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Association of Follicle-Stimulating Hormone Receptor Gene Polymorphisms with PCOS in Salah Al-Din, Central Iraq

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Abstract
This study was conducted on (100) samples of women, including (70) samples of women with PCOS after diagnosis by the specialist of the Women's Consultation Clinic and Hospital in Tikrit and Al-Alam District - Salah Al-Din Governorate - Iraq, and (30) samples from healthy women Non-affected with the syndrome for the period from November (2021) to February (2022), the ages of women ranged between (17-44) years, the phenotypic characteristics of the affected women included 78.5% suffering from irregular menstruation, 54.2% having acne, 52.8% baldness of the forehead and hirsutism, 74.2% and 95.7% of the women having cysts on the ovaries as well as the weight characteristic, as 28.5% had an increase In weight and 44.2% were obese, and 14.2% of women with PCOS were infertile. regarding the concentration of follicle stimulating hormone, there was no significant difference between the group of women with PCOS (0.450)mlU/ml compared to the group of healthy women whose concentration reached (0.371)mlU/ml. the results of the genotyping of the FSHR gene, represented by the genotypes (CC, CT, TT), which are present in a percentage (29%, 22%, 49%), respectively, and we noticed that there was no significant difference when comparing the value of chi-square (X2 = 2.42) for the repetitions with their tabular value At a significant level (0.05) and a degree of freedom (2) for women with PCOS with healthy women, the study indicates that the critical ratio (OR=0.6875) for the genotype (CT) and the critical ratio (OR=0.9307) for the mutant allele (T) is less than one. We conclude from this that there is no association between the FSHR gene and the incidence of PCOS, and that the (T) allele does not represent a risk factor for the disease, and the results of the association between the FSHR gene and the hormonal variables indicate that the genotype (TT) has the highest value for follicle-stimulating hormone (0.4910), and the genotype (CC) has the highest value for the hormone. Luteinizing and testicular adipose hormone (0.5198, 52.58), respectively, and the genotype (CC) has the highest value for gonadotropin concentration (0.1744).

Introduction

PCOS is a common infertility disorder that affects a large proportion of the world's population. As it represents an endocrine disorder in females, especially in women of childbearing age. It is the main cause of ovulatory infertility in women with a prevalence of 5-10% worldwide (Krysiak *et al*., 2006) . PCOS can be diagnosed by infertility, acne,

amenorrhea, hirsutism, insulin resistance, obesity, hyperandrogenism, and confirmed by ultrasound. According to infertility studies, PCOS is responsible for 40% of infertility cases in women. moreover, it is a major cause of endometrial cancer, in addition to reproductive abnormalities. PCOS is also associated with a wide range of metabolic disorders, such as hepatic steatosis, glucose intolerance, dyslipidemia, T2DM, and hypertension (Liu *et al*., 2017). In PCOS, abnormalities of LH secretion are prominent, and abnormal serum LH/FSH ratio may be the initiating factor. These latter cases of PCOS are likely to be initiated by primary disturbances in androgen production, particularly those that appear within the ovary, or by disturbances that directly affect follicle maturation and ultimately cause atresia and lysis of the follicle. It is possible that the disturbances characterized by insulin resistance give rise to PCOS through the latter mechanisms (Mulaikal *et al*., 1987).The *FSHR* gene encodes the protein G-coupled receptor, which is essential for gonadotropin development, and this gene stimulates the secretion of follicle-stimulating hormone (FSH). A defect in this hormone leads to the syndrome (Gromoll & Simoni, 2005).

Follicle Stimulating Hormone

FSH is secreted from cells called basophilic located in the anterior lobe of the pituitary gland (Bansal, 2004). In the form of pulses, depending on the secretion of the gonadotropin-stimulating hormone (GnRH), which is produced by the pituitary gland - hypothalamus, and the frequency of its pulse secretion is every (60-90) minutes, and the amount of secreted hormone varies during the different stages of the menstrual cycle of women (Smelllie, 2007). this hormone is a glycoprotein hormone with a molecular weight of about 30,000 Daltons (Marya, 2003). Its chemical composition is 15% carbohydrates and 85% proteins, and the protein molecule of the hormone consists of two α and β units. the α unit in FSH is similar in chemical structure to that of HCG, TSH, LH and its protective function as well. As for the β unit, it is responsible for regulating the physiological activity of this hormone, whose function is to stimulate the growth of ovarian follicles (Graaf & Rhees, 2001). during the corpus luteum, hypertrophy of granulosa cells, secretion of follicular fluid and differentiates the ovarian stroma into theca interna. One of the most important factors that contribute to the regulation of FSH secretion are the levels of steroid hormones in the blood via a negative feedback mechanism. Negative back feed into the hypothalamus and pituitary gland. the secretion of the follicle-stimulating hormone (FSH) is stimulated before ovulation through the high levels of the positive feedback mechanism, as the low levels of the hormones progesterone and luteinizing hormone (LH) will raise the level of FSH and conversely, the high level of these two hormones in the blood will lead to a decrease in its level in the blood. (Klein *et al*., 1996) added that after ovulation, the secretion of the follicle stimulating hormone (FSH) is inhibited due to the high levels of estradiol and progesterone hormones through the negative feedback mechanism and mediated by the hormone Inhibin secreted from the ovaries. and the secretion of this hormone is pulsating with the average of the basic levels that are maintained throughout the month, except for the obvious cyclical changes as they occur during the different stages of the menstrual cycle (Castro *et al*, 1998).

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The FSHR receptor gene for FSH

The FSHR gene is located on the short arm of chromosome 2 (2p16.3) and consists of 14 exons and its genetic sequence consists of 2080 nucleotides. the gene encodes the G protein-coupled receptor consisting of 695 amino acids, which is essential for gonad development (Gromoll *et al*., 1996). the mutation in the gene disrupts the structural protein, causing a hormone imbalance and causing a hormonal imbalance in PCOS. histological studies of the ovaries of polycystic patients carrying mutations in the FSHR gene showed the presence of furrows, as well as ovarian hypoplasia, with a weakness in the development of primary and primary follicles (Meduri *et al*., 2013). similarly, the presence of polymorphism in the FSH receptor gene was associated with PCOS, and the comparison of polymorphism in healthy and affected individuals in northern Iraq showed a higher gene frequency among affected individuals (Baban *et al*., 2018). The FSHR receptor gene for follicle-stimulating hormone spans 54kb of the chromosome and consists of 10 exons and 9 introns. the segment of chromosome 2 carrying the FSHR gene may be subject to point mutations that cause differences in the amino acid sequence of the follicle-stimulating hormone receptor protein (FSHR protein). these structural changes will affect the functional properties of the follicle-stimulating hormone receptor, and these mutations cause early ovarian dysfunction (Kim *et al*., 2017). polycystic ovaries, which constitute a risk factor.

Determination of concentration Follicle Stimulating Hormone (FSH)

FSH was measured using the ready-made kit of ELIZA technology and according to the instructions of the manufacturer, the American company Monobind (Vitt, *et al*.1998).

Results & Discussion

The incidence of polycystic ovary syndrome leads to an imbalance in the pulsating release of luteinizing hormone, where the ratio of luteinizing hormone (LH) to follicle stimulating hormone (FSH) increases, meaning that the ratio of LH / FSH increases by more than 2.5 and this is what was indicated by the study of (Vale *et al*.,2004 ; Altuntase *et al*., 2006 ; ASRM, 2005) therefore, ovulation does not occur, and therefore the egg becomes cystic in the ovary and causes a delay in menstruation or a delay in pregnancy in women (Banaszewska *et al*., 2003). and that this increase in LH and decrease in FSH occurs because the ovaries are impotent the secretion of ovarian reproductive hormones, such as gonadotropins, as estrogen, through negative feedback, which is under the control of the pituitary axis hypothalamic. this is consistent with what was previously mentioned and with the results of other research as well, which confirm the inability of the ovaries to produce and form ovarian hormones, which are estrogen and progesterone, by granulose cells, which leads to a decrease in their level in the blood and in turn an increase in androgenic hormones, which are secreted from the ovary (Koracs & Norman, 2007). the results of the current study agreed in Table (1).

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Control	Patient	Hormone
0.371 ±0.150	0.450 ±0.120	**FSH (Mean±SD) mUl/ml**
1.51		***T-value***
0.135		***P-value***

Table (1)

where the results showed that there were no significant differences in the effectiveness of the follicle stimulating hormone (FSH) among the group of women with PCOS and healthy women, and the results were (0.450 ± 0.237) (0.371 ± 0.150) mlU/ml, respectively in Ovarian follicle-stimulating hormone concentration. The presence of a decrease in FSH/LH hormones was observed in patients with pituitary gland dysfunction, and this was reached by (Al-Tikrity, 2017) and (Farhood, 2017) but it did not show a significant difference between the group of women with PCOS and the healthy group as a group. Control, and this slight decrease in the level of follicle stimulating hormone (FSH), which did not show a significant difference, may be due to the fact that the high concentration of prolactin hormone may lead to inhibition of the secretion of follicle stimulating hormone (Eldar-Geva *et al*., 2001). Melmed and his group (2004) also indicated that the decrease in the level of FSH may be due to the production of adrenaline from the adrenal gland, which in turn affects the concentration of FSH through the secretion of androgens (Testosterone). An increase in FSH or LH indicates a defect in the ovaries (hypogonadism). Normal or low FSH levels indicate a defect in the pituitary gland - hypothalamus, and this is evident in the results of our current study, and it is consistent with the study (Sahin *et al*., 2007), which found that there were no clear significant differences between infected and uninfected women in the effectiveness of The FSH hormone is attributed to the reason that it leads to a delay/non-occurrence of ovulation, which leads to a cyst.

Polymorphism of the FSH receptor gene *FSHR*

A single nucleotide polymorphism (SNP) was found for the FSHR receptor gene, using the T-ARMS PCR indicator, where the DNA segments to be studied and detected were multiplied using specialized primers manufactured by the Korean Macrogen Company that were designed according to demand, then PCR reactions were performed to detect whether or not the mutation. PCR reactions: The reaction was carried out using a PCR-PreMix kit supplied by Bioneer, with a final volume of 20 µl, using four specialized primers according to what was mentioned by (Anderson et al., 1999).

Nucleotide sequence (5` → 3`)	Primers name	No.
5`-GGGACAAGTATGTAAGTGGAACAAC-3`	IF66 Forward	1
5`-TTCAGCTCCCAGAGTCACAAA-3	Forward IR66	2
5`-CAGCTTCCTAATGTATCACATGGA-3`	Revers OF66	3
5`-AAAACTTTCGCAGAGATTTCTTCA-3`	Revers OR66	4

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To amplify the required piece of DNA, the components of the reaction were as in the following table:

Component	20 µl Reaction	Concentration
PreMix	20 µl	-
Free-nuclease water	14 µl	-
DNA Sample	2 µl	50 ng/ µl
Primers (IF,OF,IR,OR) Forward & Revers	4 µl	10 pmol/ µl

PCR reactions were carried out using the following program:

Steps	Temperature	Time	Number of cycles
Initial denaturation	95 C	4 min	1
Denaturation	95 C	30 sec	
Annealing*	58 C	45 sec	35
Extension	72 C	45 sec	
Final extension	72 C	5 min	1

***The optimum temperature for docking**

After that, the PCR product is transferred onto the Agaros gel according to the above-mentioned steps, and when photographed with a UV Transilluminator, three types of bands appear according to the genotypes of the samples, as follows:

- Normal Homozygous (173bp)
- Heterozygous (373bp)
- Mutant Homozygous (246bp)

Results & Discussion

The results of the genotyping of the FSHR gene amplified by Tetra ARMS-PCR technology showed the presence of two alleles, c, t, as well as the presence of three genotypes (CC, CT, TT) in the PCOS sample and the control sample , the C allele is At the 173 bp band, and when this band appears, the genotype is CC (Normal Homozygous) and is present in a percentage (29%), and the T allele is represented by the 246 bp band. When this band appears, the genotype of the sample is TT (mutant homozygous). There is a percentage of (22%), but when the two bundles appear, the genotype is CT (heterozygous) and its percentage is (49%). These bands appear when the Tetra ARMS-PCR product is migrated onto a 2% agarose gel. And as in Figures (1) and (2), respectively.

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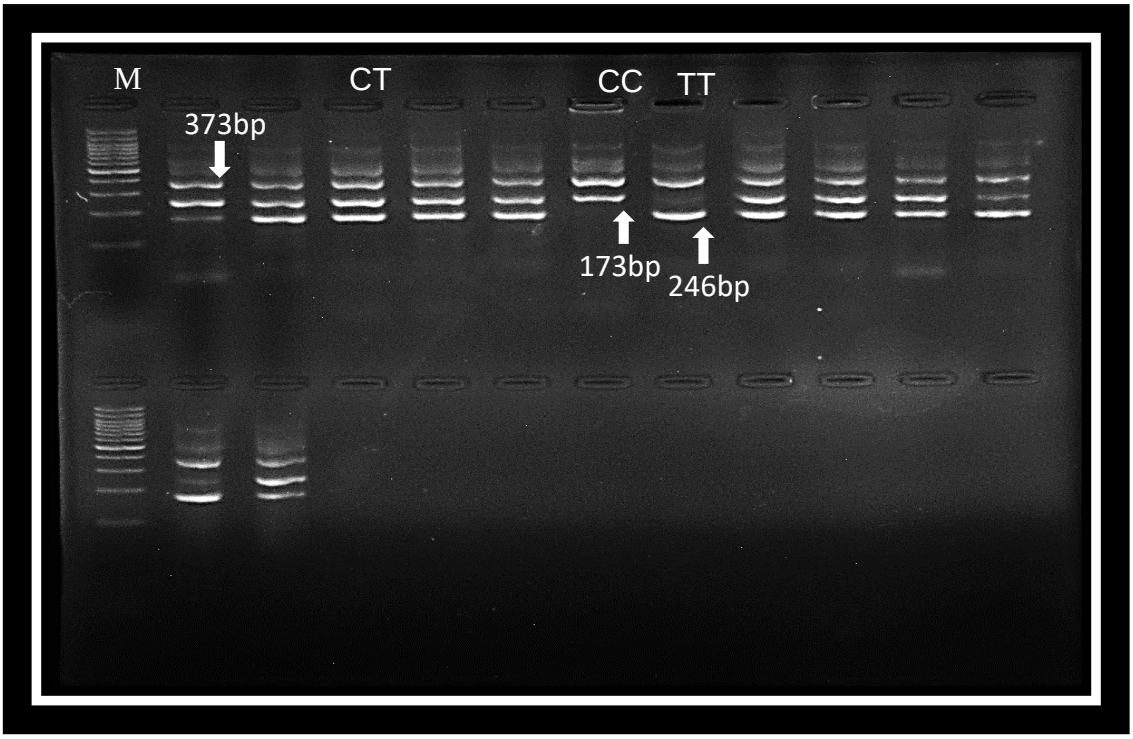

Figure (1) represents the electrophoresis of the ARMS-PCR product of the FSHR gene, showing the two alleles C and T in a sample of women with PCOS, and the migration was carried out on agarose gel at a concentration of 2%

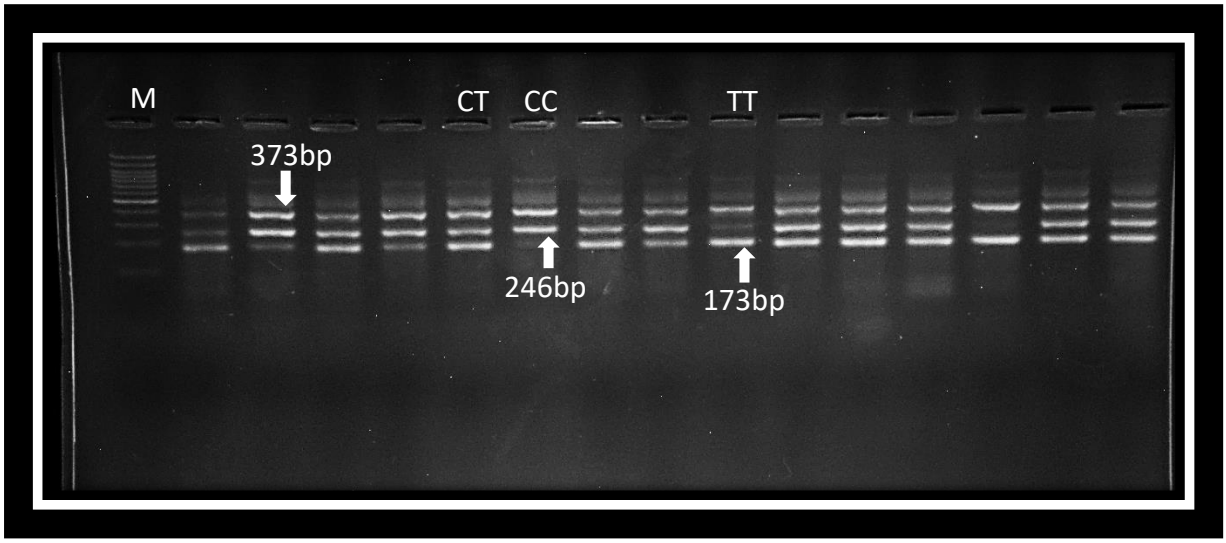

Figure (2) Electrophoresis of the ARMS-PCR product of the FSHR gene, showing the two alleles C and T in the standard sample, and the migration was carried out on agarose gel at a concentration of 2%

We assume that p represents the frequency of the normal non-mutant allele C and that q represents the frequency of the mutant allele T' so that the population when it is in Hardy-Weinbrg equilibrium is (p+q=1) and the frequency of the genotypes is (p2(CC)+2pq (CT) + q2 (TT)) .

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When genotyping the gene and using the Hardy-Weinberg equilibrium law for a sample of patients, the observed number of patients with a homozygous normal genotype CC (22) and a frequency (0.31), and the observed number of patients with a heterogeneous CT genotype (32) and a frequency (0.45).) While the observed number of patients with the homozygous mutated genotype TT was (16) and with a frequency of (0.53) where the frequency of the C allele was (0.54) and the frequency of the T allele was (0.45). Table (2) and (3).

Expected Number (Frequency)	Observed Number (Frequency)	Genotype
21	22	**CC**
(0.29)	(0.31)	**(p^2)**
35	32	**CT**
(0.49)	(0.45)	**(2pq)**
15	16	**TT**
(0.20)	(0.53)	**(q^2)**
70	70	**Total**
Calculated X^2 = 2.42 < tabulated X^2= 5.99 level of significance 0.05 d.f= 2		

Table (2)

Frequency	Allele
0.54	**C (p)**
0.45	**T (q)**
1.000	**Total (p+q)**

Table (3)

The results of genetic analysis when applying the Chi-Square chi-square test on a group of women with PCOS, it was found that the calculated value of X2 (2.42) is less than its tabular value (5.99) at the degree of freedom of 2 and the level of significance 0.05. These results indicate that the population of women with PCOS was balanced for the FSHR gene, and Hardy-Weinberg's equilibrium law applies to it represented by the equation (p2+2pq+q2=1) (Klug *et al*., 2010). As for the group of unaffected women, the observed number of the homozygous normal genotype CC was (7) with a frequency (0.23), and the number observed for the non-infected sample with the heterogeneous CT genotype (17) and with a frequency of (0.56), while the number observed for the TT genotype was (6) and by repeating (0.2). As for the allelic frequency, it was in the non-affected group for the C allele (0.52) and the allelic frequency for the T allele (0.49), Table (4) and (5). The calculated Chi-Square value (0.06) was less than the tabular value of X2 (5.99) at a degree of freedom of 2 and a significance level of 0.05. This result indicates that the population of women without PCOS for the FSHR gene is balanced according to Hardy-Weinberg law (p2+2pq+q2) .

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Expected Number)Frequency(	Observed Number)Frequency(	Genotype
8	7	**CC**
(0.26)	(0.23)	**(p^2)**
15	17	**CT**
(0.49)	(0.56)	**(2pq)**
7	6	**TT**
(0.24)	(0.2)	**(q^2)**
30	30	**Total**

Calculated X^2 = 0.06 < tabulated X^2= 5.99 level of significance 0.05 d.f= 2

Table (4)

Frequency	Allele
0.51	**C (p)**
0.49	**T (q)**
1.000	**Total (p+q)**

Table (5)

The results of genotyping for the FSHR gene show the presence of all genotypes (CC, CT, TT) in the samples in the affected and unaffected group, and the highest percentage of the normal genotype was between the two groups, while the mutant genotype was the lowest among the genotypes by (5%) and it appeared In the unaffected group, one TT is a homozygous mutant genotype, and this indicates the association of the mutant gene with a defect in the ovulation process in women, Figure (4-3) and (4-4). There is no difference in the allelic frequency of the C allele between the group of affected women, whose value was (0.54) and the group of unaffected women (0.51). As for the allelic frequency of the T allele, there was no difference between the allelic frequency of the group of women with PCOS whose value was (0.45) compared to the control group (0.49), table (3) and (5).

The results of (Braven *et al*., 2018) also indicated that all genotypes appeared in the group of women with and without PCOS of the Sri Lankan women community, and the mutant genotype was the lowest among the genotypes, while the majority of genotypes were for the asymmetric CT genotype, as well as Braven indicated and his group (2018) indicated that there was no difference in allelic frequencies between the group of women with PCOS and the control. The results of the current study also agreed with (Lingyuan *et al*., 2013) when studying the two polymorphisms (Ala307Thr, Ser680Asn) of the FSHR gene in women with PCOS in the North Chinese Han women population. With slims (control), It was found that polymorphism (Ser680Asn) is not associated with PCOS, but it increases the level of FSH hormone in the blood of women carrying the mutation and leads to a decrease in the level of E2 estradiol. This was also indicated by (Mohiyiddeen *et al*., 2012) that (Ser680Asn) is a mutation that is not associated with the disease, but it raises the level of follicle-stimulating

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hormone (FSH). (0.4910) Table (6), and its results also agreed with our current study, which indicated that there were no differences between the allelic frequencies of the FSHR gene for women with PCOS with high levels of AMH and non-infected.

P-value	**Genotypes**						**Hormones**
	Control			**Patient**			
	TT	**CT**	**CC**	**TT**	**CT**	**CC**	
	N=6	N=17	N=7	N=16	N=32	N=22	
0.007	0.5460 a	0.3130 b	0.3170 b	0.1318 c	0.1744 c	0.1367 c	**GnRH**
0.876	0.4288 a	0.3639 a	0.3180 a	0.4910 a	0.4516 a	0.4182 a	**FSH**
0.045	0.3260 b	0.2523 b	0.2275 b	0.2657 b	0.3715 b	0.5198 a	**LH**
0.0005	0.1762 c	0.2087 c	0.2561 c	0.3811 b	0.4928 ab	0.5258 a	**Testo.**

*** Similar letters mean there are no moral differences between them**

Table (6)

The results of the study show that there are no significant differences between all phenotypes CC, CT, TT in women with and without PCOS with regard to the hormonal characteristics of each of the hormones (GnRH, FSH, LH and Testo.). As we mentioned previously, the homozygous TT mutated genotype in women with PCOS carries the highest value for FSH, and this is consistent with the study of (Lingyuan *et al*., 2013; Mohiyiddeen *et al*., 2012) previously mentioned. The homozygous CC genotype of the FSHR gene had the highest value for Testo. And luteinizing hormone LH (0.5198, 0.5258), respectively. As for the heterogeneous CT genotype of the FSHR gene, it has the highest value for GnRH, and its value was (0.1744) Table (6). (Valkenburg *et al*., 2009) showed an increase in the level of FSH and LH for women with the syndrome in the homozygous TT genotype of the FSHR gene and a decrease in the level of testosterone.

Table (7): shows the allelic frequency and distribution of FSHR gene genotypes in the group of women with PCOS and the control group.

rs 6166 Genotype	Patients No (70)		Control No.(30)		*P* Value	OR	(95% CI)
	No.	Freq.	No.	Freq.			
CT	32	45.71%	7	%35	0.5135	1 Ref.	-
CC	22	31.42%	7	%35		0.6914	0.2285 to 2.0920
TT	16	22.85%	6	%30		0.6875	0.2113 to 2.2373
Alleles	No.	Freq.	No.	Freq.	*P* Value	OR	(95% CI)
C	76	54.28%	21	52.5	0.8416	1 Ref.	0.4603 to 1.8819
T	64	45.71%	19	47.5		0.9307	

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C = wild allele , T = mutant allele. OR > 1 = Significant or(risk factor)

The results of the study show that the value of the critical ratio (OR) for women carrying the TT genotype (OR=0.6875, CI=0.2113 to 2.2373) and the critical ratio (OR) for the mutant allele T OR=0.9307 (CI=0.4603 to 1.8819)) that the value of the critical ratio Odds ratio less than the correct one with a confidence interval (CI) of not less than 95%, we conclude that the FSHR gene is not associated with PCOS and that the mutant T allele is not a risk factor for PCOS. These results are in agreement with several studies (Mohiyiddeen *et al*., 2012) and (Lingyuan *et al*., 2013) who indicated that there is no association between FSHR and PCOS. . It did not agree with (Braven *et al*., 2018), which proved that there is an association between the FSHR gene and PCOS, where the critical ratio value was OR > 1 and that the mutant allele T represents a risk factor for the disease where the critical ratio value of the mutant allele T was higher than the correct one. There are two researchers who found a relationship between the FSHR gene and PCOS (Dolfin *et al*., 2011) and (Du *et al*., 2010), while the majority of researchers failed to find a relationship between polymorphism of the gene and PCOS.

The follicle-stimulating hormone receptor gene, FSHR, encodes the G protein-coupled receptor, which consists of 695 amino acids, which is essential for gonadotropic development (Gromoll et al., 1996). The segment of chromosome 2 carrying the FSHR gene may be subject to point mutations that cause differences in the amino acid sequence of the follicle-stimulating hormone receptor protein (FSHR protein). These structural changes will affect the functional properties of the follicle-stimulating hormone receptor and these mutations cause early ovarian dysfunction (Kim et al., 2017) and Kim et al. (2017) indicated that the two mutations (Asn680Ser, Thr307Ala) of the FSH receptor gene are associated with FSHR syndrome. Polycystic ovaries, which constitute a risk factor.

Conclusion

A slight decrease in the level of follicle-stimulating hormone and an increase in the level of luteinizing hormone in women with polycystic ovary syndrome, which leads to a delay / lack of ovulation and a cyst occurs. the mutant TT genotype of the FSHR gene in women with PCOS carries the highest value for FSH, which indicates that the mutation is not related to the disease, but it raises the level of FSH. and we conclude that the FSHR gene is not associated with PCOS and that the mutated T allele does not constitute a risk factor for PCOS.

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