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ORIGINAL INVESTIGATION

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Missense mutations in the *BMP15* gene are associated with ovarian failure

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Abstract Premature ovarian failure (POF) is an unexplained amenorrhoea (>6 months) with raised levels of gonadotropins (FSH > 40 U/L) occurring before the age of 40 years. Recent studies have elucidated the role of oocyte derived growth factors (BMP15 and GDF9) in maintenance of folliculogenesis, granulosa cell (GC) proliferation and overall fertility. Our recently published work showed presence of two rare missense variants in the *GDF9* gene associated with ovarian failure (Dixit et al. 2005, Menopause 12:749–754). The present case-control study has been structured to establish the role of *BMP15* germline status associated with ovarian failure. Sequence analysis of the coding region of the *BMP15* gene was carried out in a cohort of women with POF ($n=133$), primary amenorrhoea ($n=60$), and secondary amenorrhoea ($n=9$) compared with control females ($n=197$). This study revealed a total of 18 germline variants in the coding region of *BMP15* gene, including 16 novel variants. These novel variants include one intronic variant, one 3' flanking variant, one silent variant, and 13 missense variants. Eleven missense variants were present only in cases with complete absence in the control females. The remaining two missense variants viz. c.308A > G (p.Asn103Ser) and c.788_789insTCT (p.Leu263_Arg264insLeu) were present both in the cases and in the controls. The c.788_789insTCT variant was significantly higher in primary amenorrhoea cases than in the controls (Fisher's exact test, $P=0.034$). Three frequent variants c.-9C > G, c.308A > G, and c.852C > T were chosen for haplotyping. The haplotype G-G-C was found to be significantly associated with ovarian failure ($P=0.0075$). In a nutshell, the *BMP15* gene is highly associated with etiology of ovarian failure.

Keywords Ovarian failure · Mutation · *BMP15*

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Introduction

Premature ovarian failure (POF, MIM#311360) is an unexplained amenorrhoea before the age of 40 years with raised levels of gonadotropin (FSH > 40IU/L) and low levels of estrogen. The Indian population is the world's second largest population and prevalent consanguinity makes it ideal for population studies. Cases of ovarian failure recruited in the Indian clinics are mainly reported as sporadic cases, while a few are familial. It occurs in ~1% of women. Though extensive studies have been carried out at the chromosomal as well as the gene level to explain its etiology, they are still not well defined. Many reports have shown abnormalities in various regions of the X-chromosome in association with ovarian failure (Schlessinger et al. 2003). Candidate genes of the hypothalamus-pituitary-ovarian axis have been studied extensively in various worldwide populations. Missense variant Ala189Val in the extracellular domain of *FSHR* gene was shown to be strongly associated with ovarian failure in the Finnish population (Aittomaki et al. 1995). This variant of the *FSHR* gene is rare in other world populations. Truncated mutations of the *FOXL2* gene were also shown in familial BPES cases where females inherit ovarian failure (Crisponi et al. 2001). Genetic studies of the *INHα* gene revealed a very significant missense mutation c.769G > A (p.Ala253Thr) in the mature peptide region, which was present in ~10% cases of ovarian failure in various populations including the Indian population (Dixit et al. 2004; Marozzi et al. 2002; Shelling et al. 2000). These studies strongly recommend the implication of this missense mutation of the *INHα* gene as a genetic marker for ovarian failure.

The significance of the *INHα* gene as a marker thus urges germline studies of other key molecules networked around inhibins. Inhibins are the most important factors involved in downregulation of FSH levels by a negative feedback mechanism. The production of inhibins by granulosa cells (GCs) in the ovarian follicle is positively regulated by two oocyte derived growth factors, namely, Growth Differentiation Factor 9 (GDF9) and Bone Morphogenetic Protein 15 (BMP15) (Roh et al. 2003; Kaivo-Oja et al. 2003; McNatty et al. 2005a). Noteworthy, the *Gdf9* null mouse shows increased inhibin alpha levels in GCs at the one layer follicle stage which may be due to the stage-dependent role of GDF9 in inhibin regulation (Elvin et al. 1999). Both the growth factors play a pivotal role in the cellular communications of oocytes with surrounding somatic cells. Both the factors are imperative for the maintenance of folliculogenesis, GC proliferation, and overall fertility (Moore and Shimasaki 2005). Thus, germline variation in any of these two growth factors would probably lead to ovarian failure. We have recently reported association of two rare missense variants in the *GDF9* gene associated with ovarian failure (Dixit et al. 2005). The present study is mainly focused on the germline status of the *BMP15* gene in association with ovarian failure. The *BMP15* gene is located in Xp11.2, in the POF critical region, and thus represents an important X-linked candidate gene associated with ovarian failure (Dube et al. 1998).

The BMP15 (MIM300247) protein is a member of the TGFβ superfamily and is exclusively expressed in the oocytes (Dube et al. 1998). This growth factor is a potent stimulator of GC proliferation, largely involved in the progression of folliculogenesis from the primary stage till the FSH-dependent stage (Otsuka et al. 2000). Progression of BMP15 preproprotein processing, dimer formation, and signaling process are akin to other TGFβ family members. The initially synthesized BMP15 preproprotein is processed by cleavage of the signal peptide, which results in the secretion of BMP15 proprotein into the extracellular matrix. This proprotein non-covalently binds with either identical proprotein or GDF9 proprotein, resulting in homodimer or heterodimer formation. The proregions of these dimers get cleaved by a specific furin like endoprotease, resulting in the formation of mature dimeric peptides. These dimers bind to specific receptors located on the GCs of follicles and activate Smad1/5/8 signaling pathway. The BMP15 ligand binds and activates its Type II receptor Bone Morphogenetic Protein Receptor II (BMPRII), which later interacts with the Type-I receptor ALK6. The activated Type-I receptor subsequently initiates intracellular Smad signaling (Moore et al. 2003).

Extensive mutational studies of the *BMP15* and *GDF9* genes have been carried out in sheep as a breeding marker for its prolificacy. The earliest report of two naturally occurring *BMP15* gene mutations, p.Glu291X (C > T transition, 23rd residue of the mature peptide), and p.Val299Asp (A > T transversion, 92nd residue of the mature peptide), in the Inverdale and Hanna sheep, respectively depicted its role in ovarian physiology and fertility (Galloway et al. 2000). Heterozygous mutations in the *BMP15* gene resulted in increased ovulation rates, whereas homozygous mutations resulted in primary ovarian failure with increased levels of gonadotropins due to blockage of follicle development. So far, five missense variants in the *BMP15* gene and one missense variant in the *GDF9* gene have been reported in sheep (McNatty et al. 2005b). The earliest mutational study of the human *BMP15* and *GDF9* genes in ovarian failure cases did not reveal any missense mutation except the c.852C > T silent variant in the *BMP15* gene in the Japanese population (Takebayashi et al. 2000). The first missense variant c.704A > G (p.Tyr235Cys) in the *BMP15* gene was shown in a sister pair with primary amenorrhoea. Functional analysis of this missense variant was performed using in vitro GC proliferation assays. Wild type BMP15 protein exhibits a dose-dependent stimulatory effect on the GC growth, whereas p.Tyr235Cys mutant protein exerts complete loss of stimulatory effect in a dominant negative manner (Di Pasquale et al. 2004). The present study revealed an astonishing spectrum of mutations with an exclusive presence of 11 missense variants in the *BMP15* gene in cases of ovarian failure, whereas completely absent in all the healthy control females. The present study provides indispensable information about the naturally occurring variants of the *BMP15* gene and would further stimulate research for these variants both in vitro and in model organisms. These results would also provide a better understanding about etiology of ovarian failure among human geneticists and clinicians.

Materials and methods

Patient and control recruitment

A total of 202 patients with ovarian failure were recruited, which included non-familial POF cases ($n = 121$), familial POF cases ($n = 12$), non-familial primary amenorrhoea cases ($n = 54$), familial primary amenorrhoea cases ($n = 6$), and secondary amenorrhoea cases ($n = 9$). These patients were recruited at the Infertility Institute and Research Centre (IIRC), Hyderabad and the Institute of Reproductive Medicine (IRM), Kolkata. Patients recruited as sporadic without any familial history of ovarian failure were considered as non-familial. Patients with family history of ovarian failure in the same generation or parental generation were considered as familial cases. The diagnostic criteria for POF following the definition include at least 6 months of amenorrhoea before the age of 40 years, with elevated serum FSH levels (> 40 IU/L). Primary amenorrhoea is defined as a condition with complete absence of menses or only induced menses. Secondary amenorrhoea is defined as a cessation of menses with previous history of menses before the age of 40. All the patients were assessed clinically, with complete medical

and gynecological history including history of menses, age at menopause, serum FSH levels (three times at 1 month intervals), LH levels, and TSH levels with no history of any autoimmune disease. All ethnic information was collected including their family, religion, caste, race, and origin during the patient's recruitment. Respective consent forms from these patients were collected by the concerned clinic. Karyotyping with a high-resolution GTG banding was carried out for all the patients and controls for chromosomal anomalies. Patients and controls with chromosomal abnormalities were excluded from the study. Normal healthy females with regular menstrual history, normal FSH levels, and successful pregnancies were recruited as controls ($n = 197$). Recruitment of the controls was entirely population-based to support the study. The Institutional Review Board of the Centre for Cellular and Molecular Biology (CCMB), Hyderabad approved the study.

DNA extraction and karyotyping

A 5 ml aliquot of peripheral blood was collected in EDTA vacutainers for genomic DNA isolation and another 5 ml of peripheral blood was collected in heparin vacutainers for chromosomal analysis. DNA was extracted using the Nucleon BACC2 DNA extraction kit (Amersham Pharmacia Biotech., NJ, USA) according to the manufacturer's protocol. Chromosomal analysis was performed on phytohaemagglutinin (PHA)-stimulated peripheral lymphocyte cultures using standard conventional cytogenetic methods.

PCR

The *BMP15* gene comprises of two exons of 328 and 851 bp length, respectively. Primers were synthesized and PCR conditions were followed as described by Takebayashi et al. (2000). The first exon was amplified using BMP15F4 (5'AGTGACGTCCCTTGGGCT TG3') and BMP15R4 (5'CAAAGCCTGACAGTAAA CCC3') primers. The first half of the second exon was amplified using BMP15F5 (5'GGGCTGATTATAGC TATCAGTC3') and BMP15R5 (5'GGAAGAGGCAG TAACCTCAGCTG3') primers, whereas the second half was amplified using BMP15F6 (5'GGGAATCTCTTCT CCGGAGAACC3') and BMP15R6 (5'CTAGCTCAC AAGTGGGGGAAGAGAC3') primers. Presence of all sequence variants was confirmed by performing three independent PCR reactions and subsequent DNA sequencing.

DNA sequencing and analysis

PCR products were obtained by amplifying the coding region of the *BMP15* gene using the above mentioned primers. Sequencing was performed using the big dye terminator sequencing protocol, supported by Applied Biosystems using an ABI prism 3730×l DNA analyzer. The sequence data obtained was analyzed using sequence analysis and autoassembler software in MacOS. The reference BMP15 cDNA sequence ID NM_005448.1 was downloaded from the NCBI. The Human Genome Variation Society (HGVS) guidelines were followed for all variant nomenclature and genotype representation throughout the text and the tables.

Statistical analysis

STAT-SAK 2.0 program was used for performing Fisher's exact test, odds ratio, confidence intervals, and chi-square test. The SNPAlyzer^{TM} software was used for case-control haplotype analysis and Hardy-Weinberg disequilibrium. $P < 0.05$ was considered as significant.

Results

Sequencing data collection and analysis was successfully performed for the *BMP15* gene (coding region) in all the cases and controls, which included non-familial POF cases ($n = 121$), familial POF cases ($n = 12$), non-familial primary amenorrhoea cases ($n = 54$), familial primary amenorrhoea cases ($n = 6$), secondary amenorrhoea cases ($n = 9$), and control females ($n = 197$). The patients as well as the control populations were in Hardy-Weinberg equilibrium for all the genetic variations. The mean age of attaining amenorrhoea in all POF cases was 26 years (range 14–40 years), the mean FSH level was 59.3 IU/L, and the mean LH level was 33.7 IU/L. The range of FSH and LH levels in POF cases was from 40–150 to 20–91 IU/L, respectively. Median of FSH and LH levels in POF cases was 75 and 39 IU/L, respectively. The controls had a mean age of 37 years (range 30–45 years) and normal FSH levels (3–20 IU/L).

This study revealed a total of 18 sequence variants in the coding region of the *BMP15* gene containing 16 novel variants and two earlier reported polymorphisms viz. c.-9C > G (rs3810682) and c.852C > T (rs17003221). All the sequence variants were confirmed by repetition of three independent PCR and sequencing reactions, including sequencing in reverse direction. Details of all the variants are given in Table 1. The 16 novel variants include one intronic insertion variant c.328 + 44_328 + 45insG, one 3' flanking duplication variant c.*40dupG, one silent variant c.381A > G, and 13 missense variants namely c.181C > T, c.182G > A, c.226C > T, c.227G > A, c.308A > G, c.538G > A, c.538G > T, [c.538G > T(+)c.539C > T], c.617G > A, c.631C > T, c.661T > C, c.727A > G, and c.788_789insTCT. Out of these 13 missense variants, 11 variants viz. c.181C > T, c.182G > A, c.226C > T, c.227G > A, c.538G > A, c.538G > T, [c.538G > T(+)c.539C > T], c.617G > A, c.631C > T, c.661T > C, and c.727A > G were present only in ovarian failure cases. None of these

Table 1 Summary of mutation analysis of *BMP15* gene

dbSNP refer ID	Location	Sequence variation	Theoretically deduced amino acid variation	Protein Domain	POF	PA	SA	CNT
Rs3810682	Promoter	[c.-9C > G] + [c.?]	Promoter region		44/133	17/60	2/9	49/197
Novel	Exon 1	[c.181C > T] + [c. =]	p.Arg61Trp	Propeptide	2/133	0/60	0/9	0/197
Novel	Exon 1	[c.182G > A] + [c. =]	p.Arg61Glu	Propeptide	1/133	0/60	0/9	0/197
Novel	Exon 1	[c.226C > T] + [c. =]	p.Arg76Cys	Propeptide	3/133	2/60	0/9	0/197
Novel	Exon 1	[c.227G > A] + [c. =]	p.Arg76His	Propeptide	1/133	0/60	0/9	0/197
Novel	Exon 1	[c.308A > G] + [c.?]	p.Asn103Ser	Propeptide	13/133	6/60	0/9	10/197
Novel	Intron 1	[c.328 + 44_328 + 45insG] + [c.328 + 44_328 + 45insG]	Intronic		0/133	0/60	0/9	1/197
Novel	Exon 2	[c.381A > G] + [c. =]	Silent	Propeptide	1/133	0/60	0/9	0/197
Novel	Exon 2	[c.538G > A] + [c. =]	p.Ala180Thr	Propeptide	1/133	2/60	0/9	0/197
Novel	Exon 2	[c.538G > T(+)c.539C > T]	p.Ala180Phe/Ser + Val	Propeptide	1/133	0/60	0/9	0/197
Novel	Exon 2	[c.588T > A] + [c. =]	p.Asn196Lys	Propeptide	1/133	0/60	0/9	0/197
Novel	Exon 2	[c.617G > A] + [c. =]	p.Arg206His	Propeptide	1/133	0/60	0/9	0/197
Novel	Exon 2	[c.631C > T] + [c.631C > T]	pGlu211X	Propeptide	1/133	0/60	0/9	0/197
Novel	Exon 2	[c.661T > C] + [c. =]	p.Trp221Arg	Propeptide	1/133	0/60	0/9	0/197
Novel	Exon 2	[c.727A > G] + [c. =]	p.Iso243Gly	Propeptide	1/133	0/60	0/9	0/197
Novel	Exon 2	[c.788_789insTCT] + [c.788_789insTCT]	p.Leu263_Arg264insLeu	Propeptide	4/133	5/60	0/9	4/197
rs17003221	Exon 2	[c.852C > T] + [c.?]	Silent	Mature peptide	9/133	6/60	0/9	20/197
Novel	3' Flanking region	[c.*40dupG] + [c.*40dupG]	3' Flanking region		1/133	0/60	0/9	0/197

POF: premature ovarian failure; *PA*: primary amenorrhoea; *SA*: secondary amenorrhoea; *CNT*: controls

variants were observed in the controls. Out of these 11, one missense variant c.631C > T was homozygous, while the remaining ten variants were heterozygous. These 11 variants were present in different patients discretely. Clinical details of the 19 individuals carrying these 11 variants are described in Table 2. Aligning the human BMP15 protein sequence with that of other mammals revealed that these 11 variants were not conserved. The remaining two missense variants, c.308A > G and c.788_789insTCT, were present both in the cases and the

Table 2 Clinical details of patients carrying missense mutations

Patient ID	Variant	Phenotype	Age (years)[a]	FSH (IU/L)	LH (IU/L)	TSH (mIU/L)	Additional information
11	p.Arg61Trp	POF	18	82	64	3	Pubic and auxiliary hair absent
26	p.Arg61Trp	POF	24	50	13	2.8	Prolactin 6, silent variant c.942C > T in *INHβ B* gene
123	p.Arg61Glu	POF	22	59	20	NA	Heterozygous c.769G > A variant *INHα* gene
81	p.Arg76Cys	POF	25	67	28	1.9	Streak ovaries
90	p.Arg76Cys	POF	29	82	43	2.6	Ovaries not visualized
107	p.Arg76Cys	PA	18	17	8	2.1	Mullerian agenesis, bilateral pelvic kidneys, deficient secondary sexual characters
148	p.Arg76Cys	POF	26	67	20	1.6	
162	p.Arg76Cys	PA	16	15	17	0.9	
15	p.Arg76His	POF	22	110	41	1.4	
78	c.381A > G	POF	23	59	21	3.8	
181	p.Ala180Thr	PA	20	9	6	0.7	
193	p.Ala180Thr	PA	14	11	8	NA	Short stature
213	p.Ala180Thr	POF	28	91	29	3.2	
211	p.Ala180Phe/Ser + Val	POF	19	105	40	2.0	
281	p.Asn196Lys	POF	22	88	28	1.8	Ovaries not visualized
105	p.Arg206His	POF	27	90	35	3.7	Streak ovaries
201	pGlu211X	POF	28	85	37	3.7	Facial paralysis, only withdrawl bleeding upon medication, 2 failed IVF, ovaries not visualized
236	p.Trp221Arg	POF	20	98	40	2.0	
66	p.Iso243Gly	POF	18	61	26	2.7	

POF: premature ovarian failure; *PA*: primary amenorrhoea
[a]Age at menopause

Table 3 Haplotype frequencies in patient groups compared with control females

Haplotype	Overall frequency	CasePOF	CasePA	CaseSA	Controls	Chi-squ	*P*-values
C-A-C	0.7769	0.7487	0.7717	0.8888	0.7893	3.11	0.3747
G-A-C	0.1339	0.1611	0.1116	0.1111	0.1319	2.12	0.5472
C-A-T	0.0441	0.0376	0.0348	0.0000	0.0533	2.13	0.5447
C-G-C	0.0256	0.0258	0.0268	0.0000	0.0254	0.48	0.9225
G-G-C	0.0185	0.0268	0.0315	0.0000	0.0000	11.95	0.0075[a]

POF: premature ovarian failure; *PA*: primary amenorrhoea; *SA*: secondary amenorrhoea
[a]Significant *P* value

controls. One missense variant c.788_789insTCT was significantly higher in the primary amenorrhoea cases than the controls (Fisher's exact test, $P = 0.034$). Haplotype analysis was performed for three frequent variants c.-9C > G, c.308A > G, c.852C > T using the SNPAlyzer software. Haplotype G-G-C was found to be significantly associated with ovarian failure ($P = 0.0075$) as shown in Table 3.

Discussion

Premature ovarian failure is largely portrayed as a heterogeneous genetic disorder but its etiology still remains elusive. Recent studies have elucidated the extensive role of two oocyte derived growth factors, GDF9 and BMP15, as the main driving force for the proliferation and progression of somatic follicle cells (Moore and Shimasaki 2005). Our recent report illustrated the association of two rare missense variants in the *GDF9* gene with etiology of ovarian failure (Dixit et al. 2005). The present study provides remarkable major evidence about naturally occurring missense variants of the *BMP15* gene associated with ovarian failure. The present study revealed a total of 18 variants including 16 novel variants and two earlier reported polymorphisms. These novel variants include one intronic insertion variant, one 3' flanking duplication variant, one silent variant, and 13 missense variants as detailed in the results and Table 1. Most importantly, 11 missense variants were exclusively associated with cases of ovarian failure. None of the controls had these variants.

The BMP15 protein is exclusively expressed in the oocytes starting from primary follicle stage till ovulation (Dube et al. 1998). This protein is considered as a major determinant of ovulation quota and dominant follicle selection in mammals (Moore et al. 2004). It inhibits major FSH actions by directly suppressing the mRNA expression of FSH receptor in GCs. The suppression of FSH receptor message results in the inhibition of FSH-induced mRNA expression like StAR, P450scc, 3β-HSD, LHR, and inhibins (Otsuka et al. 2001). The suppression of FSH responsive messages results in the inhibition of FSH-induced progesterone production but not of estradiol (Otsuka et al. 2000). These molecular events elucidate the role of BMP15 in early follicular growth while preventing premature luteinization via partially inhibiting FSH-induced responses (Moore et al. 2005). In contrast, FSH downregulates the BMP15 production in a dose-dependent manner via the Kit mediated pathway. Precocious downregulation of BMP15 has been reported to result in impaired oocyte growth in the presence of higher FSH levels (Thomas et al. 2005). Notably, the elevated FSH level is a principal clinical feature of POF. Effects similar to those observed at higher FSH level have also been revealed by repeated ovarian stimulation of mice with FSH and LH in vivo, which results in the reduced meiotic competence of oocytes (Combelles et al. 2003). Therefore, any loss in the activity of BMP15 protein would result in defective follicle proliferation, increased FSH sensitivity, and precocious luteinization in a dose-dependent manner (Moore and Shimasaki 2005).

Identification of missense or truncation mutations in the *BMP15* gene in sheep has established it as an important candidate gene for female fertility (Galloway et al. 2000; McNatty et al. 2005b). The *BMP15* heterozygous missense mutations in sheep resulted in higher ovulation rates and increased litter size, whereas the *BMP15* homozygous missense mutations led to complete infertility because of blockage of follicles at the primary follicle stage (McNatty et al. 2005b). Probably, the higher ovulation rates are due to the early maturation of developing follicles as their increased responsiveness to the FSH and earlier acquisition of LH receptors by the GCs. Sheep harboring the *BMP15* homozygous missense mutations show hypoplastic/streak ovaries consist of follicles with antrum and abnormal oocyte surrounded by abnormally dispersed layers of cells and oocyte with thickened zona pellucida (McNatty et al. 2005b). In vitro incorporation of the sheep missense mutations in the human *BMP15* cDNA resulted in an impaired post-translational activity and defective secretion of its homo/heterodimers. Intriguingly, these effects were realized when mutant BMP15 was co-expressed with the GDF9, indicative of a synergistic role of their function (Liao et al. 2004). Missense mutations in the *GDF9* or *BMP15* gene do not inhibit the homo/heterodimer formation. Rather, proprotein heterodimer of the BMP15 and GDF9 is significantly less susceptible for proteolytic cleavage than the individual homodimers of BMP15/GDF9. Hence the proprotein heterodimer of BMP15/GDF9 becomes unstable and undergoes rapid degradation. Another possibility

suggested is that the germline missense variants in these genes can lead to misfolding of the protein during protein synthesis itself. It is also striking that the *BMP15* missense mutations in sheep can affect the GDF9 biosynthesis in a dominant negative fashion (Liao et al. 2004).

A more severe phenotype of the *BMP15* missense variant was observed in the human than in sheep. The human heterozygous missense variant p.Tyr235Cys represented similar phenotype as observed due to the homozygous missense mutations of sheep (Di Pasquale et al. 2004). The functional study of this first human missense variant in the *BMP15* gene illustrated the complete functional loss of mutant BMP15 protein using GC proliferation assays in vitro. Results also corroborated abnormal processing of the mutant protein, which antagonizes the activity of wild type protein in a dominant negative fashion (Di Pasquale et al. 2004). This missense mutation results in the secretion of unprocessed monomers and abnormal dimeric products, which probably antagonize the normal protein functions via receptor interference (Di Pasquale et al. 2004, Moore and Shimasaki 2005). Functional analysis of a BMP15-like protein, cartilage derived morphogenetic protein-1, harboring p.Cys400Tyr variant also leads to abnormal dimers, producing potent dominant negative effects by preventing the secretion of bioactive protein in an autosomal dominant form of chondrodysplasia (Thomas et al. 1997).

Construction of the *Bmp15*$^{-/-}$ null mice and *Bmp15*$^{-/-}$*Gdf*$^{+/-}$ double mutant mice further provide a molecular insight into the *BMP15* gene function in fertility maintenance (Yan et al. 2001). The *Bmp15* knockout male mice are normal and fertile. In contrast to the severe phenotype seen in sheep and human, knockout female mice are subfertile with reduced levels of ovulation and fertilization rates. The ovarian and follicular development appears normal in the *Bmp15* knockout female mice. These female mice show disruption of cumulus cell-oocyte complexes. The female mice heterozygous for both *Bmp15* and *Gdf9* were fertile but with smaller and less frequent litters. The *Bmp15* knockout mice with heterozygous *Gdf9* gene showed normal follicular growth but ovulation and fertilization rates were dramatically impaired, resulting in infertility (Yan et al. 2001). The differences in the *Bmp15* mutant phenotype of mice versus sheep and human might be due to an increased bioactivity of BMP15 over GDF9 in sheep and human. Another reason for the differences in phenotype may be the monoovulatory nature of sheep and human, as against the polyovulatory nature of mice. It is also remarkable that mouse *Bmp15* shows significant sequence variation from those of sheep and human *BMP15* (Yan et al. 2001; McNatty et al. 2005b).

The present study reveals eleven missense variants in the proregion of *BMP15* gene exclusively associated with ovarian failure. Of these 11 missense variants, one variant c.631C > T was found to be a homozygous truncation mutation. This truncated variant would probably behave as a knockout of the human *BMP15* gene, which is the first report in literature. The patient carrying the c.631C > T homozygous variant creates a premature stop in proregion at 211th position in the place of Glutamic acid. This truncation mutation would result in the complete lack of mature BMP15 peptide. This patient presented severe hypoplastic ovaries with complete infertility. She never attained menarche and expressed high gonadotropin levels (FSH 85 IU/L, LH 37 IU/L). She had facial paralysis with skewed lower mandible, which may be indicative of a developmental role for the *BMP15* gene. The patient underwent two IVF but failed both the times. The remaining ten missense variants associated with ovarian failure were heterozygous and resulted in diverse amino acid alterations as depicted subsequently. Missense variants viz. c.181C > T substitutes arginine^{61} a strong basic amino acid to tryptophan^{61} a bulky stereotype aromatic amino acid, c.182G > A substitutes arginine^{61} to glutamic acid^{61} a strong acidic amino acid, c.226C > T substitutes arginine^{76} to cysteine^{76} a sulfur-containing amino acid, c.227G > A substitutes arginine^{76} to histidine^{76} which is frequently involved in oxidoreduction reaction, c.538G > A substitutes alanine^{180} an aliphatic amino acid to threonine^{180} a hydroxyl group containing amino acid, [c.538G > T(+)c.539C > T] substitutes alanine^{180} to phenylalanine^{180} an aromatic amino acid, c.588T > A substitutes asparagine^{196} to highly basic lysine^{196}, c.617G > A substitutes arginine^{206} to histidine^{206}, c.661T > C substitutes tryptophan^{221} to arginine^{221} and c.727A > G substitutes isoleucine^{243} to glycine^{243}. These above mentioned missense variants were non-conservative among mammals. Recently we have reported two rare missense variants, c.199A > C (p.Lys67Glu) and c.646G > A (p.Val216Mat) in the proregion of *GDF9* gene (Dixit et al. 2005). Similar to the *BMP15* gene missense variants reported here, both *GDF9* gene missense variants were also exclusively present in cases of ovarian failure, while these variants were completely absent in all the controls. The missense variant c.646G > A in the *GDF9* gene was found to be non-conservative among mammals. None of the patients had missense mutation in both *GDF9* and *BMP15* genes concurrently. Both missense mutations in the *GDF9* gene were discretely present in six POF cases, whereas 11 missense mutations in the *BMP15* gene were discretely present in fifteen POF cases and four primary amenorrhoea cases. Thus 21 out of 133 (15.8%) POF cases had missense mutation either in the *GDF9* or the *BMP15* gene accounting for significant association of both the genes with ovarian failure. None of the primary amenorrhoea cases had missense mutation in the *GDF9* gene, while four primary amenorrhoea cases out of 60 (6.6%) had missense mutation in the *BMP15* gene. These proregion missense mutations in the *BMP15* and *GDF9* genes may result in complete or partial functional loss of mutant protein due to erroneous processing, misfolding, defective secretion, loss of bioactivity, and reduced protein stability, either as reported by

Di Pasquale et al. (2004) or by an alternate mode. It was remarkable that the first human missense variation p.Tyr235Cys was heterozygous in both sisters and confined to the *BMP15* proregion similar to the variants identified in this present study. In the present study, only one missense variant c.226C > T (p.Arg76Cys) resulted in substitution by cysteine as seen in the p.Tyr235Cys variant. Interestingly, out of these eleven missense variants, the c.226C > T was most frequent with its presence in three POF cases ($n = 3/133$) and two primary amenorrhoea cases ($n = 2/60$). Five variants out of these 11 missense variants involved in the substitution of wild type arginine to other amino acids but its significance is not known. These missense variants are located across the *BMP15* proregion suggesting the probable importance of the proregion overall. Out of the total 18 variants, three variants had high frequency (more than 5%) of mutants (heterozygous and homozygous). These three variants viz. c.-9C > G, c.308A > G, c.852C > T were chosen for haplotyping to investigate the presence of any haplotype combination with cumulative effect associated with ovarian failure. The haplotype analysis revealed significant association of G-G-C haplotype with ovarian failure cases ($P = 0.0075$).

In conclusion, this present study provides a major evidence for naturally occurring variants in association with ovarian failure. It is also noticeable that so far only two variants have been reported in the *BMP15* gene in all the SNP databases. This study points to the immense importance of *BMP15* as a crucial candidate gene, which should also be studied in other populations. These variants will stimulate research among medical geneticists, protein crystallographers, and scientists working on rodent/mice models. Further population studies are required to establish the *BMP15* gene as a genetic marker for ovarian failure.

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References

Aittomaki K, Lucena JLD, Pakarinen P, Sistonen P, Tapanainen J, Gromoll J, Kaskikari R, Sankila EM, Lehvaslaiho H, Engel AR, Nieschlag E, Huhtaniemi I, De la Chapelle A (1995) Mutation in the follicle stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. Cell 82:959–968

Combelles CMH, Albertini DF (2003) Assessment of oocyte quality following repeated gonadotropin stimulation in the mouse. Biol Reprod 68:812–821

Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, Bisceglia L, Zelante L, Nagaraja R, Porcu S, Ristaldi MS, Marzella R, Rocchi M, Nicolino M, Lienhardt-Roussie A, Nivelon A, Verloes A, Schlessinger D, Gasparini P, Bonneau D, Cao A, Pilia G (2001) The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus/inversus syndrome. Nature Genet 27:159–166

Di Pasquale E, Beck-Peccoz P, Persani L (2004) Hypergonadotropic ovarian failure associated with an inherited mutation of Human Bone Morphogenetic protein-15 (BMP15) gene. AJHG 75:106–111

Dixit H, Deenadayal M, Singh L (2004) Mutational analysis of the mature peptide region of inhibin genes in Indian Women with ovarian failure. Hum Reprod 19:1760–1764

Dixit H, Rao KL, Padmalatha VV, Kanakavalli M, Deenadayal M, Gupta N, Chakraborty BN, Singh L (2005) Mutational screening of coding region of growth differentiation factor 9 gene in Indian women with ovarian failure. Menopause 12:749–754

Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ, Matzuk MM (1998) The Bone Morphogenetic Protein 15 Gene Is X-Linked and Expressed in Oocytes. Mol Endocrinol 12:1809–1817

Elvin JA, Clark AT, Wang P, Wolfman NM, Matzuk MM (1999) Paracrine actions of growth differentiation factor-9 in the mammalian ovary. Mol Endocrinol 13(6):1035–1048

Galloway SM, McNatty KP, Cambridge LM, Laitinen MPE, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH, Ritvos O (2000) Mutations in an oocyte-derived growth factor gene (*BMP15*) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nat Genet 25:279–283

Kaivo-Oja N, Bondestam J, Kamarainen M, Koskimies J, Vitt U, Cranfield M, Vuojolainen K, Kallio JP, Olkkonen VM, Hayashi M, Moustakas A, Groome NP, Dijke P, Hsueh AJW, Ritvos O (2003) Growth differentiation factor-9 induces smad2 activation and inhibin B production in cultured human granulosa-luteal cells. J Clin Endocrinol Metab 88:755–762

Liao WX, Moore RK, Shimasaki S (2004) Functional and molecular characterization of naturally occurring mutations in the Oocyte-secreted factors bone morphogenetic protein-15 and growth and differentiation factor-9. JBC 279:17391–17395

Marozzi A, Porta C, Vegetti W, Crosignani PG, Tibiletti MG, Dalpra I, Ginelli E (2002) Mutation analysis of the inhibin alpha gene in a cohort of Italian women affected by ovarian failure. Hum Reprod 17:1741–1745

McNatty KP, Juengel JL, Reader KL, Lun S, Myllymaa1 S, Lawrence SB, Western A, Meerasahib MF, Mottershead DG, Groome NP, Ritvos O, Laitinen MPE (2005a) Bone morphogenetic protein 15 and growth differentiation factor 9 co-operate to regulate granulosa cell function in ruminants. Reproduction 129:481–487

McNatty KP, Smith P, Moore LG, Reader K, Lun S, Hanrahan JP, Groome NP, laitinen M, Ritvos O, Juengel JN (2005b) Oocyte-expressed genes affecting ovulation rate. Mol Cell Endocrinol 234:57–66

Moore RK, Otsuka F, Shimasaki S (2003) Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. JBC 278:304–310

Moore RK, Erickson GF, Shimasaki S (2004) Are BMP-15 and GDF-9 primary determinant of ovulation quota in mammals? Trends Endocrinol Metab 15:356–361

Moore RK, Shimasaki S (2005) Molecular biology and physiological role of the oocyte factor, BMP-15. Mol Cell Endocrinol 234:67–73

Otsuka F, Yamamoto S, Erickson GF, Shimasaki S (2001) Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. JBC 276:11387–11392

Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF, Shimasaki S (2000) Bone morphogenetic protein-15 identification of target cells and biological functions. JBC 275:39523–39528

Roh JS, Bondestam J, Mazerbourg S, Kaivo-Oja N, Groome N, Ritvos O, Hsueh AJW (2003) Growth differentiation factor-9 stimulates inhibin production and activates smad2 in cultured rat granulosa Cells. Endocrinology 144:172–178

Schlessinger D, Herrera L, Crisponi L, Mumm S, Percesepe A, Pellegrini M, Pilia G, Forabosco A (2003) Genes and translocations involved in POF. Am J Med Genet 111(3):328–333

Shelling AN, Burton KA, Chand AL, van Ee CC, France JT, Farquhar CM, Milsom SR, Love DR, Gersak K, Aittomaki K, Winship IM (2000) Inhibin: a candidate gene for premature ovarian failure. Hum Reprod 15:2644–2649

Takebayashi K, Takakura K, Wang HQ, Kimura F, Kasahara K, Noda Y (2000) Mutation analysis of the growth differentiation factor-9 and -9B genes in patients with premature ovarian failure and polycystic ovary syndrome. Fertil Steril 74:976–979

Thomas FH, Ethier J, Shimasaki S, Vanderhyden VC (2005) Follicle stimulating hormone regulates oocyte growth by modulation of expression of oocyte and granulosa cell factors. Endocrinology 146:941–949

Thomas JT, Kilpatrick MW, Lin K, Erlacher L, Lembessis P, Costa T, Tsipouras P, Luyten FP (1997) Disruption of human limb morphogenesis by a dominant negative mutation in CDMP1. Nat Genet 117:58–64

Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ, Matzuk MM (2001) Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol Endocrinol 15:854–866