Hypomethylation of the GSTM1 promoter is associated with ovarian endometriosis

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STUDY QUESTION: Is the methylation status of the glutathione S-transferase M1 (GSTM1) promoter region altered in patients with ovarian endometriosis, and does this affect the expression of GSTM1 in their endometrial tissues?

SUMMARY ANSWER: The promoter region of GSTM1 was significantly hypomethylated in the ectopic and eutopic endometrium of patients with ovarian endometriosis and this was associated with higher expression of GSTM1 mRNA.

WHAT IS KNOWN ALREADY: GSTM1, a member of the glutathione S-transferase family, is primarily known as a detoxification enzyme, but it has also been shown to negatively regulate apoptosis-related signalling cascades through protein–protein interactions with apoptosis signal-regulating kinase-1.

STUDY DESIGN, SIZE, DURATION: This is a case–control study between September 2013 and December 2016, involving 65 patients with ovarian endometriosis and 53 women without endometriosis. We analysed the methylation status and expression levels of GSTM1 in the ectopic and eutopic endometrium of patients with ovarian endometriosis and the endometrium of women without endometriosis. In addition, we collected endometrial samples from 12 women without endometriosis for endometrial epithelial cell cultures.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Methylation levels of the GSTM1 promoter region in the ectopic and eutopic endometrial tissues of patients with ovarian endometriosis and the endometrial tissues of women without endometriosis were analysed by pyrosequencing. The expression of GSTM1 mRNA and protein in endometrial tissues was investigated by RT-qPCR and immunohistochemistry, respectively. Primary cell culture, gene transfection, Cell Counting Kit-8 assay and flow cytometry were used to analyse the effect of GSTM1 on viability and apoptosis in endometrial epithelial cells.

MAIN RESULTS AND THE ROLE OF CHANCE: Compared with that in the endometrium of women without endometriosis, the GSTM1 promoter region was significantly hypomethylated in the ectopic and eutopic endometrium of patients with ovarian endometriosis. Additionally, GSTM1 mRNA and protein levels were significantly higher in the ectopic and eutopic endometrium than in the control endometrium. Moreover, the methylation levels of the GSTM1 promoter region were significantly negatively correlated with the mRNA expression of GSTM1. Furthermore, in vitro results suggested that the over-expression of GSTM1 could significantly increase viability and inhibit apoptosis in endometrial epithelial cells following hormone treatment and withdrawal.

LIMITATIONS, REASONS FOR CAUTION: Due to restrictions in the isolation and culture of pure populations of endometrial epithelial cells, as well as limitations in the number of passages possible in primary cells, we could not explore the underlying molecular mechanism by which GSTM1 modulates apoptosis in endometrial cells.

WIDER IMPLICATIONS OF THE FINDINGS: This study provides new evidence to support the notion that endometriosis may be an epigenetic disease.
Introduction

Endometriosis is a chronic and painful gynaecological disorder affecting ~10% of reproductive-age women (Guidice, 2010). It is characterized by the extraterine growth of endometrial-like tissue. The most common symptoms of this disease are menstrual irregularity, chronic pelvic pain, dysmenorrhoea, dyspareunia and infertility, which greatly affect the psychological well-being and quality of life of patients (Moradi et al., 2014; Lagana et al., 2017a). Although numerous theories have been proposed to elucidate the possible mechanisms responsible for the development of endometriosis (Sampson, 1927; Sofo et al., 2015; Lagana et al., 2017b; Vitale et al., 2018), the exact aetiology of this disease remains elusive. Recent studies have reported that the expression of certain genes, including those involved in regulating sex steroid hormone levels, proliferation, apoptosis, implantation, cell adhesion, etc., is commonly altered in the eutopic and ectopic endometrium of women with endometriosis (Bischoff and Simpson, 2004; Vigano et al., 2007). Alterations in gene expression in endometrial tissues may play an essential role in the development of endometriosis.

DNA methylation, an epigenetic modification, is now recognized as a crucial regulator of gene expression in mammals. Methylated cytosines of CpG dinucleotides in gene promoter regions change the structure of the major groove of DNA, preventing transcription factors from binding to their binding sites and thereby inhibiting gene transcription (Costello and Plass, 2001). Accumulating evidence suggests that aberrant DNA methylation may be related to the molecular features of endometriosis and thus may be associated with the occurrence and development of endometriosis. In our previous study, we used Illumina HumanMethylation 450 K BeadChip array to analyse the genome-wide DNA methylation profile of patients with ovarian endometriosis, and identified that the glutathione S-transferase M1 (GSTM1) gene was significantly hypomethylated in both the eutopic and ectopic endometrium compared with that in the control endometrium (unpublished data).

GSTM1, a member of the glutathione S-transferase (GST) family, is primarily known as a detoxification enzyme, but it has also been shown to regulate negatively apoptosis-related signalling cascades (Kalina et al., 2014). The gene encoding GSTM1 is highly polymorphic. Inactivating genetic variation in GSTM1, which is associated with a loss of enzymatic activity, can change the susceptibility of an individual to carcinogens and toxins and affect the toxicity and efficacy of certain drugs. GSTM1 null mutation has been linked to an increased risk of various diseases, including endometriosis (Singh et al., 2008; Hosseinizadeh et al., 2011; Chen et al., 2015; Huang et al., 2018). However, our initial experimental data revealed significant hypomethylation of the GSTM1 gene promoter region and high expression of GSTM1 mRNA in the eutopic and ectopic endometrium of patients with ovarian endometriosis. Several studies have shown that over-expression of GSTM1 could significantly inhibit cytokine- and stress-induced cellular apoptosis by repressing apoptosis signal-regulating kinase-1 (ASK1)-mediated apoptotic signalling pathways (Ichijo et al., 1997; Hosono et al., 2010). Decreased cell apoptosis in the endometrium is an important factor for the development and progression of endometriosis (Wang et al., 2015; Song et al., 2016). Thus, we hypothesized that hypomethylation of the GSTM1 gene promoter region, leading to the upregulation of gene expression, may be involved in the pathogenesis of endometriosis through the inhibition of endometrial cell apoptosis.

To verify this hypothesis, we analysed the methylation status of the GSTM1 gene promoter region and GSTM1 expression levels in paired eutopic and ectopic endometrial tissues procured from patients with ovarian endometriosis and in endometrial tissues from women without endometriosis. Furthermore, we did a preliminary exploration of the effect of GSTM1 on the viability and apoptosis of endometrial cells in vitro.

Materials and Methods

Tissue sampling

All study subjects were recruited from the Department of Gynecology at the Fourth Hospital, Hebei Medical University, China (September 2013–December 2016). Paired eutopic and ectopic endometrial tissues were collected from 65 patients (average age 37.6 ± 6.89 years) undergoing surgical treatment for ovarian endometriosis. The selected patients had ovarian endometriosis Stage III–IV according to the revised American Fertility Society (rAFS) classification, and patients with superficial endometriosis or deeply infiltrating endometriosis were not selected. As controls, endometrial tissues were collected from 53 subjects (average age 38.9 ± 6.78 years) without endometriosis who underwent total laparoscopic hysterectomy for cervical intraepithelial neoplasia III. All study participants were premenopausal and underwent surgical treatment during the secretory phase of their menstrual cycle, without having received steroid medications. The phase of the menstrual cycle at the time of tissue harvest was estimated according to the start of the last menstrual period and confirmed by histological examination. All samples were stored in RNAlater solution (Ambion, Carlsbad, CA, USA) immediately after surgical removal and then preserved at −20°C.

For endometrial epithelial cell (EEC) culture, endometrial samples (n = 12, average age, 39 years; range, 32–46 years) were obtained at the time of hysterectomy for cervical intraepithelial neoplasia III. The samples were obtained from patients in the secretory phase of the menstrual cycle. The endometrial samples were placed in Hank’s balanced salt solution (HBSS) and transported to the laboratory for culture within 30 min. Written informed consent was obtained from all recruited subjects. The study was reviewed and approved by the Ethics Committee of the Fourth Hospital, Hebei Medical University (2013MEC04). This case–control study is conducted in accordance with Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines (von Elm et al., 2007).
Genomic DNA extraction and pyrosequencing methylation analysis

Genomic DNA was extracted from tissue samples using the Wizard Genomic DNA Puriﬁcation Kit (Promega, Madison, WI, USA). Extracted DNA was then bisulﬁte converted using the EpiTect Fast Bisulﬁte Conversion Kit (Qiagen, Hilden, Germany). Based on the results of our 450 K BeadChip array, two fragments in the GSTM1 promoter region were measured in the sodium bisulﬁte-converted DNA. One fragment includes four CpG sites (−116, −111, −104 and −98 from the transcription start site), and the other contains three CpG sites (−40, −23 and −16 from the transcription start site) (Fig. 1). Polymerase chain reaction (PCR) ampliﬁcation was performed with the PyroMark PCR kit (Qiagen). The primer sequences used for PCR ampliﬁcation and pyrosequencing are listed in Table I. Pyrosequencing was performed on a PyroMark Q96 instrument using PyroMark Gold Reagents (Qiagen) according to manufacturer’s instructions. After pyrosequencing was complete, CpG methylation percentages were analysed using Pyro Q-CpG software (Qiagen). During methylation analysis, the average methylation level of all CpG sites within each fragment was analysed among eutopic, ectopic and control endometrial tissues.

RNA extraction and quantitative real-time reverse transcriptase-PCR (RT-qPCR)

Total RNA was isolated using the TRIzol-chloroform extraction method (GENERAY BIOTECH CO., LTD., Shanghai, China). Total cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientiﬁc, USA). First-strand cDNA was stored at −80°C until use. For qPCR, the primers for GSTM1 and GAPDH were designed by Sangon Biotech Co., Ltd. (Shanghai, China). GAPDH was used as an internal control. qPCR was carried out using QuantiNova SYBR® Green PCR Kit (Qiagen). The primer sequences for PCR ampliﬁcation are listed in Table I. The relative expression of GSTM1 mRNA was calculated with the $2^{-\Delta\Delta Ct}$ method, and all experiments were repeated three times.

Immunohistochemistry (IHC)

Parafﬁn-embedded endometrial tissue samples (secretory phase) collected in the Pathology Department of the Fourth Hospital of Hebei Medical University were used for immunohistochemical staining of GSTM1. Brieﬂy, 4-μm thick sections were dewaxed in xylene and dehydrated through a graded series of ethanol. After blocking endogenous peroxidase and nonspeciﬁc binding, the sections were incubated overnight at 4°C with primary antibody (rabbit polyclonal anti-GSTM1 antibody, Abcam, ab113432, Cambridge, UK; dilution 1:100) and then with biotinylated secondary antibody and streptavidin-peroxidase complex. After the sections were washed in PBS, they were incubated with DAB reagent and counterstained with haematoxylin. Negative control sections were incubated with PBS instead of primary antibody. The sections were independently examined by two pathologists, who were blinded to the clinicopathological information. Immunohistochemical staining was evaluated using a previously reported scoring method (Umemoto et al., 2001). For binary analysis, scores between 0 and 2 were classiﬁed as negative expression, and scores between 3 and 6 were classiﬁed as positive expression.

Cell culture

Cell culture was performed as previously reported (Zhu et al., 2018). Fresh endometrial tissues at the secretory phase were cut into 1 mm³ pieces and digested with type I collagenase (1 g/L; Solarbio Science & Biotechnology Co., Ltd., Beijing, China) at 37°C for 30 min. The digested material was progressively ﬁltered through sterile 150- and 38-μm sieves. Epithelial cells remained on the upper surface of the 38-μm sieves as residual material. Thus, the 38-μm sieves were reverse-washed into a plastic petri dish with Epithelial Cell Medium (ScienCell, catalogue number: 4101), and then, the medium was pipetted into a cell culture ﬂask coated with ﬁbronectin (ScienCell, catalogue number: 8248). The cells were
cultured in a humidified atmosphere with 5% CO₂ at 37°C, and the medium was first replenished after 72 h.

Immunofluorescence

The primary cultured EECs were fixed with 4% (v/v) formaldehyde for 20 min, washed with PBS, and then permeabilized in 0.2% (v/v) Triton X-100 for 5 min. After nonspecific binding was blocked with 5% (w/v) BSA, the cells were incubated with rabbit anti-CK 19 monoclonal antibody (Abcam, ab52625, Cambridge, UK; 1:100 dilution) overnight at 4°C. After incubating with Fluorescein-conjugated Anti-rabbit IgG (Proteintech Group, Inc., Wuhan, China; 1:20 dilution) for 2 h at room temperature, the cells were incubated with DAPI for 5 min. After nonspecific binding was blocked with 5% (w/v) BSA, the cells were incubated in 0.2% (v/v) Triton X-100 for 10 min in the dark. Then, 400 μL of 1× binding buffer and subsequently 5 μL Annexin V-FITC and 5 μL PI Staining Solution at room temperature were added and mixed with medium, followed by incubation in the dark for 2 h. The absorbance was measured at 450 nm using a microplate spectrophotometer (Varioskan LUX, Thermo Scientific).

Gene transfection

The pENTER-GSTM1 vector and empty vector were purchased from Vigene Biosciences (Shandong, China). Transfection was performed using Lipofectamine™ 2000 transfection reagent (Invitrogen, MA, USA) according to manufacturer’s instructions. Primary EECs were seeded in a six-well plate at a density of 4 × 10⁴ cells/mL in a volume of 2 mL/well. When the EECs reached 70–80% confluence, they were transfected with either the GSTM1 expression plasmid (pENTER-GSTM1) or empty vector, used as a control. At 48 h after transfection, the effect of pENTER-GSTM1 was confirmed by analysis of GSTM1 mRNA and protein expression.

Table I Primer sequences used for PCR amplification, pyrosequencing, and RT-qPCR.

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<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
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<tr>
<td>Fragment one for −116/−111/−104/−98 CpG site</td>
<td>Forward PCR Primer: 5′-GGGAGGAAGTTTATT GAGTGTAGT-3′</td>
</tr>
<tr>
<td></td>
<td>Biotinylated Reverse PCR Primer: 5′-CCCAGTACGCCC AATATCATACATACATCTC-3′</td>
</tr>
<tr>
<td></td>
<td>Forward Sequencing Primer: 5′-GTTTTAGGGTGT G-3′</td>
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<tr>
<td>Fragment two for −40/−23/−16 CpG site</td>
<td>Forward PCR Primer: 5′-GGGAGGAAGTTTATT ATTGAGTGT-3′</td>
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<td></td>
<td>Biotinylated Reverse PCR Primer: 5′-TATCCCAATACCCC AATATCATACATACATCT-3′</td>
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<td>Forward Sequencing Primer: 5′-TTTTTAGGAGTTTT ATATTTTGA-3′</td>
</tr>
<tr>
<td>GSTM1 RT-qPCR</td>
<td>Forward PCR Primer: 5′-GACTTCTATCTCCGCC TTGAG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse PCR Primer: 5′-CCCAGACAGCCACC TTGAG-3′</td>
</tr>
<tr>
<td>GAPDH RT-qPCR</td>
<td>Forward PCR Primer: 5′-ACCACAGTTCC ATGCCATAC-3′</td>
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<tr>
<td></td>
<td>Reverse PCR Primer: 5′-TCACACCACCC TGTTGCTGTA-3′</td>
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Western blot analysis

Collected cells were lysed in ice-cold RIPA buffer, and protein lysates were quantified with the BCA Protein Assay Kit. Equal amounts of protein (50 μg) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., CA, USA). After blocking in 5% (w/v) non-fat milk at room temperature for 1 h, the membranes were incubated with rabbit monoclonal antibodies against GST mu (Abcam, ab108524, Cambridge, UK; 1:2000 dilution) and β-actin at 4°C overnight, followed by washing with Tris-buffered saline with Tween-20 and incubation with anti-rabbit secondary antibody (Rockland, Gilbertsville, PA, USA; 1:10 000 dilution) for 1 h at room temperature in a dark room. The membranes were scanned with an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Hormone treatment

Endometrial cell growth and apoptosis are regulated by changes in sex steroid hormone levels. The induction of apoptosis by hormone withdrawal has been well documented in the endometrium (Nayak et al., 2000). Thus, in the present study, we treated EECs with oestradiol (10⁻⁶ M; MCE Co., NJ, USA) and progesterone (10⁻⁶ M; MCE Co.) for 48 h and then removed the hormones from the culture for another 24 h to mimic physiological hormone withdrawal in humans. Thereafter, cell viability and apoptosis assays were performed to investigate the effect of GSTM1 on EEC viability and apoptosis. Oestradiol and progesterone concentrations and the period of incubation were selected based on previous report (Song et al., 2002).

Cell viability assay

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8) (MCE Co.). Briefly, at 24 h after transfection with pENTER-GSTM1 vector or empty vector, EECs were seeded in a 96-well plate (5000 cells/well) and incubated for another 24 h. Then, oestradiol (10⁻⁶ M) and progesterone (10⁻⁶ M) were added to the cultures for 48 h. After removing the sex hormones and incubating the cells for another 24 h, CCK-8 solution (10 μL/well) was added and mixed with medium, followed by incubation in the dark for 2 h. The absorbance was measured at 450 nm using a microplate spectrophotometer (Varioskan Lumina, Thermo Scientific).

Apoptosis assay

Apoptosis of EECs was analysed using an Annexin V-FITC-PI apoptosis detection kit (Vazyme BioTech Co., Ltd, Nanjing, China). At 48 h after transfection with pENTER-GSTM1 vector or empty vector, EECs were treated with oestradiol (10⁻⁶ M) and progesterone (10⁻⁶ M) for 48 h; following the removal of hormones from the medium, the cells were incubated for another 24 h. Then, adherent cells were trypsinized without EDTA and collected by centrifugation. After being washed twice with PBS, the cells were resuspended in 100 μL 1× binding buffer and subsequently incubated with 5 μL Annexin V-FITC and 5 μL PI Staining Solution at room temperature for 10 min in the dark. Then, 400 μL of 1× binding buffer was added and the fluorescence intensity was determined by a Beckman Coulter FC500 type flow cytometer (Beckman Coulter, FL, USA).

Statistical analysis

Statistical analysis was performed using the SPSS 21.0 (Chicago, IL, USA). Methylation levels and mRNA expression of GSTM1 were expressed as the median (quartile range). Non-parametric unpaired Mann–Whitney U tests were used to determine whether there was a difference in the median values between the eutopic or ectopic endometrium and the control endometrium, and non-parametric Wilcoxon signed-rank tests were performed using the SPSS 21.0 (Chicago, IL, USA).
used to compare paired eutopic and ectopic samples. Spearman’s rank tests were performed to analyse the correlation between the mRNA expression and methylation levels of \textit{GSTM1}. Chi-square tests were conducted to determine whether there was a difference in the frequency of positive \textit{GSTM1} expression between the eutopic or ectopic endometrium and the control endometrium. McNemar’s test was conducted to compare paired eutopic and ectopic samples. Data for cell viability and apoptosis assays were analysed using Student’s t tests. $P < 0.05$ was used as the criterion for statistical significance.

**Results**

\textbf{GSTM1} methylation patterns in the case and control groups

The four CpG sites ($-116, -111, -104$ and $-98$) in one fragment of the \textit{GSTM1} gene promoter region showed strong correlations among one another (all $r > 0.55$, $P < 0.001$), and similar results were observed among the three CpG sites in the other fragment ($-40, -23$ and $-16$) (all $r > 0.65$, $P < 0.001$). Therefore, the average methylation level of all CpG sites within each fragment was used for further analysis. The average methylation levels of the $-116/-111/-104/-98$ CpGs were significantly lower in the eutopic ($P = 0.046$) and ectopic ($P < 0.001$) endometrium of patients with ovarian endometriosis than in the endometrium of women without endometriosis. Furthermore, the average methylation levels were significantly different between the eutopic and ectopic endometrium ($P < 0.001$) (Fig. 2A). The average methylation levels of the $-40/-23/-16$ CpGs were significantly lower in the ectopic endometrium than in the eutopic endometrium of cases ($P < 0.001$) and controls ($P < 0.001$) but were not significantly different between the eutopic endometrium of cases and controls ($P = 0.504$) (Fig. 2B).

\textbf{GSTM1} expression in endometrial tissues from the case and control groups

The RT-qPCR results showed that the mRNA levels of \textit{GSTM1} were significantly higher in the eutopic ($P = 0.011$) and ectopic ($P < 0.001$) endometrium of patients with ovarian endometriosis than in the endometrium of women without endometriosis. Moreover, the difference between eutopic and ectopic endometrial tissues was statistically significant ($P < 0.001$) (Fig. 2C).

To evaluate the protein expression of \textit{GSTM1}, we examined 28 paired eutopic and ectopic endometrial samples from patients with ovarian endometriosis and 23 control endometrium samples from women without endometriosis by IHC. Immunohistochemical staining showed that \textit{GSTM1} was mainly expressed in the cytoplasm and nuclei of glandular epithelial cells within endometrial tissues (Fig. 2D). The frequency of positive \textit{GSTM1} expression in the eutopic ($P = 0.026$)

![Figure 2](https://academic.oup.com/humrep/article-abstract/34/5/804/5466132)

*Figure 2* The expression and methylation analysis of \textit{GSTM1} in eutopic and ectopic endometrium of patients with ovarian endometriosis and the endometrium of women without endometriosis. (A, B) The average \textit{GSTM1} methylation levels of each fragment in the eutopic, ectopic and control endometrial tissues. $^*P < 0.05$, $^{***}P < 0.001$. (C) Relative mRNA expression of \textit{GSTM1} in the eutopic, ectopic and control endometrial tissues. $^*P < 0.05$, $^{***}P < 0.001$. (D) Representative immunohistochemical staining of \textit{GSTM1} in the endometrial tissues (SP $\times$ 200). a: a case of endometrium from women without endometriosis demonstrating weak \textit{GSTM1}; b: a case of eutopic endometrium from patients with ovarian endometriosis demonstrating moderate levels of \textit{GSTM1}; c: a case of ectopic endometrium from patients with ovarian endometriosis demonstrating positive expression of \textit{GSTM1}. 

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endometrium (P < 0.001) endometrium of cases was significantly higher than that in the control endometrium (Table II). Moreover, the frequency of positive GSTM1 expression in the ectopic endometrium was significantly higher than that in the eutopic endometrium (P = 0.001).

### Association between GSTM1 mRNA expression and its methylation levels

Spearman’s correlation analysis showed that GSTM1 mRNA expression was significantly negatively correlated with the methylation levels of the GSTM1 promoter region (average of the −116/−111/−104/−98 CpGs: r = −0.61, P < 0.001; average of the −40/−23/−16 CpGs: r = −0.52, P < 0.001).

### Effect of pENTER-GSTM1 on GSTM1 expression in EECs

The RT-qPCR results demonstrated that the mRNA level of GSTM1 in the pENTER-GSTM1 transfection group (pENTER-GSTM1 group) and empty vector transfection group (control group) was significantly higher than that in the empty vector transfection group (P < 0.001) (Fig. 3A). This result was further validated by protein expression using Western blot analysis (P = 0.001) (Fig. 3B).

### Effect of GSTM1 on EEC viability and apoptosis

CCK8 assays were used to assess the proliferation of GSTM1-expressing EECs following hormone treatment and withdrawal. EEC viability in the pENTER-GSTM1 transfection group was significantly higher than that in the empty vector transfection group following hormone withdrawal (P = 0.010) (Fig. 4), indicating that the upregulation of GSTM1 enhances EEC viability. In addition, flow cytometry analysis demonstrated that the percentage of apoptotic cells in the pENTER-GSTM1 transfection group was significantly lower than that in the empty vector transfection group following hormone treatment and withdrawal (6.15 ± 0.34 versus 9.44 ± 0.62, P = 0.010) (Fig. 5), suggesting that the over-expression of GSTM1 could suppress EEC apoptosis.
apoptosis. These data implied that GSTM1 is involved in the regulation of endometrial cell proliferation and apoptosis.

**Discussion**

In the present study, we demonstrated that the promoter region of GSTM1 was significantly hypomethylated in both the ectopic and eutopic endometrium of patients with ovarian endometriosis compared to that in the endometrium of women without endometriosis. Moreover, the methylation level of the GSTM1 promoter region was negatively correlated with GSTM1 mRNA expression, which was significantly higher in ectopic and eutopic endometrial tissues. To the best of our knowledge, this is the first study on the relationship between methylation of the GSTM1 promoter and ovarian endometriosis.

It was previously reported that the methylation status of several genes, such as H0XA10, CDH1, ESR2, NRSA1 and COX-2, is aberrant in the ectopic and eutopic endometrium of patients with endometriosis (Wu et al., 2005; Xue et al., 2007, 2011; Wang et al., 2012; Li et al., 2017). Genome-wide methylation analysis also indicated that significant differences between the methylation profiles in the ectopic and eutopic endometrium of endometriosis patients and those in the endometrium of women without endometriosis (Dyson et al., 2014; Naqvi et al., 2014; Yotova et al., 2017). In this study, the results showed that the methylation levels of the GSTM1 gene promoter region in the ectopic and eutopic endometrium of patients with ovarian endometriosis were significantly lower than those in the endometrium of women without endometriosis. RT-qPCR analysis showed that the mRNA levels of GSTM1 in the ectopic and eutopic endometrium were higher than those in the control endometrium. In addition, a clear inverse relationship was observed between the methylation levels of the GSTM1 promoter region and GSTM1 mRNA expression. Moreover, immunohistochemical staining confirmed that GSTM1 protein expression in the ectopic and eutopic endometrium of patients with ovarian endometriosis was significantly higher than that in the control endometrium within the cytoplasm and nucleus of glandular epithelial cells. It is worth mentioning that bioinformatics analysis (http://jaspar.genereg.net/) also showed that the abnormally methylated region (from −94 bp to −113 bp) obtained by our study may be the binding site for the transcription factor AP-2α. Taken together, these results provide strong evidence that epigenetic activation of GSTM1 through hypomethylated promoter region may play an important role in the pathogenesis of ovarian endometriosis.

To date, the association between GSTM1 gene polymorphism and endometriosis has been extensively studied (Hosseinizadeh et al., 2011; Chen et al., 2015; Tuo et al., 2016). Individuals with GSTM1 null genotype are considered to be at risk for endometriosis, because the implantation of ectopic endometrium occurs in these patients as a result of the enzymatic defect in the detoxification system. However, the results of the present study demonstrated that GSTM1 mRNA and protein levels were significantly increased in the ectopic and eutopic endometrium of patients with ovarian endometriosis. Aside from the classic catalytic activity, GSTM1 also participates in regulation of cellular processes including proliferation and apoptosis via interactions with kinases (Kalninina et al., 2014). GSTM1, an endogenous inhibitor of ASK1, could inhibit the activation of ASK1, thereby suppressing ASK1-mediated cell apoptosis (Cho et al., 2001). Previous studies have reported that the over-expression of GSTM1 may contribute to drug resistance in patients with certain tumours by inhibiting cell apoptosis (Hosono et al., 2010; Barros et al., 2013). Using a mouse model of endometriosis, Han et al. (2015) demonstrated that preventing ASK1 activation significantly inhibited endometrial cell apoptosis in endometriotic lesions. Therefore, we speculate that upregulating GSTM1 in the eutopic endometrium may suppress apoptosis in endometrial cells shed during menstruation by inhibiting the ASK1 signalling pathway, thereby facilitating their ectopic survival and implantation. Similarly, the over-expression of GSTM1 in the ectopic endometrium is also closely related to the promotion of cell survival in ectopic lesions. To further confirm this speculation, we cultured primary EECs and transfected them with pENTER-GSTM1 to investigate the effect of GSTM1 on endometrial cell apoptosis. Flow cytometry analysis demonstrated that the apoptotic rate of EECs subjected to hormone treatment and withdrawal decreased by 1.5-fold in the pENTER-GSTM1 transfection group relative to that in the empty vector transfection group. Furthermore, the CCK-8 assay results revealed that EEC viability in
the pENTER-GSTM1 transfection group was higher than that in the empty vector transfection group following hormone withdrawal. Therefore, our results suggested that high expression of GSTM1 in endometrial tissues may be involved in the decreased apoptosis of endometrial cells, which is associated with the development of ovarian endometriosis.

One limitation of this study should be mentioned. Due to restrictions in the isolation and culture of pure populations of EECs, as well as limitations in the number of passages possible in primary cells, we could not explore the underlying molecular mechanism by which GSTM1 modulates apoptosis in endometrial cells.

In conclusion, the present study demonstrated that the high expression of GSTM1 due to the hypomethylation of its promoter region is associated with ovarian endometriosis and may be involved in its development through the inhibition of endometrial cell apoptosis. This study also provides new evidence to support the notion that endometriosis may be an epigenetic disease (Guo, 2009; Konincx et al., 2019). Of course, further studies are necessary to confirm our findings.

Supplementary data
Supplementary data are available at Human Reproduction online.

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Authors’ roles
S.K., Y.L. and J.Z. designed the study and carried out the experiments. J.Z., L.W. and W.Z. recruited the patients and collected the data. J.Z., L.W. and Y.L. analysed the data and prepared draft figures and tables. All authors were involved in writing the paper and provided final approval of the submitted and published versions.

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Conflict of interest
All authors declare that they have no conflict of interest.

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