

A common variant in the adiponectin gene and polycystic ovary syndrome risk

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Abstract In this study, we explored whether polymorphisms in insulin receptor (INSR), adiponectin (ADIPOQ), parathyroid hormone (PTH), and vitamin D receptor (VDR) genes are associated with polycystic ovary syndrome (PCOS). A total of 362 subjects, including 181 women with PCOS and 181 controls were enrolled in this case-control study. Two SNPs (rs2059806 and rs1799817) in the INSR gene, two SNPs (rs2241766 and rs1501299) in the ADIPOQ gene, one SNP (rs6256) in the PTH gene, and one SNP (rs757343) in the VDR gene were analyzed using PCR-RFLP method. We observed no significant difference in genotype and allele frequencies between the women with PCOS and controls for the rs2059806, rs1799817, rs1501299, rs6256, and rs757343 polymorphisms either before or after adjustment for confounding factors

including age and BMI. However, the ADIPOQ rs2241766 “TT” genotype compared with “TG and GG” genotypes was associated with a 1.93-fold increased risk for PCOS ($P = 0.006$, OR = 1.93, 95% CI = 1.20–3.11), and the differences remained significant after adjustment for age and BMI ($P = 0.039$, OR = 1.72, 95% CI = 1.03–2.86). Furthermore, the ADIPOQ rs2241766 “T” allele was significantly overrepresented in women with PCOS than controls ($P = 0.006$; OR = 1.80, 95% CI = 1.18–2.70), and the difference remained significant after Bonferroni correction. Our findings suggest that the ADIPOQ rs2241766 “TT” genotype is a marker of increased PCOS susceptibility. This study also indicates for the first time that there are no significant association between INSR rs2059806, PTH rs6256, and VDR rs757343 gene polymorphisms and PCOS risk. However, these data remain to be confirmed in larger studies and in other populations.

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Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder with a strong genetic background [1]. Insulin resistance and central obesity are prominent features of the syndrome [2, 3]. Previous epidemiologic studies have suggested that the genes involved in insulin signaling pathway are candidate genes for PCOS because as mentioned, the clinical and metabolic features of the syndrome include the increased risk of insulin resistance and obesity. In recent years, significant associations between insulin resistance and both adiponectin (ADIPOQ) [4] and insulin receptor (INSR) [5] gene variants have been found.

Furthermore, the associations between the polymorphisms in INSR [6, 7] and ADIPOQ [4, 8–11] genes and PCOS risk have been examined in previous studies and the results were contradictory.

In addition to the genes related to insulin signaling pathway, it is possible that the genes involved in calcium homeostasis are associated with susceptibility to PCOS, because insulin secretion is a calcium-dependent process [12] and positive correlations between serum calcium concentration and both insulin levels and insulin resistance have been found [13]. Furthermore, insulin resistance and obesity are associated with alterations in the hormones related to calcium homeostasis and it is known that the disorders have a negative effect on serum levels of 25-hydroxyvitamin D [25(OH) D] [14, 15] and a positive effect on parathyroid hormone (PTH) concentrations [14, 16] in the women with PCOS. In addition, our study and other recent studies have demonstrated higher serum levels of PTH [14, 16], 25(OH) D, and phosphorous [16] in women with PCOS than control women. Previous studies have reported significant associations between PTH gene polymorphisms and serum levels of PTH and calcium [17, 18]. Also, vitamin D receptor (VDR) gene polymorphisms, which are involved in the control of genomic and non-genomic effects of 1, 25(OH)₂ D, have been shown to be associated with cancer [19], tuberculosis [20], obesity [21], insulin sensitivity [22], serum levels of LH [23], PTH [24], and 25(OH) D [25]. Finally, in our previous study [26] a different distribution of VDR rs7975232 gene polymorphism between women with PCOS and controls was found.

Accordingly, these observations led us to look for the possible associations of two SNPs (rs2059806 and rs1799817) in the INSR gene, two SNPs (rs2241766 and rs1501299) in the ADIPOQ gene, one SNP (rs6256) in the PTH gene, and one SNP (rs757343) in the VDR gene with PCOS risk. These SNPs were selected based on their common use in previous genetic epidemiology studies and high degree of heterozygosity.

Materials and methods

Participants

The study population consisted of 181 women with PCOS (age range, 15–40 years) and 181 control women (age range, 18–45 years) reporting to the Royan Institute. Diagnosis of PCOS was based on the criteria proposed by the 1990 NIH-National Institution of Child Health and Human Development conference on PCOS [27]. These criteria are (a) the presence of menstrual dysfunction i.e. oligomenorrhea (fewer than six menstrual periods in the preceding year) or amenorrhea (absence of periods for

more than 6 months), and (b) clinical hyperandrogenism (i.e. hirsutism: Ferriman–Gallwey score >8) and/or hyperandrogenemia, and (c) the exclusion of related disorders such as non classic congenital adrenal hyperplasia, androgen secreting tumors, Cushing's syndrome and hyperprolactinemia. The control women were randomly selected from healthy community volunteers and none of them had clinical evidence of hyperandrogenism and all of them had normal menstrual cycles. Both patients and healthy controls were Iranian and genetically unrelated. All participants gave informed consent in accordance with the policy established by the Ethical Review Boards of the Royan Institute and the Institutional Review Board approval was obtained. The body mass index (BMI) of each subject was calculated as weight (kg)/height (m²).

Genotype analysis

Blood samples from all 362 subjects for molecular genetic studies were collected in tubes containing ethylene diaminetetraacetic acid (EDTA) as an anticoagulant and stored at 4°C. Genomic DNA was extracted from whole blood using a Genomic DNA Extraction Kit (BioNEER, Daejeon, Korea). Using PCR-RFLP method all of the six studied SNPs (Table 1) were genotyped. Digested products were run on a 2 or 3% agarose gel, and stained with ethidium bromide for visualization under UV light. For quality control reasons, we repeated the genotyping analysis of 10% of the samples with identical results. The genotyping was also confirmed by the DNA sequencing of 2% of the samples.

Statistical analysis

Differences in anthropometric factors were calculated using t-test. Testing for Hardy-Weinberg equilibrium for each of the six SNPs within cases and controls separately and comparisons of the distribution of the genotype and allele frequencies were performed using the χ^2 or Fisher's exact tests as appropriate. We used logistic regression analysis to adjust for confounding factors such as age and BMI. Odds ratios (OR) are given with the respective 95% confidence intervals (95% CI). Significance was accepted at *P*-value less than 0.05, or in case of multiple testing using the conservative Bonferroni correction for 6 SNPs, at *P* < 0.0083 (*P* < 0.05/6). Data were analyzed using SPSS software (version 15.0; SPSS Inc. Chicago, IL, USA).

Results

Selected characteristics of the study population are summarized in Table 2. Women with PCOS were younger and

Table 1 Information for the studied markers in the INSR, ADIPOQ, PTH, and VDR genes

Gene (SNP ID)	Location (base change)	Forward primer Reverse primer (Reference)	PCR program (35 cycles)	PCR fragment size (bp)	Restriction enzyme, Incubation temperature ^a	Alleles: RFLP fragments size (bp)
INSR (rs2059806)	Exon 8 (A/G)	5'-CGGTCTTGTAAAGGTAAGTACTG-3' 5'-GAATTCACATTCCCAAGACA-3' [28]	93°C 45 s, 64°C 30 s, 72°C 45 s	324	<i>NsiI</i> , 37°C	Allele G: 324 Allele A: 239 + 85
INSR (rs1799817)	Exon 17 (T/C)	5'-CCAAGGATGCTGTGTAGATAAG-3' 5'-TCAGGAAAGCCAGCCCATGTC-3' [7]	93°C 45 s, 56°C 30 s, 72°C 45 s	317	<i>PmlI</i> , 37°C	Allele T: 317 Allele C: 274 + 43
ADIPOQ (rs2241766)	Exon 2 (T/G)	5'-GAAGTAGACTCTGCTGAGATG G-3' 5'-TATCAGTGTAGGAGGTCCTGTGATG-3' [9]	93°C 45s, 56°C 30s, 72°C 45s	372	<i>SmaI</i> , 30°C	Allele T: 372 Allele G: 216 + 156
ADIPOQ (rs2241766)	Intron 2 (C/A)	5'-GGCCTCTTTCATCACAGACC-3' 5'-AGATGCAGCAAAGCCAAAGT-3' [9]	93°C 45 s, 64°C 30 s, 72°C 45 s	196	<i>BsmI</i> , 37°C	Allele A: 196 Allele C: 146 + 50
PTH (rs6256)	Exon 3 (C/A)	5'-CATTCTGTGTAAGTATAGTTT-3' 5'-GAGCTTTGAATTAGCAGCATG-3' [29]	93°C 45 s, 56°C 30 s, 72°C 45 s	600	<i>DraII</i> , 37°C	Allele A: 600 Allele C: 420 + 180
VDR (rs757343)	Intron 8 (G/A)	5'-AATACTCAGGCTCTGCTCTT-3' 5'-CATCTCCATTCCTTGAGCCT-3' [30]	93°C 45 s, 56°C 30 s, 72°C 45 s	331	<i>Tru9I</i> , 65°C	Allele G: 331 Allele A: 178 + 153

^a All the PCR products were digested overnight with the appropriate restriction enzymes (Fermentas, Leon-Rot, Germany)

had higher BMI than controls ($P < 0.001$ and $P = 0.021$, respectively). The genotype frequency distribution of all the six polymorphisms fit Hardy-Weinberg predictions in both cases and controls, suggesting that the alleles are in equilibrium ($P > 0.05$). In the present study, no significant difference was observed in genotype and allele frequencies between the women with PCOS and controls for the INSR rs2059806 and rs1799817, ADIPOQ rs1501299, PTH rs6256, and VDR rs757343 gene polymorphisms (Tables 2, 3).

However, as shown in Table 2, we observed an association between ADIPOQ rs2241766 gene polymorphism and PCOS risk. The ADIPOQ rs2241766 “TT” genotype compared with “TG and GG” genotypes was associated with a 1.93-fold increased risk for PCOS ($P = 0.006$, OR = 1.93, 95% CI = 1.20–3.11), and the difference remained significant after adjustment for age and BMI ($P = 0.039$, OR = 1.72, 95% CI = 1.03–2.86). This association becomes non-significant after the stringent Bonferroni correction for analysis of the six SNPs ($P = 0.039/6$ which is more than $P < 0.0083$). Furthermore, the ADIPOQ rs2241766 “T” allele was significantly overrepresented in women with PCOS than controls ($P = 0.006$; OR = 1.80, 95% CI = 1.18–2.70), and the difference remained significant after Bonferroni correction ($0.006/6 < 0.0083$).

Additionally, we conducted a breakdown comparison between cases and controls within different BMI categories

with respect to allele and genotype frequencies (data not shown). In the comparison between normal weight (BMI < 25 kg/m²) controls and normal weight women with PCOS, as well as in the comparison between overweight/obese (BMI ≥ 25 kg/m²) controls and overweight/obese women with PCOS, we found no differences in previous obtained results. In other words, no significant association was observed between the studied gene polymorphisms and risk of PCOS with the exception of the ADIPOQ rs2241766 polymorphism.

In this study, the risk of obesity in relation to these polymorphisms in women with PCOS was also examined; comparison between normal weight cases and overweight/obese cases (data not shown). We observed no significant difference in genotype and allele frequencies between these two groups for all of the six polymorphisms.

Discussion

We conducted a case-control study to examine the possible association between the polymorphisms in INSR, ADIPOQ, PTH, and VDR genes and risk of PCOS. In the present study, no statistically significant difference was found in the frequencies of INSR rs2059806 and rs1799817, ADIPOQ rs1501299, PTH rs6256, and VDR rs757343 gene

Table 2 Distribution of rs2059806 and rs1799817 polymorphisms from the INSR gene and the rs2241766 and rs1501299 polymorphisms from the ADIPOQ gene in women with PCOS and controls ^a

Variables	Controls (n = 181)	Cases (n = 181)	P-value
Age (years)	31.07 (5.84)	27.13 (5.29)	<0.001
BMI (kg/m ²)	25.48 (4.21)	26.80 (6.37)	0.021
Gene/SNP			
INSR/rs2059806			
Genotype-wise comparison, n (%)			
GG	93 (51.4)	96 (53.0)	0.519
GA	70 (38.7)	73 (40.3)	
AA	18 (9.9)	12 (6.7)	
Allele-wise comparison, n (%)			
G	256 (70.7)	265 (73.2)	0.456
A	106 (29.3)	97 (26.8)	
INSR/rs1799817			
Genotype-wise comparison, n (%)			
TT	8 (4.4)	12 (6.6)	0.630
TC	63 (34.8)	64 (35.4)	
CC	110 (60.8)	105 (58.0)	
Allele-wise comparison, n (%)			
T	79 (21.8)	88 (24.3)	0.427
C	283 (78.2)	274 (75.7)	
ADIPOQ/rs2241766			
Genotype-wise comparison ^b , n (%)			
TT	121 (66.9)	144 (79.6)	0.023
TG	54 (29.8)	34 (18.8)	
GG	6 (3.3)	3 (1.6)	
Allele-wise comparison, n (%)			
T	296 (81.8)	322 (88.9)	0.006
G	66 (18.2)	40 (11.1)	
ADIPOQ/rs1501299			
Genotype-wise comparison, n (%)			
AA	11 (6.1)	12 (6.6)	0.963
AC	79 (43.6)	77 (42.6)	
CC	91 (50.3)	92 (50.8)	
Allele-wise comparison, n (%)			
A	101 (27.9)	101 (27.9)	1.000
C	261 (72.1)	261 (72.1)	

^a Continuous variables presented as mean (SD); categorical variables as number (percent)

^b χ^2 test for genotype-wise comparisons: TT/TG + GG ($P = 0.006$; OR = 1.930, 95% CI = 1.199–3.105), TG/TT + GG ($P = 0.014$; OR = 0.544, 95% CI = 0.333–0.888), and GG/TT + TG ($P = 0.502$; OR = 0.492, 95% CI = 0.121–1.996) test models, respectively

polymorphisms between women with PCOS and controls. However, the ADIPOQ rs2241766 “TT” genotype appeared to be marker of increased PCOS susceptibility. Furthermore,

Table 3 Association between genotypes and alleles of PTH rs6256 and VDR rs757343 gene polymorphisms and risk of PCOS

Gene/SNP	Controls (n = 181)	Cases (n = 181)	P-value
PTH/rs6256			
Genotype-wise comparison, n (%)			
AA	16 (8.8)	10 (5.5)	0.293
AC	58 (32.1)	69 (38.1)	
CC	107 (59.1)	102 (56.4)	
Allele-wise comparison, n (%)			
A	90 (24.9)	89 (24.6)	0.931
C	272 (75.1)	273 (75.4)	
VDR/rs757343			
Genotype-wise comparison, n (%)			
GG	127 (70.2)	123 (68.0)	0.891
GA	48 (26.5)	51 (28.2)	
AA	6 (3.3)	7 (3.8)	
Allele-wise comparison, n (%)			
G	302 (83.4)	297 (82.0)	0.623
A	60 (16.6)	65 (18.0)	

the ADIPOQ rs2241766 “T” allele was more frequent among the women with PCOS compared with the controls.

At the present, PCOS is considered as a complex multi factorial disorder that might result from the interaction of predisposing and protective genomic variants under the influence of environmental factors, including nutritional factors. The association between DNA sequence variations to PCOS has become a subject of interest in recent years. The number and nature of genes that influence susceptibility to PCOS are largely unknown. However most studies have included genes involved in the secretion and/or action of insulin, and regulation of androgen biosynthesis and function [31].

Our findings are in line with previous studies [8, 10] showing a significant association between the ADIPOQ rs2241766 polymorphism and risk of PCOS. However, we observed that the ADIPOQ rs2241766 “T” allele was associated with increased risk of PCOS; while Zhang et al. [10] found that the “G” allele was related to the increased risk; but null associations also have been observed [11]. Since rs2241766 at exon 2 is a “synonymous” SNP meaning that it does not alter the amino acid sequence of adiponectin, the exact molecular mechanisms responsible for the biological effects of the variation is not known at present. However, Yang et al. [32] have suggested that, the rs2241766 polymorphism to be associated with differences in ADIPOQ mRNA expression level and the “T” allele appear to be less active than the “G” allele. The rs2241766 polymorphism may affect mRNA levels through regulation of mRNA splicing and/or stability. On the other hand,

adiponectin levels were lower in patients with PCOS than control [11], and it has been reported that the serum levels of adiponectin is significantly lower in individuals with “TT” genotype compared with those with “GG” genotype [33]. Furthermore, in women with PCOS, the individuals with “TT” genotype had higher insulin resistance [4]. In addition, the “T” allele was related to a higher risk of obesity [32]. Our finding that the ADIPOQ rs2241766 “TT” genotype appeared to be a marker of increased PCOS susceptibility is consistent with the notions above. Therefore, a possible hypothesis is that as the “T” allele is less stable and translated less efficiently into adiponectin, reduced adiponectin abundance may impede adiponectin actions and may contribute to the PCOS risk. Alternatively, the rs2241766 polymorphism may be in linkage disequilibrium with another unknown functional variant of the ADIPOQ gene that explains the association observed. In the present study, we also found that the ADIPOQ rs2241766 “TG” genotype compared with “TT and GG” genotypes was associated with an approximate 45% decreased risk for PCOS. On the other hand, in a previous study by Kaklamani et al. [34], ADIPOQ rs2241766 “TG” genotype was associated with increased serum levels of adiponectin. Therefore, it is possible that the decreased PCOS risk in the individuals with “TG” genotype is result of the higher levels of adiponectin in these subjects.

This study is also in concordance with recent studies [4, 9], where no association was found between the ADIPOQ rs1501299 polymorphism and the risk of PCOS; nevertheless, significant association has been reported [10]. Previous studies have also suggested that the subjects with “AA and AC” genotype had higher ADIPOQ mRNA levels than those with “CC” genotype [35]. Since the rs1501299 polymorphism is located in intron 2 meaning that it does not alter the amino acid sequence of adiponectin, the exact molecular mechanisms responsible for the biological effects of the variation is not known at present.

Studies of the effect of INSR gene SNPs on PCOS have been inconclusive. Our finding is in concordance with a recent study [7], where no association was found between the INSR rs1799817 gene polymorphism and risk of the syndrome. Nevertheless, Siegel et al. [6] have reported a significant association between the SNP and the risk of PCOS. These inconsistent results may contribute to false positive results, ethnic/racial differences in genetic makeup, the variation in environmental, particularly nutritional, factors, the differences in disease definition, genotyped markers and statistical methods. Furthermore, to our knowledge, in this report, the association of INSR rs2059806 gene polymorphism with PCOS risk was studied, which to our knowledge has not been examined previously. The rs2059806 polymorphism at exon 8 is a “synonymous” SNP meaning that it does not alter the amino acid sequence of INSR [36].

However, to conclude that there is no relationship between the rs2059806 polymorphism and PCOS risk, it should be further studied in other populations and larger groups.

To our knowledge, this study represents the first investigation into the association of the VDR rs757343 gene polymorphism with the risk of PCOS; no significant association was found for the polymorphism. The rs757343 polymorphism is located in intron 8 at the 3' end of the VDR gene [30]. The 3'-untranslated region (3'-UTR) of genes is known to be involved in regulation of gene expression, especially through regulation of mRNA stability [37]. Furthermore, alterations in intronic sequences may influence protein expression, but the rs757343 polymorphism was not found to influence VDR protein and mRNA levels. On the other hand, in our previous study [26], we investigated the association between the PCOS risk and VDR rs10735810, rs1544410, rs7975232, and rs731236 polymorphisms, and we demonstrated that the VDR rs7975232 “CC” genotype was associated with PCOS risk. Conflicting results such as these are unfortunately common in genetic association studies [38, 39] and discrepancy in these studies may be explained by false positive results, small sample size, variation in dietary intakes specifically calcium and vitamin D, and differences in the genetic and/or environmental factors triggering the development of PCOS.

These data also investigated for the first time the association between PTH gene polymorphism (rs6256) and PCOS risk. The rs6256 polymorphism, which is located in exon 3 of PTH gene, might contribute to the altered gene expression. It has been demonstrated that serum PTH levels were higher in subjects carrying the rs6256 “AA” genotype compared with individuals in the “AC” and “CC” genotypes [17]. Furthermore, recent studies [14, 16] have reported that the serum level of PTH is higher in women with PCOS than controls. However, we did not find any association between PTH rs6256 genotypes and alleles and PCOS risk. It is possible that our sample size was not large enough to demonstrate the possible difference in the genotype or allele distributions between these two groups. However, to conclude that PTH gene is not involved in the pathogenesis of PCOS, other PTH gene polymorphisms should also be investigated in larger studies.

There are several limitations to our study. A potential limitation of the present study is the modest sample size that precludes drawing strong conclusions. The other limitation is that only one variant per gene was genotyped and thus coverage of each gene was incomplete. Another limitation is a potential information bias from the case-control study design. Accordingly, we could not completely rule out the possibility of chance findings. Nevertheless, the possibility of true finding should not be excluded.

In conclusion, in this case-control study, the ADIPOQ rs2241766 “TT” genotype appeared to be marker of

increased PCOS susceptibility. This study also indicates for the first time that there are no significant associations between INSR rs2059806, PTH rs6256, and VDR rs757343 gene polymorphisms and PCOS risk. However, further studies are warranted to confirm these findings.

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