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The *FSHR* Polymorphisms Association with Polycystic Ovary Syndrome in Women of Erbil, Kurdistan in North of Iraq

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Abstract

Polycystic ovary syndrome (PCOS) is an endocrine disorder in women during fertilization age that reflects changing clinical symptoms. The genetic concept of PCOS is unclear and no significant genetic association with PCOS has been established. The level of Follicle stimulating hormone FSH is encoded by FSH receptor (*FSHR*) and abnormal *FSHR* affects follicle cogenesis and ovary and consist of 9 introns, 10 exons, and the region of chromosome promoter at 2p21. Sample of 93PCOS patients and 52 controls were collected from Province of Erbil in north of Iraq. Genomic DNA was extracted from the blood and genotype dissected was improved for the two population of study using PCR-RFLP with the restriction enzyme *Eam1105I*. The genotype distributions and allele frequency of *Ala307Thr* polymorphisms of *FSHR* were not statistically various between the controls and the PCOS patients. Significant elevation of body mass index with all genotype of PCOS was found when compared with controls. There were statistical differences in the BMI and most of the serum hormone and lipid profile parameters including LH, total testosterone, fasting glucose, Cholesterol, HDL and LDL, there were significant various in FSH and LH levels of hormones and HDL, LDL and VLDL with PCOS group conveying different genotypes of *Ala307Thr* polymorphisms. The variant of *Ala307Thr* was not associated with PCOS in Kurdistan women; there was no relationship between the POCS and gene of *FSHR* polymorphism at codons 307. There was a significant difference in FSH and LH levels with PCOS patients conveying different genotypes of *Ala307Thr* polymorphism.

Keywords: PCOS, *FSHR*, rs6165polymorphism, *Ala307Thr*, PCR-RFLP

1. Introduction

Polycystic ovary syndrome (PCOS) is the most common defects in endocrine system in women of fertile age. Its prevalence may reach up to 18% of women of reproductive age. The syndrome is associated with irregular menstrual cycles, indications of insulin resistance and hyper-androgenism. Women with PCOS are at increased symptoms for developing disorders of reproductive, cardiovascular and metabolic, involving infertility, hypertension, diabetes mellitus type II, insulin resistance and atherosclerosis. Moreover, it may affect daily physical activities. Management of PCOS aims to lower body weight and insulin levels, restore fertility, treat hirsutism or acne, restore regular menstruation and prevent complications[1].

FSH is one of the main hormones in women of reproductive age, necessary for the development of gonads and maturation at puberty and gamete production during the fertility period of life [2,3]. The pituitary gland produces and excretes gonadotropin as an exceedingly heterogeneous glycoprotein[4]. FSH is binding with special receptors and is found mainly in the gonads. The *FSHR* attributed to the G protein-coupled receptors family, complex transmembrane proteins Features by 7 hydrophobic helices inclusion in the plasma lemma and by intracellular and extracellular domains of changeable measurements depending on the ligand type [5].

The gene for the *FSHR* is found on chromosome 2p21 in humans consists of 10 exons, 9 introns [6]. The gene sequence of the *FSHR* consists of about 2,080nucleotides[7]. The FSH receptor gene contains a few SNP that may influence its activity. The quantity of SNPs differs with the species, in mice it has been discovered 25 SNPs, while in people it has been accounted for more than 1.000 SNPs.[8,9]. Polymorphism have been related with ovarian function and deferred adolescence in women[10]. SNP were recognized in the FSH receptor gene[11,12].

FSH receptor gene includes two great SNPs in exon 10, which are in linkage disequilibrium and variation two amino acids at *Ala307Thr* and *Ser680Asn* positions. *Ala307Thr*, arranged at the extracellular domain of *FSHR*, the site responsible for high liking hormone official, has been accounted for to influence hormone trafficking, signal transduction, and subsequent FSH efficacy[13,14].The importance of *FSHR* in the signaling transition of FSH made *FSHR* gene one of the important candidate genes for PCOS[15]. *FSHR* Mutations can prompt capture of follicle advancement at a few periods of growth[16,17]. There are various hereditary variations in the *FSHR* that affect the phenotype; these impacts incorporate variable development of secondary sex qualities, serum levels of FSH hypoplastic ovary and primary amenorrhea[18].

The present study aim was to evaluate the association of *Ala307Thr* (rs6165) polymorphism of *FSHR* gene with PCOS and with clinical features of PCOS patients in Erbil, Kurdistan women in north of Iraq**.**

2. Materials and methods

Sampling

PCOS sampling and controls women

This study included two groups of women. The first group was the PCOS group which included 93 infertile women, with mean age of 28.22 ± 8.16,Samples were collected from Maternity Hospital in Erbil within the period from “October 2015 to May 2016”and the second group was the control group, which included 52 healthy women with mean age of 29.52 ± 5.33, with regular menstrual cycles, recruited randomly from the fertility care unit, POCS patients were chosen according to the presence of polycystic ovary morphology and Oligomenorrhea or an ovulation or amenorrhea for at least 6 months[19]. Biochemical and clinical signs of hyperandrogenism, PCOS on ultrasound

[20].while healthy women were chosen according to normal ultrasonography, regular menstrual cycle and normal endocrinal hormones.

Biochemical and hormonal findings

Five milliliters from blood were collected from each subject after 12-h fast at two to three days after the menstruation start. 3 ml of each blood sample was used to obtain serum samples and the separated serum was stored at -80°C for further measurement of the biochemical parameters, the levels of fasting glucose and hormones, which measured FSH, LH and total Testosterone were measured by a minividas (bioMérieux Inc., France), and Lipid profile, were measured and investigated to determine the contrasts between the samples acquired from the patients and the controls and the result was shown in Table 1.

Blood Sampling and DNA Extraction

The rest 2 ml from the peripheral blood samples were collected in tubes containing EDTA as an anticoagulant and were stored at 4°C for genomic DNA extraction and performing genotyping working. Genomic DNA was isolated from the venous blood of PCOS patients and controls using a DNA purification kit (Geneaid, Taiwan) as indicated by the manufacturer's guidelines. Genotyping was performed without learning of the subjects' clinical status. The DNA was stored at -20 C until processing.

Genotype Analysis

Candidate Genes

Candidate gene has been ensnared in the etiology of patients (see Table 2).The one gene map tone distinct chromosomal locations. The regions of 2p16.3 were examined. Discovery of single-nucleotide polymorphism (SNP) of *FSHR*, is shown in Table 2[21].

2.2.2. Polymerase Chain Reaction Analysis

Genotyping for the *FSHR Ala307Thr* gene polymorphism (rs6165) was performed by polymerase chain reaction (PCR), and the regions encompassing the *Ala307Thr* polymorphisms of exon 10 within the gene of *FSHR* were amplified by PCR and which was achieved in a 25 µl mixture includes: 2.5 µl 1x PCR buffer, 0.75 µl MgCl2, 1 µl dNTP mixture, 0.7 µl of each primer, 0.5 µlTaq DNA polymerase, 13.85 µl double distilled H2O, and 5 µl genomic DNA. A 577 bP fragment of *FSHR* was amplified by PCR primer sequences forward and reverse were:
5'CCTGCACAAAGACAGTGATG-3'
5'TGGCAAAGACAGTGAAAAAG-3'

All reactions had an initial denaturation step of 5 min at 95 °C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min and 72 °C for 1 min, and a final elongation step at 72 °C for 7 min**.**

Restriction Fragment Length Polymorphism (RFLP)

The *Ala307Thr* of *FSHR* was detected by restriction fragment length polymorphism (RFLP) analysis[22].The *FSHR* fragment were digested with enzyme AhdI (*Eam1105I*) (Thermo Fisher Scientific Inc., USA). Digestion of the G allele delivered two sections with lengths 403 and 174 bp, assimilation of the A allele created three parts with lengths 403, 143 and 31 bp. The digestion products were settled after electrophoresis in 2% agarose gel (see Figure 1).

Statistical Analysis

Statistical analysis was performed using SPSS Statistics Version 22.0 program on windows 10. To analyze the variance between two continuous variables, a T test was used. Genotype frequencies were measured for Hardy–Weinberg equilibrium. The test of Chi squared was used Hardy–Weinberg test and measured the variables in allele frequencies and genotype distribution between patients and controls by contrasting the observed genotype frequencies with the expected ones, odds ratio (OR), and 95% confidence interval (CI) were done to assess the association between the groups. The results obtained were expressed as mean ± standard deviation (SD) and/or percentage. A p-value <0.05 was expressed statistically significant.

3. Results

Hormone

In this study a total of 52 controls and 93 PCOS patients were recruited. All subjects were from Erbil in Kurdistan, north of Iraq. The demographic data of the PCOS patients and control women are illustrated in Table 1. There was difference of statistically significant in BMI and age women with PCOS when compared with healthy control p- value (0.008) and (0.012) respectively. There was significant elevation in luteinizing hormone (LH) in (PCOS) p- value (0.002) concerning sex hormones in women with (PCOS).Follicular stimulating hormone (FSH) no significant reduction in mean of their level was found in women with PCOS when compared with healthy control p- value (0.436). Total testosterone and fasting glucose were significant in women with PCOS when compared with control group p- value (0.050) and (0.050) respectively**.**

Lipid profile

Lipid profile in PCOS patient's women was measured and compared with healthy control women. This study showed highly significant elevation in Cholesterol and LDL and highly significant reduction in HDL. with p-value 0.000 in PCOS women and there was no significant difference in the mean of Triglycerides and VLDL as shown in Table 1.

Genotype and Allele Frequencies

In this study three patterns of genotype were obtained: AA, AG and GG, of genotyping distributions of SNP rs6165 A/G of *FSHR* gene in 93 patients with PCOS and 52 controls women. *FSHR* polymorphisms of *Ala307Thr* were analyzed by PCR–RFLP, A 577bp of PCR yield might have been acquired, which for absorption with restriction enzyme *Eam1105I* offered three distinctive patterns to 919G→ A substitution. (*Ala307Thr* variant): fragment 403bp., 143 bp. and 31 bp. band demonstrates AA genotype (for 307Thr/Thr); 31 bp. 143 bp. 174 bp. 403 bp., and bands indicate AG genotype (for 307Thr/Ala); and 403 and 174bp. bands indicate GG genotype (for 307 Ala/Ala) (see Figure.1).

The allele frequencies and genotypes distribution of FHSR in controls and PCOS women were assessed (see Table 3). This table explains those amounts for allele frequencies, genotype percentage (%), relative genotypes (n), and their percentage, evaluated odd ratios (OR) at 95% confidence interval (CI)and heterozygosity alongside the P values. The SNP of genotype frequencies were confirmed by Hardy-Weinberg equilibrium test principle in both PCOS and controls. No statistically significant differences were observed in the distribution of the homozygote AA or GG and the heterozygote AG genotypes between the two groups and no statistically significant difference was observed in the allele frequencies between PCOS group and controls (OR = 0.85, 95% CI = 0.52-1.38, *P* = 0.45).

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Table 4 indicates the anthropometric and biochemical characteristics of both the control and the PCOS patients' women within their particular rs6165 genotypes. The values of age and BMI of the control group (p= 0.005 and 0.001) are higher than the PCOS group (p= 0.165 and 0.771). The endocrine values, there were statistically significant differences in the FSH and LH level among the PCOS patients (p= 0.031 and p=0.014) whereas the FSH and LH values in the control group are the opposite. There were no significant differences in two groups concerning the Total testosterone and Fasting glucose. For the lipid profile Characteristics, There were no statistical differences in Cholesterol and Triglycerides values of the patient group and control group. However, there were significantly differences in the level of HDL, LDL and VLDL in the patient group than the control.

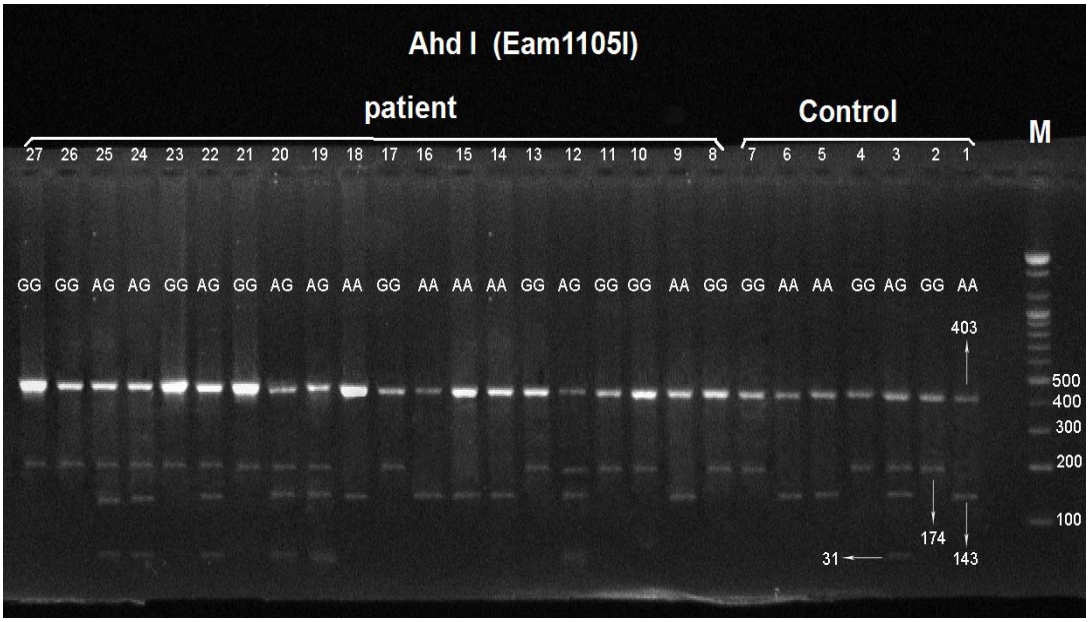

Figure (1): Electrogram of PCR-RFLP analysis of the *Thr307Ala FSHR* variant of *rs6165*. Agarose gel (2%) electrophoresis after *Eam1105I* digestion of the PCR. DNA ladder (100-2000) lane M. lane (1,6,5,9,14,15,16,18,) samples indicate to A/A homozygote was shown by the band of 31 bp. 143 bp. and 403 bp. lane (3,12,19,20,22,24,25) samples indicate to A/G heterozygote was shown by the bands of 31 bp., 143 bp., 174 bp. and 403 bp. lane (2,4,7,8,10,11,13,17,21,23,26,27) sample indicate to G/G homozygote was shown by the bands of 174 bp. and 403 bp. Lane (1-7) Control and lane (8-27) PCOS patient.

Table (1): Anthropometric Characteristics and biochemical characteristics of PCOS patients and controls.

Characteristics	**PCOS (*n* = 93) Mean + SD**	**Controls (*n* = 52) Mean + SD**	***p* value**
Age (year)	28.22 ± 8.16	29.52 ± 5.33	*0.008
BMI (kg/m^2)	27.28 ± 3.30	21.87 ± 1.89	*0.012
Serum hormone			
FSH (mIU/ml)	5.20 ± 1.27	5.70 ± 1.32	0.436
LH (mIU/ml)	6.17 ± 2.15	5.32 ± 0.90	*0.002
Total testosterone (ng/mL)	0.24 ± 0.28	0.18 ± 0.08	*0.050
Fasting glucose (mmol/l)	8.50 ± 1.61	5.58 ± 0.76	*0.050
Lipid Profile			
Cholesterol (mmol/l)	5.05 ± 1.07	5.68 ± 0.48	*0.003
Triglycerides (mmol/l)	2.13 ± 1.02	1.86 ± 0.20	0.160
HDL (mmol/l)	1.38 ± 0.52	2.01 ± 0.20	*0.000
LDL (mmol/l)	3.24 ± 1.26	3.29 ± 0.55	*0.001
VLDL (mmol/l)	0.42 ± 0.20	0.37 ± 0.04	0.160

BMI body mass index, FSH follicle-stimulating hormone , LH luteinizing hormone, HDL high density lipoprotein, LDL low-density lipoprotein, VLDL very low-density lipoprotein, SD standard deviations, *Significant values ($p < 0.05$).

Table (2): Genotyping Panel for PCOS Candidate Genes.

Marker Locus	**Gene Symbol**	**Gene ID**	**dbSNP**	**Ref. SNP Alleles**	**Chromosome**
Gonadotropin action	*FSHR* (Follicle-Stimulating Hormone Receptor	2492	rs6165	A>G	2p16.3
Chromosome Position	**Coding DNA Reference Position**	**Ensemble Accession**	**Gene Mode**	**Protein Position**	**Residue Change**
g.49191041	c.919G>A	ENST00000406846	Missense	307	Ala-Thr

PCOS, polycystic ovary syndrome; dbSNP, single nucleotide polymorphism database; Ala, alanine; Thr, threonine.

Table (3): Distribution of genotypes and allele frequencies of *FSHR* polymorphism among women with PCOS and healthy control identified in the study.

		Genotype		***p* value**		**H-W test**
A307T(rs6165)	*Thr/Thr* (AA)	*Thr/Ala* (AG)	*Ala/Ala* (GG)			
POCS (*n*=93)	19 (20.4%)	33 (35.4%)	41 (44.1%)	0.057		0.016
Control (*n*=52)	13 (25%)	18 (34.6%)	21 (40.3%)	0.111		0.036
Allele						
	Thr (A)	*Ala* (G)			**Or (95 % CI)**	
POCS (*n*=93)	71 (38.17%)	115 (61.83%)		0.490	0.85 (0.52 ~1.38)	
Control (*n*=52)	44 (42.31%	60 (57.69%)				

POCS: Polycystic Ovarian Syndrome; Thr: threonine; Ala: alanine; Or: Odds ratio; CI: confidence interval; H-W test: Hardy-Weinberg test.

Table (4): Distribution of clinical Characteristics of each rs6165 genotype in patients and controls

Characteristics	PCOS (*n* = 93) Mean + SD			*p* value	Controls (*n* = 52) Mean + SD			*p* value
	AA (*n*= 19)	AG (*n*= 33)	GG (*n*= 41)		AA(*n*= 13)	AG(*n*= 18)	GG(*n*=21)	
Age (year)	25.10 ± 5.52	29.48 ± 9.11	28.07 ± 7.87	0.165	7.07 ± 4.23	10.27±5.06	12.14±3.27	0.005*
BMI (kg/m2)	27.78 ± 2.91	27.09 ± 3.56	27.36 ± 3.26	0.771	5.61 ± 1.89	8.33 ± 2.00	7.76 ± 1.57	0.001*
Hormone								
FSH (mIU/ml)	1.91 ± 0.95	0.96 ± 0.59	2.23 ± 1.38	0.031*	1.51 ± 0.11	1.84± 1.05	2.27 ± 1.20	0.948
LH (mIU/ml)	1.46 ± 0.71	2.92 ± 1.09	1.79 ± 0.92	0.014*	1.66 ± 0.96	1.86± 1.04	1.86± 1.33	0.750
T. tes. ng/mL	0.26 ± 0.06	0.17 ± 0.04	0.59 ± 0.07	0.084	0.64 ± 0.14	0.60± 0.12	0.66 ± 0.13	0.990
F. glu. (mmol/l)	2.31 ± 1.66	3.53 ± 1.54	2. 64 ± 1.33	0.361	1.43 ± 1.00	1.86± 1.44	2.15 ± 1.62	0.703
Lipid Profile								
Chol. (mmol/l)	1.57 ± 0.02	2.00 ± 1.87	1.53 ± 0.12	0.587	1.60 ± 0.80	1.90 ± 1.21	2.04 ± 0.21	0.560
Trig. (mmol/l)	0.45 ± 0.06	0.95 ± 0.79	0.72 ± 0.08	0.082	0.44 ± 0.16	0.61± 0.55	0.71 ± 0.32	0.538
HDL (mmol/l)	0.51 ± 0.29	0.28 ± 0.20	0.55 ± 0.02	0.011*	0.43 ± 0.33	0.60± 0.47	0.99± 0.40	0.273
LDL (mmol/l)	0.78 ± 0.08	1.53 ± 1.40	0.86 ± 0.11	0.012*	0.87 ± 0.10	1.36± 1.17	0.98± 0.60	0.345
VLDL (mmol/l)	0.73 ± 0.68	0.22 ± 0.10	0.12 ± 0.02	0.015*	0.70 ± 0.17	0.20 ± 0.19	0.10± 0.04	0.100

p values from one-way ANOVA test with Bonferroni post hoc and data are shown as mean + SD,* Significant values (p is <0.05), BMI body mass index, FSH follicle-stimulating hormone , LH luteinizing hormone, T. tes. Total testosterone (ng/mL) , F. glu. Fasting glucose, Chol. Cholesterol, Trig. Triglycerides, HDL high density lipoprotein, LDL low-density lipoprotein, VLDL very low-density lipoprotein.

4. Discussion

The incidence of PCOS gradually increased during recent years and that it is a complex polygenic disorder. This is the first investigated study on *FSHR* gene in Iraqi Kurdistan women with PCOS. In the present study, the age distribution, the mean age for PCOS group was significantly less than those for control group. This is in agreement with another study which found PCOS has been identified in much younger population (see Table 1)[23]. A PCOS patient has shown significant higher level of BMI when compared with controls. The BMI were higher in PCOS patients than in controls. In Iraqi PCOS women the distribution of obesity differ in various regions of Iraq, there is a significant difference between the south and north of Iraqi women due to changes in temperature, food and different races[24].

The endocrine characteristics, there were all women with PCOS showed high significantly serum LH, total testosterone and fasting glucose, while FSH was found to be no significantly in two groups (see Table 1), which is in agreement with previous studies[25,26]. Insulin resistance as one of the PCOS manifestations can cause some degree of glucose intolerance or diabetes Type II in more than 40 percent of these patients, which these disorders can be intensified by obesity[27].In lipid profile of this study, we observed this significantly effect on cholesterol. When subjects were classified according to their BMI (≤ 25 and higher than 25 kg/m2), no significantly serum level of triglyceride TG was observed in PCOS women (P = 0.160) and VLDL. The cholesterol ester transfer protein (CETP) activity results to exchange of Triglycerides for cholesterol ester in Triglycerides embellished HDL particles that are catabolized additional quickly, and cholesterol enhanced VLDL particles that are converted into small dense LDL particles. LDL and HDL levels in women with PCOS are affected by their weight so that, obese women had higher LDL and lower HDL levels than control women[28].

The SNP genotypic analysis of A307T polymorphism of *FSHR* gene showed that the causes change Alanine to Threonine at position 307, The homozygous (AA) status in 20.4 % of PCOS and 25% in controls. Homozygous (GG) in 44.1%in PCOS and 40.3% in controls. Heterozygous (AG) in 35.4%in PCOS and 34.6% in controls (see Table 2). There were no significant differences between the values of POCS and control samples OR = 0.85, 95% CI = 0.52-1.38, P = 0.490. The result may be due to differences in the samples size between the POCS and control or this may be due to the difference of race between south, middle and north of Iraq and ethnicity may deeply influence the distribution of allelic variants. It is known that those living in northern Iraq are descended from the Aryan race and are different from those living in middle and southern Iraq who are descended from the assets of the Arabian Peninsula. An investigation starting with China, demonstrated no significant association with PCOS women's and *FSHR* polymorphisms (*Ala307Thr*) in the ethnic women's of Han city[29].Studies are focusing on the roles of Ala307of *FSHR* in PCOS, Those present conclusions are either conflicting or opposing. In Korean populations, Gu [18] have expressed that Ala307 may be not correlated with PCOS patient in Korean population. Fu [15] studied the *FSHR* genotype in 384 Chinese PCOS women and 768unselected controls furthermore discovered that *FSHR* modification were determinedly associated with the seriousness of PCOS clinical highlights, however not with disease risk.

In the present study, the association of *FSHR Ala307Thr* polymorphism with characteristics in PCOS women was examined. PCOS women with homozygotes GG, AA or heterozygote AG genotypes had comparable age and BMI, while there were significant

in control. No significant differences were observed between levels of total testosterone; fasting glucose, cholesterol and triglyceride among carriers of different *FSHR* genotypes (see Table 4). Abdel-Aziz[30]in their hereditary POCS-control reported on *FSHR* gene polymorphism didn't indicate any statistically significant difference in genotypes distribution or allele frequency between Controls and PCOS, and found a significant association of rs6165 (*Ala307Thr*) genotype. Fu [15] found that the distribution of clinical characteristics genotype frequencies of the *Ala307Thr* polymorphism of *FSHR* were not different within controls and POCS women's in Chinese women.

5. Conclusion

The Ala307Thrpolymorphism of *FSHR* is associated with PCOS in the studied Iraqi women and this was the first study to report *FSHR* polymorphisms at position 307 in Kurdistan women with POCS, we found no association between *FSHR* gene polymorphisms at positions307 and PCOS. This polymorphism correlates with the increased levels of LH, fasting glucose and total testosterone among PCOS women. In addition, the *FSHR* polymorphisms are associated with the levels of FSH and LH.

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