Mutation analysis of the inhibin alpha gene in a cohort of Italian women affected by ovarian failure

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BACKGROUND: Premature ovarian failure (POF) is a secondary hypergonadotrophic amenorrhoea affecting 1–3% of females, whose aetiology is almost unknown. However, inhibin alpha gene (INHa) has recently been indicated as candidate in POF pathogenesis. METHODS: We analysed patients affected by POF (n = 157) for the missense mutation (769G→A transition) in the exon 2 of the INHa gene. The same analysis was carried out on early menopause (EM) (n = 36) and primary amenorrhoea (n = 12) patients. RESULTS: The incidence of the mutation was significantly more frequent within both POF (7/157, 4.5%) (Fisher’s exact test, P = 0.030) and primary amenorrhoea (3/12, 25%) (Fisher’s exact test, P < 0.001) patients, compared with the control population of women (0/100), who experienced physiological menopause. No mutation was found in EM patients. Furthermore, the likelihood of finding the mutation was statistically significant in familial (5/65; 7.7%) (Fisher’s exact test, P < 0.01) but not in sporadic (2/92; 2.2%) (Fisher’s exact test, P = not significant) POF, compared with the control group. The analysis of pedigrees showing the inheritance of the 769G→A mutation and POF strengthens the concept of the disease heterogeneity, since the POF phenotype was not always associated with the mutation. Moreover, a higher prevalence of the C allele of a single nucleotide polymorphism (129C→T), located in the 5’-UTR of the INHa gene, was observed in POF patients (80.3%) than in the control group (66.7%) (Fisher’s exact test, P = 0.014). CONCLUSION: These data strengthen the concept of the INHa gene as a candidate for ovarian failure.

Key words: inhibin/mutation/ovarian failure/premature ovarian failure/sterility

Introduction

Premature ovarian failure (POF) is a secondary hypergonadotrophic amenorrhoea occurring before the age of 40 years, and affecting 1–3% of females (Coulam et al., 1986); the POF condition occurs mainly as idiopathic disease, whose aetiology is still unknown.

From the observation that some POF women carry chromosomal abnormalities and that several families show at least two relatives experiencing POF, the disease can be considered as a genetic disorder. Pedigree studies of affected families suggest that the idiopathic POF condition may be inherited as an autosomal dominant sex-limited transmission or X-linked with incomplete penetrance (Vegetti et al., 1998; Van Kasteren et al., 1999).

Although the pathogenesis of POF is still unknown, several cellular mechanisms can be suggested as responsible for the disease, including a reduced primordial follicle pool, an accelerated follicular atresia, and an alteration of follicular recruitment and maturation (Christin-Maire et al., 1998). Some of these cellular events could be correlated to high FSH levels (te Velde et al., 1998). Thus, FSH and other genes implicated in the FSH signalling, or in the control of the FSH level, have been considered responsible for POF.

Supporting this hypothesis, the analysis of the FSH receptor gene revealed a point mutation in exon 7 in Finnish families affected by primary amenorrhoea (Aittomaki et al., 1995). However, further studies on different cohorts of POF patients did not identify the above mutation and must be unique to the Finnish population (Layman et al., 1998; Conway et al., 1999).

Inhibins and activins are multifunctional hormones, belonging to the transforming growth factor-β superfamily of proteins, which inhibit or stimulate respectively, the synthesis and secretion of FSH (Ling et al., 1985; Miyamoto et al., 1985; Rivier et al., 1985; Robertson et al., 1985; Ling et al., 1986; Vale et al., 1986). Furthermore, inhibins increase the number of ovarian follicles (O et al., 1989), and inhibit oocyte meiosis (Hsueh et al., 1987), whereas activins cause follicular atresia (Woodruff et al., 1990), and induce granulosa cell proliferation in vitro (Rabinovici et al., 1990).

Inhibins (INH) are heterodimeric glycoproteins constituted by an inhibin α subunit and by one of the two related β subunits (βA or βB), to produce respectively INHA and INHB.
(Ling et al., 1985; Miyamoto et al., 1985; Rivier et al., 1985; Robertson et al., 1985). Activins are homodimeric glycoproteins composed of inhibin βA or βB subunits (Ling et al., 1985; Vale et al., 1986).

The synthesis of the three inhibin subunits (α, βA and βB) is restricted to the granulosa cells of the ovary from early stages of folliculogenesis (Drummond et al., 1996). The comparison of perimenopausal with mid-reproductive-aged women by INHA, INHB and activin assays, indicates that a decrease in both INHA and INHB, or an increase in activin A (Danforth et al., 1998; Santoro et al., 1999), is responsible for the high level of FSH characteristic of reproductive ageing. The observed variation of inhibin:activin ratio is probably due to the deficit of the α subunit production, which leads to the preferential formation of activin homodimers (Santoro et al., 1999). Thus, the POF condition could be a consequence of mutations in the INHα gene, which causes a decrease in the amount of bioactive inhibin, and consequently an increase in FSH concentration.

Recently, the analysis of the INHα gene has revealed a missense mutation (769G→A transition) in exon 2 of the gene, occurring in three out of 43 POF women (Shelling et al., 2000). The mutation has been hypothesized to impair the binding of INHA and INHB to their receptor, and therefore to inhibit the activation of the signal transduction pathway.

We analysed 205 women affected by ovarian failure to evaluate the possible association between the 769G→C transition and hypergonadotropic amenorrhea, to further test the concept that INHα can be considered a gene involved in ovarian dysfunction.

Materials and methods

Patient population

A total of 205 patients affected by POF (n = 157), early menopause (EM) (n = 36) or primary amenorrhea (n = 12) were recruited by the Reproductive Endocrinology Services of the Departments of Obstetrics and Gynaecology in Milan and Varese.

The POF status was defined as the cessation of ovarian function for a period of ≥6 months, before or at the age of 40 years, and FSH concentration ≥40 IU/l detected on two different occasions. The EM condition is defined as the occurrence of menopause before the age of 45 years.

Complete medical and gynaecological history, including age at menarche and previous menses, were undertaken for all patients. Family history was reviewed during genetic counselling, and family members were traced back three generations. All the patients included in this study were phenotypically normal and considered idiopathic because they did not show any POF-related conditions (ovarian surgery, previous chemo- or radiotherapy, autoimmune diseases or metabolic disorders such as galactosaemia). Sixty-five POF patients had a family history of premature menopause, with at least one relative experiencing POF (n = 48) or early menopause (EM) (n = 17). Conversely, the remaining 92 patients were classified as having sporadic POF. Among the patients with primary amenorrhea, six patients (four families) showed a familial condition.

None of the patients carried structural or numerical chromosome anomalies, as evaluated by karyotype analysis based on high resolution banding technique, on at least 30 metaphases. A total of 100 peripheral blood samples was obtained from woman who experienced physiological menopause; this cohort was used as the control group. All the women included in the study gave their informed consent to review their medical history and to collect a peripheral blood sample suitable for further cytogenetic and molecular analysis.

DNA extraction and PCR

Genomic DNA was extracted from 1 ml of peripheral blood by the proteinase K method as previously described (Marozzi et al., 1999). Two regions of the INHα gene were analysed by PCR. The first region of 444 bp, which includes the coding region of exon 1 (268 bp), 119 bp of the 5′-UTR, and 57 bp of the first intron, was amplified by primers INHαex1F (5′-AAGGTAGAGGGTGTTGTTG) and INHαex1R (5′-CATGCTGTGCCTTCTTCT). The amplification of a second region of 601 bp, which includes part of exon 2, was carried out by primers INHαF and INHαR, as previously described (Shelling et al., 2000). Moreover, an internal region of the exon 2 comprising 243 bp was amplified by employing primers INHαF and INHαR (Shelling et al., 2000). PCR reactions were carried out using 100 ng of genomic DNA as a template in the presence of 50 pmol of both primers, in a final volume of 50 μl.

After denaturation at 94°C for 5 min, the samples underwent 30 cycles of amplification (94°C denaturation for 45 s, 58–65°C annealing for 45 s, 72°C extension for 1 min); the last cycle was followed by 10 min extension at 72°C.

Restriction fragment length polymorphism (RFLP) analysis and sequencing

The 5′-UTR and exon 1 region of the INHα gene was analysed by sequencing the PCR product from patient and control DNA samples obtained using primers INHαex1F–INHαex1R. Conversely, exon 2 PCR products obtained by primers INHαex1F–INHαex1R were analysed for the Bst71I restriction enzyme polymorphism (RFLP), since the occurrence of the 769G→A transition abolishes the Bst71I restriction site. The digestion was performed as previously described (Shelling et al., 2000), and the products analysed by 3% agarose gel electrophoresis. Wild-type DNA yields three fragments of 85, 25 and 134 bp, whereas a homozygous sample for the mutation gives only two fragments of 85 and 159 bp. DNA samples showing the occurrence of the RFLP were further analysed by sequencing the PCR products obtained using primers INHαF and INHαR (Shelling et al., 2000). Sequencing was performed on PCR products purified by nucleospin extraction kit (Macherey-Nagel, Düren, Germany) following the BigDye terminator sequencing protocol consisting of 50 ng DNA, 2 μl Big Dye terminator mix, and 3.2 pmol primers (Applied Biosystems, Foster City, CA, USA). All fluorescent traces were analysed using the Applied Biosystem Model 3100 DNA Sequencing System.

Results

A mutation analysis of the INHα gene was carried out on 205 patients with premature menopause. The analysed POF patients belonged to three groups: (i) familial POF (48 patients), (ii) POF with at least one relative experiencing EM (17 patients), and (iii) sporadic POF (92 patients). Moreover, we analysed 36 EM patients, and 12 patients with sporadic (six) and familial (six) primary amenorrhea. The classification of the recruited patients is shown in Table I.

Molecular analysis of the patients showed 10 women heterozygous for the Bst71I RFLP in exon 2 of the INHα gene (Table I). Conversely, no Bst71I RFLP was detected in the control group, consisting of 100 women who experienced physiological menopause. DNA samples showing the occur-
Ovarian failure and mutation of \textit{INH\(\alpha\)} gene

**Table I.** Summary of patients analysed for the \textit{INH\(\alpha\)} 769G→A variant

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of analysed patients</th>
<th>No. of patients with 769 G→A variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Familial POF</td>
<td>48</td>
<td>3</td>
</tr>
<tr>
<td>POF with familial EM</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>Sporadic POF</td>
<td>92</td>
<td>2</td>
</tr>
<tr>
<td>EM</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>Familial primary amenorrhoea</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Sporadic primary amenorrhoea</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

POF = premature ovarian failure; EM = early menopause.

The obtained results indicate a higher incidence of the 769G→A missense mutation in the familial POF (three patients out of 48; 6.9\%) (Fisher’s exact test, \(P = 0.033\)), and in the POF with familial EM (two patients out of 17; 11.8\%) (Fisher’s exact test, \(P = 0.020\)), compared with the control group. In general, the group of familial POF showed a statistically significant prevalence of the mutation (five out of 65, 7.7\%) (Fisher’s exact test, \(P < 0.01\)), as well as that of primary amenorrhoea patients (three out of 12, 25\%) (Fisher’s exact test, \(P < 0.001\)). Conversely, the prevalence of carriers for the 769G→A mutation in sporadic POF was not statistically significant (two out of 92; 2.2\%) (Fisher’s exact test, \(P = 0.23\), not significant) compared with the control group.

The seven POF patients carrying the 769G→A mutation had a mean age of menopause onset of 35.6 ± 3.7 years (range 30–40), whereas the mean age of menopause experienced by their mothers was 44.3 ± 5.8 years (range 36–50) (Table II). All the women carrying the 769G→A mutation and belonging to the group of familial POF showed a maternal transmission of the disease (patients 65, 55 and 162), whereas the POF women with a family history of EM showed either a maternal or paternal transmission of the disease (patients 77 and 96 respectively) (Table II).

To verify the co-segregation of the disease with the mutation, the relatives of two familial POF probands carrying the 769G→A transition were analysed at the molecular level. The pedigrees of the two probands (patients 55 and 162) are reported in Figure 1C and D respectively. In pedigree A (Figure 1C), the proband (III4), the mother (II5) and the grandmother (I4) experienced POF at the age of 39, 40 and 40 years respectively. The proband (III4) and her mother (II5) were both heterozygous for the 769G→A mutation. In pedigree B (Figure 1D), the age at menopause of the proband (III1) and her mother (II4) was 34 and 40 years respectively. The screening for the 769G→A transition revealed that the father (II3), and two daughters and the son (III1, III2 III3), but not the mother (II4), were carriers for the heterozygous mutation. Thus, in this pedigree the mother experiencing POF does not
carry the $769G\rightarrow A$ transition. Furthermore, individual III2 is 26 years old, and until now she has had regular menses.

Almost all the patients recruited in this study were also screened for DNA variations in the 5'-UTR and exon 1 of the $\alpha$-INH gene. The analysed region spans 444 bp; the forward primer was located 25 nucleotides downstream of the transcription start, and the reverse primer located in the first intron, 57 nucleotides downstream of the end of exon 1. The analysis, carried out by direct DNA sequencing, showed a 129C→T transition in the 5'-UTR. This transition was found in 28 out of 142 POF patients (19.7%), eight out of 34 EM patients (23.5%), and in three out of 11 primary amenorrhoea patients (27%). Due to the unavailability of DNA, the same analysis was carried out on 69 DNA samples from the control group; this analysis revealed the T allele in 23 women (33%). The result indicates that the prevalence of the C variant in POF patients (80.3%) is higher than expected (66.7%) (Fisher’s exact test, $P = 0.014$).

### Discussion

Previous work has identified the possible involvement of the mutation $769G\rightarrow A$ within exon 2 of the $\alpha$-INH gene in the pathogenesis of POF (Shelling et al., 2000). This was a relatively small study including 43 patients, and the analysis of a larger population group is required. Furthermore, the study indicated a prevalence of the mutation in women experiencing POF before the age of 25 years, thus suggesting the existence of a correlation between the mutation and the early onset of premature menopause. The data provided in the present study, analysing the background, which, in association with the $769G\rightarrow A$ variant, could lead to POF.

The observed median age of POF onset in patients carrying the mutation was 35.6 ± 3.7 years. This figure is higher than that previously reported (Shelling et al., 2000) for three POF patients carrying the same mutation (20 ± 4). The small number of analysed patients may represent a possible explanation for the discrepancies observed on the median age of POF onset. Furthermore, we identified a high percentage of primary amenorrhoea patients with the mutation (three out of 12). One possibility to explain how the $769G\rightarrow A$ variant might be involved in causing infertility, is to hypothesize that in primary amenorrhoea patients the mutation is highly penetrant.

A further contribution to the concept of POF as a heterogenous disease can be derived from the analysis of families showing the inheritance of both POF and $\alpha$-INH gene mutation. In one family, we found the co-segregation of the $769G\rightarrow A$ mutation with POF (Figure 1C), but in another family the proband inherited the mutation from the father, whereas the mother affected by POF did not carry the mutation (Figure 1D). In this case, the POF manifestation in the mother is not correlated to the $\alpha$-INH gene mutation, and thus she represents a phenocopy of her daughter, probably due to the genetic heterogeneity of the disease. Conversely, the previous analysis of another POF family

### Table II. Clinical features of premature ovarian failure (POF) patients carrying the $\alpha$-INH $769G\rightarrow A$ variant

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age at menarche (years)</th>
<th>Age at menopause (years)</th>
<th>Age at menopause of mothers (years)</th>
<th>Familial transmission of POF</th>
<th>Familial transmission of early menopause</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>12</td>
<td>40</td>
<td>36</td>
<td>Yes (M)</td>
<td>No</td>
</tr>
<tr>
<td>55</td>
<td>12</td>
<td>39</td>
<td>40</td>
<td>Yes (M)</td>
<td>No</td>
</tr>
<tr>
<td>162</td>
<td>13</td>
<td>34</td>
<td>40</td>
<td>Yes (M)</td>
<td>No</td>
</tr>
<tr>
<td>77</td>
<td>14</td>
<td>38</td>
<td>44</td>
<td>Yes (M)</td>
<td>Yes (P)</td>
</tr>
<tr>
<td>96</td>
<td>12</td>
<td>36</td>
<td>50</td>
<td>No</td>
<td>Yes (M)</td>
</tr>
<tr>
<td>88</td>
<td>12</td>
<td>32</td>
<td>50</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>90</td>
<td>14</td>
<td>30</td>
<td>50</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

M = maternal transmission; P = paternal transmission.
showed the occurrence of the mutation in a woman experiencing physiological menopause (Shelling et al., 2000). The occurrence of the mutation without POF manifestation could be explained through incomplete penetrance.

To investigate whether other sequence variations within the INHβ gene might be linked to the development of POF, we also analysed the 5′-UTR and exon 1. We only detected a single nucleotide polymorphism in the 5′-UTR: a 129C→T transition. The derived frequency of the C allele (80.3%) was significantly higher (Fisher’s exact test, P = 0.014) in the POF population than in the control group (66.7%). Interestingly, none of the analysed patients simultaneously carried the 769G→A variant and the T allele. Moreover, the frequency of the C allele in the POF group is comparable with that observed in a large population of women from 326 pedigrees showing an elevated frequency of dizygotic (DZ) twin pregnancy (Montgomery et al., 2000). Mothers of DZ twins have a higher incidence of spontaneous multiple ovulation and elevated FSH concentrations (Martin et al., 1991). FSH release is controlled by a negative feedback mediated by the INHβ peptide produced by the ovary; thus, a reduced concentration of the INHβ peptide is thought to result in elevated levels of FSH and multiple ovulation. Although the INHβ 5′-UTR variant was not found in linkage with DZ twinning, it is possible to speculate that in the POF population this variant may contribute, in concomitance with variations in other genes, to the increased rate of follicular depletion (Richardson et al., 1987).

The implication of the single nucleotide polymorphism in follicle depletion could be derived by the evaluation of INHβ peptide levels (McConnell et al., 1998) in carriers of the C allele, but not affected by the disease.

Further investigations are required to confirm our preliminary observation on the preferential occurrence of the 769G→A mutation in familial POF. In the case of validation of this observation, the INHβ mutation analysis can represent a useful diagnostic marker and allow prevention of the early onset of ovarian failure by replacement of the inhibin hormone. The mutation analysis could also be important to allow family members to anticipate the decision of conception.

References
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