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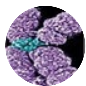

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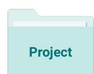

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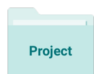

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Original Article

Inhibin α gene −16C → T and 769 G → A polymorphisms in Syrian women with idiopathic premature ovarian failure ☆

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1. Introduction

Premature ovarian insufficiency (POI) is a condition that affects about 1% of women under the age of 40 [1]. It is characterized either by early onset of amenorrhea (secondary amenorrhea) or by complete absence of menarche (primary amenorrhea). Women affected by POI usually show increased serum levels of FSH (>25 IU/L) and decreased anti-Müllerian hormone (<0.3 ng/mL). In most cases, the etiology of POI is unclear. Nevertheless, several studies showed that genetic aberrations are involved in the etiology and pathogenesis of POI [2]. It is estimated that 9% of POI are related to X chromosome aberrations [3,4]. Mutations in specific genes have also been associated with POI (e.g. *FMR1*, *FSH*, *FSHR*, *LH*, *LHR*, *BMP15* and inhibin A). With exception of the *FMR1* gene permutations [5], none of these mutations are associated with more than 2% of cases, suggesting that POI is a complex multifactorial disease probably involving many different loci [2].

Inhibin has been considered as a potential candidate for POI pathogenesis because it plays a key role in regulating FSH action by a negative feed-back mechanism [6]. As inhibin negatively regulates FSH, mutant inhibin with a decreased bioactivity could lead to increased FSH levels and consecutively to an accelerated rate of follicular depletion and to early menopause.

Inhibin exists in two heterodimeric forms in human tissues: inhibin A (α-βA) and inhibin B (α-βB), having the α subunit in common, which is encoded by the inhibin α gene (*INHA*) located on chromosome 2q33-qter [7]. A missense mutation in the inhibin α subunit gene (G769A, Ala257Thr) has been associated with POI in several populations, including New Zealand [8], India [9], Iran [10] and Egypt [11]. However, this association was not replicated in several other populations of different ethnicities, including Italy/Germany [12], Argentine [13] and Korea [14]. Moreover, controversial results has been obtained concerning whether promoter polymorphisms of the *INHA* gene (−16C → T and −124A → G) are associated with POI [12–16].

As there is no information about the association of *INHA* gene polymorphism with POI in the Syrian population, we investigated the prevalence of *INHA* gene variants (G769A and −16C → T) among a cohort of Syrian women affected with idiopathic POI, and compared allele frequencies among patients and controls.

2. Materials and methods

2.1. Subjects

90 Syrian women affected by premature ovarian insufficiency (consulting the Orient Hospital in Damascus in the period 2013–2015) were involved in this study. Inclusion criteria were: cessation of menses (secondary amenorrhea) for consecutive 6 months or more, basal FSH level over 20 IU/L, anti-Müllerian hormone level below 0.3 ng/mL, preantral follicle count less than 3. Exclusion criteria were: chromosomal aberrations, chirurgical interventions, exposure to radiations or radiotherapy, chemotherapy, FSH level under 20 IU/L, anti-Müllerian hormone level over 0.3 ng/mL. We

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also included women with signs of ovarian atresia or who did not experience menarche up to the age of 18 years (primary amenorrhea). 33 patients had primary amenorrhea, and 57 patients had secondary amenorrhea. Four patients (3 primary and 1 secondary amenorrhea) had family history. Patients (age 15–45 yrs, mean 31.1 ± 6.6) were clinically evaluated and their FSH levels were assayed by the Modular E170 platform electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany). Anti-Müllerian hormone was quantified by ELISA using a competitive inhibition enzyme immunoassay kit. Preantral follicle count was determined by vaginal ultrasound imaging (GE, VolsunE8). None of the included POI patients had chromosomal aberrations as determined by conventional GTG banding. On the other hand, 190 randomly selected Syrian women served as control group in order to determine allele distribution and frequencies among the general Syrian population. This project was approved by the review board of the Atomic Energy Commission of Syria (AECS), and informed consent was obtained from all participants.

2.2. Isolation of genomic DNA

Genomic DNA was isolated from 2 ml peripheral blood using a conventional phenol/chloroform extraction procedure. Briefly, red blood cells were lysed by the addition of 3 volumes of RBC lysis buffer (5 mM $MgCl_2$, 10 mM NaCl, 10 mM Tris-HCl pH7) and eliminated by 3 consecutive washes in the same buffer. Leukocyte pellets were resuspended in 1 ml water and lysed by the addition of 20 μl 10% SDS. After addition of 80 μl proteinase K (1 mg/ml), cell lysates were incubated at 54 °C for 30 min under shaking, and then extracted with an equal volume of Tris-saturated phenol (pH 8). After three extractions with chloroform, genomic DNA was precipitated by the addition of 2.2 ml of cold ethanol, washed with 75% ethanol, air dried and dissolved in 500 μl high quality water.

2.3. Duplex-PCR amplification of the promoter region and exon 2 of the INHA gene

The promoter region of the *INHA* gene (containing the −16C → T polymorphism site) and exon 2 (containing the 769G → A mutation site, missense Ala257Thr) were amplified by a duplex-PCR reaction using primers listed in Table 1. Each PCR reaction (25 μl final volume) contained following components (final concentrations): buffer (1×), $MgCl_2$ (3 mM), dNTPs (200 μM each), DMSO (5%), 2 pairs of primers (800 nM each, see Table 1), Taq polymerase (1 unit HotStart GoTaq, Promega) and gDNA (30–100 ng). PCR cycling conditions were as follows: initial denaturation 95 °C for 2.5 min, then 35 cycles of 94 °C for 15 s, 57 °C for 20 s and 72 °C for 30 s (final elongation cycle at 72 °C for 7 min). After electrophoresis in a 2% agarose gel containing ethidium bromide, the amplified products were visualized and photographed using a UV-transilluminator.

2.4. Detection of point mutations in the INHA gene by real time PCR

Single nucleotide changes in the *INHA* gene corresponding to the −16C → T polymorphism and the mutation Ala257Thr (nucleotide 769 G → A), were detected using 5′-FAM labeled Taqman MGB-probes (minor groove binding probes) designed to hybridize to *INHA*DNA segments encompassing each one of the tested polymorphism (Table 2). For each mutation tested, two Taqman MGB-probes were designed; one probe was fully identical with the sequence of the "wild type" allele, whereas the other probe was fully identical with the sequence of the mutant allele. Mutation detection was performed by real time PCR as previously described [17].

Table 1
Primers used in this study.

Primer	Sequence (5′ → 3′)	PCR product
INHA-Ex1-F INHA-Ex1-R	TCGCTTGAGGCGAAATCCTTCC AAGAACAGGGCCCTCACCTTGG	399 bp
INHA-Ex2-F INHA-Ex2-R	GACGCTCAACTCCCCTGATGTCCT TGTGCAGCCCACAACCACCA	204 bp

Table 2
3′-Minor groove binder-DNA probes (MGB-probes) used in this study.

Probe name	Sequence (5′ → 3′)
−16C	FAM-CCACTAG**C**AGGGCCA-MGB
−16T	FAM-CCACTAG**T**AGGGCCA-MGB
769 G-wild type	FAM-GAACCG**G**CTGCCCA-MGB
769 G → A-Mut	FAM-GAACCG**A**CTGCCCA-MGB

2.5. Statistical analysis

We used the Chi-Square Test (SPSS software, version 16.0.0) in order to evaluate the association of *INHA* gene polymorphisms with increased risk of POI. P-values were considered significant if they were below 0.05.

3. Results

In this study, two *INHA* gene polymorphisms (−16C → T and 769 G → A) have been investigated among 90 idiopathic Syrian POI patients (33 primary, 57 secondary), and among a random group of 190 women representative of the general Syrian population. In the POI group, the mean age was 31.1 ± 6.6 (SD) years, and the mean values of FSH and anti-Müllerian hormone were 45.4 ± 22.06 IU/L and 0.155 ± 0.028 ng/mL, respectively. We successfully determined genotypes of both polymorphisms in all subjects, and the genotype distributions in all groups were compatible with Hardy–Weinberg equilibrium.

Table 3 shows genotyping results of the −16C → T promoter polymorphism in our Syrian cohort. Genotype distribution among total POI patients (CC = 72.2%, CT = 22.2%, TT = 5.6%) did not differ significantly from that found in the control group (CC = 66.3%, CT = 26.8%, TT = 6.9%; *P*-value = 0.612). Similarly, no significant differences in genotype distribution were found for the primary-POI subgroup (CC = 60.6%, CT = 27.3%, TT = 12.1%; *P*-value = 0.557) or the secondary- POI subgroup (CC = 78.9%, CT = 19.3%, TT = 1.8%; *P*-value = 0.135) when compared to controls. On the other hand, comparing genotype distribution between primary and secondary POI subgroups did not show significant difference, though the *P*-value (0.060) was close to significance. Comparing allele frequencies between the two POI subgroups revealed a significant difference (*P*-value = 0.012). This implicates that the −16T-allele is significantly less frequent in the secondary POI subgroup (11.4%) than in the primary POI subgroup (25.7%). Although the T-allele was not significantly less frequent in the secondary POI subgroup compared to controls (20.3%), the *P*-value was close to significance (0.077). Taken together, our results suggest that the T-allele is less frequent among secondary POI patients and would have a possible slight protective effect against developing secondary POI in the Syrian population.

Table 4 shows genotyping results of the 769 G → A polymorphism in our Syrian cohort. Genotype distribution among total POI patients (GG = 95.6%, GA = 4.4%, AA = 0%) did not differ significantly from that found in the control group (GG = 91.1%, GA = 8.9%, AA = 0%; *P*-value = 0.136). Similarly, genotype distribution among

Table 3
The distribution of the *INHA* gene −16C > T polymorphism in POI patients and controls.

Group	n	Genotypes				Allele frequency		
		CC (%)	CT (%)	TT (%)	*P*-value*	C	T	*P*-value
Total POI	**90**	**65 (72.2)**	**20 (22.2)**	**5 (5.6)**	**0.612**	**0.833**	**0.167**	**0.186**
Primary	33	20 (60.6)	9 (27.3)	4 (12.1)	0.557	0.743	0.257	0.197
Secondary	57	45 (78.9)	11 (19.3)	1 (1.8)	0.135	0.886	0.114	0.077
Controls	190	126 (66.3)	51 (26.8)	13 (6.9)	0.612	0.797	0.203	

* Evaluated by χ^2 test in comparison with the controls.

Table 4
The distribution of the *INHA* gene G769A (Ala257Thr) polymorphism in POI patients and controls.

Group	n	Genotypes				Allele frequency		
		GG (%)	GA (%)	AA (%)	*P*-value*	G	A	*P*-value
Total POI	**90**	**86 (95.6)**	**4 (4.4)**	**0 (0)**	**0.136**	**0.978**	**0.022**	**0.141**
Primary	33	32 (97.0)	1 (3.0)	0 (0)	0.219	0.985	0.015	0.225
Secondary	57	54 (94.7)	3 (5.3)	0 (0)	0.278	0.974	0.026	0.284
Total controls	**190**	173 (91.1)	17 (8.9)	0 (0)		0.955	0.045	

* Evaluated by χ^2 test in comparison with the controls.

primary and secondary POI subgroups did not differ significantly from that found in the control group (*P*-values = 0.225 and 0.284, respectively). No significant differences in allele frequencies were found between the different groups.

Taken together, our results suggest that the −16C → T promoter polymorphism and the A-allele of the 769 G → A polymorphism of the INHA gene do not contribute significantly to increased risk of developing POI in the Syrian population (P-value = 0.195, OR = 0.735, CI 95% = 0.46–1.17, and P-value = 0.199, OR = 0.485, CI 95% = 0.16–1.46, respectively).

4. Discussion

There was no obvious correlation between the −16C → T and 769G → A *INHA* gene polymorphisms and idiopathic POI in our Syrian cohort. Regarding the −16C → T promoter polymorphism, our findings agree more with previous studies carried out in different countries as India [9], Argentina [13], Italy [12] and Korea [14], which showed no significant correlation between risk of POI development and the −16C → T *INHA* promoter variant. To our knowledge, only one study reported a correlation between −16C → T polymorphism and POI in a population from New Zealand [16], with a significantly decreased frequency of the −16T allele among POI patients, suggesting a protective role of the T allele against POI. Although we observed a decrease (to about a half) of the −16T allele frequency in the Syrian secondary POI subgroup compared to controls (11.4% vs. 20.3%, respectively), this difference did not reach statistical significance (*P*-value = 0.077). Nevertheless, our results could be explained if we assume a slight protective effect of the −16T allele against developing secondary POI in the Syrian population. Women carrying one or two copies of the −16T allele would be less prone to develop secondary POI. On the other hand, the −16T allele would not confer any protection against primary POI, because this severe condition is presumably caused by "severe" genetic aberrations causing a big damage that cannot be reduced by the −16T allele. Taken together, our results suggest that the T-allele is less frequent among secondary POI patients and would have a possible slight protective effect against developing secondary POI in the Syrian population.

Concerning the 769 G → A polymorphism, some previous studies in different countries, such as New Zealand [18], India [9], Egypt [11] and Iran [10], suggested that the A-allele might be associated with a greater risk of developing POI, while other studies on different populations did not reveal such association, as in Italy [12], Argentina [13], and Korea [14]. These discrepancies between studies could be explained by ethnic variations that might influence the contribution of *INHA* gene polymorphisms in POI patients. Interestingly, our data disagree with the two studies done on Middle Eastern POI cohorts (Egypt [11] and Iran [10]), which failed to detect the A-allele in control groups (0/20 and 0/24, respectively). As Syria is a Middle Eastern country, we had expected to find similar results as found in the Egyptian and Iranian populations. To our surprise, we found the A-allele in similar frequencies in the Syrian POI cohort and controls (2.2% and 4.5%, respectively). This discrepancy could be explained by the small number of participants in the Egyptian and Iranian studies (20/20 and 24/24 POI /controls, respectively), which makes their conclusions about a positive link between the A-allele and POI questionable.

In summary, the 769 G → A polymorphisms in the *INHA* gene failed to show any correlation with idiopathic POI in Syrian women, suggesting that this variants is unlikely to have clinically relevant consequences and might be not associated with POI in the Syrian population. On the other hand, the −16C → T polymorphism appears to contribute little (if any) to secondary POI, with the T-allele having a possible slight protective effect in the Syrian population. Further studies including larger number of patients and controls from diverse ethnicities are necessary to clarify the role of *INHA* gene polymorphisms in the etiology of POI.

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Conflict of interest

The authors declared that there is no conflict of interest.

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