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Biochemical and Genetic Studies on Inhibin as a Hormonal Candidate in Prediction and Diagnosis of Premature Ovarian Failure in the Egyptian Women

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ABSTRACT

Background: Recent studies have implicated a role for inhibin alpha (*INHα*) gene abnormalities in the etiology of premature ovarian failure (POF).The present study aimed at demonstrating the possibility that -16C>T polymorphism of *INHα* gene may enhance susceptibility to this disease among Egyptian women undergoingt *in-vitro* fertilization(*IVF*) technique.

Methods: A total of 50 POF Egyptian women at age (31.5±7.3) and 50 control women at age (29.1 ± 6.8) were included in this study. Genotyping of *INHα*-16C>T gene was performed by restriction fragment length polymorphism. Levels of inhibin, activin, FSH and LH were also assessed.

Results: Serum levels of FSH and LH showed significant increase coupled by decrease in serum inhibin and inhibin/activin ratio, however, levels of activin were within normal values in POF women comparing to control ones. The frequencies of CC, CT and TT genotypes showed no significant changes in POF women compared to control group. Moreover, there were no significant differences in frequency of C and T alleles among the POF women in comparison to controls.

Conclusion: Obtained data indicated that -16C>T polymorphism of *INHα* gene can not imply a functional effect on the current decline of serum inhibin and hence the risk of developing POF in the studied Egyptian women. Further studies on POF women are needed to expand the present data.

Keyword: Premature ovarian failure (POF); Inhibin; Activin; *INHα* gene polymorphism.

INTRODUCTION

Premature ovarian failure (POF), or poor ovarian response is a syndrome characterized by amenorrhea for duration of 4-6 months or more, hypo-estrogenism and elevated serum FSH concentration (FSH ≥ 40 IU/l) before age of 40 years[1].Women with POF can experience multiple consequences of hypo–estrogenism including, osteoporosis, accelerated cardiovascular and neurocognitive disorders[2]. However, the major consequence of POF is lowered fertility, which in some cases, is due to the inability of the follicles to respond to hormonal stimulation, and in most cases due to the actual absence of follicles[3]. Women with POF are suffering from clinical symptoms like those observed with the onset of menopause, such as hot flushes, dyspareunia, vaginal dryness, vaginitis, insomnia, mood swings, heart palpitation and headache[4]. Depression may also occur when women recognized that fertility and the femininity are possibly to be lost [5].

POF may arise through different events including: 1) reduction of primordial follicle pool; 2) acceleration of follicular atresia; or 3) alteration of primordial follicles maturation/recruitment[6]. There may be a number of contributing factors for POF, including genetic abnormalities, pelvic surgery and chemotherapy or other medications [5, 7]. In most cases, it would be logical to consider reproductive hormones including FSH and LH as contributing factors dealing with oocyte atresia [1,8] . Evidence is provided also for the role of ovarian hormones; estradiol (E2), progesterone, anti-mullerian hormone (AMH) and activin in the diagnosis of menstrual irregularity and depletion of the primordial follicular pool[9].

In this line, recent studies have implicated inhibin as a hormonal candidate in the mechanistic determinants of POF disease. Inhibin is a hormonal product of granulosa cells [10,11]that serves as marker of ovarian function and/ or follicular development due to its role in negative feedback control of FSH[12]. Inhibin structure is closely related to the multi-functional transforming growth factor (TGF)-β superfamily[13]. Bioactive inhibin is a heterodimer glycoprotein consisting of two subunits (18 kDa-alpha) and (14 kDa- beta)

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linked by disulphide bonds to generate two forms of inhibins (A&B). Each form has a common alpha subunit (α-subunit) which is attached to either beta subunits (βA) or (βB) to form inhibin A (α-βA) and inhibin B (α-βB). Inhibin subunits are encoded by three separate genes: *INHα, INHβA* and *INHβB* which map to 2q33-q36, 2cen-q13 and 7p15-p14, respectively[12].

Researchers suggested that onset of POF can be a result of *INHα* genetic abnormalities. Inhibin α (*INHα*) that found on chromosome 2 (2q33-q36) , is the gene encodes the alpha subunits of inhibin A and inhibin B. *INHα* genetic variants have suggested to be responsible for less bioactive and decreased circulating inhibin levels, causing an increase in FSH levels, with increased follicle recruitment, elevated follicle depletion and increased susceptibility for developing POF[14].

Among genetic variants of *INHα* genes, the most extensively studied is -16C>T polymorphism. However, controversial results were obtained among different populations. Marozzi *et al*.[15] examined -16C>T *INHα* gene polymorphism and illustrated lowered frequency of T allele in the Italian POF patients compared to control populations. Other study by Sundblad *et al*.[16] showed no association between -16T allele and prevalence of POF among the Argentinean women, indicating that -16C>T variants in *INHα* gene are not equally distributed among different populations. More recently, two studies were performed in Korean women with contrasting results. Data from Yoon *et al*. [17]suggested no association between *INHα*-16C>T gene polymorphism and risk of POF, whereas evidence from Kim *et al*. [18]supported that this polymorphism is a contributing factor in this disease.

Accordingly, the present study was undertaken to evaluate whether *INHα*-16C>T gene polymorphism could be a predictive marker for POF in Egyptian females undergoing *IVF*. Also, to identify role of the other gonadal protein hormone; activin in predisposition to this disease.

MATERIALS AND METHODS

Subjects

A total of 100 women (50 patients with POF and 50 healthy control) are recruited from Gynecology and Obstetrics department, Mansoura University, Egypt, during the study period from March 2013 to April 2016. All studied women were of age between 21-42 yr and with no evidence of endocrinological disorders. Patients were diagnosed as having POF based on stopped menstruation for at least 6 months with elevated serum FSH level (FSH ≥ 40 IU/l) and poor response during gonadotropin ovarian stimulation when they undergo *IVF*. Subjects of the control group are normally menstruating women, without family history of POF or early menopause, but undergo *IVF* due to male factor infertility. The protocol was approved by Review Board of Faculty of Medicine and informed consents were taken from all studied subjects.

Blood sample (5ml) was taken by venipuncture from each woman at the third day of menstruation for hormonal assay. The blood was allowed to clot for 15 min. in room temperature then centrifuged at 7000 r.p.m for 10 min .Sera were separated and stored at -20^{0}C until assay. Another sample (3ml) of peripheral venous blood was collected in a tube including K_2EDTA and mixed well for DNA extraction.

Ovarian stimulation

In both groups, ovarian stimulation started by down-regulation in the luteal phase with a gonadotropin-releasing hormone agonist triptoeline (0.1 mg/day; Decapeptyle®,Ferring, Kiel, Germany), followed by administration of recombinant human FSH (Gonal-f®, Serono, Switzerland) with doses ranging from 75 to 225 IU/day. Follicular growth was monitored by serum E2 levels and transvaginal sonography. When at least 3 follicles ≥ 16mm in diameter and the leading follicle reached a mean diameter of 18 mm, human chorionic gonadotropin (hCG; Choriomon®, IBSA, Switzerland) was administered.

Hormonal assay

All samples were assayed in duplicate using a commercial ELISA kits. FSH and LH were measured by ELISA kit (FSH, LH; Calbiotech Inc., Austin, USA) according to manufacturer's instructions.

Serum inhibin level was estimated using total inhibin ELISA kit, purchased from Ansh Labs, 44s Medical Center Blud, Webster, TX 77598-447, USA, while activin was measured by using activin A ELISA kit (hActAA-ELISA) purchased from Scien Cell Research Laboratories, USA according to the manufacturer's instructions.

Genotyping of *INHα*-16C>T gene by restriction fragment length polymorphism

Extraction of genomic DNA was done from peripheral blood lymphocytes using QIAamp DNA blood mini-kit (Qiagen, Valencia, USA). Inhibin α (*INHα*) gene was amplified in a 50 µl volume reaction containing 5µl $Mgcl_2$, 1x PCR buffer (Amersham Pharmacia Biotech, Piscataway, Nj, USA), 2mmol of each dNTP, 100mmol of each gene specific primer and 2µl of Taq DNA polymerase (Promega, Madison, WI, USA). The primer sequences were: forward, 5'-GGA AGA CTG GAT GAG AAG G-3'; and reserve 5'- GCT TTT TCT CAA AGT CAT CC-3'. (240 bp product size). The cycling stages were; initial denaturizing at 94^{o}C for 1 min, followed by 30 cycles of denaturation for 30 sec at 95^{o}C, annealing for 30 sec at 55^{o}C, extension for 30 sec at 72^{o}C and a final extension for 5 min at 72^{o}C.

PCR-products were subjected to restriction digestion *SpeI* restriction enzyme (New England Biolabs, Ipswich, MA, USA) at 37^{0}C overnight. Electrophoresis of 5µl of each PCR product was done after amplification by using 1.5% agarose gel to ensure the presence of a single band of wanted size, then visualized via Light UV Trans illuminator (Model TUV-20, OWI Scientific, Inc. 800 242-5560, France) and photographed.

Statistical analysis

Analysis of the data was done using the computer program SPSS version 17.0. Obtained data were expressed as means ± SD, where differences were considered significant at $p<0.05$. Student's *t-test* was used to compare between mean values of the groups. The relative risk of T allele and the different genotypes combination were detected by calculating the odds ratio (OR) and 95% confidence interval.

RESULTS

Clinical characteristics and hormonal profile

The prevalence of clinical characteristics (age and weight) showed non-significant variations between the two studied groups, while the number of oocytes showed significant decrease for POF women compared with the control (Table 1). All women with POF exhibited significantly increased serum FSH and LH hormone levels and significantly decreased inhibin level and inhibin/activin ratio, while activin level showed non-significant changes compared to the control (Table 2).

Distribution of *INHα*-16C>T genotypes and allele frequencies

The distribution of *INHα*-16C>T genotypes and alleles among the POF women and control are presented in Table 3. When comparing studied subjects, the frequencies of CC, CT and TT genotypes showed no significant changes in POF women in comparison to controls (52.0 % vs. 70.0%, 42.0% vs. 24.0 % and 6.0% vs. 6.0%, $p>0.05$), respectively. Moreover, there was no significant variations in the frequency of C and T alleles among the POF women in comparison to control subjects (73.0% vs. 82.0% and 27.0% vs. 18.0% $p>0.05$).

95% confidence interval and odds ratio in *INHα*-16C>T polymorphism

Obtained results (Table 3) showed no significant changes in the genotype distribution of women with POF related to control group ($P=0.052$, OR=0.24 and 95% CI=0.17-1.02) in genotype CT and ($P=1.00$, OR=0.74 and 95%CI=0.14-3.98) in genotype TT. Also, T allele showed no significant changes in POF women compared to control ($P=0.12$, OR=0.6 and95%CI=0.3-1.16).

PCR amplification for polymorphic region of *INHα*-16C>T gene

As regarding *INHα*-16C>T gene polymorphism, CC homozygous genotype (wild type) for C allele was recognized by the presence of a single 240 bp product, while CT heterozygous genotype (mutant type) was presented with two bands; 240 bp and 120 (Figure 1). The TT homozygous genotype for T allele was recognized by the presence of a single 120 bp band.

Table1.Clinical characteristics in all subjects of the studied groups

Groups	Control (n=50)	POF Patients (n=50)
age	29.1 ± 6.8	31.5 ± 7.3
weight	84.54 ± 17.12	80.98 ± 14.82
No. of oocytes	9.64 ± 1.94	3.42 ± 1.18[a]

Data are presented as mean± SD. n: number of cases, a =significant at p<0.05 on comparing with group

Table2. Hormonal profile in all subjects of the studied groups

Groups	Control (n=50)	POF Patients (n=50)
FSH	6.58 ± 2.02	12.85 ± 1.59[a]
LH	6.77 ± 1.59	9.43 ±1.53[a]
Inhibin	77.29 ± 17.95	28.91± 9.59[a]
Activin	17.76 ± 2.73	17.04 ± 1.88
Inhibin/activin ratio	4.55 ± 0.96	1.62 ± 0.42[a]

Data are presented as mean± SD. n=number of cases. a =significant at $p<0.05$ on comparing with control group

Table3. Odds ratio and 95% confidence interval in *INHα*-16CT polymorphism in all subjects in the studied groups

Groups	Control (n=50)	POF patients(n=50)	OR (CI 95%)
Genotypes	**n (%)**	**n (%)**	
CC	**26(52)**	**35(70)**	**1.00**
CT	**21(42)**	**12(24)**	**0.24(0.17-1.02)**
TT	**3(6)**	**3(6)**	**0.74(0.14-3.98)**
CT+TT	**24(48)**	**15(30)**	**0.46(0.2-1.6)**
Alleles	**n (%)**	**n (%)**	**n (%)**
C	73(73)	82(82)	1.00
T	27(27)	18(18)	0.6(0.3-1.16)

n: number of cases, (%) = percentage of cases, CI = confidence interval, OR= Odd ratio

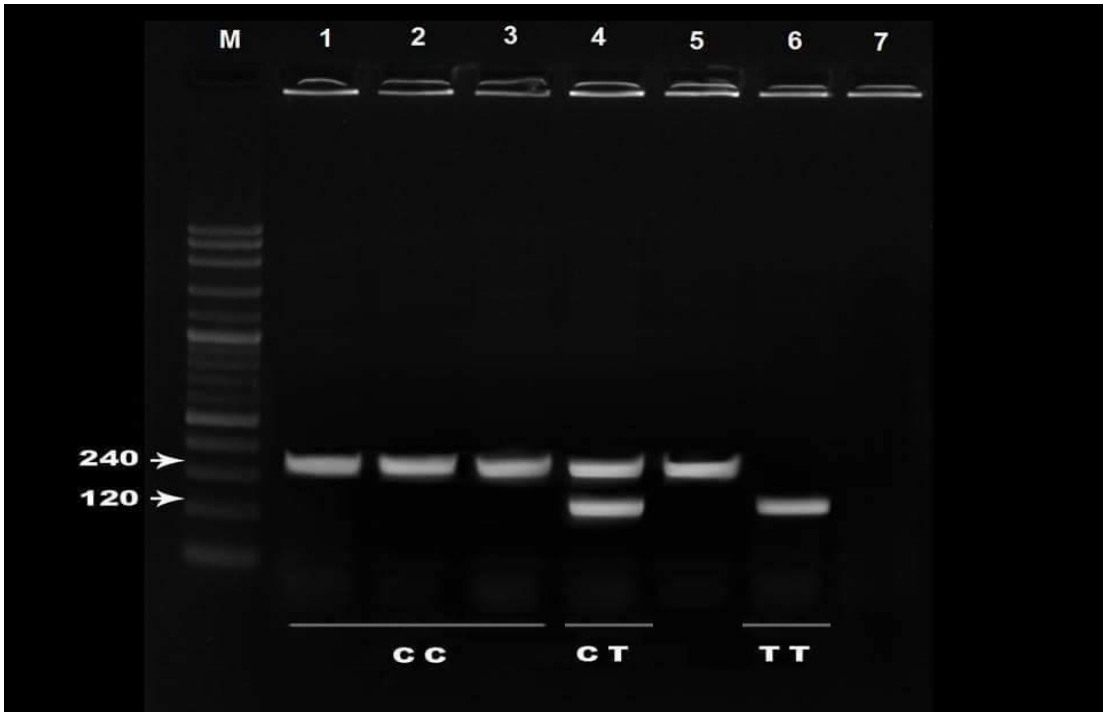

Figure1.Agarose gel electrophoresis stained with ethidium bromide, showing *INHα*-16C>T polymorphism. Lane M is 50 bp DNA ladders, lanes 1, 2, 3 and 5 represent the homozygous individuals for the C allele (CC genotype) and they were recognized by the presence of a single 240 bp product. Lane 4 shows the heterozygous individuals (CT genotype) and it was identified by the presence of both 120 bp and 240 bp products. Lane 6 shows the homozygous individuals (TT genotype) and it was identified by the presence of 120 bp.

DISCUSSION

Females diagnosed with POF are characterized by early loss of ovarian function, with failure to produce adequate follicles and increased risk of infertility. This brings out marked difficulty for proceeding *in-vitro* fertilization (*IVF*), where a large number of oocytes are desirable[19, 20].

In most cases, the main features of POF included, stopped menstruation for at least 6 months, with rise in serum level of FSH (≥ 40 IU/l) resulting in menopause pattern starting before age of 40 years [2,10]. Women with raised FSH level always exhibit lowered ovarian reserve [21] and reduced pregnancy rate with IVF technique, independent of age[22]. Loutradis *et al.*[23] also showed that women with unexplained infertility, but with higher serum FSH concentration are at risk of developing ovarian failure within short time.

Owing to the close relation between FSH and LH during the early and late follicular phases, both FSH and LH are known to act synergistically for maintaining the ovarian function. FSH plays an essential role through enhancing recruitment of the antral follicles during the early follicular phase. On the other side, LH is shown to selectively trigger development of dominant follicles supposed to ovulate, as well as to keep the smaller follicles from fully maturation in the mid and late follicular phases [24]. In the present study, tested females with POF showed significant increases in FSH and LH hormones compared to control group which in turn may be related to decreased number of oocytes and poor response during ovulation induction, confirming that both FSH and LH can be used as monitoring indicators for ovarian dysfunction with lesser probability for ovulation and pregnancy[13,21].

Inhibin and activin are gonadal hormones, structurally belonging to the transforming growth factor (TGF)-β superfamily. Both are protein hormones regulating synthesis and secretion of FSH in opposing manner [25]. Activin can directly stimulate FSH biosynthesis and secretion whereas; inhibin can negatively regulate these effects by blocking synthesis and secretion of FSH[25]. Lowered inhibin production is directly correlated to increased FSH output, with increased follicle recruitment and diminished follicular pool[26]. Evidences suggested that diminished levels of dimeric inhibin at the menopausal transition time, with increase in activin A, could be the main reason for high levels of FSH characteristic for late reproductive life. Results expanded also to include decreased inhibin / activin ratio during this period due to the deficit in production of *INH* α subunit,that leads to the preferential formation of activin homodimers[27]. Similar studies on hormonal profile of patients with POF have also implicated lowered inhibin and inhibin/activin ratio as a risk factor for incidence of POF[16].

The present study showed significantly lowered serum inhibin and inhibin/activin ratio in women with POF on comparing to control women, whereas level of activin was within normal range as in control. Comparable results were observed by Munz *et al.*[28] suggesting the association between lowered serum inhibin and the development of POF disease.

At the molecular level, several studies have indicated a relation between *INHα* gene polymorphisms and the decline in inhibin bioavailability, resulting in pre-mature depletion of ovarian function[1,12]. Previous studies described two polymorphisms in inhibin gene: 675C>T and -16C>T, the latter one is suggested to be linked with predisposition to POF[29] However, other studies found no evidence for an association between this polymorphism and POF disease [30] . In consistence, a study on Argentinean POF women by Sundblad *et al.*[16] indicated no significant variation between CC ,CT and TT genotypes in a group of women with POF comparing to control, suggesting that -16C>T variant in *INHα* gene can not influence POF disease. On contrast, *INHα*-16C>T polymorphism was significantly presented in the POF populations of New Zealand and Slovenia compared to control populations.

Subsequent studies were performed in Korean women with contrasting results. Yoon *et al.*[17] suggested no association of *INHα* gene polymorphism (-16C>T) with risk of POF, whereas evidence from Kim *et al.* [18] supported these polymorphisms with POF disease. Earlier studies have also addressed that *INHα* gene polymorphism could be responsible for developing POF in New Zealand women [15, 26]. Rah *et al.* [13] carried out another studies on Korean women, suggesting that *INHα* gene polymorphisms might be involved in the etiology of POF.

In the present study, the analysis of *INHα*-16 C>T gene polymorphism was demonstrated, where the frequencies of homozygous genotypes CC, heterozygote genotypes CT and homozygote genotypes TT of the *INHα*-16C>T polymorphism were 70% (35/50), 24% (12/50) and 6% (3/50) in the studied POF patients. However, among the fertile control women, the genotypes CC, CT and TT were 52.0% (26/50), 42.0% (21/50) and 6.0% (3/50) respectively, showing no significant variation in the genotypes distribution of women with POF compared to control group (*P*=0.052, OR=0.24 and 95%CI=0.17-1.02) in genotype CT and (*p*=1.00, OR=0.74 and 95%CI=014-3.98) in genotype TT.

Considering the allelic frequencies, the present study indicated that, C allele was presented in 82% of patients with POF and in 73% of the control group, whereas T allele was presented in 12% and 27% respectively, showing non-significant differences in the allelic distribution between the two studied groups (P=0.12, OR=0.6 and 95%CI=0.3-1.16).

Marozzi *et al*. [15] examined -16C>T polymorphism and showed that the frequency of the T allele was significantly decreased in POF patients compared to control group. As such, significant reduction in the frequency of -16T allele was detected in New Zealand and Slovenia POF patients, indicating that *INHα* -16C>T polymorphism is associated with the development of POF [30,31]. Nevertheless, other studies were unable to support these findings in the Argentinian women [16], and in a large sample of Italian and German populations [5].

The same results were obtained by other studies in which no association between *INHα* -16C>T gene variants and risk of POF disease was observed[32]. Conflicting results could be due to other polymorphisms that may have an effect on the genetic susceptibility to POF and are also in linkage disequilibrium with the examined *INHα* gene polymorphism[26]. Considering these results and others detected in this study, it can postulate that -16C>T *INHα* polymorphism may serve as a susceptibility factor for POF in several populations, however this polymorphism was not found to affect the disease in others, indicating that ethnic variations might influence the pattern of -16C>T *INHα* polymorphism in the POF patients.

CONCLUSION

Based on the current data, *INHα*-16C>T gene polymorphism seems not to account for incidence of POF among studied Egyptian women, suggesting that other gene variants may be involved in this respect. Additional studies with larger number of Egyptian POF patients are recommended for identifying responsible genetic variants, which may facilitate putative therapy for POF patients.

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