Iraqi Journal of Biotechnology, December 2019, Vol. 18, No. 3, 26-32

Allelic estrogen receptor beta (*ESR2*) gene association with Polycystic Ovary Syndrome in unmarried Iraqi female of Baghdad governorate

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Received: July 22, 2019 / **Accepted:** October 20, 2019 / **Published:** December 31, 2019

Abstract: This study was carried out in the laboratories of Institute of Genetic Engineering and Biotechnology for postgraduate Studies – University of Baghdad from 1 Novmber 2018 until May 2019, and the sample collected from several laboratories in the city of Baghdad. The total numbers of samples are100 were collected from unmarried Iraqi female aged between (20-35) years old include (50) patients were diagnosed with polycystic ovary syndrome and (50) apparently healthy subject (control).The DNA was extracted and the electrical transfer was carried out for both groups and the *ESR2* **SNP (rs4986938)** gene were detected by qRT-PCR. The result showed there was significant difference between control and patients in genotypes of wild allele (GG) (64.0 *vs*. 48.0%, respectively; odds ratio = 0.52 ; p-value = 0.0472), and that *A* allele frequency was significantly increased in patients compared to control (p-value = 0.038) and the wild allele *G* showed a significant decreased frequency in patients (p-value = 0.038). The findings indicate that the GA genotype of rs4986938 SNP was efficient in raising the serum amount of LH in PCOS patients relative to GG or AA genotype ($P<0.05$) but the distinction between GA and AA genotypes was substantial. While the AA genotype of rs4986938 SNP was effective in increasing the serum level of FSH in PCOS patients compared GG or GA genotype ($P<0.05$) and both differences were highly significant. The GA genotype of rs4986938 SNP was effective in increasing the serum level of T3 in PCOS patients compared GG or AA genotype ($P<0.05$), but the difference was significant between GA and AA genotypes. This study was concluded that the mutant allele *A* could be associated with an increased risk to develop PCOS (Odds ratio= 1.88), while the wild allele *G* might have a protective effect.

Keywords: *ESR2*gene, Genotype , LH, qRT-PCR, PCOS.

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Introduction:

Polycystic Ovary Syndrome (PCOS) is the most common heterogenous complex endocrine disorder. It is affecting approximately 15 % of the women in their reproductive age. The exact cause of the PCOS is unknown, but genetic factors, environmental and endocrine factors plays a important role in the etiology of PCOS (1, 2).

Lately the number of genes involved in susceptibility to polycystic ovary syndrome increased dramatically, each with individually effect which interacts with one another. The candidate genes are FSHR, ESR1 and *ESR2* (3, 4).

In polycystic ovary syndrome dominant follicles fail to develop consistently. During the anovulatory cycles, there is a failure to up-regulate the expression of the aromatase enzyme in GC, and the estradiol concentration in the follicular microenvironment fails to increase adequately. Estrogen extends the action of (FSH) on granolosa cells by promoting their proliferation and increasing their expression of FSH receptor. These observations raise the question of

whether abnormal Estrogen Receptor in polycystic ovaries affect (5,6).

The ER1 gene is highly polymorphic, with more than 2200 SNPs, while around 720 SNPs in ER2 have been identified (7).

The etiology of PCOS is yet to be elucidated but a number of studies have suggested that genetic factors play important role in its etiology and pathogenesis, and the ERβ gene may play important role in development of polycystic ovary syndrome(8).

Kosova and Urbanek (9) discuss the present status of PCOS genetic assessment, including the outcomes of various association research with candidate genes engaged in TGF-β and insulin signaling, type 2 diabetes mellitus and susceptibility to obesity. The aim of this study is to investigate the effect of Estrogen hormone in Iraqi female with PCOS and to evaluate whether there is a difference in the *ESR2* gene +1730 G/A polymorphism between patients with PCOS and control.

Materials and Methods:

This study was conducted during the period from 1 Novmber 2018 until the first of May 2019. The total numbers of samples are 100 were collected from unmarried Iraqi female aged between (20-35) years old from several laboratories in the city of Baghdad include (50) patients were diagnosed with polycystic ovary syndrome and (50) apparently healthy subject (control). Blood samples (5 ml) have been collected from each women of both PCOS and healthy control. Two milliliters from blood were collected in tubes containing anticoagulant EDTA and were stored at 4^{o}C for genomic DNA extraction for genotyping.

1. DNA extraction WizPrep Blood gDNA Mini kit:

a) A volume of 200 µl of blood, 20 µl of proteinase K and 200 µl GB buffer was added into 1.5 ml microcentrifuge tube and Incubate at 56°C for 10 minutes.

b) A volume of 200 µl of 100% ethanol was added and centrifuge for 1 min. at 13,000 rpm.

c) A volume of 500 µl of W1 buffer and W2 Buffer was added respectively and centrifuge for 1 min. at 13,000 rpm.

d) A volume of 100 µl of elution buffer was added and centrifuge for 1 min. at 13,000 rpm.
The DNA was stored at -20°C.

2. Gel electrophoreses

a) Agarose 1% and the rest was prepared.

b) A volume of 5 µl of the DNA ladder (100bp) was loaded in single well.

c) The electric current was allowed at 75V/cm for 90 min, UV transilliuminator was used for observation of DNA bundles.

3. Real Time PCR (qRT–PCR)

The *ESR2* **SNP (rs4986938)** gene was detected by qRT-PCR. To confirm the target gene, quantitative real time qRT-PCR Taqman assay was used. Primers sequences for *ESR2* gene was prepared according to synthesized by Alpha DNA Ltd (Canada) and stored lyophilized at (-23°C). TaqMan fluorescent oligonucleotide probes were prepared according to Alpha DNA Ltd (Canada) matched with the NCBI, synthesized by Alpha DNA Ltd (Canada) and stored lyophilized at (-23°C). Every reaction was done in a duplicate and included a non-template

control (NTC), non-amplification control (NAC) and non-primer control (NPC) as negative controls.

4. Primer and Probe sequence matching:

TaqMan fluorescent oligonucleotide probes and primers sequences synthesized by Alpha DNA Ltd (Canada) and stored lyophilized at (-23°C). The sequences of each of the probes and primers used in the allelic discrimination experiments are listed below, table (1) they included *ESR2* gene SNP (G to A).

Table (1): Primers and their sequences.

Primers	Sequence (5 ' 3 'direction)
Forward	CCAGAACCCACAGTCTCAGT
Reverse	GCAGAAAGATGAAGCCCAGG
VIC - probe	CCACAGAGGTCACAGGCTGA
FAM - probe	GCCCACAGAGGTCACAAGCTG

5. Real-time PCR run

Real time PCR was interacted with the use of Wizpure TaqMan qPCR Master Mix (Probe) kit, Korea.

Components of Real time PCR was performed in a 25 µl reaction volume includes: 12.5 µl TaqMan master mix, 0.5 µl Forward Primer (10 µM), 0.5 µl Reverse Primer (10µM), 0.5 µl Probe FAM, 0.5 µl Probe VIC, 3 µl Template DNA, 7.5 PCR grade water up to 20 µl.

All reaction had enzyme activation step of 5 min at 95ºC hold followed by 5 cycle of denaturation at 95ºC for 15 sec, annealing 62ºC for 30 sec and extension 72ºC for 20 sec, followed by 40 cycle of denaturation at 95ºC for 15 sec, annealing 62ºC for 30 sec and extension 72ºC for 20 sec.

Result and Discussion:

Figure (1) and table (2) the *ESR2* rs4986938 SNP was observed to have three genotypes (GG, GA and AA) that were correspondent to two alleles, which were *G* and *A*.

The statistical analysis showed there was significant difference between control and patients in the genotypes of wild allele (GG) (64.0 *vs*. 48.0%, respectively; odds ratio = 0.52; p-value = 0.0472).

The homozygous genotype of mutant allele (AA) showed an increased frequency in PCOS patients compared to control (12.0 *vs*. 4.0%). The odds ratio of such association was 3.27, but the difference was no significant (p-value = 0.0735).

However, when the comparison was made at the allele level, *A* allele frequency was significantly increased in patients compared to control (0.32 *vs.* 0.20; Odds ratio = 1.88; p-value = 0.038). In contrast, the wild allele (G) showed a significant decreased frequency in patients (0.68 *vs.* 0.80; p-value = 0.038). These findings suggested that the mutant allele (A) was associated with an increased risk to develop PCOS, while the wild allele (*G*) might have a protective effect.

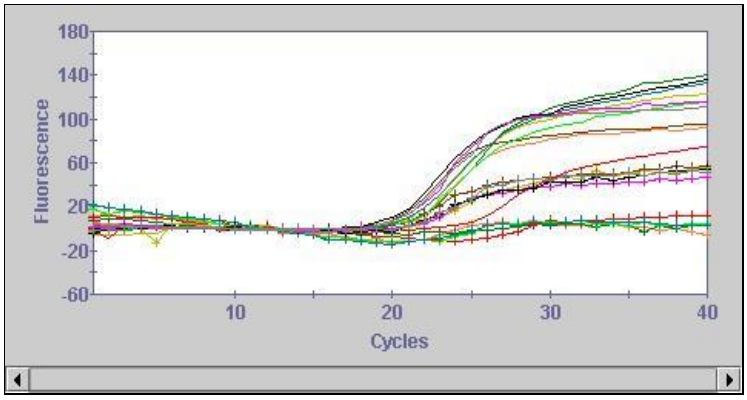

Figure (1): *ESR2* amplification plots by qPCR sample included all study groups. The photograph was taken directly from cephoid qPCR machine.

Table (2): Comparison of the Genotype and Allele Frequencies detected of *ESR2* gene polymorphism rs4986938 in patients and control

***ESR2* rs4986938**	**Control No (%)**	**Patient No (%)**	**P value**	**Odd ratio (95% CI)**
GG	32 (64.00%)	24 (48.00%)	0.0472 *	0.52 (0.24 - 1.15)
GA	16 (32.00%)	20 (40.00%)	0.0735 NS	1.42 (0.63 - 2.83)
AA	2 (4.00%)	6 (12.00%)	0.0735 NS	3.27 (0.64 - 2.06)
Total	50 (100%)	50 (100%)	---	---
Allele	**Frequency**			
G	0.80	0.68	0.038	0.53 (0.28-1.01)
A	0.20	0.32	0.038	1.88 (0.99 - 3.58)

*** (P<0.05), NS: Non- significant.**

In this study, we have selected important gene (*ESR2*) that is required for the developmental follicle, oocyte maturation, and regulation of steroidogenesis in the ovaries. Several studies have tried to correlate the frequency distribution of polymorphism in patients with PCOS.

Sundarrajan *et al.* (10) found that investigated the roles of the *ESR2* gene +1730 G/A polymorphism in patients with ovulatory dysfunctions and reported that these polymorphisms were significantly associated with ovulatory dysfunctions, especially in subjects with unknown causes. However, they found no differences between patients with PCOS and controls in terms of the *β* 1730 G/A polymorphism. The study was performed on a small scale (PCOS subjects, n = 30).

The present study provides data showing that the ER-*β* gene +1730 G/A polymorphism may be associated with PCOS, but the mechanism underlying this association remains to be elucidated. In addition, because the +1730 G/A polymorphism does not result in an amino acid change, the observed effect on PCOS may be due to a linkage disequilibrium with other functional ER- *β* gene variant(s) that were not assessed in this study.

Kim *et al.* (11) found that the genotype distribution of the ER- *β* gene +1730 G/A polymorphism in the PCOS group was significantly different from that of controls, and the ER- *β* gene +1730 G/A polymorphism may be

associated with pathophysiologic aberrancies involved in PCOS.

Khafagi *et al.* (7) found that the polymorphism of *ESR2* (rs4986938) and frequency of AG genotype of *ESR2* shows a slight different between PCOS and control group.

Al Hayawi and Abbas, (12) found that *T* and *A* variants of *ESR2* were not associated with ovarian disorder patients in Saladin women, there was no relationship between the ovarian disorder and gene of *ESR2* polymorphism at codon 307. There was a significant difference in estrogen, testosterone, progesterone, and HDL levels with ovarian disorders patients conveying different genotypes of *ESR2* polymorphism.

Estrogen signaling is mediated by estrogen receptors, which are ligand-activated transcription factors composed of several domains important for hormone binding, DNA binding and activation of transcription(13). In folliculogenesis, the proliferative actions of estrogens are mediated by ER1 (predominantly expressed in the theca layer), while the differentiation and antiproliferative effects required for reaching the antral stage require ER2 (expressed in granulosa cells of growing follicles at all developmental stages (4). Therefore, estrogen receptors (ESRs) are important candidates in PCOS patient, since direct effects of estrogens on follicle growth, maturation and oocyte release are well established. In addition to folliculogenesis, estrogens play an important role in endometrial preparation for implantation (6).

Genotype and hormone

The GA genotype of rs4986938 SNP was effective in increasing the serum level of LH in PCOS patients (24.50 ± 2.99) compared GG or AA genotype (16.12 ± 2.02 and 14.20 ± 2.78, respectively), but the difference was significant between GA and AA genotypes (Table 3).

The AA genotype of rs4986938 SNP was effective in increasing the serum level of FSH in PCOS patients (23.20 ± 16.21) compared GG or GA genotype (8.36 ± 0.55 and 8.95 ± 0.76, respectively), and both differences were highly significant (Table 4).

The GA genotype of rs4986938 SNP was effective in increasing the serum level of T3 in PCOS patients (1.86 ± 0.04) compared GG or AA genotype (1.74 ± 0.05 and1.60 ± 0.11, respectively), but the difference was significant between GA and AA genotypes (Table 6).

However, there was no significant impact of rs4986938 SNP genotypes on serum level of the hormones E2, T4 and TSH (Tables 5, 7 and 8, respectively).

Table (3): Relationship between genotype of gene and level of LH hormone in patients and control.

Genotype of SNP (rs4986938)	Mean ± SE	
	Patients	Control
GG	16.12 ± 2.02 ab	6.71 ± 0.49 a
GA	24.50 ± 2.99 a	5.50 ± 0.33 a
AA	14.20 ± 2.78 b	5.67 ± 0.33 a
LSD value	10.011 *	2.513 NS
P-value	0.034	0.230

*** (P<0.05), NS: Non-Significant.**
Means having with the different letters in same column differed significantly

Table (4): Relationship between genotype of gene and level of FSH hormone in patients and control.

Genotype of SNP (rs4986938)	Mean ± SE	
	Patients	Control
GG	8.36 ± 0.55 b	8.00 ± 0.47 a
GA	8.95 ± 0.76 b	6.68 ± 0.46 a
AA	23.20 ± 16.21 a	8.33 ± 0.33 a
LSD value	9.713 *	2.547 NS
P-value	0.025	0.181

*** (P<0.05), NS: Non-Significant.**
Means having with the different letters in same column differed significantly.

Table (5): Relationship between genotype of gene and level of E2 hormone in patients and control.

Genotype of SNP (rs4986938)	Mean ± SE	
	Patients	Control
GG	241.84 ± 20.78 a	143.84 ± 19.85 a
GA	302.15 ± 28.43 a	159.37 ± 20.89 a
AA	255.60 ± 45.85 a	101.67 ± 9.83 a
LSD value	100.65 NS	107.67 NS
P-value	0.214	0.644

NS: Non-Significant.
Means having with the different letters in same column differed significantly

Table (6): Relationship between genotype of gene and level of T3 hormone in patients and control.

Genotype of SNP (rs4986938)	Mean ± SE	
	Patients	Control
GG	1.74 ± 0.05 ab	1.77 ± 0.06 a
GA	1.86 ± 0.04 a	1.60 ± 0.078 a
AA	1.60 ± 0.11 b	1.67 ± 0.12 a
LSD value	0.232 *	0.361 NS
P-value	0.049	0.250

*** (P<0.05), NS: Non-Significant.**
Means having with the different letters in same column differed significantly.

Table (7): Relationship between genotype of gene and level of T4 hormone in patients and control .

Genotype of SNP (rs4986938)	Mean ± SE	
	Patients	Control
GG	103.84 ± 1.54 a	109.19 ± 1.71 a
GA	100.65 ± 2.07 a	107.00 ± 2.57 a
AA	107.40 ± 3.93 a	116.33 ± 6.64 a
LSD value	7.493 NS	10.622 NS
P-value	0.219	0.323

NS: Non-Significant.
Means having with the different letters in same column differed significantly.

Table (8): Relationship between genotype of gene and level of TSH hormone in patients and control.

Genotype of SNP (rs4986938)	Mean ± SE	
	Patients	Control
GG	2.26 ± 0.15 a	2.02 ± 0.18 a
GA	2.13 ± 0.08 a	2.21 ± 0.33 a
AA	2.00 ± 0.20 a	1.57 ± 0.38 a
LSD value	0.546 NS	1.194 NS
P-value	0.623	0.636

NS: Non-Significant.
Means having with the different letters in same column differed significantly.

According to the findings, this study concluded: The present study suggests the association of the *ESR2* gene+1730 G/A polymorphism with presence of PCOS, and the mutant allele (A) was associated with an increased risk to develop PCOS, while the wild allele (*G*) might have a protective effect.

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