Lack of association between intercellular adhesion molecule-1 (ICAM-1) polymorphisms and polycystic ovary syndrome

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Abstract

Purpose This study examined the possible association of *G241R* and *K469E* single nucleotide polymorphisms (SNPs) of *ICAM-1* gene with the occurrence and clinical/biochemical characteristics of polycystic ovary syndrome (PCOS).

Methods *G241R* and *K469E* SNPs in DNA from peripheral blood leukocytes of 169 PCOS and 259 healthy control women were investigated by real-time PCR combined with melting curve analysis using fluorescence-labeled hybridization probes.

Results We did not find significant differences in the distributions of *G241R* and *K469E* polymorphisms, nor in the haplotype frequencies between PCOS and control women. None of the studied polymorphisms were found to affect insulin resistance indices significantly.

Capsule There are no significant differences in the distributions of *ICAM-1 G241R* and *K469E* polymorphisms, and in the haplotype frequencies between PCOS and control women.

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Conclusions These preliminary results suggest that the *241* and *469 SNPs* of *ICAM-1* gene may not be risk factors for PCOS. Further studies with a larger cohort, analyzing other *ICAM-1* polymorphisms are necessary to support our observations before any statement can be made about the relationship between PCOS and *ICAM-1* polymorphisms.

Keywords Polycystic ovary syndrome · Intercellular adhesion molecule-1 · Polymorphism · Insulin resistance

Introduction

The polycystic ovary syndrome (PCOS) is a common and complex endocrinopathy affecting 5–10% of women of reproductive age, characterized with chronic anovulation, oligoamenorrhea, and hyperandrogenism [1]. The exact pathophysiologic mechanisms of PCOS remains still elusive. Increasing evidence suggests that ongoing low-grade chronic inflammation and endothelial dysfunction are closely involved in the pathogenesis of PCOS and its associated features such as insulin resistance (IR), dyslipidemia and atherosclerosis [2]. Cellular adhesion molecules are important biomarkers of endothelial dysfunction, and increased plasma levels in response to chronic inflammation have been reported [3]. It has been shown recently that cellular adhesion molecule levels, including intercellular adhesion molecule-1 (ICAM-1), increase in patients with PCOS [2].

ICAM-1 (CD54), a 90- kDa cell surface glycoprotein, is involved in the firm adhesion of leukocytes to endothelium and their trans-endothelial migration to sites of inflammation [4]. A number of *ICAM-1* coding biallelic polymorphisms have been described in the human *ICAM-1* gene located on

chromosome 19p13.3-p13.2, including those located in exons 4 and 6, modifying codons 241 and 469, respectively [5]. *G241R* (glycine/arginine) is located within the third Ig-like domain, shown to be macrophage antigen-1 (Mac-1) and leukocyte function associated-antigen-1 (LFA-1) binding domain [6], and therefore of potential importance during leukocyte transmigration. *K469E* (lysine/glutamic acid) is located in the membrane proximal domain after the fifth-like domain, near the site required for clustering of ICAM-1, which is necessary for efficient leukocyte transmigration [7]. Greater cell surface expression of ICAM-1 on endothelial cells carrying *G241/E469* homozygous genotype was shown [8]. ICAM-1 mRNA and protein of ICAM-1 were reported to be increased in hyperglycemia, diabetes mellitus as well as association of elevated ICAM-1 levels with diabetic complications [9–13]. *G241R* and *K469E* have been demonstrated to be associated with microangiopathies including diabetic retinopathy [14], nephropathy [15], immune disease as Graves disease [16], Behçet's disease [17], inflammatory bowel disease [18], and coronary artery disease [19].

Considering the recognized roles of increased ICAM-1 levels in chronic inflammation conditions, including diabetes mellitus and PCOS, and lack of information about the relationship between PCOS and *ICAM-1* gene polymorphisms, we aimed to investigate whether *G241R* and *K469E* polymorphisms could predispose women to PCOS, and to evaluate the possible relationship between genotypes and clinical or biochemical characteristics of PCOS.

Materials and methods

Subjects

One hundred and sixty nine women with established PCOS and 259 healthy women were studied. PCOS was defined when at least two of the following three features were present: oligo or amenorrhea (<eight menstrual cycles in the presenting year); hyperandrogenism (and/or hirsutism); and polycystic ovaries [20]. Cushing's syndrome, late-onset 21-hydroxylase deficiency, thyroid dysfunction, hyperprolactinemia, diabetes mellitus or androgen secreting tumors were excluded. Among the exclusion criteria, also, were current or previous use (within 6 months) of oral contraceptives, antiandrogens, statins, glyocorticoids, or infertility medications. Control group consisted of 259 healthy fertile women. None of the controls had symptoms of hyperandrogenism, a history of menstrual dysfunction, infertility, or sonographic sings of PCOS. All subjects (PCOS and controls) were nonpreg-

nant, non-smokers, and normotensive. All of the women were studied within the first 10 days after onset of menstruation in the case of mild oligomenorrhea or at random in those suffering from severe oligo- or amenorrhea. The characteristics of PCOS women and controls are summarized in Table 1. The study was approved by the Institutional Review Board at Şişli Etfal Research and Training Hospital. Informed consent was obtained from each subject.

IR was assessed by means of the fasting glucose/insulin ratio (GIR), homeostasis model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI). The GIR values were calculated as fasting glucose (mg/dL)/fasting insulin (μ U/mL). The HOMA index was calculated as [fasting insulin (μ U/mL) x fasting glucose (mg/dL)]/405. The QUICKI was calculated as $1/[\log \text{fasting insulin } (\mu\text{U/mL}) + \log \text{fasting glucose (mg/dL)}]$. High HOMA, low QUICKI and low GIR scores denote IR (low insulin sensitivity). Body mass index (BMI) was calculated as body weight (kg) divided by body height squared (m^2).

Laboratory methods: blood sample processing

Blood samples were taken in the morning subsequent to an overnight fast. Peripheral venous blood samples were collected in plain tubes for routine biochemical analysis, and in EDTA- K_3 for genotype analysis. Serum testosterone, DHEA-S and insulin were measured on Modular EEE Electro Elecsys Roche autoanalyzer (Roche Diagnostics, Mannheim, Germany). Serum glucose, triglyceride, cholesterol, HDL-and LDL-cholesterol measurements were performed on ISE 1800 DPP Roche autoanalyzer (Roche Diagnostics, Mannheim, Germany).

Determination of the ICAM-1 gene polymorphisms

Genomic DNA was isolated from peripheral blood leukocytes by using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). We established a real-time PCR method combined with melting curve analysis using fluorescence-labeled hybridization probes in a LightCycler (Roche Diagnostics, Mannheim, Germany) instrument for the detection of two SNPs. Primers and probes were designed and prepared by Tib MolBiol (Berlin, Germany). The sequences of PCR primers and probes are as follows:

ICAM1 G241R (rs1799969)

Forward 5'-GCGACTCCCCCACAACCTT-3'

Reverse 5'-TGAGGCCTTGGCCGAGAA-3'

Probe 1 5'-TCTGTTCCCTGGACAGGCTGT-FL

Probe 2 5'-LC640-CCCAGTCTCGGAGGCC CAGGTC-3'

Table 1 Clinical and biochemical characteristics of controls and PCOS women [median (range)]

	Control (n=259)	PCOS (n=169)
Age (years)	28 (18–38)	23 (16–40)
BMI (kg/m ²)	21.0 (18–26)	23.6 (18–44)*
Glucose (mg/dL)	88 (68–101)	88 (67–135)
Insulin (μ IU/mL)	8.1 (2.6–17.0)	11.0 (2.5–59.0)*
GIR	10.6 (5.3–29.3.0)	8.1 (1.6–34.4)
HOMA	1.8 (0.5–3.8)	2.4 (0.5–14.0)*
Subjects with HOMA ≥ 2.5 (n/%)	65/25	85/50*
QUICKI	0.38 (0.26–0.43)	0.33 (0.26–0.67)*
Testosterone (ng/mL)	0.12 (0.01–1.14)	0.81 (0.19–1.56)*
DHEAS (μ g/dL)	230 (115–415)	266 (53–3649)
Cholesterol (mg/dL)	143 (86–200)	165 (106–255)
Triglyceride (mg/dL)	73 (27–150)	80 (33–348)
HDL-C (mg/dL)	43 (26–63)	49 (27–73)
LDL-C (mg/dL)	80 (32–121)	96 (50–161)

Mann-Whitney U test; * $p < 0.05$

Abbreviations: BMI body mass index, GIR glucose/insulin ratio, HOMA homeostasis model assessment, QUICKI quantitative insulin sensitivity check index, DHEAS dehydroepiandrosterone-sulfate, HDL-C high density lipoprotein-cholesterol, LDL-C low density lipoprotein-cholesterol

ICAM1 K469E (rs5498)

Forward 5'-GCACTCAAGGGGAGATCATCC-3'

Reverse 5'-GGGGCTGTGGGGAGGATA-3'

Probe 1 5'-ATCCGCAAGGTGACCGTGAAT-FL

Probe 2 5'-LC640-CTCTGTGAGTGAGCCGG
CGGGCA-3'

The melting point analysis uses fluorescence resonance energy transfer for detecting a polymorphic site. Therefore, the 3'-end of the sensor probe was labeled with FL and the 5'-end of the anchor probe, which was placed 1 nucleotide downstream of the sensor probe, was labeled with LC Red 640 and the 3'-end was phosphorylated. The sensor probe was designed for a perfect match either to the wild-type or the variant allele sequence. Thus, in the allele with the sequence deviating from the sensor, a 1-nucleotide mismatch between sensor and target DNA sequence was formed and caused destabilization of the hybrid yielding to a melting point (T_m) shift of 5°C to 10°C.

Analysis was done in 20 μ l volumes using glass capillaries. The PCR mix contained 2 μ l of the genomic DNA, 2 μ l of LC™ FastStart DNA Master HybProbe kit (Roche Diagnostics), 0.5 μ M of each primer, 0.3 μ M of each probe (0.2 μ M for *ICAM-1 K469E*) and 2.5 mM total $MgCl_2$. The initial 10 min denaturation at 95°C was followed by 45 cycles - denaturation (95°C; 10 s), annealing (58°C; 10 s), and elongation (72°C; 10 s). Melting curve analysis was done with an initial denaturing step at 95°C for 5 s, 20 s at 40°C, and slow heating to 80°C (70°C for *ICAM-1 K469E*), with a ramping rate of 0.15°C/s and continuous fluorescence detection. Melting curves were converted to melting peaks by plotting the negative derivatives of fluorescence against temperature ($-dF/dT$) (Figs. 1 and 2). A negative control containing all reagents but water instead of the DNA template was included to each amplification set. Melting curves were evaluated by two independent observers who were blinded to the analysis of the clinical data. In addition, 10% of randomly

Fig. 1 Melting curve analysis of the ICAM G241R detection. The figure shows the melting curves converted to melting peaks by plotting the negative derivative of fluorescence against temperature. The melting points (T_m) are 57°C for G and 64°C for R alleles

Fig. 2 Melting curve analysis of the ICAM K469E detection. The figure shows the melting curves converted to melting peaks by plotting the negative derivative of fluorescence against temperature. The melting points (T_m) are 53°C for E and 60°C for K alleles

selected samples were repeated independently to verify genotyping results and 100% concordance was found.

Statistical analysis

Differences in genotype distributions and allele frequencies in the cases and the controls were compared for statistical significance using the chi-square (χ^2) test. The statistical significance for deviations from Hardy-Weinberg Equilibrium (HWE) was determined using the Pearson χ^2 -test. Odds ratios (ORs) were calculated and given with 95% confidence intervals (CIs). The wild-type genotype/allele served as a reference category. Comparison of individual clinical variables between genotypes were assessed with χ^2 -test. Mann-Whitney U, Kruskal-Wallis and Spearman correlation tests were used for the evaluation of clinical and biochemical parameters. The differences were considered significant if the value of probability (p) did not exceed 0.05. Linkage disequilibrium (LD) and haplotype frequencies were estimated using the

Haploview program [21] and compared between cases and controls using a contingency χ^2 -test. In addition the NCSS 2000 statistical package (Kaysville, Utah, USA) was used to evaluate the power analysis.

Results

In this study, *ICAM-1* G241R and K469E polymorphisms were analyzed in 169 patients with PCOS and 259 unrelated nonpregnant healthy women. Table 1 summarizes the clinical and biochemical characteristics of controls and PCOS women. We had a 97% power to detect an effect size (W) of 0.20 using a 2° of freedom ($\alpha=0.05$).

The genotypic and allelic distributions of *ICAM-1* G241R and K469E polymorphisms for cases and controls are shown in Table 2. SNP genotypes were tested for departures from HWE for controls and patients, and both polymorphisms were in HWE. The allelic frequencies of G (0.96) and R (0.04) for *ICAM-1* 241 and K (0.56) and E

Table 2 Distribution of genotypes and allele frequencies for Polycystic Ovary Syndrome (PCOS) and control group

	Controls n (%)	PCOS n (%)	OR (95% CI)	<i>p</i>
<i>ICAM1</i> G241R				
GG	238 (91.9)	156 (92.3)	1.0 ^a	–
GR	20 (7.7)	13 (7.7)	0.99 (0.48–2.05)	0.98
RR	1 (0.4)	0 (0)	0.51 (0.02–12.55)	0.42
GR+RR	21	13	0.94 (0.46–1.94)	0.88
G allele frequency	0.96	0.96	1.0 ^a	
R allele frequency	0.04	0.04	0.90 (0.45–1.81)	0.77
<i>ICAM1</i> K469E				
KK	79 (30.5)	49 (29.0)	1.0 ^a	–
KE	133 (51.4)	88 (52.1)	1.07 (0.68–1.67)	0.78
EE	47 (18.1)	32 (18.9)	1.10 (0.62–1.95)	0.75
KE+EE	180	120	1.08 (0.70–1.64)	0.74
K allele frequency	0.56	0.55	1.0 ^a	
E allele frequency	0.44	0.45	1.05 (0.80–1.38)	0.74

Each *p*-value was based on Chi-square (χ^2) analysis

^a Reference values for OR

Table 3 Insulin resistance and lipid profile parameters of PCOS women in accordance with their genotypes of the *ICAM1 G241R* gene polymorphism [median (range)]

	<i>GG</i>	<i>GR</i>
BMI (kg/m ²)	23.7 (18–45)	23.0 (18–38)
Glucose (mg/dL)	88 (67–135)	88 (74–111)
Insulin (μIU/mL)	11.1 (2.5–59.0)	10.8 (4.9–29.0)
GIR	8.1 (1.6–34.4)	8.6 (3.8–17.7)
HOMA	2.4 (0.5–14.0)	2.3 (1.1–8.0)
QUICKI	0.33 (0.26–0.67)	0.34 (0.29–0.38)
Cholesterol (mg/dL)	165 (106–255)	138 (13–199)
Triglyceride (mg/dL)	86 (33–348)	55 (49–195)
HDL-C (mg/dL)	49 (27–73)	56 (32–62)
LDL-C (mg/dL)	96 (50–161)	90 (71–127)

Mann-Whitney U test (when *GR* genotype carriers were compared to *GG*)

Abbreviations: *BMI* body mass index, *GIR* glucose/insulin ratio, *HOMA* homeostasis model assessment, *QUICKI* quantitative insulin sensitivity check index, *HDL-C* high density lipoproteine-cholesterol, *LDL-C* low density lipoproteine-cholesterol

(0.44) for *ICAM-1 469* found in our control population were similar to those reported for the French [22], English [23] and German [24] populations. We did not find any evidence for association between PCOS and the two individual SNPs (Table 2). It is seen from the Tables 3 and 4 that the alterations of the indices of IR and lipid profile parameters between genotypes of both *241* and *469* locuses of *ICAM-1* gene were not observed to be statistically significant. The Spearman correlation test was shown that there were positive correlations between BMI/insulin ($r=0.322$, $p=0.0001$), BMI/HOMA ($r=0.319$, $p=0.0001$), BMI/LDL ($r=0.217$, $p=0.038$), BMI/cholesterol ($r=0.278$, $p=0.008$); and negative correlations

between BMI/GIR ($r=-0.325$, $p=0.0001$), and BMI/QUICKI ($r=-0.319$, $p=0.0001$). As we expected, strong correlations between the indices of IR were observed (data not shown). Haplotype analysis revealed that there was not a linkage disequilibrium between *ICAM-1 G241R* and *K469E* polymorphisms. The *G241/K469* haplotype was the most frequent one among the PCOS and controls (0.561 and 0.563, respectively). The haplotype frequencies in PCOS were not different from these ones in controls.

Discussion

Insulin resistance and hyperinsulinemia are present in up to 70% of women with PCOS. It is present in both obese and nonobese PCOS women, and probably have a pivotal role in the pathogenesis of the syndrome [25]. Women with PCOS exhibit a decrease in insulin sensitivity of between 30–40% in women with PCOS, a deficit similar to that seen in subjects with type 2 diabetes mellitus [26]. It is seen from the results that IR is present in 85 (%50) of PCOS patients. In addition, our results showing increased insulin and HOMA, decreased QUICKI in PCOS women, and significant correlations between indices of insulin resistance and BMI in PCOS, agree with these observations.

Insulin resistance has been increasingly recognised as having a central role in chronic inflammation and endothelial dysfunction, which represent an early sign of atherosclerosis [27]. Cellular adhesion molecules are well accepted as markers of low-grade inflammation and endothelial dysfunction [3]. Clinical studies indicate that plasma levels of adhesion molecules are elevated in patients with diabetes mellitus (both type 1 and type 2)[9–12], and in nondiabetic subjects with insulin resistance [28] and correlated with

Table 4 Insulin resistance and lipid profile parameters of PCOS women in accordance with their genotypes of the *ICAM1 K469E* gene polymorphism [median (range)]

	<i>KK</i>	<i>KE</i>	<i>EE</i>
BMI (kg/m ²)	23.5 (18–44)	23.7 (18–44)	23.7 (18–36)
Glucose (mg/dL)	88 (68–113)	87 (67–117)	89 (74–135)
Insulin (μIU/mL)	11.0 (3.7–59.0)	10.9 (2.5–44.4)	11.7 (3.1–33.0)
GIR	8.1 (1.6–20.4)	8.2 (1.9–34.4)	7.8 (3.0–25.2)
HOMA	2.4 (0.8–14.0)	2.4 (0.5–10.6)	2.7 (0.6–8.2)
QUICKI	0.33 (0.27–0.41)	0.33 (0.27–0.67)	0.33 (0.28–0.42)
Cholesterol (mg/dL)	164 (121–234)	165 (106–232)	160 (119–255)
Triglyceride (mg/dL)	106 (40–289)	76 (33–348)	106 (44–241)
HDL-C (mg/dL)	49 (27–61)	49 (32–65)	50 (29–73)
LDL-C (mg/dL)	100 (56–140)	96 (50–158)	88 (59–161)

Kruskal-Wallis test (when *KK*, *KE* and *EE* genotypes were compared)

Abbreviations: *BMI* body mass index, *GIR* glucose/insulin ratio, *HOMA* homeostasis model assessment, *QUICKI* quantitative insulin sensitivity check index, *HDL-C* high density lipoproteine-cholesterol, *LDL-C* low density lipoproteine-cholesterol

urinary albumin and carotid artery intimal-medial thickness [29, 30]. Additionally, increased plasma levels of adhesion molecules, including ICAM-1, in PCOS were reported [2]. Mononuclear cells isolated from insulin resistant subjects have been reported to bind to endothelial cells with enhanced affinity [31]. Although the exact mechanisms are still poorly understood, this process seems to be modulated by various cell adhesion molecules which may explain the increased risk of atherosclerosis in insulin resistant state [31]. ICAM-1 protein is expressed on the surface of several cell types, including leukocytes, endothelial cells, and proliferating intimal smooth muscle cells [4]. ICAM-1, facilitating the adhesion of leukocytes and monocytes to and their transmigration through the activated endothelium, plays an important role in the early stages of vascular disease in conditions with chronic inflammation [3]. Many studies described associations between *ICAM-1 G241R* and *K469E* polymorphisms and peripheral occlusive arterial disease [32], inflammatory bowel disease [18], diabetes mellitus [14, 15] and coronary artery disease [19]. However, although there is a strong relationship between chronic inflammation and insulin resistance in PCOS, we did not find an association between *G241R* and *K469E* polymorphisms and the risk of developing PCOS in the present study, indicating that these polymorphisms alone may not play a major role in the PCOS etiopathogenesis.

There is no consensus as to which alleles of *ICAM-1 G241R* and *K469E* polymorphisms are protective or detrimental. The functional consequences of studied polymorphisms have not been elucidated yet. While the *ICAM-1 G241R* polymorphism results in a glycine to arginine change within the Ig-like domain 3, the *ICAM-1 K469E* polymorphism results in a lysine to glutamic acid change in the Ig-like domain 5. The Ig-like domain 3 mediates binding to Mac-1 and may also affect accessibility of LFA-1 binding to the Ig-like domain 1 [6]. Therefore, it seems possible that these polymorphisms, modified receptor-ligand interactions, and influencing the expression rate, may affect the adhesive and transmigration functions of ICAM-1. It has been shown that the mutation at 240 position of ICAM-1 gene enhances binding to Mac-1 [6]. So, the polymorphism at the neighboring position 241 may also modulate the affinity of Mac-1. Moreover, a functional study performed by Holder et al. [8] revealed that the *G241/E469* haplotype is associated with greater cell surface expression of ICAM-1, which in turn leads to greater adhesion of leukocytes to damaged endothelium and explains the possible association between chronic inflammatory diseases and *ICAM-1* polymorphisms. In the present study, we find that the *G241/E469* haplotype frequencies are similar in controls and PCOS cases. So this finding suggests again that these polymorphisms may not be risk factors for PCOS. In addition, while the presence of *K469* allele has been

associated with coronary artery disease [16], and peripheral artery disease [32], it has been demonstrated that the *EE* genotype predispose patients with type 2 diabetes mellitus to diabetic retinopathy [14]. Finally, the amino acid substitution resulting from studied polymorphisms may influence the three-dimensional conformation and serum concentrations of ICAM-1, as the ICAM-1 concentrations in Caucasians with the *G241G* and *E469E* were significantly higher than those in Caucasians with *R241R* [33] and *K469K* or *K469E* [14]. Since we were not able to determine the plasma ICAM-1 concentrations in all of the subjects involved, we could not estimate the relationship between genotypes and plasma ICAM-1 levels.

Another objective of our study was to investigate the probable relationship between studied polymorphism and indices of IR as well as lipid profile parameters. When PCOS patients parameters were classified in accordance with their genotype of *ICAM-1 G241R* and *K469E*, no significant differences among genotypes were found.

To the best of our knowledge, this is the first study regarding for the association between *ICAM-1 G241R* and *K469E* polymorphisms and PCOS susceptibility. There is not any association between *G241R* or *K469E* polymorphisms and susceptibility to PCOS. Additionally, there is not a significant difference among genotypes of both locuses in mean of IR and lipid profile parameters. As a conclusion, the results obtained in this study do not provide any definitive evidence that *ICAM-1 241* and *469* genotypes play a role in susceptibility to PCOS, although this cannot be definitely excluded without further analysis in a larger study group combined with analysis of further polymorphisms in the *ICAM-1* gene.

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