Abstract. – OBJECTIVE: Endometriosis is a common benign disease in gynecology, and can cause chronic pelvic pain, dysmenorrhea and even infertility. Its pathogenesis mechanism has not been fully illustrated. miRNA (miR) participates in various biological activities including cell growth, proliferation, apoptosis, organ formation, inflammation and tumor. Its role in endometriosis has not been reported. MiR-33b is involved in cell metabolism, proliferation and invasion, but with its function and mechanism in endometriosis unknown.

PATIENTS AND METHODS: Real-time PCR was used to test miR-33b expression in ectopic endometrial and normal tissues. In vitro cultured endometrial cells were transfected with miR-33b mimic or inhibitor, followed by Real-time PCR for miR-33b expression. MTT method detected endometrial cell proliferation. Caspase 3 activity was quantified by test kit. Real-time PCR and Western blot measured effect of miR-33b on vascular endothelial growth factor (VEGF) and matrix metalloprotein 9 (MMP-9).

RESULTS: MiR-33b was down-regulated in ectopic endometrial tissues (p < 0.05 compared to normal tissues). Transfection of miR-33b inhibitor facilitated endometrial proliferation, decreased Caspase 3 activity, increased VEGF and MMP-9 mRNA or protein expression (p < 0.05 compared to control group). MiR-33b mimic suppressed endometrial proliferation, elevated Caspase 3 activity, and decreased VEGF or MMP-9 expression (p < 0.05 compared to control group).

CONCLUSIONS: MiR-33b can mediate cell apoptosis, alter VEGF and MMP-9 expression and affect proliferation and apoptosis of uterus endometrial cells, thus participating endometriosis formation.

Key Words: Endometriosis, MicroRNA-33b, Cell proliferation, VEGF, MMP-9.

Introduction

Endometriosis is a common and frequent benign disease in gynecology. With rapidly increasing incidence, endometriosis severely affects women health and daily life. Some endometriosis lesions may develop tumor-like infiltrative growth behavior, with about 1.0% probability of malignant transformation and a certain rate of recurrence, therefore drawing increasing research interests. Endometriosis is manifested as implantation of endometrial tissues on ectopic side, such as extra-muscular layer of uterus including ovarian, rectum-uterus depression or bladder-uterus depression. Endometriosis leads to chronic pelvic pains, dysmenorrhea, menoxenia and even infertility. Currently both medication and surgery are available for treating endometriosis. Drugs can suppress endometriosis to certain extents, but having defects such as adverse reactions and higher recurrence. Radical surgery is usually applicable for fertile women, whilst conservative surgery also faces the issue of high recurrence. EMS has a complicated pathogenesis mechanism, which has not been fully illustrated. Therefore, the identification of endometriosis pathogenesis related molecular targets can benefit treatment efficacy of endometriosis. MicroRNA (MiR) is widely distributed in animal/plant cells and even virus, with 22-23 nucleotides length having endogenous regulatory role. MiR can negatively regulate gene expression via complementary binding with target mRNA for degradation of mRNA or post-transcriptional inhibition. MiR participates in various biological activities, including cell growth, proliferation, apoptosis, organ formation, inflammation and tumor. The role of miR in endometriosis has not been reported. MiR-33b plays a role in cell metabolism and regulation of cell proliferation or invasion, which is important for diseases, such as tumor or metabolic disorder. However, its expression or functional role in endometriosis has not been fully illustrated.
Patients and Methods

Recruitment of Research Objects and Sample Collection

Endometriosis patients who were admitted in Renmin Hospital of Wuhan University from January 2015 to June 2016 were recruited, in parallel with 15 patients having uterus prolapse, hysteroscopy or benign ovarian tumors as the control group. Patients aged between 37 and 47 years (average age: 38.6 ± 8.2 years). A total of 20 patients who were confirmed as endometriosis and having surgery in our hospital were recruited as endometriosis group (aging between 36 and 50 years, average age: 47.2 ± 9.8 years). No significant difference existed in general information between these two groups, so comparison was possible. All patients have not received related treatment before, nor did any chemo-, radio- or hormone therapy. No antibiotics have been applied within 6 months before surgery. No patients had intrauterine device implantation. Those patients with severe organ failure, malignant tumor or severe complications were excluded. Endometrial tissues collected during surgery were partially cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Hyclone, Logan, UT, USA) or frozen in -80°C fridge for further use. The study protocol was approved by the Research Ethics Committee of Renmin Hospital of Wuhan University and all patients gave their informed consent before study commencement.

Major Reagents and Equipment

Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and streptomycin-penicillin were purchased from Hyclone (Logan, UT, USA). Dimethyl sulfoxide (DMSO) and 4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) powders were purchased from Gibco (Grand Island, NY, USA). Trypsin-EDTA lysis buffer was purchased from Sigma-Aldrich (St. Louis, MO, USA). Caspase 3 activity assay kit and polyvinylidene fluoride (PVDF) membrane were purchased from Pall Life Sciences Inc. (Pensacola, FL, USA). Ethylene diamine tetra acetic acid (EDTA) was purchased from Hyclone (Logan, UT, USA). Western blotting reagent was purchased from Beyotime (Beijing, China). Enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Piscataway, NJ, USA). Rabbit anti-human vascular endothelial growth factor (VEGF) monoclonal antibody (Catalogue No. 2445; 1:3000), rabbit anti-human matrix metalloprotein 9 (MMP-9) monoclonal antibody (Catalogue No. 2270; 1:3000), and mouse anti-rabbit horseradish peroxidase (HRP)-conjugated IgG secondary antibody (Catalogue No. 7074; 1:2000) were all purchased from Cell Signaling Technology (Beverly, MA, USA). TaqMan microRNA reverse transcription kit was purchased from Thermo Electron Corp (Waltham, MA, USA). miR-33b mimic, miR-33b inhibitor and negative control (NC) sequences were synthesized by Gimma (Shanghai, China). RNA extraction kits and reverse transcription kit were purchased from Thermo Electron Corp (Waltham, MA, USA). Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Laboratories (Hercules, CA, USA). ABI7700 Fast fluorescent quantitative PCR cycler was purchased from ABI (Foster City, CA, USA). Ultrapure workstation was purchased from Sutai High-tech Materials Co. Ltd. (Shanghai, China). Thermo Scientific Forma CO₂ incubator was purchased from Thermo Electron Corp (Waltham, MA, USA). GeneAmp PCR system model 2400 was purchased from PE Applied Biosystems (Foster City, CA, USA).

Endometrial Cell Primary Culture and Grouping

Endometrial tissues were rinsed in sterile phosphate buffered saline (PBS) for 2-3 times, and were cut into 0.5-1.0 cm² tissue blocks, which were digested with 0.25% trypsin, 0.1% collagenase IV and 0.1% hyaluronidase at 37°C for 60 min. Lysate was centrifuged at 1000 r/min for 5 min centrifugation to discard the supernatant. Cells were re-suspended in 1 ml fresh DMEM, followed by 1000 r/min for 5 min centrifugation and discarding supernatant. The process was repeated and cells were re-suspended in 1 ml fresh DMEM and were cultured in 5 ml culture dish at 37°C culture with 5% CO₂ for 24-48 h. Cells at 3-8 generation at log-growth phase were selected and randomly divided into four groups: miR-33b mimic NC, miR-33b inhibitor NC, miR-33b mimic and inhibitor group.
Liposome Transfection of miR-33b Mimic and miR-33b Inhibitor

MiR-33b mimic (5'-AGGAU CGGUU UGUGC ACA-3'), miR-33b inhibitor (5'-AUCGG AUGUG GUGCA CUA-3'), miR-33b mimic NC (5'-AUUUU CCAGG UCGGA AUG-3') or miR-33b inhibitor NC (5'-AGGUC AAGCA GUUCG UUG-3') were transfected into endometrial cells. In brief, cells were cultured until reaching 70-80% confluence. MiR-33b mimic/inhibitor or negative control liposome were mixed with 200 μl serum-free medium for 15-min room temperature incubation. Lipo2000 reagent was then mixed with miR-33b mimic, miR-33b inhibitor or negative controlled dilutions for 30-min room temperature incubation. Serum was removed, followed by phosphate buffered saline (PBS) rinsing gently and the addition of 1.6 ml serum-free culture medium. Cells were then kept in a humidified chamber with 5% CO2 at 37°C for 6 h, followed by the application of 10% fetal bovine serum (FBS)-containing medium in 48 h continuous incubation for further experiments.

Real-time PCR for Detecting miR-33b, VEGF and MMP-9 Expression in Endometrial Tissues

Trizol reagent was used to extract RNA from normal and ectopic endometrial tissues. Reverse transcription was performed according to the manual instruction of test kit, using primers designed by PrimerPremier 6.0 (Table I). Real-time PCR was performed on target genes under the following conditions: 55°C for 1 min, followed by 35 cycles each containing 92°C for 30 s, 58°C for 45 s and 72°C for 30 s. Data were collected and calculated for CT values of all samples and standards based on fluorescent quantification using GAPDH as the reference. Standard curve was firstly plotted using CT values of standards, followed by semi-quantitative analysis by 2^{-ΔΔCt} method.

MTT Assay for Cell Proliferation

Uterus endometrial cells at log-phase were seeded into 96-well plate which contained Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at 5×10^5 density. After 24 h incubation, the supernatant was removed. After 24 h incubation, 20 μl sterile 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added into each test well in triplicates. After 4 h continuous culture, the supernatant was completely removed, with the addition of 150 μl dimethyl sulfoxide (DMSO) for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) values were measured at 570 nm in a micro-plate reader. The proliferation rate was calculated in each group.

Caspase 3 Activity Assay

Caspase 3 activity in cells was evaluated using test kit from all groups. In brief, cells were digested with trypsin, and were centrifuged at 600 × g for 5 min under 4°C. The supernatant was discarded, followed by the addition of cell lysis buffer and iced incubation for 15 min. The mixture was then centrifuged at 20 000 × g for 5 min under 4°C, followed by the addition of 2 mM Ac-DECD-pNA. Optical density (OD) values at 450 nm wavelength were measured to reflect Caspase 3 activity.

Western Blot for VEGF and MMP-9 Protein Expressions

Total proteins were extracted from endometrial cells. In brief, cells were lysed on ice for 15-30 min, with ultrasound treatment (5 s, 4 times). After centrifugation at 10 000 × g for 15 min, the supernatant was saved, quantified by Bradford method and was stored at -20°C for Western blot assay. Proteins were separated in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and were transferred to polyvinylidene fluoride (PVDF) membrane by semi-dry method (100 mA, 1.5 h). Non-specific binding sites were removed by 5% defatted milk powder for 2 h. Anti-VEGF monoclonal antibody (1:1000), anti-MMP-9 monoclonal antibody (1:2000) were added for 4°C overnight incubation. After phosphate-buffered saline-tween (PBST) washing, goat

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5'-3'</th>
<th>Reverse primer 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>GADPH</td>
<td>AGTACCAGTCTGTTGCTGG</td>
<td>TAATAGACCCCGAGTCTGCTG</td>
</tr>
<tr>
<td>miR-33b</td>
<td>ATTCTTTCTGAACGCTTGG</td>
<td>TCACCCCTCGTGCCTCGACA</td>
</tr>
<tr>
<td>VEGF</td>
<td>TGACACGGCTTCTTTC</td>
<td>CCGACTTTCGAGTCTT</td>
</tr>
<tr>
<td>MMP-9</td>
<td>CCACATCCGACTAGCTGTA</td>
<td>GCATTGTJACCACCCGTAATT</td>
</tr>
</tbody>
</table>

Table I. Comparison of POCD in two groups.
anti-rabbit secondary antibody (1:2000) was added for 30 min incubation at room temperature. Electrochemiluminescence (ECL) reagent was then added for developing the membrane for 1 min after PBST rinsing, followed by X-ray exposure. The film was scanned and analyzed by protein imaging system and Quantity One software for measuring band density. Each experiment was replicated for four times (n=4) for statistical analysis.

**Statistical Analysis**

All data were presented as mean ± standard deviation (SD). Comparison of means between groups was performed by Student’s t-test. SPSS 11.5 software (SPSS Inc., Chicago, IL, USA) was used for analyzing data. Comparison between the groups was made by analyzing the data with Turkey’s post hoc test. Statistical significance was defined when \( p < 0.05 \).

**Results**

**Expression Profile of miR-33b in Ectopic and Normal Endometrial Tissues**

Real-time PCR was used to analyze the expression difference between ectopic and normal endometrial tissues. Results showed relatively higher miR-33b in control group whilst endometriosis tissues had lower miR-33b expression (\( p < 0.05 \), Figure 1).

**Effects of miR-33b on Expression in Endometrial Tissues**

RT-PCR was used to test the effect of miR-33b mimic or inhibitor transfection on its expression in endometrial tissues. The transfection of miR-33b mimic significantly facilitated miR-33b expression (\( p < 0.05 \) compared to control group), whilst miR-33b inhibitor transfection inhibited miR-33b expression (\( p < 0.05 \) compared to control group, Figure 2).

**Effects of miR-33b on Endometrial Cell Proliferation**

MTT assay was used to test the effect of miR-33b mimic/inhibitor transfection on proliferation of endometrial tissues. After transfection using miR-33b mimic to facilitate its expression, proliferation of endometrial cells was suppressed (\( p < 0.05 \) compared to control group). The transfection of miR-33b inhibitor to down-regulate its expression could facilitate endometrial proliferation (\( p < 0.05 \) compared to control group, Figure 3).

**MiR-33b regulation and Caspase 3 Activity in Endometrial Tissues**

The transfection of miR-33b mimic facilitated Caspase 3 activity (\( p < 0.05 \) compared to control group). The transfection of miR-33b inhibitor to down-regulate its expression suppressed Caspase 3 activity (\( p < 0.05 \) compared to control group, Figure 4).
Regulation of miR-33b on endometriosis and expression of related factors

Effects of miR-33b on VEGF Expression in Endometrial Cells

Real-time PCR and Western blot were used to test the effect of miR-33b on vascular endothelial growth factor (VEGF) mRNA and protein expression. The transfection of miR-33b mimic decreased vascular endothelial growth factor (VEGF) mRNA and protein expression ($p < 0.05$ compared to control group). The transfection of miR-33b inhibitor facilitated VEGF mRNA/protein expression ($p < 0.05$ compared to control group, Figure 5).

Regulation of miR-33b and MMP-9 Expression in Endometrial Cells

Real-time PCR and Western blot were used to quantify the effect of miR-33b on MMP-9 mRNA/protein expression in endometrial cells. Transfection of miR-33b mimic decreased MMP-9 mRNA and protein expression ($p < 0.05$ compared to control group). Transfection of miR-33b inhibitor facilitated MMP-9 mRNA and protein expression ($p < 0.05$ compared to control group, Figure 6).

Discussion

MicroRNA has highly conserved structure, tissue-specific domain, temporal expressional profile and variable structure\textsuperscript{15}. MicroRNA as small molecule nucleotide has widely regulatory functions, and plays a critical role in cell proliferation, differentiation, apoptosis and immune response\textsuperscript{16}. Expressional profile of microRNA is under the regulation of multiple factors including transcriptional regulatory level, physiological status and environmental change. Therefore, in different tissue/cell types, microRNA expression and regulation mechanism are under influence\textsuperscript{17}. MicroRNA is closely correlated with disease type, and can be used as the important target for disease diagnosis and prognosis, thus becoming a major challenge for modern medicine\textsuperscript{18}. MiR-33b as one newly discovered microRNA, has been shown to have various physiological/pathological activities, and is involved in metabolic diseases in addition to tumor oncogenesis\textsuperscript{19,20}. In endometriosis, which is the most common disorder in gynecology, microRNA expression profile has been shown to have difference, but leaving the role of miR-33b no fully illustrated\textsuperscript{21}. Therefore this study investigated the expressional profile of miR-33b in endometriosis, and found lower miR-33b expression in endometriosis tissues than controlled endometrial tissues. By mediating miR-33b expression, we confirmed that transfection of miR-33b inhibitor inhibited its expression,
facilitated the proliferation of endometrial cells, and decreased Caspase 3 activity. Transfection of miR-33b mimic facilitated its expression, inhibited endometrial cell proliferation and elevated Caspase 3 activity. These results suggested that miR-33b could regulate endometrial tissues proliferation via mediating apoptosis. As one of the most potent angiogenesis facilitating factor, vascular endothelial growth factor (VEGF) is expressed in vascular endothelial cells, thus enhancing vessel permeability, in addition to degradation of extra-cellular matrix, thus facilitating cell proliferation, differentiation and migration. Matrix metalloproteinase (MMPs) participate in the regulation of various body pathophysiological processes, and play positive roles in embryonic implantation or wound healing. It is also involved in other diseases such as inflammatory response, autoimmune disease, invasion or metastasis of malignant tumors, and cardiovascular disease. MMP-9 has been shown to play an important role in cell proliferation and migration. Further study found that transfection of miR-33b inhibitor elevated VEGF and MMP-9 mRNA or protein expression, whilst miR-33b mimic transfection suppressed mRNA/protein expression of VEGF and MMP-9.

Conclusions

MiR-33b can affect proliferation and apoptosis of endometrial cells via mediating apoptosis and altering VEGF or MMP-9 expression, thus can work as a novel target for diagnosis and treatment of endometriosis.

Conflict of Interest
The Authors declare that they have no conflict of interests.

References
Regulation of miR-33b on endometriosis and expression of related factors


