EXPRESSION OF MIR-96 IN PATIENTS WITH UTERINE FIBROIDS AND ITS EFFECTS ON PROLIFERATION AND APOPTOSIS OF UTERINE FIBROID CELLS

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ABSTRACT

Objective*: This paper aimed to investigate miR-96 expression in patients with uterine fibroids (UFs) and its effects on the proliferation and apoptosis of uterine fibroid cells (UFCs).*

Methods: *Patients with UFs treated in our hospital from October 2018 to October 2019 were enrolled, with human uterine leiomyosarcoma cells (SK-UT-1 cells) and normal human uterine smooth muscle cells (HUSMCs) purchased from BeNa Culture Collection. qRT-PCR was adopted for detecting miR-96 and BRCA1 expression in samples, and Western Blotting (WB) was adopted for detecting BRCA1 protein expression in the cells. Cell proliferation, invasion and apoptosis were detected by MTT assay, Transwell and flow cytometry.*

Results: *Compared with adjacent tissues, miR-96 mRNA expression in uterine fibroid tissues remarkably upregulated (P<0.001), and compared with HUSMC cells, the expression in SK-UT-1 cells remarkably upregulated (P<0.001). SK-UT-1 cells were chosen for transfection. According to qRT-PCR, after transfection, miR-96 expression was remarkably lower in the miR-96-inhibitor group but remarkably higher in the miR-96-minics group compared with the NC group (both P<0.001). Cell proliferation remarkably reduced in the miR-96-inhibitor group, but enhanced in the miR-96-minics group compared with the NC group (both P<0.001); the apoptotic rate was remarkably inhibited in the miR-96-minics group (P<0.001); cell invasion remarkably reduced in the miR-96-inhibitor group, but remarkably enhanced in the miR-96-minics group (P<0.001). According to WB, BRCA1 expression remarkably downregulated in SK-UT-1 cells transfected with miR-96-minics, but remarkably upregulated in those transfected with miR-96-inhibitor (P<0.001). BRCA1 protein level in the sh-BRCA1 group remarkably rose (P<0.001), showing that the inhibitory effect of miR-96-minics on the level could be reversed by transfection with sh-BRCA1. According to the functional analysis, after transfection with sh-BRCA1, SK-UT-1 cells had remarkably inhibited proliferation and invasion, but remarkably enhanced apoptosis (all P<0.01). Compared with the NC group, S-phase cells reduced, the expression of pro-apoptotic proteins remarkably upregulated, the expression of Bcl-2, APR3 and Bcl-xl was remarkably inhibited in the sh-BRCA1 group. After transfection with miR-96-minics/sh-BRCA1, S-phase cells rose, the expression of pro-apoptotic proteins was remarkably inhibited compared with the sh-BRCA1 group, and the expression of Bcl-2, APR3 and Bcl-xl remarkably upregulated (P<0.05).*

Conclusion: *Highly expressed in patients with UFs, miR-96 can target BRCA1 to inhibit SK-UT-1 cells to proliferate and promote their apoptosis.*

Keywords: *miR-96, UFs, BRCA1, SK-UT-1, proliferation, apoptosis.*

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Introduction

With a relatively high prevalence rate, uterine fibroids (UFs) are a common tumor in females and usually accompanied by abnormal bleeding and pelvic pain[1-2]. Due to the lack of effective drug treatment, surgery has become a clear choice for treating the disease, which is usually not fatal but seriously affects the pregnancy rate of females[3-4]. Therefore, it is essential to develop new therapies for UFs, in order to enable females to suffer less from abnormal pelvic pain and to protect their normal reproductive rights and interests[5-6]. As research on targeted biomolecular regulation deepens, miRNA analysis to determine the potential molecular mechanism of metastatic cervical cancer has become an important

cornerstone of molecular targeted therapy[7-8]. As an important regulator of the pathological processes (development and metastasis) of many tumors, microRNAs (miRNAs) affect the biological pathways, tumor progression, clinical results and prognosis of many cancers[9-10]. Upregulating in cervical cancer cell lines and tissues, miR-96 is related to clinical characteristics of patients with cervical cancer[11]. According to in vivo and vitro experimental results by Ma Xiaoping et al., its overexpression promotes cervical cancer cells to proliferate[12], but its inhibition reduces this[13]. Clinically, serum miRNAs have been used as a biomarker for diagnosing females with symptoms of gynecological diseases[14]. Moreover, it has been confirmed that miR-96 is crucial for coordinating cell morphology and structure, and functions as an oncogene in various cancers, but its role in the development and progression of UFs has not been clarified[15-16].

This study is designed to investigate miR-96 expression in patients with uterine fibroids (UFs) and its effects on the proliferation and apoptosis of uterine fibroid cells (UFCs).

Materials and methods

Main instruments and reagents

A human uterine smooth muscle cell line (SK-UT-1 cells; BNCC337870) and a normal human uterine smooth muscle cell line (HUSMCs; BNCC338667) were purchased from BeNa Culture Collection. A real-time fluorescence quantitative PCR instrument (ABI 7500, Applied Biosystems, USA). lip 2000 (TaKaRa, Dalian, China). Trizol kits for RNA extraction (TaKaRa, Dalian, China). Annexin V/PI apoptosis detection kits (TaKaRa, Dalian, China). SYBR Green qPCR Mix (TOYOBO, Japan). MTT assay kits (Shanghai Zeye Biological Technology Co., Ltd.). ECL luminescence kits (BioThrive Sciences LLC, Shanghai). BCA protein assay kits (Fermentas). A Thermo Multiskan GO full-wavelength microplate reader (Thermo Fisher Scientific, China). All primer sequences were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (BioRad, Hercules, USA)

Detection methods

Cell culture and transfection

The uterine fibroid cell (UFC) strains were transfected into the DMEM which contained 10% fetal bovine serum (FBS) and penicillin-streptomycin mixed solution, for subsequent culture in a Constant Temperature & Humidity Incubator at 37°C and with 5% CO_2. miR-96-inhibitor (miR-96-inhibitor), miR-96-minics (miR-96-minics) and empty plasmids (NC) were respectively transfected into the cells according to the instruction of the Lipofectamine™2000 transfection reagent kit. Primers were transfected into the cells with the greatest difference in mRNA expression, and 6 hours later, the cells were continuously cultured in culture solution containing 10% FBS. qRT-PCR was performed to verify the cells' transfection efficiency, and then the cells were collected for subsequent experiments.

qRT-PCR

qRT-PCR was adopted for detecting mRNA expression in the tissues, serum and cells. mRNA transcripts were detected using standard SYBR Green real-time PCR assays. Total RNA in the tissues was drawn based on the instruction of the Trizol reagent and then dissolved in 20 μL of DEPC-treated water.

Then, the total RNA was reversely transcribed using a reverse transcription kit, and the synthesized cDNA was taken as a template for qRT-PCR amplification. Conditions for PCR preparation were 40 cycles at 95°C for 5 s, then denaturation (95°C, 10 s), annealing (60°C, 20 s) and extension (72°C, 15 s). Three same wells were provided for each sample. Based on the result parameters, the relative expression of the target gene was calculated, and the relative quantification of the target gene was calculated by $2^{-\triangle Ct}$.

Bioinformatics analysis and dual luciferase reporter gene assay (DLRGA) were performed. TargetScan (www.targetscan.org) database was used to predict the target gene of miR-96, with BRCA1 identified as its potential target.

Genes	Positive primers	Reverse primers
miR-96	5'-GCCCGCTTTGGCACTAGCACATT-3'	5'-GTGCAGGGTCCGAGGT-3'
BRCA1	5'-CCGCTCGAGCCGGCCTTCCAACCTTTGTC-3'	5'-TAATACGACTCACTATAGGG-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	5'-GGTCTCCTCTGACTTCAACA-3'	5'-GTGAGGGTCTCTCTCTTCCT-3'

Table 1: Primer sequences of miR-96 and internal references.

Western blotting (WB)

The lysed cells were collected for transferring to a centrifuge tube, and then centrifuged for 10 min at 12000×g and 4°C. After the supernatant was col-

lected as protein samples, the protein concentration was determined by BCA method, and the samples were diluted with lysis buffer to prepare 20 mg/mL proteins. The protein was transferred to PVDF membrane after the preparation of 8.00% separation gel and 5.00% stacking gel as well as SDS-PAGE electrophoresis. Next, the membrane was added with β-catenin, cyclin D1, c-myc (1:1000) primary antibody, and internal reference β-actin (1:3000), and then sealed all night at 4°C.

Subsequently, the membrane was added with HRP-labeled goat anti-mouse secondary antibody (1: 5000), incubated at 37°C for 1 hour, and then rinsed with TBST over 5 min for 3 times. After the membrane was developed in a dark room, excess liquid on it was absorbed dry, and it finally luminesced with an ECL reagent and was developed. The protein bands were scanned, and the gray values were analyzed using Quantity One (Molecular Devices Corp, The Bay Area, CA, USA).

MTT assay for cell proliferation

The cells transfected for 24 hours were collected. After their density was adjusted to 5*103 cells/well, they were inoculated on a 96-well plate and then incubated at 37°C. 20 μL of MTT solution (5 μmg/mL) was added at each time point. After the solution was removed, the wells were added with 150 μL of dimethyl sulfoxide. Finally, optical density (OD) values of cells in each group were measured by the spectrophotometer.

Transwell for cell invasion

Matrigel was coated in Transwell chamber for standing at 37°C for 30 min. The cells were resuspended in a tissue-free DMEM at 4×105 cells/mL. 200 μL of the cell suspension was added to the upper chamber, while 800 μL of DMEM (10% FBS) was added to the lower one, both of which were cultured for 24-48 hours.

After that, the chamber was taken out, fixed in 4% paraformaldehyde and stained with hematoxylin, with the cells in the upper chamber wiped off. Under an optical microscope, 10 high power fields were randomly selected to count the number of cells passing through the basement membrane of the chamber, so as to indicate cell invasion.

Cell cycle analysis

As mentioned earlier, the DNA content of the cells was analyzed using a flow cytometer. NC and transfected cells were collected for cleaning twice with phosphate buffer saline solution (PBS) and fixing with 70% ethanol at -20°C until analysis. Next, they were stained with 20 μg/mL PI containing 20 μg/mL RNase (DNase-free) for 2 hours. The flow cytometer was adopted for analyzing the stained cells. The percentage of cells in G1, S and G2 phases was measured using Mulicycle Cell Cycle Software.

Flow cytometry for cell apoptosis

After 48-hour transfection, the cells were collected and digested with 0.25% trypsin for 1 min, cleaned with PBS over 5 min for 3 times, and then resuspended with AnnexinV binding buffer (100 μL), to prepare a 1×106 cells/mL suspension.

After added with 5 μL of Annexin-V-FITC solution (20 μg/mL), the suspension was incubated at 4°C for 15 min. Finally, it was added with PI staining solution (5 μL) for incubation at 4°C for 2 min. Apoptosis was measured by the flow cytometer within 30 min.

Statistical methods

Statistical analysis was performed through SPSS 20.0 (IBM Corp.). Data conforming to normal distribution were expressed by mean ± standard deviation (mean±SD), and independent samples t test was used for the comparison of measurement data between groups.

Repeated measures analysis of variance (ANOVA) was used for data comparison between multiple time points, and Bonferroni was used for post hoc test. The comparison of means between multiple groups was analyzed by one-way ANOVA, and LSD-t test was used for post hoc test. Receiver operating curve (ROC) was plotted to assess diagnostic value. Pearson test was used for correlation. $P<0.001$ indicated a statistically significant difference.

Results

miR-96 expression in patients with UFs

qRT-PCR was adopted for detecting miR-96 expression in uterine fibroid tissues and adjacent tissues. miR-96 mRNA expression in the uterine fibroid tissues remarkably upregulated compared with the adjacent tissues ($P<0.001$). See Figure 1.

miR-96 expression in cells

qRT-PCR was adopted for detecting miR-96 expression in the cell lines. This miR mRNA expression in SK-UT-1 cells remarkably upregulated compared with HUSMC cells ($P<0.001$).

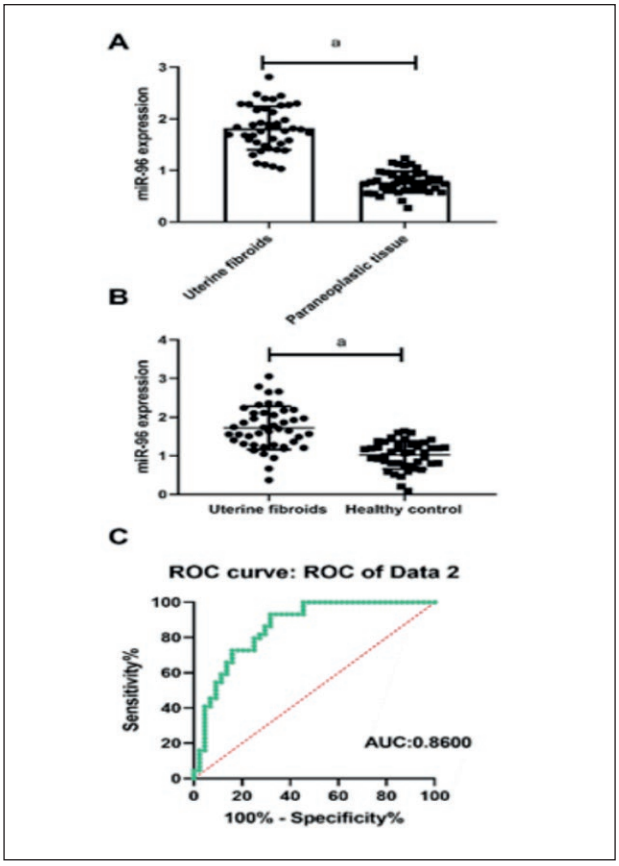

Figure 1: miR-96 expression in patients with UFs. Figure A: miR-96 mRNA expression in the uterine fibroid tissues remarkably upregulated compared with the adjacent tissues. Figure B: Serum miR-96 mRNA expression in patients with UFs remarkably upregulated compared with healthy controls. Figure C: The ROC curve.
a indicates P<0.001.

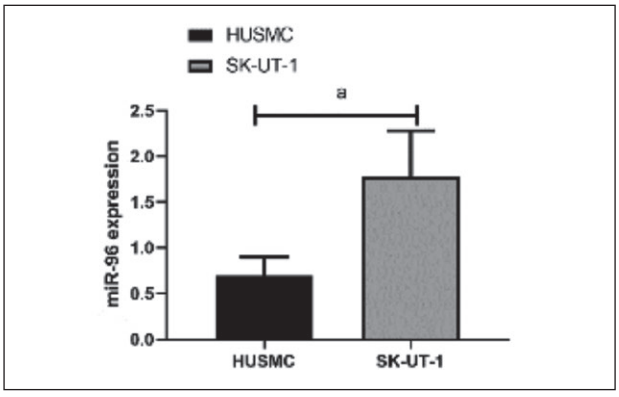

Figure 2: miR-96 expression in cells. miR-96 expression in the cells. a indicates P<0.001.

Transfection

• SK-UT-1 cells were chosen for transfection. According to qRT-PCR, after transfection, miR-96 expression was remarkably lower in the miR-96-inhibitor group but remarkably higher in the miR-96-minics group compared with the NC group (both P<0.001). See Figure 3.

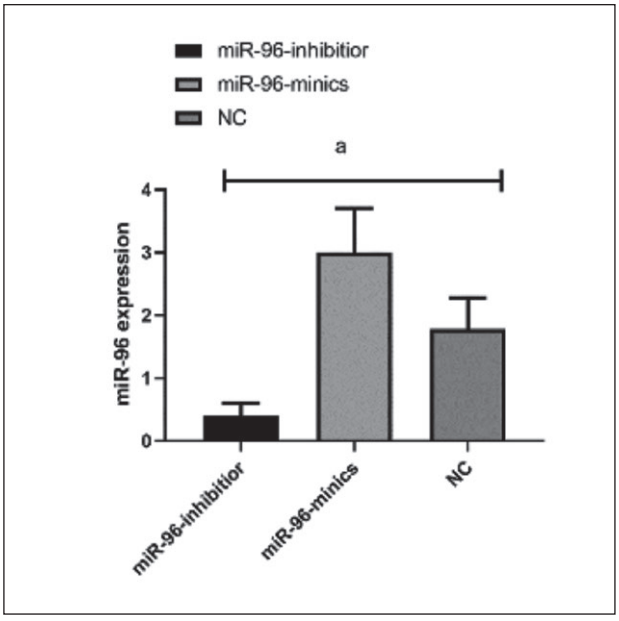

Figure 3: miR-96 expression in SK-UT-1 cells after transfection. miR-96 expression in SK-UT-1 cells after transfection.

• According to MTT assay, cell proliferation remarkably reduced in the miR-96-inhibitor group, but enhanced in the miR-96-minics group compared with the NC group (both P<0.001). According to flow cytometry, the apoptotic rate was remarkably inhibited in the miR-96-minics group compared with the NC group (P<0.001). According to Transwell, cell invasion remarkably reduced in the miR-96-inhibitor group, but remarkably enhanced in the miR-96-minics group compared with the NC group (P<0.001).

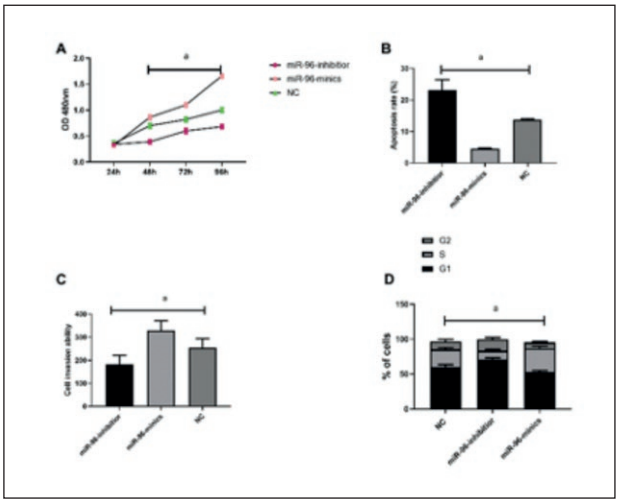

Figure 4: Effects of miR-96 on biological functions of SK-UT-1 cells. The proliferation of SK-UT-1 cells after transfection (A). The apoptosis of SK-UT-1 cells after transfection (B). The invasion of SK-UT-1 cells after transfection (C). The cell cycle of SK-UT-1 cells after transfection (D).
Note: a indicates P<0.001.

BRCA1 was a functional target of miR-96 for inhibiting UFCs

• Firstly, the prediction by Targetscan6.2 showed that there were complementary binding sequences between BRCA1 and miR-96 in their 3'-UTR, respectively, so we carried out DLRGA. BRCA1 overexpression remarkably reduced miR-96-Wt luciferase activity (P<0.001), with no effect on miR-96-Mut luciferase activity (P>0.05). See Figure 5.

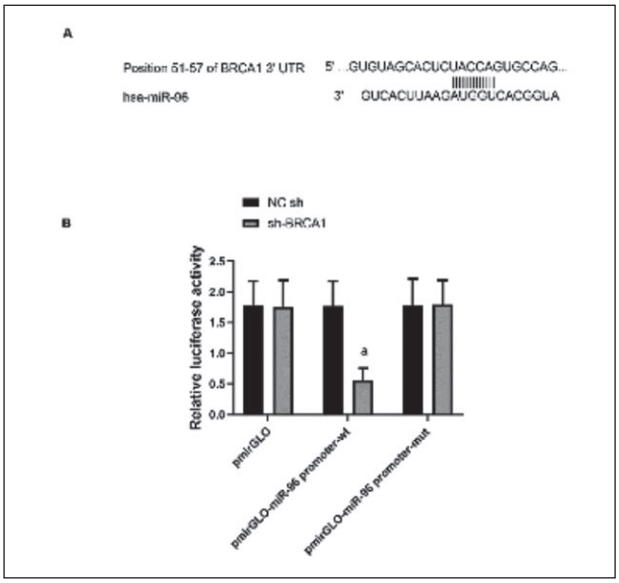

Figure 5: DLRGA. Figure A: There was a binding site between miR-96 and BRCA1, according to relative luciferase activity-DLRGA. Figure B: The viability of SK-UT-1 cells after transfection.
Note: a indicates P<0.001.

• For verifying the relationship between BRCA1 and miR-96, we explored the role of BRCA1 in UFs by co-transfecting miR-96-minics and sh-BRCA1 into SK-UT-1 cells. According to WB, compared with the NC group, BRCA1 protein level in the sh-BRCA1 group remarkably rose (P<0.001), showing that the inhibitory effect of miR-96-minics on the level could be reversed by transfection with sh-BRCA1.

According to the functional analysis, after transfection with sh-BRCA1, SK-UT-1 cells had remarkably inhibited proliferation and invasion, but remarkably enhanced apoptosis (all P<0.01). These reveal that BRCA1 is a functional target of this miR in SK-UT-1 cells. See Figure 6.

Effects of miR-96 on SK-UT-1 apoptosis and related proteins

We further transfected miR-96-minics/sh-BRCA1 into SK-UT-1 cells to detect their biological functions. Compared with the NC group, S-phase cells reduced, Bax and Bak remarkably upregulated, and Bcl-2, APR3 and Bcl-xl were remarkably inhibited in the sh-BRCA1 group. After transfection with miR-96-minics/sh-BRCA1, S-phase cells rose, Bax and Bak was remarkably inhibited compared with the sh-BRCA1 group, and Bcl-2 and Bcl-xl remarkably upregulated (P<0.05). See Figure 7.

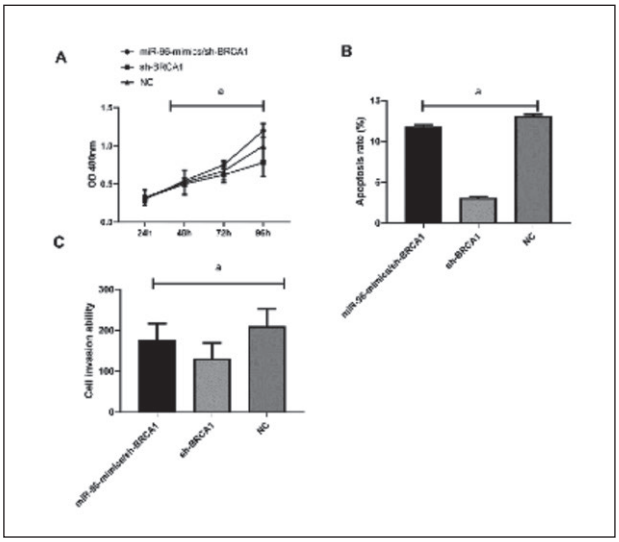

Figure 6: Cell viability. Figure A: Cell proliferation. Figure B: Apoptosis. Figure C: Cell invasion.
Note: a indicates P<0.001.

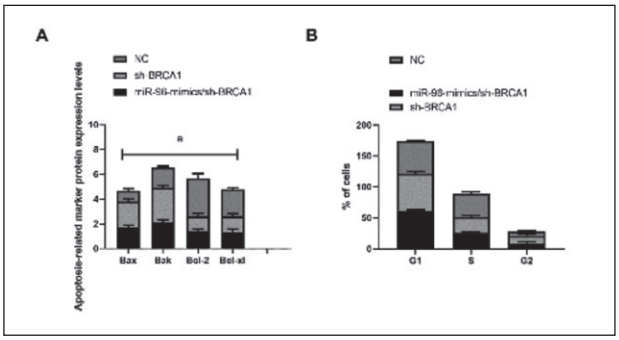

Figure 7: Effects of miR-96 on SK-UT-1 cell cycle and apoptosis-related proteins. Figure A: Changes in cell cycle after miR-96-minics/sh-BRCA1 transfection. Figure B: Apoptosis-related proteins.
Note: a indicates P<0.001.

Discussion

miRNAs, whose regulatory effect has been gradually valued in clinical research on the development of new molecular targeted therapy, affect cancer cell development via regulating gene expression[17-18]. According to many cancer reports, miR-96 exerts a carcinogenic function in various cancers, but its impact on the development and progression of UFs has not been clarified[19-20]. This study is designed to investigate miR-96 expression in patients with UFs and its effects on UFC proliferation and apoptosis, further providing a new theoretical ba-

sis for the molecular targeted therapy of UFs. In this study, we used qRT-PCR technologies to detect miR-96 expression in the uterine fibroid tissues, and found that this miR abnormally upregulated in the patients' tumor tissues and serum. Additionally, the AUC of this miR was >0.8 based on the ROC curve. As reported by present studies, this miR expression increases in cholangiocarcinoma tissues and cell lines, respectively, and its high expression is related to poor prognosis, so this miR has been identified as an important clinical and biological predictor of oncogenes in cholangiocarcinoma[21-22]. Other miRs of the miR-96 family are also considered as inducers of tumors, since they show upregulation in cancer tissues. For example, miR-96-5p expression in breast cancer tissues and cell lines remarkably rises[23]. miR-96 upregulates in serum samples of ovarian cancer patients, and its overexpression promotes ovarian cancer cells to proliferate and migrate[24]. We detected the TCGA database and this miR expression in UFCs, finding that this miR was highly expressed in the cells, which is consistent with the results of this study. This suggests that miR-96 may have a regulatory effect on UFs.

In cell experiments, we compared SK-UT-1 with HUSMC cells, and found that this miR was highly expressed in the former. Subsequently, we silenced and overexpressed miR-96 in SK-UT-1 cells, and observed results of biological functions, founding that the cell proliferation and invasion were remarkably inhibited after miR-96 was inhibited. This indicates that this miR can be used as a potential target for treating UFs, with its downregulation inhibiting UFCs to proliferate and invade. Acting as a tumor suppressor through targeting BCR-ABL1 oncogene during the transformation of chronic myeloid leukemia[25], miR-96 induces the progression of non-small cell lung cancer via competitive endogenous RNA network and affecting EGFR signaling pathway. This further demonstrates the influence of miR-96 changes on UFCs[26].

Finally, we observed biological functions of the cells and changes in the apoptosis-related proteins by transfecting miR-96-minics/sh-BRCA1 into SK-UT-1 cells. Compared with the NC group, S-phase cells reduced, Bax and Bak remarkably upregulated, and Bcl-2, APR3 and Bcl-xl were remarkably inhibited in the sh-BRCA1 group. After transfection with miR-96-minics/sh-BRCA1, S-phase cells rose, Bax and Bak were remarkably inhibited compared with the sh-BRCA1 group, and Bcl-2, APR3 and Bcl-xl remarkably upregulated. This suggests that miR-96 can target and regulate BRCA1. There is evidence that miRNAs are regulated by a variety of mechanisms and involved in tumor development and progression through binding to target mRNAs' 3'-UTR[27]. Silencing miR-96-5p can remarkably reduce cell activity, increase apoptosis, and inhibit cell migration, cell invasion and Bcl-2 and COX-2 expression, as well as promote Bax, cleaved caspase-3 and cleaved caspase-9[28]. The DLRGA was conducted to further verify the correlation with miR-96 with BRCA1. The luciferase activity of BRCA1-3'UTR Wt remarkably rose after miR-96 overexpression, which had no effect on that of BRCA1-3'UTR Mut. Moreover, miR-96 expression remarkably increased after si-BRCA1 transfection, which indicates a targeted regulatory relationship between the two. Therefore, we believe that inhibiting miR-96 expression can promote BRCA1 expression, thus affecting the apoptosis of UFCs.

In this study, we have confirmed that miR-96 is highly expressed in UFs, and that its overexpression can inhibit cell proliferation mediated by this miR. However, this study still has shortcomings and lacks animal experiments. The regulatory network of miR-96 is still unclear, so further research is needed to determine whether it can affect the development and progression of tumors through other ways. Therefore, we hope to explore the regulatory network through bioinformatics analysis in future research, so as to provide more basis for our experiments.

In summary, miR-96 can regulate the growth and apoptosis of UFCs by regulating BRCA1, so it is expected to become a potential therapeutic target for UFs. Moreover, this miR may be a useful marker for evaluating UFs' diagnosis and prognosis.

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