Screening of FSH receptor gene in Argentine women with premature ovarian failure (POF)

Victoria Sundblad \(^a,^*\), Violeta A. Chiauzzi \(^a\), Maria Eugenia Escobar \(^b\), Liliana Dain \(^c,^d,^1\), Eduardo H. Charreau \(^a,^d,^1\)

\(^a\) Instituto de Biología y Medicina Experimental (IBYME), Vuelta de Obligado 2490, C1428ADN Buenos Aires, Argentina
\(^b\) Centro de Investigaciones Endocrinológicas, Hospital de Niños Ricardo Gutierrez, Buenos Aires, Argentina
\(^c\) Centro Nacional de Genética Médica (ANLIS), Buenos Aires, Argentina
\(^d\) Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

Received 16 January 2004; received in revised form 28 April 2004; accepted 9 May 2004

Abstract

Diverse mutations in FSH-receptor (FSHR) gene have been described as possible cause of premature ovarian failure (POF). To investigate the presence of mutations and/or polymorphisms in FSHR gene, DNA from 20 POF, 5 of which were diagnosed as resistant ovary syndrome (ROS), and from 44 controls was isolated from peripheral lymphocytes. The complete coding sequence was analysed by PCR followed by SSCP, direct sequencing or restriction enzyme analysis. No mutations in FSHR gene were identified in the patients studied. The two already described polymorphisms in exon 10, A919G and A2039G, cosegregated in all the homozygous individuals, indicating that FSHR presents two isoforms: Ala307 –Ser 680 and Thr 307 –Asn 680. OR results suggest that the 919G–2039G allelic variant or the homozygous genotype is not associated to disease risk. In addition, a heterozygous substitution T1022C (Val341Ala) was found in two control subjects. We suggest that mutations in FSHR gene are rare in women with POF in Argentine. Presence of a particular FSHR isoform does not appear to be associated with this disease.

Keywords: Premature ovarian failure; Resistant ovary syndrome; FSH-receptor gene

1. Introduction

Premature ovarian failure (POF) is a syndrome clinically defined by failure of the ovary before the age of 40. It is characterised by primary or secondary amenorrhea, hypoestrogenism and elevated gonadotropin serum levels (de Moraes-Ruehsen and Jones, 1967). This syndrome is very heterogeneous with a multicausal pathogenesis, and any of the following: chromosomal, enzymatic, iatrogenic, autoimmune or infectious aberration, may be the cause of the disease (Hoek et al., 1997). Prevalence of POF among women in reproductive age is estimated in 1% (Coulam et al., 1986). Resistant ovary syndrome (ROS) has been described by Jones and de Moraes-Ruehsen (1969), who called it “Savage Syndrome”. At present, it is proposed as a follicular form of POF, with ovaries in which numerous primordial follicles are present (Hoek et al., 1997). We have previously demonstrated that circulating immunoglobulins that inhibited binding of FSH to its receptor, could explain the gonadotropin resistance in two patients with ROS and myasthenia gravis (Chiauzzi et al., 1982; Escobar et al., 1982). Because of its familial tendencies, it was suggested that POF may be a genetic disorder (Coulam et al., 1983; Matison et al., 1984; Conway et al., 1995).

The follicle stimulating hormone receptor (FSHR) has been considered the foremost candidate gene for premature ovarian failure. Defects in the FSHR could diminish the ability of the receptor either to bind FSH or to activate signal transduction pathways, thus becoming a possible cause of ovarian dysfunction. Diverse mutations and polymorphic sequences have been described in this gene in women with POF. The first reported mutation, the C566T missense mutation of exon 7, was described by Aittomäki et al. (1995), in
women of six Finnish families with hypergonadotrophic ovarian dysgenesis. There are eight other inactivating FSHR gene mutations that have all been reported only once: Asn191Ile (Gromoll et al., 1996a), Ile160Thr and Arg573Cys (Beau et al., 1998), Asp224Val and Leu601Val (Tournaye et al., 1999), Ala419Thr (Doherty et al., 2002), Pro348Arg (Allen et al., 2003), and Pro519Thr (Meduri et al., 2003). On the other hand, an activating mutation of the FSHR gene has been identified in a hypophysectomized male (Gromoll et al., 1996b). So far, no activating mutations have been described in women (Gromoll et al., 1996a; Batista et al., 2000). In addition, the presence of a heterozygous point mutation, T1022C, changing Val in codon 341 into Ala, has been reported in one infertile man (Simoni et al., 1999). Finally, the presence of polymorphic sites in the FSHR gene have been reported initially by Whitney et al. (1995), and were subsequently identified as: A919G and A2039G, both in exon 10 (Aittomäki et al., 1995; Simoni et al., 1997).

The purpose of the present study was to determine the presence of mutations and/or polymorphic sites in the FSHR gene in Argentine patients with premature ovarian failure.

2. Materials and methods

2.1. Subjects

The population studied comprised twenty patients with POF, 5 of which had been diagnosed as ROS, all with normal 46 XX karyotype. Among these patients, five presented significant familial history. Forty four normally menstruating women with proven fertility were also included in this study as controls. The Protocol was approved by the Institutional Review Board of the Instituto de Biología y Medicina Experimental. Informed consent was obtained from all patients and controls.

Patients had been characterised as POF due to amenorrhea for over a year starting before the age of 40 (range, 16–38-year-old), and serum FSH level above 40 mIU/ml (normal follicular phase levels: 2–9 mIU/ml) in two consecutive determinations. Analysis of their menstrual history showed that some patients had had episodes of eumenorrhea/oligomenorrhea since menarche. Eight patients presented primary amenorrhea and twelve secondary amenorrhea. Serum LH levels ranged from 7 to 68 mIU/ml (normal follicular phase levels: 2–9 mIU/ml) in two consecutive determinations. Analysis of their menstrual history showed that some patients had had episodes of eumenorrhea/oligomenorrhea before POF, and other patients had had oligomenorrhea since menarche. Eight patients presented primary amenorrhea and twelve secondary amenorrhea. Serum LH levels ranged from 7 to 68 mIU/ml (normal follicular phase levels: 2–10 mIU/ml). Plasma 17β-estradiol was under 15 pg/ml (normal follicular phase levels: 2–10 mIU/ml). Among these POF patients, five had been previously diagnosed as ROS on the basis of hypergonadotrophic amenorrhea, resistance to ovarian stimulation with exogenous gonadotropins, and ovarian examination. Three of them had been submitted to biopsy, which had evidenced the presence of numerous cortical primordial follicles (van Weissenbruch et al., 1991; Arici et al., 2002). The remaining two ROS patients had been examined by ultrasonography, which evidenced both ovaries of normal size with hyperechogenic stroma and with numerous small follicular images (≤3 cm³) at the periphery (Pache et al., 1990; Mehta et al., 1992).

2.2. DNA analysis

DNA was isolated from peripheral blood leukoytes by standard methods. Exons 1–10 of the FSHR gene were amplified from genomic DNA by polymerase chain reaction (PCR) with specific oligonucleotide primers (Table 1). PCR conditions were specifically adjusted for each fragment.

The C566T inactivating mutation was screened by restriction enzyme analysis using BsmI (New England Biolabs) in all the samples, as described by Aittomäki et al. (1995). Briefly, a fragment of 78 base pair (bp) was amplified and ten microlitrers of the PCR product were digested overnight with 5 U of BsmI, electrophoresed on 10% polyacrylamide gels, stained with ethidium bromide, and photographed. The restriction site that renders two fragments of 51 and 27 bp, is abolished in the mutated allele.

Exons 1–9 were screened for mutations by PCR-single stranded conformation polymorphism (SSCP). For each exon, at least eight different electrophoresis conditions were initially evaluated, and three were selected among them, based on pattern discrimination. The SSCP doubtful results were confirmed by direct sequencing.

For mutation screening in exon 10 of the FSHR gene, three separate reactions with overlapping PCR products (10A, 10B and 10C) were performed (Table 1). PCR conditions were specifically adjusted for each fragment. PCR products from all patients and from five controls were purified by GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia), and sequenced by Cancer Research Center, Chicago University, Chicago, Illinois, USA.

Presence of the different polymorphisms of exon 10 was analysed in the non-sequenced control samples as follows. The A919G polymorphism was studied by digestion with AflII restriction enzyme (New England Biolabs). Fragment 10A of exon 10 was amplified by PCR, the product was digested overnight with 5 U of AflII, run on 8% polyacrylamide gels, stained with ethidium bromide, and photographed. The uncleaved fragment has a size of 577 bp, whereas the cleaved fragment, homozygous for A, gives rise to 403, 143 and 31 bp. The homozygous for G, gives rise to 403 and 174 bp. To analyse the A2039G polymorphism, fragment 10C of 305 bp was amplified by PCR. Ten microliters of the PCR product were digested overnight with 5 U of BstXI, and analysed as described above. The presence of the 305 bp fragment indicated a homozygous variant for A, whereas the presence of two fragments, namely 217 and 88 bp, corresponded to homozygous variant G.

The change of T into C in nucleotide 1022 identified in a control subject, was subsequently studied in the non-sequenced subjects by mismatch-PCR (Table 1) that creates a BsoXI restriction site in the wild type allele. The
Table 1

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequence</th>
<th>Fragment</th>
<th>Product size (bp)</th>
<th>Primer location</th>
<th>Subsequent reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>566</td>
<td>5′-GTATTTTCAGATGTGTACAAG-3′</td>
<td>Exon 7</td>
<td>78</td>
<td>Intronic-592</td>
<td>Digestion BsmI</td>
</tr>
<tr>
<td>F</td>
<td>R: 5′-CCCTATGTTGGTCTTCTG-3′</td>
<td>Exon 2</td>
<td>118</td>
<td>Intronic</td>
<td>SSCP</td>
</tr>
<tr>
<td>3′</td>
<td>1</td>
<td>F: 5′-TCAGGATCTGCTAACAGCTG-3′</td>
<td>Exon 3</td>
<td>146</td>
<td>Intronic</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>F: 5′-TTGAGGCACTTACACAGC-3′</td>
<td>Exon 5</td>
<td>277</td>
<td>Intronic</td>
</tr>
<tr>
<td>3′</td>
<td>3</td>
<td>F: 5′-TCAAGGATCTGCTAACAGCTG-3′</td>
<td>Exon 4</td>
<td>153</td>
<td>Intronic</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>F: 5′-TCCTCGACTCTCAACACTTGT-3′</td>
<td>Exon 10</td>
<td>209</td>
<td>Intronic</td>
</tr>
<tr>
<td>3′</td>
<td>5</td>
<td>F: 5′-GTTGCGATCTGCTAACAGCTG-3′</td>
<td>Exon 6</td>
<td>282</td>
<td>Intronic</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>F: 5′-CCTCGACTCTCAACACTTGT-3′</td>
<td>Exon 10A</td>
<td>209</td>
<td>Intronic</td>
</tr>
<tr>
<td>3′</td>
<td>7</td>
<td>F: 5′-TCCTCGACTCTCAACACTTGT-3′</td>
<td>Exon 10B</td>
<td>583</td>
<td>Sequencing</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>F: 5′-TCAAGGATCTGCTAACAGCTG-3′</td>
<td>Exon 10C</td>
<td>305</td>
<td>Sequencing</td>
</tr>
<tr>
<td>3′</td>
<td>9</td>
<td>F: 5′-TCAAGGATCTGCTAACAGCTG-3′</td>
<td>Exon 10D</td>
<td>152</td>
<td>Digestion BsmI</td>
</tr>
</tbody>
</table>

* Primers described by Aittomäki et al. (1995).
* Primers designed for the present work.
* Underlined base corresponds to mismatched base.

PCR product of 152 bp (fragment 10 D) remains uncleaved in the mutated allele and renders two fragments of 127 and 25 bp in the wild type allele.

2.3. Statistics analysis

Odds ratio (OR) and 95% confidence interval (CI 95%) have been calculated for the allelic frequencies and genotypes between patients and controls.

3. Results

The 78 bp PCR product comprising nucleotide 566 in exon 7 was cleaved with BsmI in two fragments of 51 and 27 bp in all patients and controls (Fig. 1, panel A). The absence of any 78 bp fragment demonstrated that no mutant alleles were present in any patient or control subject.

In addition, no abnormal SSCP migration pattern was detected in exons 1–9 in most patients. The SSCP doubtful results were analysed by direct sequencing, and no mutations of the FSHR gene were found in any patient or control.

Screening of exon 10 was performed by directed sequencing, as sections of this exon share high homology with other receptors, thus indicating important functional domains. Indeed, several disease-causing mutations occurring in the transmembrane domain of the G protein-coupled receptors have been reported (Themmen and Huhtaniemi, 2000). Our analysis revealed the two polymorphisms already reported: A919G (amino acid 307) and A2039G (amino acid 680) (Aittomäki et al., 1995; Simoni et al., 1997). At codon 307, two alternative sequences were found, ACT or GCT, corresponding to the amino acid substitution Thr307Ala. At codon 680, the sequence alternatives were AA T or AGT, corresponding to the amino acid change Asn680Ser.

These polymorphisms were analysed by restriction enzyme digestion in the 39 control samples that were not
Fig. 1. Analysis of FSHR sequence by digestion with restriction enzymes. (A) To study C566T inactivating mutation, a PCR product of 78 bp comprising nucleotide 566 was digested with BsmI. Since all PCR products rendered two fragments of 51 and 27 bp, only wild type allele (C) was present. (B) A919G polymorphism was studied by digestion with AhdI. Homozygous A/A gave rise to 403 bp, 143 bp and 31 bp. Homozygous G/G gave rise to 403 and 174 bp. (C) A2039G polymorphism was studied by digestion with BsrI. Presence of a 305 bp fragment indicated homozygous variant for A, whereas presence of two fragments, 217 and 88 bp, corresponded to homozygous variant G. (D) Change T to C in nucleotide 1022 was studied by mismatch-PCR that creates a BstXI restriction site in the wild type allele. PCR product of 152 bp remained uncleaved in the mutated (C) allele and rendered two fragments of 127 and 25 bp in the wild type allele (T). (A and D) (−) PCR product incubated overnight without restriction enzyme. (+) PCR product incubated overnight with restriction enzyme.

The 357 bp PCR product comprising nucleotide 919, fragment 10A, was digested with AhdI (Fig. 1, panel B). The allelic frequencies and genotypes found in POF patients and in control individuals are shown in Table 2. The 305 bp PCR product comprising the nucleotide 2039, fragment 10C, was digested with BsrI (Fig. 1, panel C). The allelic frequencies and genotypes found are shown in Table 2. This polymorphism cosegregates in all homozygous individuals with the A919G allelic variant, and all heterozygous subjects were indeed heterozygous for both loci.

Table 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control (n/total)</th>
<th>POF (n/total)</th>
<th>POF-ROS (n/total)</th>
<th>ROS (n/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>919–2039</td>
<td>A–A–A–A</td>
<td>14/44</td>
<td>5/20</td>
<td>5/15</td>
</tr>
<tr>
<td>(B)b</td>
<td>Allele33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>919</td>
<td>A</td>
<td>0.545 (48/88)</td>
<td>0.500 (20/40)</td>
<td>0.533 (16/30)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.454 (40/88)</td>
<td>0.500 (20/40)</td>
<td>0.466 (14/30)</td>
</tr>
<tr>
<td>2039</td>
<td>A</td>
<td>0.545 (48/88)</td>
<td>0.500 (20/40)</td>
<td>0.533 (16/30)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.454 (40/88)</td>
<td>0.500 (20/40)</td>
<td>0.466 (14/30)</td>
</tr>
</tbody>
</table>

Two previously reported polymorphisms, A919G and A2039G, were analyzed in all individuals by directed sequencing and/or by restriction enzyme digestion. (A) Genotypes counts. Odds ratio (OR) and 95% confidence interval (CI 95%) have been calculated comparing patients (POF) and controls. (B) Allelic frequencies. Odds ratio and 95% confidence interval have been calculated comparing patients and controls.

a OR919–A/A = 1.80; IC95% = 0.23–13.46; OR919–A/G = 1.49; IC95% = 0.25–7.87.

b OR = 1.20; IC95% = 0.53–2.71.
et al., 1999; Tong et al., 2001) comprising the whole coding region of FSHR gene in a representative number of POF patients. Moreover, as to the South American populations, there is only one study carried out in Brazil on POF patients (da Fonseca Kohek et al., 1998), where mutation screening was restricted to exons 7 and 10 of this gene.

In accordance with other studies in different populations (Conway et al., 1997, 1999; da Fonseca Kohek et al., 1998; Jiang et al., 1998; Layman et al., 1998; Takakura et al., 2001), we were unable to demonstrate the presence of the C566T mutation in exon 7 in any of the subjects studied. Moreover, only one mutation carrier out of 1162 samples from Switzerland was identified, in a large scale screening study (Jiang et al., 1998). Thus, our results further support the hypothesis that the C566T mutation is restricted to Finland, and may represent a founder effect in this country.

On the other hand, we did not observe any difference between POF patients and the control group, in migration patterns on gel electrophoresis of SSCP in exons 1-9. This technique has been proposed as allowing the identification of a change (mutation/polymorphism) with high sensitivity (close to 100%) under two different conditions (Forrest et al., 1995). We used three running conditions in the polyacrylamide gels, to ensure detection of almost all the changes that would be present. Our results would indicate the absence of alterations in the nucleotide sequence in the DNA of patients in the sequences studied, though failure to detect changes due to limitations in the technique sensitivity cannot be ruled out. In accordance with our results, several other groups were unable to identify any mutations specific to patients with ovarian disorders that might be invoked to explain abnormal FSH responsiveness (Whitney et al., 1995; Yin Liu et al., 1998; Conway et al., 1999; de la Chesnaye et al., 2001; Takakura et al., 2001; Tong et al., 2001).

Direct sequencing analysis of exon 10 revealed two previously reported polymorphisms, A919G and A2039G (Aittomäki et al., 1995; Simon et al., 1997) with similar allelic frequencies in the groups studied. In accordance with the report by Simoni et al. (1999), our results suggest that A919G and A2039G polymorphisms occur in two possible arrangements, supporting the existence of two discrete allelic variants—and isoforms—for FSHR: Ala307-Ser680 and Thr307-Asn680. Unlike ours findings, the two polymorphic variants were found to occur in linkage disequilibrium in UK and Brazilian subjects (da Fonseca Kohek et al., 1998; Conway et al., 1999). We cannot rule out that populations ethnic differences or the number of patients analysed could account for these discrepancies. In addition, we were unable to demonstrate an association between a particular isofrom and the risk of being affected with POF in the population studied. Even though the number of patients analysed in this work was probably not high enough to detect such association, our results are in accordance with those obtained by Conway et al. (1999) who studied a group of 49 POF and 93 PCOS English women. Furthermore, Simon et al. (1999) found that these two FSHR isoforms showed similar distribution in 86 proven fathers and 75 infertile men. Binding studies in transiently transfected COS-7 cells showed similar binding affinity and comparable cAMP production for these two receptor variants (Simon et al., 1999), suggesting...
that two different FSHR isoforms with similar functional properties exist. However, the possibility that different activities of the two receptor isoforms might become evident under pathophysiological conditions cannot be excluded. We found a heterozygous substitution T1022C, changing GTG (Val) in codon 341 into GCC (Ala) in two control subjects. This substitution has been previously described only once in an infertile man. In vitro studies in transiently transfected COS-7 cells showed that the mutated receptor was normally responsive to FSH stimulation (Simoni et al., 1999). In view of these previous results and given the fact that we observed this substitution in normal controls, we suggest that this variant has no functional significance.

In conclusion, we suggest that mutations in FSHR gene are rare in women with premature ovarian failure in Argentine population. Presence of a particular FSHR isoform would not seem to be associated with this disease and polymorphisms of FSHR gene might not be invoked to explain abnormal ovarian function. Although we naturally acknowledge the fact that we were unable to find a novel mutation in our screening, we believe that the findings of our present investigation mean a significant contribution to further the concept of FSHR gene as a putative cause of premature ovarian failure in patients from Argentina, whose population had not been studied until our research.

Other possibilities for the presence of 46XX ovarian failure, such as defects in regulatory regions of FSHR gene promoter, in untranslated regions of exons 1 and 10, and within introns, should be also considered. Nevertheless, in view of ours and others’ results, and given the fact that C566T mutation might be restricted to Finland and that the other eight FSHR mutations so far described were unique POF cases, we suggest that the FSHR gene might not longer be considered the “foremost” candidate gene for premature ovarian failure. Other genes likely to be relevant to normal ovarian function that have been proposed as candidate genes for ovarian failure (Christin-Maitre et al., 1998; Shelling et al., 2000; Takebayashi et al., 2000) should also be studied in order to elucidate the pathophysiology of the disease.

Acknowledgements

The study was supported by grants from the Argentine Ministry of Health, and from Alberto J. Roemmers Foundation.

References


58

V. Sundblad et al. / Molecular and Cellular Endocrinology 222 (2004) 53–59


