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ESR1 and FSHR Gene Polymorphisms Influence Ovarian Response to FSH in Poor Responder Women with Normal FSH Levels

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Abstract

In COS (Controlled Ovarian Stimulation), individual response are highly variable. A number of studies have evaluated the ovarian response to FSH in women most often aged over 35 years and with high FSH level. But we see also a poor response to ovarian stimulation in young women (<32 years) with normal FSH levels.The aim of this study was to evaluate the relationship between FSHR and ESR1 gene polymorphism in this young women population.

For 70 patients, the FSHR p.Ala307Thr (rs6165) and p.Ser680Asn (rs6166) single-nucleotide polymorphisms (SNPs), the ESR1 PvuII T/C (rs2234693) SNP, and the (TA)n microsatellite polymorphism were studied alone or in combination. We found that the frequency of the p.307[Ala;Ala] FHSR genotype was 3-fold higher in poor responders (PRs) to COS (Controlled Ovarian Stimulation) than in good responders (GRs).The frequency of ESR1 polymorphism was not correlated with the FSH response. In the combined analysis of FSHR and ESR, ESR1 polymorphism seems to improve the efficiency of prediction of the FSHR polymorphism. The more specifics genotypes were FSHR307[Ala;Ala]/ ESR1 (TA)[>16;<17] for PRs, FSHR307[Thr;Thr]/ ESR1 (TA)[>16;<17] and FSHR307[Thr;Ala]/ ESR1 (TA)[>16;>16] for GRs.

Our study show the involvement of ESR1 and FSHR polymorphisms and the multi-genic spectrum of ovarian response to gonadotropin in women with normal gonadotropin levels. Moreover, the poor response to FSH linked to the 307GG genotype was not associated with an increase in plasma FSH.

Keywords: FSH; IVF; FSHR; ESR1; Ala307Thr; (TA)n microsatellite

Introduction

Stimulation of ovarian follicular development is crucial for successful in vitro fertilization (IVF)-embryo transfer therapy. Several ovarian stimulation protocols have been developed. The mainly responsible actor of follicular maturation is the follicle-stimulating hormone (FSH) [1]. Individual response to FSH stimulation are highly variable among women and is difficult to predict. Poor ovarian response, defined as the production of a low number of collectable oocytes (ranges from <3 to <6) despite using appropriate doses of FSH is one of the main reasons for treatment failure. By definition, the term POR refers to the ovarian response and, therefore, one FSH stimulated cycle is considered essential for the diagnosis of POR [2]. FSH exerts its stimulatory effect by binding to its receptor (FSHR). Estrogens augment the action of FSH by promoting the proliferation of granulosa cells and increasing the expression of FSH receptor in these cells [3-5]. Several single-nucleotide polymorphisms (SNPs) in the FSHR and estrogen receptor alpha (ESR1) genes have been identified, but only a few of them are associated with ovarian response to FSH [6-8].

Two SNPs in exon 10 of the FSHR gene, p.Ala307Thr (rs6165) and p.Ser680Asn (rs6166), have been studied in the context of IVF outcome. These polymorphisms are in linkage disequilibrium, resulting in two major receptor variants: Ala307-Ser680 and Thr307-Asn680 [9-11]. A number of studies have evaluated the relationship between basal FSH, FSHR polymorphism at codon 680, and ovarian response to FSH [8,12]. Despite some discrepancies, the general consensus is that women with the FSHR variant p.Ser680Ser have higher basal FSH and are poor responders (PRs) to FSH stimulation (meta-analysis [7,13-16]).

Two polymorphisms in ESR1, the SNP ESR1 PvuII T/C (rs2234693) and the microsatellite length polymorphism ESR1 (TA)n, have been studied [17]. The longer ESR1 (TA)n microsatellite polymorphism associated with the PvuII*C allele predicts better ovarian response to FSH.

Recently, Anagnostou et al. [18] showed that the combination of genotypes of these polymorphisms is involved in the control of ovarian stimulation in a Greek population. They investigated normally ovulating IVF or intra-cytoplasmic sperm injection patients under the age of 46 and suggested that the combination of ESR1 PvuII CC and FSHR 680 [Asn;Asn] was associated with the poorest ovulation induction profile. In this study, the FSHR 680 allele associated with a poor response was in contradiction with the general consensus [7].

The purpose of the present study was to determine the possible roles of the FSHR p.Ala307Thr and p.Ser680Asn variations in a women population with poor responders criteria different from those proposed by ferraretti [2]. Indeed, the studied population consists of young ovulating women who were undergoing IVF treatment with normal FSH levels, normal antral follicular count, but with a number of oocytes retrieved <5 in response to FSH stimulation. Likewise a preliminary study of the ERS1 PvuII and (TA)n polymorphisms was realized in combination with the FSHR polymorphisms.

Materials and Methods

Subjects

SNPs of the FSHR and ESR1 genes were analyzed in 70 women undergoing IVF treatment recruited in Caen (France) (19 women) and in Byalistok (Poland) (51 women), all of whom provided written informed consent. The study was approved by the local Ethical Committees. The inclusion criteria for the study were as follows: tubal factor or male factor infertility necessitating IVF treatment, less than 32 years of age, FSH below 8 UI/L at cycle day 3, and antral follicle count > 10. Exclusion criteria were the presence of any ovulation anomalies (according to the World Health Organization criteria for classification of anovulation), and previous treatment failure due to poor response.

Controlled ovarian hyperstimulation (COH) was conducted according to the long gonadotropin-releasing hormone agonist protocol. Pituitary desensitization was induced using a 100 µg daily dose of subcutaneous triptorelin (Gonapeptyl, Ferring Pharmaceuticals,Kiel, Germany) administered beginning on day 21 of the previous cycle. COH started on cycle day 5. In all patients, a daily dose of 150 IU of recombinant FSH (Gonal F, Merck Serono, Lyon, France) was maintained throughout the stimulation cycle and continued until at least one ovarian follicle reached 20 mm in size.

Transvaginal ultrasound-guided oocyte recovery was performed 36 hours after human chorionic gonadotropin (hCG) injection. Oocyte maturation was induced by subcutaneous injection of 250 µg recombinant hCG (Ovitrelle, Merck Serono, Lyon, France).

Patients from whom fewer than 5 oocytes were collected were classified as PRs. Patients who produced 5 or more oocytes were classified as good responders (GRs) and served as the control group.

PCR analysis

Genomic DNA was extracted from peripheral EDTA-treated blood using a NUCLEON BACC2 (GE Healthcare Life Sciences, Caen, France). To analyze the FSHR A to G transition at position 919 (p.Thr307Ala variant) and the G to A transition at position 2039 (p.Asn680Ser variant), polymerase chain reaction (PCR) amplification of the fragment of exon 10 was performed using specific oligonucleotide primers (primer-F: 5’-TTTGTGGTCATCTGTGGCTGC-3’; primer-R: 5’-CAAAGGCAAGGACTGAATTATCATT-3’ for FSHR680 and primer-F: 5’-CAAATCTATTTTAAGGCAAGAAGTTGATTATATGCCTCAG-3’; primer-R: 5’-GTAGATTCCAATGCAGAGATCA-3’ for FSHR307) as described by Sudo et al. [9]. PCR analysis of the two ESR1 variants, PvuII (T/C) in intron 1 and the (TA)n microsatellite polymorphism in the promoter region, was performed with the primers (primer-F: 5’-CTGCCACCCTATCTGTATC-3’; primer-R: 5’-ACCCTGGCGTCGATTATCTG-3’ for ESR1 PvuII (T/C) and Primer-F: 5’-AGACGCATGATATACTTCACC-3’; primer-R: FAM-5’-CCTACAACTCGATCTTCTCG-3’ for ESR1 (TA)n microsatellite) as described by Altmäe et al. [17].

The PCR for the SNP analysis was performed in a final volume of 25 µl containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM of each dNTP, 1 mM of each primer, 0.5 units of Taq DNA polymerase (Bioline, Paris, France), and 200 ng of the gDNA template. The cycling conditions were as follows: initial denaturation at 95°C for 5 min, 35 cycles consisting of denaturation at 95°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min, and a final elongation step at 72°C for 7 min. The lengths of the PCR products were as follows: 520 bp for the FSHR 680 variant, 364 bp for the FSHR 307 variant, and 339 bp for the ESR1 PvuII variant.

For the ESR1 (TA)n microsatellite amplification, in contrast to the primers described by Altmäe et al. [17], the reverse primer was not labeled, and the forward primer was labeled by Cy5® at its 3’ end. PCR was performed as described above, and the fluorescence-labeled PCR products were analyzed for size using a CEQ8000 automated DNA sequencer (Beckman Coulter, Paris, France). The sizes of the PCR products were determined by CEQ8000 software (Beckman Coulter, Paris, France).

RFLP analysis of the p.Asn680Ser and p.Thr307Ala FSHR variants and the ESR1 PvuII variant. The G to A transition in p.Asn680Ser variant creates a restriction site for the endonuclease BsrI. For the p.Thr307Ala variant, a nucleotide mismatch was introduced in one of the primers [9]. This mismatch and the A to G transition created a Bsu36I restriction site. The C to T transition in intron 1 of the ESR1 gene created a restriction site for PvuII. The PCR products were digested using the appropriate endonucleases in 40 µl reactions containing 1X reaction buffer, 15 units of the restriction enzyme, and 20 µl of purified PCR product with incubation at 65°C for 1 hour for p.Asn680Ser variant and at 37°C for 1 hour for p.Thr307Ala and ESR1 PvuII variants. The restriction endonuclease digestion products and a DNA size marker were visualized on a 3.0% MetaPhor® agarose gel (Tebu, Paris, France) and photographed. We analyzed 67 patients for FSHR polymorphism and 69 patients for ESR1 polymorphism. The other samples were no analyzed for technical problems

Statistical analysis

The $\chi 2$ test and Fisher’s exact test were performed with the software suite R2.3.1 (Free Software Foundation, Boston, MA, USA). $p<0.05$ was considered significant.

Results

This study of 70 patients shown no difference either in their age (GR 30 ± 2.6; PR 31.22 ± 2.3) or in their basal FSH level (GR 6.74 ± 1.26; PR 6.8 ± 1) between both studied groups. The oocyte yield was significantly different (t-test; $p<0.001$) between GR and PR (11.46 ±

3.5 and 2.67 ± 1.2 respectively). The observed estradiol level is in concordance with the number of oocytes obtained (about 250 pg / oocyte in the two groups). No difference between Polish and French women was observed concerning the distribution of polymorphisms studies.

Frequency of the FSHR gene allelic variants

The SNP analysis of FSHR revealed strong but incomplete linkage between the 307 and 680 amino acid transitions (χ2 test; $p<0.001$). In our study, three subjects presented a recombination between these two loci (Table 1).

FSHR307/680	Total	GR	PR
	(n=67)	(n=33)	(n=34)
[Thr;Thr]/[Asn;Asn]	16 (23)	11 (31.4)	5 (14.7)
[Ala;Thr]/[Asn;Ser]	28 (40.6)	16 (45.7)	12 (35.3)
[Ala;Ala]/[Ser;Ser]	20 (29)	6 (17.1)	14 (41.2)
[Ala;Ala]/[Asn;Asn]	1 (1.4)	0	1 (2.9)
[Ala;Ala] /[Asn;Ser]	2 (2.9)	0	2 (5.9)

Table 1: Frequency of FSHR gene polymorphisms

Number of patients (percentage). GR: good responder; PR: poor responder with normal FSH levels. The distribution of genotypes was significantly different between GRs and PRs (χ2 test; $p<0.05$).

We observed a significant difference (p=0.028) in the frequency of genotypes between these two populations, with a higher frequency of the genotype combination p.307[Ala;Ala]/c.680[Ser;Ser] in the PR group. This difference was greater for variant 307 (P=0.012), for which the p.307[Ala:Ala] genotype was more common in the PR group (17.1% for GR vs. 50% for PR). Thus, the p.307[Ala;Ala] genotype was associated with a poor response to FSH stimulation.

Frequency of the ESR1 gene allelic variants

The distribution of the (TA)n microsatellites among patients shown two allele groups, one inferior to 17 repeats and an other superior to16 repeats of TA. The first group was the allele (TA)S and the second group allele (TA)L. The ESR1 PvuII SNP and the ESR1 (TA)n microsatellite analysis showed tightly linkage between these two polymorphisms (χ2 test $p<0.001$). The variant T of ESR1 PvuII was linked to the shorter (TA)S microsatellite polymorphism and the ESR1 PvuII*C allele to the longer (TA)L variant (Table2).

No significant difference (p=0.4) was observed in the frequency of genotypes between these two populations (Table 2).

PvuII/TA	GR	PR	Total
TT/SS	5 (15%)	8 (24.2%)	13 (19.7%)
TT/SL	1 (3%)	2 (6%)	3 (4.6%)
TT/LL	0	0	0
TC/SS	1 (3%)	0	1 (1.5%)
TC/SL	17 (51.5%)	18 (54.5%)	35 (53%)
TC/LL	2 (6%)	1 (3%)	3 (4.6%)
CC/SS	0	0	0
CC/SL	4 (3%)	1 (3%)	5 (3%)
CC/LL	6 (18.2%)	3 (9.1%)	9 (13.6%)
total	36	33	69

Table 2: Frequency of ESR1 gene polymorphisms

Haplotype of ESR1 PvuII (allele C or T) associated to the haplotype ESR1 (TA)n (S for allele <17 repeats and L for allele >16 repeats).

Number of patients (percentage of each group). GR: good responder; PR: poor responder with normal FSH levels. The distribution of genotypes was not significantly different between GRs and PRs (χ2 test; p=0.4).

FSHR and ESR1 gene polymorphisms in combination

We combined the results obtained for the FSHR p.Ala307Thr and ESR1 (TA)n genotypes. Only 66 samples were combined, the other were exclude for ESR1 or FSHR analysis failure.

The results in Table 3 show a significantly different genotype distribution between the GR and PR patients (χ2 with p=0.009). We retrieved the difference of genotype distribution between the two groups of women for the FSHR polymorphism, [Ala;Ala] associated to the PR population and [Thr;Thr] associated to the GR population, but for each FSHR haplotype, the ESR1 (TA)n polymorphism seems to define a prevalent genotype, [Ala;Ala]/SL for PR, [Thr;Thr]/SL and [Thr;Ala]/LL for GR.

	GR	PR	Total
[Thr ;Thr]/SS	4 (12.1%)	2 (6.1%)	6 (9.1%)
[Thr ;Thr]/SL	6 (18.2%)	0	6 (9.1%)
[Thr ;Thr]/LL	0	3 (9.1%)	3 (4.5%)
[Thr ;Ala]/SS	2 (6.1%)	1 (3.1%)	3 (4.5%
[Thr ;Ala]/SL	10 (30.3%)	10 (30.3%)	20 (30.3%)
[Thr ;Ala]/LL	5 (15.1%)	0	5 (7.6%)
[Ala ;Ala]/SS	0	5 (15.1%)	5 (7.6%)
[Ala ;Ala]/SL	3 (9.1%)	11 (33.3%)	14 (21.2%)
[Ala ;Ala]/LL	3 (9.1%)	1 (3%)	4 (6.1%)
Total	33	33	66

Table 3: Frequency of good responders and poor responders who had the FSHR307 and the ESR1 [TA]n polymorphism.

Number of patients (percentage). GR: good responder; PR: poor responder with normal FSH levels. The distribution of genotypes was significantly different between GRs and PRs (χ2 test; p=0.009).

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Discussion

The results of the present study show that the FSHR307 or FSHR680 polymorphisms were efficient markers in predicting poor ovarian response to FSH stimulation in IVF in young population with normal FSH level.

As previously described [9], we observed a tightly linkage disequilibrium ($\chi 2$ with $p<0.001$) between the FSHR307A and FSHR680A alleles. This linkage was not complete (three subjects presented a recombination between these two loci), contrary to the previous description by Sudo et al. [5] in a Japanese population. In our study, the FSHR SNP at position 307 was more indicative than the SNP at position 680. Some studies of this latter polymorphism [8,9,19], but not all [20-21], show a relationship between ovarian response to FSH and the FSHR680Ser allele. However, a meta-analysis [7] found that the FSHR p.680Ser allele was associated with higher circulating FSH, although it does not seem to have any real impact on the number of oocytes produced. This result could be attributed to the use of a higher dose of exogenous FSH, which is required in patients undergoing IVF who have increased basal FSH. In our study, in which the same exogenous FSH dose was used for all patients, the FSHR p. 307Ala allele was associated with a lower number of oocytes. The FHSR p.307 [Ala;Ala] genotype was three times as frequent in PRs than in GRs. Our results show that the FSHR p.307Ala allele predicted a lower number of oocytes in women with normal levels of circulating FSH.

Similarly, our investigation of ESR1 polymorphisms confirmed a linkage between the ERS1 PvuII*C allele ($\chi 2$ with $p<0.001$) and the longer ESR1 (TA)L microsatellites (17 repeats or more), as previously reported by Altamäe [17]. The linkage between the FHSR680Asn and FSHR 307Thr alleles, as well as the linkage between the ESR1 PvuII*C allele and the longer ESR1 (TA)n variant, allow for comparison with the other previously reported data. The discrimination between PRs and GRs using ESR1 (TA)n was less clear than that observed using the FSHR polymorphism (1.3 times as many GRs had alleles with longer ESR1 (TA)n microsatellites) and the ESR1 polymorphisms do not appear to be goods markers in predicting poor ovarian response to FSH stimulation in IVF. However, in association with the FSHR polymorphism, it seems to improve the efficiency of prediction of the FSHR polymorphism. As we observe with the haplotype [Ala;Ala]/SL or [Thr;Thr]/SL. This result could explain the apparent discrepancies of the study by Anagnostou et al. [18] with the general consensus for the FSHR allele associated to the poor response. Indeed, in this publication, Anagnostou et al. [18], indicating that the FSHR 680 [Asn;Asn]/ESR1 PvuII CC genotypes present the worst ovulation induction profile in normally ovulating patients. The linkage between the FHSR680Asn and FSHR 307Thr alleles, as well as the linkage between the ESR1 PvuII*C allele and the longer ESR1 (TA)n variant, allow for comparison between the FSHR 680 [Asn;Asn]/ESR1 PvuIICC and the FSHR 307 [Thr;Thr]/LL genotypes.

The FSHR polymorphism seems to involved in the FSH response and the ESR1 polymorphism seems regulate this FSHR polymorphism effect. The link between estradiol and FSHR exists because FSH induce an increase in estradiol production which activates ESR1 expression which in turn increase the expression of FSH receptor [22,23]. However the role of FSH receptor polymorphism or ESR 1 polymorphism gene in this mechanism is not understood.

According to our results, poor response to FSH associated with the FSHR307Ala allele does not result in increased serum FSH, as observed in other studies on infertile women. Even if this effect of the ESR1 polymorphism must be confirmed by a larger number of case, this study confirms the involvement of FSHR gene polymorphisms in the ovarian response to FSH, highlighting the multigenic nature of this mechanism. We showed that genotypes combining ERS1 (TA)n variants and FSHR307 polymorphisms are good markers for predicting poor ovarian response in IVF patients in particular for women without usual poor responder criteria. This is the first study demonstrating an association between genotype and poor response to FSH in women with normal gonadotropin levels. These results could improve the management of patients entering an IVF cycle.

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