Follicle-stimulating hormone receptor gene polymorphisms of ovarian reserve markers in Romanian population

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Abstract. Association between phenotype and follicle-stimulating hormone (FSH) receptor and FSH beta chain genotype was evaluated in women with ovarian dysfunction. FSH receptor gene single nucleotide polymorphisms (SNPs) were analyzed by restricted fragment length polymorphism (RFLP) technique. Three groups were analyzed: two groups formed of poor responders (women with ovarian dysfunctions caused by endometriosis and patients who underwent ovarian stimulation protocols) and a third good responders group (normal-ovulatory women who gave birth to naturally conceived children). A higher average level of basal FSH values were found in mutants in the A919G/Ala307Thr/rs6165 or A2039G/Asn680Ser/rs6166 tests (7.16±1.09; P=0.659). Anti-mullerian hormone (AMH) below 1.2 ng/ml was associated with a higher frequency of mutations: 33.3% A919G/Ala307Thr and A2039G/Asn680Ser (P=0.137) and also in 66.6% FSH receptor less frequent polymorphism (c.-29G>A) rs 1394205 (P=0.522). The age, day 3 FSH, and AMH levels are widely used to investigate female infertility. However, we have not yet found the ideal biomarker to determine the best outcome and treatment plan for our patients. We cconsider that genetic markers will become the future in the personalization of controlled ovarian stimulation treatment in the upcoming period.

Introduction

Follicle-stimulating hormone (FSH) plays a key role in reproduction and infertility. FSH activates the FSH receptor on granulosa cells in the ovary. The polymorphisms of the FSHR gene influence the ovarian action of the endogenous and exogenous FSH. Studies have shown that there are more than 1,800 polymorphisms of the FSHR gene (1).

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This topic becomes important in clinical research because specific genotype patterns of the FSH receptor, estrogen receptors ESR1 and ESR2 have been found as possible causes involved in the poor response to exogenous FSH in *in vitro* fertilization (IVF) protocols (2).

The POSEIDON (Patient-Oriented Strategies Encompassing Individualized Oocyte Number) group introduced a new term of success in controlled ovarian stimulation (COS), meaning the ability to retrieve the number of oocytes needed to obtain at least one euploid blastocyst per transfer. The age of the female is a crucial factor in the POSEIDON classification because age is inversely correlated with embryo aneuploidy (3).

Thus, the POSEIDON criteria classify patients in 4 groups, as groups 1 and 3, if aged less than 35 years, and groups 2 and 4 if aged more than 35 years of age (4,5). This is an important care factor in pregnancies that further require prenatal genetic diagnosis (PGD) because in assisted reproductive techniques (ART) implantation rates reach ideally 50-60% (6).

This investigation evaluated the effect of different FSHR polymorphisms on the ovarian function, investigating ovarian biomarkers such as basal FSH, AMH, and other hormones such as estradiol, progesterone, and prolactin during COS. The association between variants in the FSH receptor gene and current markers of ovarian reserve (AMH, antral follicle count, FSH) and hormonal markers (estradiol, progesterone, prolactin), during ovarian stimulation protocols were evaluated.

Subjects and methods

The study group consisted of 132 patients divided into three groups, depending on the diagnosis. Two poor prognosis groups formed by 54 patients who underwent ovarian stimulation protocols and 44 patients with different degrees of endometriosis that required surgical treatment and a good prognosis group formed by 34 pregnant women who gave birth at term (control group). The study was approved by the Ethics Committee of the 'Grigore T. Popa' University of Medicine and Pharmacy (Iasi, Romania). All patients signed an informed consent prior to the study.

DNA testing. Genomic DNA was extracted from peripheral blood using Wizard Genomic DNA Purification kit (Promega Corp.). FSHR polymorphism c.-29G>A and FSHB

Table I. Frequency of genetic test results according to study groups [n (%)].

Test	FSH R/FSH B polymorphism results	IVF group (n=54)	Endometriosis group (n=44)	Control (pregnancy) group (n=34)	P-value
A919G	W (AA) M (GG)	20 (37.0)	17 (38.6)	8 (23.5)	0.295
	H (AG)	9 (16.7)	7 (15.9)	3 (8.8)	
		25 (46.3)	20 (45.5)	23 (67.6)	
A2039G	W (AA) M (GG)	20 (37.0)	17 (38.6)	7 (20.6)	0.282
	H (AG)	9 (16.7)	7 (15.9)	4 (11.8)	
		25 (46.3)	20 (45.5)	23 (67.6)	
FSHR (c.-29G>A) rs1394205	W (GG) M (AA)	26 (48.1)	26 (59.1)	19 (55.9)	0.828
	H (GA)	3 (5.6)	2 (4.5)	1 (2.9)	
		25 (46.3)	16 (36.4)	14 (41.2)	
FSH B (c.-280G>T)	W (GG) M (TT)	42 (77.8)	38 (86.4)	28 (82.4)	0.213
	H (GT)	3 (5.6)	0 (0)	0 (0)	
		9 (16.7)	6 (13.6)	6 (17.6)	

Data are given as n (%). FSH, follicle-stimulating hormone; IVF, *in vitro* fertilization.

polymorphism c.-280G>T detection were performed by real-time PCR based genotyping solution, with rhAmp™ SNP Genotyping (IDT Integrated DNA Technologies, Inc.).

Statistical analysis. In the statistical analysis, both descriptive and analytical methods were used, with a threshold of significance of 95%. The results were centralized in SPSS v18.0 databases and processed with specific statistical functions.

Results

Ovarian stimulation characteristics. As a type of initial protocol, the long protocol is used in the majority in the world, but as everywhere in the world, the short protocol has become elective. In recent years, the short protocol with antagonists is the latest as a novelty in terms of ovarian stimulation protocols. Rarely, when we use the long protocol the inhibition starts from day 21 with GnRH agonist depot dose or daily injections for 14 days. We use various types of drugs for ovarian stimulation, like FSH recombinant, highly purified urinary FSH, recombinant FSH + LH. The patient was instructed by our nurses how to administer the medication. The stimulation was started (short protocol) only the progesterone was less than 0.8 ng/ml. On the 8th day the patients were called in regarding the stimulation to see how they responded to the medication (how many follicles and what size). We chose the long protocol especially in young patients with endometriosis or in patients in whom progesterone on day 2 was not less than 0.8 ng/ml for several months in a row. We use for the start of stimulation the count of the antral follicle on day 2 and the dosage of progesterone. Stimulation started from day 2, in a short protocol with antagonist, on the 5th day the patient is recalled to receive the answer, and to introduce the antagonist medication.

Depending on the follicle number and size, we also need the dosage of estradiol and progesterone (estradiol to see the

risk of hyperstimulation, which can be assessed well enough on ultrasound but primarily to evaluate the time of onset and progesterone to check the quality of inhibition). The next visit to the doctor will be on day 10-11 when the time of triggering and therefore of the puncture will be established. Triggering is done with 6500 IU recombinant HCG.

Ovarian puncture is performed under transvaginal ultrasound guidance with suction without washing and with the patient often sedated. The embryo transfer is done at 72 h or if the situation allows (sufficient number of oocytes and the patient's desire) or at 5 days (blastocysts) where the implantation rate due to selection is higher compared with 72 h. To support the classic luteal phase, we used progesterone 200 mg intravaginally 3 times per day.

Genetic testing. In the testing A919G/Ala307Thr/rs6165, the frequency of the W (AA) response was higher in the group of patients with endometriosis and IVF (37%, 38.6% vs. 23.5%), and the frequency of the H (AG) response was higher in the control group (46.3%, 45.5% vs. 67.6%) (P=0.295; Table I).

Among the results of the A2039G/Asn680Ser/rs6166 test, the frequency of the W (AA) response was higher in the group of patients with endometriosis and IVF (37%, 38.6% vs. 20.6%), and the frequency of the H (AG) response was higher in the control group (46.3%, 45.5% vs. 67.6%) (P=0.282; Table I).

Among the results of FSH receptor testing (c.-29G>A) rs1394205, the frequency of the W (GG) response was slightly higher in the group of patients with endometriosis and the control group compared with the group of IVF (48.1%; 59.1% vs. 55.9%), and the frequency of response H (GA) was slightly higher in the control group (46.3%; 36.4% vs. 41.2%) (P=0.828; Table I).

Among the results of testing rs10835638 (c.-280G>T) in FSHB, the frequency of response W (GG) (77.8%; 86.4% vs. 82.4%) and H (GT) (16.7%; 13.6% vs. 17.6%) was comparable in frequency in the study groups (P=0.213; Table I).

Table II. Markers of ovarian reserve and correlations with the genetic testing.

| Parameters | A919G/Ala307Thr/rs6165/A2039G/Asn680Ser/rs6166 | | | |
	W (AA)	M (GG)	H (AG)	P-value
AFC	11.12±5.74	11.38±5.58	10.45±5.21	0.896
AMH, ng/ml	1.88±1.71	2.01±1.98	2.20±1.87	0.892
FSH, mUI/ml	6.01±2.53	7.16±1.09	6.60±3.68	0.659
LH, mUI/ml	3.00±1.37	3.91±1.93	4.10±2.23	0.196
Estradiol day 3, pg/ml	29.12±8.93	50.07±14.03	34.63±23.12	**0.036**
Estradiol day 5-7 of stimulation	720±503	671±260	762±228	0.850
Estradiol trigger day	1574±940	1551±836	2026±827	0.313
Progesterone day 3, ng/ml	0.23±0.10	0.33±0.19	0.26±0.17	0.364
Progesterone day 5-7 of stimulation	0.32±0.14	0.37±0.23	0.39±0.20	0.481
Progesterone trigger day	0.52±0.30	0.70±0.63	0.88±0.44	0.073
Prolactin	236.31±50.21	305.29±141.27	436.87±148.56	**0.019**

| Parameters | FSHR (c.-29G>A) rs1394205 | | | |
	W (GG)	M (AA)	H (GA)	P-value
AFC	9.90±4.51	5.50±0.71	12.17±5.90	**0.043**
AMH, ng/ml	2.09±1.89	0.55±0.08	2.13±1.69	**0.049**
FSH, mUI/ml	6.95±1.99	4.68±4.29	6.24±3.31	0.514
LH, mUI/ml	3.38±1.70	2.34±1.57	3.94±2.07	0.407
Estradiol day 3, pg/ml	33.92±16.83	44.50±47.38	34.96±17.86	0.752
Estradiol day 5-7 of stimulation	705±317	160±0	774±409	**0.025**
Estradiol trigger day	1666±888	492±0	1869±872	**0.029**
Progesterone day 3, ng/ml	0.28±0.16	0.20±0.05	0.25±0.15	0.703
Progesterone day 5-7 of stimulation	0.33±0.17	0.22±0	0.38±0.20	0.468
Progesterone trigger day	0.63±0.46	0.41±0	0.75±0.44	0.626
Prolactin	271.05±80.35	0±0	372.78±166.55	0.159

| Parameters | FSHB (c.-280G>T) rs10835638 | | | |
	W (GG)	M (TT)	H (GT)	P-value
AFC	9.91±5.06	18.50±7.78	13.13±4.58	**0.034**
AMH, ng/ml	1.77±1.75	1.88±0	3.00±1.74	0.263
FSH, mUI/ml	6.49±3.13	4.13±0	6.58±1.31	0.723
LH, mUI/ml	3.58±1.94	1.82±0	3.97±1.79	0.561
Estradiol day 3, pg/ml	34.26±18.81	41.00±0	37.64±18.82	0.864
Estradiol day 5-7 of stimulation	692±370	1434±0	796±306	0.119
Estradiol trigger day	1694±879	2445±1185	1710±907	**0.025**
Progesterone day 3, ng/ml	0.23±0.10	0.10±0	0.42±0.25	**0.004**
Progesterone day 5-7 of stimulation	0.36±0.19	0.35±0	0.35±0.16	0.990
Progesterone trigger day	0.62±0.40	1.51±0.90	0.68±0.29	**0.022**
Prolactin	297.78±96.66	219.17±0	458.90±213.45	**0.014**

Data are given as mean ± standard deviation. Bold font indicates statistically significant difference. FSH, follicle-stimulating hormone.

Number of antral follicles (AFC). The number of antral follicles ranged from 3 to 25, the median level (n=10) was close to the mean level of the study group (10.87±5.37), suggesting the homogeneity of the series of values.

Based on the results of mutations in tests A919G/ Ala307Thr/rs6165 and A2039G/Asn680Ser/rs6166, a slightly lower average number of antral follicles was found in heterozygotes (H) (10.45±5.21) compared with mutants (M) (11.38±5.58)

or normal results (W) (11.12±5.74) (P=0.896; Table II). In the FSH receptor test (c.-29G>A) rs1394205, the mean AFC level was significantly higher in heterozygotes (12.17±5.90) (P=0.043), while in the rs10835638 test (c.-280G) >T) in FSH beta chain, the mean level of AFC was significantly higher in mutants (18.50±7.78) (P=0.034).

Depending on the genetic testing, it was noted that the number of antral follicles below six were associated with a low frequency with heterozygotes: 16% A919G/Ala307Thr/rs6165 or A2039G/Asn680Ser/rs6166 (P=0.219); 8% FSHR (c.-29G>A) rs 1394205 (P=0.283); 0% rs10835638 (c.-280G>T) in FSHB (P=0.144). The M (AA) mutation result on the FSHR test was 33.3% associated with antral follicles below six.

Anti-mullerian hormone (AMH). The AMH value ranged from 0.04 to 6.30 ng/ml, the median level (m=1.31) was close to the mean level of the study group (2.02±3.11) and the Skewness test result (P=0.858) suggests homogeneity of the series of values. Depending on the genetic testing, it is noted that AMH below 1.2 ng/ml was associated with a higher frequency of mutations: 33.3% A919G/Ala307Thr/rs6165 or A2039G/Asn680Ser/rs6166 (P=0.137); 66.6% FSHR (c.-29G>A) rs 1394205 (P=0.522). The normal test result rs10835638 (c.-280G>T) in FSHB was 38.1% associated with AMH below 1.2 ng/ml (P=0.032).

Follicular stimulating hormone (FSH). The FSH value ranged from 0.64 to 18.22 mIU/ml, the median level (m=6.43) was close to the mean level of the study group (6.45±2.86) and the Skewness test result (P=1.815) suggests the homogeneity of the series of values. Depending on the results of the genetic testing, a slightly higher average level of FSH values was found in mutants in the A919G/Ala307Thr/rs6165 or A2039G/Asn680Ser/rs6166 tests (7.16±1.09; P=0.659) and in heterozygotes in the tests. FSHR (c.-29G>A) rs1394205 (6.24±3.31; P=0.514) and10835638 (c.-280G>T) in FSHB (6.58±1.31; P=0.723), compared with mutants (Table II).

Estradiol. On day 3, estradiol ranged from 9 to 92 pg/ml, the median level (m=32 pg/ml) was relatively close to the mean level of the study group (34.97±14.42) and the test result Skewness (P=1.082), suggests the homogeneity of the series of values. At 5-7 days, estradiol ranged from 160 to 2115 pg/ml, the median level (m=697.5 pg/ml) was relatively close to the mean level of the study group (727.38±370.54) and the result in the Skewness test (P=1.284), suggests the homogeneity of the series of values. Also, on the trigger day, estradiol ranged from 433 to 3779 pg/ml, the median level (m=1724 pg/ml) was close to the mean level of the study group (1737±886) and the Skewness test result (P=0.398), suggests the homogeneity of the series of values, so tests of statistical significance can be applied (Table II).

Progesterone. On day 3, progesterone ranged from 0.10 to 0.76 ng/ml, the median level (m=0.23 ng/ml) was close to the mean level of the study group (0.26±0.15). The Skewness test (P=1.828), suggests homogeneity of the series of values. At 5-7 days, progesterone ranged from 0.12 to 1.02 ng/ml, the median level (m=0.33 ng/ml) was close to the mean level of the study group (0.35±0.18) and the result of the Skewness test (P=1.669), suggest the homogeneity of the series of

values. Also, on the trigger day, progesterone ranged from 0.12 to 2.14 ng/ml, the median level (m=0.65 ng/ml) was close to the mean level of the study group (0.68±0.45). The result of the Skewness test (P=1.628), suggest the homogeneity of the series of values, so tests of statistical significance can be applied.

Prolactin. Prolactin ranged from 169.84 to 684.32 mIU/ml, the median level (m=270.76) was relatively close to the mean level of the study group (331.07±143.76), suggesting homogeneity of the values. Based on the tests A919G/Ala307Thr/rs6165 and A2039G/Asn680Ser/rs6166, a significantly higher mean prolactin level was found in heterozygotes (H) (436.87±148.56), compared with mutants (M) (305, 29±141.27) or normal results (W) (236.31±50.21; P=0.019).

Poseidon classification did not show significant percentage differences (P=0.869), regardless of the result of testing A919G/Ala307Thr/rs6165 or A2039G/Asn680Ser/rs6166, 20-25% of patients were in class 2b. Poseidon criteria showed significant percentage differences (P=0.039) in FSHR testing (c.-29G>A) rs1394205, 66.7% of patients with M (GG) mutations were in class 2b, while in heterozygotes 60% were class 1a and 1b.

Poseidon group showed significant percentage differences (P=0.011) in the rs10835638 (c.-280G>T) test in FSHB, 66.7% of patients with M (TT) mutations were in class 1a, while in heterozygotes only 33.3% were from class 1a.

Discussion

It is known that the prognosis of the results for IVF procedures depends on how the ovary reacts to the administration of exogenous FSH. POSEIDON criteria for 'low prognosis' patients undergoing ART were founded in 2016 to help facilitate these terms, using age, AMH, and antral follicle count.

Many authors have investigated variations in different receptors such as the estrogen receptor, with its two forms ESR1 and ESR2 genes, and analyzed the influence of these variations on the number of retrieved oocytes after an ovarian stimulation protocol.

Altmäe *et al* (7), found that ovarian response to exogenous FSH decreased in patients with endometriosis compared with other types of infertility (i.e. pelvic inflammatory disease).

Alviggi *et al* (8), demonstrated that the polymorphic variant of the FSH receptor (FSH-R) was correlated with higher basal FSH values. Recent studies showed that the most common type of polymorphism is FSHR (Asn680Ser), and in affected patients, it requires increased consumption of exogenous FSH during IVF procedures. Therefore, the term of 'ovarian resistance' (hypo-response) was postulated to exogenous FSH, in these patients.

Alviggi *et al* (9), found a common LH polymorphism (v-LH) in infertile patients to whom were administered more than 3000 IU of exogenous FSH to obtain more than five oocytes. Therefore, the present hypothesis is that women with v-LH variants are prone to be less responsive to recombinant FSH.

Valkenburg *et al* (10), investigated women with type II anovulatory infertility (according to WHO classification). They found intimate associations between the most common type of

FSH receptor polymorphism (p.N680S) and the outcome of ovarian stimulation. Using exogenous FSH, a better outcome was observed for carriers of the 680S allele during second-line treatment that was not present with classic clomiphene citrate induction of ovulation.

It appears that FSH R gene polymorphism is influenced by race distribution. Fang et al (11), investigated 5 different FSHR polymorphisms (rs1394205, rs115030945, rs6165, rs6166, 2309T>C). As expected, linkage disequilibrium was observed between rs6165 and rs6166. The allele frequencies of rs6165 and rs6166 in Chinese women were lower than in Caucasians.

In our study, depending on the genetic testing, we noted that the number of antral follicles below 6 were associated with a low frequency with heterozygotes: 16% A919G/Ala307Thr/rs6165 or A2039G/Asn680Ser/rs6166 (P=0.219); 8% FSHR (c.-29G>A) rs 1394205 (P=0.283); 0% rs10835638 (c.-280G>T) in FSHB (P=0.144). The M (AA) mutation result on the FSHR test was 33.3% associated with antral follicles below six.

In our cohort of Romanian women, a slightly higher average level of FSH values was found in mutants in the A919G/Ala307Thr/rs6165, A2039G/Asn680Ser/rs6166 tests (7.16±1.09; P=0.659) and in the heterozygotes in the tests. FSHR (c.-29G>A) rs1394205 (6.24±3.31; P=0.514) and 10835638 (c.-280G>T) in FSHB (6.58±1.31; P=0.723), compared with mutants.

On the contrary, in a Brazilian cohort of patients, the ovarian response to exogenous FSH did not vary according to the FSH receptor genotype (position-29 and 680), neither did the number of FSH ampoules required to obtain a proper ovarian stimulation. The presence of the Ala307Thr polymorphism was associated with an early onset of premature ovarian failure. Ovarian size or number of antral ovarian follicles was not associated with FSHR genetic variants (12).

Boudjenah et al (13), tested for no less than 13 gene polymorphisms: FSHR(Asn680Ser), p53(Arg72Pro), AMH(Ile49Ser), ESR2(+1730G>A), ESR1(-397T>C), BMP15(-9C>G), MTHFR1(677C>T), MTHFR2(1298A>C), HLA-G(-725C>G), VEGF(+405G>C), TNFalpha (-308A>G), AMHR(-482 A>G), PAI-1 (4 G/5 G), using PCR assay in order to genotype women undergoing ICSI program. Only the FSHR and AMH polymorphism combined were found to have a statistical difference regarding mature oocyte numbers (M2 oocyte). FSH receptor gene variations such as Asn680Ser, Ala189Val, Thr449Ile, and Ile160Thr did not seem to determine poor ovarian response to treatment. In contrast, the low ovarian reserve determined by AMH was an independent prognostic factor.

In our investigation, AMH below 1.2 ng/ml was associated with a higher frequency of mutations: 33.3% A919G/Ala307Thr/rs6165 or A2039G/Asn680Ser/rs6166 (P=0.137); 66.6% FSHR (c.-29G>A) rs 1394205 (P=0.522). The normal test result rs10835638 (c.-280G>T) in FSHB was 38.1% associated with AMH below 1.2 ng/ml (P=0.032).

Mohiyiddeen et al (14), found no evidence between variants in the FSHR receptor (FSHR) gene and routinely used markers of ovarian reserve (antimullerian hormone, antral follicle count, FSH). Extensive data have been published in the literature, for poor responder women, including high doses of gonadotropins, the luteal onset of GnRH-a, and the short protocol. However, the truth is that as yet an ideal stimulation has not been found for these patients with diminished ovarian reserve (15).

The wide variability in the poor responder patient population makes it difficult to cope with the possibility of a single treatment plan in COS to make the patient needs ideal. Modern medicine has developed new treatment options to obtain every single patient's characteristics. These could be used to cross-match patients with the best treatment plan to improve efficacy during COS. At present, female age and basal FSH levels are the most used in patient profiles in clinical practice. These can offer only a mediocre prognosis for success and can lead to general recommendations for standard COS treatment plans. In contrast, the AMH level appears to be a better predictor of ovarian reserve and response to COS. The antral follicle count is also a good predictor to plan the dose of exogenous FSH necessary during ovarian stimulation.

In the future, genetic testing will help to individualize the patient's response, according to genotype, when they decide to star ovarian stimulation. Unfortunately, we cannot offer an ideal biomarker as a guide to determine the best treatment plan. A correlation between different markers such as hormonal and genetic ones will make possible an individualized COS.

Currently there is only one polymorphism of the FSH receptor gene (codon 680) for which sufficient data have consistently found a significant association with ovarian dysfunction.

Polymorphisms of the FSHR gene may be used as markers to predict differences in FSHR function and ovarian response to FSH. They may be a pioneer in ovarian stimulation protocols and treatment personalization for every woman's need.

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Availability of data and materials

The corresponding author should be contacted for any further details regarding patient database.

Authors' contributions

AET analyzed and interpreted the patient data. MO and AL performed the ovarian stimulation protocols. RP analyzed FSHR polymorphism data. AC and RM had important contribution to the conception of the study. DN was involved in the acquisition and interpretation of the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the 'Grigore T. Popa' University of Medicine and Pharmacy (Iasi, Romania). All patients signed a consent form prior to the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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