

Mutations Analysis of the Growth Differentiation Factor 9 Gene in Syrian Women with Ovarian Failure

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KEYWORDS Amenorrhea. GDF9 Gene. Primary. Secondary. Variant

ABSTRACT Premature ovarian failure (POF) is a primary ovarian defect characterized by absent menarche or premature depletion of ovarian follicles. Growth differentiation factor 9 (GDF9) plays an important role in normal growth, differentiation, and proliferation of granulosa cells surrounding the oocyte in the ovary. The present study was to verify the involvement of GDF9 variations in a POF woman in Syrian. POF cases (n= 80) consist of (primary amenorrhea PA n=55) and (secondary amenorrhea SA n=25) compared with 200 controls. All cases with POF had a normal karyotype analysis (46XX). Genetic analysis of the GDF9 gene were showed, four variants in 23 patients. Two of novel variants were observed in two patients, the first was [c.1231G<A] and the second novel variant was [c.531T<G]. The document variants [c.447C>T] was observed in 17 patients and 15 controls. But the variant [c.546G>A] was showed in one patient and in one control. The variant [c.447C>T] appeared associated with the variant [c.546G>A] in 3 patients [c.447C>T]+[c.546G>A] (compound heterozygous genotype). this compound variant didn't detect in the controls. The researchers' findings are consistent with the critical role played by GDF9 in human folliculogenesis. The presence of these variants might indicate a higher risk for POF.

INTRODUCTION

Premature ovarian failure (POF, MIM#311360) is the loss of functional follicles in women younger than 40 years with unexplained amenorrhea (of more than 6 months duration) (Rao et al. 2012). It occurs in women with elevated gonadotropin levels (FSH \geq 40UI/l), and low estrogen levels (Mandon-Pépin et al. 2008; Vujović et al. 2012). POF is a heterogeneous disorder affecting approximately 0.1 to 1 percent of the women aged 30 and 40 years respectively. The prevalence of POF in women with primary amenorrhea (POF-I) is 10 percent -28 percent, and in those with secondary amenorrhea (POF-II) is 4 percent -18 percent (Conway 2000; Goswami et al. 2005; Jiao 2012 et al; Bricaire et al. 2013). POF is not only premature menopause, which in most cases is due to the absence of follicles, or to the inability of the remaining follicles to respond to stimulation, also adds the risk

of osteoporosis and coronary heart disease because of the low level estrogen (Yi et al. 2006; Wu et al. 2014). Problems may arise either during fetal development and maturation or with the normal menstrual cycle. The molecular mechanisms underlying POF are still unclear, but the diverse etiologies include viral or autoimmune inflammatory disease, environmental toxics, and radiation or chemotherapy (Davison et al. 2000; Wu et al. 2014). POF has been associated with chromosomal defects like deletions and translocations involving three extensive regions of the X chromosome (Xq13-22, Xq26-28 and Xp11.2-22.1) (Laissue et al. 2006; Al-ajoury et al. 2014). Few genes have been identified that can explain a substantial proportion of cases of POF. A large number of these genes are found on X chromosome and autosomes. Two closely related members of the transforming growth factor- β (TGF- β) super family: growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are interesting. They play a very important role in determining follicle growth and ovulation rate in humans (Simpson et al. 2014). The GDF9 gene is located on chromosome 5 (5q31.1). It has two exons encoding a 454 amino acid peptide. This gene plays a very important role in normal growth, differentiation, and proliferation of granulosa cells surrounding the oo-

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cyte in the ovary (Lledo et al. 2014). It is translated as a preproprotein composed of a signal peptide, a proregion and a mature peptide. After the removal of the signal peptide, the proprotein is cleaved by a furin-like protease, resulting in the mature proteins (Kovanci et al. 2007). In mammals, BMP15 and GDF9 are specifically expressed in the oocytes in a similar spatio-temporal pattern throughout folliculogenesis (Shimasaki et al. 2004; Kathirvel et al. 2013). Both genes act as granulosa cell mitogens by promoting granulosa cell (GC) proliferation from the primary follicular stage to the FSH-dependent stage. Inhibins are produced by granulosa cells surrounding oocytes in the ovary. They regulate FSH production by gonadotrophs in the anterior pituitary through a negative feedback mechanism. Inhibin production is positively regulated by two oocyte-secreted growth factors, they are GDF9 and BMP15. Recent studies indicate that mutations in any of the two factors can decrease the inhibin production and increase FSH levels and lead to ovarian failure (Shi et al. 2003; Prakash et al. 2010). In humans, the first mutational screening of the GDF9 gene was reported in 15 Japanese women with POF, but no mutations were found (Persani et al. 2014). A 4 bp deletion of GDF9 apparently leading to a premature stop codon has been reported in two sisters with twins from one family (Laissue et al. 2006). The variations in GDF9 gene previously described in some study as heterozygous, affect exclusively the pro-region with a prevalence of 1.4 percent (Zhao et al. 2007, Persani 2010). The global data indicate that the GDF9 gene is a strong candidate for POF.

The aim of this study is to identify specific gene variants, that will help in the early detection and intervention in Syrian women with premature ovarian failure and had normal karyotype (46,XX).

MATERIAL AND METHODS

Subjects and Selection Criteria

A total of eighty unrelated patients diagnosed with POF were recruited from the Department of Gynecology and Obstetrics of Damascus University Hospital, Alzihrawi Hospital, and of several clinical centers between April 2011 and September 2013. The diagnostic criteria for POF include primary or secondary amenorrhea depending on the cessation of menses (PA is defined as a complete absence of menses, SA is

defined as a cessation of menses with a history of menses before the age of 40 years) and serum FSH level above 40mIU/ml. All POF cases (PA n=55, SA n=25) had a mean age of 26 years (range 18-38 years). A detailed medical history was recorded for each patient. These patients were also interviewed regarding their medical history, family background, reproductive problems, and possible consanguinity with their parents. Physical examination was conducted in order to identify anatomical problems. None of patients was exposed to gonadotoxins, such as radiation treatment or cancer chemotherapy. The patients with hormone replacement therapy were excluded in this study. Following the previously described inclusion criteria most of them being referred with POF. Written informed consent was obtained from all participants. The study was approved by the ethic and biosafety committee of the Syrian Atomic Energy Commission.

The control population included a 200 women of Syrian origin with regular menstrual cycles (28–32 days), having at least one child and no personal history of infertility or auto-immune disease.

Cytogenetic Analysis

Karyotype analysis using GTG-banding was performed following standard procedures (Al-Achkar et al. 2010). A minimum of 20 metaphases analyzed from stimulated peripheral blood cultures were analyzed for each patient, while in cases with mosaicism this number was increased to 100 metaphases. Karyotype was described according to the International System for Human Cytogenetic Nomenclature (Shaffer et al. 2009).

Molecular Analysis

DNA Extraction

Genomic DNA was extracted from peripheral blood using the QIAamp DNA Blood Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purified DNA was run on a 0.8 percent agarose gel. The quality and quantity of the DNA was determined by spectrophotometer.

Polymerase Chain Reaction (PCR)

The coding regions of GDF9 were amplified by using four pairs of GDF9 gene-specific prim-

ers for both exons. The first pairs of primers were used for the amplification of the exon 1 and three pairs primers were used for the amplification of the exon 2. The sequences of the primers and sizes of the fragment are given in Table 1. All reactions were performed on a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA). The final volume was 25 µl containing 100 ng of genomic DNA, 12.5 µl AmpliTaq Gold PCR Master Mix (Applied Biosystems, USA), 1 µl (10 pmol) of each primer and 9.5 µl of distilled water. Cycling conditions were 95°C for 5 minutes for one cycle and 94°C for 30 seconds, 60°C for 1 minute, 72°C for 1 minute for 40 cycles, followed by an elongation cycle of 72°C for 10 minutes. The amplification products were loaded on a 2.5 percent agarose gel with 1X TAE running buffer and visualized using ethidium bromide fluorescence under ultraviolet light. The band size was determined using a 100-bp DNA ladder (Fermentas, Lithuania).

DNA Sequencing

Variants were confirmed by DNA direct sequencing in an ABI Prism 310 (Applied Biosystems, Foster City, CA) with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The genotypes of variant at the same position were determined on both forward and reverse sequences. Nomenclature of variants identified was established according to Human Genome Variation Society (HGVS, www.hgvs.org/mutnomen).

RESULTS

Karyotype Distribution

Karyotyping was performed for the 200 controls and 80 POF women. All cases with POF had

a normal karyotype analysis (46XX), based on high-resolution GTG banding technique.

Genotype Analysis

Genetic analysis of the GDF9 gene in the patients and controls were successfully performed. Total POF cases (n= 80) consist of (PA n=55) and (SA n=25). The mean FSH level of 56 IU/L (Normal FSH levels 3–20 IU/L).

Four variants in 23 patients were detected. The researchers' results are classified in Table 2. Two of these variants were novel, the first was a heterozygous substitution [c.1231G<A] at position 411 in the exon 2, and was detected in one patient. This patient had primary amenorrhea with elevated FSH levels 65 IU/L. Her age was 22- years- old.

The second novel variant [c.531T< G] was detected in heterozygous state at position 177 in the exon 2. The POF case presented a primary amenorrhea with elevated FSH level 56 IU/L. Her age was 19- years- old, she has a small uterus. The two novel variants were absent in dbSNP database and in 200 Syrian controls women.

The variants [c.447C>T] and [c.546G>A] did not give any change in amino acid sequence (silent variant). They were detected in the present study. The [c.447C>T] was observed in heterozygous state in 17 patients and 15 controls, but the variant [c.546G>A] was detected in one patient and in one control.

The variant [c.447C>T] appeared to be associated with the variant [c.546G>A] in 3 patients [c.447C>T] + [c.546G>A] (compound heterozygous genotype). These patients had PA, small uterus and small ovaries. The ages of them were 17, 20, 18 years- old. The FSH levels were high (52 IU/L, 68 IU/L and 58 IU/L). However, this compound variant was not detected in our controls.

Table 1: Primers used for amplification and sequencing of GDF9 gene

Primer name	Exon	Primer sequences (5'-3')	PCR product (bp)
1F	1	AGAGACCAAGCTAGGCTCTTCCT	579
1R		AATACAATTC AAGGA ACTAGG	
2F	2	AGTACTAATTCTTTGACTTGA	454
2-1F		AGAGTCATGTTAAACAAAC	
2-1F	2	GACCAGCCTCCTTCAACCT	433
2-1R		AAGTCTAAAGTCATGGAG	
2-3F	2	AGTTCTGAATTGAAGAAGCC	459
2-3R	2	AAGGCACACATAGGCACACA	

Table 2: Results of the sequence analysis of GDF9 in 80 POF patients. The overview includes base substitutions at the genomic level, amino acid (AA) change, number of patients and Clinical information (PA,SA)

<i>Mutation</i>	<i>Sequence variation</i>	<i>Amino acid change</i>	<i>POF no= 80</i>	<i>PA no=55</i>	<i>SA no=25</i>	<i>Controls no=200</i>
Novel	c.1231G<A	p. D 411 N	1	1	0	0
Novel	c.531T< G	p. N177 K	1	1	0	0
Rs254286	c.447C>T	Silent	17	14	3	15
Rs10491279	c.546G>A	Silent	1	1	0	1
Rs254286/ Rs10491279	c.447C>T/ c.546G>A	Silent	3	3	0	0
		Silent	3	3	0	0

PA: primary amenorrhea; SA: secondary amenorrhea.

DISCUSSION

Premature ovarian failure is largely portrayed as a heterogeneous genetic disorder, but its etiology still remains elusive. Many 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 (Gilchrist et al. 2004; Moore et al. 2005; Otsuka et al. 2011). Mutations in the GDF9 gene may adversely affect granulosa cell proliferation and differentiation, inhibin production, and subsequent FSH level modulation. The mechanism of GDF9 paracrine action determines its importance as a significant candidate gene for POF (Dixit et al. 2004; Gode 2011). The first mutational screening of GDF9 in Japanese women with ovarian disorders did not reveal any genetic variation in the GDF9 coding region (Persani et al. 2014).

In the present study, we identified four variants. Sequencing of the GDF9 revealed two novel variants [c.531T>G] and [c.1231G>A] and two earlier reported [c.447C>T] and [c.546G>A]. These variants were associated with cases having ovarian failure Table 2.

The two novel variants [c.1231G>A] and [c.531T< G] present in the propeptide region of the GDF9 protein, and associated with ovarian failure were completely absent in the controls. The first heterozygous variant [c.1231G>A] was presented in the mature peptide region of the GDF9 protein, and was absent in the controls. It leads to replace the hydrophobic amino acid residue aspartic (D) for hydrophilic asparagines (N) at the 411 position in exon 2. It was detected in one patient having primary amenorrhea, with elevated FSH levels 65 IU/L. Her age was 22- years-old, and the transvaginal ultrasonography showed a small uterus, and small ovaries devoid

of follicles. No personal history of pelvic surgery was recorded and no other case of POF was identified in the family.

The second novel variant [c.531T< G] altered asparagine (N) a basic amino acid, into lysine (K) an acidic amino acid at the 177 position. This variant is found in one patient having a primary amenorrhea, with elevated FSH levels 56 IU/L, her age was 19- years- old. The transvaginal ultrasonography for this patient showed a small uterus and small ovaries devoid of follicles and her parents and two sisters are healthy but the brother had a feminine characteristics. The aunt showed infantile uterus and streak gonads. There are many sterility cases in its family.

Amino acid substitutions are more likely to affect protein function when differences in the biochemical properties are large and the amino acids are located at residues that are highly conserved among species (Zhao et al. 2007). These new mutations have not been reported previously to SNP databases. The absence of these variants in women with normal fertility suggests a potential pathogenic effect for these variants.

Furthermore, there are common heterozygous variation of GDF9 that have been presented in many studies [(Dixit et al. 2005; Laissue et al 2006; Zhao et al. 2007). In the present study, the researchers found two polymorphisms, [c.447C>T] and [c.546G>A] in 17 cases and in one patient respectively. These variants are common polymorphisms in diverse ethnical groups since they have also been described in Indian POF patients (Dixit et al. 2005). According to literature, a high frequency of [c.447C>T] variant compared to the [c.546G>A] variant was detected (Dixit et al.2005; Zhao et al. 2007). The results also revealed a high frequency of [c.447C>T] variant compared to [c.546G>A] variant.

Surprisingly, among the cases in this study the compound heterozygous variant [447C>T] + [c.546G>A] was detected in 3 patients, this genotype was absent in our controls. These patients had PA, small uterus and small ovaries. The presence of the c.447>T and the compound heterozygous variant [447C>T] + [c.546G>A] might indicate a higher risk for POF. To the researchers' knowledge, this compound variant has not been reported previously in SNP databases.

The different mutational status of populations is probably indicative of ethnic differences. To confirm these observations we need more studies of the GDF9 coding region in our populations (Takebayashi et al. 2000; Dixit et al. 2005; Laissue et al. 2006; Zhao et al. 2007; Simpson et al. 2014).

CONCLUSION

The researchers observed five variants in GDF9 gene, including two novel variants [c.531T<G] and [c.1231G<A], and one compound heterozygote variants [c.447>T + 546G>A] associated with ovarian failure. Their results were comparable with those POF women from other countries and regions in the world. These results and the researchers' findings suggest that the GDF9 may play a role for the premature ovarian failure.

RECOMMENDATIONS

Mutational screening of the GDF9 in present study is considered as only a preliminary result and requires further substantiation with more studies.

ACKNOWLEDGMENTS

The authors would like to thank Professor I. Othman the General Director of Atomic Energy Commission of SYRIA (AECS) and Dr. N. Mir Ali Head of the Department of Molecular Biology and Biotechnology for their support. This study was financed by the Syrian Atomic Energy Commission and the Department of Biology, Faculty of Science, Damascus University, Syria.

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