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Variants of the BMP15 gene in a cohort of patients with premature ovarian failure

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BACKGROUND: Bone morphogenetic protein 15 (BMP15) is an oocyte-derived growth factor acting as a major player in follicle differentiation in mammals. Mutations in the BMP15 gene, some of which lead to defective secretion of bioactive dimers, have been associated with premature ovarian failure (POF) in humans.

METHODS: Fifty patients diagnosed with POF with a normal karyotype were included in the study. After DNA extraction and amplification by PCR, the entire coding sequence and intron–exon junctions of BMP15 gene were analysed in the cohort of POF patients and in a control group of 214 patients.

RESULTS: Nine variants of the BMP15 gene including six missense substitutions and one insertion of three nucleotides were identified in the POF group. Three of them were previously described as single nucleotide polymorphisms and were also found in the control group. Two variants (H81R and G199R) have not been previously described and were not identified among controls but were not predicted to be deleterious. One variant (A180T) was identified among two POF cases, and also in two controls. One variant (F194S), predicted as potentially deleterious, was identified for the first time in a POF patient but also identified in one control. One variant (L148P), potentially deleterious, previously reported in POF patients, was identified for the first time among controls. The variant 788insTCT, previously identified among POF patients, probably has a low biological impact as it was also found in control patients and is a common polymorphism in sub-Saharan African populations.

CONCLUSIONS: Various missense variants of the BMP15 gene were identified among patients with POF. For most variants, the impact of the amino-acid substitution on the protein structure and function was predicted to be low. The two variants predicted as potentially deleterious were also identified among controls and could be considered as rare polymorphisms. Although some of these variants could contribute to the development of POF in a complex manner, the demonstration of their role in the pathogenesis of POF requires additional functional studies.

Key words: premature ovarian failure / BMP15 / mutation / polymorphism

Introduction

Premature ovarian failure (POF), also referred to as premature menopause, is a disorder characterized by cessation of menstruation for at least 4 months and features of hypoestrogenism and elevated gonadotrophins (FSH >40 mIU/ml) before the age of 40 years (Sinha and Kuruba, 2007). It affects ~1% of women and is characterized by either a primary amenorrhea, if occurring at a pre-pubertal stage, or more frequently, by a secondary amenorrhea if occurring after puberty. POF is a heterogeneous condition that can be related to a defect in follicle formation during ovarian development, to a block in the folliculogenesis process or to an increased rate of follicle loss. There are several known causes of ovarian failure, including chromosomal defects like Turner’s syndrome, iatrogenic causes such as exposure to radiotherapy or chemotherapy and autoimmune disorders (Goswami and Conway, 2005). Familial forms of POF have
been reported with variable incidences, from 4 to 30% of all cases, suggesting a genetic aetiology of the pathology to a large extent (Beck-Peccoz and Persani, 2006). Although the list of mutations that can cause ovarian failure has rapidly increased, most of these known causes are extremely rare, and most cases of POF are still considered to be idiopathic (Goswami and Conway, 2005).

Folliculogenesis in the mammalian ovary is one of the most complex developmental processes in biology. Normal folliculogenesis requires the complex interplay of both long-range endocrine cues, as well as short-range paracrine and autocrine factors (Roy and Matzuk, 2006).

The formation of a dominant follicle is a step-wise process that involves growth, cell proliferation and cytodifferentiation. During the early phases of this process (i.e. the pre-antral, gonadotrophin-independent stages), follicle growth and development are controlled by autocrine/paracrine mechanisms. In this stage, a major role is played by members of the transforming growth factor-

b superfamily, such as growth differentiation factor 9 (GDF9) and its close X-linked paralogue, the bone morphogenetic protein 15 (BMP15). Later stages of follicular maturation require FSH-receptor signalling in granulosa cells (Shimasaki et al., 2004).

In mammals, BMP15 and GDF9 are specifically expressed in the oocyte in a similar spatio-temporal pattern during folliculogenesis. Both promote granulosa cell proliferation from the primary stages up to the FSH-dependent stages (Elvin et al., 1999; Otsuka et al., 2000; Shimasaki et al., 2004). The BMP15 gene is located on chromosome Xp11.2 and the GDF9 gene on 5q31.1. Both genes have two exons that encode a pre-proprotein (signal peptide, pre-region and mature region). After the removal of the signal peptide, the proprotein is cleaved by a furin-like protease, resulting in the mature proteins some Xp11.2 and the GDF9 gene on 5q31.1. Both genes have two exons that encode a pre-proprotein (signal peptide, pre-region and mature region). After the removal of the signal peptide, the proprotein is cleaved by a furin-like protease, resulting in the mature proteins is cleaved by a furin-like protease, resulting in the mature proteins (Shimasaki et al., 2004). It has been shown that mutations in BMP15 and GDF9 alter female sheep and rodent fertility. In sheep, natural embryos (Yan et al., 2007) and in the ability of oocytes to develop into normal mature cells (Di Pasquale et al., 2004). In mice, targeted deletions of the BMP15 gene lead to infertility, due to a block in folliculogenesis at the primary follicle stage (Dong et al., 1996). In contrast, knockout mice lacking BMP15 are subfertile due to defects in the ovulation process and in the ability of oocytes to develop into normal embryos (Yan et al., 2001). The differences observed between the phenotypes of the BMP15 mutant ewes and the BMP15 knockout mice are not yet understood. It has been suggested that these differences could be implicated in the mono versus poly-ovulatory nature of these species (Hashimoto et al., 2005).

In humans, a mutation in the BMP15 gene (substitution Y235C) has been identified at the heterozygous state in two sisters who presented primary amenorrhoea (Di Pasquale et al., 2004). The mutation affected the prodomain of the protein and functional experiments showed that the mutant BMP15 was associated with a decreased proliferation of granulosa cells (Di Pasquale et al., 2004). Other variants have been identified in the BMP15 gene of patients with POF in Caucasian, Indian and Chinese populations (Dixit et al., 2006; Di Pasquale et al., 2006; Laissue et al., 2006; Ledig et al., 2008; Lakhal et al., 2009; Lakhal et al., 2010; Rossetti et al., 2009; Wang et al., 2009). Most of these have been identified in the BMP15 gene region corresponding to the propeptide of the protein, which is essential for dimerization and subsequent post-translational processing, leading to the secretion of biologically active proteins. Although a causal link between these variants and the development of POF has been missing and disputed (Moron et al., 2007; Ledig et al., 2008), a recent publication showed that some of these variants were associated with a marked reduction of mature protein production (Rossetti et al., 2009). Using a functional luciferase-reporter assay in a human granulosa cell line, the authors showed that their biological effects were significantly reduced, providing more direct evidence for their implication in the development of POF (Rossetti et al., 2009).

Because of its crucial role in folliculogenesis, the BMP15 gene is a major player in follicle differentiation in mammals. Mutations in the BMP15 gene have been shown to be responsible for POF in humans. The aim of this study was to test the prevalence of BMP15 gene mutations and variants in POF by screening a cohort of 50 POF patients and of 214 controls for mutations in BMP15. We analysed the entire coding sequence of the gene by direct sequencing.

**Materials and methods**

**Patients**

Fifty patients diagnosed with POF were included in the study. Inclusion criteria were primary or secondary amenorrhoea occurring before the age of 40 years and a FSH serum level >40 IU/l. Patients with POF resulting from radio- or chemotherapy, surgery or chromosomal aberrations were excluded from the study.

Patients were recruited during consultations in the Department of Gynecology and Obstetrics of Erasme Hospital, most of them being referred for infertility. The study was approved by the Ethics Committee of the hospital and all participants gave their written informed consent for blood sampling and genetic investigations. Patients were asked to complete a questionnaire about their ethnic origin, up to their four grandparents. Of the patients included in this study, 42 were of Caucasian origin, 5 originated from North Africa, 1 from West Indies, 1 from India and 1 from Asia. We excluded from our study one patient originating from Sub-Saharan Africa.

Eight patients had primary amenorrhoea and 42 patients secondary amenorrhoea. Among these, four were diagnosed with POF after stopping the contraceptive pill. For the others, the mean age of onset of POF was of 28.0±6.5 years (mean±SD) (range from 15 to 38 years old). The karyotype was 46XX for all patients.

**Controls**

The control population was recruited in collaboration with the occupational medicine centre of the hospital among hospital employees. The collection of a DNA database in a control population was part of a transdisciplinary project of genetic susceptibility to diseases and treatments. The project was approved by the Ethics Committee of the hospital. All controls received written information about the project. After having signed an informed consent, they were asked to complete a questionnaire about their ethnic origins up to their four grandparents.

The control population was composed of 189 women of Caucasian origin and 25 women originating from North Africa.

**Samples**

Two blood samples of 7 ml were collected for all patients and controls in EDTA tubes for further DNA extraction. All samples were provided a code in order to insure their anonymity.
DNA extraction, sequencing and analysis

Genomic DNA was isolated from whole blood samples using the standard phenol–chloroform procedure. The entire coding sequence and intron–exon junctions of the BMP15 gene were analysed for all patients. Both exons of BMP15 were amplified by the PCR procedure; primer sequences and PCR conditions are available upon request. PCR products were treated with shrimp alkaline phosphatase and exonuclease I as described by the manufacturer (USB, Cleveland, OH, USA) and directly sequenced, using an ABI 3100 sequencer (Applied Bio-systems, Foster City, CA, USA).

Statistical analysis

To determine the potentially deleterious effect of the amino acid changes, we used PolyPhen software (http://genetics.bwh.harvard.edu/pph/index.html) and SIFT software (http://sift.jcvi.org/www/SIFT_BLink_submit.html). The PolyPhen software predicts the possible impact of an amino acid substitution on the structure and function of a human protein by considering structural parameters and three-dimensional protein structures (Sunyaev et al., 2001). The SIFT software predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids (Ng and Henikoff 2003). The score is calculated according to the current available BMP15 sequences. For multiple alignments, we used the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw/index.html).

Results

Sequence analysis

By analysing the coding region of the BMP15 gene of patients with POF, we found nine variants including six missense substitutions and one insertion of three nucleotides (Table I). Three of them were previously described as single nucleotide polymorphisms in the promoter (−9C>G) or the coding sequence of the gene (308A>G and 852C>T). All three were identified among different patients with POF and several controls. Two missense variants [443T>C (L148P) and G538A (A180T)] were previously described in patients presenting POF and several controls. Two new variants with a missense mutation in the heterozygous state [242A>G (H81R) and 595G>A (G199R)] were each identified in a patient with POF. These two variants were not found in our control population. We also found at the heterozygous state an insertion of three nucleotides (788_789insTCT) leading to an insertion of a leucine at position 263 in 3 patients from the POF group, although in the control group, the insertion was present in five individuals, four at the heterozygous and one at the homozygous state. Notably, both heterozygotes for the variant L148P (one in the POF group and one in the control group) were also heterozygous for the insertion of a leucine at position 263 (ins263L).

Clinical history of patients carrying missense variants and the insertion variant ins263L

The clinical characteristics of the patients carrying BMP15 variants are detailed in Table II.

BMP15: 242A>G (H81R)

The variant was identified in a patient of Caucasian origin who presented secondary amenorrhoea with elevated FSH levels at the age of 16. The ovarian biopsy performed at the age of 16 revealed many primary and secondary follicles, consistent with an arrest of folliculogenesis before the antral stage. However, the in silico analysis did not predict an important change in the structure of BMP15 by this substitution. The score attributed by the SIFT software was in favour of a tolerated substitution (SIFT score 0.16).

BMP15: 443T>C (L148P)

The variant was found in a patient originating from West Indies who presented a primary amenorrhoea with elevated FSH levels. The ovarian biopsy performed at the age of 21 showed streak ovaries without any follicles. In addition she had during the same year a surgical removal of a gonadotroph pituitary adenoma. A multiple sequence alignment showed that the leucine in position 148 is well conserved among different species, and that it might have an important structural or functional role. Both PolyPhen and SIFT softwares showed a potentially deleterious effect. The patient was also a carrier of the insertion mutation BMP15: 788_789insTCT (ins263L).

BMP15: 581T>C (F194S)

This variant was found in a patient of Caucasian origin with secondary amenorrhoea and high FSH levels as of the age of 30. The replacement of an aromatic hydrophobic aminoacid (phenylalanine) to a small, neutral, slightly polar residue (serine) may alter the structure or function of the protein. The alignment realized by the PolyPhen software showed that in different species, the phenylalanine is replaced by a leucine, isoleucine or valine and its replacement by a serine is considered as potentially damaging. The score attributed by the SIFT software confirmed this hypothesis (SIFT score <0.05).

Table I  Sequence variation in the BMP15 gene in POF patients.

<table>
<thead>
<tr>
<th>Sequence variation</th>
<th>AA change</th>
<th>Protein domain</th>
<th>No. of patients</th>
<th>No. of controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>−9C&gt;G</td>
<td></td>
<td>Promoter</td>
<td>17/50</td>
<td>73/214</td>
</tr>
<tr>
<td>242A&gt;G</td>
<td>H81R</td>
<td>Exon 1</td>
<td>1/50</td>
<td>0/214</td>
</tr>
<tr>
<td>308A&gt;G</td>
<td>N103S</td>
<td>Exon 1</td>
<td>7/50</td>
<td>25/214</td>
</tr>
<tr>
<td>443T&gt;C</td>
<td>L148P</td>
<td>Exon 2</td>
<td>1/50</td>
<td>1/214</td>
</tr>
<tr>
<td>538G&gt;A</td>
<td>A180T</td>
<td>Exon 2</td>
<td>2/50</td>
<td>2/214</td>
</tr>
<tr>
<td>581T&gt;C</td>
<td>F194S</td>
<td>Exon 2</td>
<td>1/50</td>
<td>1/214</td>
</tr>
<tr>
<td>595G&gt;A</td>
<td>G199R</td>
<td>Exon 2</td>
<td>1/50</td>
<td>0/214</td>
</tr>
<tr>
<td>788_789insTCT</td>
<td>ins263L</td>
<td>Exon 2</td>
<td>3/50</td>
<td>5/214</td>
</tr>
<tr>
<td>852C&gt;T</td>
<td>S284S</td>
<td>Exon 2</td>
<td>3/50</td>
<td>5/214</td>
</tr>
</tbody>
</table>

POF, premature ovarian failure; AA, amino acid.
### Table II: Clinical characteristics of the patients carrying a variant of the BMP15 gene.

<table>
<thead>
<tr>
<th>BMP15 gene variant</th>
<th>Ethnical origin</th>
<th>Amenorrhoea</th>
<th>Age at menopause</th>
<th>FSH level (mIU/ml)</th>
<th>Additional information</th>
</tr>
</thead>
<tbody>
<tr>
<td>242A&gt;G (H81R)</td>
<td>Caucasian</td>
<td>Secondary</td>
<td>16</td>
<td>75</td>
<td>Ovarian biopsy: arrest of folliculogenesis before antral stage</td>
</tr>
<tr>
<td>443T&gt;C (L148P)</td>
<td>West Indies</td>
<td>Primary</td>
<td>na</td>
<td>138</td>
<td>Ovarian biopsy: streak ovaries</td>
</tr>
<tr>
<td>581T&gt;C (F194S)</td>
<td>Caucasian</td>
<td>Secondary</td>
<td>30</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td>538G&gt;A (A180T)</td>
<td>Caucasian</td>
<td>Primary</td>
<td>na</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>538G&gt;A (A180T)</td>
<td>Caucasian</td>
<td>Secondary</td>
<td>32</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>595G&gt;A (G199R)</td>
<td>Caucasian</td>
<td>Secondary</td>
<td>33</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>788_799insTCT (ins263L)</td>
<td>Caucasian</td>
<td>Secondary</td>
<td>25</td>
<td>112</td>
<td>Ovarian biopsy: stromal hyperplasia without follicles</td>
</tr>
<tr>
<td>788_799insTCT (ins263L)</td>
<td>West Indies</td>
<td></td>
<td></td>
<td></td>
<td>Also carrier of L148P variation</td>
</tr>
<tr>
<td>788_799insTCT (ins263L)</td>
<td>Asia</td>
<td>Primary</td>
<td>na</td>
<td>88</td>
<td>Laparoscopy: streak gonads</td>
</tr>
</tbody>
</table>

no, not applicable.

### Discussion

We identified two new missense mutations of the BMP15 gene in a small cohort of 50 POF patients. These two variants were not identified in our control group.

One of these new BMP15 variants [BMP15: 242A>G (H81R)] was identified in a patient who displayed secondary amenorrhoea at the age of 16, and whose ovarian biopsy revealed a follicular arrest with numerous primary and secondary follicles. This phenotype is in contrast with the ones previously described in patients with POF and rare BMP15 variants, which were all characterized by streak ovaries and the absence of follicles when the ovarian biopsy was available. However, the in silico analysis did not predict an important change in the structure of BMP15 by this substitution. Rare inactivating mutations of the FSH receptor gene have been identified in patients with hypergonadotropic ovarian dysgenesis related to a block in folliculogenesis but appeared to be present mainly in the Finnish population (Aittomäki et al., 1995). On the other hand, rare missense mutations in the coding region of the GDF9 gene have been recently associated with POF (Dixit et al., 2005; Laissue et al., 2006; Kovanci et al., 2007). In addition, in vitro studies using recombinant human GDF9 and BMP15 carrying naturally occurring mutations in sheep showed that the co-expression of both mutant proteins resulted in lower secretion levels of both proteins compared with those of cells co-expressing the wild-type GDF9 and mutant BMP-15, suggesting potential additive effects of these mutations (Liao et al., 2004). In order to evaluate if the BMP15: 242A>G variant might contribute to POF in a complex manner in association with other alterations, the entire coding sequence of the FSH receptor and GDF-9 genes

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**BMP15: 538G>A (A180T)**

One of the patients carrying this variant was of Caucasian origin and presented a primary amenorrhea with elevated FSH levels. Puberty was induced at the age of 16 with hormonal treatment. The other patient, also of Caucasian origin, developed a secondary amenorrhea at the age of 32 with high FSH levels. The variant was not predicted to be deleterious as suggested by Polyphen and SIFT softwares.

**BMP15: 595G>A (G199R)**

This variant was identified in a patient of Caucasian origin with secondary amenorrhea and elevated FSH levels diagnosed when she was 33 years old. It consists of a heterozygous replacement of a guanine to an alanine in position 595, which leads to a substitution of a glycine with an arginine. The arginine is present at this position in several species (Sus scrofa, Bos taurus, Pan troglodytes and Monodelphis domestica) and thus it might not have any deleterious effect on the structure or function of BMP15. PolyPhen and SIFT softwares supported this finding (SIFT score 0.53).

**BMP15: 788_799insTCT (ins263L)**

This variant was observed in three patients presenting POF. One of them was of Caucasian origin, one was from West Indies and one from Asia. The patient originating from West Indies was also carrier of the L148P variant. The Caucasian patient developed secondary amenorrhea at the age of 25. An ovarian biopsy performed at the age of 25 showed stromal hyperplasia without follicles. The patient originating from South-East Asia had primary amenorrhea with elevated FSH levels. A diagnostic laparoscopy performed at the age of 16 showed an infantile uterus and streak gonads. No biopsy was performed. Puberty was induced at age 16 with hormone replacement therapy.

This variant consists of the insertion of three nucleotides (TCT) after the position 788, resulting in an insertion of a leucine in position 263 of the protein. The multiple alignment of the BMP15 protein with other species shows that the allele presenting this insertion is in fact the ancestral allele, as it is present in chimpanzee, orang-utan, sheep, pig. In macaque, mouse and rat, it is replaced by a phenylalanine.
was analysed by direct sequencing in this patient, but no mutation was identified in none of these two genes (data not shown).

The variant BMP15: 595G→A (G199R) is a new missense mutation involving the proregion of the protein. However, as the arginine is present at this position in several other species, it might not have any deleterious effect on the structure or function of the BMP15 gene. Accordingly, the SIFT (score 0.53) and Polyphen predictions did not suggest a deleterious effect. The entire coding sequence of the FSH receptor and GDF-9 genes was also analysed by direct sequencing in this patient but no mutation was identified in any of these two genes (data not shown).

The variant BMP15: 581T>C (F194S) had not previously been identified among patients with POF. This variant was also identified in one of our controls. The score attributed by the SIFT software suggested that the replacement of phenylalanine by a serine was potentially damaging (SIFT score <0.05). Recently, this mutant has been identified in a large group of mothers of dizygotic twins (5 carriers in 1693 mothers of dizygotic twins and 933 dizygotic twinning families; Zhao et al., 2008). This variant was also found in a large control group of individuals selected at random and unselected for twinning history (11 carriers among 1512 controls; Zhao et al., 2008). Our patient had no reported familial or personal history of twinning. Her mother (DNA not available) began menopause at the age of 42, which is considered physiological but is, nevertheless, earlier than the mean age of menopause in Caucasian patients, suggesting a genetic predisposition to early menopause in the family. Together, these observations suggest that this variant can rather be considered to be a rare polymorphism. The question of a potential role in the development of POF cannot be answered so far.

The variant BMP15-L148P was identified in a patient originating from West Indies with primary amenorrhoea and streak ovaries with the absence of follicles at ovarian biopsy. It has been previously identified in four patients presenting with POF (Di Pasquale et al., 2006; Laissue et al., 2006; Rossetti et al., 2009). One patient was of African-American origin and developed POF at the age of 20 (Di Pasquale et al., 2006). Another was an African woman who developed secondary amenorrhoea at the arrest of oral contraception at the age of 29. An ovarian biopsy revealed streak ovaries and the absence of follicles (Laissue et al., 2006). The two last patients were of Caucasian origin and developed secondary amenorrhoea (Rossetti et al., 2009). The multiple sequence alignment showed that the leucine in position 148 is well conserved among different species and that it might have an important structural or functional role in protein synthesis. In addition, a recent study using a luciferase-reporter assay in a human granulosa cell line elegantly showed that the biological effect of the L148P mutant protein was significantly reduced, supporting its contribution in the development of the pathology (Rossetti et al., 2009). We also identified this variant in one of our controls originating from the Maghreb, however, as our controls were unselected for their clinical characteristics and regarding the relatively high prevalence of POF, we could not exclude the pathogenic nature of this variant. As three among the five patients presenting POF and carriers of the L148P reported so far were not Caucasian and likely with African ancestors [our patient from West-Indies, one African (Laissue et al., 2006) and one African-American (Di Pasquale et al., 2006)], we also searched for this variant in a group of 62 individuals from sub-Saharan Africa and found it at the heterozygous state in four subjects (6.3%), suggesting that it is more prevalent in the population originating from sub-Saharan Africa than in the Caucasian population in which it was not identified among more than 3500 controls (Di Pasquale et al., 2006; Laissue et al., 2006; Zhao et al., 2008; Rossetti et al., 2009). Although identified in vitro as the most potentially deleterious substitution of BMP15 (Rossetti et al., 2009), the prevalence of this variant in a group of subjects from sub-Saharan Africa questions its role in the development of POF and emphasizes the importance of stratifying genetic analyses according to the ethnicity.

The variant 788insTCT has been previously identified in patients presenting POF (Di Pasquale et al., 2006; Dixit et al., 2006; Laissue et al., 2006; Rossetti et al., 2009; Wang et al., 2010). It has probably a low biological impact as it was previously identified in control patients (Di Pasquale et al., 2006; Dixit et al., 2006; Laissue et al., 2006; Zhao et al., 2008; Rossetti et al., 2009; Wang et al., 2010), particularly of African origin (Laissue et al., 2006). In the control group, we found the insertion in three subjects of Caucasian origin and in two individuals originating from North Africa. We also searched for this variant in a group of 62 subjects from sub-Saharan Africa and found it in 45 patients (73%) at a heterozygous (31/62; 50%) or homozygous (14/62; 22.58%) state confirming that it is a very common polymorphism in sub-Saharan African populations (Laissue et al., 2006). Our patient with POF, carrier of the L148P variant, was also a carrier of the 788insTCT such as the POF patient of African origin reported by Laissue et al. (2006). Interestingly, among individuals of African origin who were tested for L148P and 788insTCT, the carriers of the L148P variant were also carriers of 788insTCT (4/62; 6.5%) as well as our control originating from the Maghreb. The question of a relevant role of the association of these two variants in patients with POF remains open.

The variant A180T has been identified among POF patients at the heterozygous state by different groups (Di Pasquale et al., 2006, Dixit et al., 2006, Ledig et al., 2008; Rossetti et al., 2009). However, it was also found in an unaffected sister of one of the POF patients (Ledig et al., 2008) and among Spanish patients with proven fertility and menopause occurring after 40 years (Moron et al., 2007). More recently, this variant was identified in a group of mothers of dizygotic twins and in a control group at a comparably low allele frequency (0.014 versus 0.016), suggesting that it could correspond to a rare polymorphism (Zhao et al., 2010). We also identified this variant in two of our controls supporting this conclusion. Accordingly, in vitro functional studies using the luciferase-reporter assay showed no effect of the mutant protein (Rossetti et al., 2009).

Previous studies have identified other rare missense variants of the BMP15 gene in cohorts of patients from various origins (Di Pasquale et al., 2006; Dixit et al., 2006; Ledig et al., 2008; Rossetti et al., 2009; Wang et al., 2010). The causal role of BMP15 variants has rarely been supported by functional tests suggestive of abnormal bioactivity or secretion of the mutated protein. Recently, a heterozygous substitution (55R) located in the signal peptide of the protein was identified in a 23-year-old woman of Tunisian origin presenting with secondary amenorrhoea and high gonadotrophin levels (Lakhal et al., 2009; Lakhal et al., 2010). This variant was also found in her 46XY sex-reversed sister, suggesting that the mother, menopausal at 36 years of age, also carried the mutation (Lakhal et al., 2009; Lakhal et al., 2010). This variant was also identified in a Caucasian.
patient with secondary amenorrhea related to premature ovarian insufficiency (Rossetti et al., 2009). Although the variant was considered as potentially damaging by in silico analyses, in vitro functional studies using the luciferase-reporter assay showed a slight but significant decrease of luciferase activity which was rescued when the mutant plasmid was co-transfected with an equal amount of the wild-type cDNA, suggesting no or a minor effect of the mutation at the heterozygous state (Rossetti et al., 2009). This last example emphasizes the difficulty of clarifying accurately the pathogenicity of a BMP15 variant in the presence of ovarian insufficiency and underlines the importance of searching the co-occurrence of alterations at other loci and of developing more sensitive functional tests (Lakhal et al., 2009; Lakhal et al., 2010).

In conclusion, we have identified six missense substitutions and one insertion variant of the BMP15 gene in a small cohort of patients presenting POF. Two of these variants have never been described previously and were not identified in our control population but the probability of a deleterious effect of these mutations regarding the protein sequence similarity of different species and the effect of amino-acid substitution on the predicted three-dimensional protein structure was low. One variant (L148P), potentially deleterious, has been identified so far in four other patients with POF but not in >3500 Caucasian controls. However, this variant was identified in one of our controls originating from the Maghreb but also in 4 out of 62 subjects of African origin suggesting a higher prevalence in African populations than in Caucasians, questioning its implication in the development of POF. One variant (BMP15-A180T) was identified previously in POF patients but also in controls supporting that it is a rare polymorphism as well.

Altogether, rare naturally occurring mutations of the BMP15 gene were identified among POF patients, most of them were either not predicted to be deleterious or can be considered as rare polymorphisms as they were identified among controls. Although it is possible that these variants contribute to the development of ovarian failure in a complex manner together with other predisposing factors, in vitro functional studies will help to clarify the functional changes induced by these mutations and their potential causal role in the development of ovarian insufficiency.

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