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GENETICS

There is no complete linkage between the polymorphisms N680S and T307A of the follicular stimulating hormone receptor gene in fertile women

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Abstract
Purpose We conducted a cross-sectional study to evaluate the linkage of FSHR T307A and N680S in a group of fertile women.
Methods Peripheral blood was obtained from 51 fertile women. DNA extraction and isolation were performed. For the detection of the T307A polymorphism a set of primers (5_-TCTGAGCTTCATCCAATTTGCA-3_and 5_-GGGAAA GAGGGCA GCTGCAA-3) was used and then the product was further amplified by a second PCR-RFLP using another set of primers (5_-CAAATCTATTTTAAGGCAAGAAGTTGAT TATATGCCTCAG-3_and 5_-GTAGATTCCAATGCAGA GATCA-3). For the N680S polymorphism the primers (5_-TTTGTGGTCATCTGTGGCTGC-3_ and 5_-CAAAGG CAAGGACTGAATT ATC ATT-3_) were used. Statistical analysis for the association between the polymorphisms was performed by the Spearman test.
Results We calculated the association between the homozygosis at codon 307 and at codon 680 both for T/T-S/S and A/A-N/N. A significant association between the genotypic results at codon 680 with those at codon 307 was found (r=0.6363, P=0.001). However, a complete linkage between these two polymorphisms was rejected as there were 12 patients with discordant results from the expected A-N/T-S at codons 307 and 680, respectively.
Conclusion The current data demonstrated an association but failed to demonstrate a complete linkage between these two polymorphisms.

Keywords FSH polymorphisms · Fertile women · Linkage · FSH receptor gene

Capsule The polymorphisms T307A and N680S are significantly associated; however, there is not a complete linkage between these two polymorphisms of the FSH gene receptor in fertile women.

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Introduction

Follicular stimulating hormone (FSH) and the FSH receptor (FSHR) play a major role in the development of follicles and regulation of steroidogenesis in the ovary [1].

The FSHR can be divided into three regions; the extracellular domain, the transmembrane region and the intracellular domain [2, 3]. The gene that synthesizes the

FSHR is located on chromosome 2, more specifically in the region 2p21-16. The FSHR is a single-copy gene consisting of 10 exons and nine introns [4] and belongs to the protein G receptor group.

The FSHR's main signal transduction mechanism involves activation of adenylate cyclase and elevation of intracellular cyclic AMP [5–8]. The C-terminal part of the extra cellular domain, the transmembrane and the intracellular domain are encoded by exon 10 with more than 1,234 bp. Although exon 10 is fundamental for signal transduction, it is not necessary for ligand binding [3, 8].

Over the 700 polymorphisms have been found within FSHR, however, only five are located in coding regions [9]. Two polymorphisms that are frequent in the Caucasian population are located in exon 10; the switch of a threonine for an alanine at codon 307 (T307A) and an asparagine for a serine at codon 680 (N680S). These polymorphisms were studied in infertile women and shown to alter their hormonal status. Homozygotic patients at both codons (alanine/alanine at 307 and serine/serine at 680) have higher serum FSH levels during menstrual cycles and demand higher gonadotrophin dosages for ovulation induction [4, 10, 11].

In men, a recent study found the heterozygous genotype Thr/Ala-Ser/Asn was significantly increased in infertile patients compared with the controls. This finding showed that the combination of heterozygous FSHR could be associated for male infertility [12].

In addition, complete linkage between these two polymorphisms has been reported, suggesting that only one needs to be tested (as a TAG SNP). However, the number of subjects and the homogeneity of the studied populations have raised questions about the relationship of these polymorphisms [11, 13, 14]. Hence, for future researches involving these two polymorphisms, it is extremely important to verify the absolute linkage between them.

Therefore, we conducted a cross-sectional study to evaluate the linkage of FSHR T307A and N680S in a group of fertile women.

Materials and methods

Subjects

Peripheral blood was obtained from 51 fertile women. All subjects were southern Brazilian women who had a term pregnancy within the last year on whom tubal ligations were performed for sterilization. All patients signed consent form, as required by the protocol approved by the Research Ethics Committee of the University Hospital de Clinicas de Porto Alegre.

All patients had regular menstrual cycles of 25–35 days, body mass index of 18–35 kg/m^2, both ovaries and their ages ranged up to 40 years old. Those with evidence of endocrinological disease were eliminated from the study through interviews, physical examinations and basic laboratory tests (FSH, TSH, Prolactin). There was no evidence of any sign of pelvic inflammatory disease or any other anatomical abnormalities seen through laparoscopy procedures or transvaginal ultrasound.

DNA isolation and detection of the polymorphisms T307A and N680S

A volume of 5 ml of blood was drawn from each subject, with EDTA added as an anticoagulant. Genomic DNA was obtained from peripheral blood leukocytes with the DNAeasy® Kit (Invitrogen, USA) according to the manufacturer's instructions.

The general conditions described by Sudo et al. used for both polymorphisms were; 0.2 mM dNTP, 2.5 mM $MgCl_2$, 1X KCl buffer 1U Taq DNA Polymerase, 20 pmols from each primer and 100 ng of DNA in a final volume of 50 μL. All reagents were purchased from Invitrogen (Invitrogen, USA). All the polymerase chain reactions (PCR) were performed in an Eppendorf Personal Cycler Thermocycler (Eppendorf, Germany).

RFLP analysis of the T307A variant

Detection of the T307A variant in exon 10 of the FSH receptor gene, was performed by the nested PCR–restriction fragment length polymorphism (PCR–RFLP) method. First a 657 bp fragment of the FSH receptor gene was amplified by PCR-RFLP using a set of primers (5_-TCTGAGCTTCATC CAATTTGCA-3_and 5_-GGGAAAGAGGGCA GCTGCAA-3) at 58°C. This PCR-RFLP product (1 μL) was further amplified by a second PCR-RFLP using another set of primers (5_-CAAATCTATTTTAAGGCAAGAAGTT GATTATATGCCTCAG-3_and 5_-GTAGATTCCAATG CAGA GATCA-3) also at 58°C. The amplified fragment of 364 bp was digested with Bsu36I (New England Biolabs, USA) restriction enzyme (Table 1) and visualized on 2.5% agarose gel stained with ethidium bromide.

PCR-RFLP analysis of the N680S variant

The FSHR gene was amplified by PCR-RFLP using genomic DNA as a template and a set of primers (5_-TTTGTGGTCATCTGTGGCTGC-3_ and 5_-CAAAGG CAAGGACTGAATT ATC ATT-3_) which amplified a DNA fragment of 520 bp in size at an annealing temperature of 60°C (Table 1). Since the A-G transition creates an endonuclease BsrI recognition site; the PCR-RFLP fragment following BsrI digestion and 2.5% agarose gel electrophoresis with ethidium bromide revealed three

Table 1 Summary of follicular stimulator hormone receptor (FSHR) PCR-restriction fragment length polymorphisms (RFLP) analysis

Polymorphism	Primer sequence (5′ to 3′)	PCR product (bp)	Annealing temperature (°C)	Endonuclease
T307A—1st PCR	TCTGAGCTTCATCCAATT TGCA; and GGGAAAGA GGGCA GCTGCAA	657	58°C	none
T307A—2nd PCR	CAAATCTATTTTAAGGCA AGAAGTTGATTATATGC CTCAG-3; and GTAGATT CCAATGCAGAGATCA	364	58°C	*Bsu36I*
N680S	TTTGTGGTCATCTGTGGC TGC; and CAAAGGCAA GGACTGAATTATCATT	520	60°C	*BsrI*

different patterns. Based on this analysis, patients were classified into three groups, NN (680Asn/Asn), NS (680Asn/Ser) and SS (680Ser/Ser).

Statistical analysis

The patients were selected by a convenience method. Descriptive statistics (mean, median and standard deviation—SD) were calculated for each quantitative variable. Statistical analysis for the association between the polymorphisms was performed by the Spearman test. $P<0.05$ was considered significant. The statistical tests were carried out using the SPSS 14 (Statistical Package for the Social Science—SPSS Inc., USA). Based on previous analyses, a sample size of 40 patients was calculated to obtain a power of 80% [13].

Results

At the time of inclusion, women were aged 33.5±5.1 years and presented BMI values at 25.9±3.9 kg/m^2.The polymorphisms T307A and N680S were analyzed as previously described. We found 15 women with A/A at codon 307 and 9 S/S at codon 680 and in the heterozygotic group there were 22 women with T/A at codon 307 and 25 women with N/S at 680. Finally, we found 14 patients homozygous to the wild genotype T/T at codon 307 and 17 N/N at codon 680.

We then analyzed the linkage between these two polymorphisms. We calculated the association between the homozygosity at codon 307 and at codon 680 both for T/T-S/S and A/A-N/N. Also, the number of heterozygotic women on at least one codon was considered (T/A or N/S). A significant association between the genotypic results at codon 680 with those at codon 307 was found ($r=0.6363$, $P=0.001$).

However, a complete linkage between these two polymorphisms was rejected as there were 12 patients with discordant results from the expected A-N/T-S at codons 307 and 680, respectively.

Discussion

The aim of the present study was to determine not only the association but also the complete linkage between the polymorphisms T307A and N680S in fertile patients. The current data demonstrated an association but failed to demonstrate a complete linkage between these two polymorphisms.

The linkage between T307A and N680S was studied in infertile men and also in infertile women who have polycystic ovarian syndrome, hypothalamic primary amenorrhoea, secondary amenorrhoea and premature ovarian failure. Most of these studies suggest that there is no need to seek polymorphisms at codon 680 and at 307 once they are completely linked [11, 13, 14].

On the other hand, our pioneer study showed that although a significant association exists between the N680S and T307A polymorphisms, there is not a 100% correlation.

Studies have estimated the frequency of the FSHR gene polymorphism in infertile populations, however, to our knowledge this is the first investigation in a group of fertile women. Conway et al. have demonstrated that no difference exists in the frequency of the FSHR gene polymorphism at codon 680 in women with polycystic ovarian syndrome, ovarian FSH resistance or premature ovarian failure but no fertile patients were included in the sample [15].

Several studies demonstrated the importance of FSHR gene polymorphisms in human reproduction; and, specially, during ovarian response after controlled hyperstimulation. Recently, a heterogenic group of infertile patients, who were S/S for the FSHR gene polymorphism had a higher FSH level at the follicular-luteal transition. These patients also presented with a longer follicular stage probably due to an ovarian resistance to FSH [15].

Additionally, polymorphisms at codon 680 were also studied in infertile women without endometriosis. The authors concluded that the homozygotic patients at this codon (S/S) demanded a higher gonadotrophin dosage for

ovulation induction [10, 11, 16]. These data taken together suggests a FSH resistance in the S/S group [10].

The detection of these FSHR gene mutations is expensive and time consuming; furthermore, when two mutations are completely linked, we could rapidly and less costly perform the FSHR mutation detection searching only one nucleotide.

However, our results showed that this is not true for polymorphisms T307A and N680S in the FSHR gene, because although they are extremely correlated, they are not 100% linked. More importantly, these results showed that each polymorphism must be analyzed independently and they cannot be used as TAG-SNPs.

The main difference between our findings and previous studies may be due to the ethnic heterogeneity of the Brazilian population while Japanese [11] and German [14, 15] populations tend to be more genetically homogeneous.

In the near future, genotyping patients will be crucial for choosing the best-controlled ovarian stimulation protocol. Our findings are extremely important in physiological terms while they opened a perspective of having a portion of a population carrying one polymorphism but not the other, and it is important to know how these patients will clinically behave. In fact, we need more detailed studies regarding the reproduction physiology of this group of patients without a complete FSHR (T307A and N680S) polymorphism link.

In conclusion, this study demonstrated for the first time in a female sample a statistically significant association between the polymorphisms T307A and N680S in the FSHR gene, but demonstrated that there was no complete link between them.

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