Identification of new variants of the BMP15 gene causing Premature Ovarian Failure in Lorestan families by whole-exome sequencing and In silico analysis

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ABSTRACT

Premature ovarian insufficiency (POI) is characterized by the halt of ovarian function prior to the age of 40, presenting with diminished sex hormone levels, elevated gonadotropin levels, and resulting in infertility and recurrent miscarriages in females. Genetic elements contribute significantly to the reproductive challenges associated with POI, impacting both infertility and miscarriage occurrences. A key player in folliculogenesis, BMP15, a TGFβ superfamily member, governs this pathway and any alterations in its gene sequence can disrupt normal follicle development. BMP15, expressed in oocytes, is pivotal for reproductive processes in certain species. When POI manifests, disruptions in BMP15 gene function result in infertility, escalating the likelihood of miscarriages due to compromised oocyte quality and diminished ovarian reserves.

Genomic DNA was extracted from the peripheral blood sample after a physical examination, ultrasound. Whole Genome Sequencing were performed. In silico prediction tools utilized were Phyer-2 web server, Polymorphism Phenotyping version 2 (PolyPhen-2), GalaxyWEB and ModRefiner, UCSF Chimera software, molecular docking using ClusPro 2.0.

Exome sequencing revealed a missense mutation c.226C>T:p.Arg76Cys in exon 1 of the BMP15 gene in Lorestan families. In silico prediction tools revealed that variation Arg 76 Cys changed the binding affinity and pattern of the BMP-15 and BMPR1B complex. Changes in the binding affinity and pattern of the complex protein may cause disruptions in BMP15 gene function and can lead to infertility.

The identification of BMP15 gene variants holds promise for healthcare practitioners to devise more targeted interventions, potentially ameliorating the impact of infertility and miscarriage among affected individuals. Hence, this investigation delves into exploring BMP15 gene variations within a cohort of infertile women from Lorestan province grappling with premature ovarian insufficiency.

Keywords: *Premature ovarian insufficiency (POI), BMP15, infertility, whole-exome sequencing*

1. *INTRODUCTION*

Premature Ovarian Insufficiency (POI), commonly referred to as premature ovarian failure (POF), is characterized by the cessation of ovarian function before the age of 40. Primary amenorrhea with delayed menarche, secondary amenorrhea, and oligomenorrhea lasting over 4 months are the differentiating factors. The diagnosis of POI involves the identification of high levels of follicle-stimulating hormone (FSH) exceeding 25 mIU/ml, confirmed through two separate tests over a span of 4 weeks (1, 2). The release of antimullerian hormone (AMH) by granulosa cells within developing follicles holds significant diagnostic value in the prompt identification of POI. The evaluation of ovarian reserve in POI cases can be accomplished through transvaginal ovarian ultrasound and the evaluation of AMH levels (3). Initially, the prevalence of POI was estimated to be around 1-1.5%. However, recent findings from a meta-analysis indicate a global prevalence of 3.7%, with a disproportionately higher occurrence observed in nations with medium to low developmental status (4). Premature ovarian failure (POI) is characterized by complex causes including infectious, environmental toxins, autoimmune, iatrogenic or genetic factors, in which genetic factors play a major role in about 20-25% of cases (5, 6). In recent decades, a multitude of potential genes associated with Primary Ovarian Insufficiency (POI) have been identified, although only a limited number have been definitively linked through functional verification. The genes in question play crucial roles in the migration and proliferation of primordial germ cells (such as NANOS3), cellular apoptosis (including PGRMC1 and FMR1), transcriptional regulation specific to oocytes (for example, FIGLA and NOBOX), follicle development (such as NR5A1, WT1, and FOXL2), members of the transforming growth factor-β superfamily (like BMP15 and GDF9), as well as receptors for various hormones (including FSHR, AMH, and AMHR2) (7, 8). Among genetic abnormalities associated with POI, the FMR1 mutation is the most common, followed by defects in the bone morphogenetic protein 15 (BMP15) gene located at Xp11.2 (9). BMP15 is a member of the TGF-β superfamily that promotes follicular development and, when combined with GDF9, enhances human primordial follicle activation in vitro. The GDF-9 gene, which is positioned on the long (q) arm of chromosome 5, is exclusively synthesized as mRNA inside the oocyte from the primary 1-layer follicle stage until post-ovulation. Comparative analysis demonstrates that both genes have undergone rapid evolution compared to other members of the TGFß family, with evidence of positive selection within the mammalian clade, highlighting the significance and distinct roles

of the GDF-9 and BMP-15 proteins they produce, consequently underscoring their crucial role in female fertility (10).

Furthermore, studies on animal models have illustrated the significant role of BMP15 in the context of reproduction. An example of this can be seen in sheep, where infertility is the outcome of homozygous BMP15 mutations. While heterozygous inactivation of Bmp15 in mice results in normal ovarian function, animals with Bmp15 null alleles exhibit reduced ovulation rate and fecundity, leading to hypofertility (11-13).

Considering the importance of BMP15 in causing primary ovarian insufficiency (POI), the main objective of our study was to investigate the mutations related to this gene in a group of Lorestan women. This research ultimately led to the identification of a novel mutation in BMP15 in an individual experiencing infertility challenges.

2. Methods

2.1 Case presentation

In this context, we describe a BMP15 mutation identified during the investigation of a 33-year-old individual with premature ovarian failure (POI) in Lorestan families. This person was a married woman with a 2-year history of infertility who presented with secondary amenorrhea and high levels of plasma gonadotropins, as well as irregular menses. She has no clinical history of autoimmunity, pelvic surgery, or chemotherapy and has a normal karyotype of 46, XX. This woman's niece was suffering from NR, seizures and a speech disorder.

2.2 Next generation sequencing (NGS) and analysis

After physical examination, ultrasound and blood test, genomic DNA was extracted from the peripheral blood sample using a filter-based methodology and quantified. A total amount of 1.0µg genomic DNA per sample was used as input material for the DNA sample preparation. PCR sequencing, as part of our general protocol for the evaluation of genetic defects in young women with POI sequencing.

The creation of sequencing libraries was performed employing the Agilent SureSelect Human All ExonV7 kit (Agilent Technologies, CA, USA) in accordance with the manufacturer's guidelines, with x index codes assigned to distinguish sequences from each sample. Initially, the DNA was fragmented using a hydrodynamic shearing system (Covaris, Massachusetts, USA) to produce fragments ranging from 180-280bp. Subsequently, any remaining overhangs were modified to blunt ends through the activities of exonucleases and polymerases, followed by the removal of enzymes. Following adenylation of the 3' ends of DNA fragments, adapter oligonucleotides were ligated. DNA fragments with adapters ligated on both ends were specifically amplified in a PCR reaction. The enriched libraries were further amplified in a PCR reaction to introduce index tags in preparation for hybridization. The resulting products underwent purification using the AMPure XP system (Beckman Coulter, Beverly, USA) and were quantified using the Agilent high sensitivity DNA assay on the Agilent Bioanalyzer 2100 system. Subsequently, the qualified libraries were loaded onto NovaSeq 6000 Illumina sequencers. Subsequent to sequencing, data quality control, analysis, and interpretation were conducted on the G9 generation of HP server utilizing a unix-based operating system.

The results of Whole Genome Sequencing were evaluated. Tertiary structure of BMP15 and BMPR1B protein was modeled by using homology method Phyer-2 web server. The protein model was refined by GalaxyWEB (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE) and ModRefiner. The UCSF Chimera software was used to create mutant structures. The Polymorphism Phenotyping version 2 (PolyPhen2) analysis was utilized. Protein-protein docking was performed using ClusPro 2.0 (https://cluspro.org/login.php).

3. Results

Collection and analysis of sequencing data for the BMP15 gene in patient case, which included non-familial POI cases, was successfully performed. A variant of the BMP15 gene was found that contained a missense substitution, at position c.226C>T:p.Arg76Cys, a missense variant that changes arginine to cysteine at position 76.

All computational tools utilized indicated that the variant likely impacts the structure and function of the encoded protein and is highly conserved across species. Apart from BMP15, no other genes were found to be linked to follicular development, maturation, or atresia. Thus, the heterozygous mutation p.Arg76Cys in BMP15 appears to be the most probable pathogenic factor in our POI patient. In silico analysis demonstrated a missense substitution, at position c.226C>T has changed amino acid Arginine into Cysteine at position 76. Docking analysis revealed that the substitution of Arginine into Cysteine decreased the binding affinity of BMPR1B into BMP-15 (Figure 1).The binding energy of BMP-15 wild type (Arg76) and mutant (Cys 76) with BMPR1B are −336.5 and −281.5, respectively. The PolyPhen2 analysis indicated these changes as 'probably damaging' (Figure 2).

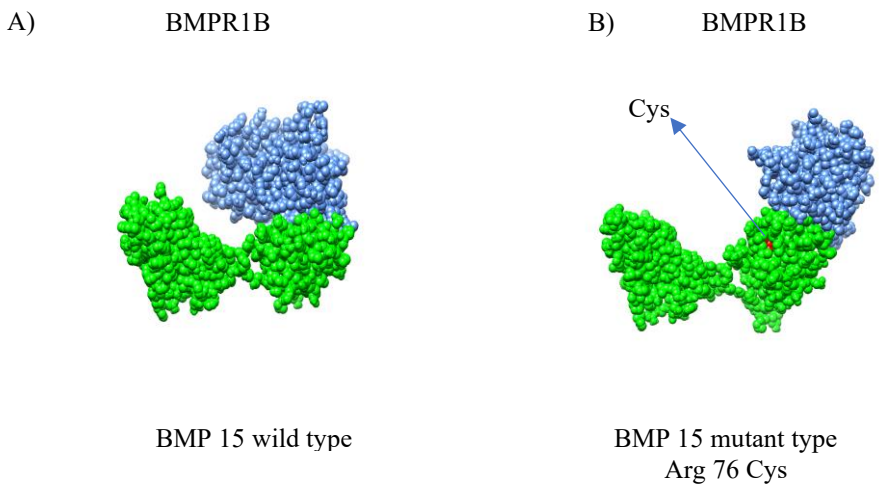

Figure 1. The variant Arg 76 Cys changed binding pattern and affinity of BMP-15 with BMPR1B. BMP-15 wild type bound to BMPR1B (A) and BMP-15 mutant type (variant) bound to BMPR1B (B) that changed binding pattern.

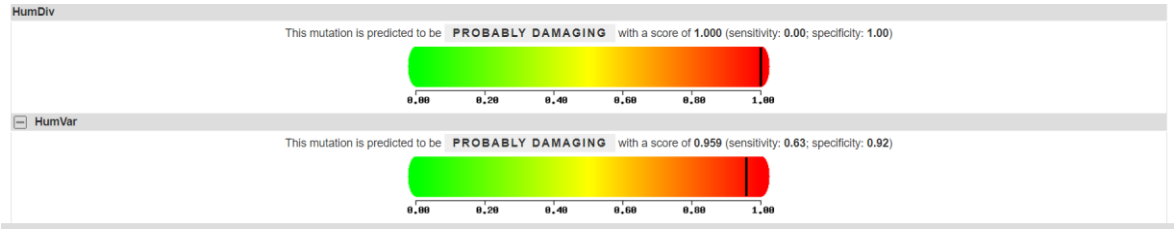

Figure2. The PolyPhen2 analysis suggested these changes are likely damaging.

4. Discussion

A diverse range of pathogenic factors can contribute to the onset and progression of Premature Ovarian Insufficiency (POI), including genetic anomalies, autoimmune responses, iatrogenic influences, and environmental triggers (14, 15). Within this investigation, a previously unreported variation in Lorestan province, c.226C>T:p.Arg76Cys, was detected in a POI patient who exhibited heterozygous mutation. Upon further investigation in our study, molecular docking revealed that variation Arg 76 Cys changed the binding affinity and pattern of the BMP-15 and BMPR1B complex. Changes in the binding affinity and pattern of the complex protein may disrupt oocyte maturation. In silico prediction the substitutions amino acid Arginine into Cysteine at position 76 with MUpro analysis display a decreased stability for BMP-15 mutant type. The PolyPhen2 analysis, on the other hand, indicated this amino acid substitutions is probably damaging. Consequently, it is plausible that the missense alteration in BMP15 could underlie the pathogenic pathways observed in this individual. This specific mutation is linked to a reduction in BMP15 protein levels, a molecule known for its role in supporting oocyte development and maturation while inhibiting granulosa cell apoptosis (16). Several naturally occurring mutations in BMP15 have been documented in POI cases, including variants H81R and G199R identified in a cohort of affected individuals (17). Variants such as c.151_152delGA and c.189_198delAGGGCATTCAinsTG in the BMP15 gene have been linked to a "knockout-like" effect on BMP15 functionality in humans, leading to premature ovarian insufficiency (POI) (18). Additionally, a homozygous missense mutation, c. G1070A (p. C357Y), in BMP15 has been identified in a POI patient, showing evolutionary conservation and a potential causative role in the disorder. Moreover, the BMP15 mutation c.406 G > C (p. V136L) has been associated with reduced activity of the mature protein in POI patients, indicating its involvement in the pathogenesis of the condition (19). These findings underscore the significance of BMP15 mutations in POI development and highlight the diverse genetic aberrations contributing to this complex disorder. This study has unveiled a missense mutation (c.226C>T:p.Arg76Cys) in the BMP15 gene within a POI patient in Lorestan families, with functional assays indicating its capability to diminish BMP15 expression and induce functional impairment.

The researchers reported a 36 KB deletion encompassing the entire BMP15 gene in a 16-year-old patient with primary amenorrhea and elevated FSH levels, along with an intragenic duplication of the TP63 gene in two sisters who also carried the BMP15 p.Y235C mutation (20). Remarkably, the p.Y235C variant represents the initial BMP15 mutation discovered in 2004 within a pair of Italian sisters with heterozygosity (13).

Subsequently, the same group of researchers identified additional alterations, namely p.R68W and p.A180T, in patients with non-familial premature ovarian insufficiency (POI) in 2006 (21). Expanding their investigation in 2009, they conducted BMP15 gene screening on a cohort of 300 patients and detected mutations p.R138H and p.L148P within the prodomain region, along with the p.S5R mutation situated in the signal peptide of the nascent protein. Through functional in vitro assays using a human granulosa cell line, it was revealed that p.R68W, p.L148P, and p.R138H induce a reduction in the production of mature BMP15 protein, while p.A180T or p.S5R do not exhibit any detrimental effects on protein secretion or function (22). Nonetheless, a study by Patiño et al. in 2017 reported a four-fold decrease in the activity of p.A180T compared to the wild-type BMP15. Their assessment of 10 BMP15 variants illustrated how these genetic variations may diminish the production, activity, or cooperative function with GDF9 of the mature peptide (23). Studies have demonstrated that mutations in the prodomain have the potential to inhibit its interaction with fully developed dimers, resulting in a decline in BMP15 functionality (24).

As previously indicated, a decrease in BMP15 levels has been linked to an escalation in ovulation frequency and the likelihood of a twin pregnancy from two separate eggs. This rise in ovulation rates could ultimately contribute to the exhaustion of ovarian reserves and the onset of Premature Ovarian Insufficiency (POI) (25). To our knowledge, this is the first report of a candidate heterozygous pathogenic mutation in BMP15 in an individual with primary ovarian insufficiency (POI) in Lorestan families. Current observations provide new perspectives on POI development. Nevertheless, the exact mechanisms by which BMP15 mutations trigger POI need to be clarified through further research.

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