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Research Article

Single Nucleotide Polymorphisms of BMP15 are Associated with Poor Ovarian Response in *In Vitro* Fertilization Programs

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Abstract

Background: Poor ovarian response represents a major negative contribution to the efficacy of *In vitro* fertilization programs. It has been shown that specific nucleotide sequence variants of *BMP15* gene, which encodes the growth factor specifically expressed by oocytes to regulate follicle development, are related to certain forms of ovarian dysfunction. The aim of the study was to search for novel variants related to poor ovarian response phenotypes by means of exon sequencing, and also to check possible relations of already known *BMP15* variants to this particular form of ovarian insufficiency.

Methods: A total of 150 patients (65 women with poor ovarian response and 85 women with normal ovarian response as a control group) participated in this retrospective case-control study. All patients received ovarian stimulation according to the protocol with follicle-stimulating hormone and gonadotropin-releasing hormone antagonist. Genotyping was carried out by polymerase chain reaction with consequent readout of melting curves by means of modified kissing probes assay. Statistical tests were two-sided, with percent values compared by χ^2 test and associations measured by odds ratio.

Results: Two novel single nucleotide polymorphisms of *BMP15* were revealed by exon sequencing. Of these, the c.607 C>T substitution was found only in the control group. In contrast, the novel single nucleotide deletion c.-8 delC in the 5' non-coding region of *BMP15* mRNA was significantly more common in the poor ovarian response group. For the previously known sequence variants, the statistical analysis revealed associations of poor ovarian response with two single nucleotide substitutions, the exonic c.308 A>G and the intronic c.328+905 A>G.

Conclusion: Two novel variants of *BMP15*, both of plausible clinical relevance, were found in this study. Examination of larger patient cohorts is required to further elucidate their connection with the phenomenon of poor ovarian response in *In vitro* fertilization programs.

Keywords: *BMP15*; In vitro fertilization; Poor ovarian response; Single nucleotide polymorphism

Abbreviations: BMP-15: Bone Morphogenetic Protein; POR: Poor Ovarian Response; ORT: Ovarian Reserve Test; AFC: Antral Follicle Counts; AMH: Anti-Mullerian Hormone; IVF: *In Vitro* Fertilization; SNP: Single Nucleotide Polymorphism(s); OD: Odds Ratio

Introduction

Poor ovarian response (POR) is a term denoting the reduced number of ultrasound-detectable growing follicles during gonadotropin stimulation (apart from specification of underlying functional condition, which represents a special issue). By European Society of Human Reproduction and Embryology consensus on POR definition (the Bologna criteria), at least two of the following three features must be present: (i) advanced maternal age (≥ 40 years) or any of the other specific risk factors, (ii) ≤ 3 oocytes retrieved using a conventional controlled ovarian stimulation protocol, and (iii) an abnormal ovarian reserve test (ORT; i.e., low antral follicle counts (AFC; less than 5–7) or low blood levels of anti-mullerian hormone (AMH; <0.5–1.1 ng/ml)). Thus, patients over 40 years of age with an abnormal ORT may be a priori classified as poor responders (or, more properly, the expected PORs); on the other hand, two episodes of poor response after maximal stimulation are considered sufficient to define a patient as a poor responder in the absence of advanced maternal age or abnormal ORT [1]. The prevalence of poor responders varies in the literature between 9 and 24%; POR is accounting for approx. 50% of total number of cancelled *In vitro* fertilization (IVF) cycles, and it is a major negative contribution to the efficacy of IVF programs [2].

The exact causes of POR are not very well understood, although its high prevalence probably correlates with the advanced maternal age. In younger women, POR is frequently conditioned by premature ovarian failure, a disease of complex epidemiology with up to 30% of the cases constituted by familial forms [3]. Associations between nucleotide sequence variation and the premature ovarian failure susceptibility have been studied for a number of genes [4]. Among these *BMP15* occupies a special place because it was cloned as a marker for premature ovarian failure [5].

The gene encodes bone morphogenetic protein 15 (BMP-15), a growth factor of the TGF-β superfamily. Specific expression of *BMP15* in oocytes begins on the early stages of follicle development. Forming complexes with another factor, GDF-9, the BMP-15 secretory proteins promote early follicle growth by enhancing granulosa cell proliferation, modulate hormone production by granulosa cells, and subsequently restrain dominant/pre-ovulatory follicle development by inhibiting the *FSHR* gene activity [6,7]. Expression of *BMP15* in oocytes is critical for recruitment and growth of follicles, and alterations in its

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nucleotide sequence are associated with ovulation rate abnormalities [8,9]. Moreover, some of the missense mutations behave as dominant (this may be due to X-chromosome localization of *BMP15*) causing the defective production of bioactive dimers and, ultimately, the premature ovarian failure [10]. Although associations of *BMP15* sequence variants with premature ovarian failure have been reported for various ethnic groups [11,12], relatively few studies focus on *BMP15* in association with outcomes in IVF programs [13-15].

We describe two novel single nucleotide polymorphisms (SNP) of *BMP15*: a substitution found only in patients with the normal response to stimulation (therefore, it may contribute to the maintenance of ovarian reserve), and a deletion that is significantly more common in POR patients. Additionally, associations of POR with two known variants of *BMP15*, rs41308602 and rs3897937, are demonstrated. We discuss possible direct molecular effects of these variations.

Materials and Methods

A total of 150 patients (65 women with POR and 85 women with the normal ovarian response as a control group) participated in the retrospective case-control study. All of them were recruited while receiving IVF treatment in a hospital of the Research Center for Obstetrics, Gynecology and Perinatology between September 2012 and August 2014. Inclusion criteria were as follows: age under 40, normal karyotype, integrity of both ovaries, and no history of ovarian surgery or pelvic radiation therapy. Thus, we excluded the gross risk factors (age in the first place), so no one of the patients was subject to the 1st of the Bologna criteria. It is an important restriction because the gene we are dealing with is specifically expressed in growing oocytes and almost nowhere else, and this was done to bring to the forefront some pathologic processes in the ovary per se but not reflections of major organic disturbances. The written informed consent was obtained from all participants; the study was approved by the Institutional Review Board at the Research Center for Obstetrics, Gynecology and Perinatology.

Ovarian stimulation was commenced using recombinant follicle-stimulating hormone, FSH (Gonal-F up to 300 IU/day, Merck Serono SA, Geneva, Switzerland) from day 3 of the cycle, and gonadotropin-releasing hormone antagonist (Cetrotid 0.25 mg, Merck Serono SA) was started when at least 1 follicle of ≥ 14 mm could be observed by ultrasound. [16]. The AMH blood levels were measured using AMH Gen II ELISA kit (A79765, Beckman Coulter, Brea, CA, USA). The genotyping was carried out using Prep-GS-Genetics kits for DNA extraction and original labeled oligonucleotides for the modified kissing probe assay (all consumables were provided by DNA-Technology JSC, Moscow, Russia). DNA amplification, detection of fluorescence, and digital analysis of melting curves were done with DT-96 Real-Time PCR Cycler (DNA-Technology JSC).

Sequencing of both exons of *BMP15* with 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Corresponding genomic fragments, 650 and 900 base pair long were amplified with specific primers. The sequences were analyzed using BioEdit Sequence Alignment Editor (Ibis Biosciences, Carlsbad, CA, USA) by alignment to *BMP15* reference sequence NC_000023.11 (NCBI, Bethesda, MD, USA).

The data were analyzed using Statistica 10 software (StatSoft, Tulsa, OK, USA); p-values <0.05 obtained by two-sided tests were considered significant. Categorical variables were converted into percent values and assessed by χ^2 test, while associations were measured by odds ratio within 95% confidence interval.

Results

No significant differences in morbidity or obstetrical history were observed between the groups; however, the patients of the POR group turned to be older than the controls (34.7 ± 3.9 and 31.9 ± 3.9, respectively, p<0.05; Table 1) and to have significantly longer histories of infertility, as well as higher FSH levels and lower AMH levels. Ultrasound examinations revealed lower ovarian reserve (decreased ovarian volume and AFC ≤ 5–7) for most women of this group as compared to their control counterparts (Table 1).

Allele frequencies for seven *BMP15* SNP are given in Table 2. Minor allele frequencies ranged from 0.007 to 0.526. Statistical analysis revealed an association of POR with the exonic SNP rs41308602 (c.308 A>G; the POR group was significantly enriched with the rare G allele: three cases of heterozygosity versus none in the control group, p<0.05; Table 3), and probably also with the intronic SNP rs3897937 (c.328+905 A>G; an increased proportion of the G allele was observed in the POR group: 0.62 versus 0.46 in the control group, p ≈ 0.05; Table 3). Combined frequency of c.308G and c.328+905G was 2.6 ± 0.1 times higher in POR group as compared to the control group, whereas frequencies of other previously known SNP did not differ significantly between the groups.

Two novel SNP of *BMP15* were revealed by exon sequencing and submitted for registration in ClinVar database. These were c.607 C>T (it was assigned rs796052131) and c.-8 delC (assigned rs796052132). The c.607 C>T substitution was found only in the control group, thus it may associate with functional preservation of ovaries. In contrast,

Indicator	POR group (n=65)	Control group (n=85)	p-value
Age, years	34.7 ± 3.9	31.9 ± 3.9	<0.0001
History of infertility, years	7.06 ± 3.8	5.4 ± 3.6	0.0078
Follicle-stimulating hormone level, IU/mL	8.6 ± 3.4	6.7 ± 2.2	<0.0001
AMH level, IU/mL	0.64 ± 0.52	2.29 ± 1.90	<0.0001
Low ovarian reserve*	53 (81.5%)	8 (9.4%)	<0.0001
Pregnancy rate*	12 (18.5%)	31 (36.5%)	0.0156
Live birth rate	8 (12.3%)	25 (29.4%)	0.0122

The Table describes the age, the hormonal status and the outcomes of IVF cycles of the participants, with numerical data presented as means ± standard deviations compared by t-test and the percentages compared by χ^2 test. The patients of the POR group were older than the controls; they also had longer histories of infertility, and manifested higher follicle-stimulating hormone levels and lower AMH levels, as compared to the controls. Note a reduced ovarian reserve for the majority of the POR group.

*as assessed by ultrasound examination

Table 1: Clinical features of the patients with poor and normal ovarian responses.

SNP	Location	Position at NCBI assembly 38.1.	Amino acid substitution	Nucleotide substitution (coding strand)	Minor allele frequency
rs41308602	Exon1	50911091	Asn103Ser	c.308A>G	0.020
rs138281369	Exon 1	50910829	Val16Met	c.46 G>A	0.020
rs104894767	Exon 2	50915966	Ala180Thr	c.538 G>A	0.013
rs104894763	Exon 1	50910985	Arg68Trp	c.202C>T	0.007
rs3897937	Intron 1	50912016		c.328+905A>G	0.526
rs796052131	Exon 2	50916035	Leu203Leu	c.607 C>T	0.020
rs796052132	Exon 1	50910775		c.-8delC	0.040

Description, localization, contents (in terms of nucleotides and amino acids, where applicable), and calculated allele frequencies for a set of *BMP15* SNP. Of these rs796052131 and rs796052132 (bottom lines) are novel, the rest have been described elsewhere.

Table 2: SNP description and allele frequencies in combined sample.

SNP	Minor allele frequency		Allelic χ^2	p-value
	POR, n=65	Control, n=85		
rs41308602	0.046 (n=3)	0	4.003	0.0454
rs138281369	0.015 (n=1)	0.023 (n=2)	0.124	0.7240
rs104894767	0.015(n=1)	0.011(n=1)	0.036	0.8481
rs104894763	0.015(n=1)	0	1.316	0.2512
rs3897937	0.615 (n=40)	0.459 (n=39)	3.621	0.0570
rs796052131	0	0.035 (n=3)	2.340	0.1260
rs796052132	0.061 (n=4)	0.023 (n=2)	1.385	0.2391

POR was associated with the exonic rs41308602 and the intronic rs3897937. The novel rs796052131 substitution was found only in the control group, thus it may associate with functional preservation of ovaries, unlike the novel rs796052132 deletion that was more common in the POR group.

Table 3: Comparison of allele frequencies between POR and control groups.

Haplotype	POR, n=65	Control, n=85	OR, 95% confidence interval
rs41308602; rs138281369; rs104894767; rs104894763; rs3897937; rs796052131	0.767	0.550	2.7 (1.25; 5.96)
rs41308602; rs104894763; rs3897937	0.676	0.458	2.47 (1.20; 5.14)

The Table comprises the results of haplotype analysis. The associations were measured by odds ratio (OR) within 95% confidence interval.

Table 4: Combined frequencies of minor variant-comprising haplotypes in POR and control groups.

the c.-8 delC, a novel deletion in the 5' non-coding region of *BMP15* mRNA, was more common in the POR group (Table 3).

Haplotype analysis was done for rs41308602, rs3897937, rs104894763, rs138281369, rs104894767, and the novel rs796052131 (all mapping to *BMP15* exons 1 or 2, or their vicinity). Odds of having any minor variant of this haplotype were 2.7 times higher for the POR patients as compared to the control group (Table 4). We also checked haplotype frequencies for combination of three common SNP mapping to exon 1 or its vicinity (rs41308602, rs104894763, and rs3897937), given the reported association of rs3810682/rs3897937 haplotypes with increased follicle numbers (≥ 12) during ovarian stimulation [17]. Similarly, the odds turned to be higher for the POR patients than for the control group (Table 4).

Discussion

The results indicate association of POR with two previously known *BMP15* variants: the exonic SNP rs41308602 (c.308A>G) and the intronic SNP rs38979307 (c.328+905A>G). The c.308A>G substitution causes asparagine-to-serine switch in 103 protein position. According to literature, the G allele can be found both in amenorrhea patients and in healthy women, though in amenorrhea patients c.308 G is more frequently combined with G (c.-9C>G) or T (c.852C>T) than in the healthy controls [18].

The c.328+905A>G relationship to ovarian function is controversial. Hanevik et al. [19] demonstrate higher frequencies of the G allele in POR samples, Mendoza et al. [20] find that women with the G allele in combination with several genetic markers of ovarian ageing have shorter fertile period, whereas Moron et al. [17] report increased frequency of TGG haplotype of c.673C>T, c.328+905A>G, and c.-9C>G for patients with enhanced responses to ovarian stimulation, including patients with ovarian hyperstimulation syndrome. Gonzalez et al. [21] suggest that AG compared to AA genotype may produce a protective effect, preventing anovulation and infertility.

Three other previously known SNP did not associate with POR in this study, despite some expectations based on published evidence. For example, a large cohort study by Di Pasquale et al. [22] demonstrates associations of premature ovarian failure phenotypes with two *BMP15* variants comprised by missense substitutions c.202C>T → p.Arg68Trp and c.538G>A → p.Ala180Thr, although Ledig et al. [23] qualify the c.538G>A as a polymorphism rather than a mutation, represented both in premature ovarian failure patients and control groups. However, the increased proportion of c.538G/A heterozygotes among premature ovarian failure patients is further confirmed in several studies [10,18]. In our setting, these heterozygotes were found in both groups, revealing no association of the G allele with POR; however, as the obvious limitation of this study is the smaller sample size, the associations will need further refinement.

The control group-specific novel c.607T variant gives no amino acid substitution (CTA, corresponding to leucine, is changed to synonymous TTA). Overall, the usage of these codons in human genome is equal (each of them accounts for 7% of the total number of leucines). However, specific tRNA for CTA is less abundant in the ovary than tRNA for TTA [24], and the c.607 C>T substitution may locally increase *BMP15* mRNA translation rates thus influencing the protein production. The novel single nucleotide deletion c.-8 delC, more common in the POR group than in the controls, is located in the 5' untranslated region of *BMP15* mRNA in position 42. It does not interfere with the reading frame, but may affect initial steps of the translation.

Conclusion

We report two novel polymorphic sites in *BMP15* gene sequence, plausibly related to POR phenotypes. Examination of larger patient cohorts is required to further elucidate their connection with the phenomenon of POR in *In vitro* fertilization programs.

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