Genetic Variation of Glutathione S-Transferase M1 Is Associated with Patients with Ovarian Endometriosis and Endometriosis-Related Primary Infertility

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Keywords

Glutathione S-transferase M1 · Endometriosis · Polymorphism · Infertility · Gene expression · Genetic susceptibility

Abstract

Background: The aim of the study was to investigate the role of the genetic variation of glutathione S-transferase M1 (*GSTM1*) in the development of ovarian endometriosis and endometriosis-related primary infertility risk. **Methods:** This case-control study included 564 women with ovarian endometriosis and 576 normal women in the control group in northern China. The polymorphism of *GSTM1* was genotyped by polymerase chain reaction (PCR)/ligase detection reaction method. To assess the biological significance of polymorphisms, the level of *GSTM1* mRNA expression in patients' endometrial tissues with different genotypes was detected by quantitative real-time PCR (qRT-PCR). **Results:** Compared with the positive genotype, the null genotype of *GSTM1* was associated with the risk of developing ovarian endometriosis (OR = 1.29, 95% CI = 1.02–1.62). Further analysis showed that patients with a null genotype also had a

significantly higher risk of primary infertility than patients with positive genotypes (OR = 1.59, 95% CI = 1.01–2.49). In addition, we found that *GSTM1* mRNA expression was present in the endometrial tissue of all patients, but the expression level of patients with a positive genotype was nearly 10 times higher than that of patients with a negative genotype. **Conclusion:** Our results suggest that the *GSTM1* polymorphism is not only related to the genetic susceptibility to ovarian endometriosis but also a potential molecular marker of primary infertility in patients with ovarian endometriosis.

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Introduction

Endometriosis, a common and clinically important problem, has an incidence of up to 10–15% in women of reproductive age [1]. It is defined by the presence of functional endometrial glands and stroma outside the uterus [1]. The most common symptoms of endometriosis are cyclical pelvic pain and infertility. There is a strong link between endometriosis and infertility; approximately 25–

50% of infertile women may be affected by endometriosis and 30–50% of patients with endometriosis may suffer from infertility [2]. Although the etiology of endometriosis is not well-understood, it is recognized as a heritable disease that is influenced by multiple genetic and environmental factors [3, 4]. Among these risk factors, individual genetic variation in related genes has been shown to significantly alter the risk of developing endometriosis [5].

Glutathione S-transferase M1 (GSTM1), a member of the GST superfamily, is a detoxification enzyme that detoxifies electrophilic compounds, including carcinogens, environmental toxins, and oxidative stress products, by conjugation with glutathione [6, 7]. The *GSTM1* gene is located on chromosome 1p13.3 and is known to be highly polymorphic. These genetic variations can alter an individual's ability to detoxify carcinogens and toxins, leading to differences in disease susceptibility [8–12]. Previously, the relationship between the *GSTM1* polymorphism and the risk of endometriosis has been widely studied [13–19]. Despite inconsistent results, most studies [15, 20–22] have confirmed that null mutations in *GSTM1*, a genotype that may lead to a lack of corresponding enzyme activity, may significantly increase the risk of endometriosis in women. In particular, several meta-analysis results [20–26] have shown that the GSTM1-null genotype is a potentially valuable genetic marker for the risk of endometriosis. However, the role of *GSTM1* polymorphisms in endometriosis-related infertility has not been reported thus far.

In the present study, we demonstrated once again that the *GSTM1* polymorphism may significantly increase the risk of ovarian endometriosis in women in northern China. Furthermore, we analyzed the association between *GSTM1* polymorphism and primary infertility in patients with ovarian endometriosis. Importantly, we confirmed that *GSTM1* mRNA expression was also present in endometrial tissues of patients with null genotypes and that there were significant differences in the expression levels of *GSTM1* mRNA in the endometrial tissue of patients with different genotypes.

Materials and Methods

Study Participants

This case-control study was designed to include 564 patients with ovarian endometriosis and 576 patients without ovarian endometriosis. All patients with ovarian endometriosis were histologically confirmed in the fourth Affiliated Hospital of Hebei Medical University from March 2004 to May 2017. According to the

Fig. 1. Identifying GSTM1 genotype. Lane M: marker; lanes 2 and 4: GSTM1-positive genotype (294 bp); lanes 1 and 3: GSTM1-null genotype. GSTM1, glutathione S-transferase M1.

Revised American Fertility Society classification system (1997), 98 (17.38%) had minimal or mild endometriosis (stage I–II) and 466 (82.62%) had moderate or severe endometriosis (stage III–IV). All patients had regular menstrual cycles (22–36 days), and none of them had taken any hormonal medications for at least 6 months before surgery.

In the control group, women without any malignant disease or endometriosis were randomly selected at reproductive age, who were diagnosed either by surgery or ultrasonography. The control group included women who received a health checkup ($n = 365$) and hysterectomy for dysfunctional uterine bleeding ($n = 211$). All participants had regular menstrual cycles (23–37 days).

DNA Extraction

The elbow venous blood (5 mL) of each participant was drawn into a vacuum-tube containing ethylenediaminetetraacetic acid and then stored in a refrigerator at 4°C. Within 1 week after blood collection, the genomic DNA extraction process is strictly in accordance with the method of Miller et al. [27].

Genotyping of GSTM1 Polymorphism

The genotypes of the *GSTM1* polymorphism were determined by polymerase chain reaction (PCR)/ligase detection reaction [28]. The primers of the PCR products were as follows: forward primer 5'-AGCTGCCCTACTTGATTGATGGTTG-3' and reverse primer 5'-CTGGGACACTCACAATTCTG-3'. The PCR products were analyzed on ethidium bromide-stained agarose gel (2%) and visualized under UV light (Fig. 1). The presence of the 294-bp fragment confirmed the GSTM1-positive genotype, and the absence of the product marked the null genotype. PCR amplification and electrophoresis were repeated 3 times for all samples. The experiment was completed by Shanghai Generay Biotech Co., Ltd (<http://www.generay.com.cn>).

RNA Isolation and cDNA Synthesis

Trizol reagent (Generay, Shanghai, China) was used to isolate total RNA from the endometrial tissue in women with endometriosis, following the recommendations of the manufacturing instructions. The concentration of purified RNA was determined at 260/280 nm using a Nano Drop 2000C spectrophotometer. The total RNA was reverse transcribed into Complementary DNA (cDNA) according to the instructions (Thermo Scientific, Waltham, MA, USA). The cDNA was subsequently stored in the refrigerator at –20°C.

Table 1. Association between GSTM1 polymorphisms and risk of endometriosis

	N	Present, n (%)	Null, n (%)	OR (95% CI)	p value
Controls	576	311 (53.99)	265 (46.01)	1.0 (reference)	
Endometriosis	564	269 (47.70)	295 (52.30)	1.29 (1.02–1.62)	0.03
Stage I–II	98	48 (48.98)	50 (51.02)	1.22 (0.79–1.88)	0.36
Stage III–IV	466	221 (47.42)	245 (52.58)	1.30 (1.02–1.67)	0.04

OR, odds ratio; 95% CI, 95% confidence interval; GSTM1, glutathione S-transferase M1.

Fig. 2. Box plots of GSTM1 mRNA expression was present in the endometrial tissue of all patients with null and positive alleles, but the expression level in the endometrial tissue of patients with the positive genotype was significantly higher than that of patients with the null genotype. GSTM1, glutathione S-transferase M1.

Gene Expression Analysis of GSTM1 mRNA by Quantitative Real-Time -PCR

Quantitative real-time PCR (qRT-PCR) was carried out using the QuantiNova SYBR Green PCR Kit (Qiagen, Shanghai, China) with the 2-step qRT-PCR system in triplicate. The qRT-PCR primer sequence of *GSTM1* was designed and synthesized by Sangon Bioengineering (Shanghai) Co. Ltd., with GAPDH as a reaction internal reference.

The qRT-PCR primers for *GSTM1* were as follows: forward primer 5'-AGCAACGCCATCTTGTGC-3' and reverse primer 5'-GCTGCATATGGTTGTCCATGGT-3'. GAPDH was amplified with the forward primer 5'-ACCACAGTCCATGCCATCAC-3' and the reverse primer 5'-TCCACCACCCTGTTTCTGTGA-3'. Amplification reactions were performed in 20 μ L reaction volume. GAPDH was used as the internal control, and the relative expression levels of *GSTM1* were calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

Statistical analysis was carried out with Graphpad Prism (version 8.0; GraphPad Software Inc., La Jolla, CA, USA), and $p < 0.05$ was considered statistically significant. The χ^2 test was used to

compare the observed genotype frequency with the expected genotype frequency to test for deviation from the Hardy-Weinberg equilibrium. Genotype distribution in the patients and controls was evaluated using the χ^2 test. Genotype distribution in primary infertility of patients with ovarian endometriosis was evaluated using the χ^2 test. The *T* test was used to compare mRNA levels among different genotypes.

Results

General Characteristics of the Study Participants

The mean ages of the patients and controls were 34.4 ± 5.7 (range 18–50) years and 35.6 ± 6.3 (range 20–51) years, respectively. There was no significant difference in the age distribution between the control group and the patient group ($p > 0.05$). The *GSTM1* polymorphism was in Hardy-Weinberg equilibrium in controls ($p > 0.05$).

Association of GSTM1 Polymorphism with the Risk of Ovarian Endometriosis

All 564 cases and 576 controls were successfully genotyped. The *GSTM1*-null genotype frequencies in cases and controls were 52.30% and 46.01%, respectively. The results demonstrated that the *GSTM1*-null genotype among the cases was significantly different from the controls (OR = 1.29, 95% CI = 1.02–1.62). According to the AFS classification, endometriosis was divided into 2 stages to evaluate the relationship between genotypes and disease classification. The frequencies of the *GSTM1* genotypes in cases and controls are presented in Table 1.

Influence of the Gene Polymorphisms on GSTM1 mRNA Levels

In the case group, the expression level of *GSTM1* mRNA was examined in 20 cases of the positive genotype and 20 cases of the null genotype. *GSTM1* mRNA was expressed at significantly higher levels in endometrial tissues from women with the positive genotype than in endometrial tissues from women with the null genotype (Fig. 2).

Table 2. Association between GSTM1 polymorphisms and risk of endometriosis-related primary infertility

Genotype	Fertility, n (%)	Infertility, n (%)	OR (95% CI)	p value
Present	227 (85.66)	38 (14.34)	1.0 (reference)	
Null	215 (78.75)	58 (21.25)	1.61 (1.03–2.53)	0.04

GSTM1, glutathione S-transferase M1.

Association between GSTM1 Polymorphism and Primary Infertility in Patients with Ovarian Endometriosis

Except for unmarried patients, the genotype distribution of GSTM1 associated with endometriosis-related primary infertility is shown in Table 2. The GSTM1-null genotype may be associated with the risk of primary infertility in patients with ovarian endometriosis. Compared with the positive genotype, the GSTM1-null genotype may significantly increase the risk of primary infertility in patients with ovarian endometriosis (OR = 1.61, 95% CI = 1.03–2.53).

Discussion

This study focused on the potential impact of the GSTM1 polymorphism on the risk of ovarian endometriosis and its related infertility. The results showed that the GSTM-null genotype may significantly increase the risk of ovarian endometriosis and infertility. In addition, our study also confirmed that there were significant differences in the mRNA expression of GSTM1 in the endometrial tissue of patients with different genotypes of GSTM1. As far as we know, this is the first study to investigate the relationship between GSTM1 polymorphism and primary infertility in patients with ovarian endometriosis and to analyze mRNA levels in the endometrial tissue of patients with different genotypes of GSTM1.

The association between GSTM1 polymorphism and the risk of endometriosis has been extensively studied [13–19]. A recent meta-analysis [26] clearly indicated that the GSTM1-null genotype may be a potentially valuable genetic marker for the risk of endometriosis. This conclusion was confirmed again in our study. According to the AFS classification, there was a significant difference in the genotype among patients in stage III–IV, while stage I–II was no difference. The aforementioned results may be due to the small number of patients in stage I–II. Furthermore,

we analyzed the relationship between the GSTM1 polymorphism and primary infertility in patients with ovarian endometriosis. The results showed that the GSTM1-null genotype may significantly increase the risk of primary infertility in patients with ovarian endometriosis compared with the positive genotype. To date, there is no research on the relationship between the GSTM1 polymorphism and female infertility, but many studies have confirmed that the GSTM1-null genotype may significantly increase the risk of male infertility [29–32]. It is widely recognized that the oxidative stress induced by reactive oxygen species (ROS) is a major cause of male infertility [33–35]. Low and controlled concentrations of ROS are necessary for the physiological function of sperm, including capacitation, hyperactivation and acrosomal reaction [36]. Because GSTM1 is considered to play protective roles against toxic xenobiotics and ROS in tissue, functional polymorphisms in the gene may impact oxidative stress and lead to male infertility. Similarly, there is also a large amount of evidence [37–40] that the high ROS content in follicular fluid results in impaired oocyte quality and decreased final pregnancy rate in women with endometriosis. Therefore, we speculate that GSTM1 polymorphisms may play an important role in endometriosis-related infertility by affecting the level of gene expression.

Most previous studies [41–45] have suggested that the null genotype of GSTM1 may result in a lack of enzyme activity, which is related to the genetic susceptibility to many diseases, including endometriosis. For this reason, we analyzed the mRNA expression level of GSTM1 in the endometrial tissue of patients with ovarian endometriosis carrying different genotypes. The results showed that GSTM1 mRNA expression was present in the endometrial tissue of all patients, but the expression level in the endometrial tissue of patients with the positive genotype was nearly 10 times higher than that of patients with the null genotype. This finding is consistent with Koch's finding in oral squamous cell carcinoma [46], indicating that patients with the null genotype had lower levels of GSTM1 mRNA expression in cancer tissue. These results suggest that the null genotype may cause a significant reduction in its enzymatic activity but not complete inactivation, at least in some tissues. This result further supports our speculation that the lack of enzymatic activity of carriers of the null genotype of GSTM1 may lead to a reduction in high concentration ROS inhibition in patients with ovarian endometriosis, thereby increasing the risk of infertility. Of course, further research needs to confirm this finding.

In conclusion, we once again confirmed that the GSTM1-null genotype may be an independent risk factor

for developing endometriosis in this study. It was also found that the GSTM1-null genotype may be involved in primary infertility in patients with ovarian endometriosis by reducing the mRNA expression level in the patient's endometrial tissue. This may provide new mechanistic evidence for infertility in patients with endometriosis.

Statement of Ethics

This study was approved by the Medical Ethics Committee of the Fourth Hospital of Hebei Medical University. All the procedures were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Written and signed informed consent form was obtained from all of the case and control subjects before the study.

Conflict of Interest Statement

The authors declare that they have nothing to disclose and that they have no financial or nonfinancial conflict of interest.

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Author Contributions

Hai-Bo Zhang, Yan Li, Jian Zhao, and Yun-Jie Tian performed statistical analysis and performed sample collection. Shan Kang designed the manuscript. Hai-Bo Zhang and Jian-Lei Wu performed experiments and drafted the manuscript.

Data Availability Statement

All data included in this study are available upon request by contact with the corresponding author.

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