

# Existence of Inhibin $\alpha$ -Subunit Gene Mutation in a Population of Iranian Women with Premature Ovarian Failure

Fallahian M<sup>a</sup>, Pouresmaeili F<sup>b</sup>, Azizi F<sup>c</sup>, Zali MR<sup>d</sup>, Samani EM<sup>a</sup>, Kharaziha P<sup>d</sup>

<sup>a</sup>Department of Obstetrics and Gynecology, <sup>b</sup>Department of Genetics, and Infertility & Reproductive Health Research Center (IRHRC); <sup>c</sup>Endocrine Research Center, Research Institute for Endocrine Sciences, and <sup>d</sup>Research center for gastroenterology and liver diseases, Taleghani Hospital, Shahid Beheshti University of Medical Sciences, Tehran. I.R.Iran

**P**remature ovarian failure (POF) is characterized by hypergonadotropic amenorrhea, before the age of 40, for which the Inhibin  $\alpha$ -subunit (INH $\alpha$ ) gene is proposed as a candidate gene, due to its role in negative feedback control of FSH. In this study we aimed at demonstrating the candidate mutation as a gene variation associated with POF in Iranian population.

**Materials & Methods:** Using DNA sequencing, DNA samples of 24 women with POF and 24 controls, aged below 40 years, were screened for mutations in the Inhibin gene.

**Results:** The 769G→A mutation in exon 2 of the Inhibin- $\alpha$  gene was found in four out of 24 idiopathic POF patients.

**Conclusion:** The results obtained in this study have shown that this variation is more frequent in patients with POF than in normal fertile populations of Iran.

**Keywords:** Inhibin  $\alpha$ -subunit gene, Premature ovarian failure, Mutation

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*Correspondence:* Farkhondeh Pouresmaeili, PhD, Genetics Department, Shahid Beheshti University of Medical Sciences, Evin Ave, Velenjak Street, Tehran 19395-4719, Iran

*E-mail:* [fpoures@yahoo.com](mailto:fpoures@yahoo.com)

## Introduction

Premature Ovarian Failure (POF) or premature menopause is a syndrome that is clinically defined by failure of the ovary before the age of 40 yr. The frequency of POF is about 1% in all women, sometimes affecting very young women in their late teens or twenties.<sup>1</sup> It has a multicausal pathogenesis, which includes chromosomal abnormality, genetic disorder, enzyme deficiency, iatrogenic condition or infectious.<sup>2</sup>

Inhibin, a glycoprotein, is a potential candidate for POF due to its role in the negative feedback control of FSH, which has a cardinal role in the folliculogenesis. Inhibin is structurally related to a group of multi-functional transforming growth factors, the TGF- $\beta$  super family. The mature Inhibin is a 31–32 kDa heterodimer glycoprotein consisting of an 18 kDa  $\alpha$ -subunit linked by two disulphide bonds to one of two 14 kDa  $\beta$ -subunits. There are two forms of Inhibin; Inhibin A ( $\alpha$ - $\beta$ A), and Inhibin B ( $\alpha$ - $\beta$ B). The Inhibin subunits are encoded by three separate genes: INH $\alpha$ , INH $\beta$ A and INH $\beta$ B, which map to 2q33-qter, 2cen-q13 and 7p15-p14, respectively.<sup>3</sup> It is proposed that a functional

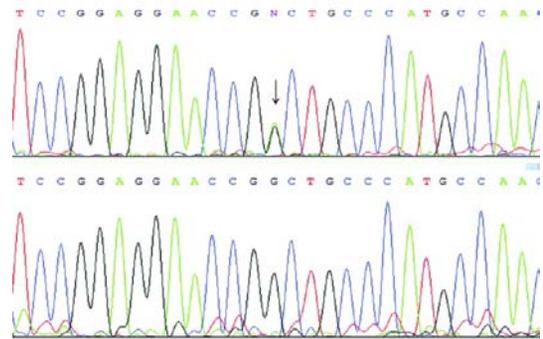
mutation in any of the Inhibin genes would lead to a decrease in the amount of bioactive Inhibin, a loss that would cause an increase in concentrations of FSH by removing the negative feedback on pituitary gland, resulting in the premature depletion of follicles. Two polymorphic sites of this gene:  $-16C>T$  in the 5'UTR and  $675C>T$  in exon 2, are in complete linkage disequilibrium with each other.<sup>4</sup> Marozzi et al. studied  $-16C>T$  polymorphism and found a significant relation between T allele frequency in the patients rather than in normal group.<sup>5</sup> On the other hand, although Shelling et al. identified a missed mutation ( $769G>A$ ) in exon 2 of the gene, in 3 of 43 women with POF,<sup>6</sup> this substitution was subsequently described in Indian and Italian women with POF,<sup>7</sup> suggesting an association between the  $769G>A$  variant and the development of POF. Jung et al found no association between the  $769G>A$  variant and the development of POF in Korea.<sup>8</sup> Recently, Sundblad et al showed no significant difference for POF development due to the  $-16 T$  allele when comparing idiopathic POF ( I-POF ) with  $C > 40$  (Odds ratio = 1.46; 95% confidence interval = 0.63–3.19). These results indicate that  $-16C>T$  and  $769G>A$  variants in the  $INH\alpha$  gene may not be associated to POF disease.<sup>9</sup> The purpose of this paper is to study the existence of Inhibin  $\alpha$ -subunit gene mutation in Iranian women with premature ovarian failure.

## Materials and Methods

### Study Sample

From June 2004 to January 2008, 24 patients with idiopathic premature ovarian failure and 24 healthy fertile women were recruited by university general hospital. The sample size was forty eight with 90% power at the level of 95%. All the women in this study were examined at the gynecology clinic and were diagnosed as having premature ovarian failure, based on missed periods more than 6 months accompanied by hot flushes and a serum FSH level  $\geq 40$  IU. Biochemical and hormonal assays were performed for

both control and case groups. For this purpose, levels of FSH, LH, PRL, TSH, Ca and Ph were measured. Both groups were investigated whether they displayed the mutation in  $INH \alpha$  769 transition of G to that alters GCT to ACT, resulting in Alanine substitution by Threonine  $INH \alpha$  subunit (Fig.1). Informed consent was obtained from all participants and the Ethical Review Boards of the center approved the study protocol.



**Fig.1.** The electropherogram indicates the sequence analysis comparison between patients (upper panel) and controls (lower panel) PCR products. The nucleotide change in a heterozygous form is seen in  $INH\alpha$  gene of patient samples (arrow).

POF was defined as cessation of menses for duration of 6 months or longer before age of 40 years and serum FSH measurements over 40mIU/mL. Controls were selected according to the following criteria: Age below 40; regular menses (cycle 25-35 days); no family history of premature or early menopause; no family history of autoimmune disorders; and no consumption of oral contraceptives or other hormonal medications at the time of inclusion in the study.

### DNA Extraction

Overall 48 DNA samples (24 from each group) were collected. Genomic DNA was extracted from peripheral blood lymphocytes using QIAamp DNA blood mini-kit (Qiagen, Valencia, USA); 10mL blood collected in Falcon tubes including EDTA. A pair of pri-

mers was designed by Primer 3 and Gene Runner software (from MWG company Germany) to amplify considered sequence, following which we examined two identified mutations of premature ovarian failure in the Inhibin alpha (INHA) gene in the control and patient groups.

#### Amplification

For PCR, performed using Super Taq (Gene Fanavaran, Tehran-Iran), we used 100 ng of DNA, 2.5 mmol/L MgCl<sub>2</sub>, and 1 pmol/primer. The sequences of primers were: 5' GCCTGCTGGCACTGTCAC 3' and 5' GGAAGGGACAGGTTTGGTG 3'. The cycling stages for amplification were initial denaturizing at 94°C for 10 minutes, following 30 cycles at 94°C for 30 seconds, 61°C for 30 seconds, 72°C for 20 seconds and the final extension of 72°C for 10 minutes.

#### Sequencing

The PCR- products were subjected to direct sequencing, performed using a Big Dye Tm Terminator Cycle Sequencing Kit (Applied Bio systems, Foster city, CA, USA) and a genetic Analyzer PRISM TM 3130x (Applied Bio systems, Foster city, CA, USA). DNA templates were sequenced in both directions and in case of any variations; results were confirmed by sequencing at least two independent PCR products. All sequences were analyzed by three software i. e. Chromas version 1.45 (Queen lands, Australia), Laser gene ver.6 and Bio Edit.

#### Statistical Analysis

SPSS program (SPSS, software 11.0, Chicago, USA) was used for data analysis and performing Fisher's exact test. P value less than 0.05 was considered as significant.

## Results

The mean and range of the participants' age was 34.4±12.3 years in cases and 37.2±11.3 years in controls; age at menarche was 13.2±1.2 and 13.3±1.1 years respectively; they had fertility scores of 3.2±1.5 and 2.4±1.1, and body mass indexes of 26.09±4.4 and 23.1±3.6 respectively.

The mutation in INH  $\alpha$  769 transition of G to that alters GCT to ACT (G> A), resulting in Alanine substitution by Threonine INH  $\alpha$  subunit (Fig.1) was significantly more frequent in POF patients (n=24) compared to the control group (n=24). The INH  $\alpha$  G769 A variation was 4/24 (16.7%) in POF patients and 0/24 (0%) among normal fertile women. (Fisher's exact test, P value < 0.05). According to the sequencing results given in table 2, close to 17% of patients were found to carry the mutation G>A at ages 19, 21, 24 and 25 yr; however none of the controls showed the mutation in the same sequence of the gene Inhibin, respectively.

The mean age of menopause in POF cases was 28.9±9.1 yr(range 16-39 years). Mean level of FSH was 72.1±59.9 mIU/mL in patients with POF. Clinical details of the 4 patients with POF, carrying the variant, are described in Table 1.

**Table 1: Clinical details of POF\* patients carrying the G>A mutation**

Patient ID	Age of menopause (yrs)	Familial	FSH (IU/L)	LH (IU/L)	TSH (mIU/L)	PRL (pmol/l)
2	24	-	91.6	21.3	0.8	161
9	25	-	105	33.4	1	211
18	19	+	190	52.5	1.9	597
21	21	-	40.8	9.8	1.6	499

\* POF= Premature Ovarian Failure

## Discussion:

We have studied the involvement of the INH $\alpha$  gene as one of the factors involved in

the etiology of POF patients (16.7 percent) in Iran. Although the results of this study showed significant difference for inhibin mu-

tation, it cannot necessarily rule out other co-factors that contributing to POF.

The INH  $\alpha$  G769A variant was first reported in New Zealand patients with POF (7%) compared with 0.7% in controls.<sup>6</sup> This mutation has also been found in 9 of 80 and in 7 of 157 POF patients from India and Italy, respectively.<sup>5,7</sup> However, it was found neither in 84 POF patients from Korea<sup>8</sup> nor in 43 subjects in Auckland.<sup>9</sup> In accordance with the studies by Shelling,<sup>6</sup> Marozzi<sup>5</sup> and Dixit,<sup>7</sup> we were able to demonstrate a significant association between this mutation and ovarian failure. In the present study, we investigated the presence of substitution 769G>A in exon 2 of INH $\alpha$  gene in order to determine whether it could further explain the involvement of one of the factors involved in the etiology and/or pathophysiology of POF patients in our population.

Some isolated cases of 769G>A substitutions have previously been described in normal women.<sup>5,6,9</sup> Even though the occurrence of mutation without POF manifestation was explained by these authors to be due to incomplete penetrance, these findings could imply that this substitution could also be present in control subjects, becoming a putative polymorphism with no clinical consequences. In view of these considerations, we therefore included a group of 24 normal cycling women below 40 years of age in the analysis, and did not find this substitution in any of them. In agreement with the results of Shelling and Dixit,<sup>6,7</sup> we found that all POF patients with 769G>A substitution developed POF before the age of 25, findings that indicate the mutation is associated to a relatively severe and early onset of POF in Iranian women, respectively.

INH  $\alpha$  has a seven conserved cysteine residue, like the other members of the TGF- $\beta$  super family. This region is thought to be involved in receptor binding, because the amino terminal region, upstream in respect to first cysteine, is distinguishable from other members of the TGF- $\beta$  super family.<sup>8-10</sup> The 769G $\rightarrow$ A transition in the INH $\alpha$  gene was

identified in 16.7 percent of Iranian patients with POF in this study, a mutation which causes the non-conservative substitution of alanine for threonine at codon 257, and results in the addition of an aliphatic hydroxyl group in the side chain of the functional group. Moreover, the functional significance of the amino acid variant at codon 257 is still unknown.<sup>9-11</sup> The human INH  $\alpha$  gene has 80% homology compared to equine, bovine, porcine, ovine, rat and mouse gene sequences.<sup>10,11</sup> The only way to be certain that a mutation causes a functional effect is to perform functional testing. Most functional studies involve the expression of receptors in a recombinant expression system accompanied by an assay of the protein of interest. However, the Inhibin receptor has to be characterized. The activity of inhibin is currently investigated by an in-vitro bioassay based on the effect of graded doses of inhibin on FSH cell content of rat pituitary cells in culture.<sup>8</sup> When the amino acid sequences of the INH $\alpha$  protein of these species were compared, the alanine at codon 257, the site of the mutation was conserved in horse, porcine, ovine, mouse, bovine, possum and chicken species; however, the rat sequence contained serine. The amino acid sequence surrounding codon 257 is also highly conserved. These data suggest that alanine at codon 257 has an important role in the function of the protein. Analysis of the protein structure suggests that it could interfere with receptor binding.<sup>6</sup> The alanine to threonine change occurs within this putative receptor binding region of the final mature peptide. It has been hypothesized that this mutation may be sufficient to impair binding affinity of Inhibin to its putative receptor, leading to a subsequent inability to activate the subsequent signal transduction pathway and deregulate the FSH level by negative feedback.<sup>6</sup> Alternative hypotheses for a functional effect of this transition mutation include preventing dimer formation, cleavage of the mature peptide, or altering glycosylation.<sup>7,8,12</sup>

We have investigated whether a G769A transition in the INH  $\alpha$  gene exists in Iranian women with POF and our results. The results obtained in this study have shown that the nucleotide change in patients with POF is more frequent than in normal fertile Iranian women.

POF could be a consequence of the INH $\alpha$  gene mutations which may cause a decrease in the amount of bioactive Inhibin, and consequently, an increase in FSH concentration. Further investigations with a larger number of patients from different Iranian populations will define whether there is a significant relation between specific INH $\alpha$  gene mutation and the etiology of the disease. The understanding of the involvement of this particular Inhibin alpha gene mutation and polymorphism in the etiology of POF will be useful

in finding a diagnostic tool for rapid screening of women susceptible to the disease, before its appearance. Moreover, the results will open a new window to physicians to perform a molecular test to identify POF variants to facilitate putative therapy for such patients.

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