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Межгенные взаимодействия FSHR и LHCGR ассоциированы с риском ановуляции у женщин Ростовской области (Россия)

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Введение. Ановуляция является широко распространённой причиной женского бесплодия. Сигнальные пути, опосредованные рецепторами фолликулостимулирующего и лютеинизирующего гормонов, имеют важное значение для успешной реализации репродуктивной функции у женщин.
Цель: изучение вклада межгенных взаимодействий генетических вариантов *FSHR* rs6166 и *LHCGR* rs2293275 в патогенез ановуляции.
Методы. В исследование включены 208 женщин из бесплодных пар, проходивших процедуру лечения бесплодия с применением вспомогательных репродуктивных технологий в Центре репродукции человека и ЭКО г. Ростова-на-Дону, в том числе женщины с ановуляцией и нормально овулирующие женщины с трубным и мужским фактором. Женщины со сниженным уровнем ФСГ и/или АМГ в сыворотке были исключены из исследования.
Результаты. Распределение частот генотипов варианта *FSHR* rs6166 составило: *AA* (40%), *AG* (43%), *GG* (17%). Распределение частот генотипов варианта *LHCGR* rs2293275 составило: *AA* (9%), *AG* (52%), *GG* (39%). В настоящем исследовании выявлена двухлокусная комбинация *FSHR* rs6166 *AG* / *LHCGR* rs2293275 *GG*, ассоциированная с повышенным риском ановуляции (OR (95% CI) = 2,43 (1,48-4,01), p<0,001). Кроме того, среди женщин с генотипом *LHCGR* rs2293275 *AA* не было ни одной с ановуляцией в анамнезе. Настоящее исследование также выявило различия среди женщин с комбинированным генотипом риска rs6166 *AG* / rs2293275 *GG*: у ановулирующих женщин наблюдалось повышение уровня АМГ, соотношения ЛГ/ФСГ и количества антральных фолликулов, а также снижение уровня ФСГ по сравнению с нормально овулирующими женщинами.
Заключение. Настоящее исследование предполагает, что варианты *FSHR* rs6166 и *LHCGR* rs2293275 являются хорошими кандидатами для ассоциативных исследований и могут обеспечить эффективную модель для прогнозирования риска ановуляции.
Ключевые слова: *FSHR* rs6166, *LHCGR* rs2293275, ановуляция, рецептор ФСГ, рецептор ЛГ.

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FSHR and LHCGR gene-gene interactions are associated with the risk of anovulation in women of the Rostov region (Russia)

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Background. Anovulation is a common cause of female infertility. Signal transduction by follicle-stimulating and luteinizing hormones receptors is substantial for the successful reproduction.
Aim: to study the contribution of intergenic interactions of *FSHR* rs6166 and *LHCGR* rs2293275 candidate gene variants to the development of anovulation.

Methods. The present study included 208 women from infertile couples who underwent assisted reproductive technologies, including women with anovulation and normally ovulating women with tubal and/or male factor. Women with reduced FSH and/or AMH serum levels were excluded from the study.
Results. The genotypes frequency distribution for *FSHR* rs6166 and *LHCGR* rs2293275 gene variants was *AA* (40%), *AG* (43%), *GG* (17%), and *AA* (9%), *AG* (52%), *GG* (39%), respectively. The present study revealed the two-locus *FSHR* rs6166 *AG* / *LHCGR* rs2293275 *GG* combination associated with the increased risk of anovulation (OR (95% CI) = 2.43 (1.48-4.01), p<0.001). In addition, among women carried genotype *LHCGR* rs2293275 *AA*, none experienced anovulation. The present study also revealed differences among women carrying the risk genotype. Anovulatory women with rs6166 *AG* / rs2293275 *GG* combined genotype had an increased AMH level, AFC and LH/FSH ratio and a decreased FSH level, compared to normally ovulating women with the same genotype of risk.
Conclusion. Present study proposes that *FSHR* rs6166 and *LHCGR* rs2293275 variants are good candidate markers for association studies, and together may provide an effective model for predicting the risk of anovulation.
Keywords: *FSHR* rs6166, *LHCGR* rs2293275, anovulation, FSH receptor, LH receptor.

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Introduction

Currently, infertility is a widespread problem, and disorders of ovulation account for about 30% of it [1]. The most common causes of anovulation are associated with disturbances in the production of gonadotropins and androgens leading to polycystic ovary syndrome (PCOS), hyperprolactinemia, hypogonadotropic hypogonadism [1, 2]. Gonadotropins play complementary roles in follicular development and ovulation through complex interactions in the hypothalamus, anterior pituitary gland, reproductive organs, and oocytes. Follicle stimulating hormone (FSH) is a key stimulator of granulosa cell proliferation in the growing follicle and is responsible for the dominant follicle selection [3]. Due to a decrease in FSH levels in response to an increase in estradiol levels, the follicle whose granulosa cells are most sensitive to FSH will enter the dominant follicle development stage, while the remaining follicles will undergo atresia [3]. Luteinizing hormone (LH) mediates proliferative and anti-apoptotic signals in granulosa cells, induces internal theca cells to steroidogenesis and leads the follicle growth and maturation, the ovulation, the granulosa cells luteinization, and the corpus luteum maintenance during the luteal phase of the menstrual cycle [4, 5]. Etiology of anovulation is caused by multiple conditions, including genetic factors [1]. An important representative of the genetic factors class is the gonadotropins receptors genes variation. It is assumed that FSH and LH receptors genes intergenic interactions might contribute to the combination of factors that determine the risk of anovulation.

FSH and LH are a pituitary-secreted glycoprotein heterodimers comprised of a hormone-specific beta chain linked to an alpha chain common to FSH, LH, human chorionic gonadotropin (hCG), thyroid-stimulating hormone (TSH). FSH and LH function by binding to their specific receptors, which belong to the glycoprotein hormone receptors (GPHRs) subfamily of the G protein-coupled receptor (GPCRs) superfamily. In response to FSH or LH binding, receptor-associated G proteins located on the intracellular plasma membrane surface are activated, resulting in increased intracellular levels of cyclic adenosine monophosphate (cAMP), activating signaling cascades that lead to hormone-mediated receptor-specific cellular responses [4]. FSH receptor (FSHr) is expressed on the membrane of ovarian granulosa cells. LH receptor (LHr) is expressed on the membrane of ovarian theca and granulosa cells [4]. Both FSH and LH receptors contain 695 and 699 amino acids, respectively, organized into three domains: N-terminal extracellular ligand-binding domain including hinge region; transmembrane domain; C-terminal intracellular domain responsible for the G-protein binding [4, 6]. Both FSH and LH receptors genes are located on the short arm of chromosome 2 (2p16.3) and comprises 14 and 11 exons respectively [4, 7].

Functionally important SNP rs6166 is located in exon 14 of the *FSHR* gene, which encodes the C-terminal part of the extracellular domain (in hinge region), the entire transmembrane domain, and the intracellular domain. SNP rs6166 occurs at position 2039, where nucleotide *A* is replaced by nucleotide *G*, resulting in the substitution of asparagine with serine (N680S) in the intracellular C-terminal domain of the FSHr protein [4, 8]. Studies

have shown that the SNP rs6166 alters ovarian response to ligand stimulation. Since exon 14 is essential for signal transduction, it is assumed, that the SNP rs6166 can lead to FSHr resistance [6, 9] through modulating the kinetics of cAMP activation, ERK1/2 and CREB phosphorylation [5], which entails increased FSHr sensitivity threshold [10] and, consequently, a decrease in the number of maturing follicles [6].

SNP rs2293275 is located in exon 10 of the *LHCGR* gene, which encodes 27 amino acids within the hinge region, which is an extracellular segment that is important for hormone selectivity and signal transduction [4]. SNP rs2293275 occurs at position 935 of exon 10, where nucleotide *A* is replaced by nucleotide *G*, resulting in the substitution of asparagine with serine (N312S) in the LH receptor protein near the glycosylation site and thus may affect translocation and stability of the receptor [7]. Deletion of exon 10 eliminates human LH activity [4].

Taken together, given the key role of gonadotropins in follicular development and ovulation, it is hypothesized that their receptors genes alternative variants may affect the processes of follicular maturation and corpus luteum formation, leading to anovulation. For these reasons, the two specific polymorphisms *FSHR* rs6166 and *LHCGR* rs2293275 are expected to be good candidate markers for association studies.

The **aim** of the research was to study the contribution of intergenic interactions of *FSHR* rs6166 and *LHCGR* rs2293275 candidate gene variants to the development of anovulation.

Methods

Study participants. The case-control study included 208 women who underwent controlled ovarian stimulation (COS) for IVF at the Center for Human Reproduction and IVF (Rostov-on-Don, Russia) from 2017 to 2019. The women were between 23 and 44 years of age (mean: 32±4.2). All women were undergoing their first or second IVF cycle. Regarding the diagnosis, all women were divided into 2 groups: patients with anovulation and the comparison group, including normally ovulating women with tubal and/or male factor. Anovulation has been determined by ultrasound as the absence of maturation of the corpus luteum. From a total of 27 anovulatory women, 3 were diagnosed with PCOS, 7 were diagnosed with hyperandrogenism, 4 were diagnosed with hyperinsulinemia or insulin resistance syndrome, and 2 were diagnosed with hyperprolactinemia. Patients with reduced FSH (<1.8 IU/l) and/or AMH (<0.6 ng/ml) levels were excluded from the present study. Regarding the number of aspirated preovulatory follicles, patients were divided into 3 groups: normal ovarian response (10-16 follicles); low ovarian response (<10 follicles); high ovarian response (>16 follicles).

Informed Consent. This study was carried out in accordance with the recommendations set out in the Declaration of Helsinki. The protocol was approved by the local Ethnic Committee of the Academy of Biology and Biotechnology of the Southern Federal University. Approval certificate № 0104 from 13.02.2017. All women were included after written and verbal informed consent.

Hormone assay. The early follicular phase FSH, LH, AMH and estradiol levels were determined in the blood serum on 3rd-5th day of the menstrual cycle, six months before the start of the IVF program, using corresponding kits and "Access 2" analyzer (Beckman Coulter, USA).

Genotyping. For DNA isolation and analysis, 10 ml of peripheral blood with EDTA as an anticoagulant was drawn from each patient. Genomic DNA was isolated from blood leukocytes using "DNA-EXPRESS-GENETICS" kit ("Lytech" Co. Ltd., Russia). Genotyping of the *FSHR* rs6166 variant was carried out applying restriction fragment length polymorphism (RFLP) analysis using restrictase *BsrI* and primers: 5′-TTTGTGGTCATCTGTGGCTGC-3′, 5′-CAAAGGCAAGGACTGAATTATCATT-3′ [6]. The PCR was carried out using 5x qPCRmix-HS ("Evrogen", Russia). The PCR cycle was as follows: start denaturation - 5 min at 95º; annealing for 35 cycles - 15 sec at 95º, 10 sec at 64º, 20 sec at 72º; final extension - 5 min at 72º. The restriction was carried out for 16 h at 65º, with subsequent restrictase inactivation for 20 min at 80º. Genotyping of the *LHCGR* rs2293275 variant was carried out applying allele-specific PCR using primers: forward 5′-GCAACAGCTCCGTAACCAAG-3′, reverse 5′-GTG AAAGCACAGTAAGGAAAGTGTA-3′, and reverse mut 5′-TGAAAGCACAGTAAGGAAAGTGCG-3′. The PCR was carried out using 5x qPCRmix-SYBR ("Evrogen", Russia). The PCR cycle was as follows: start denaturation - 5 min at 95º; annealing for 35 cycles - 15 sec at 95º, 10 sec at 59º, 20 sec at 72º; final extension - 5 min at 72º. PCR products were detected by horizontal electrophoresis in 2% agarose gel. Results were validated by double genotyping.

Statistical analysis. Hardy-Weinberg equilibrium (HWE) conformance was calculated applying Pearson's chi-square test. Allele frequencies were assessed using a two-tailed Fisher's exact test. For analysis of variance Kruskal-Wallis H-test and Mann–Whitney U-test were used. A two-sided p value <0.05 was admitted as statistically significant. Nemenyi's test was used as a post-hoc multiple comparisons tests. The association between genotypes and

risk of anovulation was computed using odds ratio (OR) with 95% confidence interval (CI) and chi-square test with Yates's correction. Multifactor Dimensionality Reduction (MDR) software was used to analyze the contribution of gene-gene interactions in assessing the risk of anovulation. The dataset was balanced using over-sampling technique [11]. Permutation test was performed to control type I error and evaluate the statistical significance of the MDR model [12]. Statistical analysis was carried out using the software «Statistica 13».

Results

The two-locus combination of the FSHR rs6166 / LHCGR rs2293275 variants is associated with the risk of anovulation

In this study we analyzed the genotype distribution of *FSHR* rs6166 and *LHCGR* rs2293275 variants in 208 women with anovulation and other factors of infertility (including tubal and male factors) aged from 23 to 44 years. The genotypes frequency distribution for rs6166 variant was as follows: *AA* - 40% (n=82), *AG* - 43% (n=90), *GG* - 17% (n=36). This was determined to be consistent with HWE (p=0.194). The genotypes frequency distribution for rs2293275 variant was as follows: *AA* - 9% (n=19), *AG* - 52% (n=107), *GG* - 39% (n=82), and did correspond to HWE (p=0.056). **Table 1** shows the distribution of *FSHR* rs6166 and *LHCGR* rs2293275 variants genotypes in women with anovulation (n=27) and comparison group (n=181). Chi-square test demonstrated no statistically significant differences in the distribution of genotypes between groups when genes variants investigated separately. Nevertheless, according to χ^2 test, *LHCGR* rs2293275 *G* allele was tended to be associated with the risk of anovulation (p=0.075).

The combined assessment of the *FSHR* rs6166 and *LHCGR* rs2293275 variants was carried out using MDR analysis (**Fig. 1**). The highest anovulation risk model of gene-gene interactions was determined: almost half of women carrying this combination (45%) experienced anovulation (**Fig. 1, Table 2**). It was the two-locus combination of the *FSHR* rs6166 / *LHCGR* rs2293275 variants, characterized by 100% reproducibility and prediction accuracy of 70.44%

Таблица 1. Распределение аллелей и генотипов *FSHR* rs6166 и *LHCGR* rs2293275 у женщин с ановуляцией и в группе сравнения
Table 1. The distribution of *FSHR* rs6166 and *LHCGR* rs2293275 alleles and genotypes in women with anovulation and comparison group

rs6166	**Anovulation**	**Comparison group**	**χ2**	**p**	**OR (95% CI)**	**p**
	n (%)	**n (%)**				
	n=27	n=181				
Genotype						
AA	12 (45%)	70 (39%)				R
AG	13 (48%)	77 (42%)	2.126	0.346	0.98 (0.42-2.30)	1.000
GG	2 (7%)	34 (19%)			0.34 (0.007-1.62)	0.222
Allele						
A	37 (15%)	217 (85%)	1.453	0.229		R
G	17 (10%)	145 (90%)			0.69 (0.37-1.27)	0.295
rs2293275	**n (%)**	**n (%)**	**χ2**	p	OR (95% CI)	p
Genotype						
AA	0	19 (10%)				R
AG	13 (48%)	94 (52%)	4.115	0.128	n.a.	n.a.
GG	14 (52%)	68 (38%)			n.a.	n.a.
Allele						
A	13 (9%)	132 (91%)	3.177	0.075		R
G	41 (15%)	230 (85%)			1.81 (0.94-3.50)	0.092

Note: R – reference; n.a. – not applicable.

(Table 2). The permutation test (MDR-PT) showed that the model is statistically significant (p<0.001). Analysis revealed a synergetic interaction between studied SNPs (red color) in the formation of predisposition to anovulation, and that *LHCGR* rs2293275 variant has the greatest potential (rs2293275 6.21% vs. s6166 1.97%) in predicting risk of anovulation (Figure 2). In addition, it was shown that among women with genotype *LHCGR* rs2293275 AA there was not even one woman experienced anovulation **(Table 1, Figure 1)**.

Thus, according to MDR analysis, the combined genotype *FSHR* rs6166 AG / LHCGR rs2293275 GG is a risk factor for the development of anovulation (OR (95% CI) = 2.43 (1.48-4.01), p<0.001).

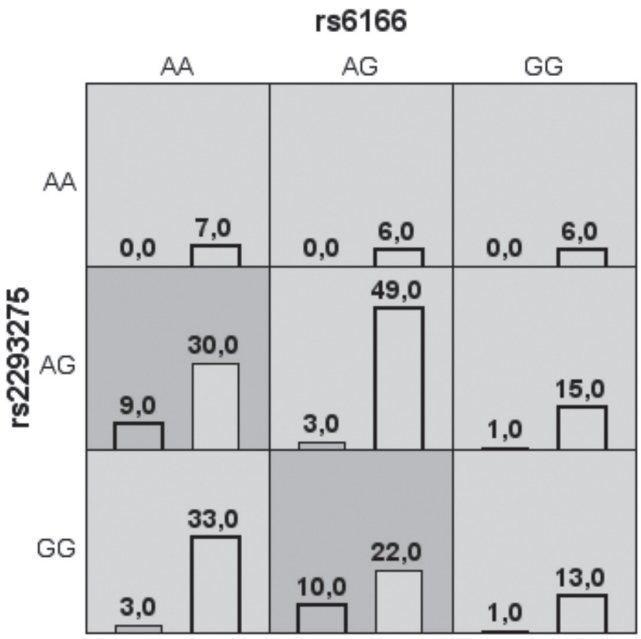

Рис. 1. Распределение женщин с ановуляцией и группы сравнения в двухлокусной модели *FSHR* rs6166 и *LHCGR* rs2293275. Темный столбец в каждой ячейке представляет собой количество женщин с ановуляцией. AA – гомозиготы по частому аллелю; AG – гетерозиготы; GG – гомозиготы по минорному аллелю

Fig. 1. The distribution of anovulatory women and comparison group in the two-locus *FSHR* rs6166 and *LHCGR* rs2293275 model. The dark column in each cell represents the number of anovulatory women. *AA* – homozygotes for the major allele; *AG* – heterozygotes; *GG* – homozygotes for the minor allele.

The clinical characteristics of studied groups

The clinical parameters of patients were analyzed based on the anovulation factor using Mann–Whitney U-test as analysis of variance (**Table 3**). The median age was 32 years in both women experienced anovulation and comparison group. LH and estradiol levels, follicles and oocytes number, body mass index (BMI) did not differ between groups. Serum FSH level was significantly lower, serum AMH level and LH/FSH ratio was significantly higher in anovulatory women (p=0.012, p=0.024 and p=0.025 respectively) (**Fig. 3**). The antral follicle count (AFC) tended to be higher in anovulatory women (p=0.074) (Fig. 3).

Since the present study revealed the two-locus *FSHR* rs6166 *AG / LHCGR* rs2293275 *GG* combination associated with the risk of anovulation, a comparative analysis of clinical parameters was carried out between *FSHR* rs6166 *AG / LHCGR* rs2293275 *GG* combined genotype carriers and the rest of genotypes carriers**.** The all previously revealed differences between women with anovulation and comparison group (Table 3) were shown only in *FSHR* rs6166 *AG / LHCGR* rs2293275 *GG* combined genotype carriers (**Table 4**). Although, the clinical parameters were investigated based on *FSHR* rs6166 and *LHCGR* rs2293275 variants separately, and there were not statistically significant differences, except FSH level was statistically significantly higher in *FSHR* rs6166 *GG* carriers, compared to rs6166 *A* allele carriers.

Previous anovulation interferes with the prediction of ovarian response

The clinical parameters were also investigated according to ovarian response in women experienced anovulation and comparison group separately. Regarding the comparison group, FSH level was statistically significantly higher in

Таблица 2. Оптимальная модель MDR для межгенных взаимодействий rs6166 *FSHR* / rs2293275 *LHCGR* в развитии повышенного риска ановуляции

Table 2. The optimal MDR model for *FSHR* rs6166 / *LHCGR* rs2293275 intergenic interactions in the development of increasing anovulation risk

Genotype of risk	Testing balanced accuracy (TBA)	Cross-validation consistency (CVC)	Sensitivity	Specificity	χ2	p
FSHR rs6166 *AG / LHCGR* rs2293275 *GG*	0.7044	10/10	69.6%	71.3%	60.525	<0.001

low responders, and AMH level was statistically significantly higher in high responders (**Table 5**). Among women with anovulation, no differences in clinical parameters were observed between groups with different ovarian response, except the number of follicles and oocytes varied respectively (Table 5). According to chi-square test, low ovarian response occurred with equal frequency in both anovulatory women and comparison group (52% in both groups). High ovarian response even was more common in women with anovulation (30%), compared to comparison group (17%), however there was no statistical significance.

Discussion

The previous studies have shown the low concentrations of FSH, LH and estradiol to be associated with hypogonadotropic hypogonadism [1] and nearly 2-fold increased AMH level to be associated with PCOS [13] leading to anovulation. The present study has also revealed a significantly lower serum FSH level in women with anovulation (p=0.012) (Table 3). Estradiol level was also lower in anovulatory women, but this was not statistically significant. The present study did not reveal statistically significant differences in LH level between anovulatory women and comparison group, although the LH/FSH ratio was significantly higher in anovulatory women (p=0.025). An increase in the LH/FSH ratio has been previously associated with an expand ovarian reserve [14], which is consistent with the trend shown in the present study: the AFC was tended to be higher in women experienced anovulation before IVF (p=0.074), and consequently, AMH level was also higher (p=0.024). Despite this, the number of follicles and oocytes obtained after COS did not differ between groups (Table 3). It is hypothesized that in anovulatory women the process of folliculogenesis is largely intact, while the process of ovulation is disrupted. This hypothesis is consistent with the MDR analysis, which resulted in an intergenic model of increasing anovulation risk: *FSHR* rs6166 *AG* / *LHCGR* rs2293275 *GG*.

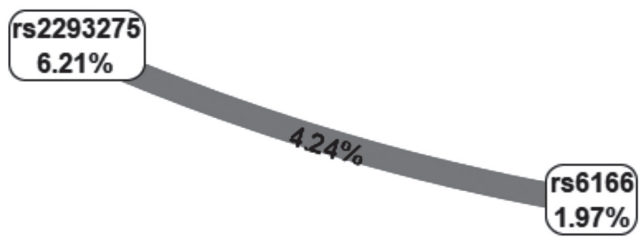

Рис. 2. График Фрухтермана-Рейгольда межгенных взаимодействий *FSHR* rs6166 / *LHCGR* rs2293275 в развитии повышенного риска ановуляции. Красный цвет обозначает синергизм.

Fig. 2. A Fruchterman-Rheigold graph of FSHR rs6166 / LHCGR rs2293275 intergenic interactions in the development of increasing anovulation risk. Red color stands for synergism

Таблица 3. Клинические показатели у женщин с ановуляцией и группы сравнения

Table 3. The clinical parameters in anovulatory women and comparison group

	Anovulation	Comparison group	p
	n=27	n=181	
Age (years)	32 (29-36)	32 (30-35)	0.578
FSH (IU/l)	5.6 (4.3-7.1)	6.4 (5.5-7.8)	**0.012**
LH (IU/l)	5.2 (3.5-6.9)	4.8 (3.7-6.7)	0.725
AMH (ng/ml)	3.8 (2.0-7.3)	2.7 (1.6-5.6)	**0.024**
Estradiol (pmol/l)	119 (81-206)	152 (101-224)	0.211
AFC (n)	14 (10-20)	11 (8-16)	0.074
Follicles obtained (n)	9 (6-18)	9 (5-14)	0.187
Oocytes total (n)	9 (5-18)	8 (4-13)	0.120
BMI	22 (20-25)	23 (21-26)	0.333
LH/FSH ratio	0.96 (0.64-1.44)	0.75 (0.57-1.00)	**0.025**

Note: Data are presented as median (25th-75th percentiles). FSH – follicle stimulating hormone, LH – luteinizing hormone, AMH - anti-Mullerian hormone, AFC – antral follicle count, BMI – body mass index. Statistically significant nominal p-values are highlighted in bold.

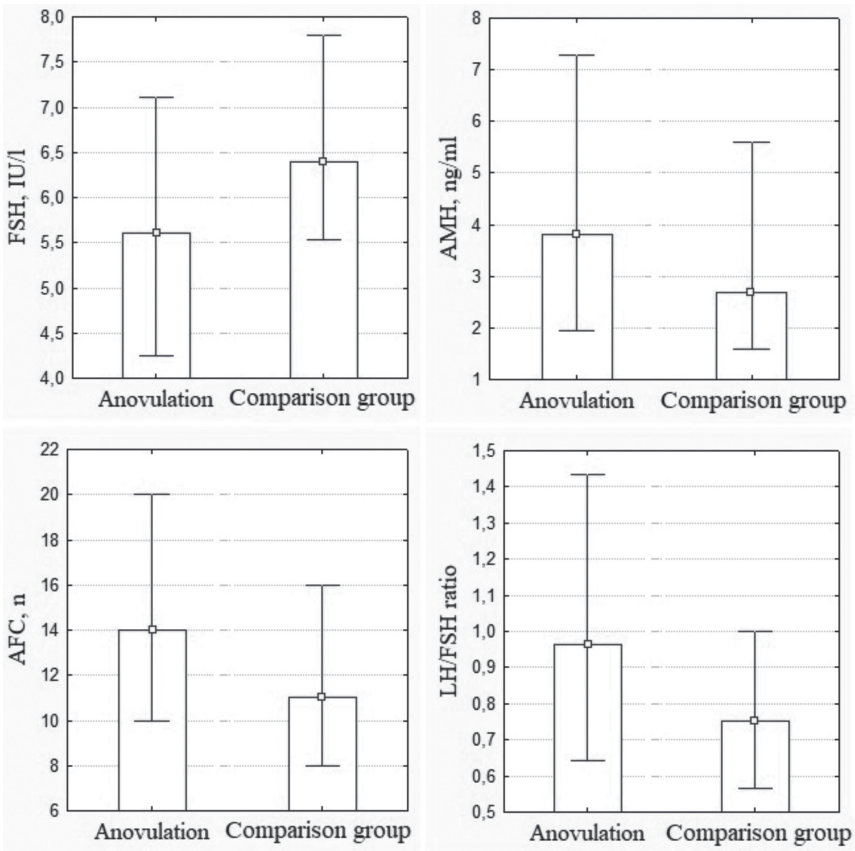

Рис. 3. Различия клинических параметров женщин с ановуляцией и из группы сравнения (p=0,012, p=0,024, p=0,074 и p=0,025 соответственно). Данные представлены в виде медианы (25-й-75-й процентили)

Fig. 3. The clinical parameters differed in anovulatory women compared to comparison group (p=0.012, p=0.024, p=0.074 and p=0.025 respectively). Data are presented as median (25th-75th percentiles)

Таблица 4. Клинические показатели у женщин с ановуляцией и группы сравнения в зависимости от генотипа риска

Table 4. The clinical parameters in anovulatory women and comparison group according to the risk genotype

	rs6166 *AG* / rs2293275 *GG* carriers only (n=32)		p	The rest genotypes carriers (n=176)		p
	Anovulation	**Comparison group**		**Anovulation**	**Comparison group**	
	n=10 (31%)	n=22 (69%)		n=17 (10%)	n=159 (90%)	
Age (years)	33 (30-36)	33 (30-36)	1.000	31 (28-32)	32 (29-35)	0.317
FSH (IU/l)	5.1 (4.3-6.9)	7.1 (5.4-7.5)	0.070	5.6 (4.3-7.1)	6.4 (5.5-7.8)	0.093
LH (IU/l)	4.7 (4.1-6.9)	4.5 (3.7-6.3)	0.704	5.6 (3.5-6.5)	4.8 (3.8-6.8)	0.812
AMH (ng/ml)	5.2 (2.9-7.4)	2.5 (1.1-4.2)	**0.031**	3.4 (2.0-7.0)	2.7 (1.6-5.8)	0.193
Estradiol (pmol/l)	122 (77-206)	159 (130-209)	0.163	117 (85-192)	147 (95-228)	0.477
AFC (n)	14 (12-20)	10 (7-12)	**0.012**	12 (10-18)	11 (8-17)	0.319
Follicles obtained (n)	10 (5-14)	7 (4-10)	0.190	9 (7-18)	10 (5-14)	0.203
Oocytes total (n)	10 (5-14)	6 (3-9)	0.140	9 (6-18)	8 (5-14)	0.142
BMI	23 (20-25)	22 (20-24)	0.535	21 (20-24)	23 (21-26)	0.194
LH/FSH ratio	0.92 (0.78-1.61)	0.75 (0.54-0.89)	0.052	1.03 (0.61-1.43)	0.75 (0.57-1.06)	0.200

Note: Data are presented as median (25th-75th percentiles). FSH – follicle stimulating hormone, LH – luteinizing hormone, AMH - anti-Mullerian hormone, AFC – antral follicle count, BMI – body mass index. Statistically significant nominal p-values are highlighted in bold.

Previous data on the association of *FSHR* rs6166 and *LHCGR* rs2293275 variants with anovulation are contrary. Some studies did not reveal an association [15], however some other studies showed an association between studied variants and conditions that may lead to anovulation. The rs6166 substitution adds a potential phosphorylation site in the intracellular domain of the receptor and affects the kinetics of steroid synthesis [5, 16]. The *FSHR* rs6166 minor *G* allele have previously been shown to be associated with hyperandrogenism [2], at the same time *FSHR* rs6166 *GG* genotype have been shown to provide protection against PCOS [17]. Previously *LHCGR* rs2293275 substitution has also been shown to be associated with PCOS [4, 7]. *LHCGR* rs2293275 *AA* genotype have also been previously associated with increased PCOS [18]. However, no statistically significant differences in the distribution of genotypes between women with anovulation and the comparison group were shown in the present study when the genes variants were investigated separately (Table 1). Nevertheless, the present study revealed the two-locus *FSHR* rs6166 *AG / LHCGR* rs2293275 *GG* combination associated with the increased risk of anovulation (OR (95% CI) = 2.43 (1.48-4.01), p<0.001) (Table 2). The comparative analysis of clinical parameters carried out between anovulatory associated *FSHR* rs6166 *AG / LHCGR* rs2293275 *GG* combined genotype carriers and the rest women revealed that earlier demonstrated increasing AMH level, AFC and LH/FSH ratio and decreasing FSH level in anovulatory women were shown only in *FSHR* rs6166 *AG / LHCGR* rs2293275 *GG* carriers (Table 3, Table 4). Clinical parameters in anovulatory women did not differ from it in comparison group in the merged group carrying the rest of genotypes (Table 4). Thus, it is assumed, that the revealed differences in clinical parameters between anovulatory women and comparison group are due to the risk genotype.

Previously, *FSHR* rs6166 variant has been shown to be associated with ovarian function. Most previous studies have shown *FSHR* rs6166 minor *G* allele or *GG* genotype to be associated with reduced ovarian response [8, 16, 19, 20]. It is expected that the FSH receptor impaired due to

Таблица 5. Клинические параметры у женщин с ановуляцией и группы сравнения в зависимости от реакции яичников
Table 5. The clinical parameters in anovulatory women and comparison group according to ovarian response

Comparison group	Normal responders	Low responders	High responders	p
	n=56	n=95	n=30	
Age (years)	32 (29-34)	34 (30-37)	31 (29-34)	0.636
FSH (IU/l)	6.3 (5.6-7.6)	7.1 (5.7-8.5)	5.9 (4.7-6.6)	**0.025**
LH (IU/l)	4.9 (3.8-6.6)	4.6 (3.7-6.5)	5.4 (3.7-7.6)	0.550
AMH (ng/ml)	2.8 (1.9-5.1)	2.1 (1.1-3.5)	7.6 (5.7-10.0)	**<0.001**
Estradiol (pmol/l)	141 (102-198)	154 (91-241)	139 (103-231)	0.731
AFC (n)	13 (10-15)	8 (6-10)	23 (19-26)	**<0.001**
Follicles obtained (n)	12 (10-14)	5 (4-7)	23 (19-25)	**<0.001**
Oocytes total (n)	11 (10-14)	5 (3-7)	22 (17-25)	**<0.001**
BMI	22 (20-25)	23 (19-26)	24 (22-27)	0.114
LH/FSH ratio	0.77 (0.62-0.99)	0.70 (0.49-0.91)	1.05 (0.63-1.42)	0.055
Anovulation	Normal responders	Low responders	High responders	p
	n=5	n=14	n=8	
Age (years)	30 (29-33)	32 (29-36)	32 (28-34)	0.825
FSH (IU/l)	7.6 (5.6-7.9)	4.8 (3.9-6.9)	5.7 (4.1-6.6)	0.213
LH (IU/l)	4.6 (4.2-6.9)	5.0 (3.0-6.5)	5.6 (5.0-6.8)	0.558
AMH (ng/ml)	5.9 (3.4-10.1)	2.9 (1.9-4.5)	5.9 (3.2-8.3)	0.770
Estradiol (pmol/l)	61 (57-119)	124 (99-231)	149 (82-199)	0.143
AFC (n)	14 (12-17)	10 (8-13)	26 (19-28)	**0.012**
Follicles obtained (n)	12 (12-14)	7 (5-8)	22 (18-27)	**0.003**
Oocytes total (n)	12 (12-14)	6 (4-8)	22 (18-27)	**0.003**
BMI	24 (22-25)	20 (19-23)	24 (21-25)	0.766
LH/FSH ratio	0.72 (0.61-0.78)	1.00 (0.64-1.44)	1.16 (0.79-1.68)	0.188

Note: Data are presented as median (25th-75th percentiles). FSH – follicle stimulating hormone, LH – luteinizing hormone, AMH – anti-Mullerian hormone, AFC – antral follicle count, BMI – body mass index. Statistically significant nominal p-values are highlighted in bold.

a genetic alteration becomes less sensitive to FSH [5, 6], resulting in an increase in the threshold required for follicle growth. So, it is likely that in rs6166 *GG* carriers, FSH level will be elevated to maintain normal ovarian function [4, 8, 14, 16]. Nevertheless, the present study showed no differences in FSH level among *FSHR* rs6166 genotypes carriers, hence FSH level have not been increased in *FSHR* rs6166 *AG* carriers and, therefore, did not compensate the impaired FSHr function. However, the process of folliculogenesis supposed to be only partially disrupted, due to the possibility of receptor oligomerization and trans-activation via intermolecular cooperation [4], including allosteric modulation [3], which may compensate the effect of minor allele in a heterozygous genotype. Unaffected receptors may be transactivated by signaling-deficient receptors bound to the hormone [3]. Therefore, it does not seem likely that altered FSHr contributes to the ovulatory disfunction [2]. While FSH is more important for folliculogenesis, LH is crucial for ovulation. *LHCGR* rs2293275 variant has been shown to make a more significant contribution to anovulation pathogenesis (Figure 2). It has previously been shown that SNP rs2293275 leads to loss of LHr function [4]. Since sustained LHr-mediated cAMP signaling is required to unlock the oocyte from meiotic blockade [5] and to induce steroidogenesis [8], the presence of both minor *G* alleles of rs2293275 *LHCGR* is assumed to result in ovulation failure, which is in consistent with the present study results. Accordingly, the present study has shown that among rs2293275 *AA* carriers there were no women experienced anovulation (Figure 1).

Thus, anovulation may result from receptor mutations that affect membrane expression, control of receptor-mediated signaling. Moreover, anovulation is a complex multifactorial disease, and treatment strategies are limited. Therefore, despite the small studied sample size, regional sample localization and limited studied SNPs set, *FSHR* rs6166 and *LHCGR* rs2293275 variants propose to be good candidate markers for association studies, and together they may provide an effective model for predicting the risk of anovulation.

Conclusion

This investigation reports on *FSHR* rs6166 and *LHCGR* rs2293275 variants in Russian women with anovulation, compared to normally ovulating women. The present study presents the two-locus *FSHR* rs6166 *AG* / *LHCGR* rs2293275 *GG* combination associated with the nearly 2-fold increased risk of anovulation. The present study also revealed increasing AMH level, AFC and LH/FSH ratio and decreasing FSH level in anovulatory women carrying *FSHR* rs6166 *AG* / *LHCGR* rs2293275 *GG* combined genotype, compared to comparison group with the same genotype. Despite these advances, many unanswered questions remain regarding the pathogenesis of anovulation, and the full potential of gonadotropin action, the presence of FSHr–LHr heteromers in vivo, and the role of receptors require further research. Since *FSHR* rs6166 and *LHCGR* rs2293275 variants are considered to have pharmacogenetic potential in the treatment of female infertility, it may also facilitate a more personalized management in predicting the risk of anovulation.

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