

Distribution of FSHR-29 polymorphism among women with polycystic ovary syndrome and association with level of its receptor expression in Granulosa cells of infertile women

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Abstract Follicle stimulating hormone receptor (FSHR-gene) is the most studied genetic factor in women with Polycystic Ovarian Syndrome (PCOS). Here, the prevalence of -29 variant of FSHR and the expression level in Granulosa cells in PCOS women undergoing in vitro fertilization (IVF) was investigated. The genotype was studied in 100 PCOS patients and 100 healthy controls undergoing IVF. Moreover, the total RNA was extracted from granulosa cells in 30 PCOS patients and 20 healthy controls. The results suggested that GA haplotype of -29 of FSHR appeared more frequent in PCOS patients and might play a role in genetic susceptibility to PCOS. Likewise, the expression of FSHR at mRNA level was reduced in subjects with AA haplotype of -29, as compared with the GG genotype. Also, the expression of mRNA in granulosa cells was upregulated in PCOS patients. Our findings demonstrated a statistically significant association between haplotype of -29 of FSHR with PCOS.



 Crossref  [10.36371/port.2023.1.2](https://doi.org/10.36371/port.2023.1.2)

Keywords: Follicle stimulating hormone receptor, FSHR-29 polymorphism, Polycystic ovary, Granulosa cells.

1. INTRODUCTION

Follicle stimulating hormone (FSH) plays a central role in oogenesis, where its main effects are related to granulosa cell proliferation, oocyte maturation, recruitment of the dominant follicle, and estrogen synthesis via activation of the aromatase gene (1, 2). It is well established that the physiological action of FSH depends on the activation of its receptor (FSHR), localized in granulosa and sertoli cells (3). FSHR acts by binding to the subgroup of G-protein coupled receptor (4) and is divided by the intracellular domain, transmembrane region, and extracellular domain, which consist of ten exons and nine introns at chromosome 2p21.(5)

Particularly, a great number of genetic variants in the FSHR gene have been reported to affect the phenotype. These effects include variable development of secondary sex characteristics, primary amenorrhea, hypoplastic ovary, and high serum levels of FSH (6). Several polymorphisms of the FSHR gene have been described so far and those that are located in nucleotides codifying for amino-acids at positions 307 and 680 are the most clinically relevant variants (7). Interestingly, these single nucleotide polymorphisms (SNPs)

were observed to be associated with altered response to FSH during in vitro fertilization (IVF) treatment, and both variants were reported to be in almost complete linkage disequilibrium. The significance of FSHR gene polymorphisms in ovarian response has been reported in subjects undergoing IVF treatment, and the variation at position -29 in the core promoter region of the FSHR gene might be of critical importance.(9,8)

In addition, mutations in FSHR can lead to the arrest of follicle development at several phases of growth. Naturally occurring inactivating mutations in the FSHR gene have been displayed in subjects with infertility. The phenotype of the infertile individuals has been appropriately correlated with the extent of FSHR inactivation (10). It is plausible that more subtle genetic variations of the receptor can contribute to functional perturbations, sub-fertility, and/or infertility (11). On the other hand, the relationship between allelic variants of FSHR and the presence of polycystic ovary syndrome (PCOS), has been studied in some ethnic groups (12). PCOS is the commonest cause of oligomenorrhea, hirsutism, and anovulatory infertility (13). The pathophysiology of the ovulatory disorder is still unclear. Resulting from intrinsic

thecal maladjustment with an increment of genes encoding steroidogenic enzymes, hyperandrogenism has been regarded as the main culprit (14). Hyperinsulinemia is one of the dominant features of PCOS patients, and increase in insulin level stimulates the sensitivity of granulosa cells to the release of FSH, resulting in the growth of follicle cysts and regulation of steroidogenesis in the ovary (15). In this study, we sought to obtain further evidence for distribution of FSHR polymorphism and its expression in granulosa cells in Iranian patients with PCOS.

2. MATERIALS AND METHODS

2.1 Subjects

All subjects were recruited from the Fertility Center of Shariati Hospital in Tehran, Iran. SNP analysis in FSHR gene, -29G/A (rs1394205), was carried out in 100 patients with PCOS and 100 women with normal ovulatory function undergoing IVF for treatment of tubal and/or male infertility. The diagnostic criteria were based on the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) ESHRE/ASRM consensus conference (15, 16). Patients had irregular menses, oligomenorrhea, or amenorrhea (without estrogen deficiency), particularly if there were also signs of hyperandrogenism. Women were between 20 and 40 yr old. The study required no modification of the routine IVF protocol. All patients provided informed written consent before their inclusion in this study. This study was approved by the Ethical committee of the Tarbiat Modares University, Tehran, Iran.

2.2 DNA Isolation

A volume of 100 μ L from peripheral blood was drawn from each subject with EDTA added as an anticoagulant. Genomic DNA was obtained from peripheral blood leukocytes with the DNPTM Kit Genomic DNA Purification Kit (SinaClon Co., Tehran, Iran) according to the manufacturer's instructions.

2.3 PCR-RFLP Analysis

The PCR reaction was performed in a final volume of 25 μ L containing 1 \times PCR buffer, 1.5 mM MgCl₂, 200 μ M of each dNTP, 1 μ M of each primer, 0.1 units of Taq-DNA polymerase (SinaClon Co., Tehran, Iran), and 200 ng of the DNA template were amplified for 35 cycles. A pair of primers was designed using Primer3 software (Whitehead Institute, Cambridge, Massachusetts, USA). Primer sets (Takapouzist, Tehran, Iran) and annealing temperatures, used for the PCR-RFLP assay, are shown in Table 1. Afterwards, approximately 500 ng of amplified DNA was digested with MboII enzyme (Fermentas) at their specific temperature according to protocol overnight.

2.4 Ovarian Stimulation

A standard protocol of IVF center of Shariati Hospital was used for ovulation induction during IVF treatment. Pituitary desensitization was started with Gonal-F and HMG on the 3rd day of the menstrual cycle during the luteal phase before IVF treatment. Follicle growth was stimulated after 12 d of desensitization by injecting recombinant FSH. Follicle growth was monitored according to transvaginal ultrasound. Human chorionic gonadotropin was administered when the leading follicle reached 18–20 mm in diameter together with at least three follicles greater than 16 mm detected by ultrasonography. Oocytes were retrieved after 36 h under transvaginal ultrasound guidance and mature oocytes (14 mm in diameter) were collected.

2.5 Quantitative Real-Time PCR

Granulosa cells in the follicular fluid were separated by centrifugation at 4000 \times g for 5 min at 4 °C, and cells were preserved by using RNeasy RNA Stabilization Reagent (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The cells were prepared with RNeasy Protect Mini Kit (QIAGEN, Hilden, Germany). A total volume of 25 μ L was achieved with the first-strand cDNA synthesis kit for RT-PCR, via 2 step RT-PCR kit, using 0.57 μ g RNA, avian myeloblastosis virus reverse transcriptase, and random primers p(dN) 6 as recommended by the manufacturer. Evaluation of gene expression levels was accomplished by real-time quantitative PCR kinetics using Evagreen as well as the predesigned primers and probes for human FSHR and housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The ABI 7500 Applied Biosystem instrument was used for amplification of cDNA by real-time PCR in duplicated reactions. The relative mRNA expression of FSHR was calculated by $\Delta\Delta$ threshold cycle (Ct) method, where the Δ Ct was calculated as the difference between the Ct of FSHR and Ct of GAPDH in each sample. The Δ Ct of each sample was then calculated as the difference between the Δ Ct of the sample with Δ Ct of the calibrator sample, which was normalized with GAPDH. The level of FSHR expression was compared within three genotypes at position -29 by ANOVA statistical test.

2.6 Statistical Analysis

Statistical analysis was performed using the chi-square test with SPSS software version 17.0. Comparisons of the genotypic or allele frequencies between cases and controls in addition to differences in continuous parameters between groups and genotypes were assessed with t-test. A conventional p value of ≤ 0.05 was considered as statistically significant

3. RESULT



3.1 Population

The genotype was studied in 100 polycystic ovary syndrome (PCOS) patients and 100 healthy controls undergoing IVF. Furthermore, the mRNA expression investigation was accomplished in 30 PCOS patients and 20 healthy controls. Main clinical parameters in normoovulatory controls and patients with PCOS are summarized in Table 2. No difference was found between the two populations regarding their age. As well, mean body mass index, LH levels, and E2 levels were higher in the PCOS group.

3.2 Polymorphism At Position -29 of The FSHR Gene

To examine the association between PCOS and the polymorphisms and to investigate the frequency of genotype, we utilized RFLP analysis as a tool to study the FSHR gene in 100 PCOS patients and 100 healthy controls. The patients' mean age was 29.4 ± 4.9 years, while the mean age of the controls was 30.8 ± 3.9 years (Table 2). PCR results were confirmed by agarose gel electrophoresis method (Figure 1). The results of RFLP analysis for AA genotype of FSHR -29G/A (rs1394205) demonstrated a frequency of 17.0% in PCOS patients and higher frequencies in control group (45.0%). The GG genotype had similar frequency in two groups, however the heterozygote GA was significantly more frequent in women with PCOS (PCOS=51.0%, Control=21.0%) (Figure 2).

3.3 FSHR Gene Expression in Gcs

The relative mRNA expression was monitored and compared by quantitative real-time PCR. The FSHR expression at transcript level exhibited a 1.5 fold-change increase in PCOS patients compared with control group ($P=0.006$, Figure 3). Also the mRNA expression was observed to be variable among the three genotypes at position -29 (Figure 4). Expression of FSHR at mRNA was reduced in subjects with AA haplotype of -29 as compared with the GG genotype, and the difference was observed to be statistically significant ($P=0.005$).

4. DISCUSSION

Polycystic ovary syndrome (PCOS), a condition with not completely well understood genetic contribution, is the most common endocrine disorder in women (5, 17). There is clear evidence for an underlying genetic cause for PCOS based on familial clustering of cases and collectively data consistent with the concept that a gene or more likely several genes predispose to PCOS susceptibility (18). Despite involvement of numerous genes in PCOS patient, it is clear that FSHR is one of the most important genes involved in this disease (7, 10, 17). The regulation of FSH level is controlled by FSHR, and it is known that the aberrant FSHR expression affects

ovary and folliculogenesis. Genetic studies of FSHR gene in specific populations have been described in the past. To date, the common FSHR polymorphism of Asn680Ser, mostly in linkage disequilibrium with Ala307Thr, has been declared to be a well-established determinant of response to FSH in IVF programs. In women, in vivo findings suggest that the Ser680 genotype is a factor of major "resistance" to FSH stimulation, resulting in higher FSH serum levels and, thus, leading to prolonged duration of the menstrual cycle. Nevertheless, its role in vitro remains to be determined (7), (20, 19, 12)

A study conducted on 50 women undergoing an IVF cycle demonstrated that women with the AA genotype in position -29 required higher doses of FSH, had lower E2 levels, produced fewer follicles, and showed a lower number of retrieved oocytes (21). In this study, one of the most impressive polymorphisms in FSHR gene was selected, and we examined the prevalence of this polymorphism at position -29 in 100 controls and 100 PCOS patients undergoing IVF. One logical reason for such study relies on the fact that polymorphisms of the FSHR seem to determine an evident alteration in the response of recruitable follicles to FSH. Therefore, we recruited 50 women undergoing ovarian stimulation protocol, 30 of them were PCOS patients and the rest were controls. The results indicated that the polymorphism at position -29 of the FSHR gene was associated with different FSHR expression in granulosa cell. Thus, we speculated that this polymorphism of the FSHR gene might influence the level of receptor expression. The frequency distribution of this polymorphism in subjects undergoing IVF treatment has also been reported for Indonesian, German, and Indian populations (7, 11, 12, 22). In our study, FSHR expression was significantly higher in PCOS patients than controls with GC haplotype. This is in agreement with previous studies showing that GCs from PCOS cells express high amounts of FSHRs and are highly responsive to this hormone in culture (14, 23, 24). The reduced expression of the FSHR gene in subjects with the AA genotype might be polymorphism-associated changes in the DNA structure, which may affect the binding of transcription factors because, since this polymorphism is located in the c-ETS-1 transcription factor binding site. Our observation strengthens the earlier report in which the promoter activity of the FSHR gene was found to be significantly reduced in the case of the A allele compared with the G allele at position -29 by in vitro analysis (24). To elucidate the importance of SNPs in FSHR in PCOS patients and the complexity of the c-ETS-1 transcription factor binding site in expression of FSHR, further investigations with a large population of PCOS patients, derived from different ethnic backgrounds, is required.

Table 1. Primers and PCR conditions of FSHR-29

Gene (sec)	Primers' Sequence	Length (bp)	Annealing temp. (°C)	Polymerization time
FSHR-29	Forward, gagaatggtgaacagcaaggag Reverse, tcttgaaagccacaggcaggag	601	58	60

Table 2. Clinical and biochemical characteristics between PCOS patients and normal controls.

Character	Control group	PCOS patient
No	100	100
Age	29.4 ± 4.9	30.8 ± 3.9
Body mass index (kg/m ²)	26.9 ± 5.04	24.3 ± 4.6
FSH levels (miu/ml)	7.4±3.7	6.7 ± 2.8
LH levels (pg/ml)	8.2±7.3	5.4±7.0
E2 level (pg/ml)	48.84 ± 27.0	41.8 ± 17.5
Cycle length	26.7 ± 2.5	28.5 ± 2.6

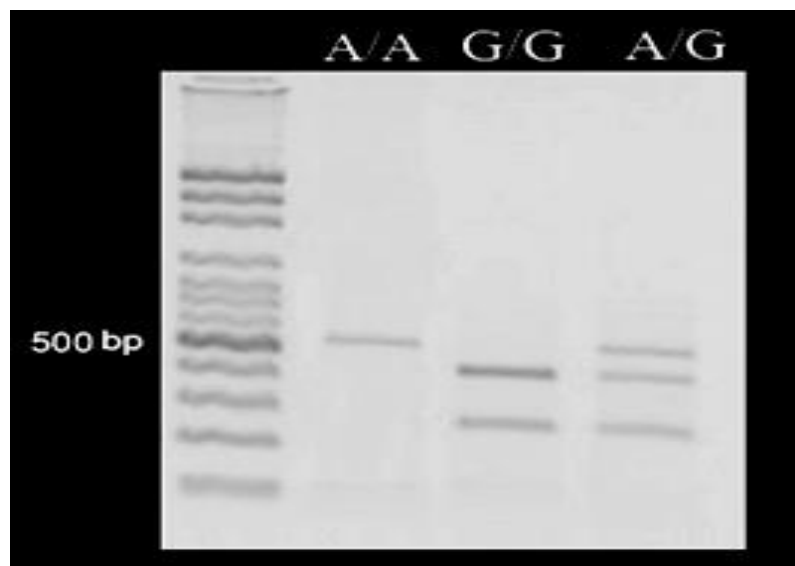


Figure 1. The rs1394205 polymorphism (A/G), product was digested with restriction enzyme MboII which gave an undigested product for A/A genotype (594 bp), two fragments for G/G genotype (330 and 260 bp), and three fragments for A/G genotype (594, 330, and 260 bp).

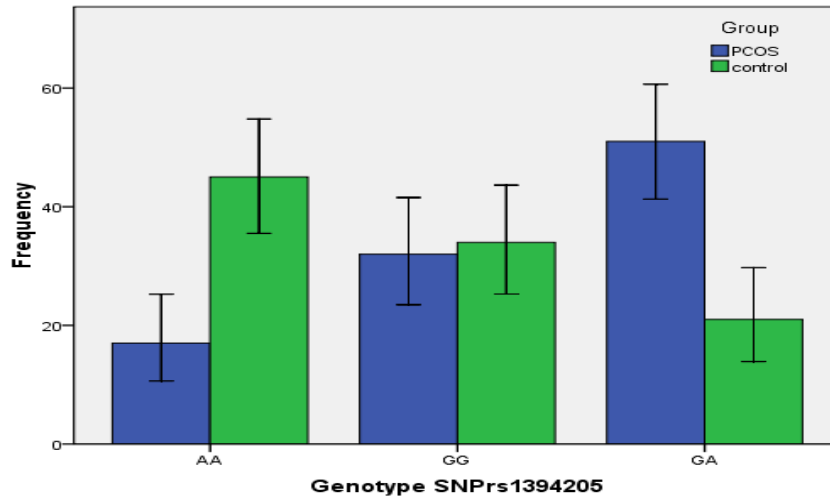


Figure 2. Distribution of the single nucleotide polymorphism at position -29 of the FSHR gene. The frequency distribution between the two groups was significantly different for all three genotypes ($P < 0.00$).

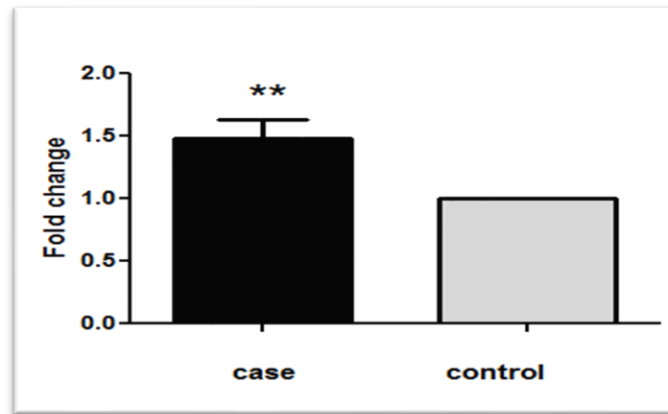


Figure 3. Comparison of expression of FSHR on granulosa cells between control and PCOS patients.

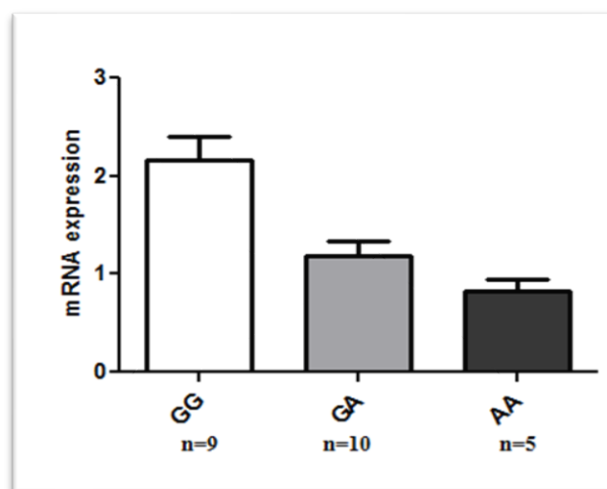


Figure 4. Expression of FSHR on granulosa cell on three genotypes at position -29 indicated statistically significant difference at $P < 0.05$ ($n=24$) analyzed by one-way ANOVA test.

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