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GENETICS

A distribution of two SNPs in exon 10 of the FSHR gene among the women with a diminished ovarian reserve in Ukraine

Ganna Livshyts · Svetlana Podlesnaja · Sergey Kravchenko · Iryna Sudoma · Ludmila Livshits

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Abstract

Purpose To evaluate the association between phenotype and follicle stimulating hormone receptor (FSHR) genotype in women with ovarian dysfunction and patients with "poor response" to gonadotropin stimulation of ovulation.

Methods FSHR gene SNPs were analyzed by PCR and RFLP. "Poor responders" (ovarian dysfunction) group and "good responders" group constituted the study group. Normo-ovulatory women who gave birth to naturally conceived children formed control groups: under 35 years of age (control I) and over 35 years of age (control II).

Results The frequency of Ala307-Ser680/Ala307-Ser680 genotype was significantly more prevalent in the ovarian dysfunction group (26%) compared to the control I (7.7%) ($P<0.001$) and a "good responders" group (12.5%) ($P<0.05$); and in a "poor responders" group (33.3%) compared to a "good responders" group ($P<0.05$), control I ($P<0.001$) and control II (17.5%) ($P<0.05$).

Conclusions Our data shows the prevalence of the Ala307-Ser680/ Ala307-Ser680 genotype in the both groups of patients. The finding should have impact on the delineation of stimulation protocols.

Keywords FSH receptor · DNA polymorphism · Ovarian reserve · FSH stimulation

FSHR receptor gene polymorphisms and diminished ovarian reserve.

Capsule The association between phenotype and Asn680Ser and Thr307Ala FSHR gene polymorphisms was found in women with ovarian dysfunction and poor response to FSH ovarian stimulation.

G. Livshyts · S. Podlesnaja · S. Kravchenko · L. Livshits (✉)
Department of Human Genomics,
Institute of Molecular Biology and Genetics
(National Academy of Sciences of Ukraine),
150 Zabolotnogo Street,
03680 Kiev, Ukraine
e-mail: livshits@imbg.org.ua

I. Sudoma
Department of Obstetrics, Gynecology and Reproductology,
P. L. Shupic National Medical Academy of Post-graduate Education,
Kiev, Ukraine

Introduction

Follicle stimulating hormone (FSH) is one of the pituitary glycoproteins that plays an important role during folliculogenesis by triggering the maturation of follicles, i.e. the proliferation of granulosa cells, and induces synthesis of the androgen-converting enzyme aromatase [1, 2]. Furthermore, it plays a pivotal role in the recruitment of the dominant follicle.

FSH action is mediated by the FSH receptor (FSHR), a member of the family of G-protein-coupled receptors expressed solely in granulosa cells, which mediates FSH signal transduction through adenylate cyclase activation and elevation of intracellular cAMP [3]. The FSHR gene contains a single large exon, which encodes the transmembrane and intracellular domains; and nine smaller exons, which encode the extracellular domain [4].

The first inactivating mutation in the FSHR gene was described in 1995 in some Finnish families with primary ovarian failure, the so-called pure gonadal dysgenesis, as a phenotype [5]. Since this mutation has been relatively identified in the Finnish population, it was originally thought that FSHR mutations in general could constitute a common defect in ovulation failure and amenorrhoea. The mutation analysis in DNA samples obtained from subjects of different ethnic origins revealed a selective enrichment of the mutation

in the Finnish population only, while such a mutation was found extremely rare in other ethnic groups [6]. A sporadic heterozygous Val241Ala mutation found in one infertile man did not impair receptor function [7] and was judged not to be responsible for the phenotype. Other mutations were revealed in rare sporadic cases of primary or early secondary amenorrhoea [8]. This finding is consistent with the indispensable role of FSH in human reproduction, so that such rare mutations abolishing gonadotropin activity lead to infertility and therefor are self-eliminating. It then became evident that partially inactivating mutations and polymorphisms of the FSHR can cause arrest at the antral or later stages of follicular growth and thereby lead to a diminished ovarian reserve. Screening of several hundreds of patients and controls world-wide led to the discovery of two SNPs (single nucleotide polymorphisms) in the FSH receptor gene and to the subsequent study of the correlation between polymorphisms and gonadal function (http://www.ncbi.nlm.nih.gov/SNP/). These two non-synonymous SNPs with frequencies of >30% in the normal population have been identified in the coding region of exon 10 of the FSH receptor gene (http://www.ncbi.nlm.nih.gov/entrez/; GeneID: 2492; Locus tag: HGNC: 3969). The first (rs6165) is located at position 919 (numbering according to the translational start codon with ATG as 1) in which A is substituted by G, changing codon 307 from threonine (ACT) to alanine (GCT). The second (rs6166) is located at nucleotide position 2039 in which G is replaced by A. This leads to an amino acid change at position 680 from serine (AGT) to asparagine (AAT) [5].

FSH has been used in the treatment of infertility since 1975, when the first ovulation induction in women by using human pituitary extracts was reported [9]. Today, the clinical use of recombinant FSH preparations in the treatment of infertility results in steadily increasing proportions of live-born children [10, 11]. It was shown that the dose of FSH given to the patient is determined by several factors, principally by age, previous response to FSH stimulation and the basal level of FSH. An adequate response indicates a normal ovarian function, also called a normal ovarian reserve. For poor response, several possible aetiologies could be suggested, but it seems that diminished ovarian reserve is the principal one [12, 13]. To this day, there is still some uncertainty as to whether FSH receptor polymorphisms play a pathophysiologic role in ovarian dysfunction or ovarian response to stimulation.

The purpose of this study is to examine the distribution of two distinct FSHR gene substitutions—919A→G (Thr307Ala variant) and 2039G→A (Asn680Ser variant), and evaluate the association between phenotype and FSHR genotype in women with ovarian dysfunction and patients with “poor response” to gonadotropin stimulation of ovulation.

Materials and methods

Subjects

SNPs analysis for FSHR307 and FSHR680 loci of the FSHR gene was performed in 374 women from Ukraine after informed consent. Group of patients with ovarian dysfunction diagnosis (102 individuals) consisted of women who had ages of less than 40 years, cessation of menses for more than 6 months and FSH levels of more than 25 IU/l. The “poor responders” (n=39) group included normogonadotrophic (FSH concentrations within normal limits (1–9.6 IU/L) patients who were younger than 40 years old with decreased antral follicle count (less than four antral follicles in each ovary if evaluated with transvaginal ultrasound) and whose ovarian response after controlled ovarian stimulation with gonadotropins was less than four follicles and/or oocytes. Patients with surgical interventions on the uterine adnexes (tubes and ovaries) and ovarian lesions (endometriomas, cysts) were not included. Controlled ovarian stimulation was performed with Gonal F in daily dosage 225–300 IU (n=39).

Control groups consisted of three groups. “Good responders” group included women who were younger than 40 years old and whose ovarian response after controlled ovarian stimulation with gonadotropins was more than ten follicles and/or oocytes. Controlled ovarian stimulation was performed with Gonal F in daily dosage 125–250 IU (n=40). Control group I consisted of normo-ovulatory women who gave birth to a naturally conceived children, under 35 years of age (n=130). Control group II consisted of normo-ovulatory women who gave birth to a naturally conceived children in age over 35 years (n=63).

Basal (day 3) serum FSH was measured time-resolved fluoroimmunoassay, using a commercial kit (DELFIA) manufactured by Wallac Finland Oy, Turku, Finland. The ultrasound examination was performed on the Accuvix (Medison, Korea)

Controlled ovarian

For controlled ovarian hyperstimulation (COH) the long luteal protocol was used. On the 19–24th day of the cycle the patients received gonadotropin releasing hormone agonist (a-GTRH) (Difereline 3.75 mg, Beaufour Ipsen International, France). In 2 weeks (after the 3rd day of the menstrual cycle), the COH with gonadotropins (Gonal-F, Serono S.A., Switzerland) was fulfilled with daily dose of 225–300 IU. When the dominant follicle diameter exceeded 18 mm, human chorionic gonadotropin (hCG) (Ovitrel, Serono S.A., Switzerland) was administered. The oocytes were retrieved approximately 34 h after hCG administration.

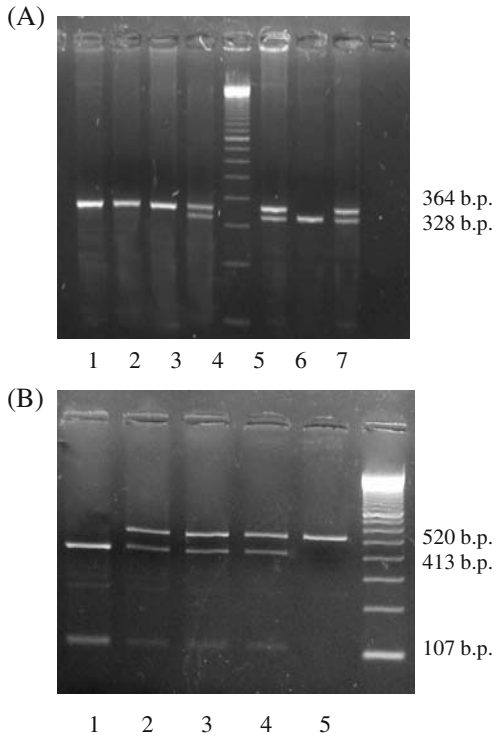

Fig. 1 **a** RFLP analysis of Thr307Ala FSH receptor variant (2% agarose gel electrophoresis) 1, 2, 3-Thr307Thr variant; 4, 6, 8-Thr307Ala variant; 5-molecular mass marker (Ladder 100 bp); 7-Ala307Ala variant, **b** RFLP analysis of Asn680Ser FSH receptor variant (2% agarose gel electrophoresis) 1-Ser680Ser variant; 2, 3, 4-Asn680Ser variant; 5-Asn680Asn variant; 6-molecular mass marker (Ladder 100 bp)

DNA isolation

DNA was extracted from the peripheral blood leukocytes by standard phenol-chloroform extraction methods with proteinase K (Fermentas, Lithuania) [14]. For DNA extraction and purification we used eppendorf centrifuge 5415C (Eppendorf, Germany).

PCR analysis

For the FSHR Thr307Ala variant, PCR amplification of a fragment of exon 10 was performed with the use of specific oligonucleotide primers described by Satoko Sudo et al [15]. To analyze the FSHR Asn680 Ser variant, polymerase chain reaction (PCR) amplification of the fragment of exon 10 was performed with the use of specific oligonucleotide primers, as described elsewhere [16]. The PCR reaction was performed in a final volume of 25 μL containing 1 × PCR buffer, 1.5 mM MgCl2, 200 μM of each dNTP, 1 μM of each primer, 0.2 units of Taq-DNA polymerase (Fermentas, Lithuania) and 200 ng of the DNA template.

The cycling conditions for Thr307Ala variant were as follows: initial denaturation at 94°C for 5 min to 2 cycles consisting of denaturation at 94°C for 45 s, annealing at 56°C for 50 s, extension at 72°C for 50 s to 5 cycles consisting of denaturation at 94°C for 45 s, annealing at 53°C for 50 s, extension at 72°C for 50 s; 23 cycles consisting of denaturation at 94°C for 45 s, annealing at 51°C for 45 s, extension at 72°C for 50 s; and a final elongation step at 72°C for 7 min. The cycling conditions for Asn680Ser variant were as follows: initial denaturation at 94°C for 5 min, 30 cycles consisting of denaturation at 94°C for 40 s, annealing at 55°C for 40 s, extension at 72°C for 40 s and a final elongation step at 72°C for 3 min. For all experiments Applied Biosystems GeneAmp PCR system 2720 Termal Cycler (Applied Biosystems, USA) was used.

The PCR products were 364 bp and 520 bp long correspondingly.

RFLP (restriction fragment length polymorphism) analysis of the Thr307Ala and Asn680Ser variants:

For the Thr307Ala variant in one of the primers, a mismatch nucleotide has been introduced [15]. This mismatch and the A to G transition create an Eco81I restriction site (Fermentas, Lithuania). The A to G transition in Asn680Ser

Table 1 Genotype frequency distribution among control groups and two patient groups for FSHR307 and FSHR680 variants

Group	Genotype					
	FSHR307 locus			FSHR680 locus		
	TT n (%)	TA n (%)	AA n (%)	NN n (%)	NS n (%)	NS n (%)
Ovarian dysfunction	19 (18.6)	55 (53.9)	28 (27.5)	19 (18.6)	55 (53.9)	28 (27.5)
“Poor responders”	7 (18.0)	19 (48.7)	13 (33.3)	5 (12.8)	20 (51.3)	14 (37.9)
“Good responders”	12 (30.0)	23 (57.5)	5 (12.5)	12 (30.0)	23 (57.5)	5 (12.5)
Control I	43 (11.5)	72 (55.4)	15 (33.1)	43 (14.6)	68 (52.3)	19 (33.1)
Control II	29 (46.0)	23 (36.5)	11 (17.5)	29 (46.0)	23 (36.5)	11 (17.5)

T - 307Thr; A - 307Ala; N - 680Asn; S - 680Ser

Table 2 Combined genotypes distribution

Genotype	Ovarian dysfunction group n=102, (%)	"Poor responders" group n=39, (%)	Control I n=130, (%)	"Good responders" group n=40, (%)	Control II n=63, (%)
AASS	27 (26)[a,b]	13 (33.3)[c,d,e]	10 (7.7)[b,d]	5 (12.5)[a,c]	11 (17.5)[e]
TTNN	17 (17)	5 (12.8)	38 (29.2)	12 (30)	28 (44.4)
ATSN	52 (51)	18 (46.2)	59 (45.4)	23 (57.5)	22 (34.9)
AASN	1 (1)	0	4 (3.1)	0	0
ATSS	1 (1)	1 (2.6)	9 (6.9)	0	0
ATNN	2 (2)	0	4 (3.1)	0	1 (1.6)
TTNS	2 (2)	2 (5.1)	5 (3.8)	0	1 (1.6)
AANN	0	0	1 (0.8)	0	0

T - 307Thr; A - 307Ala; N - 680Asn; S - 680Ser
Fisher exact test [a,c,e] $P<0.05$; [b, d] $P<0.001$

variant creates a restriction site for endonuclease BseN1I (Fermentas, Lithuania).

PCR products of exon 10 were digested with Eco81I for Thr307Ala variant and BseN1I for Asn680Ser variant. Digestion was performed in 15 μL reaction volume containing 1 × reaction buffer, 0.5 units of the restriction enzyme and 10 μL of purified PCR product, incubated at 37°C overnight for Thr307Ala variant and 60°C for 2 h for Asn680Ser variant. Restriction endonuclease digestion products were visualized, in 2.0% agarose gel, and photographed.

Statistical analysis was performed by χ^2 test, Fisher's exact test, likelihood-ratio test and Expectation-Maximization (EM) algorithm using GENEPOP [17] and ARLEQUIN [18] packages. $P< 0.05$ was considered significant.

Results

Based on RFLP analysis of substitution 919A→G (Thr307Ala variant), patients were classified into three groups of genotypes: 307Thr/Thr, 307Thr/Ala and 307Ala/Ala. The presence of three different patterns was revealed for 919A→G substitution (Thr307Ala variant): a 364 bp band (for 307 Thr/Thr), a 364 bp and a 328 bp bands (for 307 Thr/Ala) and a 328 bp band (for 307 Ala/Ala). The small 36 bp band has run out from the gel (Fig. 1a).

Three different patterns can be observed for 2039G→A substitution (Asn680Ser variant): a 520 bp band (for 680 Asn/Asn); a 520 bp, a 413 bp and a 107 bp bands (for 680 Asn/Ser); a 413 bp and a 107 bp bands (for 680 Ser/Ser) (Fig. 1b).

Based on this RFLP analysis of Asn680Ser variant, patients were classified into three groups: 680Asn/Asn, 680Asn/Ser and 680Ser/Ser.

Genotype frequency of FSHR307 and FSHR680 loci for all investigated groups are shown in Table 1. The observed genotype distributions showed no deviations from Hardy-Weinberg expectations in all investigated groups [19]. Distribution of combined genotypes (polymorphisms at position 307 and 680) is shown in Table 2.

It's important to note that Ala307-Ser680/Ala307-Ser680 genotype was more prevalent in patients with ovarian dysfunction (26%) and in "poor responders" (33.3%) group, compared to "good responders" (12.5%), control I (7.7%) and control II (17.5%) groups.

Pairwise comparisons of investigated groups using Fisher exact test revealed statistically significant differences of Ala307-Ser680/ Ala307-Ser680 genotype frequencies between ovarian dysfunction group and "good responders" group ($P<0.05$) as well as ovarian dysfunction group and control group I ($P<0.001$). Moreover, pairwise comparisons of "poor responders" and all three control groups revealed statistically significant differences of Ala307-

Table 3 Distribution of FSHR gene haplotypes TN, AN, TS and AS among control groups and two patients groups

Group	FSHR gene haplotypes			
	TN n (%)	AN n (%)	TS n (%)	AS n (%)
Ovarian dysfunction	90 (44.1)	3 (1.5)	3 (1.5)	108(52.9)[a,c,d]
"Poor responders"	30 (38.5)	0 (0)	3 (3.8)	45 (57.7)[b,e,f]
"Good responders"	47 (58.7)	0 (0)	0 (0)	33 (41.3)[a,b]
Control I	144 (55.4)	10 (3.8)	14 (5.4)	92 (35.4)[c,e]
Control II	79 (62.7)	1 (0.8)	1 (0.8)	45 (35.7)[d,f]

T - 307Thr; A - 307Ala; N - 680Asn; S - 680Ser
Fisher exact test [a,b] $P<0.05$; [c,d,e,f] $P<0.001$

Ser680/ Ala307-Ser680 genotype frequencies between "poor responders" and "good responders" groups($P<0.05$), between "poor responders" and control I groups ($p<0.001$), as well as between "poor responders" and control II groups ($P<0.05$) (see Table 2).

Using likelihood-ratio test, the strong linkage disequilibrium between FSHR307 and FSHR680 loci was revealed in all investigated groups ($P<0.0001$).

Concerning the two Asn680Ser and Thr307Ala polymorphic variants in exon 10, they can be associated in four possible combinations: Thr307-Asn680, Ala307-Ser680, Ala307-Asn680 and Thr307-Ser680. We calculated maximum likelihood haplotype frequencies using EM algorithm for two loci.

It has been revealed that haplotypes Thr307-Asn680 and Ala307-Ser680 are very common (55.4% and 35.4%), while the haplotypes Ala307-Asn680 and Thr307-Ser680 are found only in 3.8% and 5.4% of normo-ovulatory group from Ukraine (control group I), correspondingly.

The frequency of Ala307-Ser680 haplotype was significantly higher both in ovarian dysfunction group and in "poor responders" group, compared to "good responders" ($P<0.05$), control groupI and control groupII ($P<0.001$). The distribution of FSHR gene haplotypes among control groups and two patients groups is shown in Table 3.

Discussion

Our data revealed the statistically significant differences of Ala307-Ser680/Ala307-Ser680 genotype frequencies between two groups of patients with diminished ovarian reserve (women with ovarian dysfunction and "poor responders") and controls. The distribution of several allelic variants of the FSHR gene has been described in different populations [6, 15, 16, 20]. Several studies have attempted to correlate the frequency distribution of FSH receptor polymorphisms and ovarian function [21–24]. The studies based on large numbers of patients identified a significant correlation between the heterozygous Thr307-Asn680/ Ala307-Ser680 genotype and polycystic ovary syndrome [15] and between the homozygous Ser at position 680 and World Health Organization type II amenorrhoea. Moreover, the identification of FSHR allelic variants led to the investigation of their potential role as predictors of ovarian response to a gonadotropin stimulation protocol [16].

Previously, it has been shown that the difference between the basal level of serum FSH in the persons with 680Ser/Ser genotype and in the persons with 680Asn/Asn genotype was not significant [15], suggesting that this receptor variants might result in a mild 'resistance' to the gonadotropin.

Our results are in agreement with those obtained in other studies, and suggest that ovarian response to exogenous gonadotropin may depend on the FSH receptor genotype of treated patients [15, 20, 25]. However, in the study of Laven et al. the increase of medium serum FSH levels was shown in anovulatory infertile woman with Ser/Ser 680 variant, but the ovarian response after ovulation induction did not differ among the patients with different FSH receptor genotypes. Such contradictions can be connected in the work of Laven et al. where another group of patients was investigated. These were normogonadotropic anovulatory patients (with WHO class II disease and PCOS). In spite of the fact that the response of the ovary to exogenous FSH varies considerably among such patients and sometimes is rather poor, they have normal or even increased ovarian reserve [26, 27]. In our study the "poor responders" group consisted of normogonadotropic women with decreased antral follicle count, which reflects the diminished ovarian reserve. The difference in protocols of stimulation in two studies is also obvious: in our study the supraphisiological doses of gonadotropins were used to achieve the multifollicular growth, while in the study of Laven et al. the induction of monofollicular growth was fulfilled and the doses of FSH were closer to physiological ones [16].

The results obtained in previous studies showed that all heterozygotes for the mutations so far studied have been free from phenotypes, indicating that a single functional FSH receptor gene allele is sufficient for normal reproductive function [28].

The statistically significant prevalence of Ala307-Ser680 observed in the ovarian dysfunction and "poor responders" groups is an additional evidence that the polymorphisms in exon 10 are associated with dimished reserve.

It has been shown that Thr30Ala substitution is located in extracellular domain of protein. On the other hand, Asn680Ser is located in intracellular domain and can change the phosphorylation by cAMP-dependent protein kinases. Strong linkage disequlibrium between these loci observed in our study and in other investigations [12] obscures the role of each polymorphism on ovarian response. Further studies of FSHR protein structure can clarify the role of each polymorphism in FSHR protein bioactivity.

Conclusions

Our data show the significant prevalence of Ala307-Ser680 allele in both patients groups. The results of our study and the investigations of other authors show a coupling between the polymorphisms and the phenotype of infertility. The finding that the FSHR genotype modifies ovarian response to FSH ought to have an impact on the delineation of stimulation protocols.

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