

## ORIGINAL ARTICLE

# Effect of *GnRHR* polymorphisms on *in vitro* fertilization and embryo transfer in patients with polycystic ovary syndrome

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We investigated the relationship between gonadotropin-releasing hormone receptor (*GnRHR*) gene polymorphisms and outcome of patients with polycystic ovary syndrome (PCOS) undergoing *in vitro* fertilization and embryo transfer (IVF-ET). PCOS patients undergoing IVF-ET were selected, and infertile patients due to dysfunctional oviducts served as controls. *GnRHR* gene polymorphisms were detected using the polymerase chain reaction-restriction fragment length polymorphism assay. Gene–gene interaction and linkage disequilibrium tests were performed using the SHEsis software. Logistic regression analysis was performed to evaluate factors affecting outcome of patients undergoing IVF-ET. The PCOS group showed more patients with CC+CT genotypes rs12644822, rs3756159 and rs13138607 than the control group, and CC+CT genotypes and C alleles from three positions enhanced risk of PCOS. Patients with CC+CT genotypes from three positions exhibited increased serum luteinizing hormone (LH), LH/follicle-stimulating hormone (FSH), testosterone (T) and follicles than those with TT genotypes. The haplotype analysis indicated that CCC, CCT and TCC haplotypes increased the risk of PCOS, while TCT, TTC and TTT haplotypes lowered the risk. After IVF-ET treatment, patients with CC+CT genotypes of three positions in the *GnRHR* gene had a lower pregnancy rate than patients with TT genotypes. Logistic regression analysis indicated that CC+CT genotypes rs12644822, rs3756159 and rs13138607 were risk factor for patients undergoing IVF-ET. In conclusion, these findings demonstrate that CC+CT genotypes rs12644822C>T, rs3756159C>T and rs13138607C>T in the *GnRHR* gene may contribute to a decreased pregnancy rate for PCOS patients after IVF-ET.

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## INTRODUCTION

Polycystic ovary syndrome (PCOS) is often characterized by hyperandrogenism, hyperinsulinemia, insulin resistance, ovulatory dysfunction and polycystic ovaries. It is a complex and heterogeneous disorder that occurs in reproductive aged women. It can lead to defects in reproduction and metabolism.<sup>1,2</sup> The prevalence of PCOS is estimated to be as high as 15–20% according to the European Society for Human Reproduction and Embryology and American Society for Reproductive Medicine.<sup>3</sup> PCOS has a complex and unclear pathogenesis and risk factors, including obesity, genetics and early childhood growth patterns.<sup>1,4,5</sup> According to a prior meta-analysis, women with PCOS face an elevated risk of developing serious pregnancy complications such as preeclampsia and pregnancy-induced hypertension.<sup>2,6</sup> Furthermore, PCOS is reported to be the most common factor for anovulatory infertility and is responsible for women pursuing fertility treatment.<sup>7</sup> PCOS also leads to long-term metabolic complications such as obesity, diabetes and cardiovascular disease.<sup>1</sup> Studies show that women with PCOS are more likely to have increased coronary artery calcium scores and increased carotid intima–media thickness.<sup>8,9</sup> It is

believed that proper diagnosis and treatment of PCOS is essential in preventing future endocrine, metabolic and cardiovascular complications.<sup>3</sup>

Gonadotropin (Gn)-releasing hormone receptor (*GnRHR*) is a member of the G-protein-coupled receptors (GPCRs) and is mainly expressed in the gonadotrope membrane of the anterior pituitary.<sup>10,11</sup> The binding of *GnRH*, *GnRHR*-associated G proteins, can activate several signaling cascades downstream including the inositol 1,4,5-triphosphate, mitogen-activated protein kinases, diacylglycerol and adenylyl cyclase pathways.<sup>12</sup> The expression of *GnRH* and its receptor has been clearly observed in numerous extrapituitary tissues such as cancer cell lines, ovary, placenta, breast and so on.<sup>13</sup> However, the effects of *GnRHR* on extrapituitary tissues, particularly the ovary, remain ambiguous. The high prevalence of PCOS was found in family lines suggesting a genetic etiology.<sup>14</sup> Furthermore, PCOS has a complicated and uncertain pathogenesis and is inherently difficult to study genetically.<sup>4</sup> In the present study, we discuss the relationship between *GnRHR* gene polymorphisms, steroid hormones and the

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outcome of IVF-ET in patients with PCOS. Finally, we hypothesized a molecular basis for the therapeutic function of IVF-ET.

## MATERIALS AND METHODS

### Ethics statement

This study was approved by the Ethics Committee of Linyi Cancer Hospital. All patients signed informed consent forms.

### Study subjects

From 2012 to 2014, 634 cases of infertility patients underwent IVF-ET at the Linyi Cancer Hospital. A total of 314 of these cases were recruited as the PCOS group, and the remaining 320 non-PCOS cases who received IVF-ET treatment at the Linyi Cancer Hospital were treated as the control group. The inclusion criteria<sup>15</sup> for the PCOS group were: (1) female aged between 21 and 38 years old; (2) infertile patients diagnosed with PCOS or PCOS complicated with tubal factor; (3) patients able to ovulate after follicle-stimulating hormone (FSH) treatment. Inclusion criteria for the control group were: (1) infertility caused by dysfunctional oviducts; (2) patients with regular menstrual cycle and normal endocrine parameters; (3) patients have no clinical manifestation or biological/chemical performance of hyperandrogenism; (4) patients have no manifestation of polycystic ovaries under mode-B ultrasonography. The exclusion criteria for both PCOS and control groups were: (1) patients have abnormality in the reproductive tract; (2) patients have endometriosis or uterine adenomyosis; (3) both husband and wife have known chromosomal abnormality; (4) patients are unable to become pregnant due to male infertility.<sup>16</sup> PCOS is often diagnosed with manifestations including oligomenorrhea or amenorrhea, hyperandrogenism or hyperandrogenemia and polycystic ovarian changes (in accordance with the criteria created by experts from the European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine on the Rotterdam meeting).<sup>17</sup> The diagnosis of tubal factor infertility was confirmed through hysterosalpingography or laparoscopy.

### IVF-ET therapeutic regimen and serum/follicular fluid collection

In the midluteal phase (patients with oligomenorrhea or irregular menstruation received artificial cycle treatment) of the last menstrual cycle before IVF-ET treatment, PCOS patients were treated with long-acting Gn-releasing hormone agonist and injected with imported leuprorelin acetate microspheres (1.35–1.875 mg) (Takeda Chemical Industries, Osaka, Japan). On the third day of the menstrual cycle, patients were examined to see if they met the pituitary downregulation standard (in the transvaginal ultrasound detection, the endometrium thickness is no more than 5 mm; the diameter of the largest follicle in the bilateral ovaries is <10 mm; the content of serum luteinizing hormone (LH) is no more than  $5 \text{ IU l}^{-1}$  and the estrogen (E2) content is  $<50 \text{ pg ml}^{-1}$ ). If met the standard, patients were treated with daily intramuscular injection of Gn (75 U), imported FSH (75 U) (IBSA Institut Biochimique SA, Lugano, Switzerland) and FSH (75 U) (Li Min Pharmaceutical Factory of Livzon Pharmaceutical Group, Zhuhai, Guangdong Province, China). Patients underwent regular transvaginal ultrasonography and LH, E2 and progesterone levels were monitored to determine uterus and follicle development. The Gn dose was adjusted over time according to the number of follicles, level of serum E2, growth rate of follicles and ovarian response. If there was a dominant follicle with a diameter of 18 mm, two follicles with a diameter of 17 mm or the serum E2 level reached  $200\text{--}300 \text{ pg ml}^{-1}$  in a dominant follicle, patients were treated with intramuscular injection (5000–10 000 U) of human chorionic Gn (HCG) (Li Min Pharmaceutical Factory of Livzon Pharmaceutical Group). This injection occurred at 8 p.m. to 10 p.m. on the same day, and 36–38 h after the HCG injection, follicle puncture was performed under the guidance of a transvaginal ultrasonography for oocyte retrieval. After 3–4 h of culturing, the oocyte–cumulus–corona complex was moved to the *in vitro* fertilization culture liquid and an *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) was performed according to the sperm status. The fertilization status was assessed 16–20 h later. The oocytes were then transferred in oil-capped culture droplets and cultured in an incubator at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  and 20%  $\text{O}_2$ . The embryo development was observed on the second and third day. Three days after fertilization, two embryos that had high morphological scores were selected for transplantation and the other transplantable embryos were frozen

and reserved. Progesterone (40 mg per day) was intramuscularly injected or orally taken in combination with progesterone tablets (Duphaston; Solvay Pharmaceuticals BV, Weesp, The Netherlands) at a dose of 20–40 mg per day for luteal phase support. This process lasted for 17 days from the day of oocyte retrieval. On the fourteenth day after embryo transfer, the HCG was detected for pregnancy determination. If a positive result was detected, luteal support was prolonged until clinical pregnancy was confirmed by transvaginal ultrasonography 2–3 weeks later when the gestational sac, yolk sac and primitive heart tube pulsation in the uterine cavity was observed. The E2 value, number of eggs, number of fertilized eggs, effective HCG embryos and occurrence rate of ovarian hyperstimulation syndrome (OHSS) was also recorded on that day. The criteria of OHSS were defined as reported previously.<sup>18</sup> Intrauterine embryo transfer was performed 48–72 h after oocyte retrieval. Two embryos were conventionally transplanted and then the transplant cancellation rate, clinical pregnancy rate, abortion rate and child birth weight were recorded.

On the second or third day of menstruation and on the day of HCG injection, 4 ml of venous blood was obtained from all patients after fasting in the morning. The blood sample was kept at room temperature for 30 min and then centrifuged ( $2000 \text{ r.p.m. min}^{-1}$ ) at room temperature for 10 min. The upper serum was transferred into a sterile EP tube, sealed with sealing film and then stored in a refrigerator at  $-80^\circ\text{C}$  for the later detection of hormone levels. The Gn treatment was stopped and HCG (r-HCG; Merck Serono, Geneva, Switzerland) was injected when one dominant follicle with a diameter of 18 mm or two follicles with a diameter of 17 mm or three follicles with a diameter of 16 mm appeared. When promoting ovulation, the amount of FSH and human menopausal Gn was adjusted in accordance with the follicular development.

Guided by the transvaginal ultrasonography, the follicular fluid (which contained follicles with a diameter  $\geq 15 \text{ mm}$ ) was collected via negative pressure suction. The first tube of follicle liquid without any blood or flushing liquid was immediately centrifuged ( $3000 \text{ r.p.m. min}^{-1}$ ) at room temperature for 10 min. The supernatant was placed into a sterile tube, sealed with sealing film, labeled and stored in a refrigerator at  $-80^\circ\text{C}$  for further use.

### Hormone determination in serum and follicular fluid

Before the experiment, all samples were thoroughly mixed at room temperature (about  $25^\circ\text{C}$ ) and assigned into the PCOS or control group. Electrochemical immunoassay (2010 Automation Instrument; Boehringer Mannheim GmbH, Mannheim, Germany) was performed to determine the E2, progesterone and LH levels in the serum. The variation within one group or between any two groups was  $<10\%$ . The follicular fluid was diluted (1:100 or 1:10 000, respectively) according to the results of the preliminary experiments and FSH, prolactin and testosterone levels in the serum. Follicles were determined using the Enzyme-Linked Immunosorbent Kit (DRG Instruments, Marburg, Germany; DRG International, NJ, USA).

### DNA extraction from whole blood

After informed consent was obtained, 4 ml of peripheral venous blood was drawn from all study subjects. It was then reserved in negative pressure non-anticoagulated blood tubes and labeled with the number and name of the patient. After 10 min of centrifugation at  $3000 \text{ r.p.m. min}^{-1}$ , the whole blood DNA was extracted from the peripheral blood using a DNA Extraction Kit (Takara Bio, Shiga, Japan). Its concentration was detected using a DNA Concentration Detector (NANODROP2000) (Thermo Fisher Scientific, Waltham, MA, USA) and its purity was detected using  $\text{OD}_{260 \text{ nm}}$ . The concentration was  $100 \pm 20 \text{ ng } \mu\text{l}^{-1}$  and  $A_{260 \text{ nm}}/A_{280 \text{ nm}}$  was between 1.6 and 1.8. The extracted DNA was stored at  $-20^\circ\text{C}$ .

### Genotyping identification

The primers were synthesized by Lejin Biotech (Nanjing, China).<sup>19</sup> Detailed primer sequences<sup>19</sup> are shown in Table 1. The Polymerase Chain Reaction (PCR) Kit was purchased from Shanghai Univ Biotechnology (Shanghai, China). The total reaction system was 20  $\mu\text{l}$ , made up of 1  $\mu\text{l}$  of extracted DNA, 1  $\mu\text{l}$  of upstream and downstream primers ( $10 \text{ pmol ml}^{-1}$ ), 10  $\mu\text{l}$  of PCR mixture and 7  $\mu\text{l}$  of deionized water. The reaction conditions were: pre-denaturation at  $95^\circ\text{C}$  for 5 min, followed by a total of 40 cycles of denaturation at

**Table 1** Primer sequences for qRT-PCR

Primer	Sequence (5'–3')	Length (bp)
Rs12644822C>T	F:TGTCTCTGCTTCACCTTCCTCAACCATA	26
Rs12644822C>T	R:AGAATGCCTTGAAGGGATTGGGAAATA	28
Rs3756159C>T	F:TTCAACAACCTAGAGCTCCTCAAATGCG	26
Rs3756159C>T	R:TGTAGCATACAGAGAATGCCTTGAAGGG	29
Rs13138607C>T	F:TATTTGTATGCTTTCCAATGGTTATCC	25
Rs13138607C>T	R:CTGAGCTCCTTTTGTACTGACTATAT	32

Abbreviations: F, forward; qRT-PCR, quantitative real-time polymerase chain reaction; R, reverse.

95 °C for 30 s, annealing at 65 °C for 40 s and extension at 72 °C for 1 min; and a last extension at 72 °C for 10 min. The samples were added into an even mixture of 3% agarose gel (containing 0.5 µg ml<sup>-1</sup> ethidium bromide), 4 µl of amplification products and 1 µl of bromophenol blue buffer. This was followed by electrophoresis (75 V) for 15 min in 0.5×Tris-boric acid-ethylenediaminetetraacetic acid buffer. Subsequently, the amplified bands were observed under ultraviolet light. The PCR products of rs12644822C>T, rs3756159C>T and rs13138607C>T positions were digested using restriction enzyme *Hga*I (37 °C), *Sml*I (37 °C) and *Hpy*CH4IV (37 °C) (purchased from Beijing Kingnor Agtech Corp., Beijing, China) in accordance with the product specifications. Next, electrophoresis was performed for 40 min in 120 V in 3.5% agarose gel and the amplified bands were once again observed under an ultraviolet light. Randomly selected samples (15%) were sent to Sangon Biotech (Shanghai, China) for bidirectional sequence detection to verify the results of the polymerase chain reaction-restriction fragment length polymorphism.

### Statistical analysis

All data were analyzed using the SPSS 20.0 Software (SPSS, Chicago, IL, USA). Quantitative data were expressed as mean ± s.d. Measurement data between two groups were validated using the *t*-test and data comparisons between three groups were detected by one-way analysis of variance. Categorical data were expressed as percentage and were compared using  $\chi^2$ . The genotype distribution and allele frequency of the control and PCOS groups were obtained by direct counting and verified using the Hardy–Weinberg Equilibrium. The Shesis Software (<http://analysis.bio-x.cn/myAnalysis.php>) was used to analyze haplotypes. Non-conditional logistic regression was used to analyze the risk factors and then the odds ratio and 95% confidence intervals were calculated. A *P*-value < 0.05 is considered statistically significant.

## RESULTS

### The PCOS group and the control groups are comparable

There was no significant difference in the age, duration of infertility and basal serum FSH, E2 and prolactin levels between the PCOS and control groups (*P* > 0.05). Body mass index, basal serum LH, LH/FSH and testosterone levels and the number of basal follicle were all significantly lower in the control group than those in the PCOS group (all *P* < 0.05) (Table 2).

### The PCOS group has higher occurrence rate of OHSS and transplantation cancellation rate than the control group

There was no significant difference in the Gn dosage and stimulation time between the PCOS and control groups (*P* > 0.05). The PCOS group had a significantly higher serum progesterone level before HCG injection, a greater amount of retrieved oocytes and embryos and a lower fertilization rate than the control group (all *P* < 0.05). There was no significant difference in pregnancy or abortion rate between the two groups (both *P* > 0.05). The PCOS group had a significantly higher occurrence rate of OHSS and transplantation cancellation rate than the control group (both *P* < 0.05) (Table 3).

### CC+CT genotypes and C alleles from rs12644822C>T, rs3756159C>T and rs13138607C>T enhanced the risk of PCOS

The frequency distribution of rs12644822C>T, rs3756159C>T and rs13138607C>T genotypes in the *GnRHR* gene was consistent with the results of Hardy–Weinberg equilibrium test (*P* > 0.05). This suggested that the genotype frequency of three positions in the sample was in equilibrium and was a good representation of the population. According to the correlation analysis, the CC+CT genotypes and their alleles in the rs12644822C>T, rs3756159C>T and rs13138607C>T showed a significantly increased risk of PCOS than the TT genotypes and their alleles. This suggested that there was a significant correlation between *GnRHR* polymorphisms and PCOS (all *P* < 0.05). It also suggested that the C alleles of the three positions were risk factors for PCOS (Table 4).

### CT+CC genotypes are associated with the clinicopathological characteristics of PCOS patients

There was no evident difference in average age, body mass index, duration of infertility and basal serum FSH, E2 and prolactin levels between the TT genotypes and (CC+CT) genotypes in rs12644822C>T, rs3756159C>T and rs13138607C>T (all *P* > 0.05). However, the CC+CT genotypes displayed higher basal serum LH, LH/FSH and testosterone levels and a greater number of basal follicle than the TT genotypes (all *P* < 0.05) (Table 5).

### CCC, CCT and TCC are risk factors of PCOS, and TCT, TTC and TTT are protective factors of PCOS

The rs12644822C>T, rs3756159C>T and rs13138607C>T haplotypes in the *GnRHR* gene of both groups were analyzed using the Shesis Software (any haplotype with a frequency < 0.03 was excluded). It was found that there were seven common haplotypes and among these haplotypes, there was a significant difference in the frequency distribution of CCC, CCT, TCC, TCT, TTC and TTT between the two groups (all *P* < 0.05). Moreover, it was demonstrated that CCC, CCT and TCC were risk factors for PCOS and that TCT, TTC and TTT were protective factors for PCOS. There was no distinct difference in the frequency of CCT between the two groups (all *P* > 0.05) (Table 6).

### CT+CC genotypes are associated with lower pregnancy rate of patients after IVF-ET

Out of the 314 PCOS patients who underwent IVF-ET treatment, there were 115 confirmed to be successful pregnancy cases that were classified into a pregnancy group and 199 to be non-pregnancy cases that were classified into a non-pregnancy group (Table 7). We found that patients with CT+CC genotypes of the three positions had a lower pregnancy rate after IVF-ET treatment than patients with TT genotypes (*P* < 0.05). The results indicated that *GnRHR* polymorphisms were associated with lower pregnancy rate of patients after IVF-ET.

### CT+CC genotypes of rs12644822C>T, rs3756159C>T and rs13138607C>T are risk factors influencing the outcome of PCOS patients after IVF-ET

Using pregnancy outcome and the three positions genotypes as dependent variables, logistic regression analysis was performed to further study the relationship between *GnRHR* polymorphisms and outcome of PCOS patients after IVF-ET treatment (Table 8). It was found out that rs12644822C>T, rs3756159C>T and rs13138607C>T from the CT+CC genotypes were risk factors influencing the outcome of PCOS patients undergoing IVF-ET.

**Table 2** Baseline characteristics of subjects between the PCOS group and the control group

	PCOS group (n = 314)	Control group (n = 320)	P-value
Age (years)	32.56 ± 3.70	32.13 ± 4.04	0.163
Years of infertility	5.67 ± 1.98	5.92 ± 2.15	0.129
BMI (kg m <sup>-2</sup> )	23.62 ± 5.14	22.32 ± 5.65	0.003
Number of basal follicle	23.42 ± 4.40	14.07 ± 2.24	<0.001
LH (pg ml <sup>-1</sup> )	7.23 ± 1.16	4.68 ± 1.15	<0.001
FSH (pg ml <sup>-1</sup> )	6.12 ± 1.71	5.96 ± 1.13	0.164
LH/FSH	1.32 ± 0.74	0.78 ± 0.05	<0.001
E2 (pg ml <sup>-1</sup> )	32.24 ± 3.52	31.86 ± 5.23	0.285
PRL (ng ml <sup>-1</sup> )	18.63 ± 2.65	19.14 ± 4.65	0.091
T (ng ml <sup>-1</sup> )	0.62 ± 0.11	0.44 ± 0.13	<0.001

Abbreviations: BMI, body mass index; E2, estrogen; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PCOS, polycystic ovary syndrome; PRL, prolactin; T, testosterone.

**Table 3** The outcomes of PCOS patients after IVF-ET between the PCOS and control groups

Outcome	PCOS group (n = 314)	Control group (n = 320)	P-value
Gonadotropin dosage	25.77 ± 8.35	27.39 ± 6.47	0.064
Stimulation time	12.39 ± 1.60	12.68 ± 2.79	0.110
Number of oocytes	17.46 ± 3.32	9.47 ± 3.00	<0.001
Number of embryos	10.68 ± 2.45	5.62 ± 1.20	<0.001
Fertilization rate (%)	170 (54.1)	229 (71.6)	<0.001
Clinical pregnancy rate (%)	115 (36.6)	148 (46.25)	0.014
Abortion rate (%)	24 (17.78)	23 (15.54)	0.614
Occurrence rate of OHSS (%)	67 (21.34)	20 (6.25)	<0.001
Transplantation cancellation rate (%)	62 (19.75)	12 (3.75)	<0.001
Progesterone (ng ml <sup>-1</sup> )	2.15 ± 0.26	1.63 ± 0.31	<0.001

Abbreviations: IVF-ET, *in vitro* fertilization and embryo transfer; OHSS, ovarian hyperstimulation syndrome; PCOS, polycystic ovarian syndrome.

## DISCUSSION

In the present study, we found that the CT+CC genotypes of rs12644822C>T, rs3756159C>T, rs13138607C>T increased the risk of PCOS and were risk factors influencing the outcome of PCOS patients undergoing IVF-ET.

Our study revealed that patients in the PCOS group had higher BMI, basal serum LH, LH/FSH and testosterone levels and a larger number of basal follicle than the control group. PCOS is regularly associated with obesity and being overweight.<sup>7</sup> Obese women with PCOS are reported to have less retrieved oocytes, lower clinical pregnancy rates and higher miscarriage rates than non-obese patients with PCOS.<sup>20,21</sup> This result may be explained by the pathogenesis of PCOS as it is regarded as an endocrine disease that can naturally lead to obesity and a hyperstimulated status.<sup>22</sup> Studies have shown that obesity can affect the size and quality of oocytes and embryos in PCOS women.<sup>23,24</sup> Huang *et al.*<sup>7</sup> reported that this may be associated with insulin resistance, elevated leptin levels and the alteration of follicle androgen profiles. Increased circulating LH levels are a common biochemical feature of PCOS and a recent study revealed that LH mutation may influence the susceptibility to PCOS.<sup>25</sup> Diurnal alteration of LH serum concentration is often high in women with PCOS. Previous data have demonstrated that insulin strengthens the

amplitude of serum LH pulse opposed to its frequency in overweight women with PCOS.<sup>26</sup> Although testosterone levels in the serum decrease as women with PCOS grow older, they still remain higher than those of the healthy individuals.<sup>27</sup>

Many factors can potentially affect the outcome IVF-ET treatment such as FSH, progesterone and E<sub>2</sub>.<sup>28</sup> Our results demonstrated that the PCOS group had a higher progesterone level in the serum before HCG injection, more retrieved oocytes and embryos, higher occurrence rates of OHSS and transplantation cancellation, as well as a downregulated fertilization rate compared with the control group. Dai *et al.*<sup>29</sup> reported that the progesterone levels on the day of HCG injection had no association with the clinical pregnancy rate, but the duration of progesterone elevation before HCG injection does have a negative association with the clinical pregnancy rate. According to Li and co-workers,<sup>30</sup> an increased progesterone level on the day of HCG injection may affect the outcome of IVF-ET treatment. This hypothesis is based on evidence that patients with an increased progesterone level displayed lower clinical pregnancy and embryo implantation rates compared with patients whose progesterone level did not increase. Implantation is an important step for the successful application of assisted reproduction techniques.<sup>31</sup> Three main factors have been reported to affect its effectiveness, namely, embryo quality, endometrial receptivity (ER) and a well-balanced embryo–endometrium interaction.<sup>32,33</sup> Under assisted reproduction techniques cycles, controlled ovarian hyperstimulation has an adverse effect on endometrial receptivity; however, this was mediated by the supraphysiologic levels of E2 and progesterone in the follicular phase. These altered hormone levels can lead to an asynchrony between the endometrium and transferred embryos, thus generating an endometrial environment that may result in implantation failure.<sup>34,35</sup>

The most important finding in our study is that CC+CT genotypes of the rs12644822C>T, rs3756159C>T and rs13138607C>T in the *GnRHR* gene increase the risk of PCOS, and that these three positions are all risk factors that affect the outcome of PCOS patients undergoing IVF-ET. Polymorphisms of the *GnRHR* gene were detected using polymerase chain reaction–restriction fragment length polymorphism. Most mutations in the *GnRHR* gene were demonstrated to be responsible for multiple reproductive genetic disorders.<sup>36</sup> A study conducted by Wu *et al.*,<sup>37</sup> showed that ducks with the CT or CC genotypes had earlier egg laying ages and laid a greater number of eggs than ducks with the TT genotype of the *GnRHR* gene. This suggests that *GnRHR* polymorphisms have an important role in reproduction. Similar reproductive traits were also reported in humans and bovines with *GnRHR* polymorphisms.<sup>38–40</sup> Based on a study on Holstein bulls, Yang *et al.*<sup>41</sup> reported that *GnRHR* may serve as a potential marker for improvement in sperm quality, and that bulls with GA or CT genotypes should be selected for breeding. There is a speculation that polymorphism (C177T) existing in the regulatory regions of genes that exert an effect on the potential promoter and transcriptional start sites. This may affect the role of *GnRHR* in follicle growth and development.<sup>37</sup> Moreover, a recent study suggested that *GnRHR* polymorphisms (rs12644822C>T, rs3756159C>T and rs13138607C>T) have a potential role in the pathogenesis of breast cancer.<sup>19</sup> On the basis of such a speculation, we can naturally predict the potential role of these three polymorphisms in PCOS. Previously, researchers observed that one or two copies of the Ser allele are involved in the regulation of hyperandrogenism and carbohydrate metabolism by mildly decreasing testosterone and increasing insulin levels.<sup>42</sup> Furthermore, positive links between reproduction outcomes and the *GnRHR* gene may indicate that IVF-ET is a good treatment option. However, the specific mechanism behind this process remains

**Table 4 Allele frequency and genotype distribution of rs12644822, rs3756159 and rs13138607 in the GnRHR gene**

SNP	PCOS group (n = 314)	Control group (n = 320)	P-value	OR	95% CI
<i>Rs12644822</i>					
TT	35 (11.15%)	101 (32.17%)			Reference
CT	174 (55.41%)	154 (49.04%)	<0.001	3.260	2.097–5.070
CC	105 (33.44%)	65 (20.70%)	<0.001	4.662	2.846–7.636
CT+CC	279 (88.85%)	219 (69.75%)	<0.001	3.676	2.408–5.614
T allele	244 (38.85%)	356 (55.63%)			Reference
C allele	384 (61.15%)	284 (44.38%)	<0.001	1.973	1.577–2.468
<i>Rs3756159</i>					
TT	25 (7.96%)	97 (30.89%)			Reference
CT	190 (60.51%)	172 (54.78%)	<0.001	4.286	2.637–6.966
CC	99 (31.53%)	51 (16.24%)	<0.001	7.532	4.326–13.110
CT+CC	289 (92.04%)	223 (71.02%)	<0.001	5.028	3.133–8.071
T allele	240 (38.22%)	366 (57.19%)			Reference
C allele	388 (61.78%)	274 (42.81%)	<0.001	2.159	1.725–2.703
<i>Rs13138607</i>					
TT	17 (5.41%)	92 (29.30%)			Reference
CT	215 (68.47%)	180 (57.32%)	<0.001	6.464	3.714–11.250
CC	82 (26.11%)	48 (15.29%)	<0.001	9.245	4.932–17.330
CT+CC	297 (94.59)	228 (72.61%)	<0.001	7.050	4.085–12.160
T allele	249 (39.65%)	364 (56.88%)			Reference
C allele	379 (60.35%)	276 (43.13%)	<0.001	2.007	1.605–2.511

Abbreviations: CI, confidence intervals; OR, odds ratio; PCOS, polycystic ovarian syndrome; SNP, single-nucleotide polymorphism.

**Table 5 The relationship between clinicopathological characteristics of PCOS patients and rs12644822, rs3756159 and rs13138607**

	<i>Rs12644822</i>			<i>Rs3756159</i>			<i>Rs13138607</i>		
	TT (35)	CT+CC (279)	P-value	TT (25)	CT+CC (289)	P-value	TT (17)	CT+CC (297)	P-value
Age (years)	32.13±4.04	32.52±2.70	0.450	33.13±4.14	32.26±3.20	0.205	32.14±3.04	32.52±3.10	0.623
Years of infertility	5.82±1.15	5.67±1.38	0.538	5.72±1.15	5.76±1.28	0.880	5.42±1.15	5.62±1.78	0.648
BMI (kg m <sup>-2</sup> )	21.32±4.65	22.62±4.14	0.085	24.32±5.35	24.62±5.04	0.777	23.32±5.65	23.62±4.14	0.776
Number of basal follicle	14.17±2.24	23.43±2.36	<0.001	14.07±2.24	22.43±2.36	<0.001	14.07±2.14	25.43±4.36	<0.001
LH (pg ml <sup>-1</sup> )	4.28±1.15	7.95±1.16	<0.001	4.18±1.15	7.39±1.16	<0.001	4.03±1.15	7.43±1.26	<0.001
FSH (pg ml <sup>-1</sup> )	5.99±1.13	6.15±1.71	0.591	5.97±1.13	6.02±1.71	0.886	5.95±1.23	6.02±1.31	0.830
LH/FSH	0.68±0.05	1.34±0.24	<0.001	0.78±0.15	1.44±0.24	<0.001	0.73±0.05	1.54±0.24	<0.001
E2 (pg ml <sup>-1</sup> )	31.46±5.23	32.04±3.52	0.388	32.86±4.23	32.14±3.52	0.335	33.86±5.23	32.21±3.52	0.069
PRL (ng ml <sup>-1</sup> )	19.24±4.65	19.63±2.65	0.459	19.14±4.25	18.63±3.65	0.509	19.04±4.65	18.93±2.65	0.874
T (ng ml <sup>-1</sup> )	0.44±0.13	0.72±0.11	<0.001	0.34±0.13	0.64±0.11	<0.001	0.34±0.13	0.62±0.11	<0.001

Abbreviations: BMI, body mass index; E2, estrogen; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PCOS, polycystic ovary syndrome; PRL, prolactin; T, testosterone.

**Table 6 Haplotype analysis of rs12644822, rs3756159 and rs13138607 between the PCOS group and the control group**

Haplotype			PCOS group (n = 314)	Control group (n = 320)	P-value	OR	95% CI
<i>Rs12644822</i>	<i>Rs3756159</i>	<i>Rs13138607</i>					
C	C	C*	48 (0.471)	106 (0.331)	<0.001	1.772	1.410–2.227
C	C	T*	25 (0.080)	12 (0.038)	0.003	2.077	1.263–3.416
C	T	C	16 (0.051)	14 (0.044)	0.739	1.092	0.649–1.839
T	C	C*	18 (0.057)	2 (0.006)	<0.001	8.257	3.096–22.018
T	C	T*	3 (0.010)	16 (0.050)	<0.001	0.16	0.064–0.398
T	T	C	6 (0.019)	15 (0.047)	0.002	0.359	0.180–0.715
T	T	T*	96 (0.306)	144 (0.450)	<0.001	0.514	0.408–0.649

Abbreviations: 95% CI, 95% confidence intervals; OR, odds ratio; PCOS, polycystic ovarian syndrome.

**Table 7 Genotype frequency of rs12644822, rs3756159 and rs13138607 in the GnRHR gene between the pregnancy and the non-pregnancy group**

SNP	Pregnancy group (n = 115)	Non-pregnancy group (n = 199)	P-value	OR	95% CI
<i>Rs12644822</i>					
TT	25 (21.74%)	10 (5.03%)	<0.001	0.191	0.088–0.414
CC+CT	90 (78.26%)	189 (94.97%)			
<i>Rs3756159</i>					
TT	20 (17.39%)	5 (2.51%)	<0.001	0.122	0.045–0.336
CC+CT	95 (82.61%)	194 (97.49%)			
<i>Rs13138607</i>					
TT	15 (13.04%)	2 (1.01%)	<0.001	0.068	0.015–0.302
CC+CT	100 (86.96%)	197 (98.99%)			

Abbreviations: CI, confidence intervals; OR, odds ratio; SNP, single-nucleotide polymorphism.

**Table 8 Logistic regression analysis for risk factors for the outcome of PCOS patients after IVF-ET**

SNP	B	s.e.	Wald	Sig.	OR	95% CI	
						Lower	Upper
Rs12644822	1.356	0.424	10.229	0.001	3.879	1.690	8.901
Rs3756159	1.830	0.542	11.411	0.001	6.232	2.156	18.016
Rs13138607	2.313	0.795	8.470	0.004	10.107	2.128	47.994

Abbreviations: B, partial regression coefficient; CI, confidence intervals; IVF-ET, *in vitro* fertilization and embryo transfer; OR, odds ratio; PCOS, polycystic ovary syndrome; s.e., standard error; Sig., significance; SNP, single-nucleotide polymorphism.

unclear. A previous study headed by Caburet *et al.*<sup>43</sup> discovered that genetic changes in the hypothalamo-pituitary axis can affect PCOS occurrence. The relationship and relevant mechanism behind *GnRHR* and PCOS patient endocrine functioning was further evidenced in our study.

The haplotype analysis indicated that CCC, CCT, TCC haplotypes were risk factors for PCOS and that TCT, TTC and TTT were protective factors for PCOS. Haplotype analysis is valued as a reputable method in genetic studies of variant complicated diseases. This consensus may contribute to the finding of more efficient strategies in identifying genetic variants that can increase the risk of human diseases.<sup>44</sup> In our study, results of haplotype analysis truly haplotype CCC (all variant) was not superior to other haplotypes with regard to odds ratio (PCOS susceptibility). It potentially can be explained that: (1) relatively small sample size; (2) SNPs of other PCOS-related genes are interacted. A genome-wide association study<sup>45</sup> reported on PCOS patients found that multiple gene polymorphisms functionally interacted with each other. Further study is guaranteed to validate whether CCC haplotype is superior to other haplotypes with regard to odds ratio (PCOS susceptibility) in large sample size.

In summary, we found that the CC+CT genotypes of rs12644822C>T, rs3756159C>T and rs13138607C>T positions in the *GnRHR* gene increased the risk of PCOS and that polymorphisms of the three positions affected the outcome of IVF-ET treatment in PCOS patients. However, the underlying molecular mechanism needs to be further validated based on larger sample sizes.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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