A large-scale association study to assess the impact of known variants of the human INHA gene on premature ovarian failure

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BACKGROUND: Three variants of the human INHA gene have been reported to be associated with premature ovarian failure (POF) in case–control studies involving a small number of patients and controls. Since inhibin has a fundamental role in the control of ovarian function, it is important to establish the relevance of the reported variants for disease risk.

METHODS: Three independent POF cohorts, recruited in Northern and Central Italy and in Germany consisting of a total of 611 patients and 1084 matched controls, were genotyped for the three variants: 216C>T, 2124A>G and 769G>A.

RESULTS: No significant difference was detected between allelic frequencies of the INHA promoter variants between POF patients and controls. The rare allele in the coding variant appeared to be more frequent among the control populations.

CONCLUSIONS: The association between the INHA promoter variants and POF could not be replicated, and our results suggest that this discrepancy is likely to be due to the small sample size of previous studies. The rare allele of the coding variant seems to exert a protective effect against loss of ovarian function, which should be confirmed in additional large and ethnically diverse cohorts.

Key words: premature ovarian failure / inhibin variants / genetic risk factor / infertility

Introduction

Premature ovarian failure (POF) is a common disorder affecting around 1% of the females in the Caucasian population (Luborsky et al., 2003; Parazzini, 2003) and characterized by loss of ovarian function before the age of 40 years. POF results in infertility and lifelong steroid deficiency, and it is potentially associated with the severe health risks of natural menopause, such as cardiovascular and neurological disorders and osteoporosis (Christin-Maitre, 2008). POF may be the consequence of environmental factors such as surgery, infections and chemotherapy, but it also has a major genetic component and it is frequently found in families. Few genes have been identified as carrying mutations responsible for POF [FSHR (Aittomaki et al., 1995; Doherty et al., 2002); BMP15 (Di Pasquale et al., 2004; Di Pasquale et al., 2006; Dixit et al., 2006b; Laissue et al., 2006); FOXL2 (Harris et al., 2002); NOBOX (Zhao et al., 2005; Qin et al., 2007); PGRMC1 (Mansouri et al., 2008); FIGLA (Zhao et al., 2008)] and inherited as Mendelian factors. A more relevant role in the etiology of POF has the premutated allele at the FMR1 gene in Xq27.3, as demonstrated by an increased occurrence of POF females among FMR1 premutation carriers (15–20%), and by the 5–10% frequency of the premutation allele in POF females in different populations
DNA variants of the INHA gene have been proposed as risk factors for POF by several groups (Shelling et al., 2000; Marozzi et al., 2002; Harris et al., 2005; Dixit et al., 2006a; Woold et al., 2009). The INHA gene encodes the α-subunit of inhibin A and B, both negative regulators of follicle-stimulating hormone (FSH). A reduction in circulating inhibin or in inhibin activity may result in an increased level of FSH, an increase in the number of follicles recruited at each cycle and, ultimately, in early depletion of the follicle pool and POF. Three variants were reported as significantly associated with the disorder. One was a coding variant, 769G > A, whose rare allele A was found significantly associated with POF in New Zealand (Shelling et al., 2000), India (Dixit et al., 2006a) and Italy (Marozzi et al., 2002). Two promoter alleles were also reported, one, −16T, was significantly under-represented in New Zealand and Italian POF (Marozzi et al., 2002; Harris et al., 2005; Woold et al., 2009) but not in a POF panel from Slovenia or Argentina (Sundblad et al., 2006; Woold et al., 2009). A second allele, −124G, was found enriched in New Zealand and Slovenian POF, but it reached statistical significance only if the two populations were considered together (Woold et al., 2009).

In general, the significance of the different studies was not very high, mainly because of the limited number of cases and controls analyzed. In a few cases, the association was not replicated (Sundblad et al., 2006; Woold et al., 2009). We have recently collected a large cohort of POF patients and genetically matched control women, through the collaboration of Gynecology and Endocrinology Clinics in Italy and in Germany and of Patients and Women Organizations in Italy. Using our cohorts, we could not replicate the association of the promoter variants to POF. Interestingly, the rare allele at the coding variant seemed to be negatively associated with POF, thus harboring a protective effect.

Materials and Methods

Patients information

Three cohorts were used for the study. A total of 468 POF patients were collected in Northern (n = 299) and Central Italy (n = 169) through collaboration with gynecologists and endocrinologists participating in the NIDO (Italian Network for the study of Ovarian Dysfunction) and recruitment through women (ONDA, National Observatory of Women Health) and patients associations (A.M.P., Premature Menopause Association, and Italian Fragile X Syndrome Association). The patients were all of Italian origin. They had undergone amenorrhea for at least 6 months before 40 years of age, with serum FSH levels > 40 IU/l and luteinizing hormone > 20 IU/l. All patients were selected for having a normal karyotype and no autoimmune disease. Patients carrying FMR1 premutation were not included in the analysis. The Northern Italy cohort included 33 cases affected with primary amenorrhea (11.0%) and 266 with secondary amenorrhea (89.0%, mean age at menopause 31.2, range 12–39). The Central Italy cohort included 5 cases affected with primary amenorrhea (3.0%) and 164 with secondary amenorrhea (97.0%, mean age at menopause 29.7, range 12–39). Italian anonymous controls were 782 geographically matched women (387 from Northern and 395 from Central Italy) who had undergone physiological menopause after 48 years of age.

The third cohort was from Germany and was composed of 143 POF patients selected with the same criteria described above (Fassnacht et al., 2006). Patients carrying FMR1 premutation were not included in the analysis. The German cohort included 7 cases affected with primary amenorrhea (4.9%) and 136 with secondary amenorrhea (95.1%, mean age at menopause 29.8, range 15–39). The German control population included 105 samples collected among women older than 40 years of age and not yet in menopause and 197 samples from the general female population. Considering the frequency of POF and of early menopause, we can expect to include a maximum number of 19 patients among the unselected control group: given the frequencies of each variant in patients and controls, this would not change the allelic frequency of the control population (McCarthy et al., 2008). The project was approved by the Human Subjects Research Ethics Committee, and all patients gave their informed consent to participate to the study.

Genotyping

The DNA of patients and controls was either provided by collaborators or extracted from blood by conventional methods. In some cases, DNA was extracted from saliva samples collected in Oragene DNA Self-collection Kit (DNA Genotek, Ottawa, Canada) and extracted according to the manufacturer’s instructions. DNAs of the Italian cohorts were whole-genome amplified by the GenomiPhi v2 Kit (GE Healthcare, Uppsala, Sweden).

Genotypes for the three SNPs were assessed by sequence analysis. A first amplicon of 399 bp containing the two promoter variants was amplified using primers 5′-TCGTTAGGGCGAATCTCTCC (forward) and 5′-AAGACAGGCCCTTACCTTG (reverse). A second amplicon of 364 bp containing the coding variant was amplified using primers 5′-TCTGCGCTGTGTCCTGCTG (forward) and 5′-TAAGGCTGGGCTGAGTTAGG (reverse). The amplification reactions were carried out using 25 ng of whole-genome amplified DNA for the Italian samples or genomic DNA for the German samples. The amplifications were performed in a final volume of 10 µl containing 0.5 µM of each primer; 2.5 mM of Mg²⁺, 0.2 mM of each dNTP and 0.25 U of Taq Polymerase on a 9700 Thermal cycler (Applied Biosystems, Carlsbad, CA, USA). Amplification cycles were 94°C for 30 s, 64°C (promoter region) or 56°C (coding variant) for 30 s, 72°C for 30 s repeated 40 times preceded by an initial denaturation at 94°C for 5 min and followed by a final elongation step at 72°C for 5 min.

0.25 µl of the amplification product were used as a template in a 5 µl final volume sequencing reaction containing 0.16 µM of each respective reverse primer and 0.75 µl of BigDye v1.1 sequencing reaction mix (Applied Biosystems). Sequencing reactions were purified by Sephadex and mixed to 5 µl of Formamide before electrophoresis in an Applied Biosystems 3730 apparatus. Sequences were analyzed using the Seqscape v2.5 software (Applied Biosystems).

Sequencing results were in part independently confirmed for 233 (6.9%) samples: 96 patients and 42 controls from the German cohort by restriction fragment length polymorphism, and 95 controls from the Northern Italian cohort by DNA high pressure liquid chromatography.

Statistical analysis

The genotyping results were analyzed through the PLINK 1.04 software (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al., 2007). Allele frequencies, deviations from Hardy–Weinberg equilibrium, single SNPs association under different genetic models and haplotype association were tested by conventional tests implemented in PLINK. Allelic association of the rare 769G > A variant was calculated by Fisher’s exact test.
Results

Promoter variants

The two promoter variants, −124A > G and −16C > T, were genotyped in the three POF cohorts. All control populations were in Hardy–Weinberg equilibrium (P > 0.1). Both SNPs had allelic frequencies similar to those reported in controls of the New Zealand study (−124A: 56.6% in New Zealand controls, 56.0% in our controls and 55.8% in our patients; −16C: 78.5% in New Zealand controls, 80.8% in our controls and 81.2% in our patients). No statistically significant differences between allelic frequencies of cases and controls could be found in our populations (Tables I and II).

Frequencies of haplotypes resulting from the combination of the two promoter variants were determined by the standard E-M algorithm implemented in PLINK. None of the promoter haplotypes showed association to the disease in the cohorts studied (data not shown).

Coding variant

The frequency of the A allele at position 769 of the coding region of the INHA gene was determined in cases and controls in the different populations tested (Table III). All control populations were in Hardy–Weinberg equilibrium (P = 1). The frequency of the A allele was found significantly higher in controls compared with POF patients in the Central Italy cohort (f(A) = 0.049 in controls and f(A) = 0.009 in patients; Fisher’s exact test P = 0.00045 and odds ratio (OR) = 0.17 (95% confidence interval (CI): 0.05–0.56)]. The difference was smaller for the other two populations and did not reach statistical significance, but the frequency of the A allele was always higher in controls compared with cases, suggesting a trend in the same direction.

The association to the disease was significant if the three populations (611 patients and 1084 controls) were considered together [f(A) = 0.036 in controls and f(A) = 0.017 in patients; Fisher’s exact test P = 0.0019 and OR = 0.47 (95% CI: 0.29–0.77)]. Haplotype analysis showed that the A allele is completely contained in the AC haplotype carrying the two promoter variants. Thus, haplotype analysis did not add any more information to the results.

Discussion

The data report an association study of previously identified variants in the INHA gene, in a large cohort of POF patients from Italy and a smaller cohort from Germany. Using a statistically more powerful study design, we were unable to confirm the association of the previously reported promoter variants. In a cohort of 611 cases and 1084 geographically matched controls, we could not replicate the association described in small cohorts from New Zealand, Slovenia and Italy (Marozzi et al., 2002; Harris et al., 2005; Woad et al., 2009). The allelic frequencies we observed were similar to those reported in controls of the New Zealand population, but no difference was found among patients and controls in our study sample and we suggest that the association previously described may be due to the limited sample size (≤150 samples per cohort) (Harris et al., 2005; Woad et al., 2009).

The analysis of the coding SNP showed that the rare allele A of the 769G > A variant may be a protective factor for early loss of ovarian function. The variant, 769A, leading to an amino acid change (Ala257Thr) was first found significantly increased in patients from New Zealand (Ala257Thr) was first found significantly increased in patients from New Zealand: the significance of the association was not very high (P = 0.036), and the number of patients and controls analyzed was quite small (POF patients n = 43; controls n = 150) (Shelling et al.,

<table>
<thead>
<tr>
<th>Table I Association analysis of the promoter variant −124A &gt; G</th>
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<tbody>
<tr>
<td>Population</td>
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<tr>
<td>Central Italian</td>
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<td>Northern Italian</td>
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<td>German</td>
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<td>All populations together</td>
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*aIn brackets are the A allele counts.

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<tr>
<th>Table II Association analysis of the promoter variant −16C &gt; T</th>
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<tr>
<td>Population</td>
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<tr>
<td>Northern Italian</td>
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<tr>
<td>German</td>
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<td>All populations together</td>
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*aIn brackets are the C allele counts.*
Table III  Association analysis of the 769G > A coding variant

<table>
<thead>
<tr>
<th>Genotype count</th>
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<th>German</th>
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<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Patients</td>
<td>Controls</td>
<td>Patients</td>
</tr>
<tr>
<td>GG</td>
<td>357</td>
<td>166</td>
<td>364</td>
<td>285</td>
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<tr>
<td>AG</td>
<td>37</td>
<td>3</td>
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<td>AA</td>
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Allele frequency

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<th>Patients</th>
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<th>Patients</th>
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<tbody>
<tr>
<td>G</td>
<td>0.951</td>
<td>0.991</td>
<td>0.970</td>
<td>0.977</td>
<td>0.975</td>
<td>0.986</td>
<td>0.964</td>
<td>0.983</td>
</tr>
<tr>
<td>A</td>
<td>0.049</td>
<td>0.009</td>
<td>0.030</td>
<td>0.023</td>
<td>0.025</td>
<td>0.014</td>
<td>0.036</td>
<td>0.017</td>
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Fisher’s exact test P-value

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<tr>
<td></td>
<td>4.5 × 10⁻⁴</td>
<td>0.506</td>
<td>0.456</td>
<td>1.9 × 10⁻³</td>
<td>0.47</td>
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OR (95% CI)

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<tr>
<td></td>
<td>0.17</td>
<td>(0.05–0.56)</td>
<td>0.014</td>
<td>0.036</td>
<td>0.047</td>
<td>(0.29–0.77)</td>
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2000). The New Zealand result may represent a real difference due to the analysis of different ethnic groups but should be confirmed by analysis of a larger cohort. Similar findings were reported in two additional small populations (Marozzi et al., 2002; Dixit et al., 2006a) but could not be reproduced in a cohort from Argentina (Sundblad et al., 2006a). In South Korea, the rare variant was absent (Jeong et al., 2004). Similar to the results reported by the Argentina study, we report here that the rare A allele was significantly under-represented in the whole POF cohort compared with the controls (−1.9%, Fisher’s exact test P = 0.0019, OR = 0.47). Thus, the presence of the rare A allele appeared to significantly decrease the risk of POF to about half. Among the three relatively large cohorts studied, the association was highly significant only in the cohort from Central Italy (Fisher’s exact test P = 0.00045, OR = 0.17). It should be noted here that some of the patients in Northern Italy cohort (157 of 299) were previously analyzed, and the results were reported elsewhere (Marozzi et al., 2002). The number was much smaller for both patients and controls, underscoring the importance of a large sample set for reproducible association studies. For the same reason, and considering the very low frequency of the A variant, we think that the three populations described here may be still too limited in size to assess with certainty a significant association.

Larger sets of patients and controls from the same, as well as different ethnic origins, should be analyzed to confirm the association, as well as to definitively rule out a possible involvement of the promoter variants.

The 769A variant was reported to inhibit the activity of inhibin B (Chand et al., 2007). It was suggested that it may reduce inhibin B-dependent suppression of pituitary FSH secretion. This hypothesis does not account for the potential protective effect of the variants from POF. However, a change in the inhibin bioactivity may have different consequences (de Kretser et al., 2002) that should be defined once the role of the variant is elucidated.

In conclusion, this study confirms that POF is a complex genetic disorder caused by a combination of numerous risk factors, which are yet to be fully identified. We suggest here that very large patient cohorts will have to be recruited and studied, to better define disease etiology and thus eventually establish a predictive diagnosis of POF.

Acknowledgements

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Appendix

Italian Network for the study of Ovarian Dysfunctions (NIDO) participants (listed by alphabetical order): Associazione Menopausa Precocie (http://www.menopausaprecoce.splinder.com/), Associazione Italiana Sindrome ‘X-Fragile’ (http://www.xfragile.net/), ONDA (http://www.ondaosservatorio.it), M. Biondi (Centre for Medical Genetics, San Giuseppe Moscati Hospital, Avellino), S.B. (Institute of Molecular genetics, CNR, Pavia), V. Bruni (Pediatric and Adolescent Gynecology Unit, University of Florence, Florence), C. Brigante (Department of Obstetrics and Gynecology, San Raffaele Hospital, Milan), M. Cisternino (Department of Pediatrics, San Matteo Hospital, Pavia), I. Colombo (Department of Pediatrics, San Raffaele Hospital, Milan), P.G. Crosignani (Department of Obstetrics and Gynecology, University of Milan, Milan), M.G. D’Avanzo (Centre for Medical Genetics, San Giuseppe Moscati Hospital, Avellino), L. Dalprà (Department of Neurosciences and Biomedical Technologies, University of Milan-Bicocca, Monza), C. Danesino (Department of General Biology and Medical Genetics, University of Pavia, Pavia), F. Di Prospero (Gynecology Endocrinology Unit, Civitanova Marche General Hospital, Civitanova Marche-AN), E. Doni (Department of Clinical and Experimental Medicine, University of Perugia, Perugia), A. Falorni (Department of Internal Medicine, University of Perugia, Perugia), F. Fusi (Department of Obstetrics and Gynecology, San Raffaele Hospital, Milan), I.G. (Department of Genetic Diagnosis, Careggi University Hospital, Florence),
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