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Association of Adiponectin Gene Polymorphisms on Circulating Adiponectin in Iraqi Women with obese Polycystic Ovary Syndrome

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Abstract

The possible association of adiponectin gene Polymorphisms with polycystic ovary syndrome (PCOS) and their influence on serum adiponectin and insulin resistance indexes in Iraqi women with PCOS was studied .Samples from 100 healthy controls women and 100 with obese PCOS characterized with respect to body mass index (BMI), glucose and insulin concentrations during an oral glucose tolerance test (OGTT), lipid profile, and serum adiponectin concentrations were genotyped for the 45T>G and 276G>T polymorphisms in the adiponectin gene. Results revealed that the distributions of genotypes and alleles of +276(G/T) polymorphisms were different in women with PCOS and controls, indicating that the individual polymorphisms were associated with increased risk for PCOS. However, the two polymorphisms were found to be associated with insulin resistance indexes among women with PCOS and to influence adiponectin production. No association was found between the +45(T/G) SNP and PCOS (P=0.3558, OR=0.83, 95%confidence interval), per contra to the association between +276(G/T) SNP and PCOS (P=0.0126, OR=0.60, 95% confidence interval). These results indicated that the SNP of +276(G/T) is strongly associated with Iraqi women with PCOS. However, the +45(T/G) SNP is not associated with PCOS.

Introduction

The polycystic ovary syndrome (PCOS) is considered as an endocrine syndrome with polymorphic clinical manifestations, including hyperandrogenism and chronic anovulation (**1-5**). The current diagnostic criteria are based on the 2003 American. Society for Reproductive Medicine/European Society of Human Reproduction and Embryology (ASRM/ESHRE) Rotterdam consensus (**6,7**). So far, the aetiology of PCOS has not been fully elucidated, but correlation studies have found that PCOS is likely to be hereditary, since a sister with PCOS increases the risk of PCOS up to 46% (**8**). Therefore, the study of the PCOS candidate gene will reveal the fundamental mechanism and complication of PCOS.

Several studies have shown that adiponectin plays an important role in the process of glucose regulation and lipid metabolism. Adiponectin is one of the most abundant adipokines and accounts for 0.01% or 3-30 μg/ml of the total plasma protein (8). Adiponectin is secreted by lipocytes and is composed of 224 amino acid peptides. Moreover, adiponectin can increase skeletal muscle fatty acid oxidation and peripheral tissue sensitivity to insulin and can inhibit hepatic gluconeogenesis.It has been suggested that adiponectin has a protective effect on the development of atherosclerosis (**9.10**). In different animal models for obesity and diabetes, adiponectin was found to correlate with

the insulin sensitivity index (**11**).Furthermore, adiponectin has been shown to be an important inflammatory response regulator (**12**).

Adiponectin is a collagen-like protein which is secreted and produced by adipose tissue and its coding gene is located on human chromosome 3q27. It has alternative names, including ACDC, APM1, ACRP30 and GBP28. Studies of the genetic association have shown that the adiponectin gene polymorphisms are closely related to obesity, type 2 diabetes (T2D) and IR. Approximately 6 adiponectin gene polymorphisms have been identified and have been associated with metabolic disorders in different ethnic populations of different environmental factors and genetic backgrounds. Most studies have focused on two polymorphisms, a silent T-to-G substitution in exon 2 (45T>G)and a G-to-T substitution in intron 2 (276G>T), that have been associated with obesity, insulin resistance, and the risk of type 2 diabetes (**13–16**). Furthermore, these two polymorphisms were selected because of their high frequencies in all populations tested, whereas other reported polymorphisms were rare. In a recent study, the 45T>G polymorphism was also studied in women with PCOS and was related to 4-androstenedione concentrations (**17**). The aim of the present study was to investigate the possible association of SNPs +45(T/G) and +276(G/T) in the adiponectin gene with PCOS in the Iraqi population and to examine the contribution of these two polymorphisms to insulin resistance indexes in women with PCOS.

Materials and methods

The study population consisted of 100 Iraqi women [age range, 22–39 years; mean (SD) age, 23.7 (6.4) years] with PCOS. Diagnosis of PCOS was based on the criteria based on the criteria proposed by the 2003 ASRM/ESHRE Rotterdam consensus(**4)**.These criteria are ovulatory dysfunction, clinical evidence of hyperandrogenism and/or hyperandrogenemia, and exclusion of related disorders such as congenital adrenal hyperplasia, hyperprolactinaemia, or Cushing syndrome. Hyperandrogenism was defined by the clinical presence of hirsutism (Ferriman–Gallwey score ≥8), acne or alopecia, and/or increased androgen concentrations. Menstrual dysfunction was defined by the presence of oligomenorrhea or amenorrhea. In those patients who were on medication, treatment was discontinued at least 6 months before their inclusion in the study. Women with obese PCOS were selected based on their body mass index (BMI) values. (BMI ≥25 kg/m^2) with PCOS. A second group of 100 healthy normal weight women with regular menstrual cycles (22–35 days) and no signs of hyperandrogenism were also used as controls for the distribution of the various adiponectin genotypes. The control group consisted of medical school students and staff of our hospital [mean (SD) age, 24.6 (6.7) years.

All women with PCOS were studied in the early follicular phase (days 3–5) of a spontaneous or progestin induced menstrual cycle. The BMI of each patient was calculated as weight (kg)/height (m)^2. Blood samples were drawn after overnight fasting for the measurement of fasting serum glucose and insulin, lipid profile, serum gonadotropin [luteinizing hormone (LH) and follicles stimulating hormone (FSH)], total testosterone, and sex hormone-binding globulin (SHBG). The free androgen index (FAI) was calculated using the formula: [total testosterone (nmol/L)/SHBG (nmol/L)] / 100.

All patients underwent a 75-g oral glucose tolerance test (OGTT). Blood was sampled for serum glucose and insulin concentrations before and at 30, 60, 90, and 120 min after glucose load. The fasting glucose-to-insulin ratio was estimated. Adiponectin concentrations were also measured in women with PCOS after overnight fasting using ELISA kit .The study protocol was approved by the Hospital Ethics Committee, and all women studied gave informed consent. Blood samples were collected in tubes containing EDTA as an anticoagulant and stored at 4˚C. Genomic DNA was extracted from the blood of PCOS patients and control women. The study was approved by the Institutional Review Board.

Genetic analysis. Two SNPs were genotyped by the PCR based RFLP analysis for all subjects. Primers and restriction enzymes used are shown in Table I. The PCR amplification conditions 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. PCR conditions for +276(G/T) were as follows: 95˚C for 5 min, followed by 35 cycles of 30 sec at 95˚C, 30 sec at 62˚C, and 30 sec at 72˚C. The PCR products were purified using Bioneer's AccuPrep PCR purification kit (Bioneer, Daejeon, Korea) and were digested with *Sma*I (New England Biolabs, Beverly, MA, USA) for 6 h at 25˚C or *Bsm*I (New England Biolabs) for 14 h at 65˚C for SNPs +45(T/G) and +276(G/T), respectively. The digested DNA fragments were electrophoresed on a 2% agarose gels containing ethidium bromide and visualized by an ultraviolet transilluminator.

In regard to the SNP +45(T/G), a single 390-bp band indicates homozygosity for the T allele; the two bands, 217- and 173-bp, indicate homozygosity for the G allele; the presence of three fragments, 390-, 217- and 173-bp bands, indicate heterozygosity for the T or G allele (Fig. 1).

As for the SNP +276(G/T), presence of a single 468-bp band indicates homozygosity for the G allele; presence of two fragments, 320- and 148-bp, indicates homozygosity for the T allele; and three fragments, 468-, 320- and 148-bp indicate heterozygosity for the G and the T allele (Fig. 2).

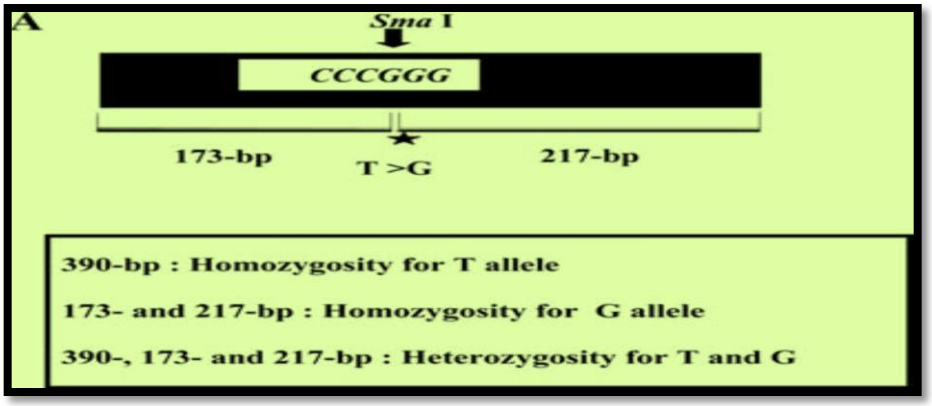
Statistical analysis

Genotype and allele frequencies were compared among study groups by use of the $\chi 2$ test. Hardy–Weinberg equilibrium for each polymorphism was also tested, comparing the observed genotype frequencies with those expected ($\chi 2$ test). For the genotypes present in statistically significant different frequencies, the odds ratios (ORs) and 95% confidence intervals (CIs) were also estimated. Gaussian distribution of continuous variables was tested by the Kolmogorov–Smirnov test. Logarithmic transformations were applied to insulin, triglyceride, and adiponectin concentrations to ensure Gaussian distribution of these variables, and the values presented were back-transformed. Biochemical differences between two continuous variables were estimated with the Mann–Whitney *U*-test or *t*-test as appropriate. Analysis of covariance was also performed with age, BMI, and insulin resistance indexes. Simple an d partial Pearson correlations were used to establish associations between adiponectin concentrations and features of PCOS alone and after adjustment for age and BMI. Continuous variables are expressed as the mean (SD). $P \leq 0.05$ was set as statistically significant. All analyses were performed with the Statistical Software Package (Ver. 14. Statsoft Inc.).

Results :

The analysis of the clinical and biochemical characteristics of the healthy control subjects and of obese patients with PCOS are shown in Table 2. In regard to the diagnostic criteria of PCOS, we followed the 2003 ASRM/ESHRE Rotterdam consensus (4). According to the criteria, patients are diagnosed with PCOS when they have two of the following three features: oligo- or amenorrhea, clinical or biochemical hyperandrogenism and ultrasonography polycystic ovarian morphology (4). As shown in Table 2, there was difference in the level of TSH, DHEA-S and testosterone for the PCOS patients and control groups. In addition, the level of LH in PCOS patients was almost twice as much as in the control group. In the PCOS patient group, 28 patients had hyperandrogenism and oligo- or amenorrhea, 18 patients had hyperandrogenism and polycystic ovaries, 36 patients had oligo- or amenorrhea and polycystic ovaries, and 18 patients had hyperandrogenism, oligo- or amenorrhea and polycystic ovaries. To analyze the frequency of the genotypes for SNPs in the adiponectin gene, we performed PCR-RFLP analysis.

Table 3 shows the SNP allele frequency of the TT, TG, GG genotypes of the exon 2 T/G polymorphism and of the GG, GT, TT genotypes of the intron 2 G/T polymorphism for the adiponectin gene in patients with PCOS and control subjects. In the case of +45 (T/G), the frequency of the TT genotype was slightly higher in the PCOS patient group than in the control group (56.0% vs. 47.0%, respectively); the TG genotype was more frequent in the control group (50.0%) than in the PCOS patient group (40.0%); the frequency of the GG genotype was similar between the two groups (patient group, 4.0%; control group, 3.09%). Also, for the +276(G/T) SNP, significantly higher frequency of the GG genotype was shown in the PCOS patient group than in the control group (44.0% vs. 31.0%, respectively); the rate of the GT genotype was slightly higher in the control group than in the PCOS patient group; and the frequency of the TT genotype was remarkably higher in the control group (16.0%) than in the PCOS patient group (7.0%). In addition, as indicated in Table 3, there was no association between the SNP +45(T/G) and the occurrence of PCOS.($P>0.05$) In contrast, the data demonstrate the association between the SNP +276(G/T) and PCOS patients in Iraqi obese women($P<0.05$).



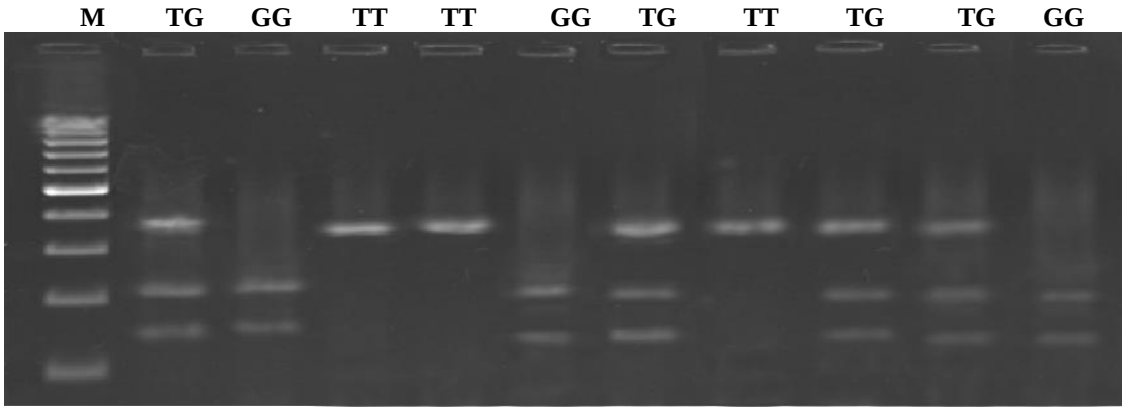
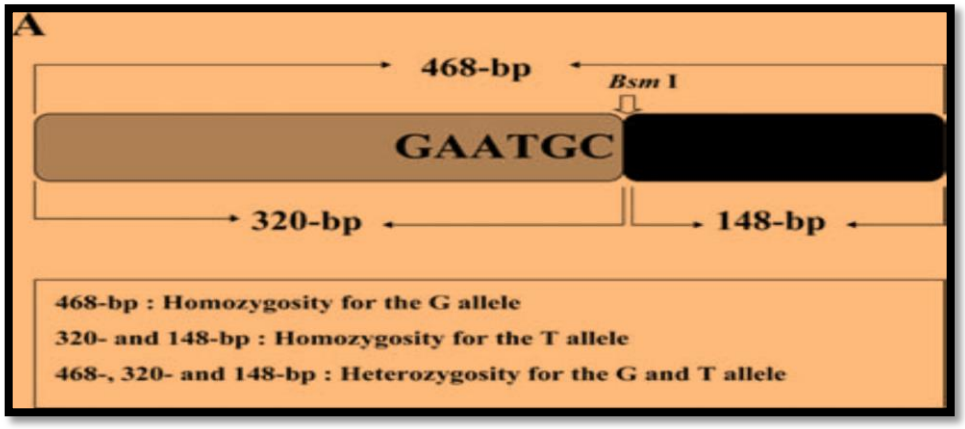


Figure 1. (A) Structure of exon 2 of the adiponectin gene. The arrow indicates the restriction site of *Sma*I. The site of the adiponectin +45(T/G)polymorphism is marked with an asterisk. When the sequence contains the G allele, *Sma*I restricts the site and produces two fragments, 217-bp and 173-bp. Presence of the T allele makes one fragment, 390-bp. (**B) RFLP** analysis of the T/G polymorphism in exon 2 of the adiponectin gene.



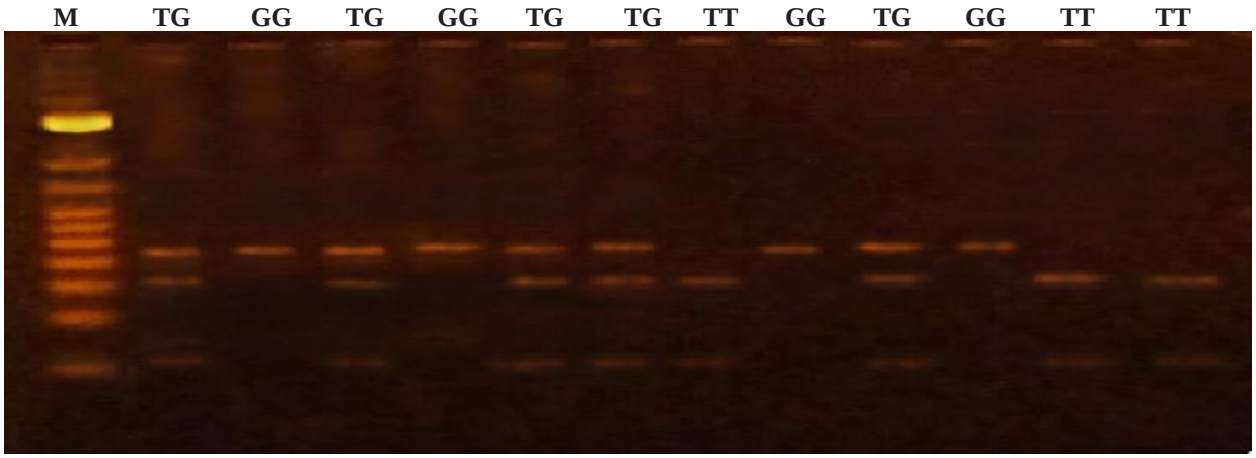


Figure 2. (A) Structure of intron 2 of the adiponectin gene. When the sequence has the T allele, *Bsm*I recognizes the site and make two fragments, 320-bp, 148-bp. Presence of the G allele makes one fragment, 468-bp. (B) RFLP analysis of the G/T polymorphism in intron 2 of the adiponectin gene.

Table 1.Primer sequences for detection of adiponectin gene SNP+45(T/G) and +276(G/T).

Primer sequence	Length	T.M	Restriction .E	Incubation time
+45(T/G) SNP primers				
(F) AGGTGGGCTGCAATATTCAG	20	58.8	*Sma*I	6h
(R) CCTGGATCTCCTTTCTCACC	20			
+276(G/T) SNP primers				
(F) TCTCTCCATGGCTGACAGTG	20	62	*Bsm*I	14 h
(R) AGATGCAGCAAAGCCAAAGT	20			

Table 2. Biochemical and clinical characteristics of normal controls (n=100) and PCOS patients (n=100).

Characteristics (n=100)	Controls (n=100)	PCOS patients	P value
BMI (kg/m2)	20.73±2.36	25.22±3.88	<0.05
Waist/hip ratio (WHR)	0.80±0.07	0.82±0.06	NS
polycystic ovaries	n=0	n=15	<0.001
FSH levels (mIU/ml)	6.55±1.82	5.81±1.83	NS
LH levels (mIU/ml)	3.24±1.48	8.37±6.55	<0.05
E2 levels (pg/ml)	32.47±15.13	42.93±34.58	<0.05
Prolactin levels (ng/ml)	12.24±6.48	13.42±9.64	NS
TSH levels (µIU/ml)	1.88±0.94	4.25±1.43	<0.05
DHEA-S levels (µg/dl)	157.86±55.46	198.07±76.54	<0.01
Test. levels (ng/ml)	0.25±0.15	0.58±0.21	<0.01
Adiponectin, mg/l	11.55±1.82	6.62±1.82	<0.001

Table 3. Genotype and allele frequencies of +45(T/G) and +276(G/T) polymorphisms of the adiponectin gene in women with PCOS and control subjects.

Polymorphism	PCOS (n=144)	Controls (n=159)	P
+45(T/G) Genotypes	**n (%)**	**n (%)**	
TT	56 (56.0)	47 (47.0)	
TG	40 (40.0)	50 (50.0)	P > 0.05
GG	4 (4.0)	3 (3.0)	
Alleles, n (%)			
T	150 (75.0)	143 (71.5)	
G	50 (25.0)	57 (28.5)	
+276(G/T) Genotypes,			
GG	44 (44.0)	31 (31.0)	P< 0.05
GT	49 (49.0)	53 (53.0)	
TT	7 (7.0)	16 (16.0)	
Alleles, n (%)183			
G	126 (63.0)	110 (55.0	
T	74(37.0)	90 (45.0)	

- **There was association between PCOS and the SNP +276(G/T), but not with the SNP +45(T/G)**

Discussion

Molecular genetic studies have shown that hormone metabolism and energy regulation-related genes, such as CYP19, INSR, LH are involved in the pathogenesis of PCOS (**17-22**). the present study, we assessed another metabolism-related gene, adiponectin, and examined the association between the SNPs +45G15G(T/G) and +276 (G/T) in the adiponectin gene and PCOS in Iraqi women. An association was found between the +276(G/T) SNP and PCOS, while the +45(T/G) SNP was not associated with PCOS in Iraqi women. To a certain extent, variation of the adiponectin gene determines the overexpression of the phenotype of the metabolic syndrome. However, it has been reported that the polymorphism of the adiponectin gene is not the real reason for the metabolic disorders of PCOS (**21**); it has been suggested that a certain degree of interaction may exist between adiponectin and steroid hormone activity and synthesis. At present, 13 SNPs have been identified in the adiponectin gene (**22**), and most of the studies have focused on two SNPs: +45(T/G) and +276(G/T).However, the influence of these two polymorphisms on the adiponectin gene expression or biological function is not fully known, because the SNP +45(T/G) is a synonymous polymorphism while the SNP +276(G/T) is an intronic one.

It has been reported that SNPs + 45(T/G) and +276(G/T) in the adiponectin gene are significantly associated with PCOS in Chinese women (**23**). These two SNPs have been analyzed in various diseases and in different ethnic backgrounds. It has been reported that fasting glucose levels are influenced by the adiponectin +276(G/T) polymorphism in Korean and Italian populations (**24,25**). In French and Swedish Caucasians, the +45(T/G) and +276(G/T) polymorphisms have not been associated with T2D (26). However, it has also been indicated that SNPs +45(T/G) and +276(G/T) are strongly associated with type 1 diabetes (T1D), but not with diabetic nephropathy (DN) in Swedish patients (**27**). Studies have illustrated that the SNP in intron 2 of the adiponectin gene plays an important role in the regulation of adiponectin levels, although the exact cellular mechanism is still not known (**28,29**).

This is the first report on the association of two SNPs of the adiponectin gene with PCOS patients in Iraqi population. Our results provide insight into the role of the adiponectin gene in the pathogenesis of PCOS in different ethnic backgrounds and also provide an important basis for the early diagnosis and prevention of PCOS.

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