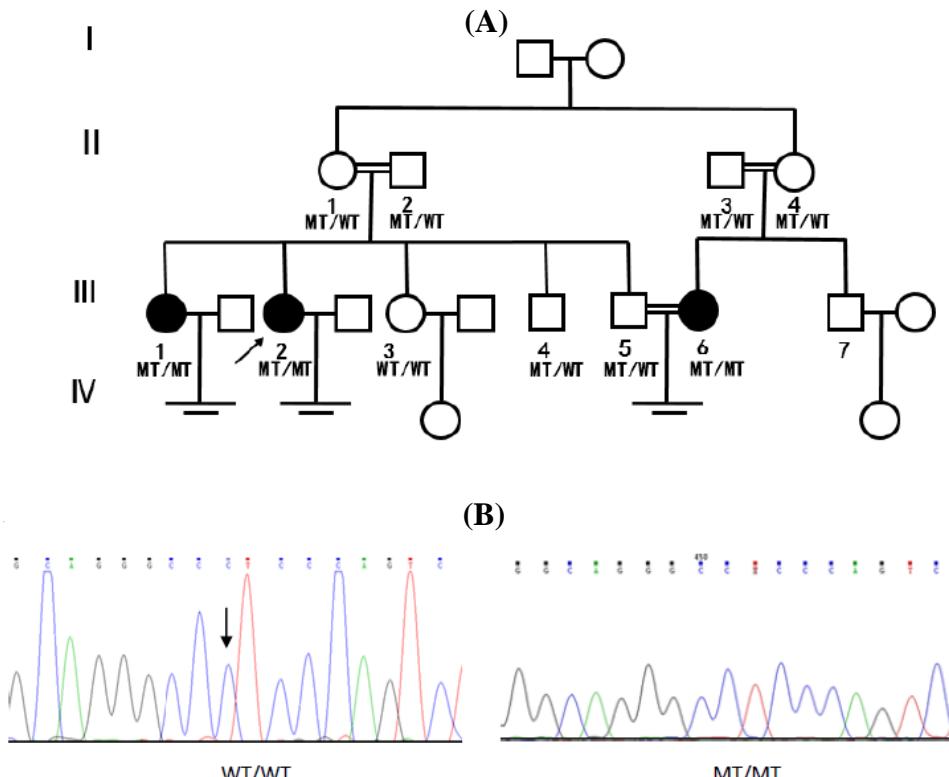
three years, and she has an 18-month old girl. She had regular menstrual with normal serum gonadotropin level. Both parents (II-1 and II-2; Fig. 1A) reported normal puberty and no fertility problem. Two brothers (III-4 and III-5; Fig. 1A) were evaluated at the age of 18 and 30 years. Both of them had normal weight and height, were fully pubertal and had adult testicular volume. Serum gonadotropin levels were in the normal adult range. The third patient was the proband's cousin (III-6; 24 years of age; Fig. 1A) who married four years ago with the proband's brother (III-5; Fig. 1A). The results of this patient study are listed in the Table 1. At the age of 21, she was consulted for infertility. Additionally, she had been referred to a physician three years ago because her menses had stopped, and she was diagnosed as premature menopause.

A homozygous frameshift mutation identified, FCN3 + 1637delC, c.349delC (p.Leu117SerfsX65) located in exon 5 of *FCN3* gene (ENST00000270879.8) in the proband (III-2; Fig. 1A and 1B), by WES and was approved by Sanger sequencing. We screened the affected and unaffected individuals in this family for the detected mutation. Results showed that the two other affected cases (III-1 and III-6; Fig. 1A) were homozygous for the mutation exactly like the proband (III-2; Fig. 1A and B). Therefore, all cases in this family could be considered as POF patients. The proband's parents (II-1 and II-2; Fig. 1A) and parent of the third patient (II-3 and II-4; Fig. 1A), as well as two healthy sons of this family (III-4 and III-5; Fig. 1A) were identified by heterozygous state, and proband's healthy sister with normal puberty and fertility was wild-type homozygous. These results indicated an autosomal recessive inheritance pattern for the detected mutation in this family. *In silico* protein prediction programs demonstrated that this mutation, FCN3 + 1637delC, resulted in the creation of a nonsense protein sequence,

**Table 1.** Demographic, karyotype, and hormone studies of the patients in the family

Indicator	III-2	III-1	III-6
Karyotype	46 XX normal female	46 XX normal female	46 XX normal female
Age (y)	27	30	24
Height (cm)	162	160	156
Weight (kg)	52	50	56
Ovaries volume size	small	small	small
Uterus volume size	normal	small	small
Storage follicles	5 follicles	2 follicles	empty
LH (IU/L)	32.3 and 32.	49.5 and 46.8	140
FSH (IU/L)	138.0 and 128.0	132.0, 155.0 and	80.5
E2 (pmol/L)	4.0	3.8	3.0

LH, luteinizing hormone normal: 11.0–14.7 IU/L; FSH, 2-follicle-stimulating hormone normal: 1.0–14.7 IU/L; Estradiol (E2) after menopause <20.



**Fig. 1.** (A) The pedigree of the family with POF having (p.Leu117SerfsX65) mutation in *FCN3* gene. The genotypes are shown with WT (wild type) and MT (mutant); (B) sequence chromatograph of (p.Leu117SerfsX65) mutation with homozygous state and also wild type form in *FCN3* gene. The arrow indicates the position of the mutation.

in the residue position of 117 to position 180, due to a shift in the reading frame, and modified the amine acids composition. This change in the C-terminal region can lead to the loss of the fibrinogen-like domain and the production of truncated FCN3 protein (p.Leu117SerfsX65).

## DISCUSSION

In this study, we identified (p.Leu117SerfsX65) mutation in *FCN3* gene in a consanguineous Iranian family with POF by using WES. All affected family members who provided samples for analysis were homozygous, and all unaffected family members were heterozygous or wild type for this mutation. To our knowledge, the identification of (p.Leu117SerfsX65) mutation in *FCN3* gene in the women with POF was not reported before. Ficolins proteins function as a part of the innate immune system. Three different forms of ficolins have been described in human, M-ficolin (ficolin-1), L-ficolin (ficolin-2, P35), and H-ficolin (ficolin-3, Hakata antigen). All of them and mannose-binding lectin can activate complement via the lectin

pathway. However, H-ficolin has higher complement activating potential compared to M-ficolin, L-ficolin, and mannose-binding lectin<sup>[22]</sup>. H-ficolin is encoded by the *FCN3* gene, which has been mapped to chromosome 1p36.11 with eight exons. The polypeptide chains of H-ficolin contain 299 amino acids<sup>[22,23]</sup>. *FCN3* is expressed mostly in the liver and the lung; nonetheless, lower expression is also detected in the heart, kidney, spleen, pancreas, brain tissue, placenta, and ovary<sup>[22,24,25]</sup>. Although various mutations have been identified in the *FCN3* gene, the frameshift mutation of (p.Leu117SerfsX65) has only been reported in a few cases in the literature<sup>[21,26,27]</sup>. This mutation was related to the lower levels of H-ficolin in the heterozygous state and total lack in the homozygous state<sup>[26]</sup>. In 2008, Munthe-Fog *et al.*<sup>[26]</sup> reported (p.Leu117SerfsX65) mutation with a heterozygous state among healthy Danish Caucasians, with an allele incidence of 0.01. In another study by the same author in 2009<sup>[27]</sup>, among the 1282 patients with various immunodeficiency diseases, 23 cases were heterozygous, and one subject was homozygous for the (p.Leu117SerfsX65) mutation. The homozygous patient, a 32-year-old man, had healthy parents with

heterozygous state and two healthy sisters (one heterozygous and one wild type). The patient was the son of unrelated parents with Macedonian and Albanian origins. A history of severe recurrent infections was observed in this patient with no detectable levels of H-ficolin in his serum. Schlapbach *et al.*<sup>[28]</sup> in Switzerland reported another patient who was a male premature baby diagnosed with necrotizing enterocolitis and recurrent infections with *Staphylococcus aureus* who was homozygous for (p.Leu117SerfsX65) mutation. In addition, Michalski *et al.*<sup>[21]</sup> in Poland detected one male prematurely born infant who was homozygous for the (p.Leu117SerfsX65) mutation with a perinatal infection caused by *Streptococcus agalactiae*.

In the present study, following the identification of (p.Leu117SerfsX65) mutation in a consanguineous family, the homozygous and heterozygous members were examined immunologically, and all were healthy. Based on previous reports, FCN3 deficiency was associated with increased susceptibility to infections<sup>[21,27,28]</sup>. However, this finding was not found in our study. The lack of correlation between this mutation and infection was also observed in the study of Michalski *et al.*<sup>[29]</sup> who reported two cases with primary FCN3 deficiency with no severe infection. One of them was a 50-year-old male, with membranous nephropathy, who carried both mutated alleles of FCN3. Another reported patient was an 11-month-old male infant with congenital heart disease and homozygous mutation of (p.Leu117SerfsX65).

All reported cases of FCN3 primary deficiency so far were male with a variety of clinical manifestations<sup>[21,27-29]</sup>. Therefore, we can conclude that the clinical association of FCN3 gene (p.Leu117SerfsX65) mutation is still unclear. Moreover, ethnic or geographical background and gender may influence the frequency of the variant allele. It is likely that sex hormones may be effective in preventing clinical symptoms in our patients. Therefore, we suggest the study of larger Iranian populations, accompanied with supplementary molecular approaches, to widen our understanding of POF genetics.

In conclusion, finding the molecular defect as the cause of disease is important for diagnosis confirmation and treatment management in POF. Our results will help to increase our knowledge about these patients. However, further studies are required to better understand the molecular mechanism underlying this disease and all the relevant clinical consequences of various congenital FCN3 mutations.

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## CONFLICT OF INTEREST.

None declared.

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