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ORIGINAL ARTICLE ***Gynaecology***

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.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by grants from the Natural Science Foundation of Hebei Province (Grant number: H2018206200) and the Department of Education of Hebei Province (Grant number: CXZZBS2017114). The authors have no conflicts of interest to declare.

Key words: endometriosis / DNA methylation / GSTM1 / apoptosis / epigenetics / endometrium / proliferation

Introduction

Endometriosis is a chronic and painful gynaecological disorder affecting ~10% of reproductive-age women (Giudice, 2010). It is characterized by the extrauterine 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 (Kalinina *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; Hosseinzadeh *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 overexpression 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).

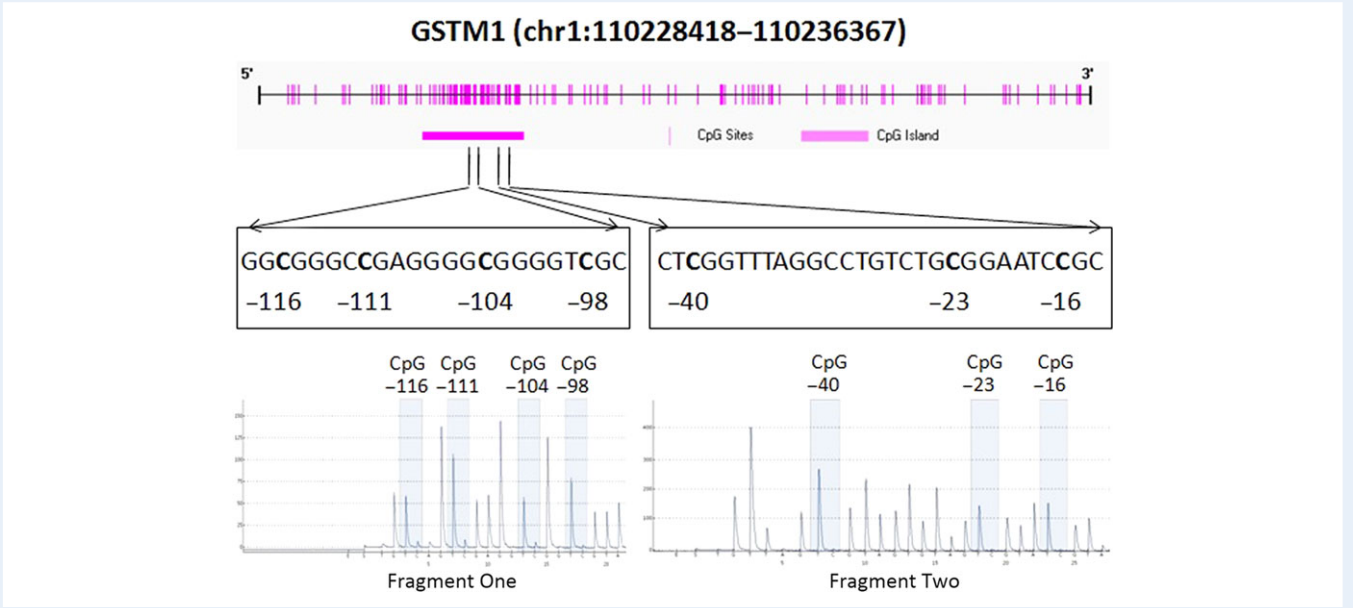

Figure 1 Two measured fragments in the CpG island of *GSTM1* promoter region. One fragment includes −116, −111, −104 and −98 CpG sites; the other includes −40, −23 and −16 CpG sites.

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 ^{TM}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 non-specific 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 mm^3 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

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

Primers	Sequences
Fragment one for −116/−111/−104/−98 CpG site	
Forward PCR Primer	5'-GGAGGAAGTTTTATTGAGTGTAGTT-3'
Biotinylated Reverse PCR Primer	5'-CCCAATACCCCAATATCATAA ACAT-3'
Forward Sequencing Primer	5'-GTTTTTAGGGTTGTG-3'
Fragment two for −40/−23/−16 CpG site	
Forward PCR Primer	5'-GGGAGGAAGTTTTATTGAGTGT-3'
Biotinylated Reverse PCR Primer	5'-TATCCCAATACCCCAATATCATAAACAT-3'
Forward Sequencing Primer	5'-TTTTTTAGGAGTTTTTATATTTTGA-3'
GSTM1 RT-qPCR	
Forward PCR Primer	5'-GACTTCATCTCCCGCTTTGA-3'
Reverse PCR Primer	5'-CCCAGACAGCCATCTTTGA-3'
GAPDH RT-qPCR	
Forward PCR Primer	5′-ACCACAGTCCATGCCATCAC-3′
Reverse PCR Primer	5′-TCCACCACCCTGTTGCTGTA-3′

cultured in a humidified atmosphere with 5% CO_2 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 AffiniPure Goat Anti-Rabbit IgG (Proteintech Group, Inc., Wuhan, China; 1:20 dilution) for 2 h at room temperature in a dark room, the cells were incubated with DAPI for 5 min at room temperature, washed twice with PBS, and imaged with a fluorescence microscope (Supplementary Figure S1).

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 4×10^5 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.

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^{-8} 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^{-8} 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 LUX, 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^{-8} 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 X 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 X 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

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 *GSTM1*. Chi-square tests were conducted to determine whether there was a difference in the frequency of positive 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

GSTM1 methylation patterns in the case and control groups

The four CpG sites (−116, −111, −104 and −98) in one fragment of the *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).

GSTM1 expression in endometrial tissues from the case and control groups

The RT-qPCR results showed that the mRNA levels of *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 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 GSTM1 was mainly expressed in the cytoplasm and nuclei of glandular epithelial cells within endometrial tissues (Fig. 2D). The frequency of positive GSTM1 expression in the eutopic ($P = 0.026$)

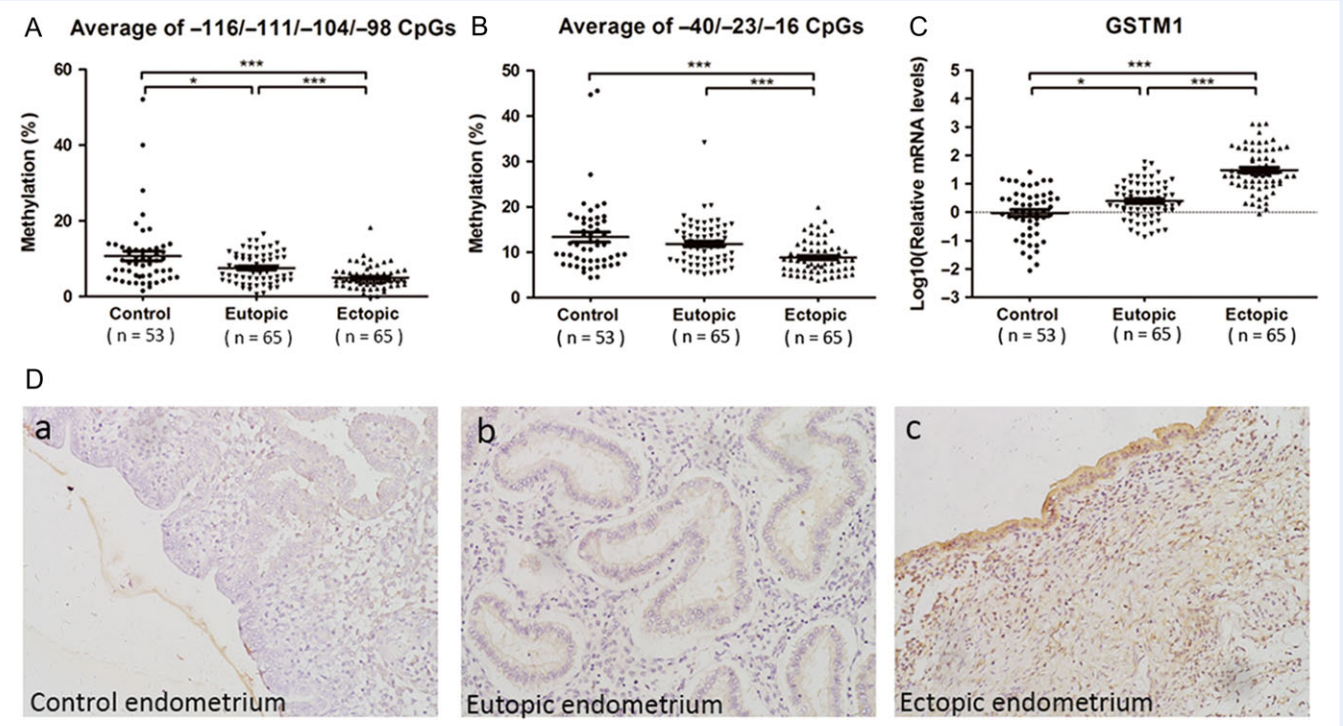

Figure 2 The expression and methylation analysis of GSTM1 in eutopic and ectopic endometrium of patients with ovarian endometriosis and the endometrium of women without endometriosis. (**A**, **B**) The average *GSTM1* methylation levels of each fragment in the eutopic, ectopic and control endometrial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

Table II Protein expression of GSTM1 in eutopic and ectopic endometrium of patients with ovarian endometriosis and the endometrium of women without endometriosis.

Groups	*N*	Negative expression	Positive expression	χ^2	*P*
Control Endometrium	23	17 (74%)	6 (26%)	Reference	
Eutopic Endometrium	28	12 (43%)	16 (57%)	4.97	0.026
Ectopic Endometrium	28	2 (7%)	26 (93%)	24.08	9.23×10^{-7}

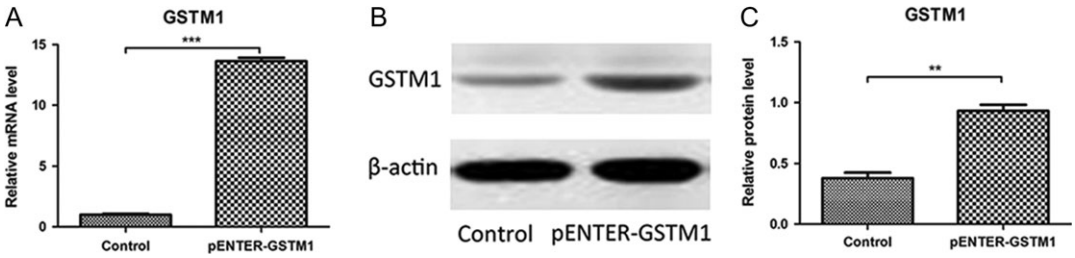

Figure 3 Effect of pENTER-GSTM1 on GSTM1 expression in endometrial epithelial cells (EECs). (**A**) Relative mRNA levels of *GSTM1* in the pENTER-GSTM1 transfection group (pENTER-GSTM1 group) and empty vector transfection group (control group). (**B**) Protein expression of GSTM1 in the pENTER-GSTM1 transfection group (pENTER-GSTM1 group) and empty vector transfection group (control group). Data were expressed as mean ± SEM, and analysed by Student's *t* test. All experiments were repeated three times. $^{**}P < 0.01$, $^{***}P < 0.001$.

and ectopic ($P < 0.001$) 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 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

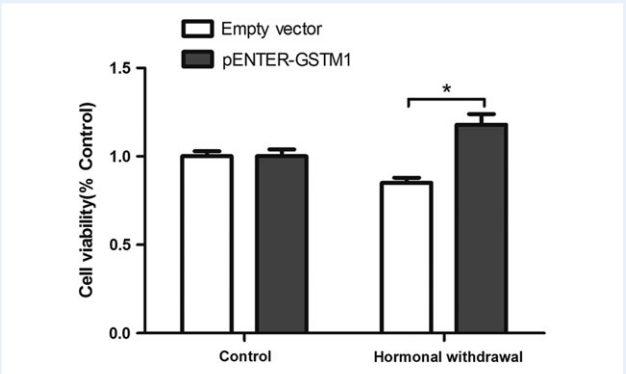

Figure 4 Effect of GSTM1 on endometrial epithelial cell (EEC) viability following hormone treatment and withdrawal. Empty vector and pENTER-GSTM1 transfected EECs were treated with oestradiol and progesterone for 48 h; following the removal of hormones from the medium, cells were incubated for another 24 h. Thereafter, CCK-8 assays were performed to evaluate cell viability. Relative EEC viability in the two groups was analysed as a percentage compared to their respective controls. Controls: EECs were not treated with oestradiol and progesterone. All experiments were repeated three times. Values were expressed as mean ± SEM, $^{*}P < 0.05$.

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

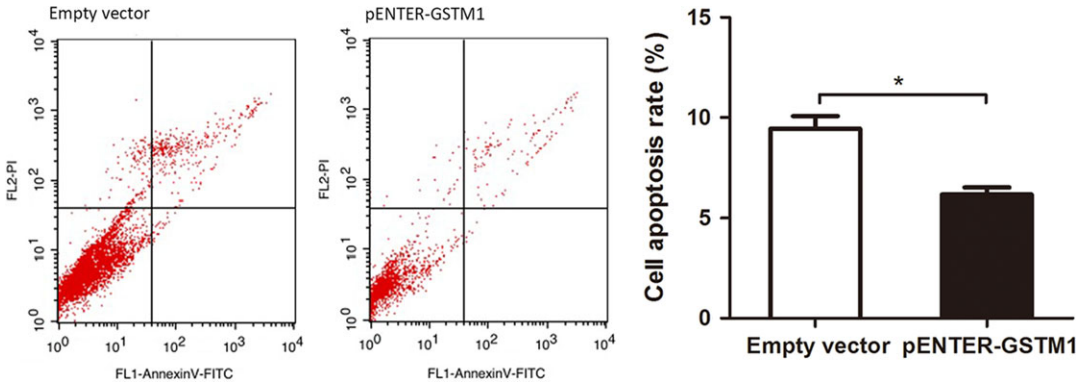

Figure 5 Effect of GSTM1 on endometrial epithelial cell (EEC) apoptosis following hormone treatment and withdrawal. Empty vector and pENTER-GSTM1 transfected EECs were treated with oestradiol and progesterone for 48 h; following the removal of hormones from the medium, cells were incubated for another 24 h. Thereafter, the flow cytometry analysis was performed to analyse cell apoptosis. All experiments were repeated three times. Values were expressed as mean ± SEM, $^*P < 0.05$.

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 *HOXA10*, *CDH1*, *ESR2*, *NR5A1* 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 (Hosseinzadeh *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 (Kalinina *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; Koninckx *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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