ORIGINAL ARTICLE

Genetic Analysis of Inhibin Alpha (*INHα*) Mutation (769G>A) in Patients with Premature Ovarian Failure in a Local Population

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ABSTRACT

Background and Objective: Premature ovarian failure is a worldwide concern effecting 1% of females of reproductive age. The objective of the present study was to analyze the role of inhibin alpha (*INHα*) gene mutation (769G>A) in patients with premature ovarian failure (POF) in the local population.

Methods: This case-control association studywas conducted in Department of Gynecology, Jinnah Hospital and The Children's Hospital and Institute of Child Health from July 2015-July 2016. A total of n = 100 were recruited for this study and divided into two gropus females with equal number (n = 50)of patients andnormal controls of reproductive age (14 – 40 years). The screening of the*INHα* for 769G>A variation in exon 2 was done through DNA sequencing.

Results: A higher frequency of the major allele G was seen in both the patients (99%) and the controls (87%) while comparing to minor allele A (1% in patients and 13% in controls). None of the patients was found to be homozygous (AA = 0%) for allele A, whereas, four of the controls were homozygous (AA = 8%). The frequency of the minor A allele in controls was found to be statistically significant (P-value = 0.002).

Conclusion: An association of decreased risk of POFwith A allele of the 769G>A variant rather than increasing the risk of development of ovarian failure.

KEYWORDS: Ovarian physiology, Inhibin alpha (*INHα)* gene, Hypothalamic- pituitary-ovarian axis, Premature ovarian failure (POF).

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INTRODUCTION

Premature ovarian failure (POF) is the early loss of functional ovarian follicles in women under the age of 40 years. POF is a common clinical condition affecting 1 – 3% of women of the reproductive age group.[1,2] The condition is usually characterized by amenorrhea of 4 – 6 months along with abnormal levels of gonadotrophins (FSH > 30 IU/l) and hypoestrogenism. Women with POF may not experience a menstrual cycle at all (primary amenorrhoea) or may experience cessation of ovarian function after a period of menstrual cycling (secondary amenorrhoea). The common presentation is in the form of secondary amenorrhea or oligomenorrhea. However, severe

forms of POF can presentwith absent pubertal development or primary amenorrhea. Also, women with POF may complain irregular menstrual cycles, palpitations, hot flushes, insomnia, vaginal dryness, infertility, and psychological disturbances especially depression resulting from the perception that fertility and femininity havebeen lost.[3] There are elevated risks for cardiovascular disease and low bone density (osteoporosis) due to the low levels of estrogen experienced by these women at an earlier stage in life than normal women. Additionally, women with POF have a nearly 2-fold age-specific increase in mortality rate. Ultrasonography may reveal streak ovaries accompanied with uterine hypoplasia.[4]

The etiology of POF is a heterogeneous with genetic, autoimmune, and iatrogenic (surgery, chemotherapy, and radiotherapy) causes being the main contributors. The interaction of multiple genetic defects and environmental factors are considered to be involved in the pathogenesis of POF.[5]Mutations in different genes are associated with POF, some of these are X-linked like BMP15, FMR1 and FMR2.While others are autosomal genes including LHR,FSHR, INHA, FOXL2,FOXO3 etc.[6]

The pivotal role of inhibin (INH) hormone in the negative feedback regulation of Follicle Stimulating Hormone (FSH) makes it a potential candidate for POF. FSH is essentially required for the development of ovarian follicles.[6] Inhibin protein regulates the secretion of FSH by inhibiting the stimulatory actions of activins. Inhibins are heterodimers 18kDa α-subunit which is connected through a disulfide bond to one of the two 14kDa β-subunits (βA and βB), resulting in inhibin-A (INHA) or inhibin-B (INHB) proteins, respectively. The Inhibin gene located on chromosome 2q33-36 encodes the alpha subunit of the inhibin protein. DNA variants of the three inhibin (*INHα, INHβA & INHβB*) genes are associated with an increased risk for POF.

In INHA protein a G to A transitionat position 769 results in an amino acid substitution from alanine to threonine at position 257 (A257T). The amino acid alanine at codon 257 was found to be highly conserved among species depicting the importance of alanine at this position in the proper functioning of the protein.[7,8] This alteration in the amino acid sequence decreases the amount of bioactive inhibin which in turn increases the FSH levels. The rise in FSH leads to increased rate of follicular depletion thereby increasing the risk of developing POF.INH α 769G>A mutation is quite common worldwide and linked with premature ovaraian failure.[9]

Nosuch study regarding the role of inhibin gene mutations in POF patients has been conducted in Pakistan. Keeping this in view, the present study was designed to investigate the association between *INHα* 769G>A mutation and disease phenotype in women with POF from the local population. Due to the heterogeneity of the condition, it is important to identify genes that impact ovarian function so they can be linked to idiopathic POF. Also there is a need to understand the molecular basis of the disease, so that we might be able to diagnose the disease earlier.

METHODS

This case control study was conducted in the Department of Gynecology, Jinnah Hospital and The Children's Hospital and Institute of Child Health (CH & ICH) and Department of Human Genetics and Molecular Biology at University of Health Sciences, Lahore (UHS), Pakistan after getting approval from Institutional Review Board. A total of n = 50 patients diagnosed with POF and equal number of normal controls of the same reproductiveage were recruited from July 2015 – July 2016. Patients (n = 50) presented with primary amenorrhea, secondary amenorrhea, unexplained infertility and 46, XX karyotype were included in the study. While the patientswith the history of chemotherapy, radiotherapy, autoimmunity, metabolic disorders and X chromosomal abnormalities were excluded. The control group comprised of n = 50 females of the same age group with normal menstrual cycle and the females with a family history of premature ovarian failure were excluded.

Sample collection and DNA isolation: Demographic variables along with weight, height, body mass index (BMI) and laboratory levels of follicle stimulating hormone (FSH) and luteinizing hormone levels (LH) were recorded on a proforma for each individual. After taking writteninformed consent, 5 ml blood samples from each patient and control was drawn and collected in properly labeled EDTA vacutainer tubes for genetic analysis.

Genomic DNA was isolated using a Phenol chloroform protocol involving the treatment with Proteinase-K enzyme followed by extractions with the organic solvents.[10]

Genotyping for the G769> A variation: After DNA isolation, the specific region of 601 bp in the exon 2 of the inhibinα subunit gene was amplified using the INHα forward (GCTGCTGCGCTGTCCCCTCTGTA) and reverse (TATTTCCCAACTCTGCCTTTCCTC) primers as described by Shelling et al.[7] The primers were synthesized commercially by Eurofins mwgoperon. Genomic DNA (200ng) was amplified in a 25 µl volume reaction containing 10 × PCR buffer, 25mM MgCl2, 0.1mM of each dNTP, 20mM of each specific primer, and 5Uµl of *Taq* DNA polymerase. Cycling conditions for amplification were initial denaturation at 94°C for 4 min followed by 35 cycles of amplification (94°C denaturation for 30 sec, 58°C annealing for 30 sec, 72°C extension for 30 sec); thelast cycle of the final extension was for 7 min at 72°C. Specific amplification was confirmed by electrophoresis on 2% agarose gel. The genotyping for *INHα*769G>A variant was carried out by using fluorescence-based chain terminator (di-deoxy) sequencing method. All the sequencing data was analyzed using Chromas Lite software v2.01.

STATISTICAL ANALYSIS

Data entered and analyzed using the Statistical Package for Social Sciences (SPSS ®) version 19 for windows®. Demographics of the patients and controls were determined as mean ± SEM. The normality of the quantitative data was assessed by the Shapiro Wilk test. The independent sample T-test was applied for normally distributed data, whereas, Mann Whitney U test was used for skewed data (FSH and LH levels). The association of *INHα* 769G>A variant alleles with POF and clinical parameters wasinvestigated using the Chi-square test (with Yate's correction). The strength of a genetic association was measured by odds ratio and 95% confidence interval. For all statistical analyses, P-value less than 0.05 was considered to be significant.

RESULTS

Mean age was found to be 23.5 ± 1.06 for n = 50 patients with POF.Weight, height and BMI distribution among patients and normal controls are shown in (Table-1). For all POF patients, serum follicle stimulating hormone (FSH) levels were within the range of 4.8 – 98 IU/L (mean 34.94 IU/L). The levels of serum FSH in the controls were in the normal range (4.5 – 21.5 IU/L). The range of serum leutinizing hormone (LH) was (2 – 115.6 IU/L) and (2.0 – 9.0 IU/L) for POF patients and normal controls respectively.

Table-1: Baseline data in POF patients and controls.

Parameters	*POF Patients n = 50 Mean ± SEM*	*Controls n = 50 Mean ± SEM*
Age (years)	23.5 ± 1.06	24.1 ± 0.94
Weight (kg)	52.7 ± 1.82	52.5 ± 1.22
Height (cm)	153.0 ± 1.66	153.3 ± 1.40
BMI	22.5 ± 0.69	22.5 ± 0.47
FSH (IU/L)	34.9 ± 3.20	8.16 ± 0.46
LH (IU/L)	21.7 ± 2.36	6.12 ± 0.26

Statistical analysis revealed a non-significant difference of mean age, weight, height and BMIamong patients and controls.Mann Whitney U test revealed a significant (P = 0.000) difference of the FSH and LH levels in patients and controls.Out of n = 50 pateints with POF 58% presented with primary amenorrhea, and 42% with secondary amenorrhea.

Direct DNA sequencing of the amplified product by using the automated genetic analyzer showed only one patient heterozygous for the 769G>A variant. Interestingly, many women from the control group harbored the minor allele (A). Of these, five controls were heterozygous (G/A) and four were homozygous for the A allele. Fig.1 (A-C) shows the selected electropherograms of patients with POF and controls.

According to the dbSNP database, at least 20 SNPs are present in the 601bp fragment of the *INHα* gene that we amplified for the 769G/A variant. It was observed that all the analyzed samples were positive for the major alleles of these SNPs and none was positive for any minor allele.

The allele frequencies were estimated among cases and controls. In patients, the frequency of the G allele was 99% as compared to 87% in the

controls. Whereas, frequency of the allele A was 1% and 13% in patients and controls respectively. The statistical analysis revealed that the frequency of allele A in controls is significantly higher as compared to the patients (Table-2). Rare allele A might hold a protective role against development of premature ovarian failure.

The three genotypes (GG, GA and AA) of the 769G>A variant was interpreted and compared among patients and controls. Chi-square test revelaed significant (P-value = 0.002) association of genotypes among two groups (Table-2).

DISCUSSION

The present study was designed to determine the association of the *INHα*769G>A variation with POF in patients from the local population. In the current study most of the patients presented with primary or secondary amenorrhea with or without other manifestations of the disease like infertility, menstrual irregularity, and psychological disturbances. Primary amenorrhea is mostly prevalent in the early reproductive age groupin Indian females.[11] In contrast, a study conducted on Argentine population reported females presenting with primary amenorrhea at relatively later reproductive phase of life approximately at the age of 29 years.[12] Reasons for this variation need to be elucidated whether it is due to genetic or environmental differences.

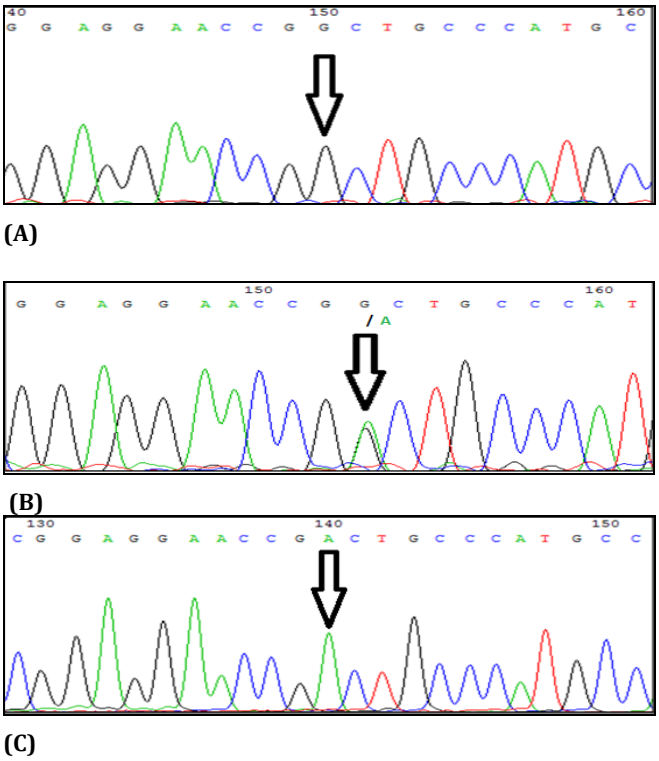

Fig.1: Sanger Sequencing Electropherogram showing the wild type sequence (A)with arrows indicating the site of G>A substitution in a heterozygous (B) and homozygous (C) 769 G>Avariant of the *INHα*gene in comparison with the corresponding nucleotide in the wild- type sequence.

Table 2: Distribution of allele frequency among POF patients and controls.

	Allele Frequency (%)				
Allele	*POF Patients*	*Controls*	*Odds Ratio (95% CI)*	*Chi-square with Yate's Correction*	*P value*
G	99 (99%)	87 (87%)	0.067		
A	1 (1%)	13 (13%)	(0.008-	9.29	0.002
Total	100 (100%)	100 (100%)	0.527)		

Further, in current study FSH levels in females wih POF were high as compared to the controls that were in the normal range. This is in contrast to that observed in an Indian study with a mean FSH of 5.66 IU/L.[13] This difference may be due to variation in the sample size. The patients with secondary amenorrhea in the present study had a mean FSH level of 28.4 IU/L which is higher than the normal value. This is following a previous study which also showed a raised mean FSH in Indian patients, however, the value of FSH was much higher (69.88 IU/L) than current study.[13] These findings

indicated the association of raised FSH levels with the disease phenotype and also supported the results obtained in an other study showing the involvement of raised FSH pathophysiology of POF.[14] Similarly, the LH levels were also high in POF patients in present study. The mean LH level was 22 IU/L in patients as compared to 6 IU/L in

controls. These results correspond to those observed in previous studies on POF patients by Dixit et al[11] and Sundblad et al.[12]

The present study showed increased frequency of allele A in controls as compared to the cases (1%). Another study conducted by Corre et al.[15] on Italian population reported this minor allele A more frequent in controls thus supporting the protective role of this rare allele A effect against loss of ovarian function. The findings of the current study were also found to be consistent with the work done by Sundblad et al.[14] on Argentine population that the 769G>A substitution was more frequent in their controls (8/149, 5%) as compared to the patients (1/59, 2%).However, another study conducted by Marozzi et al.[16] on Italian patients showed significant association (7/157, 4.5%, Fisher's exact test, P = 0.030) of this variant in patients with the ovarian failure while comparing to controls. So 769G>A mutation may be considered as a polymorphism with no clinical consequences. Furthermore, none of the patients was homozygous for the allele of A this variant as compared to four homozygote individuals from the control group. The study conducted in Syrian population also showed no significant association of allele A with increased risk of POF.[17] These results were in contrast to the work done by Shelling and co-workerin New Zealand.[7] They reported heterozygous mutation 769G>A more frequent in patients (7%) than controls (0.7%), ($p < 0.035$). Falahianet et al.[18] andZargar et al.[19] reported an association of the INHα 769G>A mutation with POF in patients from in Iranian and Kashmiri population respectively.

The frequency of major allele G was observed in 99% of patients in current study which is in accordance to the dbSNP database that showed major allele G for the 769G>A variant in 100% patients in Japanese and Chinese Han populations.[20] Another study in the Korean population reported the absence of the allele A and significantly lower frequency of allele G in patients.[22] In contrast, the results of the present study demonstrated the presence of the minor allele A in both patients and controls. These findings suggest a difference in the genetic constitution among different ethnic groups and the variant 769G>A as polymorphism and not a mutation.

CONCLUSION

A rare allele A of the 769G>A variantmight be associated with a decrease in risk for POF rather than increasing the risk of development of ovarian failure.There is genetic heterogeneity regarding the *INHA* gene in different populations and among the causes of premature ovarian insufficiency.

LIMITATIONS OF THE STUDY

Due to financial and time constraints, it was not possible to conduct this study on a large cohort of POF patients. Given the interesting results of the study, it is recommended that the study should be conducted on a large number of POF patients from different populations to define the exact role of *INHα* 769 G>A substitution in the pathogenesis of the disease.

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CONFLICT OF INTEREST

None to declare.

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Author's Contribution

SF: Acquisition and analysis of data, drafting of manuscript.

ZU: Analysis of data.

SM: Conception and design of study and revising it critically.

SK: Conception and design of study,revising the manuscript critically for intellectual content.

ALL AUTHORS: Approval of the final version of the manuscript to be published.