OnLine Journal of Biological Sciences

Original Research Paper

Identification of Novel Mutations of *AMH* Gene in Iraqi Women with Premature Ovarian Failure

[1]Farah Amer Abed, [2]Raya E. Maroof and [3]Ulfat Mohammed Ali Al-Nakkash

[1]*Ministry of Science and Technology, Iraq*
[2]*College of Health and Medical Technology, Baghdad, Iraq*
[3]*Consultant Gynecologist, Director of Elwyia Maternity Hospital, Baghdad, Iraq*

Article history
Received: 07-10-2018
Revised: 04-12-2018
Accepted: 10-12-2018

Corresponding Author:
Farah Amer Abed
Ministry of Science and Technology, Iraq
Email: ahmed.suleiman@uoanbar.edu.iq

Abstract: An over all of thirty females experiencing idiopathic POF and thirty controls have been studied and among them only six patients and four control were sent to sequence. The *AMH* gene fragments *AMH1*, *AMH2* and *AMH5* generates 530 bp, 288 bp and 1080 bp respectively were amplified. Fragments were sequenced and the results show six out of eight observed SNPs was appeared missense mutations rs764149385, rs753970896, rs10407022, rs61736575, rs1438510393 and rs950459635 Whereas, the novelty of two discovered SNPs were validated, including g.2189550T>G and g.2189667T>G. the novel two SNPs has found to cause missense effect of V73G and L112W, respectively present in *AMH1* fragment. While *AMH2* fragments how two SNPs, including rs17854573 and rs8112524, in *AMH5* fragment, five SNPs were observed, including rs1254589021, rs1478367998, rs7252789 and rs10417628, while only one SNP (g.7156-7157insC) was proven to be novel insertion-deletion SNP, synonymos and missense mutation in POF patient. Our results indicated that the POF Iraqi patients has genetic variant in *AMH* gene which participate in POF pathophysiology.

Keywords: Premature Ovarian Failure, Anti-Mullerianhormone, Follicular Stimulating Hormone

Introduction

Premature ovarian failure, is identified via amputation of ovarian assignment with decrease in AMH, increase in Follicular Stimulating Hormone (FSH) and low concentration of oestrogen prior to age 40.this state influence sacrificial 1 percent of females in childbearing age (Panay and Kalu, 2009). There is an abundant agents participate to POF, including genetic factors, chromosome abnormalities, autoimmune disorders, iatrogenic agents (Pelvic surgery, chemotherapy and radiation) (Nelson, 2009; Shelling, 2010), but in most cases the cause remain un known. Because of the complexity and indecision of POF, the irreversible prejudice of the ovarian reserve, it is important to identify the risk factors for ovarian damage. AMH is belong to Transforming Growth Factor-beta family (TGF-beta), a dimeric glycoprotein, it does have an essential role in the development of ovarian follicle. AMH is produced in females through the ovary via the granulosa cells of small-antral and pre-antral follicles, while in men produced by Sertoli cells, AMH considered a perfect indicator for ovarian function, also it is ideal for ovarian reserve (Li *et al*., 2016; Karagiorga *et al*., 2015). It has a relation with the size of primordial follicle pool and the number of follicles in ovary. Usually in ladies suffering from POF, there is a decrease in the follicle reserve, this could be as a reason for deficiency in oocyst apoptosis process, leading to either fading oocyst formation during ovarian evolution or follicle loss (Qin *et al*., 2014).

Concerning the recruitment of primordial follicles, the AMH has an important repressive function, the missing of AMH result in a follicle pool that is prematurely exhausted and then the cycle will be prematurely terminated (Durlinger *et al*., 1999). AMH is considered as a small gene that consist of 5 exons in chromosome 19p13.3 (Josso and Di Clemente, 1999), also its protein product operate through AMH type II receptor (*AMHR2*), a threonine/serine, member of the family of type II receptors for proteins related to TGF-beta (Josso and Di Clemente,1999).

This research has been designed to investigate the function of AMH in pathophysiology of idiopathic POF Iraqi women and study the genetic variations in AMH gene.

Materials and Methods

Subjects: Thirty infertile premature ovarian failer women were enrolled, aged between 18-40 years, these patients were collected from Kamal Al-Samarray hospital in Baghdad city for period December 2017 to March 2018.

Table 1: The specific primers' pairs selected to amplify three loci within human genomic DNA sequences

Primer	Sequence (5′-3′)	Amplicon size	Accession Number	Reference
*AMH*1-F	AAACACCCCACCTTCCACTC	530 bp	NG_012190.1 (5157-5176)	Nishi *et al*. (2012)
*AMH*1-R	CCGGCCCACCTGAAGGAA		(5669-5686)	
*AMH*2-F	CAGGGACAGATCCCAAAGAT	290*(1) bp	NG_012190.1 (6149-6168)	Nishi *et al*. (2012)
*AMH*2-R	TACTGCAGACCCTGCAACAA		(6419 – 6438)	
*AMH*5-F	CTGGACACCGTGCCCTTC	1059*(2) bp	NG_012190.1 (6867-6884)	Nishi *et al*. (2012)
*AMH*5-R	TGGGGTCCGAATAAATATGG		(7906-7925)	

F: forward R: reverse
*(1) The NCBI blastn engine has indicated that the actual length of the *AMH*2 amplicon is 290 bp instead of 288 bp.
*(2) The NCBI blastn engine has indicated that the actual length of the *AMH*5 amplicon is 1059 bp instead of 1080 bp.

In this study, informed consent was obtained from all patients and control. The control group divided into two groups first group included Thirteen infertile women without suffering of premature ovarian failer, the second group seventeen fertile women. The POI's diagnostic standards (Qin *et al*., 2011) are: At least six months of experiencing amenorrhea a prior to the age of forty, with at least 2 concentrations of >40 IU/l. Patients having related autoimmune disorders, endocrinopathies, iatrogenic agents like infections, radio-therapy, chemo-therapy and pelvic surgery have been omitted, each patient have been examined, with a comprehensive gynecological and medical record, consisting history of pelvic ultrasound, age at menopause, menses and concentrations of FSH (Two times at intervals of one month each).

From each samples involved in this research, 5 milliliter of blood has been taken from vein puncture.

DNA Extraction

DNA samples were extracted using Genaid Kit according to manufacturer's instructions (using a mammalian genomic DNA extraction kit (Geneaid Biotech, Taiwan).

PCR Protocol

Three PCR fragments were selected for amplification, which supposed to cover three loci, including 530 bp, 290 bp and 1080 bp, respectively, within *Homo sapiens* genome using the following primers (Table 1). The lyophilized primers were purchased from Bioneer (Bioneer, Daejeon, South Korea), The amplification was begun by initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 97°C, annealing (at 57,54°C for both *AMH*1 and *AMH*2 loci and 54°C for *AMH*5 locus) and elongation at 72°C and was finalized with a final extension at 72°C for 10 min.

DNA Sequencing of PCR amplicons

The resolved three types of PCR amplicons were commercially sequenced from forward termini according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). The sequencing results of the PCR products of different samples were edited, aligned and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA).

In sequencing analysis, we select the letter A for *AMH1* gene, the letter B for *AMH2*gen, letter C for *AMH5*gene. We chose 10 samples, 6 patients and 4 control, from the control 2 infertile and 2 fertile.

Results

Sequence variants detected in *AMH* gene Thirty samples of infertile premature ovarian failer, Thirteen infertile women without suffering of POF, the result were compared with 17 apparently healthy individuals as a control group. For sequencing we select 10 samples 6 patients and 4 control. In *AMH1*gene, The result of sequencing for this genes six out of eight observed SNPs was appeared missens mutations namely rs764149385, rs753970896, rs10407022, rs61736575, rs1438510393 and rs950459635 Whereas, the novelty of two discovered SNPs were validated, including g.2189550T>G and g.2189667T>G. the latter two SNPs has found to cause missense effect of V73G and L112W respectively. The genetic varients appear in patients and controle were identified in Table 2 and Fig. 1. The result of sequencing for*AMH2* gene only two SNPs were observed, including rs17854573 and rs8112524 are detect synonymous mutation and intronic variant, the genetic varients appear in patients and control were identified in Table 3 and Fig. 2. in *AMH5* gene, five SNPs were observed, including rs1254589021, rs1478367998, rs7252789 and rs10417628, while only one SNP (g.7156-7157insC) was proven to be novel insertion – deletion SNP, synonymos and missense mutation in POF patient, in control group C1 and C7 there is no genetic variation were observed, the date given in Table 4 and Fig. 3.

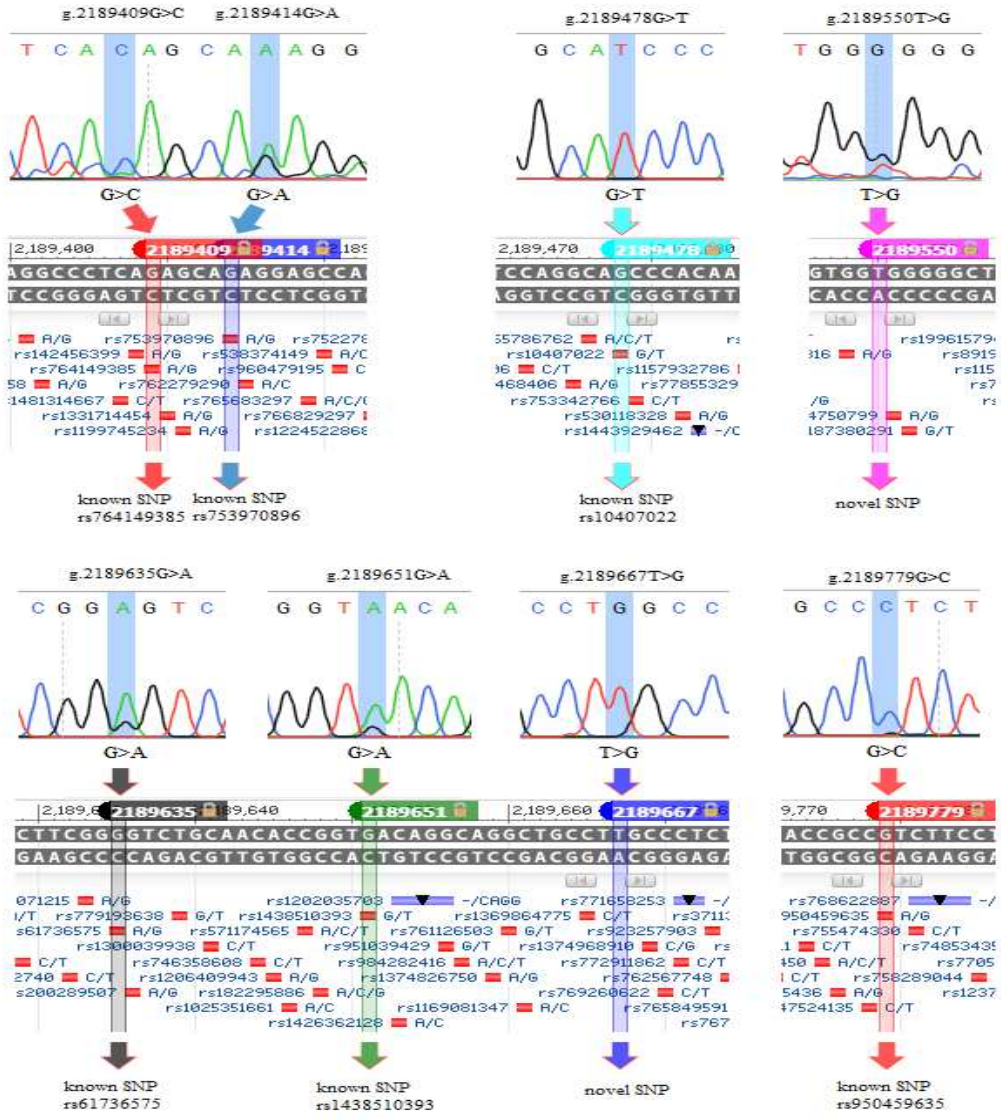

Fig. 1: The *AMH* PCR amplicons' SNPs novelty checking using dbSNP server. Each different color refers to its corresponding SNP. Two novel SNPs were observed, namely g.2189550T>G and g.2189667T>G

Table 2: The pattern of the observed SNPs in the identified *AMH1* sequences in comparison with the NCBI referring sequences of 530 bp amplicons (GenBank acc. no. NT_011295.12), in which, the annotation of all observed mutations were described

No	Mutation	Sample No.	Position in the PCR amplicon	Position in the protein	Position in the referring genome NT_011295.12	Type of SNP	SNP summary
1	G>C	A5, A7, A8	140	Arg (R)26	g.2189409G>C	Missense (R26K)	rs764149385
2	G>A	A2, A5, A8	145	Glu(E)28	g.2189414G>A	Missense (E28K)	rs753970896
3	G>T	A1, A2, A3, A5, A6, A7, A8, A9, A10	209	Ser(S)49	g.2189478G>T	Missense (S49I)	rs10407022
4	T>G	A10	281	Val(V)73	g.2189550T>G	Missense (V73G)	Novel SNP
5	G>A	A3	366	Gly(G)101	g.2189635G>A	Synonymous (G101G)	rs61736575
6	G>A	A2, A4, A8	382	Asp(D)107	g.2189651G>A	Missense (D107Y)	rs1438510393
7	T>G	A9	398	Leu(L)112	g.2189667T>G	Missense (L112W)	Novel SNP
8	G>C	A4, A8, A9	510	Downstream of exon 1	g.2189779G>C	Intronic variation	rs950459635

Table 3: The pattern of the observed SNPs in the identified *AMH* sequences in comparison with the NCBI referring sequences of 290 bp amplicons (GenBank acc. no. NG_012190.1), in which, the annotation of all observed mutations were described

No	Mutation	Sample No.	Position in the PCR amplicon	Position in the protein	Position in the referring genome NG_012190.1	Type of SNP	SNP summary
1	G>A	B9	209	Pro(P)182	g.6357G>A	Synonymous (P182P)	rs17854573
2	G>A	B1, B2, B3, B4, B5, B7 B8, B10	268	Downstream of exon 2	g.6416G>A	Intronic variant	rs8112524

Table 4: The pattern of the observed SNPs in the identified *AMH* sequences in comparison with the NCBI referring sequences of 1059 bp amplicons (GenBank acc. no. NG_012190.1), in which, the annotation of all observed mutations were described.

No	Mutation	Sample No.	Position in the PCR amplicon	Position in the protein	Position in the referring genome NG_012190.1	Type of SNP	SNP summary
1	ins C	C2, C4, C6, C9, C10	274-275	Leu(L)327	g.7140-7141insC	Indel (L327P)	rs1254589021
2	ins C	C2, C5	290-291	Asp(D)332	g.7156-7157insC	Indel(D332A)	Novel INDEL SNP
3	C>T	C8	442	Leu(L)349	g.7208C>T	Synonymous	rs1478367998 (L349L)
4	T>A	C2, C3, C4, C5, C6, C9, C10	534	Gly(G)413	g.7400T>A	Synonymous (G413G)	rs7252789
5	C>T	C8	839	Ala(A)515	g.7705C>T	Missense (A515V)	rs10417628

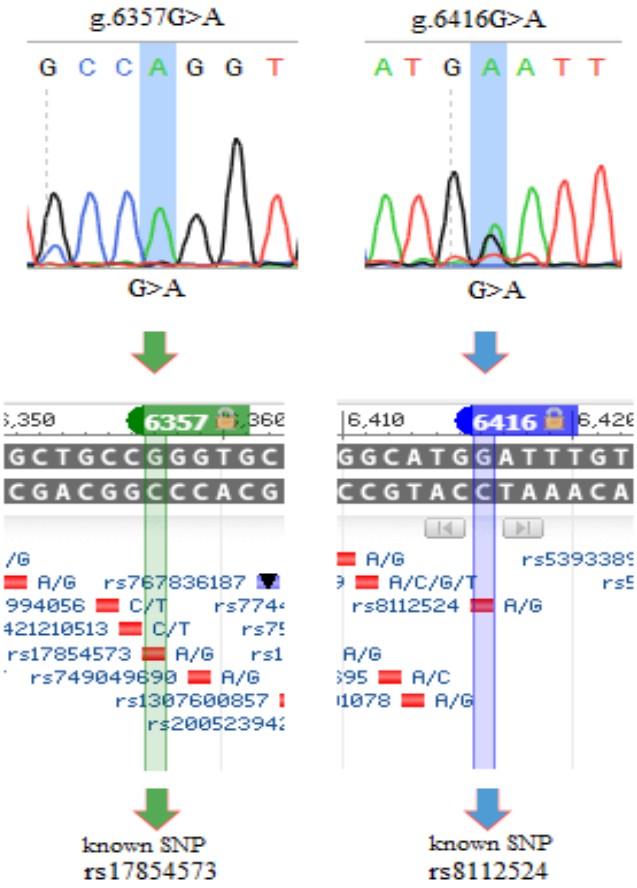

Fig. 2: The *AMH* PCR amplicons' SNPs novelty checking using dbSNP server. Each different color refers to its corresponding SNP. No novel SNPs were observed

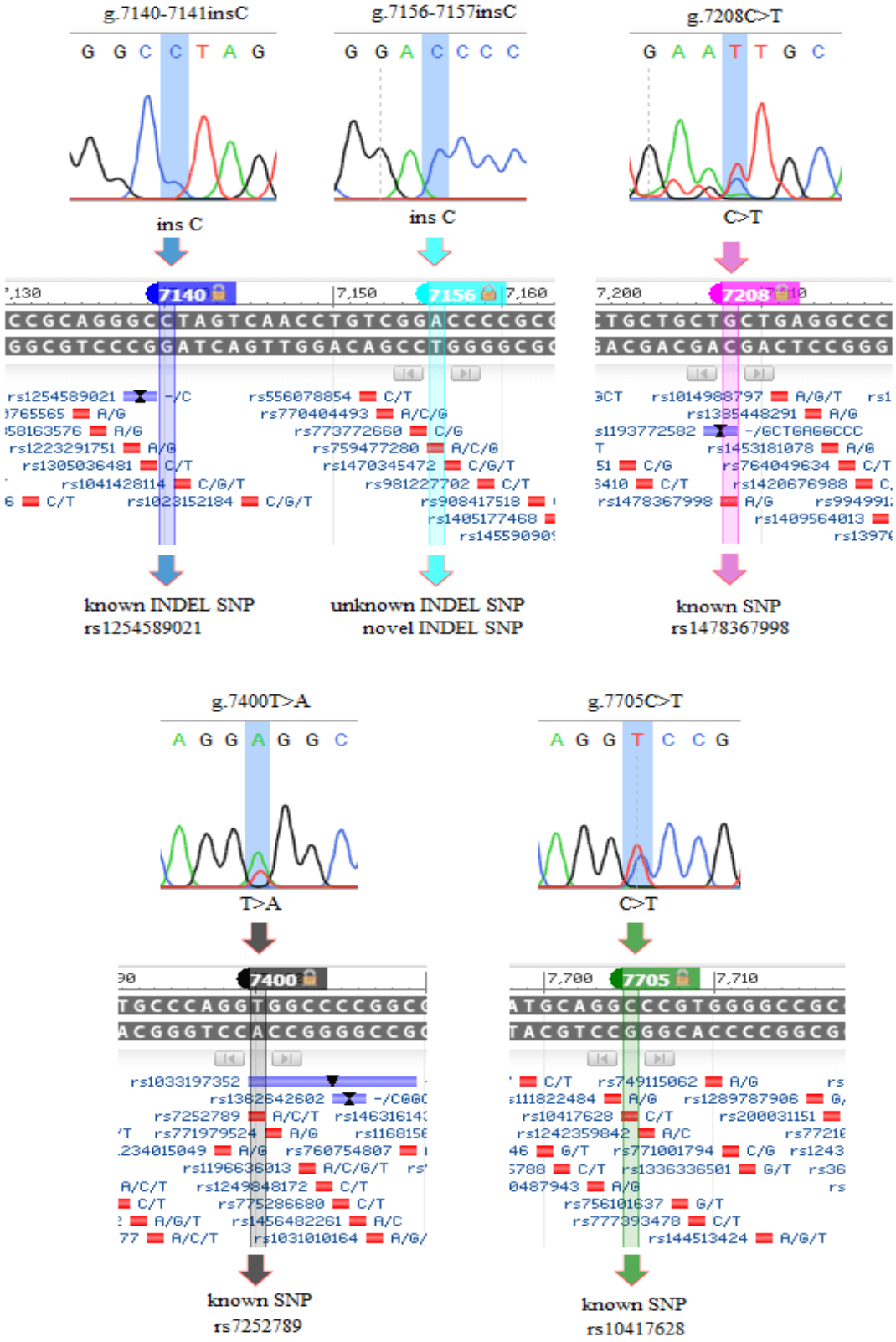

Fig. 3: The *AMH* PCR amplicons' SNPs novelty checking using dbSNP server. Each different color refers to its corresponding SNP. One novel INDEL SNP was observed, namely g.7156-7157insC

Discussion

The study of *AMH* in females has become widespread, the first who indicate the importance value of AMH gene in the follicular reserve estimation was Fauser and his colleagues (De Vet *et al*., 2002; Van Rooij *et al*., 2002; Weenen *et al*., 2004). The conclusive function of AMH and its effect on follicular development, AMH polymorphisms could lead to POF.

The sequencing analysis of *AMH1* gene is summarized in Table 2. The two patients A9, A10 with fresh AMH variant experienced secondary amenorrhea at the age of 31 and 24 following stopping pills of birth-control at the age of 24. This is based on the AMH's role in the ovary, as this hormone does not act as a blocker of follicle progress, however it acts as inhibitor of follicular depletion (Durlinger *et al*., 1999; Visser *et al*., 2007). Afterwards, the anticipated clinical presentation will be well-matched with a secondary amenorrhea: The ovaries have the ability to complete their role but throughout a shorter time, even when the number of primordial follicles has been normal at birth. Compared with control group (A4, A5) control fertile there is a Missense mutation g.2189478G>T in A5 and g.2189779G>C in A4, the observed SNPs in A9, A10 control infertile was g.2189779G>C, g.2189478G>T, g.2189550T>G with Missense and an Anintronic variation, This variant can alter splicing of the gene and this 'splice-variant gene expression' can change the level of gene expression. This may result in infertility, while the variation in control fertile with less effect.

The sequencing analysis of *AMH2* gene is summarized in Table 3, in control group (B1,B5,B7,B9) and patients (B1,B3,B4,B6,B8,B10)there is a genetic variation in both groups, in B9 control fertile, a Synonymous (P182P) mutation in Pro(P) 182, is an alteration in the sequence of DNA which codes for amino acids in a protein sequence, however, it doesn't alter the encoded amino acid. Because of the redundancy of the genetic code (multiple codons code for the same amino acid), so these alterations does not impact the protein structure. Anintronic varient in B1, B2, B3, B4, B5, B7, B8, B10 patients on g.6416G>A, The introns of a gene contains downstream regulatory elements known as DREs and the intron variants either loose these elements or accquire a new type of elements which ultimately affects the gene signaling and regulation involving post transcriptional alterations, in this manner the general expression level in addition to the pattern of expression to a tissue type get influenced, leading to infertility and low level of AHM.

The sequencing analysis of *AMH5* gene is summarized in Table 4. Control group (C1,C7,C8,C10), patient (C2,C3,C4,C5,C6,C9) in C2, C4, C6 patients samples an Indel (insertion deletion) mutation observed in Leu(L) 327 on g.7140-7141insC, a Cytosine nucleotide insertion between two regions 7140-7141, leading to chang in reading frame, these can have devastating effects because the m RNA is translated in new groups of three nucleotides and the protein being produced maybe useless. A two novel mutation in C2, C5 patients were identified in Asp(D)332 on g.7156-7157insC, a Cytosine nucleotide insertion between two regions 7156-7157, leading to chang in reading frame. A two Synonymous mutations in C8, C2, C3, C4, C5, C6,C9, C10 in patients and control group(C8,C10) control infertile, were identified in Leu(L)349, Gly(G)413, on g.7208C>T, g.7400T>A, the triplet codes for leucine, a single base change in the DNA can give rise to one of nine other codones. As the resulting codons still code for leucine, *AMH* polymorphism could affect biological activity of hormones which influence the follicle development.

A missense mutation in C8 control infertile in Ala(A)515 on g.7705C>T, leading to produce another type of amino acids and change the protein structure and function causing infertility cases.

Conclusion

POF is a complicated merit, the genetic variants in *AMH* gene impact AMH signaling pathway, the genetic factors and the SNPs interacting with each other and evolution of POF.

Acknowledgment

My special thanks and appreciation to staff members of PCR Laboratory in the Collage of Health and Medical Technology/Baghdad, staff of Al-musaibbridge company, staff of Zenat Al-hayat center My special thanks and appreciation go to Dr. Ahmed A. Suleiman for his helping and support.

Author's Contributions

All authors contribute equally in this work.

Ethics

All work was done under recommendation of ethical committee of Collage of Health and Medical Technology/Baghdad

References

De Vet, A., J.S. Laven, F.H. De Jong, A.P.A. Themmen and B.C. Fauser, 2002. Antimüllerian hormone serum levels: A putative marker for ovarian aging. Fertil. Steril., 77: 357-362. DOI: 10.1016/S0015-0282(01)02993-4

Durlinger, A.L., P. Kramer, B. Karels, F.H. De Jong and J.T. Uilenbroek *et al*., 1999. Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary. Endocrinology, 140: 5789-5796. DOI: 10.1210/endo.140.12.7204

Josso, N. and N. Di Clemente, 1999. TGF-beta family members and gonadal development. Trends Endocrinol. Metab., 10: 216-222. DOI: 10.1016/S1043-2760(99)00155-1

Karagiorga, I., G.A. Partsinevelos, D. Mavrogianni, E. Anagnostou, I. Zervomanolakis *et al*., 2015. Single nucleotide polymorphisms in the Anti-Müllerian hormone (AMH Ile49Ser) and Anti-Müllerian hormone type II receptor(AMHRII −482 A>G) as genetic markers in assisted reproduction technology. Assist. Reprod. Genet. J., 32: 357-367. DOI: 10.1007/s10815-014-0403-0

Li, L., X. Zhou, X. Wang, J. Wang and W. Zhang *et al*., 2016. A dominant negative mutation at the ATP binding domain of $AMHR_2$ is associated with a defective anti-Mullerian hormone signaling pathway. Molec. Hum. Reprod., 22: 669-678. DOI: 10.1093/molehr/gaw040

Nelson, L.M., 2009. Primary ovarian insufficiency. N Engl. Med. J., 360: 606-614. DOI: 10.1056/NEJMcp0808697

Nishi, M.Y., S. Domenice, A.T. Maciel-Guerra and A.Z. Neto 2012. Analysis of anti-Müllerian hormone (AMH) and its receptor (AMHR2) genes in patients with persistent Müllerian duct syndrome. Arq Bras. Endocrinol. Metab., 56: 473-477. DOI: 10.1590/S0004-27302012000800002

Panay, N. and E. Kalue, 2009. Management of premature ovarian failure. Best Pract., Res. Clin. Obestet. Gynaecol., 23: 129-140. DOI: 10.1016/j.bpobgyn.2008.10.008

Qin, C., Z. Yuan, J. Yao, W. Zhu and W. Wu *et al*., 2014. AMH and AMHR2 genetic variants in Chinese women with primary ovarian insufficiency at normal menopause. Repro. Biomed. Online, 29: 311-318. DOI: 10.1016/j.rbmo.2014.05.003

Qin, R., S.H. Chen, J. Yao, W. Wu and J. Xie, 2011. Identification of novel missense mutations of the TGFBR3 gene in Chinese women with premature ovarian failer. Reprod. Biomed. Online J., 23: 697-703.

Shelling, A.N., 2010. Premature ovarian failure. Reproduction, 140: 633-641. DOI: 10.1530/REP-09-0567

Van Rooij, I.A., F.J. Broekmans, E.R. Te Velde, B.C. Fauser and L.F. Bancsi *et al*., 2002. Serum anti-Müllerian hormone levels: A novel measure of ovarian reserve. Hum. Reprod., 17: 3065-3071. DOI: 10.1093/humrep/17.12.3065

Visser, J.A., A.L.L. Durlinger, I.J.J. Peters, E.R. VandenHeuvel and U.M. Rose *et al*., 2007. Increased oocyte degeneration and follicularatresia during the estrous cycle in anti-Mullerian hormone null mice. Endocrinology, 148: 2301-2308. DOI: 10.1210/en.2006-1265

Weenen, C., J.S. Laven, A.R. Von Bergh, M. Cranfield and N.P. Groome *et al*., 2004. Anti-Müllerian hormone expression pattern in the human ovary: Potential implications for initial and cyclic follicle recruitment. Mol. Hum. Reprod., 10: 77-83. DOI: 10.1093/molehr/gah015