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MiR-129 is involved in the occurrence of uterine fibroid through inhibiting TET1

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Abstract. – OBJECTIVE: To detect the expressions of micro ribonucleic acid (miR)-129 and its target gene in uterine fibroid tissues and to investigate the role of miR-129 in the occurrence of uterine fibroid.

PATIENTS AND METHODS: The expressions of miR-129 and its target gene ten-eleven translocation 1 (TET1) were detected *via* quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Dual-luciferase reporter gene and Western blotting were used to verify the regulatory relation between miR-129 and target gene. The effects of miR-129 on the proliferation, apoptosis, cycle and extracellular matrix (ECM) of uterine fibroid cells were investigated *via* transfection with miR-129 mimics and TET1 small-interfering RNA (siRNA).

RESULTS: MiR-129 was lowly expressed in uterine fibroid. The expression of miR-129 was regulated by sex hormones. The highly expressed miR-129 promoted apoptosis and inhibited proliferation through reducing the low expression of TET1. At the same time, miR-129 affected the accumulation of ECM.

CONCLUSIONS: The expression of miR-129 in uterine fibroid is lower, and the proliferation capacity of tumor cells is enhanced, thus promoting the occurrence and development of uterine fibroid.

Key Words

MiR-129, Uterine Fibroid, TET1, Proliferation, Extracellular matrix.

Introduction

Uterine fibroid is the most common benign tumor in women, and nearly half of women aged 35-49 years old suffer from myometrial smooth muscle cell-derived uterine fibroid[1,2]. The disease is often accompanied by long-term massive bleeding similar to that in menstrual period, so it will cause moderate anemia. In addition, it can lead to moderate to severe pain[3]. It is also very common that the oppression of uterine fibroid results in pelvic discomfort, and intestinal and bladder dysfunction. More seriously, it affects fertility, such as infertility and habitual abortion[1]. Uterine fibroid grows very quickly during pregnancy, which may cause delivery obstruction, so pregnant women are forced to receive caesarean section. In addition to postpartum hemorrhage, uterine fibroid may also lead to malpresentation and abnormality of fetus. All of these clinical complications seriously affect the health of women[4]. Micro ribonucleic acid (miRNA) is a class of non-coding small RNA, which is ubiquitous and highly conserved in many species. MiRNA regulates cell proliferation, differentiation and apoptosis[5]. Some genes, such as estrogen receptor-α (ER-α), high-mobility group protein A1 (HMGA1), HMGA2, insulin-like growth factor 1 (IGF1) and collagen 4 (COL4) family members, play important roles in uterine fibroid tissues, and the 3'-untranslated regions (3'-UTRs) of these genes contain multiple sites, which are speculated to be related to miRNA target genes[6]. The most common uterine fibroid chromosome loss [del(7)(q22-q32)] may be encoded by miRNA. Due to the tissue-specific expression of miRNA, it may also be a specific group in female reproductive organs (cervix, uterus and ovary)[7]. Therefore, it is speculated that the abnormal expression of miRNA may be closely related to the occurrence and development of uterine fibroid. It has been reported[8,9] that the Let-7 family in uterine fibroid tissues affects uterine fibroid through its target gene HMGA2.

MiR-129-5p is one of the mature forms of miR-129 gene coding. Scholars[10,11] have shown that the expression of miR-129 is down-regulated in a variety of tumors and involved in the regulation of tumor development. Bandres et al[12] studied and found that the expression of miR-129 is down-regulated in colon cancer, and its overexpression can inhibit colon cancer cell proliferation. In the endometrium, miR-129 can inhibit

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cell proliferation by targeting sex-determining region Y box gene-4 (SOX4)[13]. However, the expression and role of miR-129 in uterine fibroid have not been reported yet.

In this work, the expressions of miR-129 and its target gene mRNA and protein in uterine fibroid tissues and corresponding extra capsular myometrial tissues were detected, and the relationship of miR-129 expression level with occurrence and development of uterine fibroid was analyzed.

Patients and Methods

Sample Collection

A total of 20 cases of uterine fibroid tissues and 20 cases of corresponding normal myometrial tissues were collected from patients receiving myomectomy or total hysterectomy from January 2017 to December 2017 in our hospital, and confirmed as benign leiomyoma *via* pathological examination. Patients were aged (41.94±6.25) years old, and they did not take hormonal drugs within 3 months before operation. Patients also signed the informed consent before operation. Tissues were stored in liquid nitrogen for standby application. This study was approved by the Ethics Committee of The Second Hospital of Dalian Medical University.

Cell Culture

Tissue samples were washed with phosphate-buffered saline (PBS), cut into small pieces, and centrifuged at l000 rpm for 5 min. The supernatant was discarded. After that, Dulbecco's modified Eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing type I collagenase (final concentration of l m/mL) was added for digestion *via* thermostatic water bath at 37°C for 4-6 h, and the mixture was filtered using a 300-mesh (with a mesh diameter of 38 μm) stainless steel cell filter screen. The filtrate was centrifuged at l000 rpm for 5 min, and the supernatant was removed. The sediment was suspended using an appropriate amount of DMEM culture solution containing 10% fetal bovine serum, inoculated into a 25 cm cell culture flask, and incubated with 5% CO_2 at 37°C. After adhering to the wall overnight, cells were observed microscopically. The culture flask was shaken slightly, and the culture solution was fully replaced to remove other extraneous cells that did not adhere to the wall. After that, the culture solution was replaced every 3 days.

Cell Transfection

At 1 day before transfection, an appropriate number of cells were inoculated into antibiotic-free medium, followed by cell transfection according to the instructions of Lipofectamine2000. The liposome, miR-129 mimic, ten-eleven translocation 1 (TET1) small-interfering RNA (siRNA) and negative control (NC) were diluted using an appropriate amount of serum-free culture solution, incubated at room temperature for 5 min, mixed and let stand for 20 min. The mixed solution was added into each well of a culture plate, and the plate was gently shaken to mix the solution and placed into the incubator. After 6 h, the original medium was replaced, followed by incubation in the incubator.

Cell Proliferation

The cell proliferation was detected using the cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan). The above cells after transfection were cultured for 48 h. 10 μL CCK-8 solution were added into each well and mixed evenly with cells, followed by incubation in the incubator at 37°C for another 1 h. The optical density (OD) value of each well was measured at a wavelength of 450 nm using a microplate reader. The average OD values of 5 wells were taken, and then averaged after the independent experiment was repeated for 5 times.

Cell Cycle

The above cells after transfection were cultured for 48 h, digested, centrifuged, suspended using pre-cooled PBS and rinsed twice. 3 mL pre-cooled 75% ethanol were added, and cells were collected and let stand at 4°C overnight. Then cells were centrifuged, washed with PBS and added with 400 μL propidium iodide (PI) staining solution and 100 μL RNase A (100 mg/L) for incubation in a dark place at 4°C for 30 min. Finally, the mixture was filtered using a 300-mesh nylon screen, and the cell cycle was detected *via* flow cytometry.

Cell Apoptosis Detection

The above cells after transfection were cultured for 48 h and collected, and three parallel wells were set in each group. Cells were washed with PBS and digested with trypsin. After the reaction was terminated using cold medium, cells were transferred into an Eppendorf (EP) tube, and centrifuged at 1000 rpm for 5 min. The supernatant was discarded. Next, cells were

washed with 1 mL cold PBS and centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. 200 μL 1 × Binding Buffer were added into each tube to suspend cells (cell density of 1×10^6/mL). 3 μL Annexin V/fluorescein isothiocyanate (FITC) and 2 μL PI were added into each tube in a dark place and mixed evenly, followed by incubation on ice at room temperature in a dark place for 15 min. The apoptosis was detected *via* flow cytometry.

Western Blotting

At 48 h after transfection, cells were lysed using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China), and centrifuged at 12,000 rpm for 5 min. The supernatant was taken. After 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and membrane transfer under constant flow, the protein was sealed *via* blocking solution (1×Tris-Buffered Saline, 0.5% Tween-20, 5% w/v skimmed milk powder) for 1 h, and incubated with mouse anti-human TET1 monoclonal antibody (diluted at 1:200) at room temperature for 2 h. After the membrane was washed with Tris-Buffered Saline with Tween-20 (TBST), horseradish peroxidase (HRP)-labeled goat anti-mouse secondary antibody (1:1000) was added for incubation at room temperature for 2 h. After the membrane was washed again with TBST, HRP substrate was added, followed by exposure using chemiluminescence method. β-actin was used as an endogenous reference.

Prediction of miRNA Target Genes

In this paper, three databases [TargetScan (http://www.targetscan.org/), miRPathDB (https://mpd.bioinf.uni-sb.de/) and miRDB (http://www.mirdb.org/)] were used for target gene prediction.

Treatment of Cells with Ovarian Steroids

Primary tumor cells were inoculated into a 6-well plate, washed and incubated with the medium containing activated carbon-treated fetal bovine serum for 24 h. Then cells were treated with 17β-estradiol (E2), progesterone (P) or E2 + P4 at a concentration of 10^{-8}M for 24 h[14].

Dual-Luciferase Reporter Gene

TET1 3'-UTR containing the target of miR-129 and mutant-TET1 3'-UTR in the target of miR-129 were connected between the two digestion sites (SacI and XbaI) of dual-luciferase reporter plasmid pMIR-GLO to construct the dual-luciferase reporter plasmid. Plasmids containing TET1 3'-UTR and mutant-TET1 3'-UTR were constructed. Cells in the logarithmic growth phase were taken and inoculated into a 48-well plate, followed by plasmid transfection after 24 h according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 250 μL serum-free medium were added into each well, and the medium contained pMIR-GLO plasmid at a final concentration of 1 μg/mL, 0.2 μg/mL pRL-SV40 plasmid and 100 nmol/L miR-129 mimic. After transfection for 48 h, Renilla luciferase activity was detected using the dual-luciferase reporter gene analysis system (Promega, Madison, WI, USA) according to the instructions.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

According to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA), cells or tissues were lysed using TRIzol reagent to extract total RNA. RNA was separated *via* chloroform, precipitated via isopropanol, washed with 75% ethanol and dissolved by 201 × diethylpyrocarbonate (DEPC)-treated water. ABI7500 fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA) was used for the quantitative detection of gene levels, and SYBR green was used as the fluorescent dye. The reaction conditions are as follows: 50°C for 2 min, 95°C for 10 min, a total of 40 cycles, 95°C for 15 s, 60°C for 60 s. Three parallel wells were set up in the experiment, and all samples were tested repeatedly for 3 times, followed by quantitative analysis using the quantitative PCR platform. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference for gene, and U6 as an internal reference for miRNA. The relative quantitative method was used to present the expression fold as -ΔCT relative to the control. All operations for samples were performed on ice. Primer sequences are shown in Table I.

Statistical Analysis

All data were analyzed using statistical product and service solutions (SPSS) 17 software (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± standard deviation, and *t*-test was used to assess the differential expression. $p<0.05$ suggested that the difference was statistically significant.

Table I. Primers used in this study.

Gene	Sense primer	Antisense primer
U6	CTCGCTRCGGCAGCACA	AACGCTCACGAATTGCGT
miR-129	AAAAAAAAAAGCCCGCAGCTACATTGTGCTG	AAAAAAAAAAGTGCAGGGTCCGAGGT
COL1A1	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
COL1A2	GTTGCTGCTTGCAGTAACCTT	AGGGCCAAGTCCAACTCCTT
COL3A1	GGAGCTGGCTACTTCTCGC	GGGAACATCCTCCTTCAACAG
COL5A1	TTGAAGGAGGATGTTCCCATCT	ACAGACACATATTTGGCATGGTT
COL5A3	TGACCGGGCATTCAGAATTGG	CGGGCACCCCTTTCATCAT
COL7A1	GTGGCCGTCAGCATAGATGG	TGAATGTCTCCCTCGAAAGTCTT
TET1	CATCAGTCAAGACTTTAAGCCCT	CGGGTGGTTTAGGTTCTGTTT
GAPDH	TCCATGACAACTTTGGTATCG	TGTAGCCAAATTCGTTGTCA

Results

Expression and Function of miR-129 in Uterine Fibroid

Compared with that in normal myometrial tissues, the expression of miR-129 in uterine fibroid tissues was significantly decreased (Figure 1A), suggesting that differentially-expressed miR-129 may be involved in the occurrence of uterine fibroid. The addition of exogenous estrogen and progesterone into uterine fibroid cells cultured *in vitro* could significantly down-regulate the expression of miR-129, and the combined application of the two hormones could further reduce the expression of miR-129, suggesting that the expression of miR-129 is regulated by sex hormones (Figure 1B). Transfection with miR-129 mimics significantly increased the expression of miR-129 (Figure 1C). Highly expressed miR-129 could significantly inhibit cell proliferation (Figure 1D) and promote apoptosis (Figure 1E); however, it had no significant effect on cell cycle (Figure 1F), indicating that the abnormally low expression of miR-129 may affect the occurrence of uterine fibroid through regulating cell proliferation and apoptosis.

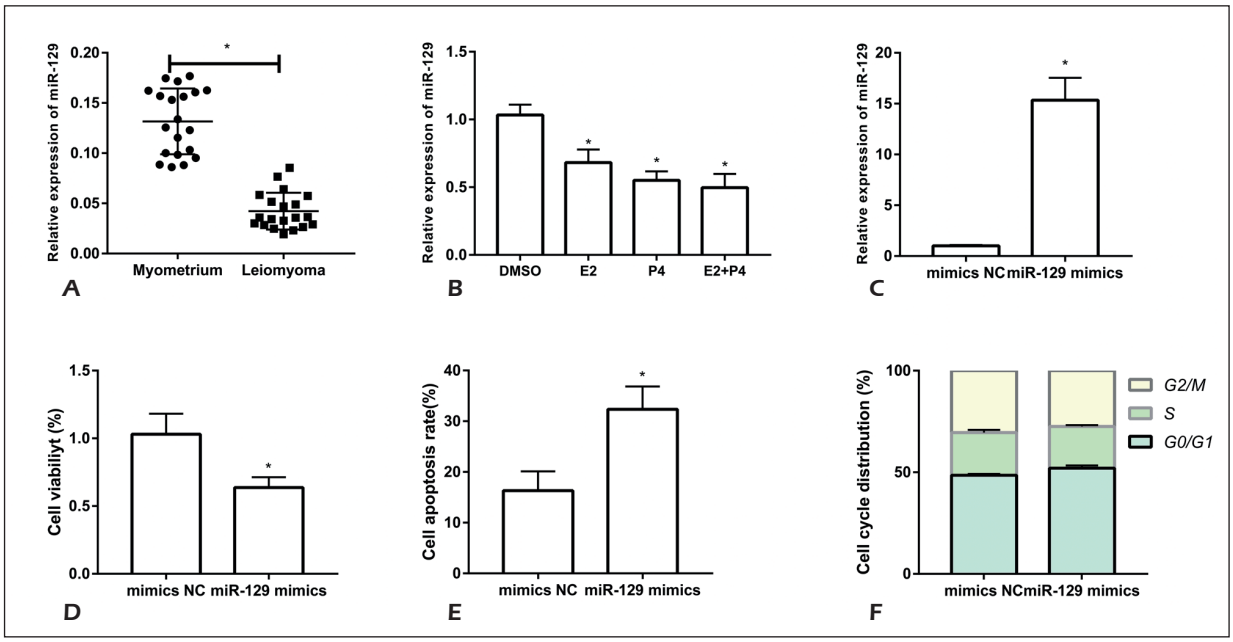

Figure 1. Expression and function of miR-129 in uterine fibroid. **A**, The expression of miR-129 in uterine fibroid tissues is decreased. **B**, Expression of miR-129 at 24 h after uterine fibroid cells are treated with DMSO, E2, P and E2 + P. **C**, The expression of miR-129 is significantly increased in miR-129 mimics group. **D**, Highly expressed miR-129 significantly inhibits cell proliferation. **E**, Highly expressed miR-129 significantly promotes apoptosis. **F**, Highly expressed miR-129 has no significant effect on cell cycle; *$p<0.05$.

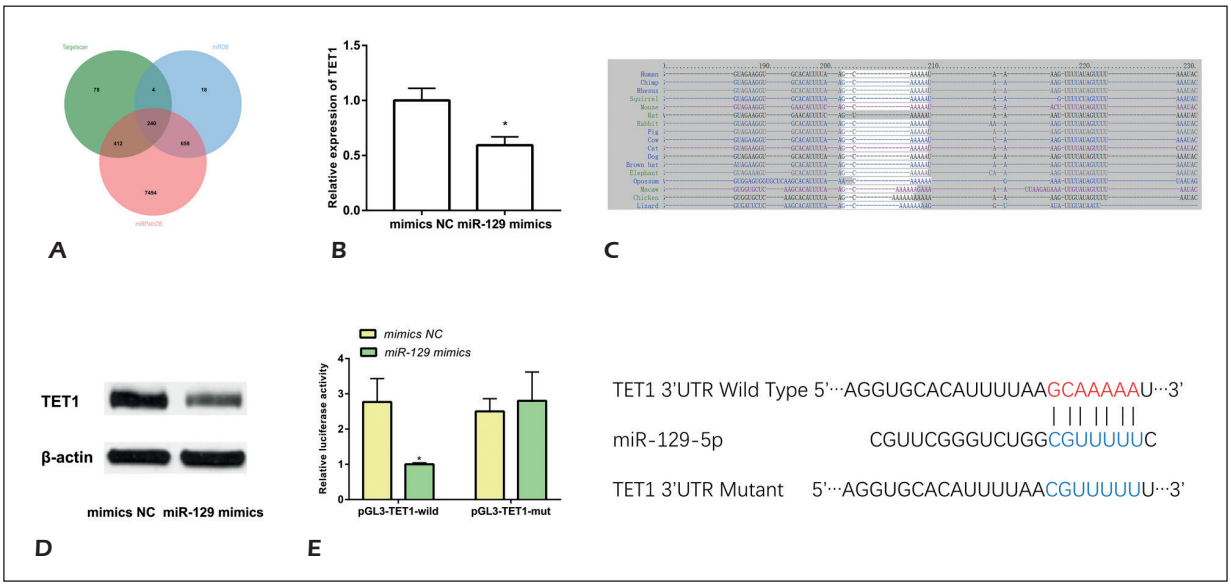

Figure 2. MiR-129 target gene prediction. **A**, Target gene prediction at different sites. **B**, MiR-129 inhibits TET1 protein expression. **C**, MiR-129 inhibits TET1 mRNA expression. **D**, After transfection with TET1 wild-type and mutant-type plasmids, miR-129 can only bind to wild-type TET1 3'-UTR. E, Conservative analysis of binding site of miR-129 to TET1 in vertebrates; *p<0.05.

MiR-129 Target Gene Prediction and Validation

A total of 240 potential target genes (Figure 2A) were obtained through three online prediction sites: TargetScan, miRPathDB and miRDB. It was found through further literature review that TET1 had been proved to be involved in the occurrence of a variety of tumors[15,16]. Therefore, TET1 was chosen as a target gene for research. Highly expressed miR-129 could significantly reduce TET1 protein (Figure 2B) and mRNA (Figure 2C) expressions. Dual-luciferase reporter gene analysis showed that miR-129 could bind directly to the TET1 3'-UTR (Figure 2D). Further analysis revealed that the binding site of miR-129 to TET1 was highly conserved in vertebrates (Figure 2E). The above results suggest that miR-129 can directly bind to TET1 and inhibit its expression.

Expression and Function of TET1 in Uterine Fibroid

Primary uterine fibroid cells were transfected with TET1 siRNA, and it was found that TET1 expression was significantly reduced (Figure 3A). Furthermore, whether low-expression TET1 could simulate the effect of miR-129 on cell function was verified. Results showed that lowly expressed TET1 could also significantly inhibit tumor cell proliferation (Figure 3B) and

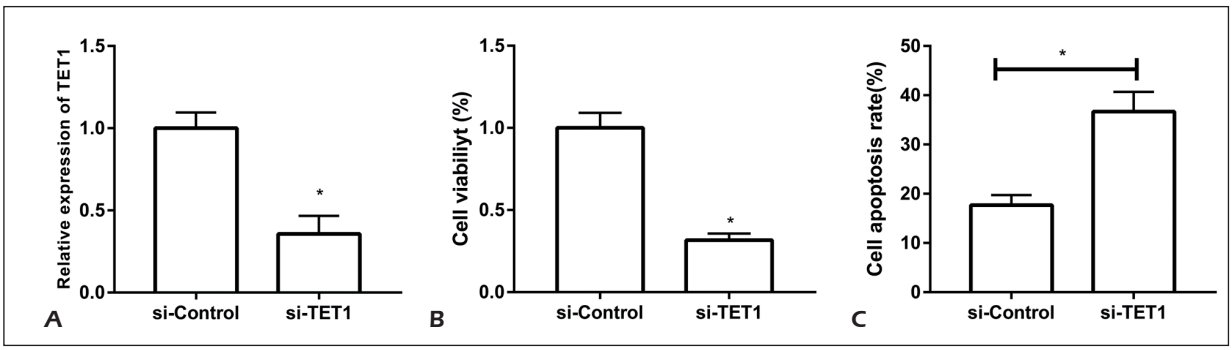

Figure 3. Expression and function of TET1 in uterine fibroid. **A**, TET1 expression is decreased in TET1 siRNA-treated group. **B**, Low-expression TET1 inhibits cell proliferation. **C**, Low-expression TET1 promotes apoptosis; *p<0.05.

promote apoptosis (Figure 3C). The above results indicate that miR-129 can affect cell proliferation and apoptosis through regulating the expression of TET1, thus participating in the occurrence and development of uterine fibroid.

Effect of miR-129 on Extracellular Matrix (ECM)

Accumulation of ECM is one of the important features of uterine fibroid, so whether miR-129 would affect ECM was explored[17]. It was found that highly expressed miR-129 could significantly down-regulate expressions of COL1A1, COL1A2, COL3A1, COL5A1, COL5A2 and COL7A1 (Figure 4), suggesting that miR-129 can significantly regulate the production of collagen in uterine fibroid, thus affecting the progression of uterine fibroid.

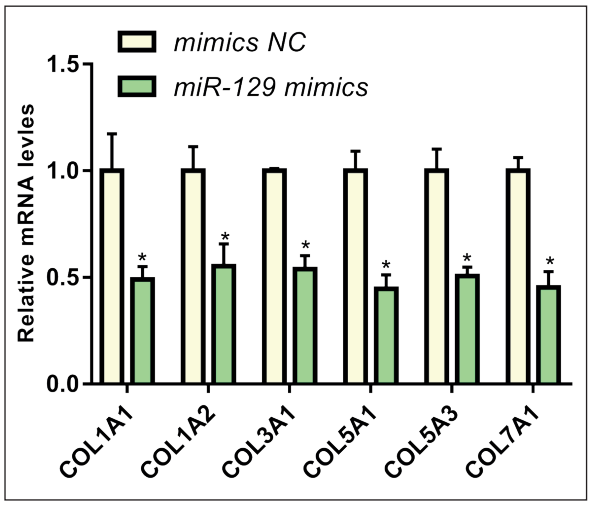

Figure 4. Effect of miR-129 on ECM. Highly expressed miR-129 can significantly reduce expressions of collagen-related genes.

Discussion

Uterine fibroid is a kind of common benign gynecological tumor, whose incidence rate shows an increasing trend year by year. Despite of great improvement in treatment techniques, clinical studies have shown that traumatic surgery still causes great pain and impact on patients. This disease cannot be completely cured by hormone therapy[18]. So far, there are still no satisfactory treatment means for uterine fibroid. Exploring the pathogenesis and controlling the development of uterine fibroid are problems to be solved in its treatment.

The occurrence and development of uterine fibroid is a multi-step and multi-factor complex process, but its pathogenesis has not been very clear yet. Current data have shown that uterine fibroid is a kind of steroid hormone-dependent tumor, and the growth of uterine fibroid relies on the ovarian steroid estrogen and progesterone, which is related to the gene and cell proliferation and apoptosis, abnormal cell signal transduction pathway, cytokines, etc. Recent studies[19] have shown that progesterone has a growth-promoting effect on uterine fibroid. As scholars wordly focus on the study on epigenetic origin of human diseases, miRNA, as a target for diagnosis and treatment, has drawn wide attention. Results in this paper suggest that estrogen and progesterone can significantly reduce the expression of miR-129 and enrich the incidence matrix of uterine fibroid. Moreover, in addition to the classic pathway, miRNA, as a downstream factor of hormone, can also be involved in the occurrence of uterine fibroid, and serve as a bridge to connect environmental factors with metabolic activity of tumor cells. ECM is a highly-organized network composed of macromolecules, which is synthesized and secreted by animal cells outside cells, and distributed on the cell surface or among cells. It is mainly composed of polysaccharides, proteins or proteoglycans, affecting the survival, death, proliferation and differentiation of tissue cells. ECM possesses complex signal transduction, functional regulation and other functions. Cell proliferation mediated by a variety of factors can promote the production of ECM[20]. Uterine fibroid is rich in ECM, and the occurrence of uterine fibroid is closely related to the formation and accumulation of ECM[21]. In this study, it was found that highly expressed miR-129 could reduce the expressions of ECM-related genes, such as COL1A1, suggesting that in addition to proliferation and apoptosis, miR-129 may also be involved in the occurrence of uterine fibroid through regulating ECM.

Apoptosis refers to the autonomous and orderly death of cells controlled by genes to maintain homeostasis. Unlike necrosis, apoptosis is not a passive process, but an active process involving the activation, expression, regulation, etc., of a series of genes. Apoptosis is not a phenomenon of autologous injury under pathological conditions, but a kind of death process that is actively pursued to better adapt to the living environment[22]. It has been found that multiple genes are related

to apoptosis, such as p53, B-cell lymphoma-2 (Bcl-2), Cmy-c and factor associated suicide-L (Fas-L). Fas and Fas ligand are located in the cell membrane, and they bind to each other to induce cell apoptosis, thereby inhibiting tumor growth. Bcl-2 resists apoptosis and prolongs cell lifespan[23]. The expression of Bcl-2 protein is weakly positive in normal myometrium, and strongly positive in uterine fibroid. Besides, it is also found that Bcl-2 mRNA is highly expressed in uterine fibroid, showing a periodic change, and its level in secretory phase is significantly higher than that in proliferative phase[24]. In this study, it was found that highly-expressed miR-129 could significantly promote cell apoptosis. Therefore, miR-129 can participate in occurrence and development of tumor through regulating apoptosis and proliferation. However, significant effects of miR-129 on cell cycle were not found. Further research on miR-129 in the apoptotic pathway will provide new suggestions for investigating the mechanism and treatment of uterine fibroid.

In addition, our study found that TET1 could be a target gene of miR-129, which was confirmed through three online prediction sites and Dual-luciferase reporter gene analysis. Moreover, we showed that highly expressed miR-129 could significantly reduce TET1 protein and mRNA expressions *via* Western blotting and qRT-PCR, respectively. Besides, the results of this work confirmed that lowly expressed TET1 could also significantly inhibit tumor cell proliferation and promote apoptosis. The above results indicate that miR-129 can affect cell proliferation and apoptosis through regulating the expression of TET1, thus participating in the occurrence and development of uterine fibroid.

Conclusions

We found that estrogen and progesterone reduce the expression of miR-129 in uterine fibroid, thereby increasing the expression of TET1, leading to increased proliferation and decreased apoptosis of tumor cells, and increasing the accumulation of ECM, ultimately promoting the occurrence and development of uterine fibroid.

Conflict of Interest

The authors declared no conflict of interest.

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