Association study of Glutathione S-Transferase polymorphisms and risk of endometriosis in an Iranian population

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Abstract
Background: Endometriosis influenced by both genetic and environmental factors. Associations of glutathione S-transferases (GSTs) genes polymorphisms in endometriosis have been investigated by various researchers; however, the results are not consistent.

Objective: We examined the associations of GSTM1 and GSTT1 null genotypes and GSTP1 313 A/G polymorphisms with endometriosis in an Iranian population.

Materials and Methods: In this case-control study, 151 women with diagnosis of endometriosis and 156 normal healthy women as control group were included. The genotyping was determined using multiplex PCR and PCR-RFLP methods.

Results: The GSTM1 null genotype was significantly higher (p=0.027) in the cases (7.3%) than the control group (1.3%). There was no significant difference between the frequency of GSTT1 genotypes between the cases and controls. The GSTP1 313 AG genotype was significantly lower (p=0.048) in the case (33.1%) than the control group (44.4%).

Conclusion: Our results showed that GSTM1 and GSTP1 polymorphisms may be associated with susceptibility of endometriosis in Iranian women.

Key words: Endometriosis, Association study, GSTM1, GSTT1, GSTP1, Polymorphism.

Introduction

Endometriosis is a multifactorial disease which is characterized by presence and growth of endometrial glands and stroma outside the uterine. It affects approximately 5-10% of reproductive age women in USA (1-3). Evidences of immunological, environmental, endocrine and genetic factors involved in endometriosis pathogenesis are available, however, its etiology and pathogenesis remain unknown (4, 5). Contribution of genetic factors to endometriosis pathogenesis was confirmed by higher risk of endometriosis among monozygotic twins compared to dizygotic twins as well as higher risk of recurrence in first-degree relatives (5, 6).

Genome-wide association studies showed that there is remarkable consistency in endometriosis (7). Several candidate genes in endometriosis including phase I and II detoxification genes, sex steroid pathways, cytokine signaling pathways, adhesion molecules and growth factor have been associated with endometriosis (8, 9). The glutathione S-transferases (GSTs) are the key phase II xenobiotic-detoxifying enzymes, which are upregulated in response to oxidative stress and overexpressed in many tumors (10, 11). The GSTs gene family encodes proteins that are critical for certain life processes, detoxication and toxification procedures. It is done by conjugation of reduced glutathione (GSH) with numerous substrates such as pharmaceuticals and environmental pollutants (11).

To date, the most significant evidence linking specific polymorphisms to endometriosis comes from studies investigating phase II detoxification enzymes (9). GSTM1 and GSTT1, two member of GST
gene family, have null allele variants, which their homozygosity causes a complete lack of enzyme activity (12, 13). A consistent association of a GSTT1 polymorphisms and endometriosis, with a 29% increased risk of endometriosis in GSTT1 null deletion carriers has been reported (9). The GSTP1 313 A/G polymorphism would result an amino acid substitution from isoleucine to valine at codon 105, which modifies the catalytic activity and heat stability of the enzyme (14).

According to our knowledge, the results of GSTP1 313 A/G polymorphism and GSTM1 null deletions with the endometriosis are not consistent. Accordingly, we investigated the association between GSTM1, GSTT1 and GSTP1 variations and susceptibility to endometriosis in Iranian women.

Materials and methods

Subjects

In this case-control study, 151 non-relative women with endometriosis were included, who referred to the Avicenna Infertility Clinic and Tehran Clinic Hospital, Tehran, Iran. The study protocol was approved by the Ethics Committee of the Avicenna Research Institute, and written informed consent was obtained from all participants. The diagnosis was made by visual inspection of the pelvis organs at laparoscopy. The sample size was estimated from previous study (15).

Endometriosis women were classified to stage I to IV according to the revised American Society for Reproductive Medicine (ASRM) classification and they were found to have minimal (stage I), mild (stage II), moderate (stage III), and severe (stage IV) of endometriosis. The controls were 156 non-relative healthy women with no history of endometriosis and without any lesion as confirmed by laparoscopy. Because stage I and II of endometriosis are commonly found in asymptomatic women, therefore, in all controls absence of endometriosis was confirmed by laparoscopy (16). These people underwent laparoscopy for conditions other than endometriosis such as benign ovarian cyst.

Genotyping

To investigate the association between GSTM1, GSTT1 and GSTP1 variations and susceptibility to endometriosis in Iranian women, the genotyping was performed by multiplex PCR and PCR-RFLP. Genomic DNA was extracted by salting out procedure from 5 ml of peripheral blood samples (17). Two multiplex PCR reactions were designed for the analysis of GSTM1 and GSTT1 null genotypes. The ZFX (495 bp) and GAPDH (113 bp) genes were used as internal controls in multiplex reactions containing GSTM1 and GSTT1, respectively. Multiplex PCR technique could not distinguish between wild homozygous and heterozygous genotype of the GSTM1 and GSTT1 genes.

Accordingly, after electrophoresis the presence of GSTM1 and GSTT1 bands indicate that there is at least one copy of these genes. Each multiplex PCR reaction was performed in a final volume of 25 µl containing: 10 X PCR buffer, 1.5 mM MgCl2, 1U Taq DNA polymerase (CinnaGen, Iran), 0.5 mM dNTPs (Fermentas, Germany), 5 pmol of each primer, 50 ng template DNA, and sterile distilled water up to 25 µl. Amplification was performed with an initial denaturation at 94°C for 3 min, followed by 35 cycles of amplification which was performed at 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec and a final extension at 72°C for 5 min. The PCR products were analyzed on 1.5% agarose gels and stained with ethidium bromide. The presence of a 459 bp or 219 bp bands indicated that there is at least one copy of the GSTT1 and GSTM1 genes, respectively, whereas the absence of these bands indicated the null genotype for these genes (Figure 1). The primer sequences and related product sizes are shown in table I.

The GSTP1 313 A/G polymorphism (rs1695) was analyzed by PCR-RFLP. The PCR amplification was carried out in a reaction mixture containing 10X PCR buffer, 2 mM MgCl2, 1U Taq DNA polymerase (CinnaGen, Iran), 0.5 mM of dNTPs, 5 pmol of each primer, 30 ng template DNA, and sterile distilled water up to 25 µl. Amplification was performed with an initial denaturation step at 94°C for 3 min, followed by 30 cycles at 94°C for 30 sec, annealing at 62°C for 30 sec and extension at 72°C for 30 sec, and a final extension at 72°C for 5 min. The PCR products of GSTP1 were digested with the restriction enzymes BsmAI at 37°C overnight. The 313G allele produced two fragments with the length of 83 bp and 93 bp, while the 313A allele was not digested (176 bp). DNA
fragments were subjected to 10% polyacrylamide gel electrophoresis and stained with silver nitrate (Figure 2).

**Statistical analysis**

Statistical analysis was performed by IBM SPSS Version 20 (IBM Corporation, Chicago, IL, USA). Genotype and allele frequencies of each variation were compared between the case and control group by Fisher's exact test, Chi- square and logistic regression analysis. p<0.05 was considered statistically significant. All analyses were estimated by odds ratio and their 95% confidence intervals (CIs).

**Results**

Descriptive analysis of 151 endometriosis women and 156 controls showed that the mean age of endometriosis and control groups were 31.4±6.0 and 29.3±5.3 years old, respectively. The mean BMI in the case and control groups were 25.0±4.7 and 25.6±5.6, respectively. The GSTM1 null genotype was significantly higher (p=0.027, OR=5.76, 95% CI:1.22-27.11) in the cases (7.3%) than the control group (1.3%).

This finding suggested that GSTM1 null polymorphism may be associated with susceptibility to endometriosis. In the endometriosis group, homozygous women for the GSTM1 null allele showed a six-fold increased risk of endometriosis than the controls (Table II). On the other hands, there was not a significant difference between the frequency of null and present genotype of GSTT1 between the cases and controls (Table II).

Genotype distribution in the control group for the GSTP1 313 A/G polymorphism was in Hardy-Weinberg equilibrium (p>0.05). Genotype and allele frequencies for GSTP1 313 A/G are summarized in Table III. Our results showed that there was significant difference in the genotype distributions of GSTP1 313 A/G between the case and control groups. The GSTP1 313 A/G genotype was significantly lower (p=0.048; OR=0.61, 95% CI:0.37-0.99) in the case (33.1%) than the control group (44.4%).

### Table I. Primer sequences and their related sizes for each polymorphism

| Gene     | Primer sequences (5′→3′)                  | Size (bp) |
|----------|------------------------------------------|-----------|
| GSTP1(27) | ACCCCAGGGCTCTCTAGGAAGAAAGCCCT            | 176 pb    |
|          | TTCTTGACTGGTCTCCACTATCTC                 | 459 pb    |
| GSTT1(27) | GCACCTCTTTTGATCGAGAAATG                  | 495 pb    |
| GSTM1(27) | GAACTCCCTGAAAAGCTAAAGC                  | 219 pb    |
| ZFX      | ACCGCTGTACCTGACTGATTACAC                 |           |
| GAPDH    | CCGGGGTCTATACTGTCTGC                     | 113 pb    |

### Table II. Genotype distribution and allele frequency of the GSTM1 and GSTT1 polymorphisms in endometriosis women and controls

| Gene     | Genotype   | Cases (n=151) | Controls (n=156) | p-value | OR (95% CI) |
|----------|------------|---------------|-----------------|---------|-------------|
| GSTM1    | Present*   | 140 (92.8%)   | 150 (98.7%)     | Reference group |             |
|          | Null deletion | 11 (7.3%)   | 2 (11.3%)       | 0.027** | 5.76 (1.22-27.11) |
| GSTT1    | Present*   | 117 (77.5%)  | 120 (76.9%)     | Reference group |             |
|          | Null deletion | 34 (22.5%)  | 36 (23.1%)     | 0.785   | 0.93 (0.53-1.62) |

* There is at least one copy of wild allele ** Fisher's exact test.

### Table III. Genotype distribution and allele frequency of the GSTP1 313A/G polymorphism in endometriosis women and controls

| Genotype/Allele | Cases | Controls | p-value | OR (95% CI) |
|-----------------|-------|----------|---------|-------------|
| AA              | 85 (56.3%) | 72 (47.5%) | Reference genotype |
| AG              | 50 (33.1%) | 67 (44.4%) | 0.048* | 0.61 (0.37-0.99) |
| GG              | 16 (10.6%) | 12 (7.9%)  | 0.863   | 0.93 (0.39-2.21) |
| A               | 220 (72.8%) | 211 (69.9%) | Reference allele |
| G               | 82 (27.2%)  | 91 (30.1)  | 0.418   | 0.864 (0.607-1.23) |

* Logistic regression (p< 0.05), n=151
Table IV. Summary of studies which evaluated the GSTM1, GSTT1 and GSTP1 variations in endometriosis. Positive and negative results abbreviated as P and N, respectively

| No. | Country   | Study                  | Year | SNP  | Case | Control | Race   |
|-----|-----------|------------------------|------|------|------|---------|--------|
| 1   | Russia    | Baranov et al.         | 1996 | P    | 42   | 67      |        |
| 2   | France    | Baranova et al.        | 1999 | P    | 65   | 72      |        |
| 3   | UK        | Hadfield et al.        | 2001 | N    | 148  | 148     |        |
| 4   | UK        | Baxter et al.          | 2001 | N    | 84   | 219     |        |
| 5   | Turkey    | Ertunc et al.          | 2005 | P    | 150  | 150     | Caucasian |
| 6   | Iran      | Hosseinzadeh et al.    | 2011 | P    | 120  | 200     |        |
| 7   | Iran      | Seifati et al.         | 2012 | N    | 101  | 142     |        |
| 8   | Italy     | Vichi et al.           | 2012 | N    | 181  | 162     |        |
| 9   | Brazil    | Frare et al.           | 2013 | P    | 50   | 46      |        |
| 10  | China     | Peng et al.            | 2003 | P    | 76   | 80      |        |
| 11  | China     | Lin et al.             | 2003 | P    | 68   | 28      |        |
| 12  | Japan     | Morizane et al.        | 2004 | N    | 114  | 179     |        |
| 13  | Taiwanese | Hsieh et al.           | 2004 | P    | 150  | 159     | Asian  |
| 14  | South Korea | Hur et al.       | 2004 | N    | 194  | 259     |        |
| 15  | South Korea | Jeon et al.       | 2010 | N    | 260  | 194     |        |
| 16  | Japan     | Matsuzaka et al.       | 2012 | N    | 100  | 143     |        |

This study

Discussion

The aim of this study was to evaluate whether the GSTM1, GSTT1 null genotypes and GSTP1 313 A/G polymorphism are associated with susceptibility to endometriosis. In Caucasians, the frequencies of homozygous deletions of GSTM1 and GSTT1 are approximately 50% and 10-20%, respectively. Most studies investigating the effect of GSTM1 and GSTT1 null polymorphisms do not distinguish between individuals with one or two copies of the genes; therefore, the effects of functional gene dosage could not be explored (17). The GSTP1 313 A/G polymorphism (Ile105 Val at codon 105), resulting in an enzyme with altered substrate affinity. Approximately 10% of Caucasians are homozygous for this mutation and 40% are heterozygous (18).
Our results showed that the GSTM1 null genotype might be associated with the risk of endometriosis in Iranian women. The endometriosis women with GSTM1 homozygous null genotype had a six-fold increased risk of developing endometriosis (p=0.027, OR=5.76). Also, GSTP1 313 A/G polymorphism was associated with the endometriosis, however, 313 A/G genotype had a protective effect (p=0.048, OR=0.61), which decreases the risk of the disease. In contrast, no significantly differences between the GSTT1 null deletion and endometriosis was observed. To compare our findings, we search the PubMed database for studies that examined the association between GSTM1, GSTT1, and GSTP1 313 A/G polymorphisms with endometriosis up to July 2014 (Table IV).

Altogether, we found 15 publications, in which nine and eight studies were performed in Caucasian and Asian populations, respectively. Briefly, positive and negative results were found in seven and six of these studies, which evaluated the GSTM1 null genotype and endometriosis, respectively (18-23, 25-30). Only one out of five studies, in a Turkish population, reported that there is a positive association between GSTP1 313 A/G and endometriosis (14, 27, 28, 30, 31). Two out of seven publications found an association between GSTT1 null genotype and the disease (21, 23, 26-30). Positive association of GSTM1 null genotypes and endometriosis in our study is consistent with the results of Hosseinzadeh et al which performed an association study in an Iranian population (21). In contrast, in another study in Iranian population by Seifati et al, no association was found between this null polymorphism and endometriosis (32).

As table IV clearly shows, the results of association studies in different populations are inconsistent, which could be attributed to small sample sizes, poorly matched control group and heterogeneity within populations. Because minimal and mild stages of endometriosis may be found in asymptomatic women, therefore, in control group absence of endometriosis should be confirmed by laparoscopy (16). However, Morizane et al used umbilical cord blood from female newborn infants as population controls for an association study of GSTM1 and GSTT1 variations in women with endometriosis in a Japanese population (29). If the case and control groups are not well matched for ethnicity or geographic origin then false positive association may be occurred because of the confounding effects of population stratification (33).

Two meta-analysis evaluated the glutathione S-transferase variations in endometriosis women (13, 34). Guo et al performed a meta-analysis involving 14 studies investigating GSTM1 and nine GSTT1 studies. The results of their analysis demonstrated an association of GSTT1 polymorphism and endometriosis, with a 29% increased risk of endometriosis in individuals homozygous for GSTT1 null genotype. They found no evidence that women with GSTM1 null genotype have increased risk of developing endometriosis (13). However, there was evidence of publication bias in this meta-analysis, indicating that the size of the increased risk associated with the GSTT1 deletion variant may actually be smaller or non-existent (9). In a recent meta-analysis by Chen et al they concluded that the GSTP1 313 A/G may not be associated with endometriosis risk (34).

In conclusion, the GSTM1 and GSTT1 null variations may be associated with the increased risk of endometriosis in Iranian population. Additional studies on different populations are necessary to further confirm the role of glutathione S-transferase variations in the pathogenesis of endometriosis.

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Conflict of interest

The authors report no conflicts of interest.

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