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Assessment of *GSTO1* (A140D) and *GSTO2* (N142D) Gene Polymorphisms in Iranian Women with Polycystic Ovarian Syndrome

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Abstract

Background: Glutathione S-transferases (GSTs) protect cells from oxidative stress (OS). In humans, the GST omega class contains two expressed genes, *GSTO1* and *GSTO2*. Because OS is involved in the pathogenesis of polycystic ovary syndrome (PCOS), the aim of this study was to investigate the relationship between *GSTO1* A140D (rs4925) and *GSTO2* N142D (rs156697) polymorphisms in PCOS patients.
Methods: 175 PCOS patients and 161 healthy controls were selected among women in Kermanshah province, Iran. *GSTO1* and *GSTO2* were genotyped using allele-specific PCR (AS-PCR) and PCR-RFLP, respectively.
Results: For *GSTO1*, the DD genotype and the D allele led to 2.17- (P= 0.02) and 1.5-fold (P= 0.01) increases, respectively, in the odds ratios for PCOS. No significant difference was found between control and patient groups for the *GSTO2* N142D genotype or allele frequency. *GSTO1* and *GSTO2* genotype interaction analysis showed that individuals with the *GSTO1* AD or DD genotypes and the *GSTO2* NN or DN genotypes had a 1.53-fold (P= 0.007) increase in PCOS risk over *GSTO1* AA and *GSTO2* DD individuals.
Conclusions: The *GSTO1* A140D polymorphism is a risk factor for PCOS.

Keywords: Gene polymorphism, *GSTO1*, *GSTO2*, Oxidative stress, PCOS.

Introduction

Polycystic ovary syndrome (PCOS), a common endocrine disorder, affects about 5 to 10% of females before menopause (1). PCOS was first reported in 1935 by Stein et al. (2), but ambiguities remain regarding the etiology of the disease. Studies have shown that the PCOS phenotype involves a combination of genetic and environmental factors. Family studies confirmed a genetic basis for this disease; however, its exact genetic mechanisms are unknown (3). More than 100 genes have been investigated in terms of the relationship between single nucleotide polymorphisms (SNPs) and PCOS (4). This disorder, which is considered a chronic systemic disorder, is often related to insulin resistance, hyperandrogenemia, chronic inflammation, and oxidative stress. Although the mechanisms of PCOS pathogenesis are not fully identified, some studies propose a role for oxidative stress (OS) in the disorder (5, 6). Oxidative stress results from an imbalance in the production and removal of reactive oxygen and nitrogen species (ROS and RNS, respectively). Excessive ROS accumulation can lead to lipid, protein, and cellular damage (6). Oxidative stress can induce genetic and epigenetic changes directly through DNA damage, and both play a role in disease pathogenesis (7, 8). Oxidative stress changes in PCOS seem to decrease gene stability and increase the risk of mutations involved in female-related cancers (6).

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An antioxidant defense system consists of non-enzymatic antioxidants, including vitamin E, ascorbate, and glutathione and enzymatic antioxidants, including superoxide dismutase, catalase, glutathione peroxidase, and glutathione S-transferases (GSTs) (9, 10).

Glutathione S-transferases are detoxifying enzymes that scavenge free oxygen radicals. Loss of the ability of these enzymes to detoxify carcinogens leads to increased cancer rates (11, 12). Types of GSTs include alpha, kappa, mu, pi, theta, microsomal, MGST, and omega (11). Glutathione S-transferase omega (GSTO) has different functions and characteristics than other GSTs (13). It has been reported that abnormal OS in PCOS patients leads to genetic instability and increased cancer risk (6). Because GSTO plays an important role in neutralizing ROSs (14), several studies have investigated polymorphisms in these genes, including *GSTO1* A140D and *GSTO2* N142D, as risk factors for the disease.

The *GSTO1* polymorphism at nucleotide 419 (rs4925) changes amino acid 140 from alanine to aspartate (A140D) and the *GSTO2* polymorphism at nucleotide 424 (rs156697) changes amino acid 142 from asparagine to aspartate (N142D) (15). The aim of the current study was to investigate a possible relationship between *GSTO1* A140D (rs4925) and *GSTO2* N142D (rs156697) polymorphisms and PCOS, which, to our knowledge, has not yet been studied.

Materials and methods

Sample collection

Whole blood samples were collected from 175 PCOS patients and 161 healthy controls who were homogenous in terms of age. Patients and healthy controls were selected among women in Kermanshah Province, which is located in western Iran (2015-2016). After signing informed consent forms all the participants were examined by a gynecologist based on clinical parameters and Rotterdam Criteria (16) Based on the Rotterdam Criteria, the patient should have at least two of these three criteria: anovulation, clinical or laboratory hyperandrogenism, and polycystic ovary identified by ultrasound (16). Study methods were approved by the Ethical Committee of Kermanshah University of Medical Sciences.

DNA extraction

Genomic DNA was extracted from 1 ml whole blood according to the method of Moradi et al. (17). The integrity, purity, and concentration of the extracted DNA were analyzed using agarose gel electrophoresis and a NanoDrop spectrophotometer (Thermo, USA).

PCR–RFLP analysis

GSTO1 (A140D) was genotyped using allele-specific PCR (AS-PCR) with four specific primers (Table 1). The 25 µl PCR consisted of 2.5 µl of 10X PCR buffer, 20 pmol of each primer (Table 1), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U of Taq DNA polymerase (SinaClon BioScience Co., Iran), and 2 µl of DNA (100-200 ng/ul). The PCR was performed using an Eppendorf Mastercycler Gradient thermal cycler (Germany) as follows: 95 °C for 6 min, 34 cycles of 95 °C for 42 s, 60 °C for 42 s, and 72 °C for 45 s, followed by 72 °C for 6 min. The PCR products were electrophoresed on a 2.5% agarose gel. *GSTO2* (N142D) was genotyped by PCR-RFLP. The PCR program was as follows: 94 °C for 6 min, 34 cycles of 94 °C for 32 s, 57 °C for 42 s, and 72 °C for 42 s, followed by 72 °C for 6 min. Approximately 10 µl of PCR product was digested by 1 unit of the MboI restriction enzyme (Takara, Korea) at 37 °C. To control the quality, approximately 10% of the samples were selected randomly and re-evaluated by electrophoresis.

Table 1. Primers used to detect *GSTO1* and *GSTO2* polymorphisms.

NCBI rs#	Gene	Location	Primer sequences	Detection method
rs4925	*GSTO1*	Exon	CF: 5'-gaagccaaaataaagaagactaaga-3' CR: 5'- ttgatttatatcttaagcaggac-3' WF: 5'- attattctctgtctaggtgc-3' MF: 5'- ttacgaaattcttcttttaggcaag-3'	AS-PCR
rs156697	*GSTO2*	Exon	F: 5'- aggcagaacaggaactggaa-3' R: 5'- gagggaccccttttgtacc-3'	RFLP-PCR

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Statistical analysis

SPSS v. 16 software was used for statistical analysis. The genetic distribution between patient and healthy control groups was determined using Pearson's chi-square ($\chi 2$) test. Odds ratios with 95% confidence interval (95% CI) were calculated using unconditional logistic regression. The results were considered significant at $P < 0.05$. Body mass indexes (BMIs) were calculated by dividing subjects' body weights by their squared heights.

Results

All patients and controls were women in their reproductive ages from Kermanshah Province, Iran. No significant difference in mean ages was found between the patient and healthy control groups (patient: 29.6 ± 3.56; control: 27.9 ± 3.6); but the two groups were significantly different in terms of BMI (patient: 29.9 ± 3.49; control: 23.15 ± 3.61). Tables 2 and 3 show the genotypic distribution for *GSTO1* and *GSTO2* polymorphisms in patients and healthy controls, respectively. The genotype frequencies in patients and healthy controls were consistent with the Hardy-Weinberg equilibrium (*GSTO1*: χ^2 = 0.0158, P = 0.899; *GSTO2*: χ^2 = 0.374, P = 0.54).

The PCR product lengths from *GSTO1* (A140D) genotyping were as follows: 304- and 263-bp bands were observed in a homozygous AA individual, 304-, 263-, and 93-bp bands in two heterozygous AD individuals, and 304- and 93-bp bands in two homozygous DD individuals (Fig. 1). The band arrangement from *GSTO2* (N142D) genotyping was as follows: 122- and 63- bp bands were observed in an NN homozygous individual, 185-, 122-, and 63-bp bands in an ND heterozygous individual, and a 185 bp band in a homozygous DD individual (Fig. 2). The DD genotype frequency was significantly greater in the patient than in the healthy control group (P = 0.02), resulting in a 2.17-fold increase in the relative risk of PCOS (Table 2). Also, the D allele frequency in the patient group was significantly greater than in the control group (P = 0.01), resulting in a 1.5-fold increase in the relative risk for PCOS (Table 2). No significant difference was found between the patient and control groups in terms of genotype or allele frequencies for the *GSTO2* N142D polymorphism (Table 3). *GSTO1* and *GSTO2* genotype interaction analysis showed that individuals with *GSTO1* AD or DD genotypes and *GSTO2* NN or DN genotypes had a 1.53-fold (P = 0.007) increase in PCOS risk over *GSTO1* AA and *GSTO2* DD individuals (Table 4).

Table 2. *GSTO1* (rs4925) genotype frequencies

***GSTO1* Allele/Genotypes**	**Controls n (%)**	**Patients n (%)**	**OR (95% CI), *P* value**	**χ^2, *P* value**
A	219 (68)	205 (58.6)	1	
D	103 (32)	145 (41.4)	1.5 (1.09-2.06), 0.01	6.42, 0.01
AA	76 (47.2)	62 (43.4)	1	
AD	67 (41.6)	81 (46.3)	1.48 (0.93-2.36), 0.1	
DD	18 (11.2)	32 (18.3)	2.17 (1.12-4.24), 0.02	6.09, 0.048

Table 3. *GSTO2* (rs156697) genotype frequencies

***GSTO2* Allele/Genotypes**	**Controls n (%)**	**Patients n (%)**	**OR (95% CI), *P* value**	**χ^2, *P* value**
D	216 (67.1)	211 (60.3)	1	
N	106 (32.9)	139 (39.7)	1.34 (0.98 - 1.84), 0.07	3.34, 0.08
DD	73 (45.3)	64 (36.6)	1	
DN	70 (43.5)	83 (47.4)	1.35 (0.85-2.15), 0.20	
NN	18 (11.2)	28 (16)	1.77 (0.90-3.50), 0.1	3.29, 0.193

Table 4. Interaction between *GSTO1* (rs4925) and *GSTO2* (rs156697)

GSTO1	***GSTO2***	**OR (95% CI), *P* value**
AA	DD	1
AD+DD	DD	1.32 (0.96-1.82), 0.084
AA	DN+NN	1.25 (0.90-1.72), 0.174
AD+DD	DN+NN	1.53 (1.12-2.08), 0.007*

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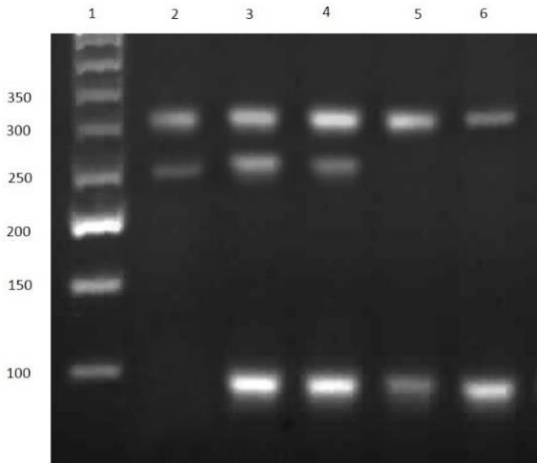

Fig. 1. *GSTO1* AS-PCR products on a 2% agarose gel. Lanes (1) 50-bp DNA Ladder, (2) 304- and 263-bp bands from an AA homozygous individual, (3 and 4) 304-, 263-, and 93-bp bands from AD heterozygous individuals, and (5 and 6) 304- and 93-bp bands from DD homozygous individuals.

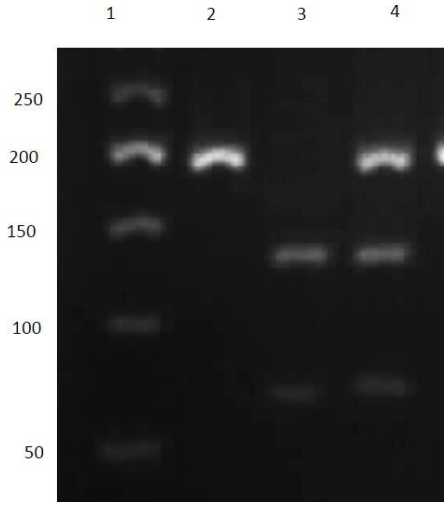

Fig. 2. *GSTO2* PCR-RFLP products on a 2% agarose gel. Lanes (1) 50-bp DNA Ladder, (2) 185-bp band from a DD homozygous individual, (3) 122- and 63-bp bands from an NN homozygous individual, and (4) 185-, 122-, and 63-bp bands from an ND heterozygous individual.

Discussion

This case-control study investigated the relationship of *GSTO1* (rs4925) and *GSTO2* (rs156697) with PCOS in reproductive-aged women in Kermanshah province, Iran. The *GSTO1* DD genotype frequency was significantly greater in the patient than in the control group, indicating that this genotype may be involved in the development of PCOS. In addition, the interaction of *GSTO1* (AD/DD) and *GSTO2* (DN/NN) genotypes increased the disease risk by 1.53-fold. Oxidative stress has been suggested as a potential inducing factor in PCOS pathogenesis, although its mechanism is not well-identified (6). Oxygen free radical production caused by hyperandrogenism in early PCOS may be associated with insulin resistance and other PCOS-related metabolic disorders (18). GSTO enzymes, which can form disulfide bonds with GSH, play important roles in cell resistance to oxidative damage. They are also involved in biosynthesis regulation and intracellular hormone transportation (19). Glutathione and related enzymes are involved in the detoxification and metabolism of ROS and cytotoxic and carcinogenic compounds. Much evidence exists for the involvement of ROS in the physiology and pathology of reproductive systems (20).

It has been shown GSTOs scavenge free radicals in various ways, including DHA down-regulation and catalyzing the reduction of

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inorganic arsenic (14). Several studies have been conducted on GST omega-class polymorphisms in malignant diseases. The *GSTO1* and *GSTO2* genetic polymorphisms are associated with bladder, breast, and ovarian cancers (21, 22); however, no relationship between these polymorphisms and PCOS has been reported. In addition to its role in infertility disorders, PCOS has been identified as the underlying cause for a variety of reproductive cancers, including endometrial malignancies and ovarian and breast cancers (23).

A relationship was found between the *GSTO1* (A140D) polymorphism and increased risk of hepatocellular carcinoma, cholangiocarcinoma, acute lymphoblastic leukemia, and breast, urothelial, and non-small-cell-lung cancers (24). Previous studies have shown that *GSTO2* D142 genotype carriers are at risk for hypothyroidism. These results can be related to the role of the GSTO2 enzyme in metabolite detoxification (25). Stamenkovic et al. reported an increased risk for age-related cataract in smokers with the *GSTO2**Asp genotype (26). *In silico* analysis demonstrated that *GSTO1* A140D and *GSTO2* N142D polymorphisms can influence protein primary structures (27), suggesting that the pathogenic effects of these SNPs may result from structural changes in the GSTO protein.

This study is the first to report the *GSTO1* (A140D) and *GSTO2* (N142D) polymorphisms in PCOS patients. Our results indicate that the *GSTO1* A140D polymorphism is a risk factor for PCOS. One limitation of this study is the small sample size; further studies with more subjects and different populations are needed to confirm our results.

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