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Fadhil Jawad Al-Tu'ma[1]; Noor HadiFarhan[1] and Wasan Ghazi Al-Safi[2]

[1]Department of Biochemistry, College of Medicine, University of Kerbala / Holy Kerbala – Iraq.
[1]Department of Biochemistry, College of Medicine, University of Kerbala / Holy Kerbala - Iraq.
[2]Department of Gynecology and Obstetrics, College of Medicine, University of Kerbala / Holy Kerbala - Iraq.

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ABSTRACT

Background: Poly Cystic Ovary Syndrome (PCOS) is a complex challenging disorder with strongly supported genetic roles and represents the most endocrine disturbance of women in reproductive age affecting 5-10% of the women and for understanding the etiological molecular genetics at mass and obesity-associated (FTO) gene with rs9939609 polymorphism has been investigated that recently been associated with obesity, the most risk factor for metabolic consequences in PCOS. Materials and methods: This case control study investigates genetic susceptibility of 70 women in Iraqi/ Karbala population with PCOS for the presence of SNPs (Single Nucleotide Polymorphisms) of FTO rs9939609 polymorphisms compared with 30 healthy control women. The presence or absence of the polymorphism was determined. Results: The association between rs9939609 variant of FTO gene and PCOS susceptibility was examined. The A allele was significantly more common in PCOS patients than in the control group (AA allele 65.71% compared with 31.42% of (TT) allele with no (AT) allele found in PCOS patients compared with 43.33% (AA) allele, 26.67% (TT) allele in control group. FTO gene polymorphism shows significant correlation with BMI in PCOS patients. Conclusion: FTO rs9939609 gene variant shows significant association with PCOS women and its related with BMI as a probable cause for PCOS susceptibility in Iraqi population.

INTRODUCTION

Polycystic ovary syndrome is an endocrine-metabolic disturbance which has features of multiple hormonal imbalances that produce short and long term consequences on women health (Kollmann et al. 2014). The prevalence of PCOS affecting about 9–18% of studied population depending on the criteria of diagnosis applied (March et al., 2010). Its heterogeneous disorder characterized by oligo/ amenorrhea, clinical and/ or biochemical features of hyperandrogenism and polycystic ovaries detected by ultrasound (Baldani et al. 2012). The etiology of PCOS is not fully identified, but it can be described as an oligogenic disorder as the interaction of a number of genetic and environmental factors may determine the heterogeneous, clinical, and biochemical phenotype of this syndrome (Fauser et al. 2011).

A genome-wide association study identified the first major success in obesity genetics with the discovery of the fat mass and obesity-associated (FTO) gene as an obesity susceptibility gene (Frayling et al. 2007). The FTO gene is a very large gene with nine exons span more than 400 kb located on the long (q) arm of chromosome 16 at position 12.2 and its expressed in a wide range of tissues, including the adipose tissue and specific areas of the brain and muscles, suggesting its potential role in body weight regulation (Wehr et al., 2010).

FTO is highly expressed in brain regions such as the hypothalamus and act by controlling feeding and energy expenditure (Taggart et al., 2011). Several polymorphisms of the gene have been described. The variant FTO rs9939609 is the most extensively studied, located within the first FTO intron has two alleles, A and T, the former has been linked to an increased risk of both obesity and type 2 diabetes mellitus (Liu, et al. 2010).

The FTO gene has recently been shown to influence a person's predisposition to obesity and is now the first gene to be associated convincingly with susceptibility to PCOS. PCOS is strongly associated with obesity, and FTO risk allele was associated with a significant increase in BMI among PCOS. It is thought that the prevalence of PCOS will increase with rising levels of obesity (Barber et al., 2008). Many studies have been appeared to investigate the association between FTO gene polymorphism and the susceptibility of PCOS. Some studies reported that FTO rs9939609 polymorphism was positively associated with PCOS (Yan et al., 2009; Li et al.,

2013). While others showed no significant association of FTO gene polymorphism with PCOS (Ewens et al., 2011; Kim et al. , 2014).

Data regarding these associations of rs9939609 genotype of FTO polymorphism with PCOS in Iraqi population have not been studied before. In this study, the association of FTO gene rs9939609 polymorphism with PCOS susceptibility has been investigated in Iraqi PCOS patients and the effect of this genetic variant on other biochemical parameters has been determined.

MATERIALS AND METHODS

This study was carried out from Dec. 2014 till June 2015 a period of case-control study of a total number of 100 women within the reproductive age (18-45 years old). Seventy women patient out of 100 were attended from gynecological and obstetric hospital in Karbala province, and they all diagnosed by their physicians as Polycystic Ovary Syndrome (depending on Rotterdam criteria), the patient meets two of the following three criteria: (i) oligomenorrhea or amenorrhea for at least 6 months; (ii) clinical and/or biochemical signs of hyperandrogenism; (iii) polycystic ovaries on ultrasound (Rotterdam ESHRE/ASRM PCOS Consensus Workshop Group, 2004) and they compared with thirty healthy control women with comparable age. Thirty, healthy, regular menstrual cycle women, fertile control group with age range (18-45 years). The history recorded for the infertility type (primary, secondary), menstrual history, history of previous medical condition, drug history in addition to biochemical, hormonal, ultrasound records. Exclusion criteria includes postmenopausal women (age >45 years), other disorders that may affect menstrual regularity and hyperandrogenism such as adrenal tumor, congenital adrenal hyperplasia, Cushing syndrome, thyroid dysfunction, and pituitary disease that related with hyperprolactinemia, causes of infertility other than PCOS (female and/or male factors) (Vause et al., 2010). BMI of each patient defined as weight (kg) / height (m^2) was calculated.

Blood Samples collection

Five milliliters of venous blood were aspirated by using disposable syringe after an overnight fasting and divided as follow:

Four m were put in-plane tube and left to clot for 15 min. and then centrifuged at 3000 rpm for 10 min. for serum collection, then serum was divided into plastic curettes one to estimate fasting

glucose, lipid profile and the second serum tube for hormonal estimation. The remaining volume (1.0 ml) of venous blood that remaining was put in EDTA tube and preserved at -4^{o}C for DNA extraction.

Biochemical parameters measurement

Serum hormonal analysis of LH, FSH, and total testosterone were measured by using MiniVidas instrument. Serum levels of glucose, total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol were measured by a commercially available kit (all kits from Biomerieux, Sa, France) and it's measured by spectrophotometrically by using UV-Visible spectrophotometer. Low-density lipoprotein-cholesterol (LDL-C) and very low-density lipoprotein-cholesterol (VLDL-C) levels were calculated using the formulae (Friedwald et al., 1972).

Genotype analysis

Genomic DNA was isolated from blood samples using a DNA purification kit (Bioneer-Korea). The FTO SNP rs9939609 was genotyped using ARMS-PCR. Primers were derived from genomic rs9939609 polymorphism of FTO gene are: Fout: 5'-TGG CTC TTG AAT GAA ATA GGA TTC AGA A-3'; Rout: 5'-AGC CTC TCT ACC ATC TTA TGT CCA AAC A-3' ; Fin: 5'-TAG GTT CCT TGC GAC TGC TGT GAA TAT A-3' ; Rin: 5'-GAG TAA CAG AGA CTA TCC AAG TGC ATC TCA-3' (product size outer primers: 321 bp, T-allele: 178 bp, A-allele: 201 bp) (Müller et al, 2008). PCR was carried out in a 20 µl premix tube mixture containing: added 1 µL from outer F primer, 1 µL from outer R primer, 0.7 µL of inner F (A) primer and 0.7 µL of inner R (G) primer 5 µ L of DNA product and complete the volume up to 20 µL with distilled water.

The PCR run cycles are:

Cycle 1	93^{o}C	5 minutes
	93^{o}C	30 seconds
Cycle 2	53^{o}C	25 seconds
	72^{o}C	25 seconds
Cycle 3	72^{o}C	5 minutes

Electrophoresis by using agarose gel stained by ethidium bromide used for validity of genotype of PCR, ladder (100 bp) used as standard for alleles comparison.

Statistical analysis

SPSS (Statistical Package for Social sciences) version 22 software program was utilized to perform data analysis. Numeric variable were presented as Mean± SE (Standard Error), Mean ± SD (Standard Deviation) while nominal variables were expressed as number and percent. Independent samples t-test was used for comparison between means of numeric variables. Chi-square test was used for comparison of frequency for genotype analysis and it's also used for describing the correlation between genetic alleles with other numeric variables. Pearson's correlation coefficient was used to study correlation between numeric variable in a specified group (for patients and control group respectively). P value was considered significant when it is less than 0.05.

RESULTS

Regarding BMI, table (1.1) shows significant difference between patients and control groups (P <0.05). The mean BMI for patients group (mean± SE 31.3± 0.64) was significantly higher than control group (mean± SE 27.27± 0.45). The higher percentage of PCOS patients (42.28%) were found among obese women with BMI (30-34.9 Kg/m^2) compared with (2%) of healthy normal women at the same range. PCOS patients lowest percentage was found among those with morbid obesity (> 40 kg/m^2) with BMI (2%) compared with absence of healthy sample at the same range. No significant difference has been found in serum triglyceride, VLDL-C, fasting glucose level when comparison between patients and control occur as below in table (P >0.05). Table (2) show significant correlation of BMI with triglyceride, LDL-C, VLDL-C with no correlation appeared of BMI with other biochemical parameters. Significant difference found in LH, FSH and total testosterone between PCOS as compared with healthy women (P<0.05).

Significant difference in FTO gene mutation has been found with frequent association in PCOS patients when compared with control group, allele AA is the major allele with percentage of 65.71% in contrast with 31.42% for TT allele in PCOS women with significant correlation has been found between FTO gene and BMI (Chi-square value=22.48, P < 0.05) while there is no correlation found with other parameters with FTO gene in patient group and control group.

Table (1): Comparison between patients and control ages using independent samples t-test

	Patient (n=70)		Control (n=30)		Total (n=100)		P< 0.05
Age interval	No.	%	No.	%	No.	%	
<25 years	35	50	4	13.3	39	39	
25-35years	31	44.3	21	70	52	52	
35-45years	4	5.7	5	16.7	9	9	
Total	70	100	30	100	100	100	
Mean±SE	24.87±0.54		29.5±0.85				4.496**
Mean±SD	24.87±4.52		29.5±4.79				

**: independent sample t test, SE: standard error, %: percentage, No.: number, SD: standard deviation

Table (2): The mean serum concentration of biochemical parameters between PCOS and healthy control group

Biochemical Parameters	Patients (n=70)			Control (n= 30)			
	mean	**SE**	**SD**	**mean**	**SE**	**SD**	**P Value**
Total cholesterol (mg/dl)	151.357	4.13	34.58	131.63	1.99	10.91	4.299*
Triglyceride (mg/dl)	101.32	4.24	35.49	93.56	3.88	21.63	1.35
HDL-C (mg/dl)	33.95	0.76	7.09	42.8	1.58	4.32	5.035*
LDL-C (mg/dl)	97.15	3.99	33.43	68.26	3.25	17.83	5.605*
VLDL-C (mg/dl)	20.26	0.85	7.09	18.64	0.79	4.32	1.398
Glucose (mg/dl)	95.16	3.09	25.85	100.03	4.12	22.59	0.895
LH	8.941	0.424	3.547	5.4	0.252	1.383	7.173*
FSH	4.63	o.202	1.694	9.39	1.526	1.526	13.246*
LH/FSH ratio	2.316	0.228	1.908	0.57	0.015	0.087	7.637*
Testosterone	0.78	0.04	0.35	0.26	0.02	0.13	10.744*

*: significant difference (P<0.05), P-value: level of significance, SE: standard error, SD: standard deviation, HDL-C: high density lipoprotein- cholesterol, LDL-C: low density lipoprotein-cholesterol, VLDL-C: very low density lipoprotein-cholesterol, LH: luteinizing hormone, FSH: follicle stimulating hormone.

Table (3): FTO allelic distribution comparison between patients and control groups using Chi-square test

Sample	Outer allele		AA allele		TT allele		AT allele	Total	P value
	No.	%	No.	%	No.	%			
Patient	2	2.85	46	65.71	22	31.42	0	70	16.007*
Control	9	30	13	43.33	8	26.67	0	30	
Total	11	11	59	59	30	30	0	100	

*: degree of significance when p< 0.05, No.: number, %: percentage, A: Adenine, T: thymine

Figure (1): Detection of rs9939609 gene polymorphism by ARMS-PCR. PCR products with three possible genotypes (AT, AA, or TT).

DISCUSSION

PCOS is a complex disorder that may result by the susceptibility of gene variants under the influence of environmental factors (Deepika et al., 2012).

Fat mass and obesity associated gene (FTO) was the first gene discovered by Frayling et al. 2007 in three independent genome wide association studies found to be associated with common forms of obesity. Although FTO gene has large size, variants implicated in obesity and weight gain are largely located in the first intron with the association of common FTO variants, especially rs9939609 (Shabana & Shahida, 2015). It has been suggested that it may regulate the expression of genes involved in metabolism and that disturbance in regulation of this process may lead to obesity (Wehr et al, 2010).

This study find out a high significant difference of FTO gene rs9939609 polymorphism between patients and control group and it found that allele AA is the major allele with percentage of 65.71% in contrast with 31.42% for TT allele and this finding was supported by up to date studies that performed to investigate the association between FTO gene polymorphism and the PCOS susceptibility which indicates that FTO rs9939609 polymorphism was positively associated with PCOS. A German study concluded that the stronger effect on body weight of the FTO SNP in PCOS may have an association with the etiology of the disease (Susanne et al., 2010). While other study showed no significant association of FTO gene polymorphism with this disorder (Kimm et al. 2012). The differences in association of FTO gene with PCOS in different studies may be due to many factors, including the limitation in statistical strength and if BMI was adjusted in each study population. The FTO gene has recently been shown to influence a person's predisposition to obesity, and is now the first gene to be associated convincingly with susceptibility to PCOS. PCOS is strongly associated with obesity, and FTO risk allele was associated with a significant increase in BMI among PCOS it is thought that the prevalence of PCOS will increase with rising levels of obesity (Barber et al., 2008).

This study show significant correlation between FTO gene polymorphism and BMI in PCOS group and these results are consistent with researchers in UK study about whether genetic variation in FTO influences risk of PCOS, have shown at the first time that FTO gene that implicated in the development of obesity is also associated with susceptibility to polycystic

ovary syndrome (Barber et al.2008). An Egyptian study concluded that rs9939609 variant in the FTO gene is associated with PCOS susceptibility in the Egyptian population, probably because of its effect on body mass index (Hussein et al., 2014). Another European study found a significant association with PCOS only in patients exhibiting obesity or metabolic syndrome (Attaoua et al., 2008). Other studies show consistent result of association of FTO gene in PCOS and its two times greater than normal control population (Wojciechowski et al., 2012). While other Chinese study reported an initial significant association between rs9939609 and PCOS was but not related to BMI (Yan et al., 2009).

FTO gene polymorphism shows no significant correlation with biochemical parameters in this study while other studies show that FTO variants not only in obesity and diabetes but also related to hyperandrogenism in women with PCOS (Wehre et al. 2010). But most studies reported significant associations with BMI in PCOS patients; therefore, it seems that possible effects of FTO in PCOS are related to the metabolic phenotypes.

The nature of the relationship between FTO and PCOS, however, remains unclear because of possible improvement of the reproductive phenotypes of PCOS after weight loss (Kowalska et al., 2009). The differences can be explained depending on BMI and variation in the identification of cutoff category of obesity, ethnicity, sample size, and behavioral and environmental factors (Shabana & Shahida, 2015). The etiology of obesity in PCOS remains unclear, but it shows pathologic role in the progress of this syndrome (Ronald et al., 2003). The mostly women that diagnosed as PCOS was found to be either overweight or obese at percent between (20-85%) (Bays, 2009). This study found out that higher percentage of PCOS is found with obese patients BMI (30-34.9 kg/m^2) with (42.28%) and it shows significant difference when compared with healthy control group that shows (2%) in this category group. Obesity and the abnormality of lipid profile parameters in PCOS women suggesting increased risk of developing metabolic consequences and Cardio Vascular Disease.

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