RESEARCH ARTICLE

Relationship Between the AMHR2 SNP (–482 A >G) with the Level of Hormone, Oxidative Stress Status and Interleukin-18 (IL-18) in PCOS Iraqi Woman

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ABSTRACT

The polycystic ovarian syndrome is a heterogeneous endocrine disorder that affected young women in child-bearing age and has severe consequences on women's health. Many studies on the polycystic ovary syndrom (PCOS) found that genetic variation is an essential factor that leads to the development of PCOS. The genetic variation in the AMHR2 and their association with PCOS gives inconstant results. The current study revealed an increment in concentration of AMH among the PCOS, and this elevation may reflect a disturbance in the normal signaling of AMH and thereby, the genetic polymorphism of Anti-mullerian hormone type 2 receptor and their potential association with the pathogenesis and phenotype of PCOS. This is the first study in Iraq concerning AMH receptor-related- PCOS. IL-18 and oxidative stress have a great impact on the severity of PCOS.

Keywords: AMHR2, IL-18, MDA, PCOS, TAOC.

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INTRODUCTION

Anti-Müllerian hormone (AMH), is a glycoprotein hormone secreted from granulosa cells in the primary ovarian follicles in the ovary and it's considered an indicator of ovarian aging and reserve.[1] The AMH hormone is one of the most hormones that have a relation to PCOS and its increment is regarded as a marker for the diagnosis of this disease.[2] Studies on this topic found that the rise in anti-mullerian hormone is not only reflecting the elevation in the number of primary follicles in the PCOS syndrome, but also there is an increase in the secretion of the hormone from these follicles.[2] AMH reduce follicular sensitivity to FSH hormone and disrupt folliculogenesis and that lead to anovulation in PCOS women.[3]

It was denoted that the gene responsible for AMH is located on chromosome 9,[4] and the gene for the AMH receptor (AMHR2) is found on chromosome.[12] This receptor was reckoned as a specific receptor for AMH cloned in 1994.[5] Among factors that disrupt the functioning of the hypothalamic-pituitary-ovary axis; hormones and their receptors is genetically varied in the form of single nucleotide polymorphism (SNPs)[6] and among these SNPs (rs 2002555) –482 A > G which located in the promotor region of AMHR2 gene. The previous studies on the SNP relationship with PCOS showed inconstant results.[7-9]

Malondialdehyde (MDA) is an excellent biomarker of lipid peroxidation. It was reported that serum MDA elevates in oxidative stress status. Increased reactive oxygen species (ROS) may cause intracellular damage causing an elevation in MDA levels.[10] Total antioxidant capacity (TAOC) is the serum ability to suppress free radical production that leads to protect the cell from molecular damage. Total antioxidant capacity measures the antioxidant power of all components, including proteins, vitamins, glutathione, etc.[11] PCOS is associated with an increase in insulin resistance, body mass index (BMI) and lipid profile; all these factors may lead to an elevation in oxidative stress and increased severity of PCOS.[12]

PCOS is a pro-inflammatory disorder accompanied by chronic low-grade inflammation and rise of cytokine and inflammatory mediator, this chronic inflammation leads to dysfunction in the ovaries and abnormality in the metabolism of the patients and also effect on endometrium implantation. Interleukin-18 (IL-18) is proinflammatory cytokine, which belongs to the IL-1 superfamily and has M.wt. 18–19 kDa work to prompt production of TNF-α, which in turn activates the synthesis of IL-6, the later regulate CRP synthesis from the liver. The recent studies correlate between polymorphism in its gene and PCOS pathogenesis, were reported that the IL-18-137G/C polymorphism increased risk of the disease.[13-15]

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Due to the importance of this disease and its spread in the Iraqi society with its great impact on Iraqi women life; this study was designed to investigate the relationship between this SNP (rs 2002555) and the hormonal fluctuations that occur in polycystic ovarian disease and the effect of this polymorphism on oxidative stress status and inflammatory cytokine IL-18 among a sample of Iraqi women, with Ethnic homogeneity, especially in south of Iraq.

MATERIALS AND METHOD

This study included 80 patients with PCOS and 60 apparently healthy women as controls. Specialist gynecologists diagnosed polycystic ovarian woman according to Rotterdam criteria, where each patient should have at least two of those criteria to be considered as a PCOS patient: [1) irregularity of menstrual cycle (oligo-ovulation or anovulation); 2) hyperandrogenemiaa (clinically or by biochemically); and 3) PCOS on USG]. The blood samples were taken from all women involved in the study in the 2nd to 3rd day of the menstrual cycle. The BMI was calculated by measurement of height, and weight of patients and control then applied the following equation: BMI = (weight kg/height m2).

The levels of the following hormones in the serum {follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), Testosterone) were measured by VIDAS method while the level of Anti-mullerian hormone was estimated by enzyme-linked immunosorbent assay (ELISA kit from Anshlab, USA) and total antioxidant capacity by colorimetric method and IL-18 by ELISA (kits supplied by Elabscience, USA). The concentration of MDA measured by the thiobarbituric acid (TBA) method.

DNA isolation and genotyping

DNA was extracted from the blood samples for patients and controls by the Promega DNA extraction kit, and the DNA concentrations were measured by Quantiflor DNA System, Promega. Genotyping then performed by the real-time PCR in Mic qPCR cycler with the technique of allelic discrimination analysis, where we examined SNP on the gene of the receptor of the anti-mullerian hormone *AMHR2* (rs2002555).

RESULTS

The result in Table 1 showed that there was a statistically significant difference between patients and the control group in the serum levels of hormones FSH, LH, LH/FSH ratio, testosterone, prolactin, and AMH ($p < 0.01$ for each). The table also showed that there was an elevation in the BMI in PCOS patients, too, in comparison with the control group ($P < 0.01$).

The results in Table 2 shows there is statistically important difference between group of PCOS women and control according to oxidative stress marker (MDA and TAOC) and inflammatory marker (IL-18) with $p < 0.01$.

Receiver Operator Curve (ROC) Analysis

To differentiate between group of PCOS patients and groups of controls group by using the vital investigated parameters, the ROC analysis was applied. Such analysis allows to arrange the parameters according to the ROC area that can occupy and if such occupation is significant or not, where AUC best measure of the overall accuracy, value of AUC if equal to 0.5 represents random discernment; values between 0.7 and 0.8 are reflected acceptable discernment; 0.8–0.9 as excellent discernment, and values larger than 0.9 are considered outstanding discernment. From the area under the curve and confidence intervals, can see that FSH and LH is inferior to the other because the entirety of its interval lies below the others. AMH Show the larger AUC (0.994, CI 98-100), optimal cut-off for AMH was 7.48 ng/ml, yielding 100% sensitivity and 92 % specificity. AMH test shows the highest sensitivity and specificity compared to other parameters. While other parameters ordered as following according to AUC (testosterone = 0.97; IL-18 = 0.96; MDA = 0.95; TAOC = 0.94; PRL = 0.87, LH = 0.80; FSH = 0.78) and the MDA, Testosterone, IL-18 and TAOC sensitivity and specificity at the optimal cut off point high compared to the FSH, LH and Prolactin. This cutoff point was defined as the

Table 1: demographic characteristic and Serum level of hormones in patients and control

			Mean ± SE					
Group	*Age*	*BMI*	*FSH (mIU/ ml)*	*LH (mIU/ ml)*	*LH/FSH ratio*	*Prolactin (ng/ml)*	*Testosterone (ng/ml)*	*AMH (ng/ml)*
Patients Group No. 80	24.23 ± 0.64	30.83 ± 0.61	4.25 ± 0.17	7.09 ± 0.48	1.844 ± 0.17	22.63 ± 1.31	1.302 ± 0.07	14.65 ± 0.46
Control Group No. 60	25.68 ± 0.81	25.79 ± 0.54	5.90 ± 0.19	3.37 ± 0.12	0.585 ± 0.02	9.02 ± 0.54	0.425 ± 0.1	5.02 ± 0.26
T-test	1.408 NS	5.900 **	6.211 **	7.347 **	7.162 **	9.570 **	11.667 **	18.112 **
P-value	0.161	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

** ($p<0.01$), NS: Non-Significant.

Table 2: Compare between patients and control in oxidative stress marker and IL-18

	Mean ± SE		
Group	*MDA*	*TAOC*	*IL-18*
Patients	3.557 ± 0.08	15.77 ± 0.93	225.08 ± 8.09
Control	2.098 ± 0.06	38.16 ± 1.54	101.26 ± 3.30
T-test	14.05 **	12.41 **	14.17**
p-value	0.0001	0.0001	0.0001

** ($p < 0.01$).

closest point to ''full sensitivity'' and "full specificity" on the ROC curve. Table 3 and Figure.1 illustrates the ROC curve, AUC, sensitivity and specificity, optimal cutoff point, P-value, and confidence interval.

The results in Table 4 illustrate the positive correlation between AMH and BMI, Testosterone and IL-18. Testosterone and IL-18 strongly correlated with AMH (r = 0.484; p = 0.0001), (r = 0.476; p = 0.0001) respectively. The table also shows the negative correlation between AMH and TAOC (r = –0.329; p = 0.003). Whereas other parameters did not reach to significantly related.

The Table 5 and Figure 2 showed the frequency of AMHR2 gene SNP (rs2002555) is more common in patient with polycystic ovary then control, where (31.3% for patients and 20% for control) (P = 0.033). The table also showed the allele frequency where A allele has 0.81 and 0.88 in patients and control respectively, while G allele have 0.19 and 0.12 in group of patients and control, respectively.

Table 6 show the demographic characters and serum levels of hormones (AMH, FSH, LH, LH/FSH, PRL, and Testosterone), oxidative stress marker (MDA and TAOC) and inflammatory marker(IL-18) affected by *AMHR2* -482 A > G Polymorphism in PCOS and control groups. The serum concentration of the LH hormone significantly decreased (p <0.01) in patients carry G allele (3.58 ± 0.55) in homozygous polymorphism GG. There is a statistically significant decrease in LH/FSH ratio in a group of patients who carrying this polymorphism but not in control. We also note a significant decrease in the level of prolactin hormone (p < 0.01). The result of this study also indicated that the level of total antioxidant capacity significantly increased (p < 0.05), in the patient group who carry this polymorphism. The results show there is no

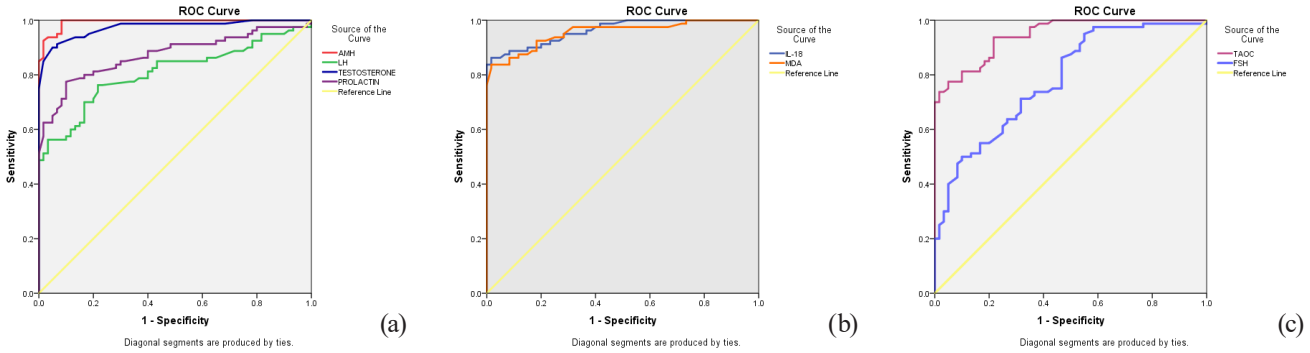

Figure 1: Receiver Operator curve analysis. (a)and (b) ROC curve of AMH, LH, prolactin, testosterone, MDA, and IL-18, more significant test result indicates more positive results. (c) ROC curve of FSH and TAOC, smaller test result indicates more positive result

Table 3: ROC curve analysis for the investigated parameters and sensitivity and specificity

						Asymptotic 95% Confidence Interval	
Test result variable(s)	*AUC*	*Optimal cut-off value*	*Sensitivity*	*Specificity*	*p-value*	*Lower Bound*	*Upper Bound*
AMH	0.994	7.48	100%	92%	0.0001**	0.99	1.000
MDA	0.955	2.53	92%	82%	0.0001**	0.924	0.987
LH	0.808	4.05	76%	79%	0.0001**	0.736	0.880
Testosterone	0.974	0.66	90%	93%	0.0001**	0.951	0.997
prolactin	0.873	13.22	80%	83%	0.0001**	0.814	0.932
IL-18	0.963	127.90	90%	85%	0.0001**	0.936	0.989
FSH	0.782	5.05	70%	69%	0.0001**	0.707	0.857
TAOC	0.945	25.34	85%	82%	0.0001**	0.913	0.978

** (P < 0.01)

Table 4: Correlation between AMH with other parameters in PCOS patient group

Parameters	*Pearson correlation*	*p-value*
BMI	0.268	0.016*
FSH	-0.016	0.886 NS
LH	0.113	0.320 NS
LH/FSH	0.063	0.579 NS
Prolactin	-0.096	0.395NS
Testosterone	0.484	0.0001**
MDA	0.074	0.516 NS
IL-18	0.476	0.0001**
TAOC	-0.329	0.003**

*Correlation is significant at the 0.05 level.
**Correlation is significant at the 0.01 level.
NS: Non-Significant .

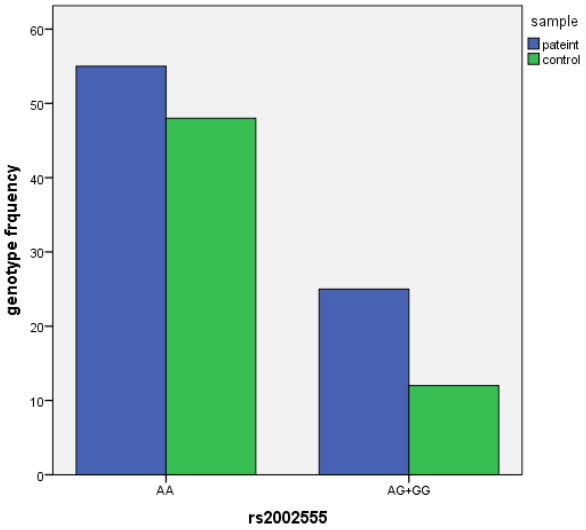

Figure 2: The genotype frequency of AMHR2 -482 A > G SNP in PCOS women and control

Table 5: Polymorphism and allele frequency of AMHR2 -482 A > G in patients and control

	Patients		*Control*		
Polymorphism	*No*	*%*	*No*	*%*	*P-value*
AA	55	68.8	48	80	0.490 NS
AG+GG	25	31.3	12	20	0.033*
Total	80	100%	60	100%	---
Allele frequency					
A	0.81		0.88		NS
G	0.19		0.12		NS

* ($p < 0.05$), NS: Non-significant.

Table 6: Serum levels of hormones and other biochemical marker affected by AMHR2 -482 A > G Polymorphism

	Genotype (patient)			*LSD*	*Genotype (control)*			*LSD*
Parameters	*AA*	*AG*	*GG*	*(P-value)*	*AA*	*AG*	*GG*	*(p-value)*
Age	23.76 ± 0.752	24.55 ± 1.396	28.20 ± 2.557	5.352 (0.252 NS)	26.02 ± 0.919	24.30 ± 2.129	24.50 ± 1.50	9.215 (0.715NS)
BMI	30.88 ± 0.77	30.72 ± 0.94	30.78 ± 3.63	5.186 (0.994 NS)	25.72 ± 0.643	26.62 ± 1.038	23.34 ± 1.0	6.151 (0.593NS)
FSH	4.28 ± 0.23	4.12 ± 0.23	4.41 ± 0.72	1.491 (0.898 NS)	5.94 ± 0.22	5.74 ± 0.45	5.58 ± 0.28	2.207 (0.890 NS)
LH	8.35 ± 0.63	4.48 ± 0.28	3.58 ± 0.55	3.715 (0.0001**)	3.32 ± 0.14	3.65 ± 0.31	3.20 ± 0.30	1.460 (0.613 NS)
LH/FSH	2.20 ± 0.23	1.107 ± 0.5	0.88 ± 0.16	1.382 (0.008**)	0.571 ± 0.02	0.657 ± 0.06	0.571 ± 0.02	0.238 (0.325 NS)
Prolactin	25.35±1.46	18.96±2.64	7.41±2.41	10.15 (0.001**)	9.58 ± 0.90	6.55 ± 1.19	8.00 ± 4.00	5.983 (0.111 NS)
Testosterone	1.31±0.09	1.29±0.14	1.30±0.30	0.618 (0.995 NS)	0.438 ± 0.02	0.376 ± 0.03	0.360 ± 0.04	0.180 (0.273 NS)
AMH	15.16±0.56	13.65±0.93	13.11±1.10	3.824 (0.263 NS)	4.97 ± 0.29	4.73 ± 0.63	7.51 ± 0.71	2.887 (0.196NS)
MDA	3.49 ± 0.106	3.73 ± 0.13	3.47 ± 0.22	0.684 (0.450 NS)	2.13 ± 0.07	2.01 ± 0.16	1.76 ± 0.42	0.716 (0.480 NS)
TAOC	14.36 ± 1.14	17.67 ± 1.58	23.64 ± 2.98	7.498 (0.027*)	38.49 ± 1.85	36.31 ± 2.28	39.61 ± 9.28	17.55 (0.862 NS)
IL-18	226.66 ± 10.02	224.65 ±14.92	209.40±38.13	68.04 (0.880 NS)	101.82 ± 3.88	97.29 ± 6.97	107.61 ± 0.62	37. 52 (0.829 NS)

** ($p < 0.01$),* ($p < 0.05$), NS: Non-Significant .

association with the rest of marker in groups of patients or control**.**

DISCUSSION

Comparison between PCOS and Control Groups according to different parameters

PCOS is one of the most complicated and debilitating disease associated with hyperinsulinemia and insulin resistance, and there is chronic low grad inflammation and increasing in oxidative stress status. The insulin resistance acts directly on increasing testosterone production (hyperandrogenemia) and have strong correlation with OS and inflammations.[16] Obesity also have a negative effect on PCOS and associated with increasing oxidative stress and inflammation ,result of this study supports these finding where shows increased in IL-18 and MDA in the serum of PCOS patients when compared with control, while level of TAOC decrease in PCOS due to decreasing in antioxidants in PCOS.[17,18] The AMH hormone also show to have main role in this syndrome as a diagnostic and mirror marker for PCOS status and severity as shown in results the AUC in the ROC curve (0.99 at the CI 0.99-1.000) with high sensitivity and specificity, the optimal advantage of this assay the women can preformed in any day in menstrual cycle not limited in follicular phase as in other hormones,these finding in line with several studies, this increased reflect multiple number of small follicles that's feature of PCOS and the recent study prove there is increased for production of hormone from each follicle.[2,19] The AMH show to have a positive correlation with BMI in a group of the patient where its level increased in an obese woman that reflects increased severity of the disease and prove the bad effect of obesity on women with PCOS.[20] The strong positive correlation between AMH and testosterone related to the fact that AMH have inhibitory effect on aromatase activity that lead to reduce activity of this enzyme in the granulosa cell and elevation androgen secretion.[21] Metabolic disturbance and obesity associated with increased severity of PCOS lead to increment in the oxidative stress and elevation in the inflammatory markers. IL-18 Shows to have large AUC and high sensitivity and specificity that indicate great discrimination power along with TAOC a marker for oxidative stress condition.[17]

Correlation between AMHR2-482 A > G polymorphism and other Parameters in PCOS

Genetic factors play a crucial role in the initiation and development of PCOS. Studies on twins have shown that genetic variations contribute to the development of PCOS disease.[22,23] Genetic variation in several candidate genes in PCOS development has been elucidating, including genes associated with gonadotropic secretion and action, steroid biosynthesis and action, folliculogenesis, and insulin action.[24] In this study, it was focused on single nucleotide polymorphism in the gene of anti-mullerian hormone receptor two -482 A>G and their association with PCOS according to the Rotterdam criteria. The result showed that the frequency of AMHR2 polymorphism is more in woman with PCOS compared to norm ovulatory control, the results of studies about the association of genetic polymorphism in the AMHR2 and PCOS is inconstant, where the Kevenaar *et al*.[25] fail to prove association between this polymorphism and PCOS, this failure may be due to the ethnic diversity of patients and control and very small number of population include in the study, while our study was conducted in southern Iraq, where there is ethnic homogeneity and also shared the same environment and residential area and this is consistent with the study conducted by Georgopoulos et al, where it proved that there is a relationship between this genetic polymorphism and PCOS disease.[7] There is an increase in the concentration of the AMH hormone in the patient serum compared to a normal woman. Although the current study does not show a significant difference in the concentration of AMH among genotype of AMHR2 polymorphism, this need further investigation, but the results support the incidence endogenic dysfunction in the follicles through alteration in the AMH signaling associated with this polymorphism. The studies on the AMHR2rs 2002555 were found that have association with high level of estradiol hormone during follicular phase of menstrual cycle in women that have normal ovulation[26] signifying the important role of AMH receptors signaling during the regulation of AMH for FSH sensitivity in the ovary and this polymorphism also have correlation with age at menopause and this spotlight on important of AMH signaling on the consumption of ovarian reserve.[26]

The 482 A > G polymorphism reported to have an important location at transcription factor –binding site (c-Myb and c-Myc)[27] and consequently may modify the activity of the promoter. The study on the effect of LH on the expression of AMHR2 showed the expression of AMHR2 reduce in the granulosa cell only in a woman with norm ovulatory, and there is no effect on women with PCOS.[28]

We can assume that alteration in a hormonal profile associated with PCOS has a major effect on the G allele of AMHR2 in transcription and bioactivity but no on A allele, proposing that the G allele have enhancing role on the pathogenesis and phenotyping of PCOS. In the serum of women with PCOS there is increased in the concentration of LH hormone and this correlated with increased in AMH hormone, in spite of the fact that the AMH level is not correlated with AMHR2 -482 A > G genetic polymorphism but this SNP decreasing AMH signaling,[7] and their association with lower LH and LH: FSH ratio need further investigation and the mechanism that lining is not clear. Also there is lower level of prolactin associated with this SNP is more difficult to explain .when study association of oxidative stress marker MDA and Total antioxidant capacity(TAOC) found the concentration MDA not affected by the polymorphism while the TAOC elevated in the patient who carries G allele and this may be explain ameliorated of the phenotyping of PCOS with G allele and this increase in antioxidant status may have positive effect on markers in PCOS, where the increased in oxidative stress indicate severity of PCOS recent research has found that giving

patient with PCOS doses of antioxidant help in management of disease and reduce severity.[29]

In conclusion, the AMHR2 (rs2002555) genetic polymorphism have effects on both phenotyping and pathogenesis of PCOS, and we need to further studies on this polymorphism to highlight all their relationships in order to better understanding of these variations in the hormones associated with this genetic polymorphism in women with polycystic ovaries.

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