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# Association of single-nucleotide polymorphisms in the *ESR2* and *FSHR* genes with poor ovarian response in infertile Jordanian women

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**Objective:** Poor ovarian response (POR) refers to a subnormal follicular response that leads to a decrease in the quality and quantity of the eggs retrieved after ovarian stimulation during assisted reproductive treatment (ART). The present study investigated the associations of multiple variants of the estrogen receptor 2 (*ESR2*) and follicle-stimulating hormone receptor (*FSHR*) genes with POR in infertile Jordanian women undergoing ART.

**Methods:** Four polymorphisms, namely *ESR2* rs1256049, *ESR2* rs4986938, *FSHR* rs6165, and *FSHR* rs6166, were investigated in 60 infertile Jordanian women undergoing ART (the case group) and 60 age-matched fertile women (the control group), with a mean age of 33.60±6.34 years. Single-nucleotide polymorphisms (SNPs) were detected by restriction fragment length polymorphism and then validated using Sanger sequencing.

**Results:** The *p*-value of the difference between the case and control groups regarding *FSHR* rs6166 was very close to 0.05 ($p=0.054$). However, no significant differences were observed between the two groups in terms of the other three SNPs, namely *ESR2* rs1256049, *ESR2* rs4986938, and *FSHR* rs6165 ($p=0.561$, $p=0.433$, and $p=0.696$, respectively).

**Conclusion:** The association between *FSHR* rs6166 and POR was not statistically meaningful in the present study, but the near-significant result of this experiment suggests that statistical significance might be found in a future study with a larger number of patients.

**Keywords:** Estrogen receptor; Follicle-stimulating hormone receptor; Ovarian stimulation; Single-nucleotide polymorphism

## Introduction

About 72.4 million couples suffer from infertility worldwide; accordingly, almost three million children have been conceived through assisted reproductive treatment (ART) [1]. ART is a multistep process that involves oocyte collection, oocyte fertilization, and embryo implantation [2]. The first step of ART is the collection of oocyte-containing follicles after ovarian stimulation with follicle-stimulating hormone (FSH) to obtain high-quality oocytes [3]. The response to this hormonal stimulation varies among women. Women producing 6–15 oocytes are considered normal responders, while those with not more than 4–5 oocytes are referred to as poor responders and women producing more than 15 oocytes are classified as hyperresponders [4].

Several factors, such as age, hormonal status, and ovarian reserve, play a role in the prediction of ovarian response [5,6]. In addition to

previously identified predictors, various genetic polymorphisms have been proposed as markers predicting ovarian response. These variations have been observed in many genes, such as estrogen receptor 2 (ESR2) and follicle-stimulating hormone receptor (FSHR) [7,8]. It is believed that polymorphisms in the FSHR and ESR2 genes cause differences in the ovarian response and folliculogenesis [9]. FSHR is a G protein-coupled receptor (GPCR) that leads to the activation of adenylate cyclase through its main signal transduction pathway by increasing intracellular levels of cyclic adenosine monophosphate [10,11].

It is well-known that single-nucleotide polymorphisms (SNPs) in genes that play a fundamental role in oogenesis and folliculogenesis have an impact on female reproduction. To date, two different mechanisms have been proposed for this effect. Specifically, this impact can be induced by changes in the biochemical properties of a protein or at the level of transcription, which subsequently affects the activity of the promoter of a specific gene [12,13]. ESR2 and FSHR are known to influence the number of mature oocytes; therefore, they can affect the outcomes of in vitro fertilization (IVF). Boudjenah et al. [3] described that the variant of FSHR (FSHR 2039 A > G) with a G allele at position 2039 may have no effect on young people; however, it might affect people at an older age. It was also observed that patients with an A allele variant in ESR2 (ESR2 1730 G > A) had a significantly higher number of mature oocytes.

Poor ovarian response (POR) can be precisely defined as occurring when two of the three clinical criteria proposed by the European Society of Human Reproduction and Embryology (ESHRE) are present. These criteria include advanced maternal age ($\geq 40$ years), a low antral follicle count (AFC; $\leq 3$ oocytes with conventional stimulation), and abnormal ovarian reserve test results (i.e., an anti-Müllerian hormone [AMH] level of 0.5–1.1 ng/mL) [13]. As a part of ART, gonadotropin therapy is used to stimulate ovarian function. This therapy has been reported to be successful in several aspects. With this background in mind, the present study was conducted to investigate the association of multiple variants of ESR2 and FSHR genes with POR among Jordanian women.

## Methods

The current study was carried out according to the Declaration of Helsinki guidelines and approved by the Institutional Review Board of King Abdulla University Hospital in Jordan (IRB No. 2912015). Furthermore, written informed consent was obtained from all subjects or their guardians before enrollment.

### 1. Study population

The cohort analyzed in this study has been described in detail elsewhere [14]. To summarize, 60 female partners of selected couples undergoing ART for infertility were enrolled in the present study. The mean age of the participants was $33.60 \pm 6.34$ years (range, 20–46 years). The study population was selected from couples referred to different medical centers in Jordan (King Hussein Medical Center, Islamic Hospital, Prince Rashid Hospital, and Al-Amal Maternity Hospital) to undergo controlled ovarian stimulation for IVF/intracytoplasmic sperm injection during 2014–2017. Patients with a history of endometrioma, ovarian surgery, and chemotherapy were excluded from the study.

Ultrasonography was performed on the second day of the menstrual cycle to evaluate the anatomical characteristics of the female reproductive system and to identify the AFC. On the third day of the menstrual cycle, 5 mL of venous blood was collected from each participant in two tubes, including 2.5 mL in a plain tube and 2.5 mL in a tube containing tripotassium ethylenediaminetetraacetic acid (K3-EDTA). The samples in the plain tube were immediately centrifuged to separate the serum and then used for the assessment of FSH and AMH following the manufacturer's recommendations (Beckman Coulter, San Jose, CA, USA). In addition, the blood samples in the K3-EDTA tubes were utilized to investigate the SNPs located in ESR2 and FSHR genes as shown in Table 1.

Women were included in the case group if they met two or more of the POR criteria defined by the ESHRE before the initiation of the study. The inclusion criteria were: (1) FSH level of > 10 mIU/mL on the third day of the menstrual cycle, (2) AFC of < 9, (3) AMH level of < 1.1 ng/mL, and (4) < 5 retrieved oocytes in metaphase II (MII). The subjects were divided into 10 groups based on these categories as summarized in Table 2.

**Table 1.** Summary of the four studied single-nucleotide polymorphisms

| dbSNP-ID | Sequence variation | Position | Consequence |
| --- | --- | --- | --- |
| rs1256049 | G > A | Chr 14:64257333(GRCh38.p12) | ESR2: synonymous variant |
| rs4986938 | G > A | Chr 14:64233098(GRCh38.p12) | ESR2: noncoding transcript variant |
| rs6165 | 919 A > G | Chr 2:48963902(GRCh38.p12) | FSHR: missen se variant (p.Thr307Ala) |
| rs6166 | 2039 A > G | Chr 2:48962782(GRCh38.p12) | FSHR: missense variant (p.Asn680Ser) |

dbSNP, single-nucleotide polymorphism database; Chr, chromosome; ESR2, estrogen receptor 2; FSHR, follicle-stimulating hormone receptor.

In addition, 60 age-matched healthy volunteers were included in the study as controls. The participants of the control group were selected from female partners with proven fertility (i.e., with normal laboratory test results showing the potential for normal pregnancy without medical assistance). A comparison between the laboratory results of the cases and controls is presented in Table 3.

## 2. DNA extraction and polymerase chain reaction-restriction fragment length polymorphism detection of four SNPs

Genomic DNA was isolated from the peripheral blood samples of the case and control groups using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruc-

tions. The concentration and purity of the isolated DNA were measured by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Polymerase chain reaction (PCR) was used to identify the 4 different SNPs in *ESR2* and *FSHR*.

Two of the SNPs were located on *ESR2*, and the other two were on *FSHR*, as shown in Table 4. Each SNP was covered by its own set of primers, and all four sets of primers were designed to detect the target SNP using Primer3 Input (version 0.4.0) based on the sequences obtained from NCBI for *ESR2* (NC_000014.9) and *FSHR* (NC_000002.12). The primers were synthesized at the Princess Haya Biotechnology Center of Jordan. Table 4 presents the primer sequences, product size, DNA variation, sequence variation, and PCR conditions for each SNP.

PCR was performed in a monoplex fashion for each primer set as indicated in Table 4. Specifically, PCR was carried out in a 0.2-mL PCR

**Table 2.** Number of included women in each selected category

| Category | No. of samples |
|---|---|
| 1. AFC/AMH | 4 |
| 2. MII/FSH | 9 |
| 3. AFC/MII | 13 |
| 4. FSH/AMH | 6 |
| 5. MII/AMH | 19 |
| 6. AFC/FSH/AMH | 1 |
| 7. MII/FSH/AMH | 1 |
| 8. AFC/MII/FSH | 3 |
| 9. AFC/MII/AMH | 3 |
| 10. AFC/MII/FSH/AMH | 1 |

AFC, antral follicle count; AMH, anti-Müllerian hormone; MII, metaphase II; FSH, follicle-stimulating hormone.

**Table 3.** Laboratory results of cases and controls

| Parameter | Infertile women with poor ovarian response (n = 60) | Control fertile women (n = 60) | *p*-value[a] |
|---|---|---|---|
| FSH (mIU/mL) | 19.55 ± 13.7 | 5.3 ± 0.93 | < 0.001 |
| AMH (ng/mL) | 0.344 ± 0.257 | 2.4 ± 0.47 | < 0.001 |
| AFC (< 9) | 3.52 ± 1.64 | | |
| MII (< 5) | 2.25 ± 1.27 | | |

Values are represented as mean±standard deviation.
FSH, follicle-stimulating hormone; AMH, anti-Müllerian hormone; AFC, antral follicle count; MII, metaphase II.
[a]Nonparametric Mann-Whitney test; statistical significance, $p<0.05$.

**Table 4.** Summary of the studied single-nucleotide polymorphisms

| Primer set | Primer sequence | Included polymorphism | | | PCR program |
|---|---|---|---|---|---|
| | | Product size | DNA variation | Sequence variation ID | |
| Set 1 | F: AGCTGAGGAGGAGGGGTG | 152 bp | rs1256049 | G > A | 97℃ for 30 sec |
| | R: CCGGGGTGGTCAATTGAG | | | | 55.6℃ for 30 sec |
| | | | | | 72℃ for 30 sec |
| | | | | | 35 Cycles |
| Set 2 | F: CCAGAACCCACAGTCTCAGT | 169 bp | rs4986938 | G > A | 97℃ for 30 sec |
| | R: GGTGGAGGGAAGGATGGTAC | | | | 52℃ for 30 sec |
| | | | | | 72℃ for 30 sec |
| | | | | | 45 Cycles |
| Set 3 | F: TCTGAGCTTCATCCAATTTGCA | 176 bp | rs6165 | A > G | 97℃ for 30 sec |
| | R: ACGTCAACCACTTCATTGCA | | | | 51℃ for 30 sec |
| | | | | | 72℃ for 30 sec |
| | | | | | 45 Cycles |
| Set 4 | F: CCCCTCATCACTGTGTCC | 374 bp | rs6166 | A > G | 97℃ for 30 sec |
| | R: GCACTGTCAGCTCTTTGTGAC | | | | 59.5℃ for 30 sec |
| | | | | | 72℃ for 30 sec |
| | | | | | 35 Cycles |

PCR, polymerase chain reaction; F, forward; R, reverse.

tube with a 20-μL reaction volume, containing 2 μL of template genomic DNA (~200 ng), 10 μL of 2X PCR Master Mix (New England Biolabs, Hitchin, UK), 2 μL (10 μmoL) of each primer, and 20 μL of nuclease-free water. The amplification reaction for each set was conducted in a programmable thermal cycler (Thermo Fisher Scientific) as shown in Table 4. Nuclease-free water was used instead of genomic DNA as a blank to check for any DNA contamination.

The generated PCR product was run on a 2% (w/v) agarose gel prepared in 1X Tris-borate-EDTA (Sigma-Aldrich, St. Louis, MO, USA), containing ethidium bromide (Promega Corp., Madison, WI, USA). Moreover, a 50-bp DNA ladder (GeneDireX Inc., Taoyuan, Taiwan) was concurrently applied with each electrophoretic run to confirm the product size. After electrophoresis at 120 V for 45 minutes, the results were visualized and recorded using the UVP GelDoc-It$^2$ 310 Imaging System (Thermo Fisher Scientific).

Restriction fragment length polymorphism (RFLP) was carried out on the cleaned PCR products using restriction endonuclease enzymes (New England Biolabs). To determine the genotype for each sample, the PCR product was incubated with different restriction endonuclease enzymes overnight at 37℃ (except for *Bsr*I, which was incubated overnight at 65℃), as shown in Table 5. The samples were then run on a 1% agarose gel at 90 V for 1 hour. Three samples of each polymorphism with different genotypes were sent to Macrogen Inc. in South Korea to be purified and sequenced for confirmation of the RFLP results.

### 3. Data analysis

Patients with *GG, GA*, and *AA* alleles were considered to be normal homozygous, heterozygous, and abnormal homozygous, respectively, except for the rs6165 substitution mutation, for which subjects

**Table 5.** Restriction endonuclease enzymes utilized for restriction fragment length polymorphism

| SNP ID | Enzyme name | Product size | |
| --- | --- | --- | --- |
| | | Genotype | Band size |
| rs1256049 | *Rsa*I | GG | 152 bp |
| | | GA | 152, 72, and 80 bp |
| | | AA | 72 and 80 bp |
| rs4986938 | *Alu*I | GG | 169 bp |
| | | GA | 169, 107, and 62 bp |
| | | AA | 107 and 62 bp |
| rs6165 | *CviKI*-1 | AA | 101, 69, and 6 bp |
| | | AG | 101, 69, 58, 43, and 6 bp |
| | | GG | 69, 58, 43, and 6 bp |
| rs6166 | *Bsr*I | AA | 374 bp |
| | | GA | 374, 239, and 135 bp |
| | | GG | 135 and 239 bp |

SNP, single-nucleotide polymorphism.

with *AA, AG*, and *GG* genotypes were regarded as normal homozygous, heterozygous, and abnormal homozygous, respectively. GraphPad Prism software ver. 7.0 (GraphPad, La Jolla, CA, USA) was used for the statistical analysis of Hardy-Weinberg equilibrium (HWE) [15]. All other statistical analyses were performed using version 8 of this software. The chi-square test, the Fisher exact tests (used when cells had counts of less than 5), and odds ratios (ORs) (variant homozygotes were compared to the sum of the homozygotes for the wild-type alleles plus heterozygotes) were utilized to compare differences between genotype frequencies. A *p*-value less than 0.05 was considered to indicate statistical significance, and 95% confidence intervals (CIs) were used to describe the strength of associations.

## Results

### 1. Study design and clinical/demographic characteristics

A total of 60 female partners of selected couples who underwent ovarian stimulation during IVF were included in the current study. The mean levels of AMH and FSH were 0.344 ± 0.257 ng/mL and 19.55 ± 13.7 mIU/mL, respectively. The mean AFC and number of MII oocytes were 3.52 ± 1.64 and 2.25+1.27 (range, 0–4) after stimulation, respectively.

### 2. Allele frequency distribution of the investigated genes

The SNPs of the two genes under investigation (*ESR2* [rs1256049 and rs4986938] and *FSHR* [rs6165 and rs6166]) were assessed in the case and control groups to determine whether the genotype frequencies of these polymorphisms were in HWE and to investigate the association of these polymorphisms with the development of POR. Table 6 tabulates the frequency of SNP genotypes in both the case and control groups.

The investigation of the genotype frequencies of the *ESR2* SNPs (rs1256049 and rs4986938) revealed that the majority of the infertile women (92.73%) were homozygotes for the wild-type allele (*GG*) of rs1256049, while 5.45% of the subjects were heterozygotes for the rs1256049 allele (*GA*) and 1.82% of the cases were homozygotes for the AA variant allele of rs1256049 (Table 6). The *AA* homozygous genotype was not observed in the control group. Regarding rs4986938, fewer than half of the subjects in the case (41.86%) and control (32.69%) groups were homozygotes for the wild-type allele (*GG*). Furthermore, 46.51% and 59.62% of the infertile and fertile women were heterozygotes for rs4986938 (*GA*), respectively. Additionally, 11.63% and 7.69% of the case and control groups were homozygotes for the *AA* variant allele of rs4986938, respectively.

Assuming random mating of the population in Jordan and applying the HWE for the distribution of the alleles, the observed genotype frequency of rs1256049 was significantly different from that

**Table 6.** Hardy-Weinberg equilibrium analysis of the four studied SNPs

| SNP ID | Genotype | Infertile women with poor ovarian response | | | | Control fertile women | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Observed (%) | Expected (%) | $\chi^2$ | $p$-value | Observed (%) | Expected (%) | $\chi^2$ | $p$-value |
| rs1256049 | GG | 92.73 | 91.12 | 13.83 | 0.001 | 93.22 | 93.33 | 0.12 | 0.941 |
| | GA | 5.45 | 8.68 | | | 6.78 | 6.55 | | |
| | AA | 1.82 | 0.21 | | | 0 | 0.11 | | |
| | G | 95.46 | | | | 96.61 | | | |
| | A | 4.54 | | | | 3.39 | | | |
| rs4986938 | GG | 41.86 | 42.4 | 0.06 | 0.972 | 32.69 | 39.06 | 7.39 | 0.025 |
| | GA | 46.51 | 45.43 | | | 59.62 | 46.88 | | |
| | AA | 11.63 | 12.17 | | | 7.69 | 14.06 | | |
| | G | 65.11 | | | | 62.5 | | | |
| | A | 34.89 | | | | 37.5 | | | |
| rs6165 | AA | 60.46 | 46.46 | 43.29 | < 0.001 | 55.53 | 44.83 | 22.58 | < 0.001 |
| | GA | 13.95 | 41.74 | | | 23.21 | 44.23 | | |
| | GG | 23.25 | 9.35 | | | 21.42 | 10.9 | | |
| | A | 69.05 | | | | 66.97 | | | |
| | G | 30.95 | | | | 33.03 | | | |
| rs6166 | AA | 28.57 | 33.67 | 4.388 | 0.112 | 41.67 | 36.006 | 5.57 | 0.062 |
| | GA | 58.89 | 48.688 | | | 36.67 | 47.998 | | |
| | GG | 12.5 | 17.6 | | | 21.66 | 15.996 | | |
| | A | 58.04 | | | | 60 | | | |
| | G | 41.96 | | | | 40 | | | |

SNP, single-nucleotide polymorphism.

predicted by HWE ($p = 0.001$) (Table 6). However, there was no statistically significant difference among the infertile women in terms of rs4986938 ($p = 0.972$) (Table 6). The HWE analysis of the *FSHR* SNPs (rs6165 and rs6166) revealed that more than half of the subjects in the case (60.46%) and control (55.35%) groups were homozygotes for the wild-type allele (*AA*) of rs6165. Moreover, 13.95% and 23.21% of the infertile and fertile women were heterozygotes for the rs6165 allele (*AG*), respectively, and 23.25% and 21.42% of the case and control groups were homozygotes for the *GG* variant allele of rs6165, respectively.

With regard to rs6166, fewer than a third of the infertile (28.57%) and fertile (41.67%) women were homozygotes for the wild-type allele (*AA*). In addition, 58.89% and 36.67% of the case and control groups were heterozygotes for the rs6166 allele (*AG*), respectively, and 12.50% and 21.66% of the infertile and fertile women were homozygotes for the *GG* variant allele of rs6166, respectively. The observed genotype frequency of rs6165 was significantly different from that expected based on HWE ($p \leq 0.001$) (Table 6) for both infertile and fertile women.

To determine significance of the associations of SNP allele and genotype frequencies with POR, the chi-square test was performed for genotypes, and $p$-values were calculated for each SNP. The results revealed no significant associations ($p \geq 0.05$) (Table 7), although it should be noted that rs6166 had a $p$-value very close to 0.05 ($p = 0.054$) (Table 7). Fur-

thermore, a comparison of allele frequency between the case and control groups demonstrated no significant difference in the four studied SNPs.

In addition, ORs were calculated for each polymorphism, with an OR of > 1 indicating an association between the homozygous variant of the allele and disease. Moreover, 95% CIs were calculated to indicate how reliable the ORs were in 95% of the occasions, with a wider interval indicating greater uncertainty. The ORs calculated for rs1256049, rs4986938, rs6165, and rs6166 were 1.07 (95% CI, 0.07–17.6), 1.58 (95% CI, 0.4–6.29), 1.15 (95% CI, 0.44–2.98), and 1.79 (95% CI, 0.82–3.87), respectively (Table 7).

## Discussion

It is generally believed that the outcomes of ART depend on how a woman responds to the administered gonadotropin dose. In this study, genetic variants in *FSHR* and *ESR2* genes were investigated in infertile Jordanian women with POR and control fertile women using RFLP and Sanger sequencing (as a confirmative method). Out of the four investigated SNPs (*ESR2* rs1256049, *ESR2* rs4986938, *FSHR* rs6165, and *FSHR* rs6166), the $p$-value for the difference between the two groups regarding the rs6166 SNP in the *FSHR* gene was very close to 0.05 ($p = 0.054$).

Previous studies have investigated the associations of genetic vari-

**Table 7.** Association of poor ovarian response with *ESR2* rs1256049 and rs4986938 and *FSHR* rs6165 and rs6166 alleles, and genotype frequencies

| SNP ID | Genotype | Poor ovarian response women | Control fertile women | $\chi^2$ | *p*-value | OR (95% CI) | Relative risk |
|---|---|---|---|---|---|---|---|
| | | Frequency (%) | Frequency (%) | | | | |
| rs1256049 | | (n = 55) | (n = 59) | 1.16 | 0.561 | 1.07 (0.07–17.6) | |
| | GG | 51 (92.73) | 55 (93.22) | | | | |
| | GA | 3 (5.45) | 4 (6.78) | | | | |
| | AA | 1 (1.82) | 0 | | | | |
| | G | 95.45 | 96.661 | 0.521 | 0.471 | 1.702 (0.433–6.556) | 1.347 |
| | A | 4.55 | 3.389 | | | | |
| | GG | 51 (92.72) | 55 (93.32) | 0.011 | 0.918 | 1.078 (0.301–3.861) | 1.038 |
| | GA+AA | 4 (7.27) | 4 (6.77) | | | | |
| rs4986938 | | (n = 43) | (n = 52) | 1.675 | 0.433 | 1.58 (0.4–6.29) | |
| | GG | 18 (41.86) | 17 (32.69) | | | | |
| | GA | 20 (46.51) | 31 (59.62) | | | | |
| | AA | 5 (11.63) | 4 (7.69) | | | | |
| | G | 65.11 | 62.5 | 0.087 | 0.768 | 0.917 (0.519–1.614) | 0.958 |
| | A | 34.88 | 37.5 | | | | |
| | GG | 18 (41.86) | 17 (32.69) | 0.850 | 0.357 | 1.482 (0.651–1.537) | 1.201 |
| | GA+AA | 25 (58.14) | 35 (67.31) | | | | |
| rs6165 | | (n = 43) | (n = 56) | 0.726 | 0.696 | 1.15 (0.44–2.98) | |
| | AA | 26 (60.46) | 31 (55.35) | | | | |
| | GA | 7 (13.95) | 13 (23.21) | | | | |
| | GG | 10 (23.25) | 12 (21.42) | | | | |
| | A | 68.6 | 66.96 | 0.092 | 0.762 | 1.096 (0.602–2.009) | 1.047 |
| | G | 31.39 | 33.03 | | | | |
| | AA | 26 | 31 | 0.260 | 0.610 | 0.811 (0.371–1.878) | 0.914 |
| | AG+GG | 17 | 25 | | | | |
| rs6166 | | (n = 56) | (n = 60) | 5.845 | 0.054 | 1.79 (0.82–3.87) | |
| | AA | 16 (28.57) | 25 (41.67) | | | | |
| | GA | 33 (58.89) | 22 (36.67) | | | | |
| | GG | 7 (12.5) | 13 (21.66) | | | | |
| | A | 58 | 0.6 | 0.083 | 0.774 | 0.921 (0.526–1.644) | 0.960 |
| | G | 42 | 0.4 | | | | |
| | AA | 16 | 25 | 2.174 | 0.140 | 1.786 (0.834–3.711) | 1.307 |
| | GA+GG | 40 | 35 | | | | |

*ESR2*, estrogen receptor 2; *FSHR*, follicle-stimulating hormone receptor; SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

ants in *ESR2* and *FSHR* with response to ovarian stimulation in various populations and presented variable and conflicting results. A review of the literature was carried out to identify studies that focused on the relationship of these genetic variants with various clinical parameters. Table 8 presents a summary of the collected data in this regard. Estrogen receptors (ERs) are nuclear receptors that bind to estrogen and act as transcription factors to induce follicle growth, oocyte maturation, and oocyte release, in addition to their role in uterine endometrial thickening and preparation for implantation [16,17]. Two ERs are known in humans: ERα, encoded by the *ESR1* gene, and ERβ, encoded by the *ESR2* gene [16,17]. Several genetic variants in ER genes have been linked to different ovarian dysfunctions [18,19]. The

genetic variants of rs1256049 in *ESR2* have not been extensively studied; however, this variant was reported to be associated with the amount of recombinant FSH administered and time of medication use [7]. In another study, no significant difference was observed between recurrent spontaneous abortion and rs1256049 genetic variants [20]. The rs4986938 variant occurs in a noncoding region of the *ESR2* gene, and its clinical significance is not reported in ClinVar [17,18].

Two recent studies addressing the role of rs4986938 variants in ovarian response in Middle Eastern populations came to different conclusions. In a study conducted in Egypt, it was observed that women homozygous for the rs4986938 A allele variant had a lower

number of retrieved oocytes after stimulation and a lower rate of clinical pregnancy [21]. However, in another study carried out in Iran, no association was reported between these SNP genotypes and re- sponse to ovarian stimulation [8]. The results of the present study are in line with the findings of the Iranian study, in which no association was observed between genetic variants in the rs4986938 SNP and

**Table 8.** Summary of allele frequencies and types of associations between the four studied SNPs and clinical measurements obtained in previous studies

| SNP ID | Reference | Country | No. of samples Association | | Allele frequency (%) | | p-value | Association |
|--------|-----------|---------|---------|------|-----|-----|---------|-------------|
| | | | Control | Case | G | A | | |
| ESR2 rs1256049 | [8] | Brazil | - | 136 | 94 | 6 | 0.001 | Women with the GG genotype needed more days of medication use |
| | | | | | | | 0.011 | The GG group used a higher amount of rFSH |
| | [16] | China | 182 | 196 | 68 | 32 | | Recurrent spontaneous abortion was not significantly associated with SNP genotypes. |
| ESR2 rs4986938 | [16] | China | 182 | 196 | 86 | 14 | | Recurrent spontaneous abortion was not significantly associated with SNP genotypes. |
| | [19] | Egypt | 111 | 105 | 55 | 45 | < 0.001 | Duration of stimulation, total dose of applied gonadotrophins, number of retrieved oocytes, number of transferred embryos, and clinical pregnancy rate were lower. |
| | | | | | | | < 0.001 | Mean AMH level and number of oocytes were lower in AA genotype patients. |
| | [9] | Iran | 106 | 92 | 66 | 34 | > 0.05 | No association was found between SNP genotypes and response to ovarian stimulation. |
| FSHR rs6165 | [22] | China | - | 450 | 33 | 67 | < 0.05 | Basal FSH level was higher in GG genotype patients than in AA and AG genotype patients on the third day of the menstrual cycle. |
| | | | | | | | 0.009 | AA genotype cases needed a longer time of stimulation than other groups. |
| | [23] | Germany | - | 148 | 51 | 49 | < 0.01 | A significant difference was observed between anovulatory patients and normoovulatory controls regarding SNP genotype frequencies. |
| | [24] | Italy | | 149 | 47 | 53 | 0.037 | Heterozygotes had a higher number of embryos. |
| | [19] | Egypt | 111 | 105 | 51 | 49 | < 0.001 | The GG genotype was 2.5-fold more common in poor responders than in good responders. |
| | [9] | Iran | 104 | 90 | 54 | 46 | < 0.05 | Total number of oocytes and levels of hormones (i.e., LH, FSH, and AMH) were significant in patients with the AA genotype than in those with other genotypes. |
| FSHR rs6166 | [22] | China | - | 450 | 31 | 69 | < 0.05 | Basal FSH level was higher in GG genotype patients than in AA and AG genotype patients on the third day of the menstrual cycle. |
| | | | | | | | 0.009 | GG genotype patients required a longer time of stimulation than other groups. |
| | [25] | Italy | 25 | 17 | 58 | 42 | 0.02 | A significant difference was observed between hyporesponders and controls regarding GG and GA genotypes. |
| | | | | | | | 0.04 | |
| | [21] | Greece | 33 | 41 | 45 | 55 | < 0.05 | Total amount of gonadotropins needed in patients with the AA genotype was higher than needed for GA and GG genotypes. |
| | | | | | | | 0.057 | AA genotype women needed more stimulation days. |
| | [26] | Germany | - | 93 | 53 | 47 | < 0.05 | Women with a GG genotype required higher FSH stimulation to overcome lower $E_2$ than women with the AA genotype. |
| | [27] | Spain | 83 | 19 | 43 | 57 | 0.04 | Frequency of the G allele was higher among the poor responders. |
| | [28] | China | - | 1,250 | 37 | 63 | < 0.01 | Basal FSH level and dose of exogenous FSH were higher in GG poor responders. |
| | | | | | | | < 0.05 | Follicular fluid $E_2$ level (on the day of hCG administration) and number of retrieved oocytes were lower in GG genotype individuals. |
| | [29] | Italy | 87 | 140 | 52 | 48 | < 0.05 | Basal $E_2$ was significantly higher in women with the AA genotype than in those with the AG genotype. |
| | | | | | | | 0.03 | The AG genotype was significantly associated with the highest number of collected oocytes. |

**Table 8.** Continued

| SNP ID | Reference | Country | Number of samples | | Allele frequency (%) | | p-value | Association |
| | | | Association | | | | | |
| | | | Control | Case | G | A | | |
|---|---|---|---|---|---|---|---|---|
| | [30] | South Korea | - | 263 | 35 | 65 | 0.001 | Third-day basal FSH levels were significantly higher in the *GG* group than in the *GA* and *AA* groups. |
| | | | | | | | 0.013 | Clinical pregnancy rate per embryo transfer was significantly higher in the *AA* group than in the *GA* or *GG* group. |
| | [31] | The Netherlands | - | 105 | 40 | 60 | 0.003 | Pregnancy rate and implantation rate in *GG* patients were three times higher than those in *AA* patients. |
| | [23] | Germany | - | 148 | 61 | 39 | < 0.05 | The FSH serum concentration was significantly higher in *GG* patients than those in *GA* and *AA* subjects. |
| | [32] | Spain | - | 145 | 62 | 38 | < 0.001 | The number of retrieved eggs was higher in *AA* genotype patients than in *GG* and *GA* genotype subjects. |
| | | | | | | | < 0.001 | Patients with the *GG* genotype required higher gonadotropin doses than those with *AA* and *AG* genotypes. |
| | | | | | | | < 0.001 | Women with *AG* and *AA* genotypes needed less time for stimulation than *GG* women. |
| | [33] | Greece | 46 | 79 | | | < 0.05 | Gonadotropin dose correlated significantly with the observed levels of third-day FSH and was higher in *GG* and *AA* genotype women than in women with the *AG* genotype. |
| | | | | | | | < 0.01 | Estrogen levels on the day of hCG administration were higher in the *AG* group. |
| | | | | | | | < 0.01 | The number of preovulatory follicles and collected oocytes in the *AG* genotype group was significantly higher than in groups with other genotypes. |
| | [34] | UK | - | 212 | | | > 0.05 | No statistically significant differences were observed in the number of mature retrieved oocytes, oocyte output rates, or fertilization rates among patients with different rs6166 genotypes; no significant difference was noted in the clinical pregnancy rate per transfer. |
| | [35] | UK | - | 73 | 49 | 51 | 0.045 | *AA* genotype patients produced higher concentrations of $E_2$ than *GG* genotype patients. |
| | | | | | | | 0.005 | Peak $E_2$ correlated with the mean cycle length in *AA* genotype patients |
| | | | | | | | 0.002 | Basal FSH was correlated with basal LH in *AA* genotype patients. |
| | | | | | | | 0.002 | Age at menarche was correlated with the mean days of stimulation in *AA* genotype patients. |
| | | | | | | | 0.001 | Peak $E_2$ concentration was correlated with the number of retrieved oocytes in *AA* genotype patients, and it showed a weak correlation in *GG* genotype patients. |
| | [36] | Germany | - | 161 | 49 | 51 | < 0.01 | Basal levels of FSH on the third day were significantly different among three genotypes. |
| | | | | | | | < 0.01 | The dose of FSH ampoules required for stimulation was different among the three genotypes. |
| | [4] | Iran | - | 108 | 47 | 53 | 0.022 | The number of retrieved oocytes in the *AA* group was higher than in the other groups. |
| | [37] | Japan | - | 522 | 36 | 64 | < 0.05 | Basal FSH levels in *AG* and *GG* patients were significantly higher than in *AA* patients. |
| | | | | | | | < 0.05 | The *AG* group needed a lower dose of hMG to achieve adequate follicular growth. |
| | | | | | | | < 0.05 | The *AA* and *AG* groups showed significantly higher levels of serum $E_2$ than the *GG* group. |
| | [24] | Italy | - | 149 | 42 | 58 | - | No significant difference was observed among different genotypes in terms of FSH and $E_2$ serum levels and ovarian response. |
| | [22] | China | - | 450 | 31 | 69 | < 0.05 | The *GG* group needed more days of induction. |

(Continued to the next page)

**Table 8.** Continued

| SNP ID | Reference | Country | Number of samples | | Allele frequency (%) | | p-value | Association |
|---|---|---|---|---|---|---|---|---|
| | | | Association | | | | | |
| | | | Control | Case | G | A | | |
| | | | | | | | < 0.05 | Patients with the *GG* genotype had significantly higher rates of poor response to stimulation. |
| | [38] | Poland | - | 22 | 36 | 64 | - | *AA* homozygotes had higher rhFSH-induced expression of *FSHR* than carriers of the *GG* genotype. |

SNP, single-nucleotide polymorphism; *ESR2*, estrogen receptor 2; rFSH, recombinant follicle-stimulating hormone; AMH, anti-Müllerian hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; E₂, estradiol; hCG, human chorionic gonadotropin; hMG, human menopausal gonadotropin; rhFSH, recombinant human follicle-stimulating hormone.

ovarian response.

*FSHR*, a member of the GPCR family, is expressed in the granulosa cells of the ovary and is considered essential for proper FSH action [28]. The rs6165 *A>G* SNP is a missense variant that causes a p. Thr307Ala amino acid substitution in the *FSHR* protein. All published studies have confirmed the association between this genetic marker and ovarian response. Specifically, it has been demonstrated that different rs6165 genotypes were associated with basal FSH levels, duration of stimulation [30], number of obtained embryos [23], and total number of retrieved oocytes [9,19].

The rs6166 A>G SNP is another well-studied SNP in the *FSHR* gene that causes a p.Asn680Ser missense variation. In a number of studies, rs6166 showed an association with basal FSH levels, the time required for stimulation [21-23,28,30], the number of retrieved oocytes [4,28,29,32,39], and implantation and pregnancy rates [40]. On the contrary, in other studies, rs6166 was reported to have no association with ovarian response, especially oocyte retrieval, pregnancy rate, and FSH levels [24,34,41]. The results of the present study are consistent with the findings of the majority of previous studies regarding the important role of this SNP in determining the response of women to ovarian stimulation.

The current study was the first attempt to investigate polymorphisms in *FSHR* and *ESR2* genes in a subset of the Jordanian Arab population. The allele and genotype frequencies were determined among women with POR and their normal counterparts. Minor allele frequencies (MAFs) were calculated for the studied SNPs in the control group, including rs1256049 MAF (A = 0.039), rs4986938 MAF (A = 0.375), rs6165 MAF (G = 0.33), and rs6166 MAF (G = 0.4). A comparison of the rs6165 MAFs obtained in this study with those reported in other studies revealed that our values were similar to those reported for populations of European and Asian origins. However, they were different from the values obtained for populations of African origin (Table 8) [25].

The rs6166 MAF was also similar to those reported for many European and Asian populations (Table 8) [42,43]. Nevertheless, only 1 study could be found regarding the Middle Eastern Arab population,

which was conducted in Bahrain and reported a MAF of almost 0.5 [27]. Discrepancies among the results of various studies could be due to differences in cohort sample size, ethnicity, population stratification, and frequency of consanguinity. There are no isolated communities in Jordan; however, this country has a high rate of consanguineous marriage (20%–59%) [33]. This high consanguinity rate could explain why the genotype frequencies of the studied SNPs were out of HWE (Table 5).

The limited number of the study population and heterogeneous patients with POR are the major limitations of this study. Every year, millions of couples seek medical assistance due to infertility problems. In many ART cycles, the lack of a normal response to stimulation affects fertilization and pregnancy outcomes. Although ART is a very common therapeutic procedure in Jordan, studies on infertility and the causes of POR remain limited. The present study assessed the role of four genetic variants in two important genes, namely *ESR2* and *FSHR*. Based on the results, only one of these variants (*FSHR* rs6166) should be further studied and evaluated as a marker of POR in Jordanian women. In the present study, the association between *FSHR* rs6166 and POR was not statistically meaningful, but the present results suggest that statistical significance may be observed in a further study with a larger number of patients.

## Conflict of interest

No potential conflict of interest relevant to this article was reported.

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