

# Reactive oxygen species downregulate ARID1A expression via its promoter methylation during the pathogenesis of endometriosis

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**Abstract.** – **OBJECTIVE:** Oxidative stress caused by reactive oxygen species (ROS) plays an important role in the pathogenesis of endometriosis. The gene AT-rich interactive domain 1A (ARID1A), is frequently down regulated and inactivated in endometriosis. This report is focused on the molecular mechanism of the correlation between oxidative stress and ARID1A gene expression in endometrial cell oxidative damage model.

**PATIENTS AND METHODS:** In this study, the ARID1A gene expression level and its promoter methylation level were detected in 30 endometriosis and normal tissues. The primary endometrial cell was co-cultured with H<sub>2</sub>O<sub>2</sub>. Then, MDA and Gpx level were used to test the ROS level, RT-PCR was employed to detect the expression level of ARID1A. At last, the ARID1A gene promoter methylation level was detected by methylation-specific PCR (MSP). Finally, the expression level of DNMT1 was detected by both RT-PCR and Western blot.

**RESULTS:** The expression level of ARID1A gene was down regulated in endometriosis compared with normal tissues. The low expression level of ARID1A gene was associated with its promoter hyper-methylation. In H<sub>2</sub>O<sub>2</sub> simulated endometrial cells, ARID1A gene expression level was decreased. Finally, ROS regulated ARID1A gene expression by changing the methylation level of ARID1A gene promoter. Finally, both the mRNA level and protein level of DNMT1 increased in H<sub>2</sub>O<sub>2</sub> simulated endometrial cells.

**CONCLUSIONS:** In endometriosis, the down-regulated of ARID1A gene was highly correlated with its promoter hyper-methylation. ROS decreased the expression level of ARID1A gene via regulating methylation of its promoter which contributing to the understanding of the pathogenesis of endometriosis. The possible mechanism of ARID1A gene promoter hyper-methylation is ROS up-regulated DNMT1 gene expression.

Key Words:

Endometriosis, Reactive oxygen species (ROS), ARID1A, Methylation, DNMT1.

## Introduction

Endometriosis (EMs) is a common benign and estrogen-dependent chronic gynecological disease in women. The morbidity of women in the reproductive period is about 10% to 15%<sup>1</sup>. Endometriosis is caused by the active endometrial cells planting outside of endometrium<sup>2</sup>. The formation of ectopic nodules, dysmenorrhea, chronic pelvic pain, menstrual abnormalities and infertility is the main symptoms<sup>3</sup>. The main pathological changes of endometriosis are ectopic endometrial hemorrhage and peripheral tissue fibrosis<sup>4</sup>. The study of its molecular mechanism has theoretical and clinical significance to the treatment of endometriosis.

Oxidative stress has been proved as a pathological factor in the development of endometriosis. The harmful effects of reactive oxygen species (ROS) have been reported in a lot of diseases including cancer, osteoporosis, metabolic diseases and so on<sup>5-7</sup>. In endometriosis, the corresponding lesions will produce an endometrial inflammation response to remove foreign bodies. Neutrophils, eosinophils and other inflammatory cells involved in the inflammatory response will release ROS to promote the oxidative stress in lesions and surrounding tissues<sup>8</sup>. At the same time oxidative stress is also involved in the development of dysmenorrhea, infertility and other clinical manifestations by regulating proliferation, angiogenesis of cells in lesions<sup>9</sup>. It is unclear how oxidative stress cause damage to uterus and surrounding tissues. The possible mechanism could be concluded in two aspects. On the one hand, ROS also leads to DNA damages and mutations in endometriotic cells<sup>10</sup>. On the other hand, ROS could also affect gene expression by regulating epigenetic modification. Growing evidence shows ROS could regulate DNA methylation leading to aberrant gene expression<sup>11,12</sup>.

AT-rich interactive domain 1A (ARID1A) gene is a key factor of SWI/SNF chromatin remodeling complex, which could regulate gene expression by changing the structure of surrounding chromatin<sup>13</sup>. ARID1A has been found frequently mutated in liver cancer, breast cancer and gastric cancer<sup>14-16</sup>. In the pathogenesis of endometriosis, ARID1A loses its expression. However, the mutation frequency of ARID1A gene in endometriosis is lower than cancer cells<sup>17</sup>. Besides, mutation frequency of ARID1A gene in breast cancer does not associate with its expression level<sup>18</sup>. In the previous report, ROS could affect ARID1A gene expression level<sup>19</sup>. However, the mechanism of ROS associated to ARID1A gene silencing in endometriosis is not clear.

In this study, we focused on the mechanism of the correlation of decreased ARID1A gene expression in the pathogenesis of endometriosis. We found that in the clinical samples of endometriosis, the low expression level of ARID1A was highly correlated with its promoter methylation level. The stimulation of H<sub>2</sub>O<sub>2</sub> in endometrial cell caused down regulation of ARID1A. Further experiments showed ROS regulated ARID1A gene expression by affecting its promoter methylation.

## Patients and Methods

### *Chemicals and Materials*

MDA and GPx determination kits were obtained from Nanjing Jiancheng Biochemistry Co. (Nanjing, China). H<sub>2</sub>O<sub>2</sub> was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Gibco (Grand Island, NY, USA).

### *Sample Collection*

All tissues were obtained from patients who underwent surgical procedures for endometriosis or ovarian cancer diagnoses, with or without hysterectomy. In total, 30 endometriosis and 30 normal endometrial tissues were obtained for experiments in this study from Zhangjiagang First People's Hospital. All the tissues were collected for RNA and DNA isolation. This study was approved by the Ethics Committee of Zhangjiagang First People's Hospital. Signed written informed consents were obtained from the patients and/or guardians.

### *Cell Culture and Treatment*

Endometrial tissue was washed with sterile phosphate buffered saline (PBS) 2 to 3 times and cut

into 0.5-1.0 cm<sup>3</sup> tissue blocks, digested with 0.25% trypsin, 0.1% collagenase IV and 0.1% hyaluronic acid enzyme at 37°C for 60 min. The lysate was centrifuged at 1000 r/min for 5 min to discard the supernatant. The cells were re-suspended in 1 mL of fresh dulbecco's modified eagle medium (DMEM) followed by 1000 revolutions per minute. Next, they were centrifuged at 5 min, and supernatants were discarded. The procedure was repeated and cells were suspended in 1 mL fresh DMEM and cultured in a Petri dish in 5 mL culture, adding 2 mL fresh medium 90% high glucose DMEM containing 100 U/mL penicillin and chain Methomycin 100 µg/mL and 10% FBS (side guards) 37°C culture with 5% carbon dioxide for 24-48 h.

### *ROS Measurement*

Endometrial cells were seeded in a 6-well cell culture dish and added DMEM medium. After cells were adhered to the medium containing different concentrations of H<sub>2</sub>O<sub>2</sub>, cells were cultured for 24 h and digested with 0.25% trypsin to collect the cells. H<sub>2</sub>O<sub>2</sub> treated endometrial cells were washed twice with pre-cooling PBS. Then, cells were broken with chemical buffer and followed protein concentration determination by Bradford method. Followed the manufacturer's instructions of the MDA and GPx determination kits (Nanjing Jiancheng Biochemistry Co, Nanjing, China), the concentration of malonyl dialdehyde (MDA) and the activity of glutathione peroxidase (GPx) were detected by spectrophotometer.

### *RNA Isolation and Real-time PCR*

After endometrial cells were stimulated with H<sub>2</sub>O<sub>2</sub>, cells were collected by centrifugation, the supernatant was discarded and extracted RNA with TRIzol reagent. Extraction procedures were carried out according to the operating instructions; RNA was extracted and analyzed by UV spectrophotometer for RNA quantification and purity. The integrity of RNA was detected by 0.8% agarose gel electrophoresis. Then, 1 µg of total RNA by ABI reverse transcription kit (ABI, Foster City, CA, USA) was taken, according manufacturer's instructions. The reverse transcription reaction program was as follows: 25°C, 10 min; 37°C, 120 min; 85°C, 5 min. Real-time PCR amplification protocol was proceeded according to Roche manufacturer's instructions (Basel, Switzerland).

### *Methylation-Specific Polymerase Chain Teaction*

Endometrial cells were treated with 100 nM and 500 nM H<sub>2</sub>O<sub>2</sub> for 48h, then 1×10<sup>6</sup> cells were har-

vested by centrifugation. Genomic DNA was isolated by using Axygen's Genomic DNA Extraction Kit, and the concentration and purity of DNA were determined by UV spectrophotometer. Genomic DNA modification using Chemicon Hydrogen Sulfate Modification Kit and the specific operating procedures is followed its operating procedures. The bisulfite-modified DNA was subjected to PCR amplification, and the methylation-specific primers only amplified the methylated DNA fragments. To correct the DNA content of bisulfite-modified DNA, select the fragment without the CpG site on the  $\beta$ -Actin gene as control.

### Statistical Analysis

Each experiment in this article was repeated at least in triplicate. Results were showed as the mean value  $\pm$  standard deviation (SD). Statistical analysis was carried out using Student's t-test. Each p value less than 0.05 is thought to be with significance.

## Results

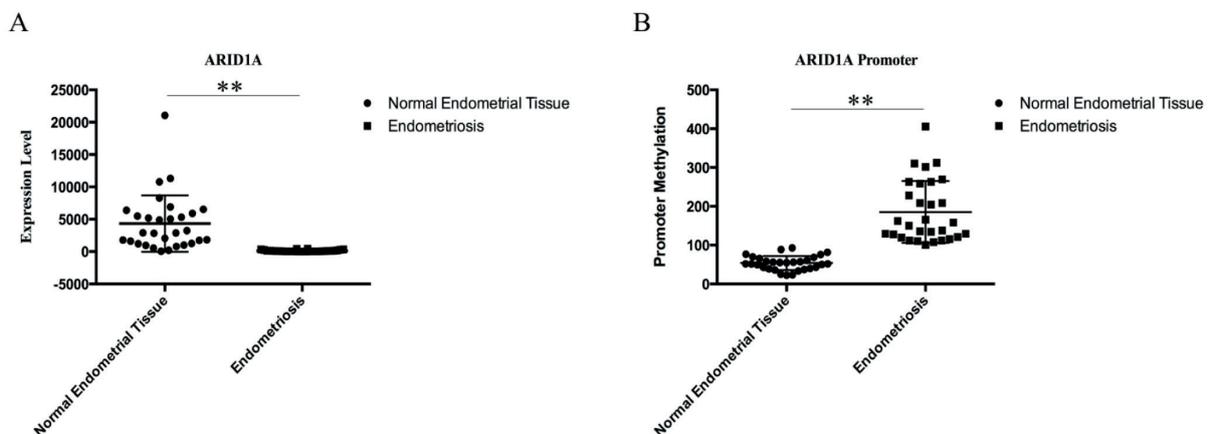
The low expression level of ARID1A gene in endometriosis samples was associated with the hyper-methylation of its promoter

30 endometriosis samples and 30 normal tissues were collected and isolated for obtaining RNA. According to the Real-time PCR result, the relative mRNA level of ARID1A was lower in endometriosis samples compared with normal tissues. More than two-fold decrease of ARID1A

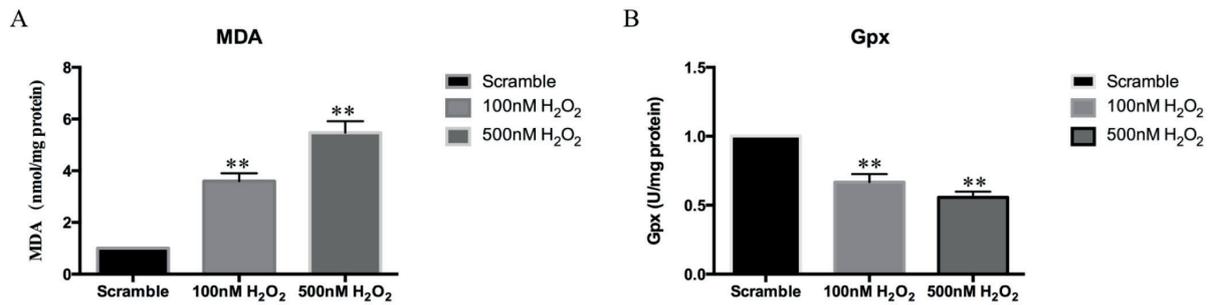
expression was seen in 70.1% endometriosis samples. The result was shown in Figure 1. In order to analyze the ARID1A gene promoter methylation level, we isolated DNA from 30 endometriosis samples and 30 normal tissues. Then, methylation specific PCR was applied to detect the methylation level of ARID1A gene promoter. As shown in Figure 1, the methylation level in ARID1A gene promoter was increased in endometriosis samples, which was consistent with the lower expression level of ARID1A.

### MDA Level was Decreased and Gpx Level was Increased in $H_2O_2$ Treated Endometrial Cells

In the next study, we investigate the effect of ROS to ARID1A gene expression. Firstly, we constructed the endometrial cell oxidative damage model. Endometrial cells were cultured and treated with  $H_2O_2$  at different concentrations (0, 100 nM, 500 nM) for 48 h. Then, endometrial cells were harvested for ROS level detection. MDA level is an indicator of oxidative stress which was significantly increased in  $H_2O_2$  treated endometrial cells in both the two  $H_2O_2$  concentration. In addition, Gpx was decreased in  $H_2O_2$  treated group, which reflects the antioxidant level. Altogether, oxidative damage both occurred in 100 nM and 500 nM  $H_2O_2$  concentration (Figure 2). However, the cell viability significantly decreased in 500 nM  $H_2O_2$  concentration, so we chose 100 nM as the suitable concentration for the following experiment.



**Figure 1.** The mRNA level and promoter methylation level of ARID1A gene in endometriosis samples. 30 endometriosis samples and 30 normal tissues were collected and analyzed. The result showed the expression of ARID1A decreased in endometriosis tissues compared with normal endometrial tissues (A). The promoter methylation level of ARID1A gene promoter increased in endometriosis tissues compared with normal endometrial tissues.



**Figure 2.** MDA level and Gpx level in H<sub>2</sub>O<sub>2</sub> treated endometrial cells. Endometrial cells were treated with 100 nM and 500 nM H<sub>2</sub>O<sub>2</sub> for 48 h. Cell were lysated for MDA and Gpx detection. The MDA level increased in both the two concentration of H<sub>2</sub>O<sub>2</sub> (A). The Gpx level decreased in both the two concentration of H<sub>2</sub>O<sub>2</sub> (B).

### ARID1A Expression Level was Decreased in H<sub>2</sub>O<sub>2</sub> treated endometrial cells

To identify whether oxidative stress could regulate ARID1A gene expression. We treated endometrial cells with 100 nM H<sub>2</sub>O<sub>2</sub> for 48 h, then detected the expression level of ARID1A. According to the result of real-time PCR, the expression level of ARID1A gene significantly decreased in the stimulation of H<sub>2</sub>O<sub>2</sub> (Figure 3).

### The hyper-methylation of ARID1A Gene Promoter was Detected in H<sub>2</sub>O<sub>2</sub> Treated Endometrial Cells

To investigate the specific mechanism of oxidative stress induced ARID1A low expression in endometrial cells, we investigated the possible mechanism in H<sub>2</sub>O<sub>2</sub> treated endometrial cells. After the endometrial cells treated with H<sub>2</sub>O<sub>2</sub> for 48 h, cells were harvested for DNA isolation. Then, the isolated DNA was modified with sulfite before applying Real-time PCR. As shown in Figure 4, the methylation level of ARID1A promoter increased

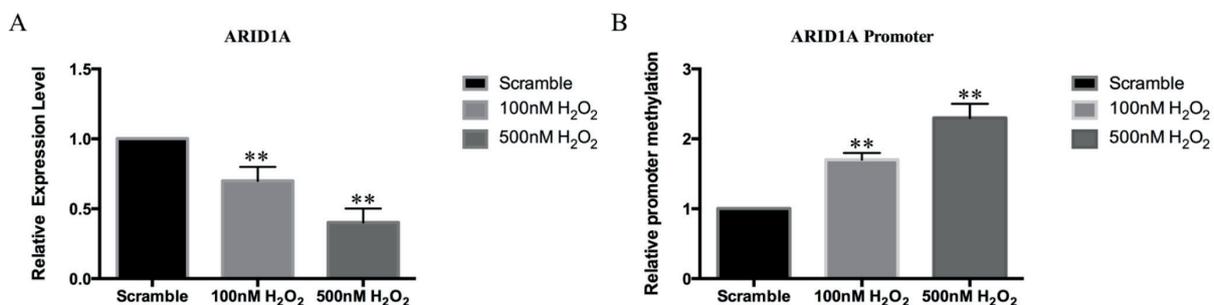
in H<sub>2</sub>O<sub>2</sub> stimulated endometrial cells. This result suggested that ROS decreased the expression level of ARID1A gene via its promoter methylation.

### DNMT1 Expression was Increased in H<sub>2</sub>O<sub>2</sub> Treated Endometrial Cells

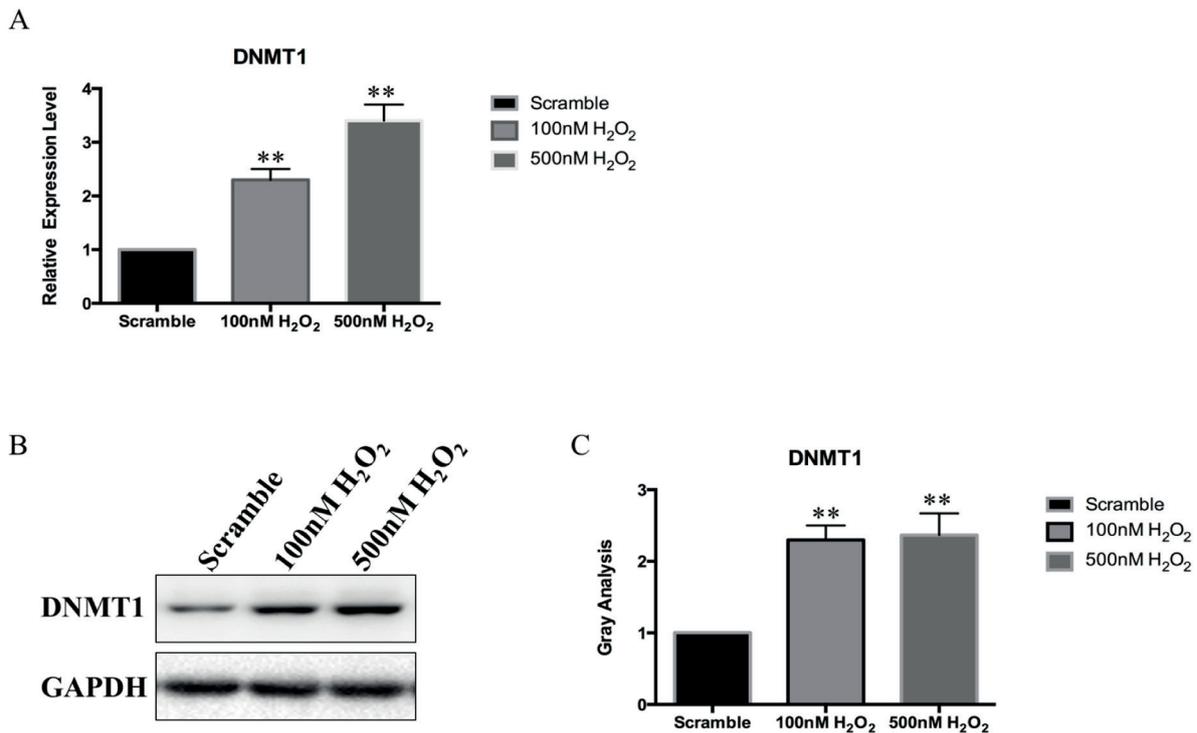
To investigate the possible mechanism of oxidative stress induced ARID1A promoter hyper-methylation, we focused on the methyl-transferase DNMT1 in the regulating of ARID1A gene methylation. After the endometrial cells treated with H<sub>2</sub>O<sub>2</sub> for 48 h, cells were harvested for RNA and protein isolation. As shown in Figure 4, both the mRNA level and protein level of DNMT1 all increased in H<sub>2</sub>O<sub>2</sub> treated endometrial cells.

## Discussion

Endometriosis (EMs) as a chronic gynecological disease in women has high morbidity and serious clinical symptom. The study of its patho-



**Figure 3.** The mRNA level and promoter methylation level of ARID1A gene in H<sub>2</sub>O<sub>2</sub> treated endometrial cells. Endometrial cells were treated with 100 nM and 500 nM H<sub>2</sub>O<sub>2</sub> for 48 h. Then, cells were collected and isolated for RNA and DNA. The ARID1A expression level was significantly decreased when endometrial cells were treated with H<sub>2</sub>O<sub>2</sub> (A). The methylation of ARID1A gene promoter was significantly increased when endometrial cells were treated with H<sub>2</sub>O<sub>2</sub> (B).



**Figure 4.** DNMT1 expression level in H<sub>2</sub>O<sub>2</sub> treated endometrial cells. Endometrial cells were treated with 100 nM and 500 nM H<sub>2</sub>O<sub>2</sub> for 48 h. Then, cells were collected and isolated for RNA and protein. The expression level of DNMT1 was detected by Real-time-PCR and Western Blot. **(A)** The mRNA level of DNMT1 was significantly increased when endometrial cells were treated with H<sub>2</sub>O<sub>2</sub>. **(B)** The protein level of DNMT1 was significantly increased when endometrial cells were treated with H<sub>2</sub>O<sub>2</sub>. **(C)** Gray analysis of DNMT1 protein level.

genesis is important to the clinical treatment to endometriosis. In this report, the ROS induced ARID1A gene down regulation was caused by its promoter hyper-methylation. The results in H<sub>2</sub>O<sub>2</sub> treated endometrial cells were in consistent with the results in endometriosis clinical samples. The hyper-methylation of ARID1A gene promoter caused by oxidative stress leads to ARID1A gene low expression is a new mechanism of endometriosis.

In this study, ARID1A mRNA was lower in endometriotic tissue compared with normal endometriotic tissue, which was consistent with other reports. Endometriosis histopathology is benign, but it is characterized by malignant biological behavior such as aberrant proliferation, invasion, metastasis and high recurrence rate<sup>20-22</sup>. AT-rich domain 1A (ARID1A) is a candidate tumor suppressor gene that participates in cell cycle progression and tumor cell inhibition<sup>23,24</sup>. ARID1A is a highly expressed gene in early embryos and embryonic stem cells, which maintain the multi-potent of stem cells. The embryonic stem cell self-renewal ability and total pluripotency are se-

verely impaired when ARID1A is absent<sup>25</sup>. Besides, ARID1A gene mutation in tumor cells is also associated with microsatellite instability, which means that the gene may be related to maintaining the stability of whole genome<sup>26</sup>. At the same time, ARID1A is also an important gene involved in Fas gene-mediated apoptosis<sup>27</sup>. ARID1A gene exists in a variety of tumor cells, such as breast cancer, gastric cancer and pancreatic cancer, especially in gynecological malignancies<sup>26,28</sup>. The malignant view of EMs has added new blood to the study of ARID1A gene. Studies have shown that aberrant ARID1A expression is common in EMs, and the possible mechanism is hyper-methylation of its promoter<sup>18</sup>. This study proved this novel mechanism in endometriosis for the first time. The depression of ARID1A gene in endometriosis and other gynecologic oncology might be presented as a new marker.

ROS increased the methylation level of ARID1A gene promoter, which leads the down regulating of ARID1A gene. Generally, the DNA methylation site occurs in the CpG island, which is the DNA sequence rich in CpG nucleotide<sup>29</sup>. DNA methyl-

tion requires the involvement of methyltransferase (DNMTs) in the regulation of gene function. Currently, the known methyltransferase are divided in three families, which are DNMT1, DNMT2 and DNMT3<sup>30</sup>. DNMT1 is the main methyltransferase, which is responsible for the methylation of semi-methylated substrate<sup>31</sup>. Oxidative stress can cause changes in the methylation level of CpG island, while oxidative stress-induced DNA oxidative damage can start DNA damage and repair process. Therefore, we speculate that DNA oxidative damage could increase the expression of DNMTs to start DNA damage repair process. The mechanism of ROS on DNA methylation is by acting on either activity or expression of DNMTs. From the reported articles, ROS could induce DNA hyper-methylation by modulating the DNA recruitment of DNMTs without changing its expression level. For example, the expression of snail, which was required to recruit DNMT1 to E-cadherin promoter, was induced for hyper-methylation<sup>32</sup>. In addition, ROS could also induce DNA hyper-methylation by changing the expression level of DNMTs. In the study of myocardial or cerebral ischemia, hypoxia-inducible transcription factor HIF1 $\alpha$  could increase the expression levels of DNMT1, DNMT3A and DNMT3B<sup>33</sup>. In this study, we only detected the expression level of DNMT1, the role of DNMT3A and DNMT3B in endometriosis should be investigated in the following research.

## Conclusions

We found the low expression level of ARID1A gene was highly correlated with its promoter hyper-methylation in endometriosis samples. ROS decreased the expression level of ARID1A gene via regulating methylation in its promoter which contributing to the understanding of the pathogenesis of endometriosis. The possible mechanism of ARID1A gene promoter hyper-methylation is the increased DNMT1 expression level caused by oxidative stress.

## Conflict of interest

The authors declare no conflicts of interest.

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