Sequence analysis of the *CDKN1B* gene in patients with premature ovarian failure reveals a novel mutation potentially related to the phenotype

Earlier reports demonstrated a key role of *Cdkn1b* during mouse ovarian development. In this study, the sequencing analysis of the complete coding region of this gene in a panel of premature ovarian failure patients and control subjects reveals a novel mutation potentially related to the phenotype. (Fertil Steril® 2011;95:2658–60. ©2011 by American Society for Reproductive Medicine.)

Key Words: Premature ovarian failure (POF), *CDKN1B*, mutations, sequencing

Premature ovarian failure (POF) is one of the most common diseases that lead to female infertility. It is estimated that POF prevalence can reach ~1.5% (1). Clinically, POF is defined as ≥6 months of amenorrhea that occurs before 40 years of age and is associated with elevated plasma levels of FSH (>40 IU/mL) (2). The hypergonadotropic hypogonadism observed in these patients indicates the lack of ovarian responsiveness to inhibit pituitary gonadotropin synthesis, an essential regulatory step of the hypothalamic-pituitary-gonadal axis. Several etiologies have been related to POF, such as autoimmune and infectious diseases, chemotherapy, metabolic disorders, and pelvic surgery. Nevertheless, the majority of cases are considered as idiopathic, which suggests genetic, epigenetic, and environmental etiologic factors.

From a genetic point of view, in syndromic and nonsyndromic POF cases, X and autosomal abnormalities have been identified (3). In Turner syndrome (45,X monosomy), the haploinsufficiency of pseudoautosomal loci, as well as the unspecific meiotic breakdown, has been proposed as molecular causative mechanisms (4). In the X fragile syndrome, premutations and microdeletions of, respectively, *FMR1* and *FMR2*, are considered to be predisposing factors (5, 6). In addition, rearrangements and deletions of the X chromosome led to identifying candidate POF regions (named *POF-1*, *POF-2*, and *POF-3*) (7–10). Some sequence variants in X-linked and autosomal genes have also been related to POF pathogenesis, as is the case for *BMP15*, *GDF9*, *FOXL2*, *LHR*, *FSHR*, *NOBOX*, *FIGLA*, *SF-1,* and *FOXO3A* (11–20).

Owing to the intrinsic difficulty in identifying familial POF cases (that allow genetic linkage analysis), the study of candidate genes issued from genetically modified mouse models is particularly interesting (21). Early studies demonstrated that *Foxo3a*, a *forkhead* transcription factor, has a key role during the earlier stages of follicular growth, because it negatively regulates their activation (22). *Foxo3a*$^{-/-}$ female mice showed massive follicular activation which results in premature follicular stock depletion and, in fine, to infertility (22). In these animals, the phenotype mimics the ovarian dysfunction observed in POF cases. More recent studies reported *FOXO3A* sequence variants potentially related to POF pathogenesis (19, 20). Interestingly, in the ovarian context, FOXO3A is a downstream mediator of the phosphatidylinositol 3-kinase signaling pathway which has an important regulatory function during mammalian follicular development (23, 24). In 2007, Liu et al. (25) created a transgenic (Tg) mouse model constitutively expressing *Foxo3a* in oocytes. Females showed infertility due to a delay in the oocyte growth, especially those of secondary follicles. In *Foxo3a*-Tg oocytes, the expression of *Cdkn1b* (a negative regulator of the cell cycle) was

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TABLE 1

Results of the sequence analysis of *CDKN1B* in 87 premature ovarian failure (POF) patients.

	Sequence variation	
	c.326T > G	c.356T > C
dbSNP-ID	rs2066827	—
Region	Exon 1	Exon 1
AA change	p.Val109Gly	p.Ile119Thr
Population		
POF		
PA	37/43 (86%)	1/43 (2.3%)
SA	39/44 (88.6%)	0/44
Total	76/87 (87.4%)	1/87 (1.1%)
Control		
A	111/137 (81%)	0/137
B	29/126 (23.01%)	0/126

Note: The overview includes base substitutions at the genomic level. Amino acid (AA) change refers to number of patients and control subjects tested and the frequency of each variant. dbSNP-ID = single-nucleotide polymorpism code (www.ncbi.nlm.nih.gov/snp); PA = primary amenorrhea; SA = secondary amenorrhea.

Ojeda. Correspondence. Fertil Steril 2011.

maintained, preserving its growth-inhibitory function. Subsequent studies, performed in *p27*$^{-/-}$ female mice, demonstrated a key role of this factor during ovarian development, because it suppresses follicular formation and activation, as well as stimulating physiologic follicular atresia before puberty (26). These animals displayed an overactivation and premature depletion of the follicular stock, leading to infertility. Therefore, *CDKN1B* is a coherent candidate gene in POF pathogenesis.

Therefore, the aim of the present study was to identify *CDKN1B* mutations that could explain the POF phenotype. For this purpose, we analyzed the complete coding sequences of the gene in a panel of 87 POF patients. The results were compared with a control population (see below). POF women were recruited from Farhat Hached University Teaching Hospital, Sousse, Tunisia. Inclusion criteria were primary (43 individuals) or secondary (44 individuals) amenorrhea, occurring before the age of 40 years, with elevated plasma levels of FSH (>40 IU/mL) and normal 46,XX karyotype. Patients with clinical antecedents of autoimmune disease, anticancer treatments, and pelvic surgery were excluded from the study. The control population group included 137 Tunisian (group A) and 126 Colombian (group B) women >50 years old without clinical antecedents of autoimmune pathology, infertility, or ovarian dysfunction. In addition, control individuals had at least one child. The study was approved by the Institutional Review Board of each participant institution. Each of the patients and control subjects signed a written informed consent.

Genomic DNA was obtained from whole blood samples using the standard phenol-chloroform protocol. The complete coding region of *CDKN1B* was amplified in all patients and control subjects using 5′ and 3′ flanking oligonucleotides. Primer sequences and polymerase chain reaction conditions are available on request. Each amplicon was purified by using shrimp alkaline phosphatase and exonuclease I, as described by the manufacturer (USB). Direct sequencing was performed with internal primers using an ABI 3100 sequencer (Applied Biosystems). Alignments of the wild type (GI: 4757962) and mutant proteins were performed by using ClustalW software.

The analysis of the coding sequence of *CDKN1B* in POF patients revealed two nonsynonymous variants, both located on the first exon of the gene (Table 1). These results were first compared with those from control group A. The c.326T>G (p.Val109Gly) variant was found in, respectively, 76 (87.4%) POF and 111 (81%) control women. This substitution was previously reported in single-nucleotide polymorphism (SNP) databases (rs2066827) as well as in women with normal fertility (see below). The second variant (c.356T>C, p.Ile119Thr) was present in one patient in heterozygous state. Because we did not find this variant in women from control group A, we expanded its screening to women with normal fertility from a distinct ethnical origin (control group B). Similarly, the p.Ile119Thr mutation was absent in the control population from Colombian origin. This variant was not found in SNP public databases. Protein alignments of this region, using available sequences at public databases, revealed that the isoleucine residue at position 119 is strictly conserved among the majority of mammalian species (*Homo sapiens*, *Sus scrofa*, *Mus musculus*, *Rattus norvegicus*, *Felis catus*; Supplemental Fig. 1, available online at www.fertstert.org). The remaining mammalian species (*Bos taurus* and *Canis lupus familiaris*) displayed at this position a leucine instead of an isoleucine.

The present study describes two nonsynonymous variants of *CDKN1B* present in nonsyndromic idiopathic POF cases. One of them (p.Val109Gly) can be considered to be an SNP that lacks an association with POF pathogenesis, because it is present in women from control populations of two distinct ethnical origins. In addition, this variant has been recently described in Chinese POF cases and women with normal fertility (27).

The novel p.Ile119Thr mutation suggests a potential deleterious effect, as the substitution Ile-to-Thr implicates a relevant change in hydrophobicity (Ile > Thr) and modifications in terms of structure. This mutation, which is located on a predicted beta-sheet region, could affect the adequate folding of the protein and its function. Furthermore, protein alignments argue in favor of a functional role of the Ile at this position, because it is conserved among mammalian species. In this context, the presence of a Leu instead of an Ile in some species may not disturb the protein function, because both have very similar structures and properties in terms of hydrophobicity.

We estimate that, to confirm the implication of *CDKN1B* in POF etiology, the new potentially deleterious mutation described here deserves further functional studies. Finally, additional screening of mutations in this gene in large panels of patients and control subjects should reveal new insights into POF pathogenesis.

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SUPPLEMENTAL FIGURE 1

Multiple protein sequence alignments of *CDKN1B* between mammalian species displaying human Ile at position 119 (*bold*).

Homo sapiens	SGSRPAAPL**I** GAPANSEDTH LVDPKTDPSD
Rattus norvegicus	SGSRQAVPS**I** GSQANSEDRH LVDQMPDSSD
Mus musculus	SGSRQAVPL**I** GSQANSEDRH LVDQMPDSSD
Sus scrofa	SGTRQAVPL**I** GSQANSEDTH LVDQKTDAPD
Felis catus	SGNRQAVPL**I** GSQANTEDTH LVDQKTDTSD
Canis familiaris	SGTRQAGPL**L** GSQANSEDTH LVDQKTDAPD
Bos taurus	SGARPAVPL**L** GSQANPEDTH LVDQKTDAPD

Ojeda. Correspondence. Fertil Steril 2011.