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MicroRNA-148a targets ADAMTS5 to inhibit proliferation of endometriosis cells

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Abstract: To examine miR-148a expression in the serum of patients with endometriosis (EMS), and to further explore the target of miR-148a in HS832.Tc cells and the effect of miR-148a on the proliferation of Hs832.Tc cells. The serum of non-EMS patients and EMS patients were collected and real-time quantitative PCR (qRT-PCR) was used to detect miR-148a in serum. The EMS cell line Hs832.Tc was cultured and transfected with miR-148aminic, miR-148a inhibitor to construct over expressing and interfering cell lines. Cell viability and apoptosis were detected. The dual luciferase assay identified a target relationship between miR-148a and ADAMTS5 and whether miR-148a regulates proliferation of endometriosis cells via ADAMTS5 was further validated. Compared with normal subjects, miR-148a was significantly reduced in serum of EMS patients ($p<0.05$). The area under the receiver operating characteristic curve for miR-148a for diagnosis of EMS was 0.91, which was statistically significant ($p<0.01$). The proliferation of Hs832.Tc cells was significantly inhibited and the cell apoptosis was increased after miR-148a over expression. The proliferation of Hs832.Tc cells was promoted and apoptosis was reduced by miR-148a down regulation. The dual luciferase report demonstrates that ADAMTS5 is a target gene of miR-148a. The addition of ADAMTS5 can directly promote apoptosis of Hs832.Tc cells. The expression of miR-148a in serum of EMS patients was decreased, and miR-148a targets ADAMTS5 to promote apoptosis in EMS cells.

Keywords: Endometriosis, miR-148a, ADAMTS5, cell proliferation.

INTRODUCTION

Endometriosis (EMS) is a common gynecological and refractory disease. The incidence rate of EMS is about 10-15% in women at childbearing age, and 70% in patients with chronic pelvic pain. Due to the periodic bleeding of the ectopic endometrium, the affected area is infiltrated by inflammation, causing adhesions and scars, which causes a series of symptoms, mainly abdominal pain (including menstrual abdominal pain, non-menstrual ectopic pain) and infertility (Marki *et al*., 2017; Berkley *et al*., 2005; Morotti *et al*., 2017), seriously affecting the physical and mental health of patients (Kvaskoff *et al*., 2015). Therefore, the search for reliable non-invasive diagnostic methods for EMS without affecting the clinical diagnosis of patients has become a hot topic of current research.

Micro RNAs (miRNAs) have been recently found having extensive gene regulation functions. miRNAs regulate gene expression in the signaling pathways and play an important role in maintaining homeostasis (Ebert and Sharp, 2012). Many studies have found differential expression of miRNA in serum of patients with EMS, suggesting that miRNA may play a significant role in the pathogenesis and progression of EMS. miR2861, miR375, miR126-5p and miR33b have all been shown related to the occurrence and development of EMS (Yu *et al.*, 2019; Zhang *et al*., 2019; Rekker *et al*, 2018; Meng *et al*., 2019). Although there have been many studies on miR and EMS, the overall mechanism of EMS development and protein expression remains unknown.

We investigated the difference of miR-148a in the serum of normal human and EMS patients. Then we explored the effects of miR-148a on the proliferation and apoptosis of Hs832.Tc cells. The relationship between miR-148a and clinical features of EMS and its value in clinical diagnosis of EMS were also explored.

MATERIALS AND METHODS

Clinical research subjects

The patients in this study were all from department of reproductive medicine in Yuhuangding hospital in Yantai city, China. A total of 23 patients with endometriosis and 20 normal female controls were included. Patients with EMS were confirmed by postoperative pathology, and patients in normal control group were patients admitted to the hospital due to tubal obstruction. All the patients were informed before this study and the patient's consent was obtained before the specimen was obtained. This study has been authorized by the ethics committee of Yuhuangding hospital (Approval No. 20180912).

Exclusion criteria of the control group were presence of other endometrial pathologies like myoma or fibroma.

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Exclusion criteria of the patients groups were the presence of signs and symptoms of endometriosis, like infertility and dysmenorrhea. Exclusion criteria for both patient and control groups were presence of systematic inflammation diseases and infections, ongoing pregnancy, history of pregnancy in the last 3 months, cancer and other major systemic diseases.

Inclusion criteria of the EMS patients underwent laparoscopic surgery, and the ectopic endometrial tissue from patients with EMS was retrieved. The pathology departments were checked by two pathologists. According to the American Fertility Association rAFs standards, 13 cases were in stage I-II and 10 cases were in stage III-IV. Inclusion criteria of the control group patients underwent laparoscopic surgery for tubal obstruction during the same period.

Venous blood (4mL) was retrieved from each patient above 2 days before surgery. After standing at room temperature for 1h, the blood was centrifuged at 3000rpm for 10min at 4°C. The separated serum samples were stored in -80°C for further RNA extraction.

Cell culture

The EMS cell line Hs832.Tc was retrieved from Shanghai Cell Bank (Chinese Academy of Sciences). The cells were cultured in RMP1640 medium with 10% fetal bovine serum (Shanghai Enzyme Bio., China), 1×10^5 µmol/L penicillin and streptomycin (Solarbio, Beijing, China) in incubator (Thermo, USA) with 5% CO_2 at 37°C till the cells reaching log phase.

Cell transfection

MiR148a interference vector, over expression vector and control vector were provided by In vitrogen (Waltham, MA, USA) and prepared by Gene Pharma (Shanghai, China). The cells were digested with 0.25% trypsin and passaged, then 2×10^5 logarithmic phase cells were seeded on 6-well plates. After 24 hours, the cells were examined using an inverted microscope. When the cell density reached 30-50%, cell transfection was started (Zhu *et al.*, 2019). The experiments were grouped as follows: BC, miR-148a mimic, mimic NC, miR-148a inhibitor, inhibitor NC. To analyze whether miR-148a regulates cell proliferation via ADAMTS5, the following groups were used: NC, miR-148a mimic, miR-148a mimic+ADAMTS5. Liposome Lipofectaxnine TM2000 was used to increase cell membrane permeability. Cells were transfected with 50nM miR-148a mimic or interference used Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA). A negative control was set up using a non-homologous RNA duplex and a blank control was set up using an empty vector.

Real-time quantitative PCR detection (RT-qPCR)

Total RNA was extracted from serum (stored in -80°C) using Trizol method. The total RNA was measured using an ultraviolet spectrophotometer and the absorbance was between 1.8 and 2.0. Reverse transcription was performed to obtain cDNA and amplification was performed on a real-time PCR machine. Exicycler TM 96 fluorescence meter (BIONEER, Korea) was used to perform fluorescence quantitative analysis. $2^{-\Delta\Delta ct}$ method was used to quantify the relative gene expression level (Livak and Schmittgen, 2001). The primers used were listed as follows: F: 5′-ACACTCCAGCTGGGTCAGTGCAC TACA-GAA-3′ and R: 5′-TGGTGTCGTGGAGTCG-3′; U6, F: 5′-TCCGATCGTGAAGCGTTC-3′ and R: 5′-GTGCAGGGTCCGAGGT-3’.

MTT assay

According to previous study (Zhu *et al.*, 2019), each group of cells was adjusted to a concentration of 1×10^4 /mL, inoculated into a 96-well flat-bottomed plate. 20µL of 5mg/mL MTT was added at 24h, 48h, 72h and 96h, respectively. After 4h, the supernatant was carefully discarded, and 200µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was added. After mixing by pipetting, the absorbance (OD value) was measured at a wavelength of 490 nm by an enzyme-linked immunosorbent assay (Bio-Rad Laboratories).

Apoptosis detection

Cells were collected by centrifuge (1000rpm, 5min). After washing twice with 4°C sterile PBS, a total number of 1×10^6 cells were resuspended in 250ul of 1X binding buffer. 195uL of cell suspension was mixed with 5uL FITC-labeled Annexin-V. After standing still for 3min, 10uL of propidium Iodide solution (20µg/mL) was added and the mixture was incubated for 10min in the dark at room temperature. 400uL of 1xbinding buffer was further added to the mixture before flow cytometry (Gallios; Beckman Coulter, Inc, Brea, California, USA) examination.

Dual luciferase reporter assay

Targeting relationship between miR-148a and ADAMTS5 was predicted by the databases of TargetScan 3.0 (www.targetscan.org). The full length of wildtype and mutant miR-148a and ADAMTS5 were amplified by overlap PCR. The sequences were inserted downstream of the luciferase gene and cloned as previously described (Zhu *et al.*, 2019).. The pmirGIO double-binding vector was used to construct pmirGIO-miR-148a and pmirGIO-ADAMTS5 recombinant vectors from miR-148a and ADAMTS5 target fragments, respectively. The cells were inoculated in 24-well plates with 2 x 10^5 cells/well. Dual luciferase reporter system (Promega, USA) was used in the determination of luciferase activity 24 hours after transfection. Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected cell sample.

Western blot detection

According to previous study (Hirano, 2012), Tissue and intracellular proteins were extracted according to kit

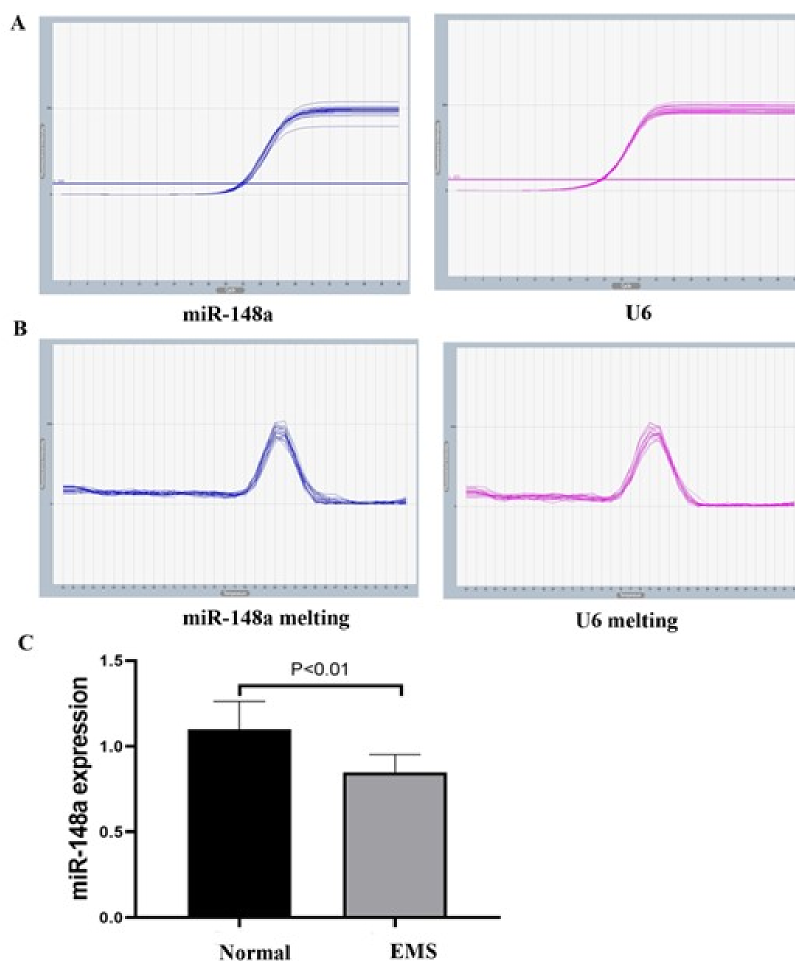

Fig. 1: MiR-148a expression in the serum of EMS patients was lower than that from normal population. (A) RT-PCR specific analysis; (B) RT-PCR melting curves; (C) The expression of miR-148a in the serum of both groups.

instructions. BCA protein quantitation kit (Thermo Fisher Scientific, Waltham, USA) was used to detect the protein concentrations. 40 ug of protein samples were separated by SDS-PAGE electrophoresis (Mini-Protean-3, Bio-Rad, Hercules, CA, USA) and transferred to PVDF membrane (Millipore, Massachusetts, USA). After blocking in 5% skin milk (diluted in 5% BSA) for 1h, the membrane was incubated with ADAMTS5 rabbit-anti-human antibody (1:1000, MAB2198, R&D Systems) overnight at 4°C. After washing with TBST (TBS, 1ml/L Tween-20) for 3 times (10min each), the membrane was incubated with goat-anti-rabbit IgG HRP (1:2000, ab6721, Abcam) secondary antibody for 2h at room temperature. The membrane was further washed 3 times for 10 each with TBST before detection by ECL chemiluminescence method. Image J (NIH) was performed in the grayscale scanning and quantification, and GAPDH was used in the normalization of protein expression levels.

STATISTICAL ANALYSIS

Statistical analysis was performed by SPSS 21.0 software. Mean ± standard deviation format was used in the representation of data. The data comparison between the two groups used *t* test, and the multi-group analysis used one-way analysis of variance (ANOVA). Tukey's test was used to subsequent analysis. A $p<0.05$ was considered as significant difference.

RESULTS

MiR-148a was down regulated s in serum of EMS patients

The results of PCR specific analysis are showed in fig. 1A, indicating that primer design/synthesis/amplification results meet the experimental requirements. RT-qPCR melting curves are shown in fig. 1B. The Tm value shows a single peak in both miR-148a-3p and snRNAU6, indicating there was no non-specific amplification or interference of primer dimmer. The relative miR-148a expression in the serum of EMS group was remarkably lower than that in the control group (fig. 1C, $p<0.01$), indicating the difference was statistically significant.

MiR-148a expression in serum is not related to the clinical stage of EMS

The relative expression of serum miR-148a in EMS patients was not found to be statistically different in different rAFS stages (table 1, p>0.05).

Table 1: Expression of miR-148a in serum of patients from different EMS stages

Stages	cases	miR-148a	P value
I-II	13	0.86±0.11	0.477
III-IV	10	0.84±0.10	

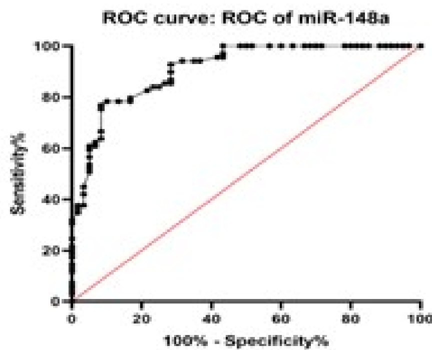

Fig. 2: ROC curve analysis in EMS diagnosis using miR-148a

ROC curve analysis in EMS diagnosis using miR-148a

The AUC was calculated to investigate the accuracy of the diagnostic test, P<0.05 was considered statistically significant. The AUC of miR-148a for the diagnosis of EMS was 0.91 and the difference of p<0.01 was statistically significant. The sensitivity of miR-148a test for diagnosis of EMS was 76.8%, the specificity was 91.7%, and the confidence interval (CI) was (0.86-0.96).

MiR148a over expression inhibited HS832TC cells proliferation and promoted apoptosis

Transfection efficiency was assessed using RT-PCR and the results indicate (fig. 3A) that miR148a mimic or inhibitors significantly up-regulated or down-regulated the expression of miR-148a in cells. The effect of different expression of miR-148a on cell proliferation was analyzed by MTT assay (fig. 3B). Compared with the negative group, the over expression of miR-148a significantly reduced the cell proliferation of Hs832.Tc cells (p<0.05). Low expression of miR-148a significantly increased cell proliferation of HS832TC cells compared to the negative group (p<0.05). The results of flow cytometry showed the effect of different expression of miR-148a on apoptosis (fig. 3C). Compared with the control group, the group of miR-148a over expression significantly increased the apoptosis of Hs832.Tc cells (p<0.05). Over expression of miR-148a significantly increased apoptosis in Hs832.Tc cells compared to the control group (p<0.05). On the contrary, compared with the control group, the low expression of miR-148a significantly reduced the apoptosis of Hs832.Tc cells (p<0.05).

MiR-148a regulates the expression of ADAMTS5

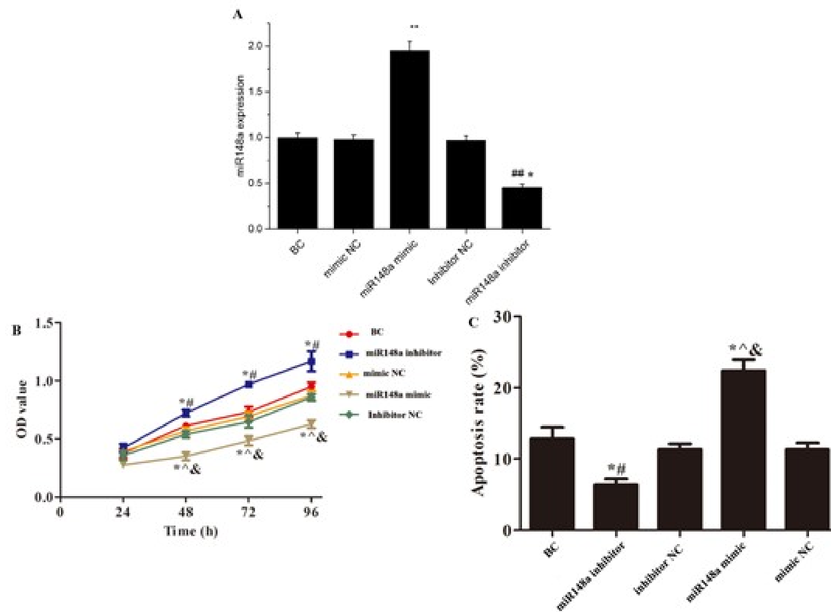

Fig. 3: The effect of different expression of miR148a on proliferation and apoptosis of HS832TC cells. (A) The expression levels of miR148a in each group were analyzed by RT-PCR; (B) The cells proliferation was detected by MTT assay; (C) Cell flowmeter was used to analyze the apoptosis of each group. Compared with mimic NC, *p<0.05, **p<0.01; Compared with inhibitor NC, #p<0.05, ##p<0.01; Compared with miR-146a inhibitor, ^p<0.05; Compared with BC, &p<0.05.

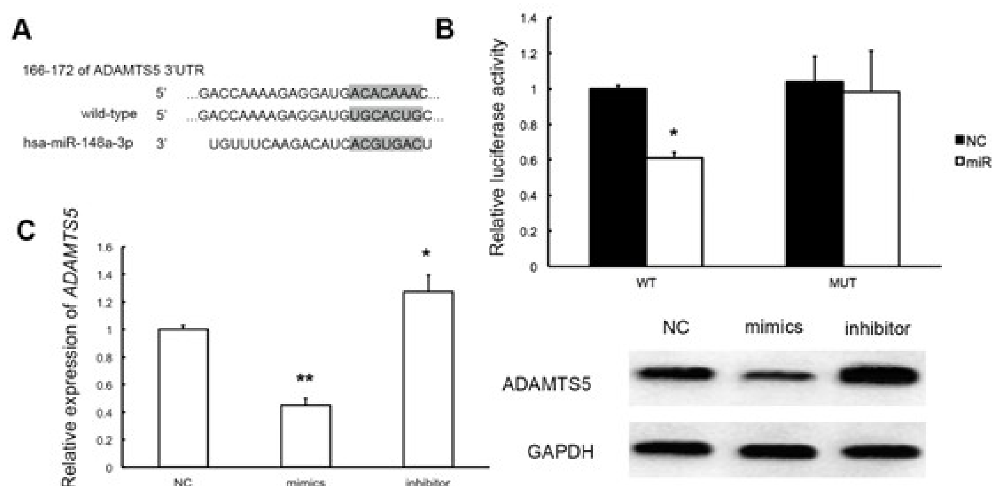

Fig. 4: MiR-148a regulates the expression of ADAMTS5. (A) the binding site of miR-148a and ADAMTS5; (B) dual luciferase report; (C) The effect of different expression of miR-148a on the expression of ADAMTS5. Comparing to NC, *p<0.05, **p<0.01.

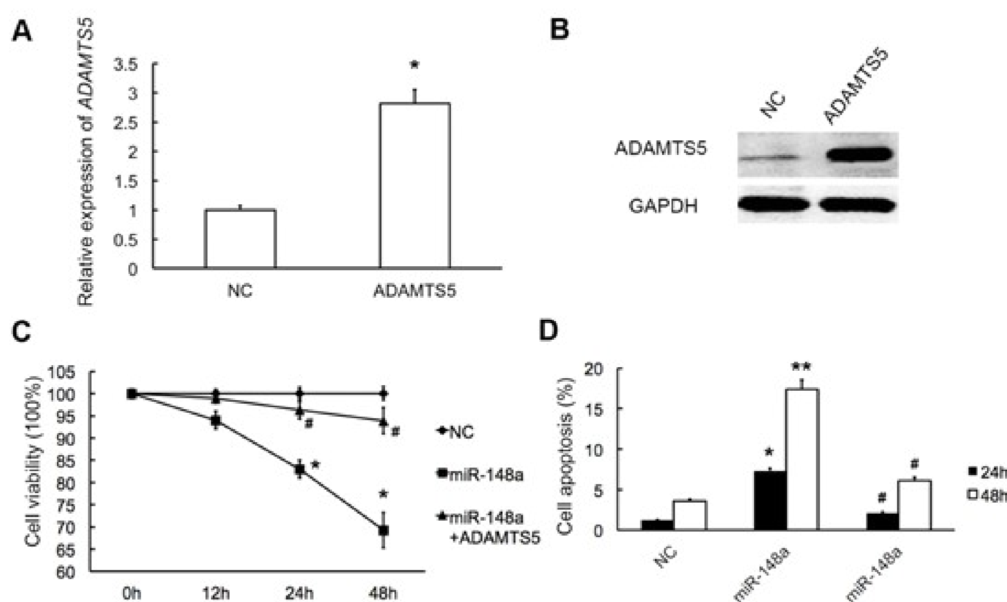

Fig. 5: Over expression of ADAMTS5 promotes apoptosis of HS832TC cells. (A, B) The expression of ADAMTS5 in HS832TC cells was analyzed by Western blot. (C) MTT assay was used for cell proliferation. (D) Cell flow analysis for apoptosis analysis. *p<0.05 compared to the NC group; #p<0.05 compared to the miR-148a group.

Targetscan software results indicated that miR-148a may be a latent target for DUXAP8 (fig. 4A), and dual luciferase reports found that miR-148a reduced luciferase activity of ADAMTS5 containing WT 3'UTR (fig. 4B, p< 0.05). The results showed that miR-148a was a target gene of ADAMTS5 in cells. Further analysis revealed that the expression of ADAMTS5 protein was reduced after miR-148a mimics transfection. Meanwhile, the expression of ADAMTS5 protein was apparently increased when transfected with miR-148a inhibitor (fig. 4C).

Over expression of ADAMTS5 promotes Hs832.Tc cells apoptosis

The construction of ADAMTS5 over expressing Hs832.Tc cells revealed that ADAMTS5 expression in Hs832.Tc cells was significantly higher than that in control cells (fig. 5AB, p<0.05). The proliferation and apoptosis were also examined in miR-148a over expression Hs832.Tc cells and miR-148a/ADAMTS5 over expression Hs832.Tc cells, and the results showed that cell proliferation was inhibited and apoptosis rate was increased in miR-148a over expression cells. Moreover, the over expression of

miR-148a and ADAMTS5 resulted in significantly higher cell proliferation and lower apoptotic rate than the miR-148a over expression group ($p<0.05$).

DISCUSSION

There have been many theories in the pathogenesis of EMS, and “menstrual blood reflux” is one of the most widely accepted theories. The detached endometrial cells flow back to the uterine cavity through the menstrual blood, requiring adhesion, invasion, and angiogenesis to complete the initial formation of the lesion, further leading to the occurrence of endometriosis (Mehedintu *et al*., 2014). The biological behavior of endometrial cell implantation, erosion and distant metastasis of endometriosis is extremely similar to that of malignant tumors. A large number of studies (Rupaimoole and Slack, 2017; Alamoudi *et al*., 2018) show that miRNAs not only have abnormal expressions in many human tumor cells, but also closely related to tumor development, invasion, metastasis, etc., as well as regulate the growth and progression of tumor cells. Recent studies have shown that miRNAs may be involved in the occurrence and development of EMS, such as miR-205-5p (Zhou *et al*., 2019), miR-17-5p (Pang and Liu, 2020), miR-451a (Li *et al*., 2019), and so on. However, the effects of miR-148a on EMS are still unclear.

As a member of miRNA, miR-148a regulates post-transcriptional gene silencing by recognizing the 3'-UTR of target gene mRNA, promoting mRNA degradation or inhibiting transcription. The study found that miR-148a is decreased in a variety of tumors, such as pancreatic cancer (Idichi *et al*., 2018), cholangiocarcinoma (Babu *et al*., 2019), gastric cancer (Pereira *et al*., 2019), colorectal cancer (Igder *et al*., 2019), and reproductive system such as ovarian cancer (Li *et al*., 2019) and prostate cancer (Peng *et al*., 2017). In these cancers, miR-148a mainly plays the role of tumor suppressor gene like, inhibiting and reversing the malignant phenotype of tumor. EMS is similar to malignant tumors in behaviors such as invasion and adhesion, so it is speculated that miR-148a is also involved in the occurrence and development of EMS. In our research, we found that miR-148a expression was decreased in EMS patients’ serum. In addition, we found that low expression of miR-148a enhanced the proliferation of Hs832.Tc cells, high expression of miR-148a, weakened the proliferation of Hs832.Tc cells and increased its apoptosis. This indicates that miR-148a plays an important role in the pathogenesis of endometriosis. It was found that (Vonk *et al,* 2014), miR-148a could target ADAMTS5 to regulate the hypertrophy of osteoarthritis cells, and miR-148a over expression could promote chondrocyte production and collagen deposition. Therefore, we speculated that miR-148a may also participate in the apoptosis of endometriotic cells through the expression of ADAMTS5. Our hypothesis was verified by double luciferase experiment that ADAMTS5 is a target of miR-148a in endometriosis cell line, and they have a negative targeting relationship.

Laparoscopy and pathological diagnosis are the main methods in the diagnosis of EMS. However, laparoscopy contains invasive procedures which have many potential risks. Moreover, previous studies have revealed that these tests may have a longer delay in the diagnosis of endometriosis and may lead to worsening of endometriosis lesions (Filip *et al.,* 2020). In addition, there hasn’t been a method for the diagnosis of endometriosis with high sensitivity and specificity. Blood has long been considered as a source of biomarkers because it is easy to obtain and reproducible (Vignali *et al*., 2021). Compared with traditional protein-based biomarkers, serum miRNAs have higher stability, higher biospecificity. In this experiment, through ROC curve analysis, miR-148a showed high sensitivity and specificity in the diagnosis of EMS, and the AUC values of the two are between 0.9 and 1.0. The accuracy of the diagnosis is high, indicating that the detection of miR-148a in serum has a certain value for the diagnosis of endometriosis, and compared with AUC=0.5 (AUC=0.5 suggests no diagnostic value), the difference is statistically significant. The sensitivity of miR-148a assay for diagnosis of EMS was 76.8%, specificity was 91.7%, and confidence interval (CI) was (0.86-0.96). These results indicate that for the diagnosis of endometriosis, serum miR-148a may become one of the indicators for new clinical diagnosis. The results also showed that there was no significant difference in the expression of miR-148a in serum of stage I-II and stage III-IV of EMS patients, and these results may be due to small sample size or the external influences on the qRT-PCR experiments. In the future studies, more samples should be involved to further verify this result.

CONCLUSION

In summary, miR-148a is low expressed in serum of endometrium patients, and the detection of miR-148a is of certain value in the diagnosis of endometriosis. Over expression of miR-148a may target the regulation of 5 ADAMTS5 to reduce the proliferation of ectopic endometrial cells and increase the apoptosis of ectopic endometrial cells. This study suggests that miR-148a may become a potential therapeutic target and predictor of endometriosis.

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