CLINICAL STUDY

Mutations and sequence variants in GDF9 and BMP15 in patients with premature ovarian failure

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Abstract

Background and objective: Mutations in bone morphogenic protein 15 (BMP15) and growth/differentiation factor 9 (GDF9) lead to altered fertility in animal models. In the human, a heterozygous point mutation of BMP15 has been associated with premature ovarian failure (POF).

Subject and methods: We have directly sequenced both genes in a cohort of 203 POF patients presenting with primary or secondary amenorrhea and high FSH levels and in a control population including 54 women with regular menstrual cycles who had at least one child.

Results: We have identified several heterozygous variants. One alteration in GDF9 (S186Y) and one in BMP15 (L148P) may have pathogenic effects as both positions are conserved in vertebrate species, ranging from the chicken to mammals. These variants were absent in the control samples. We also found synonymous and neutral substitutions.

Conclusions: We propose that although mutations in BMP15 and GDF9 are not a major cause of ovarian insufficiency, they may be involved in POF.

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Introduction

Premature ovarian failure (POF) is defined as amenorrhea for at least 6 months occurring before the age of 40 years, associated with raised plasma gonadotrophin levels (follicle-stimulating hormone (FSH)) greater than 40 mIU/ml (1). POF has a prevalence of 1%. Ovarian failure can result from viral or auto-immune oophoritis, environmental toxins and iatrogenic causes such as pelvic surgery, radiation or chemotherapy. Deletions and translocations involving three extensive regions of the X chromosome (i.e. Xq13–22, Xq26–28 and Xp11.2–22.1) have been associated with POF (2–5). In Turner’s syndrome, ovarian insufficiency is probably due to haploinsufficiency of pseudoautosomal loci of the X chromosome (6, 7). Mutations in autosomal genes (i.e FSHR, FOXL2, GALT) have also been related to POF (8–11). Nevertheless, in most cases the etiopathology of the disease remains unknown.

Mutations in bone morphogenic protein 15 (BMP15) and its close autosomal paralogue growth/differentiation factor 9 (GDF9) are able to alter female sheep and mice fertility (12–15). Recently, a point mutation in BMP15 has been associated with premature ovarian failure (POF) (16). A 4 bp deletion of GDF9 apparently leading to a premature stop codon has been reported in two sisters with twins from one family (17). Furthermore, two missense mutations in GDF9 have recently been associated with POF (18). BMP15 and GDF9 (respectively located on Xp11.2 and 5q31.1) encode soluble factors, which belong to the transforming growth factor superfamily (TGFβ). TGFβ proteins are involved in various developmental processes including reproductive functions (19–21). In mammals, BMP15 and GDF9 are specifically expressed in the oocytes in a similar spatio-temporal pattern throughout folliculogenesis (20, 22–24).

Both genes have two exons and encode preproproteins composed of a signal peptide, a proregion and a
mature peptide. The signal peptide region is encoded by the first exon, the proregion by segments of both exons and the mature peptide region by the second exon. Their biological activity depends on the formation of homo and/or heterodimers after proteolytic processing (25–27).

Here we have screened a cohort of 203 POF patients for mutations in GDF9 and BMP15. We analyzed the entire coding sequences of both genes by direct sequencing. The variants were screened in a panel of 54 women with normal fertility.

Materials and methods

Patients and control population

Women (203 subjects) were recruited from several clinical centres: Reproductive Endocrine Units from Hôpital Saint-Antoine, Hôpital Necker, Paris, France, Hôpital Jeanne de Flandres, Lille, France, a gynaecological unit in Hôpital Antoine Béclère, Clamart, France and the Department of Obstetrics and Gynaecology of Helsinki University Hospital, Helsinki, Finland. Inclusion criteria were primary or secondary amenorrhea occurring before the age of 40 years, with an FSH serum level higher than 40mIU/ml. The group of patients was composed of 83% of women of Caucasian origin, 14.9% of African origin and 2.1% of Asian origin (37 patients were Finnish). This study was approved by the institutional review board of the relevant hospitals and all participants gave their written informed consent. The control population included 54 women with regular menstrual cycles (28–32 days), having at least one child and no personal history of infertility or auto-immune disease. Among these women 40 were of Caucasian origin and 14 of African origin.

DNA extraction and sequencing

Genomic DNA was isolated from whole blood samples using the standard phenol–chloroform procedure. Both exons of GDF9 and BMP15 were amplified by PCR in all the patients (n = 203). The second exon was amplified in all control individuals (n = 54). PCR products were treated with shrimp alkaline phosphatase and exonuclease I as described by the manufacturer (USB, Cleveland, Ohio, USA) and directly sequenced using an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA). The presence of each variant was confirmed by a new round of PCR amplification and sequencing.

In silico analysis

To determine the potential deleterious effect of the amino acid changes we used SIFT (http://blocks.fhcrc.org/sift/SIFT.html) and PolyPhen softwares (http://tux.embl-heidelberg.de/ramensky/index.shtml). The SIFT software uses the protein sequence similarity of different species and the hydrophobic characteristics of amino acids to calculate the probability of a deleterious effect of specific amino acid variants (28). Scores lower than 0.05 suggest a potential pathogenic amino acid substitution. The PolyPhen program offers information about the effect of the amino acid substitution by considering structural parameters and three-dimensional protein structures (29, 30). The GoCore program version 5.0.1 (http://www.helsinki.fi/project/ritvos/GoCore/) was used for performing multiple alignments of sequences from several vertebrate species including H. sapiens, P. troglodytes, C. familiaris, R. norvegicus, M. musculus, S. scrofa, B. taurus, C. hircus, C. elaphus, O. aries, T. vulpecula, D. novemcinctus, O. garnettii, M. mulatta, P. anubis, C. jacchus, M. domestica, G. gallus and F. rupibes.

Results

Sequence analysis

The analysis of the coding sequence of GDF9 revealed, in one case, a heterozygous transversion 557C > A (second exon). This leads to the change S186Y in the proregion. This variant was not found in any control (n = 54). Furthermore, we found two rather common synonymous substitutions. 447C > T and 546G > A (summary in Table 1).

We detected, in the first exon of BMP15, a heterozygous transition G308A (with respect to the sequence AH007120 in Genbank). This variant appeared in 99% of the patients and controls. In the second exon, six different variants were detected. In one patient, we found the heterozygous 443T > C transition, leading to an L148P change in the proregion. We did not find this variant in any control (n = 54). Furthermore, this patient presented an insertion of three base pairs (788insTCT) leading to the insertion of a leucine at position 263. Ins263L was found in nine other patients and in 16.6% (nine of 54) of the control sample. All the controls presenting these variants were of African origin. Among the patients showing this mutation 60% were of African origin. A transition 588G > A (A180T) was found in two patients at the heterozygous state. This variant was not present in any control. In addition, we detected three silent substitutions, 538G > A, 831T > C and 468G > A (Table 1).

Patients’ phenotype

BMP15-L148P The patient was a 30-year-old African woman presenting with secondary amenorrhea. She was a second daughter of a non-consanguineous family. Puberty occurred when she was 13 years old, with normal secondary sex characteristics and breast development. The patient described
oligomenorrhea. When she was 19 years old, she received the oral contraceptive pill (OC) until she was 29 years old but she stopped it frequently. She then became pregnant twice but interrupted the pregnancy in both cases. When she definitely stopped taking the OC, secondary amenorrhea appeared. When she was referred to our department, the patient’s height was 163 cm and her weight was 73 kg. Plasma FSH and luteinizing hormone (LH) were high: 120 mIU/l (normal (N), 4 – 8 mIU/l) and 100 mIU/l (N, 3 – 12 mIU/l) respectively. Plasma oestradiol and inhibin B were low: 30 pmol/l (N, 70 – 1000 pmol/l) and 15 pg/ml (N, 60 – 120 pg/ml) respectively. No anti-thyroid or anti-ovary antibodies could be detected. The karyotype was normal. Pelvic ultrasonography showed a small uterus (68 £ 39 £ 27 mm), an atrophic endometrium and very small ovaries in their surface (0.56 and 0.64 cm² for the right and left ovaries respectively (N, 2 – 6 cm²). No follicle was observed. The patient received an oral oestrogen-progestagen treatment.

We did not have access to blood samples from any family members.

GDF9-S186Y

The patient, of Caucasian origin, was referred to us at the age of 37 years for primary infertility. Menarche had occurred at 9.5 years of age with regular menstrual cycles every 21 days up to the age of 33 years, then her menses suddenly stopped. At that time, her FSH, LH and oestradiol serum levels were respectively 104 mIU/ml, 46 mIU/ml and < 20 pg/ml. Anti-thyroid and anti-DNA serum antibodies were undetectable. No familial or personal history of pelvic surgery was recorded. Her height was 153 cm and her weight 60 kg. Her karyotype was 46XX. No other case of POF was identified in the family. At the age of 35 years, a coelioscopy was performed showing atrophy of both ovaries. The ovary cortex biopsy was composed of interlacing bundles of stromal spindle cells and contained some white body remnants but there were no follicles on serial sections. After the initial diagnosis of POF, the patient received hormone replacement therapy. We did not have access to familial blood samples.

Discussion

BMP15 and GDF9 play a key role in the molecular dialogue between the oocyte and the surrounding somatic cells, promoting granulosa cell mitosis and cumulus expansion by a paracrine effect (31, 32). They are synthesized as propeptides composed of a signal peptide, a proregion and a mature peptide. As for many TGFβ factors, the proregion of BMP15 and GDF9 might be necessary for a correct folding, dimerization and secretion of the mature protein (19).

In vivo, the biological role of BMP15 and GDF9 in animal fertility has been demonstrated in mice and ruminants. Naturally occurring heterozygous mutations in BMP15 and GDF9 in sheep increase the ovulation quota leading to hyperfertility, whereas homozygous mutations produce infertile animals (12, 13). The biological effect of the mutations in mammals varies among species (12, 15). For example, GDF9 −/− mice present a phenotype that is similar to BMP15 homozygous sheep mutants whereas BMP15 knockout (KO) mice have normal ovaries and subfertility (15). The double gene KO leads to infertility while other combinations of alleles display different phenotypes (15). The difference in the effects of the mutations could be explained by the intrinsic characteristics of mono-ovulatory or

Table 1 Results of the sequence analysis of BMP15 and GDF9 in 203 POF patients. The overview includes base substitutions at the genomic level (numbering refers to the coding region), amino acid (AA) change, number of patients tested and allele frequency of each variant.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence variation</th>
<th>AA change</th>
<th>Clinical information</th>
<th>No. of patients</th>
<th>Frequency in population</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP15</td>
<td>788insTCT</td>
<td>Ins263L</td>
<td>POF</td>
<td>203</td>
<td>4.93% (10/203)</td>
</tr>
<tr>
<td></td>
<td>443T &gt; C</td>
<td>L148P</td>
<td>Controls</td>
<td>54</td>
<td>16.6% (9/54)</td>
</tr>
<tr>
<td></td>
<td>538G &gt; A</td>
<td>A180T</td>
<td>POF</td>
<td>203</td>
<td>0.49% (1/203)</td>
</tr>
<tr>
<td></td>
<td>852C &gt; T</td>
<td>S284S</td>
<td>Controls</td>
<td>54</td>
<td>0% (0/54)</td>
</tr>
<tr>
<td></td>
<td>831T &gt; C</td>
<td>T277T</td>
<td>POF</td>
<td>203</td>
<td>0.49% (1/203)</td>
</tr>
<tr>
<td></td>
<td>468G &gt; A</td>
<td>V156V</td>
<td>Controls</td>
<td>54</td>
<td>0% (0/54)</td>
</tr>
<tr>
<td>GDF9</td>
<td>557C &gt; A</td>
<td>S186Y</td>
<td>POF</td>
<td>203</td>
<td>0.49% (1/203)</td>
</tr>
<tr>
<td></td>
<td>447C &gt; T</td>
<td>T149T</td>
<td>Controls</td>
<td>54</td>
<td>0% (0/54)</td>
</tr>
<tr>
<td></td>
<td>546G &gt; A</td>
<td>E182E</td>
<td>POF</td>
<td>203</td>
<td>23.15% (47/203)</td>
</tr>
</tbody>
</table>

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polyovulatory species. Moreover, the mutations in mice are loss-of-function whereas in sheep and human there are presumed to be dominant negative. Indeed, in vitro cell transfection assays using constructs corresponding to the natural mutations observed in sheep demonstrated that dominant negative interactions between these factors have an important effect on the secretion of the mature peptides (27). In the human, a heterozygous point mutation of BMP15 that has been associated with POF (Y235C) is located in the proregion and in vitro experiments have shown a diminished stimulatory activity of granulosa cell mitosis by a potential dominant negative effect (16). Recently, two missense mutations (K67E and V216M) located in the GDF9 proregion have been described in Indian POF patients and could be implicated in the ovarian phenotype (18). Sequence alignments have shown that the first variant is not likely to have any functional relevance since in the sheep and the chicken the K residue is replaced by an E. In the second case, the amino acid V is well conserved or replaced by a highly hydrophobic residue which argues in favour of a functional impact. The results using SIFT are in agreement.

In our cohort of 203 patients, we identified two novel heterozygous missense mutations, one in each gene, that could be implicated in the ovarian phenotype. Sequencing of GDF9 revealed the mutation S186Y found in one patient with secondary amenorrhea. The absence of this variant in women with normal fertility suggests a potential pathogenic effect. This was further corroborated by the evolutionary conservation of the S186Y in 17 vertebrate species, ranging from chickens to mammals (Fig. 1). Accordingly, both the SIFT (score = 0.03) and PolyPhen predictions suggested a deleterious effect. We also found two polymorphisms, 447C > T and 546G > A, in 154 (75.9%) and 47 (23.1%) respectively of our POF patients. These variants are common polymorphisms in diverse ethnical groups since they have also been described in Indian POF patients (18).

The sequence analysis of BMP15 revealed that the heterozygous transition G308A (S103N, with respect to Genbank AH007120) that appeared in the first exon is in fact the most common allele. One patient carried a 443T > C transition at a heterozygous state in the second exon. This leads to a L148P change at the protein level. This mutation was located in the proregion, closer to the N-terminus than that described by Di Pasquale et al. (16). The ovarian phenotype of the patient with the BMP15 mutation was less severe than in the two cases previously described (16). A multiple sequence alignment showed that the amino acid L148P position was also conserved across vertebrates, from chicken to mammals, suggesting a crucial functional or structural role. As expected, both Polyphen and SIFT programs suggested a potential deleterious effect. The substitution L-to-P translates into an important change in hydrophobicity (L > P) and relevant modifications in terms of structure. The presence of a P might change the rigidity of the peptidic chain and could alter protein folding itself or its kinetics. The mutation A180T, found in two patients, is not likely to be deleterious as suggested by the SIFT and PolyPhen programs. Although we did not find it in any control, the Thr appears in wild-type sequences from C. hircus and O. aries. The insertion of a leucine in position 263, found in ten patients, probably has a low biological impact, as suggested by its presence in 16.6% of the controls (nine of 54). Furthermore, it is present in rodents and the marsupial T. vulpecula (Fig. 1). It is interesting
to underline that 60% of patients and the totality of controls bearing this variant were of African origin. These results suggested that BMP15-Ins263L is a common variant in African populations.

It is possible that the adequate folding of the proregion could be necessary for a normal folding of the mature region. We hypothesize that the misfolded proregion of BMP15-L148P and GDF9-S186Y could affect the normal cleavage or inhibit/alter the folding of the mature region. This may lead to the production of abnormal dimers or could even inhibit the dimerization process. The importance of a normal structure of the BMP15 proregion has been recently demonstrated by Hashimoto et al. (33) who showed that the intrinsic molecular properties of the human BMP15 proregion are strong determinants for the correct processing of the mature protein (33). Furthermore, they highlighted the relevance of the post-translational processing of the BMP15 proregion (33).

We have concluded that although mutations in BMP15 and GDF9 are not a major cause of premature ovarian failure they can be involved in this disease. This is in line with the fact that Takebayashi et al. (34) failed to detect mutations in GDF-9 and the BMP15 in a small cohort of 15 POF patients. Furthermore, our study corroborates the recent findings of Dixit et al. (18) showing a low GDF9 mutation frequency in a large cohort of Indian POF cases. The new potentially deleterious mutation in BMP15 and GDF9 described in our POF patients deserve further functional in vitro studies. Nevertheless, these assays are particularly challenging since different studies using recombinant GDF9 display contradicting results in terms of cumulus cell expansion and regulation of gene expression (35).

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