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Report

Hypergonadotropic Ovarian Failure Associated with an Inherited Mutation of Human Bone Morphogenetic Protein-15 (*BMP15*) Gene

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Hypergonadotropic ovarian failure is a common cause of female infertility. It is a heterogeneous disorder that, in the most severe forms, is a result of ovarian dysgenesis (OD). Most OD cases are associated with major X-chromosome abnormalities, but the pathogenesis of this disorder is still largely undefined in patients with a normal karyotype. Animal models showed the important role in female reproduction played by the product of a gene located at Xp11.2 in humans (*BMP15*). BMP15 is an oocyte-specific growth/differentiation factor that stimulates folliculogenesis and granulosa cell (GC) growth. We report two sisters with a normal karyotype who are affected with hypergonadotropic ovarian failure due to OD. The familial presentation suggested a genetic origin, and candidate genes were screened for mutations. A heterozygous nonconservative substitution in the pro region of BMP15 (Y235C) was identified in both sisters but not in 210 control alleles. This mutation was inherited from the father. Mutant BMP15 appears to be processed abnormally, is associated with reduced GC growth, and antagonizes the stimulatory activity of wild-type protein on GC proliferation. In conclusion, the first natural mutation in human *BMP15* is associated with familial OD, indicating that the action of BMP15 is required for the progression of human folliculogenesis. This condition represents an exceptional example of X-linked human disease exclusively affecting heterozygous females who inherited the genetic alteration from the unaffected father. BMP15 defects are involved in the pathogenesis of hypergonadotropic ovarian failure in humans.

Hypergonadotropic ovarian failure is a heterogeneous disorder that, in the most severe forms, is a result of ovarian dysgenesis (OD [MIM 233300]). OD accounts for about half of the cases of primary amenorrhea (Timmreck and Reindollar 2003). Most OD cases are associated with major X chromosome abnormalities. Accordingly, genetic studies have identified several loci at Xq and Xp11.2–p22.1 whose functions are relevant for ovarian development (Zinn et al. 1998; Simpson and Rajkovic 1999; Marozzi et al. 2000). The etiopathogenesis of this disorder is still largely undefined in patients with a normal karyotype. Severe gonadotropin receptor defects are, indeed, a rare cause of OD in 46,XX women (Aittomaki et al. 1995; Layman et al. 1998), suggesting the possible involvement of other candidate genes. Recently, animal models showed the important role in ovarian development and folliculogenesis played by the paracrine action of growth/differentiation factors (GDF9 and GDF9b or BMP15) that originate from oocyte cells (Dube et al. 1998; Galloway et al. 2000; Yan et al. 2001; Hanrahan et al. 2004). BMP15 (MIM 300247), in particular, is the product of a gene located at Xp11.2 in humans (Dube et al. 1998). *Bmp15* knockout female mice are subfertile and show reduced ovulation rates after gonadotropin treatment (Yan et al. 2001). Natural mutations in the *Bmp15* gene in sheep, termed "fecundity X Inverdale and Hanna" ($FecX^{I/H}$), have provided insight into the role of BMP15 in female reproduction (Galloway et al. 2000). Ewes with heterozygous mutations exhibited increased ovulation and lambing rates, whereas severe infertility with an early block in folliculogenesis was seen in ewes with homozygous mutations. In humans, *BMP15* transcripts could be detected in gonads by RT-PCR. In situ hybridization localized the transcript to the oocyte of developing follicles (Aaltonen et al. 1999). BMP15 is a growth/differentiation factor, a member of the transforming growth

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factor-β (TGF-β) superfamily (Chang et al. 2002). Members of this superfamily control many aspects of development by binding and activating two types of transmembrane serine/threonine kinase receptors (Chang et al. 2002). Functional studies have shown that BMP15 stimulates GC growth and promotes the progression of folliculogenesis from the primary stage to the FSH-dependent stage (Otsuka et al. 2000; Chang et al. 2002; Shimasaki et al. 2004).

An Italian family with two female siblings affected with hypergonadotropic ovarian failure (fig. 1*A*) came to our attention recently. At 23 years old, the proband (VB) presented with primary amenorrhea and modest hirsutism (Ferriman-Gallwey score = 12 [Ferriman and Gallwey 1961]; normal values are <8). She was born at term after an uneventful pregnancy. Her physical and intellectual development were fairly normal until puberty. Owing to the lack of spontaneous menarche, the diagnosis of pubertal delay was given when she was 15 years old, but no treatment was initiated. At age 17 years, she underwent appendectomy, and laparoscopic investigation allowed the visualization of streak ovaries with a small terminal crest and hypogenesia of the uterus. She was put on estroprogestin therapy soon after surgery. Family history revealed that her younger sister (SB) was also affected with a similar menstrual defect, reporting a single episode of spotting at 13 years. At the time of this clinical evaluation, SB was 18 years old and had been receiving estroprogestins for 18 mo. Both patients had hypoplasic gonads at ultrasound (ovarian diameters <18 mm, with a homogeneous structure and without visible follicles) and a 46,XX karyotype. Their BMIs were 24 and 22 kg/m^2, respectively, and all other endocrine functions were normal. Anti-ovary autoantibodies were negative on two occasions and there was no evidence of other specific autoimmune disease. Fertility and gonadal function were unaffected in both parents. The parents of VB and SB were not consanguineous, and clinical history of the family was negative for reproductive, endocrine, or mental disorders in the two previous generations.

We decided to screen candidate genes for OD in this family. An institutional ethical committee approved the study protocol, and informed consent and blood samples for genetic investigations were obtained from all family members.

The phenotype of these patients was similar to that observed in patients with complete resistance to FSH action (Aittomaki et al. 1995). Accordingly, we had excluded mutations in the FSH-receptor coding sequence in the proband (see methods in appendix A [online only]). Because of evidence in animal models, investigations were focused on genes encoding for oocyte-specific growth/differentiation factors of the TGF-β super-

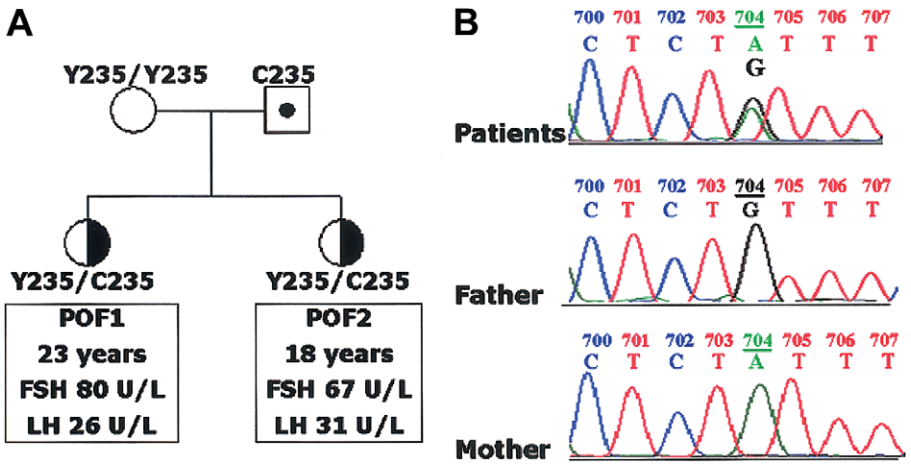

Figure 1 Pedigree and sequence analysis. *A*, Pedigree of the family. The elder sister (VB) and her younger sister (SB) were affected with idiopathic hypergonadotropic ovarian failure and presented with primary amenorrhea. After a 3-mo estrogen withdrawal, estradiol was low (<0.1 nmol/L) and gonadotropin values rose into the postmenopausal range, as shown (premenopausal female values: FSH = 1.0–8.0 U/L; LH = 0.5–9.0 U/L). The mother had regular menses and was postmenopausal at the time of the study (physiological menopause at 49 years), and the father had normal fertility and gonadal function (FSH = 5.3 U/L; LH = 2.5 U/L; testosterone = 15.6 nmol/L). *B*, BMP15 sequence analyses in VB, SB, and the parents. In the proband (VB), automated sequencing revealed a heterozygous A→G transition at base pair 704 of the *BMP15* gene located at Xp11.2. The same heterozygous transition was seen in the younger sister, whereas the father was a hemizygous carrier of the same 704A→G transition and the mother was normal. The grandmother, two uncles, and two aunts of the paternal family had normal fertility (five, one, one, four, and three births, respectively). One of these aunts had a normal BMP15 sequence; therefore, the mutation should have arisen de novo in the hemizygous father. This transition generates a missense substitution (Y235C) located in the propeptide region of the BMP15 protein.

family (Chang et al. 2002; Shimasaki et al. 2004). A Japanese group (Takebayashi et al. 2000) reported the absence of *BMP15* and *GDF9* mutations in women with postpubertal onset of hypergonadotropic ovarian failure and secondary amenorrhoea or with polycystic ovaries. In the two sisters, the *GDF9* coding sequence (GenBank accession number NM_005260) was completely normal, but we found a heterozygous A→G transition at base pair 704 of the *BMP15* gene (GenBank accession number AF082349) in the constitutive DNA of VB (fig. 1*B*; see methods in appendix A [online only]). The same heterozygous transition was seen in SB, whereas the father was a hemizygous carrier of the same transition, and the wild-type BMP15 coding sequence was seen in the mother. This transition does not represent a polymorphic variant diffuse in the white population, as it was absent in a series of 210 alleles from 120 ethnically matched controls (90 women and 30 men). Thus, this heterozygous transition of the *BMP15* gene leads to a Y235C mutation associated with prepubertal onset of hypergonadotropic ovarian failure characterized by primary amenorrhea and ovarian hypoplasia. These manifestations reflect the phenotype of sheep with homozygous mutations, rather than that of heterozygous ewes (Galloway et al. 2000; Hanrahan et al. 2004). The possibility that the affected sisters inherited additional alterations in other genes involved in ovarian function may explain such discrepancy. Nevertheless, mutations in *GDF9* and *FSHR* genes were not detected, possibly suggesting that the different modes of inheritance in humans and sheep may be a result of species diversity or the peculiar nature and location of the human mutation.

The missense mutation lies in the second exon, in a highly conserved part of the *BMP15* gene encoding the propeptide region (fig. 2). The pro region plays important biological roles, with a significant impact on secretion and action of TGF-β superfamily members (Chang et al. 2002). All TGF-βs are, indeed, synthesized as prepropeptide precursors (comprising the signal peptide, pro region, and bioactive mature domain) (Chang et al. 2002). The processing of TGF-β precursors is a critical step that modulates the availability of bioactive peptides at the target tissue level. Accordingly, TGF-βs are secreted partially as latent forms, and the pro region is cleaved by specific proteases in the intracellular or extracellular compartments as an important component of the posttranslational processing that leads to the release of the bioactive mature peptides (Gentry et al. 1988; Schultz-Cherry et al. 1994; Saharinen et al. 1999; Chang et al. 2002; Liao et al. 2003). Moreover, the pro region is involved in dimerization, and an essential role in this process was assigned to three Cys residues in the pro region of TGF-β1 (Brunner et al. 1989). The wild-type (WT) BMP15 lacks these characteristic cysteines. On the basis of all this data, the replacement of the highly conserved Tyr235 with a Cys was predicted to cause relevant modifications in the conformation of the BMP15 precur-

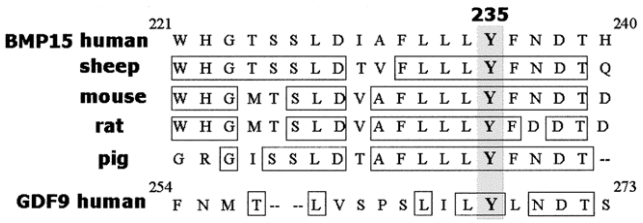

Figure 2 Alignment of the amino acid sequence of part of the BMP15 propeptide region from several mammalian species with its human homologue GDF9. The tyrosine affected by the mutation and the surrounding residues are highly conserved.

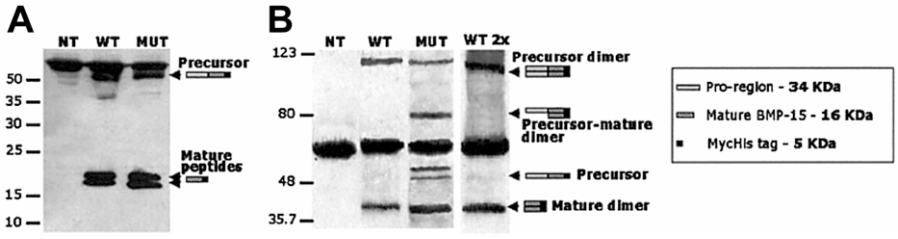

Figure 3 Western immunoblotting was performed under reducing and nonreducing conditions (with or without β-mercaptoetanol [β-ME]) by use of anti-His-HRP antibody (Roche) or anti-Myc antibody (Invitrogen), as appropriate. Immunofluorescent signals were detected with the use of the ECL system (Amersham). Western blots performed with anti-Myc antibody are shown. *A*, Analysis performed in the presence of β-ME. Bands corresponding to mature (duplet at ~21–23 kDa) and precursor (at ~50–55 kDa) BMP15-Myc-His chimeras were seen in the WT and mutant (MUT) lanes but were not seen in the control media from nontransfected (NT) cells. *B*, Analysis performed under nonreducing conditions allowing the visualization of dimeric products. Bands corresponding to mature (~45 kDa) and precursor (>110 kDa) dimers were seen in both WT and MUT lanes. Additional bands corresponding to precursor monomers (~50–55 kDa) or to stable precursor-mature dimers (~80 kDa) were detected in the mutant Y235C-BMP15 but not in WT lanes, even when double the amount of protein was tested (WT 2x). The band at 65–70 kDa in both panels corresponds to a nonspecific cross-reacting protein present in all media.

sor, possibly leading to altered processing and impaired activation of latent forms or to abnormal dimerization.

For functional studies, stable clones of human embryonic kidney 293T (HEK293T) cells expressing WT BMP15 or Y235C mutant BMP15 were obtained (see methods in appendix A [online only]). Western blotting qualitatively investigated immunoprecipitated BMP15 forms secreted by HEK293T cells. Under reducing conditions (fig. 3*A*), bands corresponding to mature and precursor BMP15-Myc-His chimeras were seen in the WT and mutant lanes, indicating that significant amounts of processed or unprocessed fusion proteins were secreted into the culture media in both cases. Under nonreducing conditions (fig. 3*B*), bands corresponding to mature and precursor dimers were seen in the WT and mutant lanes, but additional bands corresponding to precursor monomers or to stable precursor-mature dimers were visible only in the mutant lane, even when a double amount of WT protein was tested. These features are consistent with an altered processing of the Y235C mutant. The introduction of the Cys into the BMP15 pro region may confer to the mutant precursor the ability to form covalent heterodimers with mature peptides through the formation of abnormal disulfide bonds. The biological impact of the Y235C mutation is demonstrated with GC growth assays (Tapanainen et al. 1987) (appendix A [online only]). Human GCs were obtained by centrifugation of follicular fluids after the oocyte pick-up procedure in women undergoing in vitro fertilization. The women gave their informed consent. Cell count showed that GCs proliferated in the absence of recombinant BMP15 constructs (control cell count = 143% of basal). The proliferation rate of GCs was stimulated by the addition of WT (+79% or +113% of control values of 0.1 or 0.2 μg/ml, respectively), whereas no significant variation was seen in the presence of both doses of mutant (fig. 4). Accordingly, WT-BMP15 significantly stimulated ^{3}H-thymidine incorporation in GCs, with a dose-response effect between 0.1 and 0.5 μg/ml (fig. 5*A*). In contrast, GC growth was not stimulated in the presence of increasing Y235C-BMP15 doses or when GCs were incubated with equal amounts (1:1) of WT and mutant proteins. A significant increase of ^{3}H-thymidine incorporation in GCs was restored in the presence of WT-BMP15 concentrations 5-fold higher than those of Y235C-BMP15 (5:1). Despite heterogeneity among diverse GC preparations with variable basal growth rates, reciprocal differences between different BMP15 preparations were conserved in eight distinct experiments (fig. 5*B*).

In summary, these experiments showed that WT-BMP15 stimulates human GC growth in a dose-dependent manner and that Y235C-BMP15 products lost stimulatory activity. When equal amounts of WT and mutant BMP15 preparations were coincubated with

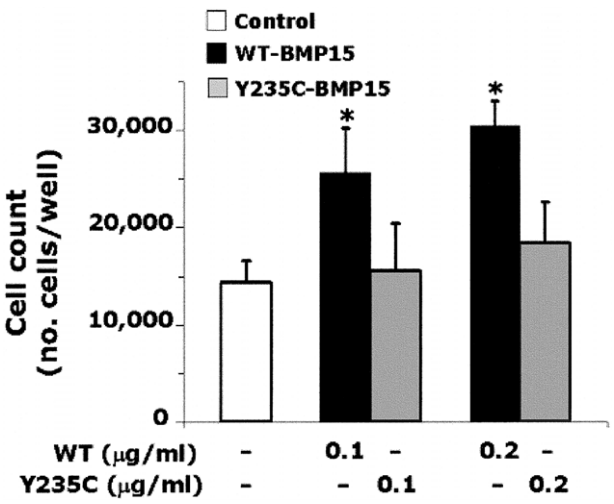

Figure 4 Granulosa cell-growth assay evaluated by direct cell count (see methods in appendix A [online only]). Results (mean ± SD) of a representative assay using different doses of BMP15-Myc-His fusion proteins. Tagged proteins were purified on nickel columns, and doses of purified proteins (0.1 or 0.2 μg/ml) were tested in triplicate wells. An asterisk (*) indicates $P < .05$ versus control and mutant preparations (two-tailed, unpaired Student's t test).

GCs, the abolition of WT stimulatory action was seen. Therefore, Y235C-BMP15 appears to generate a dominant negative effect on WT-BMP15 action. The formation of abnormal dimers producing a potent dominant negative effect by preventing the secretion of bioactive proteins was reported elsewhere in the case of the C400Y mutation of a BMP-like protein, the cartilage-derived morphogenetic protein-1 in an autosomal dominant form of chondrodysplasia (Thomas et al. 1997). Similarly, BMP15 mutations in sheep (Galloway et al. 2000) were reported to impair intracellular processing and the secretion of bioactive peptides (Liao et al. 2003). The dominant negative effect exerted by Y235C-BMP15 may be explained by means of two different molecular mechanisms: (1) impaired pro-protein processing and reduced production of bioactive peptides and (2) secretion of unprocessed monomer or dimeric mutant products with an antagonistic effect on GCs at the target level. The relevance of the latter mechanism was confirmed by GC proliferation assay through the abrogation of WT-BMP15 growth effect when WT protein was coincubated with equal amounts of mutant. Though specific BMP15 receptors are still not defined, binding to two distinct receptor types on GC membranes is expected for the generation of BMP15 signal (Chang et al. 2002; Moore et al. 2003). It is conceivable that Y235C-BMP15 unprocessed products (either monomer or abnormal dimer) may interact with one of the two receptor isoforms required for signal transduction in GCs, thus limiting receptor availability for WT-BMP15

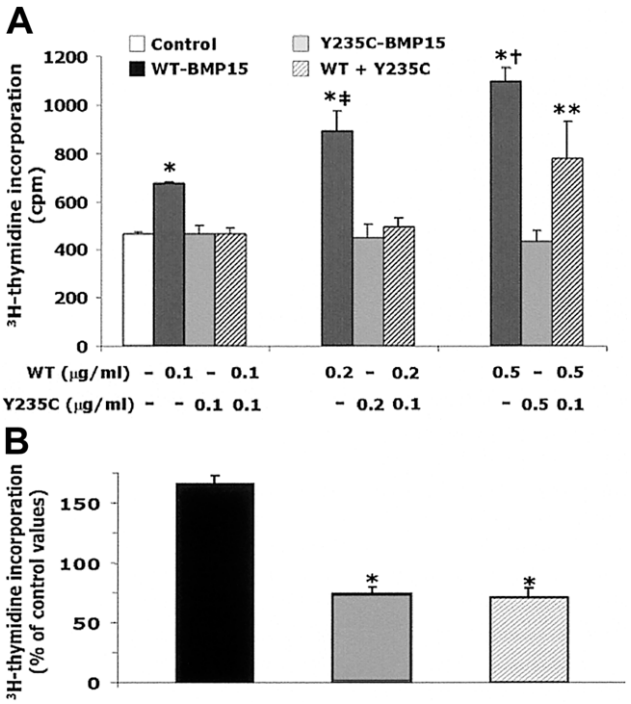

Figure 5 Granulosa cell-growth assay evaluated by ^{3}H-thymidine incorporation (see methods in appendix A [online only]). *A*, Results (mean ± SD) of a representative assay using different doses of BMP15-Myc-His fusion proteins. Tagged proteins were obtained after affinity chromatography on nickel columns. Mutant BMP15 and WT-BMP15 were tested either separately or in combination (1:1 or 1:5 mixtures) in triplicate wells. An asterisk (*) indicates $P < .01$ versus control and mutant alone; a double dagger (‡) indicates $P < .02$ versus WT 0.1 μg/ml; a dagger (†) indicates $P < .03$ versus WT 0.2 μg/ml; and two asterisks (**) indicate $P < .03$ versus control and mutant alone (two-tailed, unpaired Student's *t* test). *B*, Effects of recombinant human BMP15 tagged proteins (0.2 μg/ml) on GC growth, tested in eight experiments with the use of different GC preparations from eight women undergoing ovarian hyperstimulation. Owing to variable growth rates among these different GC preparations, results (mean ± SE; $n = 8$) obtained with WT, mutant, or 1:1 mixtures are expressed as the percentage of ^{3}H-thymidine incorporation in control wells. In WT-BMP15 wells, growth rate was 165% ± 6% (mean ± SE) of control wells ($P < .001$). In contrast, growth rates were reduced significantly, by >2-fold, when mutant BMP15 was tested separately or in the presence of equal amounts (1:1) of WT (74% ± 4% or 69% ± 6% of controls, respectively). An asterisk (*) indicates $P < .001$ versus WT-BMP15 (two-tailed, unpaired Student's *t* test).

action and creating a functional antagonism. Consistently, the antagonism exerted by mutant products can be overcome partially by the addition of 5-fold higher WT-BMP15 concentrations. A similar mechanism was described to modulate the activity of another member of the TGF-β superfamily, that is, the antagonism on activin function generated by inhibin binding to betaglycan (Lewis et al. 2000). Although evidence in animal models (Galloway et al. 2000; Hanrahan et al. 2004) suggests that additional genetic alterations may be required to generate the clinical phenotype of our patients, the present experimental findings indicate that Y235C-BMP15 could antagonize the activity of WT protein. The biological relevance in vivo of the partial processing defect may also develop from the peculiar type of BMP15 paracrine action in the restricted follicle compartment.

In conclusion, the diminished GC proliferation observed in vitro in the presence of Y235C-BMP15 can explain the in vivo phenotype of primary amenorrhoea and hypoplasic ovaries in the two 46,XX sisters with the *BMP15* mutation. The hemizygous father was apparently unaffected. Extragonadal tissues known to express BMP15 include the pituitary (Otsuka and Shimasaki 2002), but normal gonadotropin secretion in the father argues against an essential role for BMP15 at this level. Our data indicate that mutations in *BMP15* gene can be associated with hypergonadotropic ovarian failure in humans and that BMP15 is one of the factors whose function is relevant for OD in Turner syndrome. Owing to the essential role, limited to the female sex, of the growth/differentiation pathway affected by *BMP15* mutation (Dube et al. 1998; Aaltonen et al. 1999; Galloway et al. 2000; Otsuka et al. 2000; Yan et al. 2001; Chang et al. 2002; Hanrahan et al. 2004; Shimasaki et al. 2004), this condition represents an exceptional example of X-linked human disease exclusively affecting heterozygous females who inherited the genetic alteration from their unaffected fathers.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for human BMP15 genomic sequence [accession number AF082349] and human GDF9 mRNA sequence [accession number NM_005260])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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