A Case-Control Study of the Luteinizing Hormone Level in Luteinizing Hormone Receptor Gene (rs2293275) Polymorphism in Polycystic Ovarian Syndrome Females

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Keywords
Luteinizing hormone · Follicle-stimulating hormone · Polycystic ovarian syndrome

Abstract
Background: Polycystic ovary syndrome (PCOS) is a common heterogeneous disorder characterized by chronic anovulation, infertility, polycystic ovaries, and hyperandrogenic signs. Objective: The aim of this study was to determine the association of luteinizing hormone/chorionic gonadotropin hormone receptor LHCGR polymorphism (rs2293275) with oligomenorrhea, amenorrhea, hirsutism, acne, infertility, LH, LH/FSH ratio, and body mass index (BMI) among PCOS females. Methods: This genetic case-control study recruited 55 PCOS and 55 control females, diagnosed based on the Rotterdam criteria. LH and FSH were measured by the Roche cobas c 502 automated analyzer. Genotypic analysis was carried out using the polymerase chain reaction-restriction fragment length polymorphism and restriction endonuclease digestion. Results: BMI was higher for PCOS patients (28.5 ± 6.59) compared to controls (25.1 ± 5.77), and ovulatory dysfunction was seen among 90% of PCOS females. Oligomenorrhea was common in PCOS (73%), and hirsutism and acne were detected in PCOS (80% and 40%; respectively). LH ≥10 were recoded among 51%, while LH/FSH ≥1.5 was recorded among 33% PCOS females. There is a statistical difference between rs2293275 polymorphism in the AG genotype between PCOS patients and controls. PCOS patients have a significantly higher mean LH level compared to controls (8.36 ± 4.86 and 5.67 ± 2.51, respectively) and showed higher LH/FSH value (1.46 ± 0.81) compared to (0.87 ± 0.30) controls. GG and AG genotypes of LHCGR showed statistically significant higher LH (8.22 ± 4.11; 9.02 ± 3.87) and LH/FSH values (1.57 ± 0.56; 1.64 ± 0.89) compared to controls.

Conclusion: LHCGR (rs2293275) GA and GG genetic variants could modulate the hormonal levels of PCOS LH levels and the LH/FSH ratio and associated with hirsutism, oligomenorrhea, BMI, and LH/FSH ratio as risk factors.

Introduction

Polycystic ovary syndrome (PCOS) or Stein-Leventhal syndrome, is one of the most common incurable endocrinopathies among women of reproductive age [1, 2]. PCOS characterized by many clinical-biochemical fea-
tures including ovulatory dysfunction which includes oligomenorrhea or amenorrhea [3], hyperandrogenic features which are clinically characterized by the presence of excessive acne or hirsutism, and biochemical features which are characterized by the elevated serum androgen level [4]. In addition to these features, polycystic ovarian morphology showed altered ultrasonography [1], in which the ovary containing 12 or more follicles measuring 2–9 mm in diameter or an ovary that has a volume of greater than 10 cm³ on ultrasonography [5].

PCOS is in fact a heterogeneous disorder, and different PCOS phenotypes have different pathogenetic mechanisms [6]. PCOS prevalence depends on ethnicity, environmental factors, and genetic factors. Among different geographic regions, it ranges from 5% to 10% according to NIH 1990 criteria, from 10% to 15% according to the AE-PCOS 2006 criteria, and from 6% to 21% when the ESHRE/ASRM 2003 criteria applied [7]. LH and human chorionic gonadotropin (hCG) are heterodimeric glycoprotein hormones that play key roles in human reproduction.

Both LH and hCG bind to the luteinizing hormone/choriogonadotropin hormone receptor (LHCGR) [8]. The signaling cascade begins when LH or hCG binds to the LHCGR and induce conformational change, leading to activation of the G protein. This will activate adenylate cyclase which increases cAMP, which activates kinases that express genes involved in the physiological responses [9]. The LHCGR gene is located on chromosome 2p16.3 [10] and consists of 11 coding exons and 10 introns [11]. Approximately 300 SNPs were identified in the LHCGR gene [12]. Two single-nucleotide polymorphisms, rs12470652 c.872A>G/p.Asn291Ser and rs2293275 c.935G>A/p.Ser312Asn, in the LHCGR gene has been screened in healthy Bulgarian women [13]. Asn291Ser minor allele G was found in 7.5% of healthy women and 6.8% of those with PCOS [13]. rs13405728 was studied among north Chinese and controls before interviewing and sample collection. Approval was received from Hashemite University Institutional Review Board, and consent forms were signed by all participants and controls before interviewing and sample collection.

BMI was determined using height and weight (BMI = kg/m²) where kg is a person’s weight in kilograms and m² is their height in meters squared. Clinical manifestations such as oligomenorrhea, amenorrhea, hirsutism, acne, infertility, LH, LH/FSH ratio, and body mass index (BMI).

Methods

In this case-control study, a total of 55 Jordanian PCOS females and 55 age-matched controls from Al Hikma Modern Hospital (Zarqa/Jordan) were recruited (2017–2018). All procedures involving human participants were in accordance with the ethical standards of the institutional Ethical Committee and with the 1964 Helsinki Declaration and its ethical standards. An informed consent was signed by all participants enrolled in the study. Ethical approval was received from Hashemite University Institutional Review Board, and consent forms were signed by all participants and controls before interviewing and sample collection.

Several inclusion and exclusion criteria were used in this study. Inclusion criteria for PCOS patients include age-group (17–38), without complications or other diseases and identified via 2003 Rotterdam criteria, and two out of three features: clinical or biochemical hyperandrogenism, oligo-anovulation, and polycystic ovarian morphology. Exclusion criteria include women with inherited disorders like congenital adrenal hyperplasia, androgen-secreting neoplasm, thyroid disorder, obesity, diabetes, hyperprolactinemia, Cushing’s syndrome, thyroid dysfunction, and those who were taking regular oral contraceptive pills for the last 6 months.

Hirsutism was classified according to the Ferriman-Gallwey score, which is used to evaluate hirsutism. All PCOS females have a Ferriman-Gallwey score of 8 or more, while all control groups have an hirsutism score less than 8 [19]. Acne was classified by the Global Acne Grading System, and all PCOS females have an Global Acne Grading System score of a moderate to severe score [20].

Age-matched healthy control females were selected with normal menstrual cycles and not having any of the 2003 Rotterdam criteria. Healthy females were examined as free of ovarian cysts by ultrasonography.
Blood Sample

Five milliliters of blood was collected on the third day of menstrual cycle and divided as follows: 3 mL in gel separating tubes (Greiner Bio-One, Solingen, Germany), was allowed to clot for 5–10 min at room temperature, and then centrifuged at 4,000 rpm for 10 min and then stored at deep freeze for LH and FSH assays. Two milliliters in EDTA tubes were used for DNA extraction.

Hormonal Assays

The serum was collected for LH and FSH assays. Measurements were carried out using the Roche cobas c 502 automated analyzer (Roche, Indianapolis, IN, USA) in the Med Labs consultancy group. cobas c 502 works according to the electrochemiluminescence immunoassay principle.

DNA Extraction and Quantification

DNA was extracted from EDTA blood using a DNA extraction kit (QIAGEN, Hilden, Germany) and according to the manufacturer’s instructions. A NanoDrop Spectrophotometer (Thermo Fisher, Waltham, MA, USA) was used to assess the quality and the quantity of DNA, and the ratio of OD260/OD280 was used to confirm the purity (1.8–2.0). Samples were then stored at −20°C for analysis.

Polymerase Chain Reaction

Using PCR, the 111-bp region of the LHCGR gene was amplified using primers complementary to the target region of the LHCGR gene. Genomic DNA was amplified using forward primer 5′-CCTCTTCTCTTCCAGA-3′ and reverse primer 5′-CATGCAATACTTACAGTGTTTTGGTA-3′. There is site-directed mutagenesis in the reverse primer which substitutes the base number 25 to introduce recognizable sequence for the restriction enzyme.

PCR reaction mixture was prepared by mixing 25 μL consisting of 1.0 μL of the forward primer (10 pmole/μL), 1.0 μL of the reverse primer (10 pmole/μL), 12.5 μL of GoTaq® Green Master Mix (Go-Taq® Green Master Mix), 4 μL of template DNA, and 6.5 μL of nuclease free water. PCR was carried out using the Bio-Rad iCycler using the following parameters: initial denaturation at 95°C for 5 min, 30 cycles including denaturation at 95°C for 1 min, annealing at 49°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 5 min.

Agarose Gel Electrophoresis

Electrophoresis was used to verify DNA migration on agarose gel with a concentration 2%. Agarose was prepared by boiling 2 g of agarose in 100 mL 1X Tris borate EDTA buffer and stained with 5 μL red safe stain. The amplified PCR product of the LHCGR gene was confirmed by detection of a 111-bp band on 2% agarose gel stained with red safe stain. Migration of agarose was at 80 V for 60 min. A gel documentation system (compact dig-image system; Major Science, Saratoga, CA, USA) was used for visualization and photography.

Results

LHCGR gene presence was confirmed by PCR product detection of a 111-bp band on 2% agarose gel stained with red safe stain (Fig. 1). Fifty-five PCOS unrelated patients and fifty-five healthy controls were enrolled in this study. There is a significant difference in hirsutism, acne, infertility, LH, LH/FSH, and BMI between these 2 groups (Table 1).

Frequencies of (rs2293275) gene polymorphism of the AA genotype among PCOS patients and controls were 16.4% and 18.2%, respectively, while GG genotype frequencies were 47.3% and 41.8%, respectively (Table 2). The AG genotype is significantly different between PCOS patients and controls (p value ≤0.05). OR at 95%
CI is shown in Table 4. No significant association between PCOS patients and controls was found within other genotypes or alleles.

The \( t \) test was used to determine the significant differences in the LH level and LH/FSH between the means of PCOS patients and controls. There is a significant difference between the means of LH levels and LH/FSH among PCOS patients and controls, and \( p \) value is less than 0.05 (Table 5).

The results of this study showed a significant association between the LH level and LH/FSH values among AG and GG genotypes, \( p \) value ≤0.05 (Table 6). The heat map was created among different genotypes (GG, GA, and AA) for all risk factors: oligomenorrhea, amenorrhea, hirsutism, acne, infertility, LH, LH/FSH, and BMI. The white color indicates positive strong association, and the black color indicates low association, and intensity of the red color in between indicates the association according to the color scale in the right.

Figure 2 shows heat map association between different \( (rs2293275) \) genotypes (GG, GA, and AA) among polycystic ovarian syndrome patients and other risk factors such as oligomenorrhea, amenorrhea, hirsutism, acne, infertility, LH, LH/FSH (LHFBS), and BMI. Light colors white and pink indicate strong association, while the dark color (black) indicates poor association. Hirsutism, oligomenorrhea, and BMI are strongly associated with GG genotypes among PCOS patients.

### Discussion

PCOS is a heterogeneous disease, and its heredity is an important cause for its development. Determining the PCOS-related genes is still not clear. Many candidate genes...
involved in PCOS include LH, FSH, and LHCGR. LHCGR encodes a transmembrane protein that fits into the G-protein-coupled receptor family and is expressed in theca cells of the ovary [14]. The genetic association of LHCGR polymorphism with the etiology of PCOS was determined previously [22]. This study evaluated the association between LHCGR rs2293275 polymorphism and PCOS in a case-control study among Jordanian females. It showed that oligomenorrhea, hirsutism, acne, infertility, elevated LH level, elevated LH/FSH ratio, and elevated mean BMI are common among PCOS patients compared to controls (Table 1). This study showed that PCOS patients complained of irregular menstruation. Oligomenorrhea was seen in 66.7% patients [23] and may reach 73.8% [24].

Hyperandrogenism occurs due to the excessive androgen production; besides, 58–82% of hyperandrogenic women have PCOS [25]. This study revealed that hirsutism was presented among 44 (80%) of PCOS cases, compared to 5 (9%) in controls ($p$ value 0.0001), while acne was present among 22 (40%) of PCOS cases compared to 9 (16%) in controls. The prevalence of hirsutism in PCOS ranges from 13% to 89% among different studies [26, 27].

PCOS may be the primary cause of anovulatory infertility; this study showed that 20% of PCOS cases were infertile compared to 4% among controls. A previous study [28] reported that infertility among PCOS women was 72% compared to 16% among non-PCOS women and showed a 15-fold infertility increase among PCOS females. The prevalence of PCOS among ovulatory women with infertility is higher than that in the normal popula-

### Table 4. The association between rs22932775 gene polymorphism and PCOS

| Genotype            | PCOS $(n = 55), n (%)$ | Control $(n = 55), n (%)$ | CI (95%)            | Odd ratio     | $p$ value |
|---------------------|------------------------|---------------------------|---------------------|---------------|-----------|
| AA (homozygous)     | 9 (16.40)              | 10 (18.2)                 | –                   | (Ref)         | –         |
| AG (heterozygous)   | 20 (36.30)             | 22 (40)                   | 0.4393–2.2204       | 0.9877        | 0.030     |
| GG (homozygous mutant) | 26 (47.30)         | 23 (41.8)                 | 0.5399–1.753        | 0.7943        | 0.570     |

$ p $ value is statistically significant at $ p \leq 0.05 $.

### Table 5. LH level and LH/FSH value among PCOS patients and controls

| Parameter        | PCOS patients, mean ± SD | Control, mean ± SD | $p$ value |
|------------------|--------------------------|--------------------|-----------|
| LH level         | 8.36±4.86                | 5.67±2.51          | 0.0004    |
| LH/FSH           | 1.46±0.81                | 0.87±0.30          | <0.0001   |

### Table 6. Differences between the LH level and LH/FSH values among PCOS patients and control genotypes

| Parameter Category | Category | AA | AG | GG |
|--------------------|----------|----|----|----|
| LH mean ± SD       | PCOS     | 7.26±2.81 | 9.02±3.87 | 8.22±4.11 |
|                     | Control  | 5.53±3.07 | 6.41±2.43 | 5.02±2.24 |
| $p$ value          |          | 0.219 | 0.012 | 0.002 |
| LH/FSH mean ± SD   | PCOS     | 1.10±0.41 | 1.64±0.89 | 1.57±0.56 |
|                     | Control  | 0.81±0.45 | 0.91±0.30 | ±0.29    |
| $p$ value          |          | 0.162 | 0.001 | 0.0001 |

$p$ value <0.05 was considered statistically significant.
tion, suggesting that PCOS may cause subfertility among women with regular menses due to hyperandrogenemia. PCOS is the most common cause of anovulatory infertility, and nearly 90–95% of anovulatory women seeking treatment for infertility have PCOS [29].

LH stimulates ovarian androgen production, whereas an insufficiency in FSH production impairs follicular development. The imbalance in LH and FSH causes proliferation of ovarian theca cells, leading to increased steroidogenesis and hyperandrogenism among PCOS women. This study showed that 51% of PCOS females have LH ≥10, and 33% have LH/FSH ≥1.5. In healthy women, the ratio between LH and FSH is usually around one. In PCOS women, this ratio is altered, and it might reach as high as 2 or 3 [30]. Saucedo de la Llata et al. [31] showed that the LH/FSH ratio was 1.25 ± 0.85 in the PCOS group compared to 0.71 ± 0.39 in controls. Khashchenko et al. [24] reported that LH/FSH (1.6) versus 0.7 in the control group.

The exact pathogenesis of PCOS is unknown, but the main characteristic includes elevated secretion of luteinizing hormone. In women with PCOS, GnRH is altered, resulting in increased LH activity by the pituitary gland. This will stimulate theca cells and prevents normal follicular maturation and ovulation. The ovary looks full of follicles with larger ovaries. Our study showed statistical

![Fig. 2. Heat map was created among different genotypes (GG, GA, and AA) for oligomenorrhea, amenorrhea, hirsutism, acne, infertility, LH, LH/FSH, and BMI.](image-url)
significant difference in the LH level among PCOS (8.36 ± 4.86) patients compared to the control group (5.67 ± 2.51). Shah et al. [32] showed a high PCOS LH level (7.82 ± 6.11) compared to controls (3.41 ± 0.70). Saucedo de la Llata et al. [31] in 2016 showed a high level of LH (6.36 ± 4.61) among PCOS patients compared to (4.34 ± 2.12) controls. Growth arrest of ovarian follicles in the non-obese PCOS patients is associated with an elevated LH level. LH promotes follicular growth during preantral-early antral transition via increased androgen production [33]. LH stimulation harms FSH-dependent antral follicle growth by destroying FSH receptor expression in granulosa cells. So, chronically elevated LH among PCOS patients must be thought-out for improved PCOS care.

Increased synthesis of LH over FSH-altered LH:FSH ratios is typical among PCOS patients [34]. Insufficient FSH levels with regard to LH levels contribute to impaired follicular development [35]. In PCOS patients, there is discrepancy in the hypothalamic-pituitary-ovarian axis, leading to increased gonadotrophins production. This increased the β-subunit of LH over the β-subunit of FSH production. Elevated LH stimulation causes hyperplasia of theca cells and accumulation of follicular fluid, forming cyst-like structures [36]. Increased expression of the fundamental enzymes by the increased number of follicles among PCOS patients is responsible for the excessive amount of androgens. The serum LH level and the LH/FSH ratio may be more useful than the serum antimullerian hormone level for representing the status of the ovarian volume in women with PCOS [35]. LH level variation is associated with menstruation dysfunction and infertility [35]. LH is stimulating AMH production which arrests folliculogenesis and PCOS development [37]. Elevated LH and LH/FSH prevalence varies from 35% to 77% in PCOS patients [38, 39]. This study also evaluated the levels of LH in PCOS patients and found that 51% of PCOS patients had elevated LH levels.

Many SNPs in the LHCGR gene among PCOS patients were recorded (rs12470652, G935A, ins18LQ, and rs2293275) [13, 17]. Robeva et al. [13] in 2018 examined the association of LHR rs12470652 and rs2293275 in Bulgarian PCOS women and found that the rs2293275 variation modulates PCOS characteristics among obese patients. Thathapudi et al. [17] in 2015 demonstrated a significant association between the LHCGR rs2293275 GG genotype with BMI and the LH/FSH ratio in South Indian PCOS women. In contrast, lack of association between LHCGR polymorphism and PCOS was reported previously [40]. Our study determined LHCGR polymorphism (rs2293275) among 55 PCOS patients and 55 controls. A significant difference in the distribution of the AG genotype between PCOS and controls was noticed (Table 4).

The results of this study showed strong association of LHCGR rs2293275 polymorphism with high LH and with a high LH/FSH ratio (Table 6). LHCGR polymorphism among AG phenotypes affect the steroidogenesis with metabolic and transport pathways of sex steroids [22]. High LH concentrations in PCOS are important for PCOS diagnosis, and genetic understanding for different polymorphisms may contribute to a better understanding of PCOS pathophysiology. Therefore, LHCGR polymorphism may be useful as a molecular marker for early detection of high risk for PCOS.

Our results showed that the frequencies of rs2293275 polymorphism differ between among AG genotypes in PCOS (36.3%) compared to 40% among controls. Robeva et al. [13] conducted his study on Bulgarian population and compared the frequencies of rs2293275 between patients (GG: 21.7%, AG: 33.3%, AA: 45.0%) and controls (GG: 12.5%, AG: 35.0%, AA: 52.5%). Another study conducted among Egyptian population showed that the AG genotype was strongly associated with PCOS (OR: 3.4, CI 95%: 2.19–5.24) [12].

This study showed that genotypic variants of rs2293275 influence the LH and LH/FSH levels among PCOS females; the homozygous variant GG and heterozygous AG had shown a significant association with LH (p = 0.002, 0.012) and significant association with LH/FSH (p = 0.0001; 0.001), respectively. Thathapudi et al. [17] showed that the GG variant increases values of LH (PCOS: 11.69 ± 6.3, control: 6.64 ± 3) and LH/FSH (PCOS: 2.5 ± 0.8, control: 1.3 ± 1.05) when compared with the AA variant that showed LH (PCOS: 9.9 ± 4.3, control: 7.28 ± 4.8), LH/FSH (PCOS: 2.38 ± 1.47, control: 1.4 ± 0.9), and AG LH (PCOS: 11.69 ± 6.3, control: 8.18 ± 5.4) genotypes.

The heat map (Fig. 2) summarizes the association between rs2293275 genotypes (GG, GA, and AA) and PCOS risk factors. GG showed strong association with hirsutism, oligomenorrhea, BMI, and LH/FSH. The AG genotype showed that hirsutism and oligomenorrhea are risk factors, while low association was shown between these risk factors within AA variants indicated with the light color white.

Limitations of the study include the following: first, relatively small sample size; second, all samples and controls were conducted from one hospital (Zarqa/Jordan); and third, this study does not include the environmental factors exposure that may have an impact on the occurrence of PCOS. All over this study, effort was put to maintain the standardization of data collection measures across the study groups, which assured accuracy of the results.
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**Conclusion**

This study indicated for the first time the potential influence of rs2293275 LHCGR on PCOS females of Jordanian population. The genetic variants AG and GG may modulate and increase the LH level and LH/FSH ratio among PCOS females. GG rs2293275 polymorphism of PCOS females showed increased frequency of hirsutism, oligomenorrhea, BMI, and LH/FSH, while the AG genotype showed increased frequency of hirsutism and oligomenorrhea. The effect of rs2293275 polymorphism may be specific to ethnic population through its interaction with the clinical and environmental factors. The results of this study may provide a platform that can be implemented and assisted in medical PCOS diagnosis. This may highlight the value of incorporating oligomenorrhea,amenorrhea, hirsutism, acne, infertility, LH, LH/FSH, and BMI in a medical database to predict PCOS susceptibility.

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