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Integral role of *BMP-15* in premature ovarian insufficiency

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Abstract---Introduction: Premature ovarian insufficiency (POI) being a heterogeneous genetic disease involves the interaction of multiple genes and environmental factors and has been associated with several chromosomal abnormalities, single gene mutations, and genetic polymorphisms. BMP-15 (bone morphogenic protien-15) is a member of the transforming growth factor β (TGF-β) family. BMP-15 gene product (protein) has 3 domains, mature domain (c-terminal region) of BMP-15 protien binds to receptors located on the granulosa cell surface to participate in key steps regarding ovarian function, such as granulosa cell proliferation and follicle maturation, ovulation rate modulation, oocyte competence determination and regulating granulosa cell sensitivity to FSH (Follicle stimulating hormone). Single nucleotide polymorphisms (SNPs) of the BMP-15 gene are associated with POI. Materials & Methods: 30 POI patients and 30 healthy age matched controls were recruited for cytogenetic and molecular analysis in this case-control study. 10 ml of whole blood was collected for karyotyping and Molecular analysis by PCR (polymerase chain reaction). The PCR was performed for known SNPs of the BMP-15 gene (-9C>G, 538G>A, 788insTCT and 852C>T) respectively. Amplified PCR products were sequenced commercially and analyzed against BMP-15 reference sequence using ClustalW2 application. Observation/Result: Thirty cases (mean age 30 years) and thirty healthy controls (mean age 23 years) were recruited for the study. On cytogenetic analysis, we found that two cases had Mosaic karyotype for the Turner. In Molecular analysis of the rest of the cytogenetically normal cases and controls, we found 3 cases and one control with a single nucleotide

polymorphism in the promoter region (-9C>G) of BMP-15 gene. Discussion: Prevalence of mosaicism is very common among turner's patients. The prevalence of this SNP was about 10.7% (3/28) in cases & 3.3% (1/30) in healthy controls. This polymorphism in the promoter region may cause altered expression of the gene and result in POI.Discussion: Prevalence of mosaicism is very common among turner's patients. The prevalence of this SNP was about 10.7% (3/28) in cases & 3.3% (1/30) in healthy controls. This polymorphism in promoter region may cause altered expression of the gene and results in POI.

Keywords---premature ovarian failure, premature ovarian insufficiency, primary & secondary amenorrhea, *BMP15* gene polymorphism, turner mosaicism.

Introduction

Premature ovarian insufficiency (POI) refers to the development of amenorrhea due to cessation of ovarian function before the age of 40 years and is characterized by low estrogen and elevated serum gonadotropin. Elevated levels of FSH (Follicle Stimulating Hormone) (≥40 mIU/ml) detected on at least two occasions, a few weeks apart, is indicative of premature ovarian insufficiency [1,2]. The triad for the diagnosis of POI is amenorrhea, hypergonadotropism, and hypoestrogenism. POI affects approximately 1% of the female population by age of 40 years and 0.1% by age of 30 years worldwide [3]. POI is not the same as natural menopause, as natural menopause is an irreversible condition, while POI is characterized by intermittent ovarian function in one-half of young women [4]. In patients of POI, Hypoestrogenism can be satisfactorily treated by hormone replacement therapy. In contrast, fertility cannot be recovered when the diagnosis of POI (or end-stage POI- premature ovarian insufficiency) is generally reached and is often compromised in the early phases of the disease when the clinical manifestations are absent [5]. The age of onset varies widely, depending on the age of onset, the disorder can manifest as primary amenorrhea, without menarche, or secondary amenorrhea after the pubertal development [6]. Kinch et al., [7] found that there are two histopathological types of POI, afollicular, and follicular form. In the afollicular form, there is a total depletion of ovarian follicles with permanent loss of ovarian function. In the follicular form, follicular structures are still preserved and hence a possibility of either a spontaneous or an induced return of ovarian function exists.

The causes of premature ovarian insufficiency are broadly categorized into the genetic and pathological category [1]. Amongst the genetic causes numerical & structural anomalies of the X chromosome adversely affect ovarian function. The most common form of X chromosome defect is Turner's syndrome. Besides genetic causes, a wide spectrum of pathogenic mechanisms like autoimmune, metabolic (galactosemia), infections (mumps), and iatrogenic (anti-cancer treatment) may lead to the development of POI. But, the Genetic causes of POI probably comprise about one-third to one-half of all cases [8]. Chromosomal defects involving the X-chromosome are frequently seen in women with POI. The deletion of X

chromosome makes the follicle or ovum unstable.1In the majority of cases, X chromosome defects caused by the deletion or disruption of genes located on X chromosome, which are critical for ovarian function. Several genes responsible for oogenesis and normal ovarian functions are present on the X chromosome and autosomes. In a meta-analysis, D. Pu et.al reported that five genes BMP15, ESR1, FMR1, FSHR, and INH are very important for normal ovarian functions. They also found that single nucleotide polymorphisms and mutations in these genes were found to be significantly more common in patients with POI compared with controls [9].

In our study, we had evaluated the polymorphism in the BMP-15 gene, as the BMP-15 gene has a very important role in ovarian functions. The BMP-15 is a member of the transforming growth factor β (TGF-β) family, which is expressed by oocytes from the early stages of follicular maturation and during all stages of development [10]. BMP-15 proteins can form homodimers (BMP-15: BMP-15) or heterodimers (BMP-15: GDF-9). The secreted soluble dimer binds to receptors located on the granulosa cell surface to participate in key steps regarding ovarian function, such as granulosa cell proliferation and follicle maturation, ovulation rate modulation, oocyte competence determination and regulating granulosa cell sensitivity to FSH [11]. In the Sanger sequencing projects of the BMP-15 encoding region in panels of POI patients, more than 15 missense variants were identified [12]. Most of them were located in the protein pro-region. This scenario led to engage the researchers in a recent functional exploration of whether BMP-15 promoter polymorphism might be related to the POI phenotype. It has also established that this variant modifies the paired-like homeodomain transcription factor 1 (PITX1) binding site and leads to BMP-15 promoter transactivation disturbances [13]. This might suggest that fine-tuning of BMP-15 expression is critical for normal human ovarian physiology. BMP-15 gene located on the short arm of the X chromosome (Xp11.2) within a 'POI critical region' [14]. Recently, Afkhami et al (2022), also reported the association of POI and single nucleotide polymorphism (SNPs) in prodomain of BMP-15 gene [15]. BMP-15 gene has two exons of 328 bp and 851 bp. In humans, mutations in the BMP-15 gene have been found in association with both primary amenorrhea and secondary amenorrhea in several worldwide POI cohorts with a variable prevalence between 1.5 and 12% [5]. BMP-15 gene is very important in regulating the normal ovarian function in both mono-ovulatory as well as poly-ovulatory species [16]

In light of these findings, one could hypothesize that BMP-15 variations might play a predisposing role in POI. The functional mechanism by which BMP-15 variants with a proven biological impact may disturb ovarian folliculogenesis is presently unknown. It may be predicted that a diminished BMP15 paracrine signal in the follicle would involve an impairment of the anti-apoptotic effects on granulosa cells [5]. Indeed, further studies are needed to understand the exact role of BMP-15 variants in POI pathogenesis. Thus, in our study, we have evaluated the cytogenetic and molecular basis (mutation in BMP-15) of disease in cases of premature ovarian insufficiency. There are very few studies in literature showing impact of BMP-15 variants in pathogenesis of POI in the indian population. We also found some cases in this study, which do not have complete turner's phenotype although they had genotypic mosaicism for Turner in karyotyping analysis, such cases excluded from cases group after the cytogenetic

analysis (Karyotyping). We were fortunate to find such cases because genotypic mosaicism and cryptic deletions related to X chromosome many times missed in cytogenetic analysis. In such cases, further molecular cytogenetic analysis is recommended.

Materials and Methods

Study design & Setting

This study was a case-control study approved by the Institute Ethical Committee-A.I.I.M.S. New Delhi (ref-no. IESC/T-150/01.04.2015). This study was to evaluate the integral role of BMP-15 gene in POI. The study was done in All India Institute of Medical Sciences, New Delhi. The cases were recruited from the obstetrics & gynecology OPD of A.I.I.M.S. New Delhi during the period from April 2015 to September 2016, after taking the informed consent. Cytogenetic and Molecular analysis among study participants was performed in the Lab for Molecular Reproduction and Genetics, Dept. of Anatomy, All India Institute of Medical Sciences, New Delhi.

Participants

The study population consisted of 30 females with POI (Premature ovarian insufficiency) with no identifiable etiology after detailed clinical, and gynecological evaluation. These all cases were recruited from the obstetrics & gynecology OPD of A.I.I.M.S. New Delhi. The control group included 30 healthy females with normal menstrual and fertility history with matched age groups with cases. These controls were recruited from a group of healthy wives of cases with male factor infertility & healthy female volunteers from AIIMS, New Delhi. The relevant information regarding physical examination, clinical investigations, developmental milestones, and occupational or environmental exposure to heat, radiation, chemicals, or toxins, were documented in a pre-designed proforma. Written informed consent was taken from each patient and control.

- **The Inclusion criteria for the study (infertile) group were:** only Cases of idiopathic POI with primary and secondary amenorrhea after detailed gynecological & cytogenetic investigations, with no recent history of illness, fever, or medication (in the past three months).
- **The Inclusion criteria for the Controls (fertile) group were:** Controls who had normal menstrual and fertility history, with no recent history of illness, fever, or medication (in the past three months).
- **The Exclusion criteria for the study group and control group:** 1) Gross dysmorphic abnormalities, structural defects, and systemic infection of the urogenital/reproductive system. 2) History of surgical intervention or trauma of genital/reproductive tract obstruction. 3) History of miscarriage, abortion whether spontaneous or induced. 4) Syndromic cases such as Turner and Klinefelter (on cytogenetic evaluation).

Variables and Data Measurement

Cases included in this study were presented with premature ovarian insufficiency but on clinical examination, no obvious cause was determined in them and all cases were phenotypically normal. So these cases were found idiopathic and further cytogenetic (karyotyping) and molecular (PCR-Polymerase chain reaction) analyses were needed in all these cases, While Controls were presented with normal menstrual and fertility history, so cytogenetic (karyotyping) analysis was not done in controls. These genetic analyses were used to observe the variables in terms of cytogenetic and molecular abnormalities like chromosomal aberrations and SNPs (single nucleotide polymorphism) related to the BMP-15 gene respectively.

Cytogenetic analysis

In all infertile cases, the chromosomal analysis was done to identify any numerical or structural chromosomal aberration. The metaphases were captured under 100X (Olympus optical company, Ltd., Tokyo, Japan). The captured metaphases were analyzed using image analysis software Cytovision 3.7 provided by Applied Imaging (ZEISS microscope, Germany) and classified according to ISCN 2005. At least 50 metaphases were analyzed and karyotyped for each individual. Molecular analysis: SNPs of the BMP-15 gene were observed by using molecular analysis (senger's sequencing). This molecular analysis was done in 28 cases which were found clinically as well as cytogenetically normal but had the phenotype of premature ovarian insufficiency and in 30 healthy fertile controls. Total Genomic DNA was extracted from peripheral blood. Blood samples were collected from cases and controls in EDTA vials only after informed consent. DNA was isolated from 58 (28 case and 30 control) samples.

Genomic DNA was isolated from peripheral blood samples by miller's protocol of DNA isolation. DNA was quantified by using a Nanodrop spectrophotometer (Thermo Scientific Corp., USA). In the analysis of the BMP-15 gene, 2 exons (with reference to the July 2009 assembly of ensemble.org) amplicons (amplified exons) were electrophoresed using 1.8 % agarose gel. Amplified PCR products were purified using gel/PCR DNA fragments extraction kit (Geneaid Biotech Ltd., Sijhih City Taiwan. Cat no DF100). Purified PCR products were sent for sequencing at MCLAB (Molecular Cloning Laboratories) South San Francisco, CA 94080, U.S.A. (http://www.mclab.com/product.php?productid=19071). DNA sequences were analyzed against BMP15 reference sequence ENST00000252677.3 using ClustalW2 (multiple sequence alignment program for DNA available at http://www.ebi.ac.uk/Tools/clustalw2/index.html provided by European Molecular Biology Laboratory (EMBL) - European Bioinformatics Institute (EBI). To manage the bias by the cytogenetic abnormal cases, prior karyotyping was done to exclude such cases from molecular analysis.

Study Size

Study sample size i.e. no. of participants were calculated by using OpenEpi Platform (www.openepi.com) supported by Centers for Disease Control and Prevention, Atlanta.

Statistical Analysis

The data has been expressed as mean ± SD and/or median (minimum, maximum) as per the statistical conventions. Associative associations were investigated by the chi-square test or fisher's exact test. SPSS 11.5 (Texas, USA) were used for statistical analysis. Univariate and multivariable logistic regression was done to see the unadjusted and adjusted odds ratio with 95%CI. All the p-values less than 0.05 were taken as significant.

Results

Participants & Descriptive data

For this case-control study, 30 cases with a mean age of 23 ± 4.15 years were recruited from the Department of Obstetrics & Gynecology, AIIMS, New Delhi, and 30 controls with a mean age of 25 ± 3.49 years were recruited from a group of healthy wives of cases with male factor infertility & healthy female volunteers from AIIMS, New Delhi. Cytogenetic analysis (karyotyping) was performed in cases only, while the Molecular analysis was done in both cases and controls.

Outcome Data and Main Results

Cytogenetic Analysis

Cytogenetic analysis is a prerequisite for molecular analysis as many patients who have a subclinical phenotype of Turner syndrome due to mosaicism in cell line or due to other chromosomal aberrations should be excluded from the molecular analysis. At the same time, cytogenetic analysis can help to identify these mosaicism and other chromosomal abnormalities. like in our study, during karyotyping, it was observed that two cases out of 30 presented with Turner's mosaic karyotype. Although, in these two cases Turner's mosaic karyotype was observed, but their phenotype was not exactly similar to Turner's phenotype. One of these case (patients) had 45, X [56%] / 46X, +mar (?) [40%] / 46X, r (?X) [4%] chromosomal complement on karyotype analysis (fig. 1). In this case, it was found that 56 % of metaphase spreads had 45, X0 , 40% of metaphase spreads had 46X,+mar(?) [i.e. turner's genotype with one marker chromosome] and 4% of metaphase spreads had 46X,r(?X) [i.e. turner's genotype with one ring chromosome] (fig. 1). Similar to the above Case (patient) another one had 46,X,i(Xq) with 45, X0 chromosomal complement on karyotype analysis (fig. 2). In this case 46 % of metaphase spreads had 45, X0 and 54 % of metaphase spreads had 46,X,i(Xq) [i.e. turner's genotype with isochromosome form by long arm of X chromosome]. These two cases were excluded from the cases group as only cytogenetically normal, idiopathic cases were included in this study. The remaining 28 cases were cytogenetically normal.

Molecular Analysis

By using a specific set of primers for Exon-1 and 2 of the BMP-15 gene, multiple copies of amplicons were made for individual cases and controls through polymerase chain reaction (PCR). Purified amplicons were analyzed by using

sequencing analysis software (Chromas pro-2.4), it was observed that, in 3 cases, polymorphism at rs3810682: g.50910775C>G Promoter region of exon -1 of the BMP-15 gene was present. While analyzing the allele frequency on chromatogram (fig.3), we found, the single nucleotide polymorphism in three cases (patients) with respect to the reference sequence. The position (at arrow) of nucleotide in the reference sequence is showing single peak of the Cytosine i.e. homozygous cystosine nucleotide. while in one patient it is showing single peak of Guanine instead of Cytosine, which means Cytosine being replaced by Guanine homozygously. In the other patient, it is showing one peak of both Guanine and Cytosine, which means Cytosine being replaced by Guanine heterozygously.

In out of 3 Cases, in one case, it was homozygous change while in other two cases, the heterozygous changes were observed (Table no. 1). In reference sequence at position rs3810682 (Promoter region of exon-1 of the BMP-15 gene) there should be homozygous (CC) allele. But in 7.1 % cases (out of 28) it was heterozygous allele (CG) and in 3.6 % cases (out of 28) it was homozygous allele (GG), showing single nucleotide polymorphism. While in control group only one patient out of 30 (3.3%) had heterozygous allele (CG). Fisher's exact test was used to analyze the frequency of Single nucleotide variant among cases and controls (Table no. 1). It has been found the prevalence of BMP-15 SNP (rs3810682: g.50910775C>G; Promoter region of exon-1 of the BMP-15 gene) (either homozygous or heterozygous) among cases was 10.7% and in controls was 3.3 %. Although, the prevalence was more in cases as compared to controls but the observed P-value was 0.34 (at 95% significance level) which was not significant.

Discussion

The most common cytogenetic anomaly in POI or POF (premature ovarian failure) is 45, X Turner syndrome. Though our cases were phenotypically normal, 2 cases harbored 45,X chromosome complement with some mosaicism. It has been also reported that many cryptic mosaicism not found in karyotypic analysis. To analyze such low-level cryptic mosaicism molecular cytogenetic techniques like FISH (Fluorescence in situ hybridization) can be used. The possible involvement of BMP-15 in POI pathogenesis was supported by evidence in animal models [17]. Di pasquale et.al, 2004 reported the first mutation of the BMP-15 gene in two sisters with ovarian dysgenesis. They also described the significant association of heterozygous BMP-15 gene variants with the POI phenotype in humans (seven of 166 patients: 4.2%; P- 0.003 vs. controls). These findings are consistent with the critical role played by BMP-15 in human folliculogenesis [18]. Fonesca et al., 2014, were taken an in-silico approach, which identified that the PITX1 (pituitary homeobox 1 protein) factor binds to a sequence located between −14 to −8 of the BMP-15 promoter. It was shown that PITX1 and BMP-15 are co-expressed in both human adult oocytes and in adult mouse ovaries. Functional in-vitro experiments showed that both BMP-15 promoter constructs (BMP-15- prom-G and BMP-15-prom-C) were activated by PITX1. A statistically significant 1.6-fold increase in BMP-15 transcription activity conferred by the BMP-15-prom-G construct was found. This feature might lead to alterations in granulosa cell proliferation rate, which may contribute to POI molecular etiology [13].

In the present study, we, therefore, decided to verify the prevalence of BMP-15 gene alterations among the POI populations. It was observed that both heterozygous and homozygous SNPs (BMP-15 GENE Promoter region: -9C>G rs3810682: g.50910775C>G) distribution in cases and controls) were present in 3 cases among 28 cases and in one control was observed. The prevalence was higher in cases as compared to controls. By using fisher's exact t-test, we observed that although the prevalence was higher in cases but the p-value was 0.034 at 95% CI, which was not significant. The present study also observed that heterozygous SNPs were more than Homozygous SNPs. Out of 3, in one case, it was a homozygous change. The frequency of homozygous allele SNPs in cases was 3.7%. In the other 2 cases, the heterozygous change was observed. The frequency of heterozygous allele SNPs in cases was 7%. Besides cases, in one control heterozygous polymorphism was also observed. The frequency of heterozygous allele SNPs in control was 3.3% and the frequency of homozygous allele SNPs in control was 0%. All these changes were present at the same location (Promoter region of exon -1 of the BMP-15 gene).Two cases presented with secondary amenorrhea and one with primary amenorrhea. So it suggests that SNPs associated with BMP-15 gene cause both primary and secondary amenorrhea.

Limitations

In the present study, there were two major limitations. first is a small population size, we were not able to recruit a more number of cases because of time constrain and the second is no analyses for the mutation or variation spectrum among other genes which are directly involved in normal ovarian functions and may also lead to premature ovarian insufficiency.

Conclusions

In previous literature, it has been observed that there is a strong correlation between mutations or SNPs of the BMP-15 gene and premature ovarian insufficiency as the BMP-15 gene is a regulator of important actions of granulosa cells of the ovary. In the present study, it was observed that both heterozygous and homozygous SNPs of BMP-15 were present in 3 of 28 cases, and in one control, heterozygous SNPs was observed. The prevalence was higher in cases as compared to controls. By using Fisher's exact t-test, we observed that the prevalence of SNPs of BMP-15 was higher in cases. In the present study, It was also observed that heterozygous SNPs were more than Homozygous SNPs. Two cases presented with secondary amenorrhea and one with primary amenorrhea. So it suggests that SNPs associated with the BMP-15 gene cause both primary and secondary amenorrhea. it was also found that Turner's mosaic chromosomal genotype is also common in subclinical phenotypes. Further study, is needed with a large cohort of idiopathic cases of premature ovarian insufficiency, to determine the prevalence of allele frequency observed during the study and its genotype-phenotype correlation among both the primary & secondary ovarian failure patients in the Indian population and expand the mutation spectrum.

Note: This manuscript is already been posted on preprint (medRxiv) with doi: https://doi.org/10.1101/2021.08.03.21261546

Ethics Statement and Conflict of Interest Disclosures

Human subjects

Consent was obtained or waived by all participants in this study. Institute Ethics Committee for Post Graduate Research, All India Institute of Medical Sciences, New Delhi issued approval IESC/T-150/01.04.2015.

Conflicts of interest

All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work.

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