

Original Research Article

Association of T45G Genetic Polymorphism in The ADIPOQ Gene with Polycystic Ovary Syndrome Patients in Al-Najaf Province

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Abstract

This study was suggested to investigate genetic variation in ADIPOQ gene as a risk factor for polycystic ovary syndrome progression. Fifty-four Iraqi women with PCOS consulting at a Fertility and infertility treatment center were inducted into this study with a mean age was (24.23+5.19) years for these patients. Also, the study includes forty-eight normal healthy women collected randomly with mean age (23.81+6.01) years as a control group. The collection of blood samples carries out from PCOS and healthy control individuals for detection of biochemical diagnostic parameters in serum which include luteinizing hormone (LH) and follicles stimulating hormone (FSH), testosterone and adiponectin. Then, DNA was extracted from the blood of these two groups for a revelation of 45T/G genetic polymorphism in exon 2 of ADIPOQ gene for revealing of Polycystic Ovary Syndrome (PCOS) as a prognostic marker for this disease.

The result of the current study appears that the serum adiponectin levels in PCOS decreased significantly when compared its level in control. In contrast, the level of LH and testosterone increased significantly in PCOs group than control. In regarding to genetic polymorphism 45(T/G) in exon 2 of ADIPOQ gene, the TT alleles polymorphism were increased significantly in control than PCOS patients while TG, GG alleles polymorphism were increased significantly in PCOS than control. The correlation between ADIPOQ polymorphism and level of adiponectin in serum demonstrated that the level of adiponectin is decreased significantly in presence of G allele in ADIPOQ gene in PCOS patients.

This study concluded that the 45T/G polymorphism in ADIPOQ gene is highly elevated in PCOS patients especially polymorphism in GG alleles may be regarded as one of the main causes of PCOS occurrence. This result was proven the role of ADIPOQ gene in the pathogenesis of this syndrome.

Key Words: PCOS, ADIPOQ, 45T/G polymorphism

العلاقة بين التغيرات الوراثية T45G في جين الاديبيوك مع مريضات تكيس المبايض في محافظة النجف

الخلاصة

أجريت الدراسة الحالية خلال الفترة من شهر تموز ٢٠١٦ الى شهر كانون الاول ٢٠١٦ . تم جمع العينات في مركز الخصوبة ومعالجة العقم في مستشفى الصدر التعليمي في محافظة النجف بينما تحليل العينات تم في مختبر البيولوجي الجزيئي في كلية العلوم/جامعة الكوفة. شملت الدراسة اربعة وخمسون امرأة عراقية مصابة بمرض تكيس المبايض ممن تراجع مركز الخصوبة ومعالجة العقم ،معدل اعمارهن (24.23+5.19) سنة. كما شملت الدراسة اربعة وتسعون امرأة سليمة جمعت بصورة عشوائية معدل اعمارهن (23.81+6.01) سنة كمجموعة سيطرة . تم جمع عينات الدم من النساء المريضات والسليمات لتقييم مستويات هرمونات LH ،FSH ، testosterone و adiponectin كمعايير كيميائية لتشخيص المرض. بعد ذلك تم استخلاص الدنا من دم المصابات بتكيس المبايض والسيطرة لتحديد التغيرات الوراثية 45T/G في جين الاديبيونكتين كمعلم تنبؤي لتشخيص مرض تكيس المبايض.

تشير نتائج الدراسة الحالية الى وجود انخفاض معنوي في مستوى الايبونكتين في مريضات تكيس المبايض مقارنة مع مستوياته في السيطرة. بالمقابل فان مستويات هرمون LH و testosterone تزداد معنويا في مريضات التكيس مقارنة مع السيطرة. بالنسبة لدراسة التغيرات الوراثي (T/G)+45 في جين الايبونكتين في الاكسون ٢ فقد وجد بان التغيرات الوراثي في الايلات TT ازداد بشكل معنوي في السيطرة عنها في مجموعة المريضا بتكيس المبايض بينما التغيرات الوراثي في الايلات GG, TG تزداد معنويا في مجموعة المريضا عنها في السيطرة. كما تم دراسة العلاقة بين التغيرات الوراثي في جين الايبونكتين مع مستوى الايبونكتين في مصل الدم وقد تم ملاحظة وجود انخفاض معنوي في مستوى الهرمون عند وجود الايل G في جين الايبونكتين في مريضات التكيس. استنتجت الدراسة الحالية بان هناك تغير وراثي بنسبة كبيرة في جين الايبونكتين خصوصا التغيرات الوراثي في الايلات GG قد يكون هو احد الاسباب الرئيسة لحدوث مرض تكيس المبايض. نتائج هذه الدراسة توضح وجود دور لجين الايبونكتين في عملية امراضية تكيس المبايض.

الكلمات المفتاحية: تكيس المبايض، الايبونكتين، التغيرات الوراثي 45T/G.

Introduction

Polycystic ovary syndrome (PCOS) is one of the most prevalent endocrine disorders in females, affecting about 5–10% of females at age (12–45) years, and it considered as one of the prominent causes of female infertility [1]. The causes of PCOS are still unknown; PCOS has a potent genetic origin [2]. In addition, the main characteristics of this syndrome are hyperandrogenism, anovulation, acne, and obesity. There are obvious differences between women depending on the severity and symptoms of PCOS. Women with PCOS are potential to develop of diabetes, dyslipidemia, obesity and insulin resistance [3].

Adipose tissue is an endocrine organ secretes proteins called adipokines that observable influence glucose homeostasis, body metabolism, insulin sensitivity that causes the development of cardiovascular and metabolic disorders [4]. Adiponectin is among the main proteins involved in this process, modulating insulin action and metabolism that appears to be linked with reduction of free fatty acids and triglycerides (TG) and increase in energy dissipation. Adiponectin regulates a series of metabolic processes such as regulation of glucose and fatty acid catabolism [5]. Adiponectin (ADIPOQ) is the most common gene product in adipose tissue; it spanning a 17-kb region and comprises of two introns and three exons located on chromosome 3q27 and [6].

Adiponectin accounts about 0.01% of total plasma protein, it regarded as most

abundant adipocytokine [7]. Clinical evidence demonstrates that the women with PCOS have lower levels of adiponectin than a healthy one. Furthermore, adiponectin may have putative anti-atherosclerotic and insulin sensitizing characteristics [8]. Both adiponectin and insulin resistance (IR) are significant parameters in the development of this syndrome. Therefore, it is needful to investigate the role of this gene in the PCOS pathogenesis [9].

Approximately six adiponectin gene polymorphisms correlated with different disorders in populations that differ in their ethnic, genetic and environmental factors. Most studies have focused on two polymorphisms, G to T (276G-T) substitution in intron 2 and a silent T to G (45T-G) substitution in exon 2. These polymorphisms correlated with insulin resistance, type 2 diabetes and obesity [10,11]. On the other hand, (276G-T) and (45T-G) polymorphisms were chosen because of their high recurrence in investigated population, while other polymorphisms in this gene were infrequent, the 45T-G polymorphism is found with high prevalence in PCOS among women with low concentration of adiponectin in plasma [12].

In spite of various studies focused on the correlation between ADIPOQ polymorphisms and obesity or insulin resistance, yet, few studies have reported this polymorphism in PCOS women and these studies still limited. Therefore, we have investigated the possible relation

between PCOS and SNPs +45(T/G) in adiponectin gene in Al-Najaf province women in Iraq which may confirm the role of this gene variation in the susceptibility of PCOS occurrence.

Materials and Methods

Patients and control:

The present study was accomplished from July to December 2016, the collection of samples was done in Fertility and infertility treatment center in Al-Sadr Teaching Hospital in Al-Najaf province while the Molecular study achieved in the laboratory of molecular biology, Faculty of Science, University of Kufa.

Fifty-four Iraqi women with PCOS were enrolled into the study; the mean age of patients was (24.23±5.19 years) as the first group. This syndrome was diagnosed by the presence of any two of these three criteria: existence of chronic anovulation (less than six menstrual cycles in twelve months), presence of clinical and/ or biochemical signs of hyperandrogenism and/or hirsutism, and PCOS appearance at the ultrasound, as recommended by the Rotterdam criteria (2004). Exclusion criteria conditions included diabetes mellitus (DM), hypertension, and hyperprolactinemia, thyroid dysfunction, abnormal kidney or liver function and use of hormonal medications or other medications that could interfere with metabolic and hormonal measurements in the last three months. This study excluded the patients who treated with hormonal therapy. A second group was forty-eight normal healthy samples were collected randomly during collection of patients' samples (mean age 23.81±6.01 years) who had no hormonal abnormalities or reproductive concerns or history of menstrual irregularities. All control women had a regular menstrual cycle every 21–35 days. On ultrasound, none of these women had PCOS.

Biochemical Analysis:

After an overnight fast, 5 ml of intravenous blood was collected from healthy women and PCOS patients; the collection of these samples was carried out

from all women with a regular menstrual cycle between days 4 and 7 of the cycle. Blood samples drawn from all women for the measurement of total testosterone, luteinizing hormone (LH), serum follicles stimulating hormone (FSH) by using a mini-Vidas device (BioMerieux SA, France) while by using an enzyme-linked immunosorbent assay (ELISA) kit (B-Bridge International, USA), the serum adiponectin levels were determined.

Genetic Analysis

The collection of blood samples were performed in EDTA tubes as anticoagulant tubes and stored at 4°. By using the QIAGEN kit protocol, the extraction of genomic DNA carries out from the blood of PCOS patients and control women.

The genotyping of Single nucleotide polymorphism (SNP) +45(T/G) in ADIPOQ gene were determined by the Restriction fragment length polymorphism PCR (RFLP-PCR) for all samples. Primers of gene amplification and restriction enzymes as shown in (Table 1). The conditions of PCR amplification used for +45G15G(T/G) were as follows: 94°C for 5 min, followed by 30 cycles of 35 sec at 94°C, 30 sec at 58.8°C, and 30 sec at 72°C. Bioneer's AccuPrep PCR purification kit (Bioneer, Korea) used for purification of PCR products which digested with SmaI enzyme (New England Biolabs, USA) for 6 hours at 25°C for SNPs +45 (T/G). Electrophoresis of the digested DNA fragments occurred by used of 2% agarose gel containing Ethidium bromide and visualized by ultraviolet transilluminator. Concerning to the SNP +45(T/G), the two bands, 217- and 173-bp, indicate homozygosity for the G allele. A single 390-bp band indicates homozygosity for the T allele; the three bands, 390-, 217- and 173-bp, indicate heterozygosity for the T or G allele [13] as shown in (Fig. 1).

Table 1: Primer sequences for detection of adiponectin gene SNP+45(T/G):

Primer sequence	Temperature	Restriction .E	Incubation time
(F) AGGTGGGCTGCAATATTCAG	58.8	<i>Sma</i> I	6h
(R) CCTGGATCTCCTTTCTCACC			

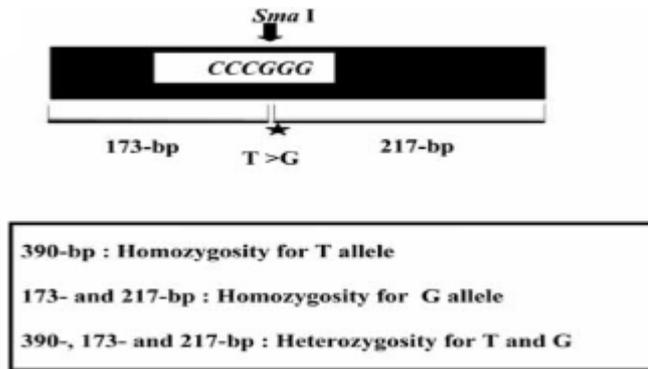


Figure 1: *Sma*I restriction enzyme

Statistical Analysis

A statistical package of the social science (SPSS) version 20 software used for analysis of data of molecular and biochemical factors. The values are expressed as mean± SD for each variable. One-Way Analysis of Variance (ANOVA) used for evaluation of Data followed by Tukey's multiple comparison test. "P" value considered significant when it was < 0.05.

Results

Biochemical analysis in PCOS patients and control

The biochemical analysis of the patients and healthy groups are illustrated in Table 1. There was a significant decreased in Adiponectin level for the PCOS patients (0.944±0.290) when compared with its level in control (3.84±2.70) (P < 0.05) while the reduction in FSH level in patients (5.44± 1.93) do not reach to a significant level when compared with control (6.42±1.79) (P > 0.05), Moreover, the level of LH and testosterone increased significantly in PCOs group than control (8.53±7.09 Vs. 4.01±1.58), (0.61± 0.27 Vs. 0.27±0.18) (P < 0.05) as shown in table 1.

Table 1: Biochemical characteristics of normal controls (n=48) and PCOS patients (n=54).

Characteristics Value	Controls (n=48)	PCOS patients (n=54)	P.
FSH (mIU/ml)	6.13± 1.79	5.44 ±1.93	P > 0.05
LH (mIU/ml)	4.01±1.58	8.53±7.09	P < 0.05
Testosterone (ng/ml)	0.27±0.18	0.61± 0.27	P < 0.05
Adiponectin (µg/l)	0.944±0.290	3.84±2.70	P < 0.05

Molecular analysis in PCOS patients and control

In regarding to genetic polymorphism +45 (T/G) in exon 2 of ADIPOQ gene (Figure 1), The genotyping distributions of TT, TG, GG in women with POCs are 16 (29.6%), 21(38.8%), 17 (31.4%) respectively , They differ from the same quantities in control: 36 (77%), 4 (8.3%),

8 (16.7%). The TT alleles polymorphism were increased significantly in control than PCOS patients (P < 0.05) while TG, GG alleles polymorphism were increased significantly in patients when compared with healthy women (P < 0.05) as shown in Table 2.

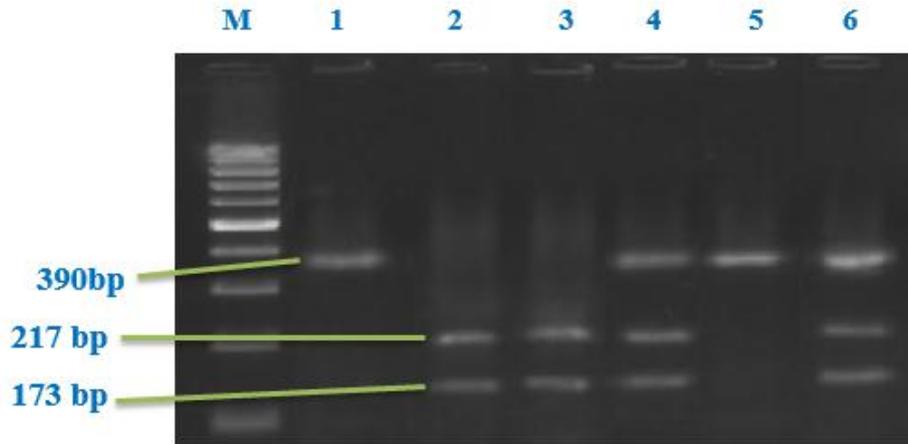


Figure 1: T/G polymorphism in exon 2 of ADIPOQ gene analyzed by RFLP-PCR method: When the GG alleles sequence present in exon 2, SmaI enzyme restricts the sequence at this site and produces two fragments, 217-bp and 173-bp bands (Lane 2& 3).When presence of the TT alleles, it makes one fragment,390-bp bands (Lane 1&5). When presence of the TG alleles, it makes three fragments 390-, 217- and 173-bp bands (Lane 4 & 6).

Table 2: Genotyping analysis of T45G genetic polymorphism in the ADIPOQ gene in PCO patients and control

Genotype		N (%)	P. value
TT	Patients	(29.6%) 16	P < 0.05
	Control	36(77%)	
TG	Patients	(38.8%) 21	
	Control	4 (8.3%)	
GG	Patients	(31.4%) 17	
	Control	8 (16.7%)	

Correlation between adiponectin polymorphism with adiponectin level:

The correlation between ADIPOQ polymorphism and level of adiponectin in serum was investigated in the current

study which demonstrated that the adiponectin level is decreased significantly in presence of G allele in ADIPOQ gene (P<0.05) as shown in Table 3.

Table 3: Correlation of adiponectin level and ADIPOQ genotyping

Genotype	Mean ± SD	P. value
TT	2.65±2.71	P < 0.05
TG	0.93±0.44	
GG	0.87±0.14	

Discussion

In this study, it was noted that the levels of serum adiponectin in the PCOS patients were decreased significantly than that in healthy women. This result is confirmed by other studies which recommended that PCOs patients have a low level of serum adiponectin than healthy women [14,15]. The low level of this hormone may be related to one of three factors that include insulin resistance, distribution of the body fat and the difference in hyperandrogenism of PCOS women as well. Serum adiponectin plays a vital role in the insulin resistance and consequently, it might deduce that hypoadiponectinemia would contribute in insulin resistance of PCOS women [16]. The silent polymorphism (T/G) in exon 2 of the ADIPOQ gene can affect one way or another plasma adiponectin levels. As well as it shows that this (T/G) polymorphism may be in linkage disequilibrium, and the G/G haplotype associated strongly with the lower concentration of adiponectin level and the metabolic disturbances of PCOS. On the other side, Glinborg *et al.* [17] and Yang *et al.* [18] were discountenance with the result of this study and documented that the level of adiponectin does not differ between PCOs patients and healthy women.

Also, the present study showed that the level of testosterone in PCOS patients increased significantly than that in healthy control women. Panidis *et al.* [19] revealed this syndrome was more public in women who have GG+TG genotype and that they had low level of serum adiponectin with higher testosterone level. This refers the existence of a potential relationship between steroid synthesis and adiponectin level. Witch el *et al.* [20] and Pangaribuan *et al.* [21] showed the level of testosterone is increased significantly in PCOs patients with TG and GG polymorphism in ADIPOQ gene and they it has been indicated that a definite degree of correlation may present between steroid hormone activity and synthesis with

adiponectin level. In other study, 45T/G ADIPOQ polymorphisms in exon 2 with three genotypes don't correlated with the high level of serum testosterone in PCOS patients [22].

So, although the number of women who have homozygous genotype for the G allele (GG) of adiponectin gene in this study was relatively small to give decisive conclusions, it has been supposed that there is a complex relationship, may be a negative feedback loop, between the hypothalamic-pituitary-gonadal axis, specifically steroid synthesis or action and adiponectin. Actually, in studies which conducted in vitro, have observed that both androgens and glucocorticoids down-regulate the adiponectin expression, and there is firm evidence suggestive of a complex correlation between gonadal function and this hormone [23].

In the current study, there was an obvious difference in the distribution of ADIPOQ genotyping between healthy and PCOS women. We demonstrate a significant correlation between ADIPOQ 45T/G polymorphisms in exon 2 and susceptibility to PCOS occurrence in TG and GG alleles; on the other hand, TT allele frequency was increased significantly in control women than patients. This result was consistent with other studies that revealed that G allele and the TG, GG genotypes were revealed at a higher frequency while T allele and TT genotype at the 45 position was revealed at a lower frequency in Chinese women with PCOS that have low level of adiponectin compared with healthy women, however, these differences between ADIPOQ genotyping and PCOS were significant, so it appears that GG genotype is a risk factor for PCOS because it increases the bioavailability of androgens in PCOS women [24]. Haap *et al.* [25] seem that the Caucasian women with PCOS have a higher prevalence of 45T/G polymorphism in the ADIPOQ gene than healthy women (GG, 13.2 vs 3%) respectively. Li *et al.* [13] showed the same result but in Asian women.

Mahdi *et al.* [26] concluded there was a significant correlation between the low concentration of adiponectin and ADIPOQ gene polymorphism in 45(T/G) position in Egyptian women with PCOS. Nambiar *et al.* [15] observed that the (GG) polymorphism in ADIPOQ gene showed a significant predisposition in PCOS patients towards a decreased concentration of adiponectin in serum. On the contrary of our results, Mohan *et al.* revealed a similar alleles genotype in 24 position of exon 2 for this gene in PCOS patients and healthy control groups [27]. Similarly, no significant difference was found in 45T/G polymorphism between Greek women with PCOS and healthy women and this polymorphism had not been correlated with a risk for development of this syndrome [28]. Escobar-Morreale *et al.* [29] indicated that the 45T/G polymorphism in ADIPOQ gene is not associated with PCOS women.

PCOS women with 45T/G polymorphisms have some metabolic alterations like (obesity and insulin resistance). Although how the polymorphisms in gene contribute to PCOS occurrence is not exactly known, the insulin resistance may have the main role in appearance of this syndrome. The hypothesis suggested that risk of PCOS, low serum adiponectin levels, hyperandrogenism and obesity in PCOS may not be directly contributed to 45T/G polymorphisms in this gene, but these polymorphisms may be correlated with hyperinsulinemia/insulin resistance in women with PCOS [30]. So, The polymorphisms in this gene may have a prevalent role in insulin resistance which is possible lead to PCOS and other diseases like obesity and type 2 diabetes [24]. The ADIPOQ gene composed of two domains, the globular domain encoded by exon 3 and collagen domain encoded by exon 2. There is no correlation of polymorphisms in exon 3 with some complex diseases including PCOS, type 2 diabetes and obesity. Instead, the polymorphisms in exon 2 that located near

exon 3 are found to give predisposition to those diseases, which reveals that the collagen domain of the adiponectin molecule may have a remarkable biological activity [31].

So, it has been documented that the polymorphism of the ADIPOQ gene may not be the actual cause of the metabolic defects of this syndrome, there is a correlation may be found between steroid hormone activity and synthesis with adiponectin. Most of the researches have focused on the role of two SNPs, +276(G/T) and +45(T/G) in the pathogenesis of PCOS. After all, the effect of these two polymorphisms on the expression of ADIPOQ gene or biological role is not completely known, because the SNP +276 (G/T) is an intronic polymorphism while the SNP +45(T/G) is a synonymous one [24].

The low frequency of GG genotype in ADIPOQ gene in healthy women may reflect the importance of this gene in the incidence of the disease since the meeting of the two mutant alleles (G) in the same woman leads to the disease when compared with increasing the percentage genotype (TT) in healthy women than PCOS women [32].

Conclusion

The assembly of the two mutant alleles (GG) in ADIPOQ gene in the same woman may leads to PCOS disease. This shows the potential role of this genetic polymorphism in this gene in the possibility of the disease occurrence.

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