6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio) hexanol ameliorates the progression of endometriosis by regulating GSTM4 expression to inhibit proliferation and induce apoptosis

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Research

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Abstract

Background: Endometriosis is a chronic disease associated with disorder of the oxidative balance and chronic inflammation. Although endometriosis is a benign disease, it has the characteristics properties similar to malignant cancer.

Methods: The present study aim to investigate the role of glutathione S-transferase Mu class 4 (GSTM4), and tested if 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio) hexanol (NBDHEX) could regulate GSTM4 expression to affect cell proliferation, migration, invasion and apoptosis in endometriosis. Expression of GSTM4 was detected by immunohistochemistry in 15 cases of endometriosis patients and compared with 15 healthy controls. Primary endometrial cells were analyzed by western blotting (WB) to determine expression of GSTM4, PCNA, MMP-9, Survivin, Bcl-xl, Bax, Keap1 and Nrf2. CCK8 and transwell assays were used to study the effects of GSTM4 and NBDHEX on endometrial cells. The effect on apoptosis was analysed by flow cytometry.

Results: The expression of GSTM4 was significantly increased in endometriosis than those from controls ($p<0.01$). The results suggested that NBDHEX negatively regulates GSTM4 expression, induces cell proliferation, migration, invasion, and promotes cell apoptosis. NBDHEX decreased the expression of GSTM4 ($p<0.05$), PCNA ($p<0.05$), MMP-9 ($p<0.01$), Survivin ($p<0.05$) and Bcl-xl ($p<0.05$), along with increased expression of Bax ($p<0.05$). The results also showed that NBDHEX decreased the expression of Nrf2 ($p<0.05$), but had no effect on the expression of Keap1 ($p>0.05$). After transfection with si-GSTM4, the protein level was down-regulated by nearly 70% ($p<0.05$). Silencing of GSTM4 depressed the proliferation, migration, invasion and gene expression of endometrial stromal cells in patients with endometriosis and controls. Knockdown of GSTM4 interacting with Nrf2 induced apoptosis by decreasing the expression of Survivin ($p<0.05$), Bcl-xl ($p<0.05$) and increasing the expression of Bax ($p<0.05$), but it did not affect the expression of Keap1 ($p>0.05$) in endometriosis and controls.

Conclusions: Inhibition of GSTM4 by NBDHEX suppresses the cell viability growth, migration, invasion and interact with Nrf2 to induce apoptosis, but has no effect on the expression of Keap1 in endometriosis. The use of siRNA to knockdown GSTM4 more accurately confirmed its ability to ameliorate the progression of endometriosis. NBDHEX may have therapeutic potential in the treatment of endometriosis.

Introduction

Endometriosis is a chronic disease caused by the presence of endometrial glands and stroma in ectopic locations, including ectopic pelvic peritoneum, ovary, fallopian tubes, broad ligament, abdomen, rectovaginal septum and some time even to lungs. It is usually associated with dysmenorrhea, dyspareunia, chronic pelvic pain, and infertility[1, 2]. Although endometriosis is a benign disease, it has the characteristics properties similar to malignant cancer. The common therapies for treatment of endometriosis include medication and surgery[3]. Medication is appropriate for most patients with

endometriosis. The first-line therapy for drug treatment include oral contraceptive pills or nonsteroidal anti-inflammatory drugs[4]. However, with these therapeutic approaches, many patients have had little efficacy, or only short-term remission, and some patients even get worse. The mechanism underlying endometriosis progression is not fully understood, but several studies demonstrated that it is associated with disorder of the oxidative balance[5, 6]. Oxidative stress is resulted from unbalanced production of reactive oxygen species (ROS) and the cell's own antioxidant defense. Cancer stem cells proliferate spontaneously taking the advantage of the aberrant redox system[7]. In addition, the imbalance of immune system resulting in the accumulation of inflammatory factors is also one of the pathogenesis of endometriosis[8]. In order to find a better therapy for endometriosis, we urgently need to find new and effective drugs to treat this disease.

Based on the biochemical, immunologic, and structural properties, GSTs have been placed into seven classes of cytosolic proteins, the most common ones are the Alpha, Mu, and Pi classes[9]. Human GSTM4 gene was first isolated from cervical carcinoma cell line HeLa and the protein forms functional dimers was comprised of 218 amino acids with a M_r of approximately 26.4kDa[10]. The genes for GSTM4 is located on chromosome 1 and contains eight exons and seven introns[11, 12]. The GST Mu family consist of at least six expressed isozyme subunits, GSTM1-6, which have 70–90% amino acid sequence identity. Despite the high level of amino acid sequence identity, the enzymatic properties of these enzymes were quite different[10]. We used tandem mass tags combined with multidimensional liquid chromatography and mass spectrometry analyses to screen the proteomic profiles of endometrial tissues from endometriosis patients and healthy women. Acquired data revealed that GSTM4 was highly expressed in endometriosis patients.

NBDHEX was reported to be synthesized by Ricci et al in 2005[13]. A study specifically targeting GSTM4 has found that NBDHEX was used to inhibit the pharmacological activity of GSTM4 and significantly limited cellular proliferation and oncogenic transformation in Ewing sarcoma cells. The combination of NBDHEX and etoposide may increase cytotoxicity, suggesting that GSTM4 may act as an inhibitor of apoptosis[14]. This compound is a representative molecule of a new class of 7-nitro-2,1,3-benzoxadiazole (NBD) derivatives, which can inhibit GSTs catalytic activity and induces apoptosis of tumor cells. Lots of evidence emphasizes that NBDHEX can be used as a new potential anticancer agent and is effective against drug-resistant tumors overexpressing[15, 16].

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is an important regulator of cellular apoptosis via inducing oxidative stress in different cell types. It encodes phase II metabolic enzymes related to detoxification, which are involved in the detoxification of various carcinogens, environmental toxins, drugs, physiological products of oxidative stress, etc[17]. Inhibition of Nrf2 promotes apoptosis and oxidative stress in multiple myeloma[18]. A research conclude that elevated hepatic iron in a mouse model activates NRF2 inducing expression of phase I/II proteins, such as GSTM1, GSTM4, and NAD(P)H dehydrogenase quinone 1[19].

The present study aim to investigate the role of GSTM4, and tested if NBDHEX could modulate GSTM4 expression to affect cell proliferation, migration, invasion and apoptosis in endometriosis.

Materials And Methods

Patients and sample collection

This study recruited nonpregnant women of reproductive age (20–46 years) at the Shandong provincial Hospital and Reproductive Hospital Affiliated to Shandong University from January 2017 to January 2019. The classification of endometriosis is referred to American Society of Reproductive Medicine (ASRM) revised classification[20]. 15 cases of human eutopic endometrial tissues of ovarian endometriosis for immunohistochemical was collected from patients who undergoing laparoscopic diagnosed as endometriosis and then confirmed by histological examination. As for the healthy control group, 15 cases of normal endometrial tissues were obtained from patients with tubal factors. Another 9 cases of fresh endometrial eutopic tissues were collected during the proliferative phase of the menstrual cycle as the experimental group and 9 cases of endometrial tissues who underwent oviduct obstruction were collected as the controls to isolate and culture primary endometrial stromal cells for subsequent experiments. These samples were collected from October 2019 to October 2020. Both the endometriosis and control groups of women did not receive hormonal treatments for 3 months prior to surgery. The studies involving human participants were approved by Institutional Review Board of the Reproductive Hospital Affiliated to Shandong University (registration number 2020-14). Written informed consent for participation was obtained from patients before surgery.

Immunohistochemical staining

Xylene dewaxing, a series of concentration gradient of alcohol hydration, EDTA repair antigen, endogenous peroxidase blocker eliminate endogenous non-specific peroxidase activity, quickblock blocking buffer block non-specific protein binding for 30 minutes. Subsequently, the samples were incubated with GSTM4 antibody (1:100 dilution, Proteintech, China) overnight at 4°C. After washing in PBS for three times, the samples were incubated with enhanced enzyme labeled goat anti rabbit IgG polymer at 37°C for 20 min. The slides were dyed with DAB agent for 5–10 min and counterstained in hematoxylin for 3 min. Finally, immunohistochemistry images were captured using an Olympus 1X51 microscope (Japan). Immunohistochemical staining was performed using the two-step rabbit detection kit (ZSGB-BIO pv-9001, China).

Isolation and culture of primary human endometrial stromal cells

Primary human endometrial stromal cells were isolated by digesting the fresh tissue fragments with 0.1% collagenase type I (Gibco, USA). Briefly, fresh endometrial tissues were washed with 10 ml of pre-warmed sterile PBS three times to remove blood and minced into small pieces using ophthalmic scissors and incubated with 0.2% type I collagenase in a water bath for 40-50min at 37°C. The collagenase activity

was terminated by adding three times the volume of PBS. The tissue suspension was first filtered through a 100- μ m monofilament nylon mesh, then filtered through a 38.5- μ m monofilament nylon mesh and centrifuged at 1000 rpm for 5 min. The stromal cells were subsequently cultured in phenol red-free DMEM/F12 (1:1) (Life Technologies, Gibco, USA) supplemented with 20% fetal bovine serum (FBS; BI, Israel), 1% penicillin and 1% streptomycin in a incubator with 5% CO₂ atmosphere at 37°C. Culture medium was exchanged every 2 days. The first passage cells were used for the subsequent experiments, and the passage number of cells used in all experiments were less than 5. The purity of stromal cells has been verified in our group's previous papers[21].

Cell Transfection Assay And Cell Treatments

GSTM4 small interfering RNA (siRNA) and negative control siRNA (si-NC) were purchased from Shanghai GenePharma (China). The sense sequence of GSTM4 siRNA (GSTM4-homo 110 siRNA) was 5'-GCUCCUGACUAUGACAGAATT-3', and the antisense was 5'-UUCUGUCAUAGUCAGGAGCTT-3'. Endometrial stromal cells were seeded in 6-well plates, cultured to 70–80% density, and transfected with the above siRNA using lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's protocol. The transfection mixture was changed after 10 hours with DMEM/F12 with 20% FBS. Cells were harvested after 48 hours for western blot and other assays.

NBEHEX treatment of primary endometrial stromal cells

NBDHEX was dissolved in DMSO at a 10 mM concentration (MecChemExpress, USA) and stock solution aliquots were stored in darkness at -80°C. The treatment concentrations ranges were determined according to the manufacturer's instructions and published papers. Immediately before use, NBEHEX was diluted to their working concentration required for the in vitro experiments with serum-free DMEM/F12. The final concentration of DMSO never exceeded 0.1%, and this dose had no cytotoxic effect on our cells. Primary endometrial stromal cells were treated with appropriate concentration of NBDHEX for 48h.

Cell counting kit-8 experiments

Cell Counting Kit-8 (CCK8; Beyotime, China) was used to assess cell viability according the manufacturer's instructions. Primary endometrial stromal cells were seeded in 96-well plates at a density of 2×10^3 cells/well. Following specific drug addition, the cells were cultured for 48 hours. CCK8 solution was supplemented with 10 μ l to each well and incubated in dark for 2 hours at 37°C. A microplate reader was used to measure color change at 450 nm, and the absorbance (optical density value) was observed to be directly proportional to cell viability. For transfected cells, each group with three replicate wells. It was incubated for 0, 24, 48 and 72 hours, with 10 μ l to each plate and incubated for 2 hours at 37°C in the dark. The OD value was observed to be directly proportional to cell viability and growth curves were drawn.

Transwell migration and invasion assays

In vitro transwell migration and invasion assays were performed using a 24-well plates with 8µm pore size inserts (Corning, USA) according to the manufacturer's protocols. For migration assay, endometrial stromal cells were implanted into the upper surface of transwell chambers after 48 hours of transfection or drug treatment. In the upper surface, Cells (1×10^5) were seeded in 200µl of serum-free phenol red-free DMEM/F12, while 600µl of phenol red-free DMEM/F12 culture medium containing 10% FBS was added into the bottom chamber. For invasion assay, matrigel(1 mg/mL, BD Biosciences, USA) was diluted with DMEM/F12 in the ratio of 1:8. 50 µL of each well was evenly coated on the upper chamber on the each transwell chamber and incubated in 37°C for 2 hours. Endometrial stromal cells were seeded into the upper surface of transwell chambers with 200 µL of serum-free medium and allowed to invade the lower chamber, which contained 600 µl of DMEM/F12 culture medium containing 10% FBS. To evaluate the migration and invasion potential, cells were allowed culture for a period of 24 and 48 hours in culture, respectively. The numbers of cells invaded into matrigel on the bottom chamber and cells migrated to the other side of the insert were counted in eight random fields and averaged.

After indicated time, the cells on the upper surface of the chambers that did not migrate or invade were removed by wiping with cotton swabs. Cells invaded into matrigel on the bottom chamber and cells migrated to the other side of the insert were fixed in 4% paraformaldehyde for 20 min, permeated in methanol for 20 min, stained with hematoxylin for another 20 min and fixed on a glass slide. The number of cells on the underside of the chambers were observed and counted under the Olympus 1X51 microscope(Japan) in three random fields.

Protein extraction and western blot analysis

Cultured cells were washed with cold PBS three times. For total protein extraction, the radioimmunoprecipitation assay(RIPA) buffer (Shanghai, China) and protease/phosphatase inhibitor cocktail(Cell Signaling Technology, USA) were mixed in a ratio of 99:1, and 100µl of mixture was added to each 10×10^6 cells lysed on ice for 30 min. The cells were scraped and centrifuged at $12,000 \times g$ at 4°C for 15 min. The supernatant was the extracted protein, which was transferred to another 1.5 ml microcentrifuge tube, diluted in 5×SDS-PAGE sample loading buffer and boiled for 15 minutes. The protein concentration was determined by BCA protein assay kit (Thermo Scientific, USA).

For nuclear protein extraction, we used Minute cytoplasmic and nuclear extraction kit for cells (Invent Biotechnologies,USA) according to the manufacturer's introduction. Add 100 µl cytoplasmic extraction buffer per 6-well plate, cultured on ice for 5 min. The lysed cells were scrape with a transfer pipette, and transferred to pre-chilled 1.5ml microcentrifuge tube and vibrated violently for 15 seconds. Centrifuge the tube at $16,000 \times g$ at 4°C for 5 min. The supernatant was cytosol fraction. Wash the pellet with cold PBS to reduce contamination of cytosolic proteins. Added 50 µl nuclear extraction buffer to the pellet, vortex vigorously for 15 seconds, incubate the tube on ice for 1 min, repeat 4 times. The nuclear extract transferred to a pre-chilled filter cartridge with collection tube and centrifuge at $16,000 \times g$ at 4°C for 30 seconds and discard the filter cartridge. The rest is the same as the total protein extraction.

The protein was separated by 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis, and then transferred to a PVDF (polyvinylidene difluoride) membrane. After blocking in 5% non-fat milk at room temperature for 60 min, the membrane was incubated in a refrigerator overnight at 4°C with the primary antibody. After washing with tris-buffered saline-tween (TBST) for 10 min three times, the membrane were incubated for 60 min at room temperature with corresponding secondary. Primary antibodies against rabbit anti-human GSTM4 (1:500 dilution, Proteintech, China), Kelch-like ech-associated protein 1 (Keap1), Nrf2, Bax, MMP9(1:1000 dilution, Cell Signaling Technology, USA), and mouse anti-human β -Actin, PCNA, Lamin B (1:5000 dilution, Proteintech, China) were used. Goat anti-rabbit and Goat anti-mouse HRP-conjugated secondary antibodies were obtained from Proteintech (1:10000 dilution, China).

Apoptosis analysis by flow cytometry

Apoptosis was evaluated using Annexin V-APC/7AAD apoptosis kit (MultiSciences Biotech, China). Endometrial cells treated with NBDHEX or transfected with si-RNA as previously described were double stained with Annexin V-APC and 7-AAD, according to the manufacturer's instruction. Cell apoptosis was detected by flow cytometry (BD Bio- sciences, USA). Briefly, the collected cells were resuspended in 500ul binding buffer. Then 5 μ l Annexin V-APC and 10 μ l 7-AAD were added and mixed for 5–10 min without light. Cell-associated fluorescence was analyzed by flow cytometry. The percentage of apoptotic positive cells including early apoptotic (annexin V-positive) and late apoptotic (Annexin V and 7-AAD- positive) cells.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 software. Values were expressed as means $\pm$ standard deviation (SD). Comparisons between two groups were performed using Student's t-tests. Anova followed by Newman-Keuls tests were used to compare differences among multiple groups. Image J software was used to count positive immunohistochemical signals, and the results were represented by optical density. $P < 0.05$ was considered statistically significant. Each experiment was repeated three times.

Results

GSTM4 protein expression and localization in endometrial stromal cells from endometriosis patients

To evaluate the protein expression of GSTM4 on endometriosis, we first performed immunohistochemistry staining to detect the protein expression level of endometrial cells between 15 eutopic endometrial samples from patients with ovarian endometriosis and 15 control endometrial samples from woman without endometrisis. We found that GSTM4 is mainly expressed in cytoplasm of stromal cells and glandular epithelial cells in endometrial tissues. The expression levels of GSTM4 was significantly increased in eutopic endometrial lesions from patients with ovarian endometriosis than those from controls(0.205 ± 0.013 vs. 0.291 ± 0.014 , $P < 0.001$; Fig. 1A). The immunocytochemical

staining results were represented by optical density (Fig. 1B). WB also detected that the protein levels of GSTM4 were increased in the eutopic endometrium compared to control endometrium (0.899 ± 0.090 vs. 1.371 ± 0.121 , $p < 0.01$; Fig. 1C). Densitometric analysis was used to calculate representative quantitative data (Fig. 1D). Taken together, the data suggested that increased expression of GSTM4 might involved in the pathological process in patients with endometriosis.

Inhibition of GSTM4 by NBDHEX suppresses the cell viability growth, migration, invasion of endometrial stromal cells from endometriosis patients

Although endometriosis is not a malignant disease, it has its own unique characteristics, but it also has similar characteristics with tumor cells, such as growth, migration, and invasion. In order to determine the mechanism of NBDHEX inhibiting the formation of endometriosis, we studied the effect of NBDHEX on the proliferation of human primary endometrial stromal cells from endometriosis patients. NBDHEX is an effective GSTs inhibitor. We treated primary endometriosis cells with different doses of NBDHEX (0.25 μ M, 0.5 μ M, 0.75 μ M, 1 μ M, 1.5 μ M, 2 μ M, 2.5 μ M, 3 μ M, 3.5 μ M and 4 μ M). According to CCK8 assay, the absorbance value of each inoculated cell well was measured. The higher the absorbance value, the more viable cells. We found that cell proliferation decreased in a dose-dependent manner after 48 hours of drug treated (Fig. 2A). The cell survival rate of the 0 μ M drug treatment well was set as 1, and the ratio of the absorbance value of other concentrations to the 0 μ M drug well was the relative survival rate. We fitted the dose-survival curve through Prism 7.0 and calculated the IC50 value of the drug. The result showed that IC50 values were about 1.44 μ M for primary endometrial cells (Fig. 2B).

We also assessed the difference effect of 0 μ M, 0.75 μ M and 1.5 μ M NBDHEX treatment on migration and invasion of endometrial cells. The cells on the lower surface of the membrane were counted. The result showed that migration and invasion were significantly reduced in 1.5 μ M NBDHEX group compared to the 0 μ M NBDHEX group. With the increase of NBDHEX concentration, the migration ability of cells decreased (Fig. 2C). There was no significant difference between 0 μ M NBDHEX group and 0.75 μ M NBDHEX group (238.3 ± 21.25 vs. 183.3 ± 22.94 , $p > 0.05$), and there was significant difference between 0 μ M NBDHEX group and 1.5 μ M NBDHEX group (238.3 ± 21.25 vs. 128.1 ± 21.98 , $P < 0.001$). In the invasion assay, the higher the concentration of NBDHEX, the weaker the invasion ability of cells (Fig. 2D). There were significant differences between 0 μ M NBDHEX group and 0.75 μ M NBDHEX group groups (154.2 ± 9.181 vs. 78.67 ± 7.315 , $P < 0.0011$), 0 μ M NBDHEX group and 1.5 μ M NBDHEX group (154.2 ± 9.181 vs. 44.67 ± 3.765 , $P < 0.001$).

Based on the above experiments, we chose NBDHEX concentration of 0 μ M and 1.5 μ M to treat the eutopic endometrial stroma cells to explore the effects on proliferation, migration and invasion. Western blot analysis was used to detect the changes of PCNA and MMP-9 protein levels (Fig. 2E). Expression of PCNA (2.487 ± 0.374 vs. 1.193 ± 0.374 , $p < 0.05$) and MMP-9 (3.153 ± 0.590 vs. 1.383 ± 0.085 , $p < 0.05$) were significantly reduced in 1.5 μ M NBDHEX group compared to the 0 μ M NBDHEX group.

Inhibition of GSTM4 by NBDHEX induces apoptosis in endometrial stromal cells from endometriosis patients

To further evaluate the inhibitor of GSTM4 by NBDHEX mediated cell death of eutopic endometrial stroma cells, the effect of NBDHEX on apoptosis was detected by Annexin V-APC/7-AAD apoptosis kit. Apoptosis and the total apoptotic rate of cells of NBDHEX exposure are shown in Fig. 3A. In 0 μ M NBDHEX group, the cell survival rate was 78.16%. However, in 1.5 μ M NBDHEX group apoptosis rate was increased by about 13.01% ($P < 0.05$). Then the apoptosis related protein levels of Survivin, Bcl-xl and Bax were measured by western blot analysis (Fig. 3B). Compared with 0 μ M NBDHEX group, Survivin (3.162 ± 0.690 vs. 1.175 ± 0.112 , $p < 0.05$) and Bcl-XL (2.529 ± 0.588 vs. 1.363 ± 0.613 , $p < 0.01$) protein levels were significantly decreased in the 1.5 μ M NBDHEX group, while Bax (2.257 ± 0.504 vs. 3.019 ± 0.598 , $p < 0.05$) protein levels were increased.

Inhibition of GSTM4 by NBDHEX interacting with Nrf2 induces apoptosis, but it did not affect the expression of Keap1 in endometrial stromal cells from endometriosis patients

Nrf2 is an important regulator of cellular apoptosis via inducing oxidative stress in different cell types. In order to further elucidate the role of GSTM4 in the progression of endometriosis, we detected the protein expression levels of Keap1 and Nrf2 and its downstream related gene in endometriosis. We first compared the protein expression of Keap1, Nrf2 and GSTM4 in endometriosis patients and controls (Fig. 4A). WB results showed that compared with the control group, Keap1 decreased (2.310 ± 0.286 vs. 1.517 ± 0.114 , $p < 0.05$), Nrf2 increased (1.500 ± 0.397 vs. 2.313 ± 0.236 , $p < 0.05$) and GSTM4 increased (1.463 ± 0.203 vs. 3.283 ± 0.090 , $p < 0.01$) in the experimental group.

We hypothesized that NBDHEX treatment did not change the expression of Keap1 and Nrf2, but changed the expression of GSTM4, indicating that GSTM4 was downstream of Keap1/Nrf2 signaling pathway. As show in Fig. 4B, WB results demonstrated that 1.5 μ M NBDHEX group could reduce the expression of GSTM4 compared to the 0 μ M NBDHEX group (2.429 ± 0.233 vs. 1.449 ± 0.092 , $p < 0.01$). However, WB results also showed that NBDHEX had no effect on the expression of Keap1 (1.363 ± 0.613 vs. 1.371 ± 0.531 , $p > 0.05$), but the expression of Nrf2 was decreased (3.49 ± 0.422 vs. 1.772 ± 0.659 , $p < 0.05$) compared to the 0 μ M NBDHEX group. We consider that Nrf2 and GSTM4 may inhibit each other, that is, Nrf2 expression decreases and GSTM4 expression is inhibited, which is consistent with the common theory. However, NBDHEX inhibited GSTM4 expression, further inhibited Nrf2 expression and promoted apoptosis.

Knockdown of GSTM4 depress the cell proliferation, migration and invasion both in eutopic endometrial stroma cells with endometriosis and control endometrial stroma cells

Because in addition to inhibiting the expression of GSTM4, NBDHEX may also inhibit the expression of other GSTs family proteins. In order to further elucidate the role of GSTM4 in the progression of endometriosis, we transfected an siRNA containing an GSTM4 targeting sequence (si-GSTM4) and negative control siRNA (si-NC) in eutopic endometrial stroma cells with endometriosis and control endometrial stroma cells.

When the cells were not transfected with si-GSTM4, the expression of GSTM4 in the eutopic group was about 60% higher than that in the control group (1.337 ± 0.152 vs. 3.306 ± 0.634 , $P < 0.001$). After cells transfected with si-GSTM4, GSTM4 protein level was down-regulated by nearly 70% in eutopic group (3.306 ± 0.634 vs. 0.987 ± 0.206 , $p < 0.001$) and down-regulated by 68% in control group (1.337 ± 0.152 vs. 0.426 ± 0.309 , $p < 0.05$). (Fig. 5A)

Silencing of GSTM4 led to proliferation inhibition of both group as assessed by CCK8 assay. The absorbance of the cells in each inoculated cell well at 450 nm was detected by enzyme-labeled analyzer. The results showed that the absorbance of the cells increased with the prolonging of culture time. The proliferation of endometriosis cells was stronger than that in the control group. After knockdown of GSTM4, the proliferation ability of cells in both groups was decreased compared with that in si-NC group (Fig. 5B).

Endometriosis cells and normal endometrial cells were cultured for 36 hours with and without si-GSTM4 and then assessed by transwell migration and invasion assay. When without si-GSTM4 transfection, the number of migration (170.3 ± 8.352 vs. 271.7 ± 9.854 , $p < 0.001$) and invasion cells (100.4 ± 3.473 vs. 139.0 ± 7.297 , $p < 0.001$) in the endometriosis group was significantly higher than that of the control group. After transfected with si-GSTM4 the number of migration cells (170.3 ± 8.352 vs. 125.3 ± 8.162 , $p < 0.05$; 271.7 ± 15.460 vs. 145.2 ± 9.854 , $p < 0.001$) and the number of invasion cells (100.4 ± 3.473 vs. 43.11 ± 3.048 , $p < 0.001$; 139.00 ± 7.297 vs. 67.89 ± 5.184 , $p < 0.001$) were significantly reduced in control group and eutopic group (Fig. 5C and D).

Additionally, we used western blot analysis to detect the changes of PCNA and MMP-9 protein levels. When without si-GSTM4 transfection, the protein levels of PCNA and MMP-9 also compared between endometrial cells and normal endometrial cells. The results showed that the expression levels of PCNA (1.457 ± 0.282 vs. 2.21 ± 0.310 , $p < 0.05$) and MMP-9 (1.190 ± 0.148 vs. 1.753 ± 0.217 , $p < 0.01$) were higher in eutopic endometrial stroma cells compared to the control group. After transfected with si-GSTM4, The protein expression level of PCNA (2.21 ± 0.310 vs. 1.437 ± 0.185 , $p < 0.05$) in endometriosis cells was significantly decreased. While the expression of PCNA (1.457 ± 0.282 vs. 1.187 ± 0.150 , $p > 0.05$) in normal endometrial cells was also decreased, but there was no statistical difference. Expression of MMP-9 (1.190 ± 0.148 vs. 0.833 ± 0.064 , $p < 0.05$; 1.753 ± 0.217 vs. 1.420 ± 0.066 , $p < 0.05$) were significantly reduced in two group cells transfected with si-GSTM4. (Fig. 5E).

Knockdown of GSTM4 induces apoptosis both in eutopic endometrial stroma cells with endometriosis and control endometrial stroma cells

The effect of knockdown of GSTM4 on apoptosis was analysed by flow cytometry. Compared with the control group, apoptosis rate of endometriosis group was statistically significant reduced ($33.26 \pm 5.878\%$ vs. $19.67 \pm 2.913\%$, $p < 0.05$). When the cells were transfected with si-GSTM4 compared to si-NC, the apoptotic rates were increased both in control group (33.26 ± 5.878 vs. $51.740 \pm 4.754\%$, $p < 0.05$) and eutopic group ($19.67 \pm 2.913\%$ vs. $36.92 \pm 9.087\%$, $p < 0.05$). (Fig. 6A)

Then the apoptosis related protein levels of Survivin, Bcl-xl and Bax were detected by western blot analysis. When the cells were not transfected with si-GSTM4, the expression levels of the anti-apoptotic proteins Survivin (2.426 ± 0.278 vs. 4.186 ± 0.728 , $p < 0.01$) and Bcl-XL (2.105 ± 0.147 vs. 2.779 ± 0.441 , $p < 0.05$) were higher in the eutopic group than those in control group, and the expression levels of pro-apoptotic protein Bax were lower (2.257 ± 0.504 vs. 1.146 ± 0.131 , $p < 0.05$). After cells transfected with si-GSTM4, the protein levels of Survivin (2.426 ± 0.278 vs. 0.854 ± 0.241 , $p < 0.05$; 4.186 ± 0.728 vs. 1.794 ± 0.752 , $p < 0.01$) and Bcl-xl (2.105 ± 0.147 vs. 1.244 ± 0.222 , $p < 0.05$, 2.779 ± 0.441 vs. 1.917 ± 0.493 , $p < 0.05$) were significantly decreased, and the protein level of Bax was increased (2.257 ± 0.504 vs. 3.685 ± 0.708 , $p < 0.05$; 1.146 ± 0.131 vs. 2.398 ± 0.560 , $p < 0.05$) in control group and eutopic group (Fig. 6B). Therefore, Knockdown of GSTM4 induces apoptosis was involved in the regulation of apoptosis in endometrial stroma cells.

Knockdown of GSTM4 interacting with Nrf2 induces apoptosis, but it did not affect the expression of Keap1 in endometriosis

We detected the protein expression levels of Keap1, Nrf2 and GSTM4 both in endometriosis and control cells by western blot analysis. The results were similar to those of inhibiting GSTM4 expression by NBDHEX. When the cells were not transfected with si-GSTM4, the protein expression levels of Nrf2 (1.802 ± 0.267 vs. 2.967 ± 0.209 , $P < 0.01$) and GSTM4 (1.337 ± 0.152 vs. 3.306 ± 0.634 , $P < 0.001$) were higher in the eutopic group than those in control group, and the expression levels of Keap1 were lower (3.623 ± 0.265 vs. 2.073 ± 0.839 , $p < 0.05$). After cells transfected with si-GSTM4, the protein levels of Nrf2 (1.802 ± 0.267 vs. 0.935 ± 0.366 , $p < 0.05$; 2.967 ± 0.209 vs. 2.349 ± 0.342 , $p < 0.05$) and GSTM4 (1.337 ± 0.152 vs. 0.426 ± 0.309 , $p < 0.05$, 3.306 ± 0.634 vs. 0.987 ± 0.206 , $p < 0.001$) were significantly decreased, and the protein level of Keap1 (3.623 ± 0.265 vs. 2.817 ± 0.410 , $p > 0.05$, 2.073 ± 0.839 vs. 1.947 ± 0.475 , $p > 0.05$) have no significant change in control group and endometriosis group (Fig. 7A).

To further confirm the activation of Nrf2 transport into the nucleus, we used nuclear protein to detect the expression of Nrf2 and GSTM4 in cell nucleus of eutopic endometrial stroma cells. Compared with si-NC group, knockdown of GSTM4 decreased Nrf2 level in cell nucleus (1.341 ± 0.550 vs. 0.896 ± 0.517 , $p = 0.0235$) and total cell (1.919 ± 0.199 vs. 1.405 ± 0.102 , $*p = 0.0394$), whereas GSTM4 was not expressed in nucleus (Fig. 7B).

Discussion

The present study aim to investigate the role of GSTM4, and tested if NBDHEX could modulate GSTM4 expression to affect cell proliferation, migration, invasion and apoptosis in endometriosis. The results demonstrate for the first time that increased GSTM4 expression may be involved in the progression in patients with endometriosis. Inhibition of GSTM4 by NBDHEX suppresses the cell viability growth, migration, invasion and which interacting with Nrf2 induces apoptosis, but it did not affect the expression of Keap1 in endometriosis. The use siRNA to knockdown GSTM4 more accurately confirmed its ability to improve the progression of endometriosis.

Many studies have reported that the expression level of GST is higher in a number of tumors. The immunocytochemical affirmed that the expression of GSTA, GSTP, GSTM4 and GSTT1 in urothelial cancer cells was stronger than the in benign cells[22]. In Yu Li's study, quantitative proteomics analysis using tandem mass tags (TMTs) coupled with liquid chromatography-mass spectrometry (LC-MS)/MS showed that GSTM4 expression was increased and GSTT1 expression was decreased in psoriasis vulgaris lesional tissues compared with healthy skin tissues[23]. Overexpression of specific GSTMs, GSTM1 and GSTM4 and elevated levels of glutathione contribute to maintaining a reduced state of cytochrome which would decrease apoptosis, thus promoting to methotrexate resistance in human MCF7 breast cancer cells[24]. A report show that GSTM4 is a direct target gene of the EWS/FLI fusion protein. Reduced GSTM4 levels resulted a decrease of oncogenic transformation and a increase of sensitivity of chemotherapeutic agents[25]. GSTM1 expression level was significantly higher in the ectopic and eutopic endometrial of patients with ovarian endometriosis, which could significantly increase cell viability and inhibit cell apoptosis[26]. In this study, for the first time, GSTM4 was found to be highly expressed in endometriosis. Our findings are consistent with above reports, but some studies have shown that the expression of GSTM4 is decreased in some diseases(aging and skin infected with dermatophytes[27, 28].

Although endometriosis is a benign disease, it has the characteristics of migratory and invasive properties similar to malignant cancer[29]. Various phytochemicals like rutin, naringenin, methyl ester of 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO-Me) have been reported for their efficacy against endometriosis[18, 30, 31]. NBDHEX appears to be a promising candidate for targeting and inhibiting GSTs[32]. GSTs displays antiapoptotic activity and is also involved in the cellular resistance to anticancer drugs. A recently published study demonstrates that NBDHEX triggers apoptosis in leukemia cell lines through the dissociation of GSTP1-1 from the GSTP1-1-JNK complex. This event revealed that cancer can be treated by hypothesizing that NBDHEX triggers a crucial perturbation in the GSTs structure[33]. NBDHEX opens up interesting prospects for cancer therapy as a suicide inhibitor of GSTs [14]. One study evaluated the acute toxicity of NBDHEX by a single injection of nbdhex into male BDF1 mice. After 15 days of follow-up, there were no significant changes in body weight, liver weight, spleen weight and red blood cell count. Only a slight increase of white cells, in particular neutrophils, was observed after treatment with NBDHEX[32]. Here, we hypothesized that NBDHEX, being an anti-inflammatory, anti-invasive,pro-apoptotic, could play a therapeutic role in the endometriosis.

We used primary endometrial cells from women with and without endometriosis to deal with the evaluation of therapeutic effect of NBDHEX and exploration of the underlying mechanism using in vitro experimental conditions. We found that cell viability growth decreased in a dose-dependent manner of drug treated. With the increase of NBDHEX concentration, the migration and and invasion ability of cells was decreased. Radhika Kapoor et al. confirmed that MMPs play a important role in the establishment and development of endometriosis. The expression of various prognostic markers such as PCNA, MMP2, MMP9, AKT1 and VEGF in endometral cells certained that endometriosis prevailed in the system[34, 35]. Western blot Analysis detected that PCNA and MMP-9 protein levels were significantly reduced with NBDHEX treatment. Flow cytometry was used to detect the effect of NBDHEX, a GSTM4 inhibitor, on cell

apoptosis in patients with endometriosis. Treatment of primary endometrial cells from endometriosis patients with NBDHEX, the apoptosis rate was increased by 13.01%. Apoptosis related protein levels of Survivin, Bcl-xl were significantly decreased, while the protein level of Bax was increased.

The role of Nrf2 transcription factor in the activation of oxidative stress has been demonstrated[36]. It plays an essential role in cytoprotection. Nrf2 resides in the cytoplasm under basal circumstances and formed complexes with Keap1. When exposed to oxidative stress, Keap1 releases Nrf2, which moves to the nucleus, binds to the antioxidant response element (ARE) and associates with Maf protein to induce the expression of cell-protective genes like phase II detoxifying enzymes[37]. Nrf2 is an important regulator of cellular apoptosis via inducing oxidative stress in different cell types. Inhibition of Nrf2 promotes apoptosis and oxidative stress in multiple myeloma[33]. In the livers of male and/or female Nrf2 knockout(KO) mice, the amounts of mRNA for Gstm1, Gstm3 and Gstm4 were reduced to between 3% and 20 % of that in wild-type (WT) mice .In female WT and female Nrf2 KO mice,The Gstm4\5 polypeptide(s) were expressed higher than genetically equivalent female WT and female Nrf2 KO mice[38]. Microsomal epoxide hyrolase 1 (EPHX1) silencing promoted ACM and MIT induced decrease in cell viability and the apoptosis of human myeloid cells. Nrf2 overexpression significantly increased EPHX1 expression and leukemic cell viability and reduced leukemic cell apoptosis[39]. A research demonstrated that MLT could protect follicle integrity and prevent apoptosis by activating Nrf2 signaling pathway, increasing the expression of heme oxygenase-1 (HO-1), glutathione S-transferase M1 (GSTM1), SOD and cat, and reducing the levels of ROS, MDA and NO[40]. Some reports suggested that Nrf2 to be activated to supports survival and chemotherapy resistance in multiple myeloma, inhibiting Nrf2 up-regulates apoptosis and oxidative stress in multiple myeloma cell lines as well as in patients' samples[41]. Our results demonstrated that NBDHEX could reduce the expression of GSTM4. However, NBEHEX had no effect on the expression of Keap1, but the expression of Nrf2 was decreased. We speculate that NRF2 and GSTM4 may inhibit each other through some mechanism. Nrf2 promotes GSTM4 expression, which is consistent with conventional theory. The specific mechanism by which NBDHEX inhibits the expression of GSTM4 and further inhibits the expression of Nrf2 to promote apoptosis remains to be further studied.

Because of NBDHEX may also inhibits the expression of other GST family proteins, besides to inhibiting the expression of GSTM4. We used primary endometrial cells from women with and without endometriosis to evaluation of effect of GSTM4 and exploration of the underlying mechanism using in vitro experimental conditions. We transfected an si-GSTM4 in endometrial cells to verify the effect of different GSTM4 expression levels on endometriosis. These results suggest that NBDHEX negatively regulates the GSTM4 expression induced proliferation, migration and invasion. The imbalance of oxidative stress has been linked to endometriosis. Keap1 frees Nrf2 in the cytoplasm, and Nrf2 enters the nucleus to induce the expression of Phase II detoxifying enzymes like GSTM4. Nrf2 inhibited apoptosis by altering the expression of Survivin, bcl-xl and bax. After transfected with si-GSTM4, the protein level was down-regulated by nearly 70%. Silencing of GSTM4 depresses the cell proliferation, migration, invasion and gene expression both in endometrial stromal cells from endometriosis patients and that from controls. Knockdown of GSTM4 interacting with Nrf2 induces apoptosis, but it did not affects the

expression of Keap1 in endometriosis. The anti-apoptotic protein levels of Survivin and Bcl-xl were significantly decreased, and the pro-apoptotic protein level of Bax was increased both in patients with and without endometriosis. In the future, the endometriosis rat model should be used to verify that NBDHEX alleviates endometriosis by inhibiting GSTM4 expression (Fig. 8).

Conclusion

In conclusion, the study found that GSTM4 is highly expressed in endometriosis. Inhibition of its expression by NBDHEX can achieve the purpose of alleviating endometriosis, which is the first study to use NBDHEX in the treatment of endometriosis. These results may provide a new therapy for endometriosis.

Abbreviations

ASRM
American Society of Reproductive Medicine
DMSO
dimethyl sulfoxide
FBS
fetal bovine serum
GSTM4
glutathione S-transferase Mu class 4
ICH
immunohistochemistry
IC50
half maximal inhibitory
NBDHEX
6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio) hexanol
Nrf2
Nuclear factor-erythroid 2-related factor 2
MLT
melatonin
NQO1
NAD(P)H dehydrogenase quinone 1
OD
optical density
PVDF
polyvinylidene fluoride
ROS
reactive oxygen species

siRNA
small interfering RNA
WB
western blotting

Declarations

Ethics Statement and Consent to participate

The study was reviewed and approved by the Reproductive Hospital Affiliated to Shandong University (registration number 2020-14). Informed consent was obtained from each patient

Consent for publication

All the authors agreed to publish the manuscript in Reproductive Biology and Endocrinology.

Availability of data

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

LY and NZ contributed to conception and design of the study. WL and XL performed the experiments. YD and XL performed the statistical analysis. WL and LY wrote the first draft of the manuscript. YD and JM wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Figures

Figure 1

GSTM4 expression in endometriosis endometrial stromal cells from endometriosis patients compared to that from controls. (A) Representative immunohistochemical analysis of GSTM4 protein localization in normal endometrium and eutopic endometrium. Images were captured at magnifications of $\times 200$ (left panels) and $\times 400$ (right panels), respectively. (B) The immunohistochemical staining results were represented by optical density. (C) Representative western blots analysis of GSTM4 protein levels in normal endometrium, eutopic endometrium. β -Actin was used as a loading control. (D) Representative quantitative data of densitometric analyses. Data were presented as the mean $\pm$ SD of three independent experiments. (* $P < 0.05$; ** $P < 0.01$; * $P < 0.001$).

Figure 2

Figure 2

The GSTM4 inhibitor NBDHEX inhibits endometrial cells proliferation, migration and invasion. (A) NBDHEX inhibits endometrial cells growth in culture. Eutopic endometrial cells were treated with different doses of NBDHEX (0.25 μM, 0.5 μM, 0.75 μM, 1 μM, 1.5 μM, 2 μM, 2.5 μM, 3 μM, 3.5 μM and 4μM). Cell proliferation was assessed by CCK8 assays. (B) The IC₅₀ values of NBDHEX in endometrial cells was presented. (C) and (D) Eutopic endometrial stroma cells were treated with increasing concentrations of

NBDHEX for cell migration (C) and invasion (D) assays using a transwell system. (E) Protein levels of PCNA and MMP-9 were determined by Western Blot. β -Actin was utilized for an endogenous reference to standardize protein levels. Densitometry analysis was carried out and normalized to β -Actin. Data were presented as the mean $\pm$ SD of three independent experiments. (* $P < 0.05$; ** $P < 0.01$; * $P < 0.001$, $p > 0.05$ no significant difference).

Figure 3

Figure 3

The GSTM4 inhibitor NBDHEX induces apoptosis in endometrial stromal cells from endometriosis patients. (A) The effect of NBEHEX on apoptosis in primary endometrial cells from endometriosis patients were detected by Annexin V-APC/7-AAD apoptosis kit. Graph represents % cells undergoing apoptosis in two groups. (B) Changes in the protein level of Survivin, Bcl-xl and Bax in different groups

detected by western blot analysis. Results are shown as mean $\pm$ SD from three independent experiments. (*P < 0.05; **P < 0.01; *P < 0.001).

Figure 4

Figure 4

NBDHEX interacting with Nrf2 induced apoptosis in endometrial stromal cells from endometriosis patients. (A) Expression of Nrf2, Keap1, GSTM4 in endometrial cells compared with controls. (B) Effects of NBDHEX on Nrf2, Keap1 and GSTM4 in endometrial cells. All experiments were performed in triplicate.

Data were presented as the mean $\pm$ SD of three independent experiments. (* $P < 0.05$; ** $P < 0.01$; * $P < 0.001$, $p > 0.05$ no significant difference).

Figure 5

Figure 5

Knockdown of GSTM4 depress proliferation, migration, invasion and gene expression both in endometriosis and controls. (A) Knockdown of GSTM4 was demonstrated to reduce its protein expression level in endometrial cells and normal endometrial cells were detected by Western Blot, and β -

Actin used as a control. (B) Effect of GSTM4 knockdown on proliferation capacity of cells were detected by CCK-8 assay. (C) and (D) Endometrial cells and normal endometrial cells were transfected with si-GSTM4, si-NC was the control. The cells were used for determining migration (C) and invasion (D) by using a transwell system. (E) Protein levels of PCNA and MMP-9 were determined by Western Blot, and β -Actin used as a control. Data were calculated as means $\pm$ SD from at least three independent experiments. (* $P < 0.05$; ** $P < 0.01$; * $P < 0.001$, $p > 0.05$ no significant difference).

Figure 6

Figure 6

GSTM4 Knockdown induced apoptosis both in endometriosis and controls. (A) The effect of GSTM4 Knockdown on apoptosis were detected by Annexin V-APC/7-AAD apoptosis kit. Graph represents % cells undergoing apoptosis. (B) Changes in the expression of Survivin, Bcl-xl and Bax protein in different groups were detected by western blot analysis, and β -Actin used as a control. Data were calculated as means $\pm$ SD from at least three independent experiments. (* $P < 0.05$; ** $P < 0.01$; * $P < 0.001$).

Figure 7

Figure 7

Knockdown of GSTM4 interacting with Nrf2 induces apoptosis both in endometriosis and controls. (A) Expression of Nrf2, KEAP1, GSTM4 in in different groups. (B) Effects of GSTM4 Knockdown on Nrf2 both in nucleus and total cells. All experiments were performed in triplicate. Results are shown as mean $\pm$ SD from three independent experiments. (*P < 0.05; **P < 0.01; *P < 0.001, p>0.05 no significant difference).

Figure 8

Figure 8

Mechanisms by which NBDHEX ameliorates the progression of endometriosis by regulating GSTM4 expression.