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ORIGINAL ARTICLE

Methylenetetrahydrofolate reductase C677T gene polymorphism in turkish patients with polycystic ovary syndrome

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Abstract Higher Levels of Hcy are associated with several clinical conditions, among them non-insulin-dependent diabetes mellitus, endometrial dysplasia and hypertension with insulin resistance, and polycystic ovary syndrome. The purpose of this study was to investigate the serum homocystein levels and other metabolic parameters in relationship with the *MTHFR* C677T gene polymorphism in patients with PCOS. Our study included 86 young women with PCOS constituting the study group and 70 healthy women constituting the control group. Homocystein levels, metabolic, and hormonal parameters were measured, and genetic analysis of the *MTHFR* C677T gene polymorphism was performed in all the subjects. A statistically significant difference was observed in mean homocystein levels between patients with PCOS when compared to the control group. The MTHFR 677 CC genotypes had significantly higher proportions in the control group compared to the PCOS patients ($\chi^2 = 21.381$, $P < 0.001$). Our data show that homocystein levels were higher than normal subjects in patients with PCOS and that the *MTHFR* C677T gene polymorphism does not influence homocystein levels of patients with PCOS.

Keywords Hyperhomocysteinemia · Methylenetetrahydrofolate reductase · MTHFR · C677T · Polymorphism · Polycystic ovary syndrome

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most encountered endocrine malfunctions, which typically occurs with chronic anovulation and hyperandrogenism [1]. Almost 15% of women in reproductive age suffer from PCOS symptoms [2].

Cardiovascular diseases (CVD), dyslipidemia, and hypertension are seen more frequently in patients with PCOS than controls [3–5]. PCOS has also been reported to be associated with an increase in subclinical atherosclerotic diseases [5]. These findings suggest that women with PCOS may be at a higher risk for early-onset CVD. Given the high prevalence of PCOS in the female population, this disorder may potentially account for a significant proportion of atherosclerotic heart diseases observed in young women.

The imbalance between homocystein (Hcy) production and metabolism might possibly due to demographic, genetic, nutritional, or metabolic factors that cause an abnormal increase in plasma and urine Hcy levels which are found to be associated with premature vascular diseases [6].

Elevated plasma Hcy levels are considered an independent risk factor for CVD [7]. Circulating Hcy levels could be influenced by genetic factors [8]. The most common cause of abnormal serum Hcy levels in the

general population seems to be the inefficiency of the methylenetetrahydrofolate reductase (*MTHFR*) enzyme, which is involved in the folate-dependent remethylation of Hcy to methionine [9]. The C677T polymorphism, i.e., cytosine to thymine transition at nucleotide 677 in the *MTHFR* gene, results in an alanine to valine substitution at codon 222 and, therefore, to an impairment of enzyme activity [10–12].

Increased Hcy levels may also cause other illnesses, which are also found to be associated with increased morbidity [13–15]. In this study, we aimed to examine the relationship of MTHFR gene polymorphism and homocysteine in PCOS patients.

Materials and methods

Subjects

Eighty-six-young women (mean age, 24.27 ± 5.44 years) with PCOS diagnosed by the Rotterdam PCOS consensus criteria [16] and 70 healthy control women (mean age, 26.41 ± 5.65 years) were included in this study, which was approved by the Ege University Hospital Ethical Committee. Baseline plasma concentrations of LH, FSH, total testosterone, 17-hydroxyprogesterone (17-OHP), dehydroepiandrosterone sulfate (DHEA-S), estradiol, SHBG, and prolactin (PRL) were determined during the follicular phase (cycle days 5–7) after spontaneous or progesterone–induced cycles in all the study and control subjects. Patients with DM, hyperprolactinemia, congenital adrenal hyperplasia (diagnosed with the adrenocorticotropic hormone stimulation test), thyroid disorders, Cushing's syndrome, hypertension, hepatic, or renal dysfunction were excluded from this study. Clinical examination, evaluation of the hirsutism score by the Ferriman–Gallwey scoring, alophesi, and body mass index (BMI) calculation was also performed for each subject [17]. All the control subjects had regular menstrual cycles and no clinical or biochemical evidence of hyperandrogenism.

Genetic polymorphism study

Genomic DNA of all the subjects was extracted from peripheral leukocytes which were obtained from blood specimens collected in tubes containing EDTA. Isolation has been performed with the High Pure PCR Template Preparation Kit (Roche Applied Science) according to the manufacturer's protocol.

The *MTHFR* C677T gene polymorphism has been studied via a real-time online PCR method. For detection of this polymorphism, primers and probes mentioned below were used together with the LightCycler-DNA Master Hybridization Probes Kit (Roche Applied Science, Mannheim, Germany): forward primer 5′-CGAAGCAGG GAGCTTTGAGGCTG-3′, reverse primer 5′-AGGACGG TGCGGTGAGAGAGTG-3′, Fluorescein probe 5′-TGA CCTGAAGCACTTGAAGGAGAAGGTGTC-Fl-3′, and LCRed640 probe 5′-LCRed640-CGGGAGCCGATTTCA TCAT-P-3′. Experiments were carried out on the LightCycler™ Instrument (Roche Applied Science; Mannheim, Germany) according to the protocol of Charalampos Aslandis and Gerd Schmitz (Institute for Clinical Chemistry and Laboratory Medicine, University of Regensburg, Regensburg, Germany). Polymorphic alleles were identified by the specific melting temperature (Tm) of the resulting amplicons. Individuals with two copies of the C allele (C/C) showed a single melting peak at 63.1°C. Individuals with two copies of the T allele (T/T) also showed a single melting peak but, at 54.6°C individuals with both alleles (C/T) showed two melting peaks at 54.6 and 63.1°C in this analysis.

Initially, ninety patients were included into this study, but four patients dropped-out due to different personal reasons (genotype success rate = 96.6%). Similarly, the 5 of 75 women in the control group dropped-out (genotype success rate = 95.8 %). Genotypes were expressed as CC for homozygous normal, CT for heterozygous, and TT for homozygous mutant.

Biochemical and hormonal assays

Serum total-cholesterol, LDL and HDL-cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyltransferase (GGT) were measured by the Olympus AU 2700 automated analyzer. Plasma insulin was determined in a two-site chemiluminescent enzyme immunometric assay for the Immulite automated analyzer. Serum glucose levels were measured with glucose oxidase method. Plasma LH, FSH, PRL, estradiol, *P*, 17-OHP, testosterone, and dehydroepiandosterone sulfate were measured by RIA assay. Serum concentrations of h-CRP were determined by an immunonephelometric assay (N-high-sensitivity CRP; Dade Behring); intra and interassay CV were 1.72 and 2.80%, respectively.

Insulin resistance was calculated using the homeostasis model assessment-insulin resistance index (HOMA-IR) according to the following formula [18]:

$$\text{HOMA-IR} = \frac{\text{Fasting serum insulin (mU/ml)} \times \text{Fastingplasmaglucose(mmol/l)}}{22.5}$$

Measurement of plasma homocysteine

Venous blood samples were centrifuged at 1000×*g* for 10 min, and the serums were stored at −80°C until the

analysis. In this study, serum total homocystein levels were measured by Fluorescence Polarization Immunoassay Method (IMX Homocysteine Assay, Abbott Diagnostics No: 7D29-20). Dilution method was used for those whose serum homocystein levels were higher than 50 μmol/l. All the samples were prepared as 200 μl. Three controls were used in each sample for calibration of the device. For each of the controls, the results including ranges were accepted as low control (5, 25–8, 75), medium control (10.0–15.0), and high control (20.0–30.0) μmol/ml.

Statistical analysis

SPSS 14.0 for Windows (SPSS Inc, Chicago USA) was used for statistical analysis of the results. $P < 0.05$ values were accepted as statistically significant. The characteristics of the PCOS patients and the mean plasma Hcy, glucose, and insulin, dehydroepiandrosterone sulfate, 17ß-estradiol, homocystein, 17-hydroxyprogesterone, prolactin, total-testosterone levels between the two clinical groups were compared by Student's *t* test for unpaired data and between and within the different groups (CC, CT, and TT) of the *MTHFR* genotypes with ANOVA.

Univariate logistic regression analysis was performed to show which of the parameters for which statistically significantly difference was found at the Student's *t* test between the study and control group was more associated with PCOS.

Allelic and genotypic frequencies were determined from observed genotype counts, and the expectations of the Hardy–Weinberg equilibrium were evaluated by χ^2 analysis. Differences in the genotype distributions between different groups were assessed by Pearson's χ^2 test of heterogeneity. All the results were expressed as means ± SD.

Results

I-Demographic, hormonal, and biochemical features in PCOS patients and controls

The demographic and hormonal data of the patients and control were shown in Table 1. In the patients, LH, FSH, 17-OHP, free and total testosterone, high-sensitive CRP (hs-CRP), and fibrinogen levels were significantly ($P < 0.05$) higher than controls. Also, the serum fasting insulin and glucose, hcy, levels were significantly higher ($P < 0.05$) in the PCOS patients than in the controls. However, HDL-cholesterol levels were significantly lower ($P < 0.05$) in patients than in the control group. However, there were statistically significant differences in HOMA-IR values ($P < 0.05$) and serum fasting insulin levels ($P < 0.05$) between the study and control groups. Nevertheless insulin, 17-OHP, HOMA-IR, HDL-cholesterol, f-testosterone parameters were found to be statistically

Table 1 Clinical, demographical and biochemical data in women with PCOS and controls

	Patient ($n = 86$)	Control ($n = 70$)	*P* value
Age (years)	24.27 ± 5.44	26.41 ± 5.65	N.S
BMI (k/m^2)	24.41 ± 5.43	23.35 ± 5.04	N.S
Fasting glucose (mmol/l)	5.065 ± 0.487	4.77 ± 0.402	<0.05
Fasting insulin (mmol/l)	104.31 ± 26.39	31.6 ± 13.12	<0.05
HOMA-IR	3.40 ± 8.2	1.29 ± 0.33	<0.05
LH (IU/ml)	6.52 ± 4.4	4.26 ± 1.5	<0.05
FSH (IU/ml)	5.43 ± 2.11	4.26 ± 2.29	<0.05
Estradiol (pmol/l)	127.1 ± 95.05	131.75 ± 139.46	N.S
DHEA-S (μg/dl)	787.69 ± 375.4	808.5 ± 335.89	N.S
TSH (μIU/l)	3.04 ± 4.66	1.98 ± 0.92	N.S
17-OHP (ng/ml)	4.40 ± 2.9	0.85 ± 0.47	<0.05
f-testosterone (nmol/l)	0.11 ± 0.07	0.05 ± 0.02	<0.05
T-testosterone (nmol/l)	1.03 ± 0.05	0.03 ± 0.02	<0.05
Prolactin (pmol)	766.07 ± 428.6	754.63 ± 322.11	N.S
Total cholesterol (mmol/l)	5.108 ± 1.1	4.9 ± 0.68	N.S
Triglyceride (mmol/l)	1.33 ± 0.76	1.32 ± 0.38	N.S
LDL-cholesterol (mmol/l)	3.0 ± 0.8	2.8 ± 0.5	N.S
HDL-cholesterol (mmol/l)	1.47 ± 0.39	1.53 ± 0.19	<0.05
Fibrinogen (mg/dl)	360.14 ± 98.5	270.53 ± 70.58	<0.05
hs-CRP (mg/dl)	0.395 ± 0.75	0.25 ± 0.30	<0.05
hcy (μmol/l)	12.315 ± 3.9	10.078 ± 2.247	<0.05

Statistically significant difference was determined between two groups ($P < 0.05$)

N.S not significant

Table 2 Genotype distribution and allele frequency of the MTHFR C677T polymorphism in PCOS patients and healthy group

Genotype	Patients		Healthy group	
	n	%	*n*	%
CC	15	17.4	35	50
CT	65	75.6	28	40
TT	6	7	7	10
Allele				
C	95	55.2	98	70
T	77	44.8	42	30

Data were compared between groups by χ^2 test

The genotype distribution of C677T in MTHFR is shown in Table 2. The allelic distribution of MTHFR genotypes was in Hardy–Weinberg equilibrium for both groups of women

n number of individuals

significant with an independent sample *t* test between the study and control groups. However, when these parameters were re-evaluated at forward logistic regression analysis, although homocystein, fibrinogen, hs-CRP, FSH, and LH parameters had statistically significant differences at inter-groups univariate analysis, no difference was found between them when they were examined at multiple regression analysis. The reason of this was thought to be that the variables found significantly different at multiple regression analysis occurred as a result of their interaction with above-mentioned parameters. Statistically significant differences were found between the patient and control group in relationship with 17-OHP ($P = 0.015$), f-testosterone ($P = 0.012$), and insulin ($P = 0.032$) (Table 2).

II-Genotype distribution in PCOS patients and controls

Genotype distribution and the allele frequencies of the *MTHFR* C677T gene polymorphism are shown in Table 3. The allelic distribution of the *MTHFR* genotypes was in Hardy–Weinberg equilibrium for both groups of women. The heterozygous CT genotype was seen 4.34 times more than the homozygous normal CC genotype in patients with PCOS ($P = 0.0001$), while the homozygous mutant TT genotype was seen two times more than the normal CC genotype, but with no statistical difference between them ($P = 0.276$). The CC genotype had significantly higher proportions in the control group than PCOS patients ($\chi^2 = 21.381$, $P < 0.001$). As shown in Table 3, the T allele is rather frequent in women with PCOS ($P = 0.008$); however, there was no significant difference between the groups as a matter of the C allele frequency (Table 3).

III-The features of PCOS patients and controls in relation to the genotypes

No differences were found between the C/C, C/T, and T/T genotypes and the HOMA-IR levels ($P = 0.397$), insulin levels ($P = 0.332$) within the PCOS patients. A statistically significant correlation was found between the C/C, C/T, and T/T genotypes and the hs-CRP levels within the PCOS patient group ($P = 0.04$) (Table 4). This difference was also significant when hs-CRP values of the study group were compared to those of the control group ($P < 0.05$). When hs-CRP levels were compared between C/C and C/T genotypes, a significant difference was found in the study group and in the control group ($P = 0.016$ and $P = 0.04$,

Table 3 Metabolic and hormonal levels of the patients with PCOS according to the MTHFR genotypes

	PCOS patients (mean ± SD deviation)			
	C/C	C/T	T/T	*P*
Fasting blood glucose (pmol/l)	90.0 ± 7.5	92.1 ± 9.2	89.6 ± 9	0.37
Fasting insulin (mmol/l)	94.3 ± 88.8	113.1 ± 11	77.6 ± 56.1	0.84
HOMA-IR	3.04 ± 2.75	3.6 ± 9.73	2.4 ± 2.17	0.82
17-OHP (ng/ml)	1.74 ± 0.54	1.7 ± 0.91	1.8 ± 0.82	0.27
HDL-cholesterol (mmol/l)	1.48 ± 0.25	1.43 ± 0.4	1.4 ± 0.4	0.40
LDL-cholesterol (mmol/l)	107.5 ± 25.0	118.9 ± 34.7	119.3 ± 23.1	0.61
Triglycerid (mmol/l)	1.363 ± 1.01	1.61 ± 0.9	1.20 ± 0.36	0.36
Total cholesterol (mmol/L)	4.65 ± 0.89	5.045 ± 1.12	4.89 ± 0.99	0.51
f-testosterone (nmol/Ll)	0.103 ± 0.05	0.107 ± 0.07	0.14 ± 0.06	0.28
hs-CRP (mg/dl)	0.37 ± 0.36	0.44 ± 0.8	0.1 ± 0.04	*0.04
Fibrinogen (mg/dl)	374.8 ± 111	363.2 ± 104	322.6 ± 12.0	0.6
Homocysteine (μmol/l)	13.2 ± 3.3	11.8 ± 4.1	14.8 ± 1.7	0.19
Right CIMT (mm)	0.42 ± 0.04	0.4 ± 0.05	0.4 ± 0.0	0.55
Left CIMT (mm)	0.40 ± 0.04	0.4 ± 0.05	0.4 ± 0.04	0.16

* Statistically significant difference was determined between two groups ($P < 0.05$)

Table 4 Evaluations with test and multiple logistic regression analysis (MLRA) of parameters in PCOS patients

Parameters	Independent sample *t* test (*P* value)	Forward logistic regression analysis
Fasting serum glucose	<0.05	
Fasting serum insulin	<0.05	*0.032
HOMA-IR	<0.05	
LH	<0.05	
FSH	<0.05	
Total testosterone	<0.05	
f-testosterone	<0.05	*0.012
HDL-cholesterol	<0.05	
Fibrinogen	<0.001	
hs-CRP	<0.05	
Hcy	<0.05	
17-OHP	<0.05	*0.015

* Parameters were significantly different ($P < 0.05$) with multiple logistic regression analyses

respectively), and the difference was higher with the C/T genotype (Table 4).

Discussion

Insulin resistance and hyperinsulinemia play central roles in multiple hormonal and metabolic derangements that occur in PCOS [19]. Hcy has taken a place among other major risk factors of CVD such as cholesterol, smoking, and obesity. However, it is now widely accepted as a major independent risk factor for cardiovascular, cerebrovascular, and peripheral vascular diseases [20].

In this current study, it was found that the hcy level was significantly higher in subjects with PCOS than in control subjects. Several studies have shown that hcy acts on the cardiovascular system with a direct toxicity on the endothelium [21–25]. In a recently published study, elevated serum hcy concentrations were found in women with PCOS, suggesting that an alteration in hcy metabolism may play a role in increased cardiovascular risk associated with PCOS [26]. Yarali H et al. [27] reported that hcy was significantly elevated in both lean and obese PCOS subjects. A more recent report has been the first to demonstrate an ethnical difference in the elevation of hcy in women with PCOS, which reflects an ethnical variation in the severity of insulin resistance of PCOS [28].

In the original reports of Kang et al. [29], a variant of the *MTHFR* enzyme characterized by thermolability and with approximately half of the normal enzyme activity has been suggested. This variant changes significantly in populations from different geographic areas [30]. For example, difference in this gene was found in 28% of the patients that had early atherosclerosis and high hcy levels, and 17 % of the CVD patients [31].

In our study, a statistically significant difference showed between hcy levels in the PCOS patient and control groups, respectively (10.078 ± 2.247, $P < 0.05$ for 12.315 ± 3.9 μmol/l). However, there was no statistically significant difference between the *MTHFR* genotypes and hcy levels ($P > 0.05$). In an Italian study, similarly no statistically significant difference of hcy levels was found between the PCOS and healthy control subjects [32].

In other studies, caucasian PCOS women had higher plasma Hcy concentrations with a 1.9 times higher frequency of the T allele than the South Asian PCOS group. The South Asians had a 1.8 times higher frequency of the C allele than the Caucasians; however, the overall frequency was comparable in the two PCOS groups [33]. In our study, we observed a high prevalence of the heterozygous CT *MTHFR* genotype in the Turkish population. However, for the homozygous TT genotype, a statistically significant difference was found between the study and control group.

Some of the previous studies reported supporting results to our findings, but some did not [34, 35]. The number of patients included in previous studies was smaller [34, 36]. The *MTHFR* C677T gene polymorphism in subjects with PCOS was found 1–2 times more frequent than in control subject by Tsanadis et al. [37].

Hcy increases DNA synthesis in vascular smooth muscle cells inducing their proliferation [38]; it blocks the regeneration of endothelial cells and causes oxidation of low lipoprotein [39]. In previous studies of coronary calcification, a risk factor of CVD was found to be significantly increased in patients with PCOS compared with healthy control subjects [40–43]. Alexandraki et al. [44] also showed that early microvascular and macrovascular angiopathy were seen similar in both the PCOS and control subjects.

The MTHFR genotypes do not appear to have significant correlation with the plasma Hcy levels in the PCOS patients. However, we seen higher homocystein levels in PCOS groups than controls. The reason for this, in our study other metabolic abnormalities (e.g., insulin resistance) and dietary inadequacies (e.g., lower taking folate and B12) may be affected the hcy levels in PCOS patients.

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