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BRIEF REPORT

Identification of New Variants of Human *BMP15* Gene in a Large Cohort of Women with Premature Ovarian Failure

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Context: Premature ovarian failure (POF) is a cause of female infertility characterized by primary (PA) or secondary amenorrhea (SA) and elevated gonadotropins. The pathogenesis is unknown in most cases. We recently reported two sisters with PA carrying a heterozygous mutation of *BMP15* gene (locus Xp11.2), but the prevalence of *BMP15* variations in the POF population is unknown.

Objective: The objective of the study was to verify the involvement of *BMP15* variations in a large POF population.

Design and Subjects: Genetic screening of 166 unrelated patients with idiopathic POF (25 PA, 141 SA) and controls (group A: 95 women with menopause beyond 50 yr of age; group B: 86 women and 30 men from the general population) of Caucasian origin.

Results: Investigation revealed four heterozygous variations affecting the proregion of BMP15. The previously reported p.Y235C mutation occurred in one and three novel variants in eight patients: two missense alterations (p.R68W in one case, p.A180T in five) and one insertion (p.262insLeu) in two cases. The p.262insLeu was found in five controls of group A, thus diminishing its potential biological impact, whereas the other three variants were not present in any of the controls. All new mutations were found in SA cases.

Conclusion: We describe the significant association of heterozygous *BMP15* gene variants with the POF phenotype in humans (seven of 166 patients: 4.2%; $P < 0.003$ *vs.* controls). These findings are consistent with the critical role played by *BMP15* in human folliculogenesis. **(*J Clin Endocrinol Metab* 91: 1976–1979, 2006)**

PREMATURE OVARIAN FAILURE (POF, Online Mendelian Inheritance in Man 311360) is a disorder associated with female infertility affecting 1–2% of women under 40 yr of age (1, 2). It is a heterogeneous disease clinically characterized by primary amenorrhea (PA) in major defects with prepubertal onset or secondary amenorrhea (SA) in women with postpubertal onset (1–3). The diagnosis is supported by biochemical findings showing the association of elevated gonadotropins and low estrogen levels.

Several mechanisms may be involved in POF pathogenesis, but the genetic contribution is a significant etiological component (1, 4). Genetic studies have identified several loci at Xq22, Xq26-q28, and Xp11.2-p22.1 whose disruption has been associated with POF (4–6). Furthermore, alterations in autosomal genes, such as *FSHR, LHR, FOXL2, INHA, GALT*, are rarely associated with POF (1, 7), and the pathogenesis remains most frequently unsolved (1, 8).

Recently attention has been focused on members of TGFβ superfamily and their potential role as local modulators of ovarian function (9, 10). Among these, growth differentiation factor-9 (GDF9) and bone morphogenetic protein-15 (BMP15) originate from the oocyte and have been shown to be critical regulators of ovulation rates and litter size and to be required for the progression of early folliculogenesis in rodents and sheep. Targeted deletion of *Gdf9* or *Bmp15* gene leads to female infertility or subfertility in mice (9, 11). Natural mutations in sheep cause increased ovulation rate in heterozygotes or early block of folliculogenesis and sterility

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Abbreviations: BMP15, Bone morphogenetic protein-15; dHPLC, denaturant HPLC; GDF9, growth differentiation factor-9; PA, primary amenorrhea; POF, premature ovarian failure; SA, secondary amenorrhea.

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in homozygous or compound heterozygous animals (9, 10, 12, 13). These genes encode prepro-proteins composed of signal peptide, proregion and mature peptide. Proteolytic cleavage of propeptides and formation of mature homo- or heterodimers represent critical steps for the bioactivity of these factors (9–14).

Based on evidence in animal models, *GDF9* and *BMP15* can be considered candidate genes in POF. Indeed, we very recently identified the first mutation in *BMP15* gene (p.Y235C) in two sisters affected with PA due to ovarian dysgenesis (15). Both sisters were heterozygous for this mutation that was inherited from the hemizygous father, representing an unusual case of X-linked disease in which the affected women inherit the alteration from a male carrier. The reported mutation was an A to G transition at position 704 of the *BMP15* gene, leading to a nonconservative substitution in the proregion that was associated with a defective processing of the mutant precursor and altered biological activity (15).

Because the prevalence of *BMP15* gene alterations is still undefined among POF patients, here we present the results of *BMP15* genetic analysis in a large series of women with idiopathic POF.

Subjects and Methods

Subjects

A total of 166 unrelated Caucasian women (79 American, 87 European) affected by idiopathic POF (aged 12–39 yr) were collected from all the clinical centers participating in this study. Inclusion criteria were represented by the presence of amenorrhea for a period more than 6 months before the age of 40 yr associated with two distinct determinations of FSH concentration in the postmenopausal range (8). The association with complex or other endocrine diseases was excluded by medical history, physical examination, and biochemical determination. The selected POF population included 25 patients with PA, 15 sporadic, and 10 probands of familial cases and 141 women with SA (age at POF: 13–39; mean ± SD: 28 ± 8; median: 29 yr), 74 sporadic, and 67 probands of familial cases. All selected patients had a normal karyotype analysis based on high-resolution banding technique.

Genetic investigation was extended to two control groups of Caucasian subjects. Group A was represented by 95 women with physiological menopause beyond 50 yr of age. Control group B included 86 women and 30 men from the general population.

Institutional ethical committees approved the study, and informed consent for blood sampling and genetic investigations were obtained from all participants.

Genetic analysis

The entire coding sequence and intron-exon junctions of *BMP15* gene were analyzed in all participants. Patients' samples were screened by denaturant HPLC (dHPLC) of PCR-amplified fragments on an automatic instrument (WAVE apparatus, Transgenomics Inc., San Jose, CA). *BMP15* sequence was divided into five fragments, two for the amplification of exon 1 and three for exon 2. Primer sequences and PCR conditions are available upon request. Each PCR sample from the POF patients was mixed 5:1 with a product obtained from a wild-type DNA. The acetonitrile gradient and temperatures for dHPLC analysis were obtained using an algorithm (WAVE Maker; Transgenomics). The appearance of additional peaks was interpreted as indicative of a mismatch in the analyzed PCR fragment. In these cases, a new PCR product was sequenced in both directions using an automatic genetic analyzer as previously reported (15). Control samples were directly sequenced. The significance of allelic distribution in the POF and control groups was determined by Fisher's exact test.

Results

Genetic analysis revealed the presence of four variants of *BMP15* gene in nine of 166 POF patients. The mutation p.Y235C was found in the previously reported proband (patient 118 in Table 1), whereas three novel *BMP15* variants were present in eight POF women. All novel alterations are nonconservative and include two missense substitutions (c.202C > T → p.R68W; c.538G > A → p.A180T) and one insertion of three nucleotides (c.788insTCT → p.262insLeu) (Fig. 1). The clinical data of the nine POF patients who were heterozygous carriers of *BMP15* variants are reported in Table 1. In particular, p.A180T variant was found in five patients (3.0%; $P < 0.02$ *vs.* 0/392 control alleles) and p.R68W in one, all presenting with SA. The p.ins262Leu variant was found in two patients, one SA and one PA. All variants were located in the proregion. The age of POF onset in SA cases with BMP15 variants was early (range 15–29 yr) (Table 1), but biochemical data of these patients did not differ from those of the others.

None of the three missense variations was found in any of the controls, whereas five of 95 women from control group A were heterozygous for the insertion p.262insLeu (P = NS *vs.* two of 332 POF alleles). On this basis, the three missense variants of *BMP15* are significantly more prevalent among the POF population (seven of 332 POF alleles *vs.* none of 392 control alleles; $P = 0.004$).

Finally, genetic screening confirmed the presence of two conflicts in the sequence of *BMP15* (GenBank no. AF082349

TABLE 1. Clinical and biochemical characteristics of patients carrying *BMP15* alterations

Patient no.	Gene variation	Protein variation	Phenotype	Age at menarche (yr)	Age at POF (yr)	FSH (U/liter)	LH (U/liter)	Familial case
36	**c. 538G>A**	**p. A180T**	SA	12	21	22	24	No
73	**c. 202C>T**	**p. R68W**	SA	16	16	28	11	Yes
79	**c. 538G>A**	**p. A180T**	SA	N.D.	27	77	112	No
92	**c. 538G>A**	**p. A180T**	SA	15	29	110	34	No
112	**c. 538G>A**	**p. A180T**	SA	14	25	100	36	No
113	**c. 538G>A**	**p. A180T**	SA	12	22	47	17	No
118	**c.704 A>G**	**p. Y235C**	PA	N.A.	N.A.	80	95	Yes
124	c.788insTCT	p.262insLeu	PA	N.A.	N.A.	52	23	No
125	c.788insTCT	p.262insLeu	SA	12	15	54	14	Yes

The alterations we did not find in the control groups A and B are in *bold*. The p.Y235C mutation was previously described (16). N.D., Not determined; N.A., not applicable.

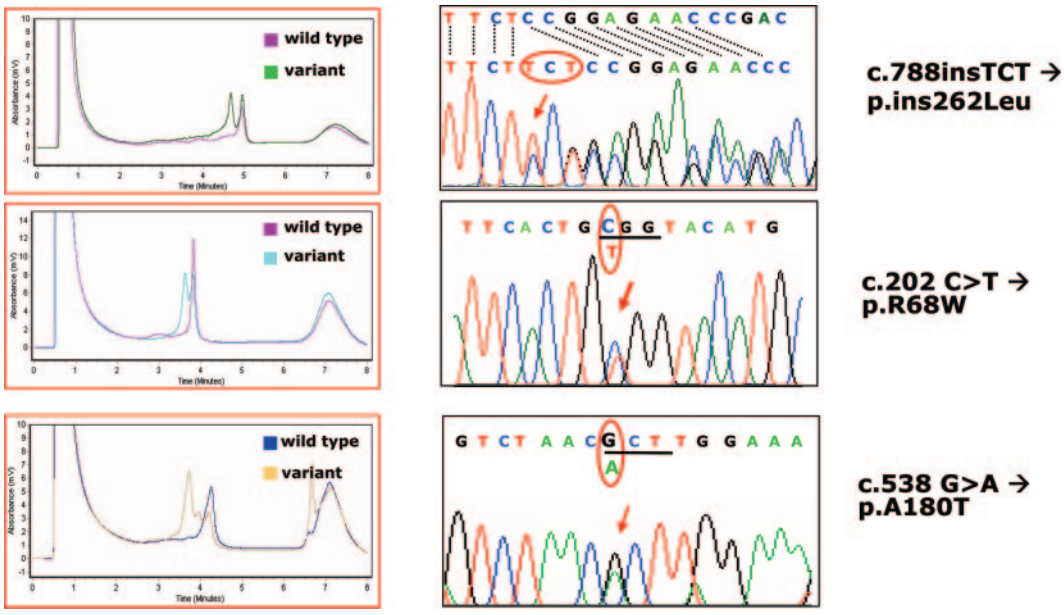

FIG. 1. The novel *BMP15* sequence variations are illustrated. The dHPLC results are represented in *left panels* and the corresponding electropherograms of the sequence are shown in the *right panels*. In these cases, the absence of additional variations was confirmed by direct automatic sequencing of the entire *BMP15* coding sequence.

and AJ132405) (16, 17). One substitution is located in the 5′ untranslated region (g.-9G > C) and was identified in 86.7% of patients and in 61.0 or 71.6% of controls from group A or B, respectively. The other substitution localizes in exon 1 (c.308G > A) leading to the variation p.S103N. This change was present in 6.7% of the POF patients and 9.5 or 8.4% of the controls from group A or B, respectively.

Discussion

The possible involvement of BMP15 in POF pathogenesis was supported by evidences in animal models (9–13). Takebayashi *et al.* (18) could not find variations in this gene, probably due to the restricted number of analyzed samples (n = 15). Recently we (15) reported the first mutation of *BMP15* gene in two sisters with ovarian dysgenesis. This study disclosed the major role of BMP15 in the progression of early folliculogenesis also in humans (14). We therefore decided to verify the prevalence of *BMP15* gene alterations among the POF populations. Samples from different centers were collected, and a large cohort of unrelated patients with PA or SA was analyzed. These analyses revealed the presence of four different variants in nine POF heterozygous patients.

All variants are located in the part of the gene encoding the proregion, which is highly conserved across species and has a critical role in correct precursor processing, dimerization, and secretion of mature protein (9, 11, 14, 19). The p.ins262Leu is located close to the endopeptidase consensus sequence (RRXR) for propeptide cleavage and was detected in one PA and one SA case with a very early onset. Nevertheless, this insertion was observed in 5% of the control women with menopause beyond 50 yr and is also present in the wild-type sequence of several other mammals, thus reducing the possibility of a relevant biological impact in POF pathogenesis. All the other variants were not found among 392 control alleles. These findings are good arguments in favor of a possible effect of these variations in BMP15 precursor processing. This possibility was indeed supported by the functional studies reported in the case of the previously reported p.Y235C mutation (15).

The introduction of a cysteine could lead to aberrant dimerization and produce major functional effects in folliculogenesis consistent with the report of this mutation in 46, XX women with ovarian dysgenesis. Two novel missense variations were instead detected among the SA cases, which may predict a minor disruption of oocyte-growth factor processing and folliculogenesis progression by these substitutions. Major alteration in precursor structure is expected by R68W variation with a basic, positively charged arginine replacing an aromatic, neutral tryptophan. Consistent with this expectation, this mutation was detected in a patient who underwent POF at 16 yr after only one spontaneous menstruation. She had a familial form of POF, with her mother, grandmother, and six maternal cousins who experienced early menopause before 28 yr of age. The A180T variation leads to the introduction of a polar residue susceptible to change the charge of the region. This variant was detected in five SA patients and is significantly associated with POF. The onset age ranged 21–29 yr and was preceded by a period of menstrual irregularity. None of these five cases was familial, but patient 92 had a 30-yr-old sister with hot flashes at the time of sampling. We also had the opportunity to study a small number of POF women with SA of other ethnicity (14 African-Americans, six Asians, four Hispanics). Two additional missense variants, p.S5R and p.L148P, were detected among the African-Americans (POF onset: 34 and 20 yr) (data not shown).

Functional studies are required to definitively support the biological impact of novel alterations, but the specific location in the proregion and early POF onset *in vivo* as with the

originally described mutation is an argument in favor of a similar mechanism in POF pathogenesis. A recent study (19) reinforced the role of BMP15 proregion in the posttranslational processing and proposed its involvement in the generation of species-specific differences in ovulation quota among mammals, suggesting that alterations in BMP15 proprotein may have direct consequences in female reproductive physiology. Dixit *et al.* (20) recently reported the significant association of *GDF9* gene variants, including two missense mutations affecting the proregion, with POF phenotype. This finding represents an additional support to the potential involvement of alterations in oocyte growth/differentiation factor pathway in human ovarian failure.

In conclusion, *BMP15* gene variants are significantly more prevalent among Caucasian women with POF, supporting the view that BMP15 plays a critical role in early human folliculogenesis. Thus *BMP15* represents a candidate for POF, either associated with primary or secondary amenorrhea. Because *BMP15* gene maps within the Xp locus linked to POF (5), the present data support the concept that *BMP15* represents one of the genes whose haploinsufficiency significantly contributes to ovarian dysgenesis in Turner syndrome.

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