Glutathione S-Transferase M1 and T1 Genetic Polymorphism And Susceptibility To Endometriosis In Egyptian Women

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Abstract: Endometriosis is a gynecologic pathology with a high prevalence and unknown etiology. Therefore, an increasing number of studies has been undertaken to search for associations between endometriosis and alterations or polymorphisms in candidate genes that interact with each other and with environmental factors to produce the phenotype, including glutathione S-transferase M 1 **(**GSTM1) and glutathione *S*-transferase T 1 (GSTT1) genes. Glutathione-S-transferases (GSTs) are enzymes involved in the metabolism of many disease-causing carcinogens and mutagens that are present in human environments. An association between the incidence of endometriosis and the GST genotypes of patients has been suggested. The objective of the present study was to investigate whether the polymorphisms of GSTM1, GSTT1 are related to endometriosis in Egyptian population. Genotyping of GSTM1 and GSTT1 was analyzed by multiplex PCR in 101 women diagnosed with endometriosis and in a control group of 101 women without complaints related to this pathology and showed that only the frequency of GSTM1 null genotype (P = 0.04, odds ratio 1.48) was statistically significant in endometriosis patients compared to controls. Analysis of the combined effect of GSTM1 and GSTT1 polymorphism found a significant association of endometriosis risk with the GSTT1/GSTM1 double-null type only (*P = 0.01*) In conclusion, the GSTM1 gene and the GSTM1/GSTT1 double-null genotype may be a risk factor for endometriosis in Egyptian patients.
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1. Introduction

Endometriosis is a gynecologic disease defined as the presence of hormonally dependent endometrial glandular and stromal cells outside uterus. Because of its negative impact on the quality of women's life in terms of pain and infertility and the economic burden for diagnosis, pharmacologic and surgical treatments, as well as for assisted fertilization practice, endometriosis has been recognized as a health priority. The etiology of endometriosis is unknown, although a multifactorial origin from a combination of immunological, genetic, and environmental factors is considered the most plausible (Vichi et al., 2012).

The prevalence of endometriosis in asymptomatic women is 2-50% depending on the diagnostic criteria used and the population studied. The prevalence in women with dysmenorrhea is 40-60%. In women with subfertility, the prevalence is 20-30% (Farquhar, 2007). Endometriosis is the most common cause of pelvic pain and occurs in 13-33% of women with infertility (Kyama et al., 2003). Its prevalence and severity are reportedly increasing in developing countries (Porpora et al., 2009). Between 20 and 40% of women with infertility have endometriosis. Among young women with chronic pelvic pain unresponsive to hormonal therapy or treatment with nonsteroidal anti-inflammatory drugs, the prevalence of endometriosis is approximately 70% (Halis et al., 2010).

The production of reactive oxygen species by peritoneal fluid seems to be increased in women with endometriosis, and altered expression of enzymes involved in bodily defense against oxidative stress has also been observed in the endometria of women with this condition. Excessive production of reactive oxygen species may also be a result of exposure to environmental compounds that disrupt the balance between pro-oxidants and antioxidants (Nakata et al, 2004).

Recently, several genetic studies have revealed an association between the development of endometriosis and certain genetic polymorphisms. However, the genes that play a role in the susceptibility to and progression of endometriosis remain unknown.Genetic susceptibility was explored also by studying mutations in genes responsible for detoxication, such as glutathione transferase (GST), as a possible risk factor to endometriosis (Attar et al., 2010).

GSTs are phase II enzymes involved in the detoxification of a broad range of toxic compounds and carcinogens, including dioxins and polycyclic

aromatic hydrocarbons (PAH), which are ubiquitous and probably the most feared environmental contaminants worldwide. In humans, six classes of GST enzymes, α,μ, ω, π, θ and ζ, have been identified, with each class being encoded by a separate gene or gene subfamily The GSTM1 and GSTT1 genes are located on chromosomes 1p13.3 and 22q11.23, respectively. Polymorphisms in the GSTT1 and GSTM1 gene loci are caused by a gene deletion, which results in the virtual absence of enzyme activity, especially in people with deletion in both genes (Guo, 2005).

The role of glutathione S-transferase (GST) polymorphisms as a risk factor for endometriosis has been extensively researched. Although the results are not entirely consistent, several studies have suggested a correlation between endometriosis and the GST mu 1 (GSTM1) or GST theta 1 (GSTT1) genotypes. Given the detoxifying properties of the GST family of enzymes, a lack of function or reduction in detoxifying enzymes owing to a deletion polymorphism may predispose women to endometriosis (Frare et al., 2013).

The aim of the present study was to determine the distribution of GSTT1 and GSTM1 null polymorphisms in Egyptian women with and without endometriosis in order to assess the risks presented by individual and combined genotypes in the development of the disease.

2. Material and Methods

Subjects

Participants were endometriosis patients attending the outpatient clinic of the Reproductive medicine department of the National Research Center, Cairo, Egypt. Clinical as well as demographic data were obtained from medical records and interviews with the patients. The study was approved by the local ethics committee, and all participants provided informed consent.

In total, 101 Egyptian women with laparoscopy-confirmed endometriosis (mean ± SD age 28.5 ± 10.2, range 18–49) were enrolled in the study. The control group comprised 101 age-matched women (mean ± SD age 30.6 ± 6.5, range 18–40), who were healthy and without medical history. There was no significant difference in age between cases and controls (P = 0.08).

Genotyping

Blood samples of 5 ml were obtained from all cases and collected in sterile EDTA tubes. Then, whole blood was stored at –20C until use. Genomic DNA was extracted from whole blood using the established protocol for DNA extraction from blood samples using DNA extraction kit (QIAGEN, Hilden, Germany).

Analysis of GSTM1 and GSTT1 polymorphisms was determined by multiplex PCR with using a housekeeping B globin gene as internal control. The primer sequence and fragments produced are listed in table 1. The primers were synthesized by Sangon and PCR amplifications were carried out in a Thermal Cycler (Perkin Elmer 4800). Main cycling parameters were 94 for 8 min, followed by 35 cycles of 94 for 30s, 60 for 40s and 72 for 1 min with a final extension at 72 for 10 min. The product obtained from each reaction was subjected to electrophoresis on 2% agarose gel in an electric field of 10 V/cm, stained with 5 μg/mL ethidium bromide, and visualized and recorded with the aid of a video documentation system (Image Master VDS®, Amersham Pharmacia Biotech). GSTM1 & GSTT1 genotypes were determined by the presence and absence (null) of bands of 157 bp and 480 bp, respectively, with an internal control of 268 bp.

Table 1: Primers Pairs used

Gene	Primers	Fragment size
GSTM1	F: 5'-GCTTCACGTGTTATGGAGGTTC-3' R: 5'-GAGATGAAGTCCTCCAGATTT-3'	157bp
GSTT1	F: 5'-TTCCTTACTGGTCCTCACATCTC - 3 ' R: 5 '-TCACCGGATCATGGCCAGCA-3'	480 bp
B globin	F: 5'-CAACTTCATCCACGTTCACC- 3 ' R: 5'-GAAGAGCCAAGGACAGGTAC-3'	286bp

Statistical analysis

The data were analyzed using the statistical package for social sciences (SPSS) for Windows, version 11.5. The chi-square ($\chi 2$) and Fischer's (F) exact test were used to compare variables between groups. The level of significance was taken as $p < 0.05$. The odds ratios (OR) with 95% confidence interval (CI) were calculated simultaneously as an estimate of the risk for endometriosis in individuals with GSTT1 and GSTM1 genotype polymorphisms.

3. Results

The distribution of persons and OR for GSTM1 and GSTT1 genotypes as well as the GSTM1 and GSTT1 genotypes in patients and healthy controls, are shown in Table 2. The proportion of patients with GSTM1 null genotype was 56.4% (57/101) and in the control sample it was 46.7% (47/101). A Statistical

significant difference was found between the endometriosis patients and healthy controls for the GSTM1 null genotype (p=0.04). The GSTM1 null genotype increased the risk of endometriosis development (OR=1.48, 95% CI=0.86-2.59, p=0.04).

The proportion of persons in the patient sample with GSTT1 null genotype was 30.7% (31/101) and in the control sample it was 22.7%. (23/101). No statistically significant difference was found between the endometriosis patients and the healthy controls for the GSTT1 null genotype (p=0.06). The GSTT1 null genotype did not increase the risk of endometriosis development (OR=1.50, 95% CI=0.80-2.82, p=0.06).

Considering different combinations of GSTT1 and GSTM1 genes among the endometriosis patients, 18.8 % (19/101) were homozygous GSTT1 null/ GSTM1 null genotypes. Among the controls, 7.9% (8/101) were homozygous GSTT1 null/GSTM1 null; this was statistically significant in comparison to the patient sample (p=0.01).

The distribution of a GSTT1 positive/GSTM1 null combination of genotypes was the highest in both patients and controls (35.6 vs. 38.6%). No statistically significant difference was found between the endometriosis patients and the healthy controls for this combination of genotypes (p=0.11). Table 3 shows the OR of endometriosis associated with each combination of genotypes. Using the GSTT1 positive/GSTM1 positive combination of genotypes as reference, a significant association of endometriosis risk with the GSTT1 /GSTM1 double-null type only was found (OR=2.694, 95% CI=1.120-6.480, P=0.01).

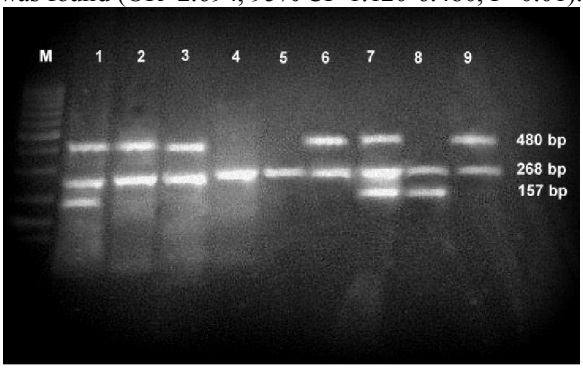

Figure 1. Genotyping of GSTT1 and GSTM1 Genes by Multiplex PCR.

M: 100–1000 bp ladder size marker. Lanes 1 & 7: show GSTT1 (480 bp), GSTM1 (157 bp) and b-Globin genes (268 bp). Lanes 2, 3, 6 & 9: show GSTM1 null/GSTT1 present genotype. Lane 8: shows GSTM1 present/GSTT1 null genotype. Lanes 4 & 5: show dual null genotype.

Table 2. Distribution of Persons and ORs for GSTT1 and GSTM1 Genotypes

Polymorphism	Patients N (%)	Controls N (%)	OR	95% CI	P value
GSTT1					
Present (+)	70(69.3)	78(77.2)	1	Reference	0.06
Absent (-)	31(30.7)	23(22.7)	1.50	0.80-2.82	
GSTM1					
Present (+)	44(43.6)	54(53.4)	1	Reference	0.04*
Absent (-)	57(56.4)	47(46.7)	1.48	0.86-2.59	

Table 3. Distribution of GSTT1 and GSTM1 Genotypes and ORs in Women with Endometriosis and Control Group

Genotype	Patients N (%)	Controls N (%)	OR	95% CI	P value
GSTT1+/GSTM1+	35(34.7)	39(38.6)	---	-----	
GSTT1+/GSTM1-	36(35.6)	39(38.6)	1.136	0.0642-2.011	0.11
GSTT1-/GSTM1+	11(10.9)	15(14.8)	1.427	0.621-3.280	0.12
GSTT1-/GSTM1-	19(18.8)	8(7.9)	2.694	1.120-6.480	0.01*

4. Discussion:

Endometriosis is a widespread disease with a frequency of~10% in the general Caucasian population. It contributes significantly to infertility problems in the industrialized world. The etiology and pathogenesis of endometriosis are still unclear. The results of epidemiological family studies and environmental investigations mean endometriosis can be considered as a multifactorial disease with a possible genetic predisposition and with the involvement of environmental toxins in its pathogenesis (Porpora et al., 2009).

Endometriosis is characterized by a general inflammatory response in the peritoneal cavity with production of reactive oxygen species (ROS), which might act by increasing growth and adhesion of endometrial cells in the peritoneal cavity, with progression of endometriosis and infertility. Contrasting results on the association between inflammation-induced oxidative stress and endometriosis have been reported. Hormonal

imbalance, genetic predisposition, failure of host immune response, and environmental factors have been suggested to concur to its onset and progression, but the association still remains controversial and no single theory seems to cover all the aspects of this disease(Vishi et al, 2012).

The GSTs enzymes, are critical in controlling free radical-associated injuries, making the association between the endometriosis and any alterations in their enzymatic activity biologically plausible. Many functional polymorphic variants at the four major loci have been described, including deletions for *GSTM1* and *GSTT1* and single-nucleotide polymorphisms in exon 5 of *GSTP1* gene (an A/G transition causing a Ile/Val substitution in position 105 of the protein) and in the proximal promoter of *GSTA1* (C-69T) (Vishi et al 2012).

The GSTM1 and GSTT1 genes are polymorphic and the presence of two o-alleles in each gene corresponds to the advent of a deletion, with consequent loss of mRNA and protein products. Yet, the deletion rates of GSTM1 and GSTT1 genes are different according to race, gender and nationality. Studies have shown that the range of GSTM1 and GSTT1 null genotype frequencies ranged from 40% to 50% and from 10% to 65% in Western and Asian countries, respectively. Both GSTM1 and GSTT1 genetic polymorphisms have been demonstrated to affect susceptibility to various cancers, and may be considered as risk modifiers for various environmentally induced diseases (Lin et al, 2003).

Because endometriosis has the characteristics of a polygenic and multifactorial disease, it is likely that both environmental and genetic determinants interact. Therefore the concurrent contributions of both factors need to be investigated, as well as the presence of a gene–environment interaction accounting for multiplicative joint effects. The lack of detoxification, which is genetically determined, might be a risk factor for endometriosis development. So, analysis of GST gene status, particularly detection of GSTM1 and GSTT1 null mutation could have a prognostic and pathologic importance.To this aim we carried a case-control study on Egyptian women to examine whether functionally relevant polymorphisms in *GSTT1 and GSTM1* genes are associated with the risk of endometriosis.

In the present study, the Egyptian control population showed a 46.7 % frequency of the GSTM1 null genotype and 22.7 % frequency of the GSST1 null genotype. These results are in agreement with a study by (Garte et al.,2001) who studied metabolic gene polymorphism frequencies in different control populations and reported that GSTM1 activity is absent in about 50% of Caucasians as a consequence of the inheritance of two null alleles while the GSTT1 activity is deficient in about 20% of Caucasians, resulting from homozygous deletion.

As regard association of GSTM1 and GSTT1 genotypes with endometriosis, Our results showed a significant association of GSTM1 null genotype in patients with endometriosis when compared to controls (56.4 %vs 46.7 %, P=0.04) but we could not elicit any significant difference between endometriosis patients and controls regarding the GSTT1 genotypes (30.7% vs 22.7%, P=0.06). However, on analysis of the combined effect of GSTM1 and GSTT1 polymorphism we found a significant association of endometriosis risk with the GSTT1 /GSTM1 double-null type (OR=2.694, 95% CI=1.120-6.480, P=0.01).

Our results agree with previous reports associating the double-null genotype with a significantly increased risk of endometriosis; (Baranova et al., 1999), (Lin et al., 2003), (Ivanshchaenko et al., 2003) and (Frare et al., 2013). However, there are differences in the correlation of endometriosis with one GST null allele: our results agree with those of (Arvanitis et al.,2003), (Hsieh et al., 2004), (Babu et al., 2005), (Abau et al., 2007) who all found a significant association of endometriosis with the GSTM1 null genotype but are different from those of (Kim et al.,2007) who found an association with the GSTT1 null genotype and endometriosis, and from those of (Morizane et al.,2004) and (Vichi et al.,2012), who did not find an association with either genotype and endometriosis. The discrepancy might be mainly due to the ethnic differences. Furthermore, whether the GSTM1 and GSTT1 genotypes in different populations results from identical deletion or from other alterations of the genes remains unknown.

Although conflicting results have been published in the past years, in this study, we observed that the genotype distribution for GSTM1 gene polymorphism was significantly different between the individuals with and without endometriosis. Our data strongly suggest that the lack of GSTM1 gene products might substantially contribute to the pathogenesis of endometriosis.

GSTM1 functions both as a detoxification enzyme and an intra-cellular drug- and hormone-binding protein.GSTM1 catalyzes the detoxification of genotoxic chemicals, including the products of chronic oxidative stress such as cytotoxic lipid and DNA species. The null condition of GSTM1 gene represented an expanded deletion of the gene, which might impair the further production of mRNA and protein. Because of the detoxification properties of the GSTM1 enzymes, and the fact that endometriosis characterized by cyclical degeneration and chronic inflammation, which will result in the production of reactive oxygen species and other toxins. It is logical

to suspect the role of GSTM1 null genotype as a risk factor in endometriosis patients (Hsieh et al., 2004).

In Conclusion, Our study support the hypothesis that the absence of the GSTM1 gene and the presence of the GSTM1 / GSTT1 double-null genotype may be risk factors for endometriosis in Egyptian women. Inability to cope with oxidative stresses due to GST deficiency may cause early onset and more extensive disease, especially in GSTM1 / GSTT1 double-null patients. Overall, this may be the first study that investigated the association of GSTM1 and GSTT1 genetic polymorphisms with susceptibility to endometriosis in the Egyptian population. However, our finding only suggested their connection as well as possibility. The related scientific proof for the underlying mechanisms is still warranted. Further larger and population-based studies including more polymorphisms in genes involved in oxidative stress are warranted to confirm these findings.

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