FSH-receptor *Ala307Thr* polymorphism is associated to polycystic ovary syndrome and to a higher responsiveness to exogenous FSH in Italian women

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Abstract

Objective Herein we analyzed FSH-R polymorphism at position 307 aiming (a) to assess the prevalence of the three allelic variants (*Ala307Ala*, *Ala307Thr* and *Thr307Thr*) in relation to the type of ovary and (b) to clarify if the allelic variant could influence the responsiveness to exogenous FSH. **Study design** We prospectively studied a group of 106 Italian women undergoing in vitro fertilization (IVF), among which 40 were subjects with polycystic ovary syndrome (PCOS) and 66 were normo-ovulatory women with a normal ovarian morphology at transvaginal ultrasound. DNA extraction, denaturing high-performance liquid chromatography (dHPLC) and DNA sequencing were used to detect the FSH-R 307 polymorphic genotype and the whole exon 10 was analyzed. **Results** The heterozygote variant *Ala307Thr* was significantly more frequent than the homozygote variants in women with PCOS, whereas in normo-ovulatory women with normal ovary the three allelic variants had a comparable prevalence. Women bearing the *Ala307Thr* variant showed a higher ovarian responsiveness to exogenous FSH than normo-ovulatory subjects. **Conclusions** The heterozygote FSH-R polymorphism *Ala307Thr* is significantly more frequent in women with

PCOS than in normo-ovulatory subjects and is more frequently associated with a higher ovarian responsiveness to exogenous FSH.

Keywords Follicle-stimulating hormone · Follicle-stimulating hormone receptor · Follicle-stimulating hormone receptor polymorphism · Polycystic ovary syndrome · In vitro fertilization

Introduction

Follicle stimulating hormone (FSH) is needed for gamete production in both genders. Its action is accomplished via binding with a specific receptor (FSH-R) situated on the plasma membrane of Sertoli cells in testes and of granulosa cells in ovarian follicles.

Several polymorphisms of the FSH-R gene have been described; those that involve nucleotides codifying for amino-acids at positions 307 and 680 are the most clinically relevant [1, 2]. The relationship between allelic variants at these positions, the presence of polycystic ovary syndrome (PCOS), and the responsiveness to exogenous FSH have been studied in some ethnic groups [3]. A different responsiveness to FSH according to the allelic variant at position 680 has been repeatedly reported, *Ser680* homozygote women being more FSH-resistant than women with homozygote *Asn680*, and heterozygote (*Ser680Asn*) patients showing an intermediate responsiveness [4–7].

The functional properties of FSH-R according to polymorphism at position 307 have been poorly studied. A couple of studies have been published, one showing that normo-ovulatory women with homozygous *Ala307* have

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the best responsiveness to exogenous FSH [8], the other showing no 307 variant-linked changes in the responsiveness to FSH in chronic anovulatory subjects [9]. A significant increase in the *Ala307Thr* genotype frequency among women with PCOS was also reported [9–11], but not confirmed by other studies [12, 13].

We analyzed herein the FSH-R polymorphism at position 307 in a group of Italian women. We aimed to clarify (a) the prevalence of the 307 allelic variants in women with normal ovary and in women with PCOS, and (b) if the 307 allelic variants affect ovarian responsiveness to exogenous FSH.

Materials and methods

Subjects

One hundred and six Italian women at their first IVF attempt were studied. None of them had previous surgery, chemotherapy or radiotherapy involving the ovary, hormone-secreting cancers, or were undergoing other hormonal treatments at the time of IVF. Sixty-six patients were normo-ovulatory with no sign of hyperandrogenism and normally appearing ovaries at transvaginal US. Forty patients had the following characteristics: a) menstrual irregularities (amenorrhea or oligomenorrhea) with proven chronic anovulation; b) clinical (hirsutism) and/or serological hyperandrogenism (increased blood testosterone); c) polycystic aspect of the ovaries at transvaginal ultrasound (at least 12 follicles/ovary, increased ovarian volume). They were classified as “full PCOS” patients, and represent a homogeneous population selected among all PCOS patients that every year are submitted to IVF in our Unit, that performs approximately 1000 IVF/year.

For each patient the following variables were recorded: age, body mass index (BMI), inter-menstrual interval, basal (day 3 of the menstrual cycle) FSH circulating level, basal antral follicle count (AFC). The study was approved by the local Ethical Committee and written informed consent was obtained from all participants.

IVF procedure

Superovulation was induced in all patients with a standard “long” protocol with GnRH agonist (Buserelin) plus recombinant FSH (rFSH) at appropriate doses (100–450 IU). Ovarian response to rFSH was monitored by transvaginal US plus serum E_2 measurement every third day from day 7 after the beginning of ovarian stimulation. Ovulation was triggered giving 10,000 IU hCG when the leading follicle reached 18 mm, with appropriate serum E_2 . Transvaginal US-guided oocyte retrieval (OPU) was

scheduled 36 hrs after hCG. Either IVF or ICSI was performed according to the clinical indication. Embryo transfer (ET) was scheduled 48 hrs after OPU. Luteal phase was supplemented by vaginally administered natural progesterone (400 mg/d).

The following variables were recorded for each IVF cycle: stimulation length, total exogenous FSH dose, number of retrieved oocytes, peak estradiol level. The ratio between total FSH dose and number of retrieved oocytes was used to describe ovarian responsiveness to rFSH; in a previous analysis based on 4800 IVF cycles in which the ovary was stimulated with the same protocol as herein, patients requiring less than 200 FSH IU/oocyte represented the 10% most responsive, and the total FSH/retrieved oocytes ratio showed a very high correlation with AMH levels (personal, unpublished data). For these reasons, patients needing less than 200 IU FSH per retrieved oocyte were defined as the “best-responders”.

DNA extraction and amplification

DNA was isolated from EDTA blood samples using a semi-automatic instrument (6100 Nucleic Acid PrepStation extractor, Applied Biosystems) and was quantified using a spectrophotometric method (BioPhotometer, Eppendorf). The sequence of exon 10 of the FSH-R gene was referred to the GenBank Gene ID 2492, whose transcript is identified by the mRNA sequence NM000145. In order to amplify exon 10, the same primers described by Gromoll [14] were used. Since exon 10 is divided into 7 fragments (from 10A to 10G), we performed three different Polymerase Chain Reactions (PCRs) to amplify the whole exon.

For fragment 10A and 10B, each PCR contained 800 μ M of deoxynucleoside triphosphates (dNTPs) (Applied Biosystems), 10X PCR buffer (Applied Biosystems), 1,5 mM $MgCl_2$ (Applied Biosystems), 0,4 μ M primer (Invitrogen) and 2 U AmpliTaq Gold® (Applied Biosystems) in 50 μ l distilled water. Denaturation at 95°C for 6 min was followed by 35 cycles at 94°C for 45 sec, 49°C for 45 sec, and 72°C for 90 sec, followed by a final elongation step at 72°C for 10 min. For fragment 10C, each PCR contained 1 mM of dNTPs, 10X PCR buffer, 2 mM $MgCl_2$, 0,4 μ M primer and 1,25 U AmpliTaq Gold® in 50 μ l distilled water. Denaturation at 95°C for 6 min was followed by 47 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 75 sec, followed by a final elongation step at 72°C for 5 min. For fragments 10D, 10E, 10F, and 10G, each PCR contained 800 μ M of dNTPs, 10X PCR buffer, 1,5 mM $MgCl_2$, 0,4 μ M primer and 1,5 U AmpliTaq Gold® in 50 μ l distilled water. Denaturation at 95°C for 6 min was followed by 35 cycles at 94°C for

45 sec, 54°C for 45 sec, and 72°C for 90 sec, followed by a final elongation step at 72°C for 10 min. All amplification cycles were performed on a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) and the PCR fragments were analysed on a 3% agarose gel.

dHPLC analysis

Exon 10 was analysed using the denaturation High Performance Liquid Chromatography (dHPLC). PCR products from exon 10 amplification were denatured at 95°C for 10 min and partially renatured with a quick decrease of temperature at 4°C. Fragment 10A was analysed at 54°C and 58°C, 10B at 57,2°C, 10C at 57,3°C, 59,3°C and 61,3°C, 10D at 59,3°C and 61,3°C, 10E at 61,2°C, 10F at 57,3°C and 59,3°C and 10G at 59,3°C. For each PCR product, 8 µl were tested for 8 min in the Wavemaker (Transgenomic). The obtained results were compared to chromatographic peaks of a well known sample (control sample) and samples with two chromatographic peaks were sequenced. The negative samples for fragments that resulted positive for more than one sample (fragment 10A and fragment 10G) were tested with a well known sample: we mixed 10 µl of the negative sample for the fragment with 10 µl of the control sample. In this way, hetero-duplex were formed if the sample was different from the control sample; differently, only one chromatographic peak was detected if the tested sample had the same DNA sequence of the control sample.

DNA sequencing

PCR products that were positive for dHPLC were purified with the QIAquick® PCR Purification Kit (QIAGEN) following the manufacturer's instructions. The sequence reaction was performed as follows: 4 ng of DNA were used as template for 4 µl of ABIPrism® BigDye® Terminator v1.1 (Applied Biosystems), 0,5 µl of BigDye® Terminator v1.1 5X Buffer (Applied Biosystems) and 0,5 µl of primer 3,2 µM (Invitrogen). The sequence reaction started with 28 cycles including a denaturation step at 96°C for 10 sec, an annealing step at 50°C for 5 sec and an elongation step at 60°C for 4 min, followed by a final step at 4°C. Products from sequence reaction were purified with the DyeEx® 2.0 Spin Kit (QIAGEN) and were analyzed using the 3100 AvantGenetic Analyzer (Applied Biosystems).

Statistical analysis

Differences among groups were tested by logistic regression analysis in case of continuous variables, by the chi-

square test in case of ordinal variables. A value of $p < 0.05$ was considered statistically significant.

Results

Among the 106 patients included in the study, 48 were heterozygote *Ala307Thr*, 34 homozygote *Ala307* and 24 homozygote *Thr307* (Table 1). Among patients with PCOS, the *Ala307Thr* variant was significantly more frequent (65%) than the homozygote variants *Ala307* (15%) and *Thr307* (20%). Differently, in normo-ovulatory patients the three allelic variants were evenly distributed (42.5% *Ala307*, 33.3% *Ala307Thr* and 24.2% *Thr307*).

Women with homozygote *Ala307*, homozygote *Thr307* or heterozygote *Ala307Thr* had comparable age, BMI, and inter-menstrual interval length, and a similar proportion of women had a partner with abnormal semen (Table 2). FSH level the third day of the menstrual cycle and basal AFC were not significantly different in the three subgroups, although heterozygotes (both those with normal ovary and those with PCOS) showed trendily lower FSH and higher AFC (Table 2).

When submitted to IVF, *Ala307Thr* women showed significantly more often than homozygote subjects a high responsiveness to rFSH. The total FSH dose/retrieved oocytes ratio was lower (although not significantly) in heterozygotes than in homozygotes (222 vs. 285 and 276 IU, respectively); this trend was observed both in women with PCOS and in women with normal ovaries (Table 3). Moreover, as a woman that requires less than 200 IU of rFSH per retrieved oocyte was defined as belonging to the “best responders” (see [Methods](#)), a significantly higher percentage of “best-responders” was found among the heterozygote subgroup than among homozygotes (62.5% vs. 29.4 and 25%, respectively; $p < 0.05$) (Table 3). This uneven distribution of the “best-responders” was observed also when PCOS women were considered separately (76.9% of women were “best-responders” among *Ala307Thr* vs. 33.3 and 25% in homozygotes *Ala307* and *Thr307*, respectively; $p < 0.05$), and when normo-ovulatory women were considered

Table 1 Frequency of the FSH-R polymorphic variants at position 307 in PCOS and normo-ovulatory women with a normally appearing ovary at US

	<i>Ala/Ala</i> n=34	<i>Ala/Thr</i> n=48	<i>Thr/Thr</i> n=24
PCOS women (n=40)	15% (6/40)	65% ^a (26/40)	20% (8/40)
Normo-ovulatory women (n=66)	42.5% (28/66)	33.3% (22/66)	24.2% (16/66)

^a $p < 0.05$ vs. *Ala307Ala* and *Thr307Thr*

Table 2 Patients' clinical characteristics in relation to the FSH-R polymorphic variant at position 307 in patients with PCOS ($n=40$) and in normo-ovulatory women with a normally appearing ovary at US ($n=66$)

			ALA/ALA $N=34$	ALA/THR $N=48$	THR/THR $N=24$
No significant differences were detected for any of the studied variables when FSH-R polymorphic variants were compared.	Age (years)	PCOS	33.3±2.5	32.7±3.7	32±1.6
		Normal	33.5±3.0	33.3±2.7	32.8±2.6
	Body Mass Index (BMI)	PCOS	23±4.1	23.6±6.8	25.1±4.5
		Normal	21.7±2.7	23.3±2.9	20.8±2.3
	Intercycle length (days)	PCOS	34.3±6.0	38.3±2.4	37.2±13.1
		Normal	28±1.3	28.1±1.6	27.7±0.7
	FSH level in cycle day 3 (IU/L)	PCOS	5.8±1.2	5.6±2.4	6.3±1.7
		Normal	8.4±4.1	6.6±1.8	7.3±1.7
	Basal antral follicle count (AFC)	PCOS	33±6.0	29.3±2.8	25.5±5.5
		Normal	11.5±1.5	13.4±1.6	11.5±1.9
	Partner with abnormal semen (%)	PCOS	50 (3/6)	46.1 (12/26)	50 (4/8)
		Normal	50 (14/28)	54.5 (12/22)	50 (8/16)

separately (45.4% of women were “best-responders” among *Ala307Thr* vs. 28.5 and 25% in homozygotes *Ala307* and *Thr307*, respectively; $p<0.05$) (Table 3).

As the lowest effective FSH dose was used for ovarian stimulation, none of the patients included in the study developed severe ovarian hyperstimulation syndrome (OHSS).

Discussion

The two most clinically relevant polymorphisms of FSH-R gene are located in exon 10 at positions 307 and 680. In the receptor protein, position 307 may be occupied by either *Ala* or *Thr*, and position 680 by either *Asn* or *Ser* [1, 2]. Most available studies were focused on position 680 [3], whereas polymorphism 307 was rarely considered. Position 307 codifies for an amino-acid

located within the extracellular domain in the FSH-binding region of the protein [1]: it can affect the hormone-binding ability of the receptor and is crucial for FSH-mediated signal transduction events [15].

Sudo [10] reported a significant increase in the *Ala307Thr* frequency among Japanese women with PCOS, 66.7% of which had such allelic variant in comparison to 43.5% of normally ovulating women. In European normogonadotropic anovulatory women (including PCOS), Laven [9] showed a significantly uneven frequency distribution: 20% homozygote *Ala307*, 57% *Ala307Thr* and 23% homozygote *Thr307*. A significant association between the polymorphism *Ala307Thr* and PCOS was also reported by a recent study on Chinese women [11]. In contrast, the existence of significant differences in the allele distribution of FSH-R polymorphism 307 in women with PCOS was not confirmed among Turkish adolescent girls [12], and on women of Caucasian origin [13].

Table 3 IVF cycle outcome in relation to the FSHR polymorphic variant at position 307 in patients with PCOS ($n=40$) and in normo-ovulatory women with a normally appearing ovary at US ($n=66$)

			ALA/ALA $N=34$	ALA/THR $N=48$	THR/THR $N=24$
Gonadotropin stimulation length (days)	PCO		14.3±4.5	13.2±4.3	13.2±2.9
	Normal		12.9±1.3	12.4±2.5	11.1±1.8
Peak estradiol preovulatory level (pg/ml)	PCO		2469±1243	1750±936	1530±727
	Normal		1727±838	1571±885	1720±969
Total FSH dose administered (IU)	PCO		1733±662	1785±318	1750±573
	Normal		2887±1641	2134±992	2364±1021
number of retrieved oocytes at ovum pick-up	PCO		13.6±4.5	11.4±2.6	12.5±7.5
	Normal		7.9±4.9	8.4±4.6	5.7±4.7
Total FSH dose/retrieved oocytes (IU)	PCO		221±85	165±84	230±66
	Normal		358±177	272±108	402±215
Proportion of women defined as the “best-responders” (%)	PCO		33.3 (2/6)	76.9 ^a (20/26)	25 (2/8)
	Normal		28.5 (8/28)	45.4 ^a (10/22)	25 (4/16)

^a $p<0.05$ *Ala307Thr* vs. homozygote allelic variants.

We studied herein an homogeneous population of women with “full” PCOS and a control population of IVF patients with normal ovary. On Italian women, we found that the allelic variants at position 307 are uniformly distributed in patients with normal ovary, whereas 65% of women with PCOS are *Ala307Thr*, a significantly higher prevalence in comparison to the homozygote variants. The heterogeneity of results observed in different studies may be due to a variable definition of PCOS and/or to some intrinsic characteristics of FSH-R polymorphism, such as incomplete penetrance, genetic heterogeneity, etc. Further, the FSH-R genotype distribution varies among different populations, and ethnicity may deeply influence the distribution of allelic variants [16]. Again, a relevant environmental component (diet, exercise, etc.) contributes to PCOS pathogenesis [17], and PCOS is likely to result from the interaction of genetic with environmental (and maybe prevalent) factors.

As far as the functional properties of FSH-R are concerned, polymorphic variants at position 307 were considered in a few studies, with inconclusive results. A trial including 50 normo-ovulatory women undergoing IVF showed that homozygotes *Ala307* required a significantly lower amount of exogenous FSH and had a higher peak estradiol level than homozygotes *Thr307* or heterozygotes *Ala307Thr* [8]. Another study on normogonadotropic anovulatory subjects (including PCOS) submitted to ovulation induction, showed that the same total dose and median daily dose of FSH were required regardless the FSH-R polymorphism at position 307 [9]; in this study, however, IVF was not performed.

Patients’ basal characteristics in our study were similar in the three subgroups, although *Ala307Thr* women had lower serum FSH on day 3 of the menstrual cycle and higher basal AFC. When stimulated for IVF, heterozygotes showed a better ovarian responsiveness, expressed by the lower FSH/oocyte ratio. Furthermore, the percentage of women considerable as the “best-responders” was significantly higher among *Ala307Thr* subjects than among homozygotes. This was evident also when PCOS and women with normal ovary were considered as separate subgroups, indicating that the higher percentage of “best-responders” among heterozygotes in the whole group was not due to a higher prevalence of PCOS among heterozygotes. Interestingly enough, results similar to ours were reported by another study [10], in which heterozygotes required a lower total hMG dose and had more retrieved oocytes. Our data suggest that heterozygosity at position 307 gives a better responsiveness to FSH: this could affect the outcome of the whole IVF treatment, as the “best-responders” are known to have a high pregnancy chance.

In conclusion, we show herein that in Italian female population the variant *Ala307Thr* of FSH-R polymorphism

is significantly more frequent among subjects with PCOS, and that *Ala307Thr* women are more frequently able to respond in a very good way to ovarian stimulation with FSH. The possible clinical implications of our observations are the following: a) the detection of FSH-R 307 polymorphism could be used to estimate the appropriate FSH starting dose for ovarian stimulation in IVF, b) it could be useful to identify women at higher risk of OHSS, c) it could be used to better tailor the stimulation protocol of each single woman submitted to IVF. Further studies are needed to develop these hypothesis and clarify the role of FSH-R 307 polymorphism detection in human IVF.

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