CLINICAL STUDY

Association of +45G15G(T/G) and +276(G/T) polymorphisms in the ADIPOQ gene with polycystic ovary syndrome among Han Chinese women

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Abstract

Objective: Polycystic ovary syndrome (PCOS) is frequently associated with insulin resistance (IR) and consequently with increased risk of metabolic disorders. Adiponectin is the most abundant adipocytokine and may play a role in the regulation of insulin sensitivity and IR in PCOS. The aim of the present study was to evaluate the genetic influence of the adiponectin (ADIPOQ) gene polymorphisms in the development of PCOS among Han Chinese women.

Methods: Two single nucleotide polymorphisms (SNPs), +45G15G(T/G) and +276(G/T), in the ADIPOQ gene were genotyped in 120 patients with PCOS and 120 healthy control subjects. All of them were Han Chinese women.

Results: Both SNPs were found to be significantly associated with PCOS ($P=0.021$, odds ratios = 1.629, 95% confidence intervals: 1.074–2.469 and $P=0.015$, 1.576, 1.091–2.279 respectively). In SNP +276(G/T), the allele G was found to be significantly associated with increased fasting insulin levels, homeostasis model assessment to assess IR index, and area under the curve glucose levels, but decreased glucose and insulin ratio in the PCOS patients. Furthermore, the patients carrying genotypes G/G and G/T had significantly decreased levels of serum adiponectin (6.16 ± 3.18 plus 5.93 ± 3.23 vs 8.96 ± 3.21 $\mu\text{g/ml}$, $P=0.030$) compared with the patients with genotype T/T.

Conclusions: The present study provides evidence that SNPs +45G15G(T/G) and +276(G/T) in the ADIPOQ gene are associated with PCOS in Han Chinese women. SNP +276(G/T) may contribute to an impact of insulin levels and IR, which are implicated in the susceptibility for PCOS.

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Introduction

Polycystic ovary syndrome (PCOS; OMIM 184700) is a multifaceted metabolic disease in women of reproductive age (1). PCOS has a strong genetic component (2), and it is often associated with obesity and insulin resistance (IR) predisposing to type 2 diabetes and atherosclerosis (3–7). Thus, identification of the susceptibility genes may offer better understanding of the molecular mechanisms underlying pathogenesis of PCOS and other related metabolic disorders.

Adiponectin (OMIM 605441) is the most abundant adipocytokine and accounts for 0.01% of total plasma protein (8, 9). Clinical evidence demonstrates that a reduction in plasma or serum adiponectin levels is commonly observed in the patients with obesity and/or type 2 diabetes, in comparison with the healthy control subjects. Similarly, the women with PCOS are found to have lower adiponectin levels than the healthy

controls (10, 11). Moreover, adiponectin may have insulin-sensitizing and putative anti-atherosclerotic properties (12–16). Both IR and adiponectin are important parameters in the development of PCOS (5, 10). Therefore, it is necessary to investigate whether the genetic influence of adiponectin plays a role in the pathogenesis of PCOS.

The adiponectin gene is encoded by ADIPOQ (adipocyte C1q and collagen domain containing), also known as adipose most abundant gene transcript 1 (APM1), gelatin-binding protein of 28 kDa (GBP28) or adipocyte complement-related protein of 30 kDa (Acrp30). This gene is located on chromosome 3q27. Genome-wide scan and linkage studies in this chromosomal region have revealed a susceptibility locus for obesity, type 2 diabetes, and coronary heart disease (17–19). In the recent years, several genetic association studies with metabolic disorders including obesity, type 2 diabetes, and PCOS have been conducted (20–23). Interestingly, two single nucleotide polymorphisms (SNPs), i.e., +45G15G(T/G)

and +276(G/T), in exon 2 and intron 2 of the ADIPOQ gene respectively, were found to be strongly associated with type 2 diabetes, obesity, and IR (24–27). These two polymorphisms are also found to be associated with PCOS among European Caucasian women (28, 29). However, information about association of adiponectin genetic polymorphisms with PCOS in Chinese women is still limited. In the present study, we have evaluated the genetic association between these two polymorphisms and PCOS among Han Chinese women, which may provide evidence for better understanding of the susceptibility of the ADIPOQ gene variation in the development of PCOS.

Subjects and methods

Subjects

A total of 240 Han Chinese women were included in the present study. Among them, 120 women had PCOS, while the other 120 were healthy control subjects. The patients with PCOS and the healthy control subjects were diagnosed based on the presence of two out of three criteria of Rotterdam European Society for Human Reproduction and Embryology (ESHRE)/American Society for Reproductive Medicine (ASRM)-sponsored PCOS Consensus Workshop Group, including oligo- and/or anovulation, and clinical and/or biochemical signs of hyperandrogenism and polycystic ovaries. Other etiologies (congenital adrenal hyperplasias, androgen-secreting tumors, and Cushing's syndrome) were excluded. Among the patients with PCOS, there were 68 cases with oligo- and/or anovulation + hyperandrogenism + PCO, 40 cases with oligo- and/or anovulation + PCO, 9 cases with oligo- and/or anovulation + hyperandrogenism, and 3 cases with hyperandrogenism + PCO. All participants gave their informed consent to take part in the study. The procedures followed were in accordance with the ethical standards of the responsible committee of human experimentation. Clinical parameters of the patients with PCOS and the healthy control subjects are shown in Table 1.

Table 1 Clinical and endocrine characteristics of the healthy control subjects and the patients with polycystic ovary syndrome (PCOS).

	Controls	PCOS	P value
N	120	120	
Age (years)	29.57 ± 3.67	28.74 ± 3.36	0.080
BMI (kg/m ²)	22.73 ± 3.56	25.68 ± 4.22	<0.001
WHR	0.79 ± 0.12	0.86 ± 0.06	<0.001
LH/FSH	0.61 ± 0.30	1.91 ± 1.56	<0.001
T (nmol/l)	1.17 ± 0.51	2.34 ± 0.93	<0.001
FAI	1.31 ± 0.82	2.32 ± 1.91	0.005
FINS (mIU/l)	6.38 ± 4.45	8.38 ± 5.29	0.036
GIR	18.76 ± 9.94	13.94 ± 8.19	0.004
HOMA-IR	1.42 ± 1.07	1.84 ± 1.23	0.065
Adiponectin (µg/ml)	8.92 ± 4.99 (n=40)	6.49 ± 3.30 (n=47)	0.008

Data are presented as means ± s.d.

Study protocol

The patients with PCOS and the healthy control subjects were diagnosed and examined at the Center for Reproductive Medicine, Shangdong Provincial Hospital, Shangdong University, PR China. Peripheral blood samples were collected on days 2–5 of spontaneous cycle or after withdrawal of bleeding from the subjects in a fasting state. Blood samples were collected at 0, 30, 60, 120, and 180 min of the 75 g oral glucose tolerance test (OGTT) in all patients with PCOS. Measurement with a chemiluminescent analyzer (Beckman Coulter Inc., Fullerton, CA, USA) was done for the following hormones: follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), and testosterone (T). Serum levels of sex hormone-binding globulin (SHBG) were measured using an IRMA kit (DSL Inc., Webster, TX, USA). Serum glucose and insulin levels were measured by enzymatic and chemiluminescent methods respectively. Serum adiponectin levels were determined using a commercially available ELISA kit (R&D Systems Inc., Minneapolis, MN, USA). The intra-assay coefficient of variation (CV) values ranged from 2.5% to 4.7%, and the inter-assay from 5.8% to 6.5%. The free androgen index was calculated according to the formula: T (nmol/l) × 100/SHBG (nmol/l). Homeostasis model assessment to assess IR (HOMA-IR) was calculated according to the following equation: fasting glucose (FBG nmol/l) × fasting insulin (FINS mIU/l)/22.5. Glucose and insulin ratio (GIR) was determined as: fasting blood glucose (FBG mg/dl)/FINS mIU/l. The glucose and insulin responses to the OGTT were analyzed by calculating the area under the curve (AUC).

Genotype analyses

Genomic DNA was isolated from the peripheral blood samples using a DNA purification kit (Tiangen Biotech Co. Ltd., Beijing, China). SNP +45G15G(T/G) in the ADIPOQ gene was genotyped by the amplification of genomic DNA using the following primers: forward, 5'-GCA GCT CCT AGA AGT AGA CTC TG-3' and reverse, 5'-TCT GTG ATG AAA GAG GCC AG-3' (Sangon Biotech Co. Ltd., Shanghai, China). The amplification conditions were used as follows: 94 °C for 10 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 57 °C, and 30 s at 72 °C, and ending with a single 10-min extension step at 72 °C. The PCR fragment was 367 bp in length, and was digested with the enzyme *Sma*I (New England BioLabs Ltd., Beijing, China) at 25 °C for 4 h. Digestion of allele G produced two fragments with lengths 204 and 163 bp. SNP +276(G/T) in the ADIPOQ gene was genotyped by the amplification of genomic DNA using another pair of primers: forward, 5'-TCT CTC CAT GGC TGA CAG TG-3' and reverse, 5'-AGA TGC AGC AAA GCC AAA GT-3' (Sangon Biotech Co. Ltd.). The amplification conditions were as follows: 94 °C for 10 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, and ending with a single 10-min extension

step at 72 °C. The resulting fragment was 468 bp in length. The polymorphism was typed using the enzyme *BsmI* (New England Biolabs Ltd., Beijing, China) at 65 °C for 4 h. Digestion of allele G produced two fragments with lengths 320 and 148 bp. In the genotyping experiments for both SNPs, the digestion products were resolved by electrophoresis in 1.5% agarose gel.

Statistical analyses

The independent segregation of alleles was tested for the Hardy–Weinberg equilibrium (HWE). Genotype and allele distribution was compared between cases (women with PCOS) and controls using Pearson's χ^2 test. Odds ratios (OR) and 95% confidence intervals (CI) were calculated to test relative risk for association. Normal probability plots were created and parameter distributions transformed to natural logarithm where necessary to improve the skewness, and to obtain a normal distribution before performing statistical analysis of the relevant phenotypes. Tests for association between genotypes and quantitative traits were performed using Kruskal–Wallis or Student's *t* analysis of ranks for traits with non-normal distributions or, alternatively, ANOVA for normally distributed traits with or without adjustment for age and BMI was performed. Results of $P < 0.05$ were interpreted as statistically significant. Multivariate logistic regression analysis was used to test for differences in genotypes and clinical features between cases and controls. Linkage disequilibrium (LD) and haplotype analysis was performed using Arlequin version 2.0. All statistical analyses were performed with SPSS statistical package, version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

We genotyped SNPs rs2241766+45G15G(T/G) and rs1501299+276(G/T) of the ADIPOQ gene in the DNA samples of Han Chinese women, consisting of 120 patients with PCOS and 120 healthy control subjects. The deviation of both SNPs remained in HWE ($P > 0.148$). In SNP +45G15G(T/G), the frequencies of genotypes T/T, T/G, and G/G were 47.5% (57/120), 45.0% (54/120), and 7.5% (9/120) in the patients with PCOS, and 61.7% (74/120), 35.0% (42/120), and 3.3% (4/120) in the healthy controls respectively. In SNP +276(G/T), the frequencies of genotypes G/G, G/T, and T/T were 46.7% (56/120),

38.3% (46/120), and 15.0% (18/120) in the patients with PCOS, and 34.2% (41/120), 41.6% (50/120), and 24.2% (29/120) in the healthy controls respectively. Furthermore, we found that in SNP +45G15G(T/G), the patients with PCOS had significantly increased frequency of allele G in comparison with the controls (30.0% vs 20.8%, $P = 0.021$, OR = 1.629, 95% CI: 1.074–2.469). In SNP +276(G/T), the frequency of allele G in the patients with PCOS was higher than that in the controls (65.8% vs 55%, $P = 0.015$, OR = 1.576, 95% CI: 1.091–2.279). Data of allelic association between these two SNPs and PCOS in Han Chinese women are presented in Table 2.

We performed an analysis for single-marker association with quantitative traits of PCOS in SNPs +45G15G(T/G) and +276(G/T). Clinical and metabolic features of the patients with PCOS according to the genotypes of these two SNPs are summarized in Table 3. The results indicated that the patients with PCOS carrying genotype G/G had higher AUC insulin levels but with borderline statistical significance in comparison with carriers with genotype T/T or T/G (250.8 vs 157.9 or 151.6, $P = 0.040$; Table 3) in SNP +45G15G(T/G). With the adjustment for age and BMI, the results became less statistically significant ($P = 0.058$). In SNP +276(G/T), the patients with genotype T/T had increased GIR in comparison with the carriers with genotype G/G or G/T (18.93 vs 13.55 or 12.28, $P = 0.013$). The statistical analyses of the differences of clinical features among the patients with different genotypes were also done with adjustment for age and BMI. This polymorphism was found to be significantly associated with several metabolic features, including FINS levels ($P = 0.037$), GIR ($P = 0.028$), HOMA-IR index ($P = 0.028$), and AUC insulin levels ($P = 0.036$) in the patients with PCOS. The patients with genotypes G/G and G/T had increased FINS levels, HOMA-IR index, and AUC insulin levels, but decreased GIR compared with patients with genotype T/T (Table 3). We further measured serum adiponectin levels in the patients with PCOS when serum samples were available ($n = 47$). Interestingly, we found that the patients with genotypes G/G and G/T had significantly decreased levels of serum adiponectin than the carriers with genotype T/T (6.16 ± 3.18 plus 5.93 ± 3.2 vs 8.96 ± 3.213 $\mu\text{g/ml}$, $P = 0.030$; Fig. 1).

SNPs +45G15G(T/G) and +276(G/T) reside on one of the LD blocks in the ADIPOQ gene in Caucasians (30). We attempted to detect the association of haplotypes constructed by these two SNPs with PCOS in Han

Table 2 Allelic association of single nucleotide polymorphisms (SNPs) +45G15G(T/G) and +276(G/T) in the ADIPOQ gene with polycystic ovary syndrome (PCOS) among Han Chinese women.

SNP ID	SNP type	Location	Group	Allele frequency	P value	OR (95% CI)
rs2241766	K = T/G	Exon 2	Controls	T (79.2) G (20.8)	0.021	1.629 (1.074–2.469)
			PCOS	T (70.0) G (30.0)		
rs1501299	M = G/T	Intron 2	Controls	G (55.0) T (45.0)	0.015	1.576 (1.091–2.279)
			PCOS	G (65.8) T (34.2)		

Table 3 Clinical features of Han Chinese women with polycystic ovary syndrome (PCOS) according to the genotypes.

Genotypes	T/T	T/G	G/G	P value	P value ^a
SNP +45G15G(T/G)					
N	57	54	9		
Age (years)	29±3	29±3	28±3	0.963	
BMI (kg/m ²)	26.1±4.4	24.9±3.8	26.7±5.1	0.214	
WHR	0.86±0.07	0.86±0.07	0.85±0.03	0.853	0.325
LH/FSH	2.08±1.70	1.82±1.52	1.50±0.83	0.512	0.527
FAI	2.42±1.49	2.09±2.35	3.19±0.31	0.641	0.965
FBG (mmol/l)	4.80±0.89	4.93±0.80	4.87±0.41	0.728	0.554
FINS (mIU/l)	8.18±4.99	8.33±5.51	9.83±6.15	0.689	0.535
GIR	14.30±9.05	13.71±7.12	13.15±9.23	0.894	0.820
HOMA-IR	1.75±1.18	1.88±1.29	2.13±1.32	0.676	0.432
AUC glucose	29.01±62.09	21.11±5.86	21.15±3.63	0.632	0.673
AUC insulin	157.9±89.7	151.6±101.7	250.8±207.1	0.040	0.058
Genotypes	G/G	G/T	T/T	P value	P value ^a
SNP +276(G/T)					
N	56	46	18		
Age (years)	29±3	29±4	30±3	0.293	
BMI (kg/m ²)	26.1±4.4	25.6±3.9	24.3±4.0	0.295	
WHR	0.87±0.07	0.85±0.06	0.85±0.06	0.343	0.894
LH/FSH	1.96±1.62	1.86±1.72	1.91±0.99	0.958	0.941
FAI	2.04±1.27	3.19±2.74	1.63±1.10	0.143	0.103
FBG (mmol/l)	4.87±0.80	4.90±0.79	4.78±0.99	0.877	0.703
FINS (mIU/l)	8.62±5.61	9.07±5.49	6.00±2.77	0.106	0.037
GIR	13.55±7.58	12.28±5.75	18.93±12.32	0.013	0.028
HOMA-IR	1.93±1.38	1.98±1.20	1.25±0.53	0.084	0.028
AUC glucose	21.07±4.57	21.74±5.90	43.16±106.91	0.144	0.162
AUC insulin	170.4±120.1	176.0±106.4	108.3±65.0	0.071	0.036

Data are presented as means±s.d. SNP, single nucleotide polymorphism; BMI, body mass index; WHR, weight-height ratio; LH, Luteinizing hormone; FSH, follicle stimulating hormone; FAI, free androgen index; FBG, fasting blood glucose; FINS, fasting insulin; GIR, glucose and insulin ratio; HOMA-IR, homeostasis model assessment of insulin resistance; AUC, area under curve.
^aAdjusted for age and BMI.

Chinese women. LD value (*D'*) between these two markers among this Han Chinese women population was 0.258. The frequencies of T-G and G-T haplotypes were 48.3% and 12.5% in the patients with PCOS, and 46.7% and 12.1% in the healthy control subjects respectively. No significant difference in haplotype frequencies between cases and controls was observed (data not shown).

Discussion

In the present study, we have evaluated the genetic influence of two common SNPs +45G15G(T/G) and +276(G/T) in Han Chinese woman with PCOS. Both SNPs were found to be significantly associated with PCOS among Han Chinese woman. In SNP +276(G/T), the allele G was found to be significantly associated with increased FINS levels, HOMA-IR index, and AUC glucose levels, but decreased GIR in the PCOS patients. Furthermore, the patients carrying genotypes G/G and G/T had significantly decreased levels of serum adiponectin compared with the patients with genotype T/T. Clinical evidence indicated that the serum adiponectin levels in the patients with PCOS were significantly decreased, compared with the healthy

control subjects (10, 11). Moreover, previous studies demonstrated that the type 2 diabetic patients with genotypes G/G and G/T in SNP +276(G/T) had decreased plasma adiponectin levels compared with the patients with genotype T/T (25). The present study, in accordance with previous reports (10, 11, 25), provides further evidence that this intronic SNP may play a role in the regulation of serum adiponectin levels. Although we have limited knowledge whether gene expression is regulated by intronic polymorphisms,

Figure 1 Serum adiponectin levels in Han Chinese women with PCOS according to the genotypes of SNP +276(G/T). Data are presented as means±s.d.

similar evidence that an intronic SNP influences the expression of calpain-10 gene has also been reported (31). Furthermore, we found that SNP +276(G/T) is related to lower GIR but higher FINS level and HOMA-IR index in the patients with genotypes G/G and G/T. Consistently, this polymorphism has been found to be strongly associated with the development of type 2 diabetes and/or IR (24–27). Thus, data from the present study may imply that genetic variations of the ADIPOQ gene confer susceptibility to IR in the development of PCOS. We hypothesize that the ADIPOQ polymorphisms may have a common role in relation to IR in the pathogenesis of PCOS and other complex diseases such as type 2 diabetes and obesity. Furthermore, investigation of the biological consequence of the polymorphisms is then taken into consideration in order to understand the specific contribution of the ADIPOQ genetic variation in the development of PCOS.

Previous studies demonstrate that the ADIPOQ gene had a low LD, and the average probability of haplotype interference across the dataset was moderate (20, 21, 25, 32). There are two relatively strong LD blocks in the gene. One LD block resides in the putative promoter. Another appears to extend over the region between SNPs +45G15(T/G) and +276(G/T) (30, 33), which is found to be associated with IR in the obese or the patients with type 2 diabetes among Caucasians (34). In the present study, we attempted to explore the association of haplotypes constructed by these two polymorphisms, but the LD value between them in Han Chinese women is low. With the approach of single-marker analyses, however, we found that both SNPs +45G15(T/G) and +276(G/T) are strongly associated with PCOS among Han Chinese women. SNP +45G15G(T/G) resides in the region of exon 2. The ADIPOQ gene consists of two domains, i.e., the collagen domain encoded by exon 2 and globular domain encoded by exon 3. Previously, several studies attempted to investigate the biological activity of the globular but not the collagen domain (35). In recent years, a number of genetic association studies have indicated that there is no association of non-synonymous SNPs in exon 3 of this gene with complex diseases, including type 2 diabetes, obesity, and PCOS. Instead, the polymorphisms in nearby exon 2 are found to confer susceptibility to those complex diseases, which implies that the collagen domain of the adiponectin molecule may have an important biological activity.

The present study provides further evidence that SNPs rs2241766+45G15G(T/G) and rs1501299+276(G/T) in the ADIPOQ gene are strongly associated with PCOS among Han Chinese women. The susceptibility of these two polymorphisms is mainly conferred to IR and insulin action, which suggests that adiponectin genetic variation may play a common role in the pathogenesis of PCOS and other complex diseases such as type 2 diabetes and obesity.

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