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MiR-93-5p promotes cervical cancer progression by targeting THBS2/MMPS signal pathway

X.-Y. SUN[1], X.-M. HAN[2], X.-L. ZHAO[3], X.-M. CHENG[4], Y. ZHANG[5]

[1]Department of Reproductive, Yantaishan Hospital, Yantai, China
[2]Department of Reproductive, Zoucheng Maternal and Child Health and Family Planning Service Center, Zoucheng, China
[3]Department of Reproductive, Yantai Yuhuangding Hospital, Yantai, China
[4]Department of Surgery, Qingzhou Maternal and Child Health Care Hospital, Qingzhou, China
[5]Department of Gynecology, Weifang People's Hospital, Weifang, China

Xueyan Sun and Xuemei Han contributed equally to this work

Abstract. – OBJECTIVE: The aim of this study was to investigate the potential effects of microRNA-93-5p (miR-93-5p) on the development of cervical cancer (CC), and to explore the underlying mechanism.

PATIENTS AND METHODS: The expression level of miR-93-5p in CC tissues and cells was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Online prediction software and Luciferase reporter assay were used to evaluate the possible target of miR-93-5p. Furthermore, the effects of miR-93-5p on siHa cells were determined by Western blot, Cell Counting Kit-8 (CCK-8), scratch-wound and transwell assays, respectively.

RESULTS: In our study, miR-93-5p was found highly expressed in CC tissues and cells. Thrombospondin-2 (THBS2) was predicted and experimentally verified as a direct target of miR-93-5p. Subsequent experiments showed that decreased expression of THBS2 resulting from miR-93-5p up-regulation could significantly promote the proliferation, invasion and migration of siHa cells. At the same time, miR-93-5p remarkably increased the expression of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), whereas decreased the expression of extracellular matrix (ECM).

CONCLUSIONS: Our research discovered the promotion function of miR-93-5p on CC by targeting THBS2/Matrix metalloproteinases (MMPS) signaling pathway. We revealed that miR-93-5p might be a potential therapeutic target for the treatment of CC.

Key Words:
MiR-93-5p, Cervical cancer (CC), Thrombospondin-2 (THBS2), Matrix metalloproteinases (MMPS).

Introduction

Cervical cancer (CC), an epithelial malignant tumor in the cervix of the uterus, is one of the most common gynecologic malignancies[1]. According to statistics, there are approximately 500,000 new cases of CC around the globe every year. Most of these cases occur in developing countries, and about 270,000 people die from CC every year[2]. The prognosis of patients with early CC is usually satisfactory after standardized treatments, with a 5-year survival rate of 80-90%. However, the 5-year survival rate of CC patients with lymph node metastasis is less than 50%. Therefore, the early diagnosis and effective treatment strategies of CC have always been the emphases of gynecologic oncology.

Micro-ribonucleic acids (miRNAs) are a category of non-coding single-stranded RNA molecules with 19-23 nucleotides in length. MiRNAs can bind to the 3'-untranslated region (UTR) of corresponding target genes by complete or incomplete pairing. Eventually, they can degrade target genes or inhibit the translation of target genes as well as regulate the expression of endogenous genes[3]. As a result, miRNA is a major breakthrough in the field of gene expression regulation[4]. By comparing the expression profiles of miRNA in normal tissues and tumor tissues, it has been discovered that some miRNAs are abnormally expressed in multiple malignant tumors[5]. Studies have manifested that the occurrence and development of malignant tumors are associated with abnormal changes in the expression of

Corresponding Author: Yan Zhang, MM; e-mail: yszy04512@tom.com

several miRNAs. Meanwhile, miRNAs could be classified as oncogenic and anti-oncogenic miRNAs, whose increased expressions target tumor suppressor and tumor promoting proteins, respectively[6]. Therefore, the functions of miRNAs have become the hotspots in cancer targeting therapy research in recent years.

MicroRNA-93-5p (miR-93-5p) is a member of the miR-106b-25 family, which is located on chromosome 11q22.1. It is relatively conserved in mammals. In the field of cancer research, miR-93-5p has been found to be widely involved in the progression of malignant tumors, especially in digestive system tumors. Low expression of miR-93 is closely associated with metastasis, differentiation and poor prognosis of colon cancer. However, the up-regulation of miR-93 significantly inhibit invasion and migration of colon cancer by inhibiting the Wnt/β-catenin signaling pathway[7]. In hepatocellular carcinoma, Ohta et al[8] has reported that miR-93 promotes the proliferation, invasion and migration of hepatocellular carcinoma cells by activating c-Met/PI3K/Akt pathway by targeting phosphate and tension homology deleted on chromosome ten (PTEN) and Cyclin Dependent Kinase Inhibitor 1A (CDKN1A). Meanwhile, miR-93-5p has been demonstrated to participate in the carcinogenesis of gastric cancer[9], esophageal cancer[10] and other non-digestive system tumors[11,12]. However, fewer studies have explored the specific role of miR-93-5p in CC. Therefore, the aim of this work was to explore the expression of miR-93-5p in clinical samples of CC and its biological role in CC cells.

Patients and Methods

CC Clinical Samples and Cell Lines

CC clinical tissue samples were obtained from 28 CC patients undergoing a surgical procedure at the Yantaishan Hospital. All collected tissues were confirmed by pathological report after the operation. Para-cancerous tissue samples were more than 5 cm from tumor tissues, which were all confirmed no cancer cells. Chemotherapy or radiotherapy treatment was forbidden. Collected CC tissue samples and para-cancerous tissue samples were preserved in liquid nitrogen for subsequent use. Declaration of Helsinki should be mentioned and respected. This study was approved by the Ethics Committee of the Yantaishan Hospital. Signed informed consents were obtained from all participants before the study.

CC cell line (siHa) together with normal human embryonic kidney cell line (293T) was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 UI/mL penicillin and 0.1 mg/mL streptomycin in an incubator with 5% CO_2 at 37°C.

Luciferase Reporter Assay

TargetScan, miRDB and microRNA websites predicted that Thrombospondin-2 (THBS2) was a target gene of miR-93-5p. Wild-type THBS2 3'-UTR (THBS2-wt) plasmid and mutant THBS2 3'-UTR (THBS2-mut) plasmid were constructed using genomic DNA of human CC cells as template. SiHa cells in the logarithmic growth phase were first collected. Meanwhile, cell density was adjusted to 2×10^5 cells/well using Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA). Subsequently, THBS2-wt, THBS2-Mut, and miR-93-5p mimics or NC (negative control)-mimics were co-transfected into siHa cells for 48 h. Finally, Luciferase activity in cells was measured.

Cell Transfection

MiR-93-5p mimics, NC-mimics and LV-THBS2 were designed and synthesized by GenePharma (Shanghai, China). The above plasmids were transfected into siHa cells according to the manufacturer's instructions of Lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA). Three groups were established for *in vitro* functional tests, including NC group, miR-93-5p mimics (siHa cells transfected with miR-93-5p mimics) group and mimics + THBS2 (siHa cells transfected with miR-93-5p mimics and LV-THBS2) group.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The expression of the genes in siHa cells was detected *via* Real Time fluorescence quantitative Polymerase Chain Reaction (qPCR). Total RNA was extracted in cells according to the instructions of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) was used to measure the expression level of miR-93-5p normalized to miRNA U6. Primer sequences used in this study were as follows: miR-93-5p, F:

5'-GCCATGTAAACATCTCGGACTG-3', R: 5'-CAATGCGTGTGGTGGAGGAG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Western Blot

Cells in each group were first collected after transfection. Total protein in cells was extracted using a protein extraction kit. Subsequently, the concentration of extracted protein was detected by the bicinchoninic acid (BCA) protein assay kit (Pierce, Waltham, MA, USA). After that, 50 μg of protein sample was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto membranes. After sealing at 4°C for 4 h, the membranes were incubated with primary antibody (1:1000, all from Abcam, Cambridge, MA, USA) at 4°C overnight. On the next day, the membranes were incubated with Horse Reddish Peroxidase (HRP)-labeled secondary antibody (1:5000) at room temperature for 2h. Finally, enhanced chemiluminescence (ECL) assay was performed in accordance with ECL kit instructions (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Proliferation

24 h after transfection, 2×10^3 cells were seeded into 96-well plates in miR-93-5p mimics group, miR-93-5p inhibitor group and NC group. Then, the cells were cultured in an incubator with 5% CO_2 at 37°C for 24 h. Subsequently, 10 μL of Cell Counting Kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was added to each well, followed by incubation for 2 h in the dark. Optical density (OD) value at the wavelength of 450 nm was measured by a microplate reader. Cell proliferation rate (%) = (OD value in the experimental group - OD value in the blank control group) (OD value in the control group - OD value in the blank control group) ×100%.

Cell Migration

Transfected cells in each group were harvested and cultured again in a constant-temperature incubator. When the cell fusion rate reached about 90%, horizontal scratch-wound was made using a 200 μL pipette tip perpendicular to the bottom of 6-well plates. Then, the cells were cultured in a constant-temperature incubator for another 24 h, followed by photography and observation under an inverted light microscope (×100). After that, Image J software (Bethesda, MD, USA) was utilized to measure the width of the scratch-wound. Finally, the scratch-wound healing rate was calculated.

Cell Invasion

After transfection, cells in each group were collected, and cell concentration was adjusted to 2×10^5 cells/mL. 200 μL of cell suspension was added into the upper chamber with pre-laid diluted Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), and the volume was supplemented to 1 mL using DMEM. Meanwhile, the lower chamber was added with 600 μL of DMEM. Subsequently, cells were cultured in a constant-temperature incubator for 24 h. After that, the chambers were taken out to wipe the Matrigel and cells on it. Next, the chambers were fixed in 4% paraformaldehyde for 20 min, air dried at room temperature and stained with Gimesa dye solution for 20 min. Then, a PC membrane was taken out, put onto a glass slide and mounted in gum. Cell invasion in chambers was observed under a light microscope. Six fields of vision were randomly selected for each sample, and the mean number of cells penetrating the Matrigel was calculated.

Statistical Analysis

Statistical analysis was performed with Student's *t*-test or *F*-test. All *p*-values were two-sided, and $p<0.05$ was considered statistically significant. Prism 6.02 software (La Jolla, CA, USA) was used for all statistical analysis.

Results

MiR-93-5p Expression was Significantly Decreased Both in CC Tissues and Cells

By detecting 31 clinical CC samples, we found that the expression of miR-93-5p in cancer tissues was significantly higher than that of para-cancer tissues ($p<0.001$) (Figure 1A). From the cellular point of view, we detected the expression of miR-93-5p in siHa cell line. As shown in Figure 1B, the expression level of miR-93-5p in siHa cells was almost 3 times higher than that of the control cells ($p<0.001$), which was consistent with the results in clinical samples. The siHa cell line was then chosen for the subsequent experiments.

THBS2 was a Direct Target of MiR-93-5p in CC

To investigate the potential target of miR-93-5p, we searched three publicly available algorithms to elucidate the putative and possible tar-

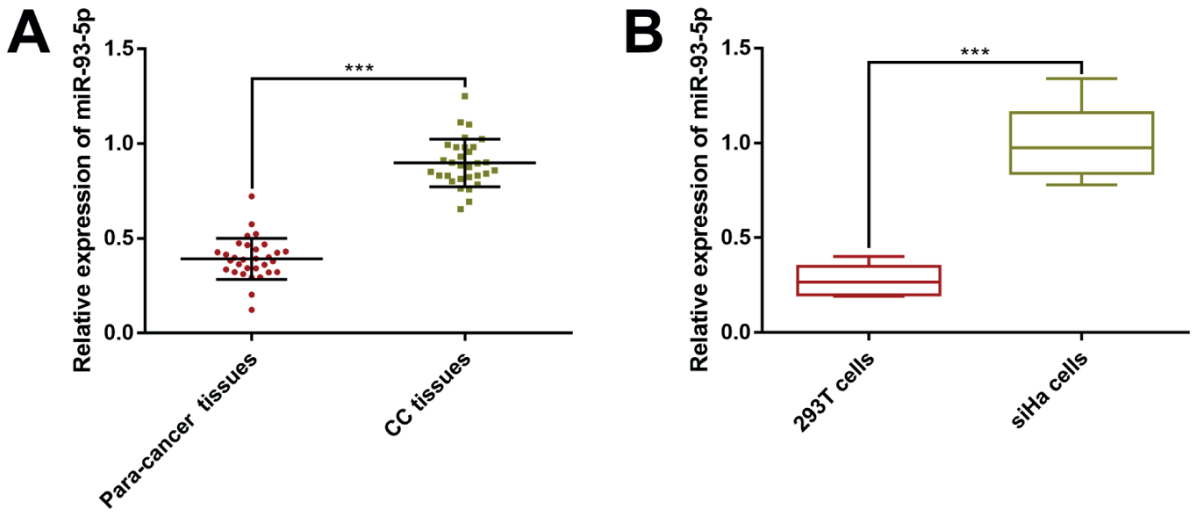

Figure 1. The expressions of miR-93-5p in cervical cancer (CC) tissue samples and cells. ***A,*** Difference in the expression of miR-93-5p between CC tissues and para-cancer tissues (***p<0.001). ***B,*** The expression of miR-93-5p in CC cells (siHa) and normal human embryonic kidney cells (293T) (****p<0.001).

gets of miR-93-5p, including TargetScan, miRDB and microRNA. The results found that THBS2 was identified as a supposed target of miR-93-5p (Figure 2A). We then confirmed the transfection efficiency of miR-93-5p in siHa cells (Figure 2B).

To further verify the effect of miR-93-5p on THBS2, we established Luciferase reporter vectors containing wild or mutant-type seed sequences of THBS2 3'-UTR. The Luciferase reporter assay demonstrated that the up-regulation of miR-93-5p with mimics transfection resulted in the decrease of Luciferase activity of THBS2-wt. However, it had no significant effect on THBS2-mut (Figure 2C). These results suggested that the expression of THBS2 was regulated by miR-93-5p.

MiR-93-5p Decreased the Expression Level of THBS2

We subsequently used Western blot assay to detect the protein expression of THBS2 in siHa cells after miR-93-5p mimics transfection. The results showed that miR-93-5p could significantly restrict the protein expression of THBS2, and its gray value was remarkably lower than that of the control group (Figure 2D).

MiR-93-5p Accelerated the Proliferation of CC Cells

Cell hyper-proliferation is a feature of tumor cell growth. To study the effect of miR-93-5p on the proliferation characteristics of siHa cells, OD values of

A

	Predicted consequential pairing of target region (top) mRNA (middle) and muntant position (bottom)
Position 1664-1670 of THBS2 3'UTR	5' ... UGUUGUUGUUUUGCU ... U ...
Hsa-miR-93-5p	3' ... GAUGGACGUGCUUGU ... C ...
Mutant type of THBS2 3'UTR	5' ... UGUUGUUGUUUUGCU GGCGUAC U ...

C

Group	The luciferase activity
THBS2-wt + NC mimics	1.103 ± 0.124
THBS2-wt + miR-93-5p mimics	0.423 ± 0.207 **
THBS2-mut + NC mimics	1.075 ± 0.132
THBS2-mut + miR-93-5p mimics	1.058 ± 0.179

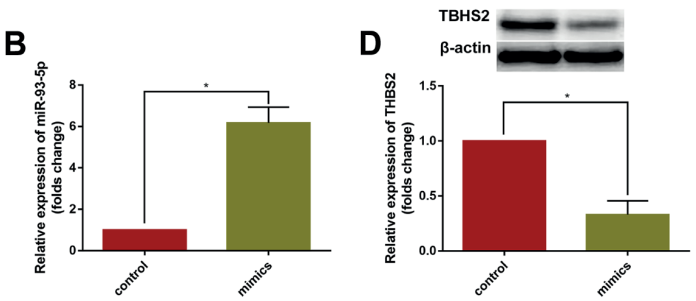

Figure 2. THBS2 was a direct and functional target of miR-93-5p. ***A,*** Diagram of putative miR-93-5p binding sites of THBS2. ***B,*** Relative activities of Luciferase reporters (**p<0.01, THBS2-wt + NC mimics group *vs.* THBS2-wt + miR-93-5p mimics group). ***C,*** Transfection efficiency detected by qRT-PCR. (*p<0.05). ***D,*** The protein expressions of TBX15 in cells after miR-93-5p mimics transfection (*p<0.05).

siHa cells at 5-time points (24 h, 48 h, 72 h, 96 h and 120 h) were detected by CCK-8 assay, respectively. The results showed that the proliferation of siHa cells in miR-93-5p overexpression group was remarkably increased than that of the control group over time. A significant difference was found on the 5th day (p<0.05). However, the proliferation curve of cells in the co-transfection group was similar to that of the control group. These findings indicated that miR-93-5p played a role in promoting the growth of CC cells (Figure 3A).

MiR-93-5p Facilitated the Invasion of CC Cells

The invasive ability of siHa cells was detected by transwell assay. By photographing and counting the number of transmembrane cells, it was found that the invasive ability of miR-93-5p overexpressing siHa cells was significantly stronger than that of cells in the NC group. The number of transmembrane cells in the miR-93-5p overexpression group was markedly more. This suggested that the invasive ability of siHa cells was remarkably enhanced after miR-93-5p up-regulation. However, the addition of THBS2 could rescue the invasive ability of siHa cells (Figure 3B).

MiR-93-5p Promoted the Migration of CC Cells

The effect of miR-93-5p on the migration ability of siHa cells was examined by scratch healing assay. The results showed that miR-93-5p up-regulation could significantly reduce the distance between scratches. However, the up-regulation of THBS2 could markedly change the effect of miR-93-5p on siHa cells and limit their migration ability (Figure 3C).

MiR-93-5p Regulated THBS2/Matrix Metalloproteinases (MMPS) Signaling Pathway

In this work, we found that, compared with siHa cells in the NC group, the protein expression level of THBS2 in siHa cells was significantly down-regulated after miR-93-5p up-regulation. This result was the same as the previous experiment. Besides, it was also observed that the expression of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) was remarkably up-regulated, while the expression of extracellular matrix (ECM) was markedly decreased in cells overexpressing miR-93-5p. This confirmed that miR-93-5p had a negative regulation effect on the THBS2/MMPs signaling pathway (Figure 4). Increased MMP-2 and MMP-9 and decreased ECM expression significantly promoted the lysis and destruction of the basement membrane, thereby promoting the invasion and metastasis of siHa cells.

Discussion

CC is a malignant tumor with an incidence rate right next to breast cancer among women worldwide. It has been confirmed that persistent infection of human papillomavirus (HPV) is the leading cause of CC attack[13]. However, it takes a very long time for persistent HPV infection to progress into CC. Only a fraction of people with persistent HPV infection eventually develops CC. This suggests that genomic alterations related to carcinogenicity after HPV infection is a crucial step in cancer progression. Therefore, it is particularly important to investigate oncogenes.

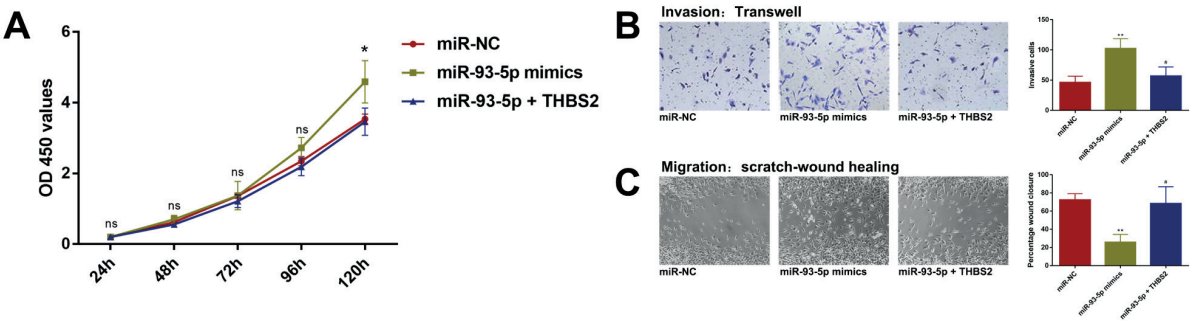

Figure 3. MiR-93-5p promoted the proliferation and invasion of CC cells. ***A,*** MiR-93-5p accelerated the proliferation of CC cells (*p<0.05, miR-93-5p mimics group *vs.* NC group). ***B-C,*** MiR-93-5p promoted the invasion and migration of CC cells. THBS2 overexpression attenuated the promotion effect of miR-93-5p on siHa cells by transwell assay (Magnification × 40) (**p< 0.01 *vs.* NC group; #p<0.05 *vs.* miR-93-5p mimics group).

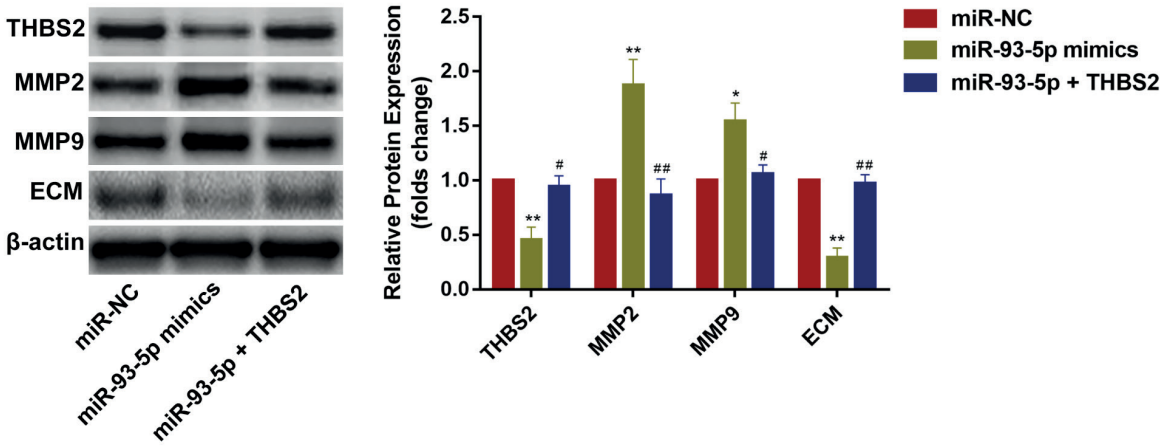

Figure 4. Effects of MiR-93-5p on related protein expression. Data were presented as means ± standard deviations (*p<0.05, **p<0.01 *vs.* NC group; #p<0.05, ##p<0.01 *vs.* miR-93-5p mimics group).

With the in-depth research of miRNAs in various tumors, it has been revealed that more than 50% of the target genes of miRNAs are located in tumor-associated genomic regions or fragile sites. It is believed that the expression profiles of miRNAs can serve as vital molecular biomarkers and therapeutic targets for multiple tumors[14]. Currently, all the studies on miRNAs focus on screening the expression profiles of miRNAs with differential expressions between tumor tissues and normal tissues (or para-carcinoma tissues). It has been revealed that differentially expressed miRNAs are directly associated with primary tumor tissues. It has been generally argued that the functions of down-regulated miRNAs are similar to those of suppressor genes, manifesting anti-cancer effects. However, the functions of up-regulated miRNAs are similar to those of oncogenes, displaying carcinogenic effects. For example, large quantities of research results have shown that the expression of classical miR-21 is up-regulated in a variety of solid tumors, including breast cancer, prostate cancer, rectal cancer, liver cancer, esophageal cancer, cervical cancer and lung cancer[15-19]. Moreover, overexpressed miR-21 can remarkably accelerate the growth of cells *in vivo* transplanted tumor models.

After the clarification of differentially expressed miRNAs, further understanding of regulated target has become another emphasis of studies on miRNAs. It has been manifested that miRNAs can regulate at least 1/3 of human genes. The same miRNA can regulate various genes, and the same gene may be regulated by different miRNAs coordinately. In this way, a complex regulatory network is formed by miRNAs and genes.

Direct infiltration and vessel metastasis are the major metastatic patterns of CC. They are high-risk factors for short-term distant metastasis and long-term local recurrence after treatment of early- and middle-stage CC. THBS2 is one of the five members of the THBS family. This family includes a category of protein factors capable of regulating the adhesion and migration ability of glycoprotein between cells and between cell and troma. Studies have shown that declined expression or lost function of THBS2 is negatively correlated with tumor neovascularization. This may eventually help to participate in the biological processes of tumors, such as incidence and progression[20,21]. THBS2 has been found to be closely associated with ECM, MMP-2, MMP-9, and other relevant factors; it can regulate their expressions[22-24].

ECM is composed of basement membrane and intercellular substances. It is a general term for macromolecular substances secreted into extracellular matrix by cells. Meanwhile, it constitutes a complicated network structure, supports and connects tissue structures, and can also serve as a crucial histologic barrier defending the invasion and migration of tumor cells. Studies have indicated that receptors on tumor cell surfaces can adhere to various molecules in ECM and destroy ECM by activating or secreting protein-degrading enzymes. Eventually, the invasion and migration can be realized. In the whole processes of invasion and metastasis, tumor cells need to

break through the basement membrane to invade normal tissues, pass in and out of vessels (mainly blood vessels and lymphatic vessels) and penetrate ECM. Therefore, the *in situ* carcinoma may develop into invasive carcinoma. Among ECM-associated factors, MMPs are considered as proteolytic enzymes playing a key role in cell invasion and metastasis. Almost all the protein components in ECM can be degraded by them. Therefore, the histologic barrier in the body is destroyed, thereby accelerating the invasion and metastasis of tumor[25]. MMPs refer to the endopeptidase family. Meanwhile, many types of malignant tumors are accompanied by disordered secretion and increased activity of MMP-2 and MMP-9, such as colorectal cancer, prostate cancer, bladder cancer and pancreatic cancer[26,27]. MMPs exerts important effects on the growth, metastasis and invasion of the cervical malignant tumor. It has been reported in the literature that the expression levels of MMP-2 and MMP-9 are significantly elevated along with the exacerbation of cervical lesions, which is related to lymph node metastasis and tumor relapse[28].

Under physiological conditions, mesangial ECM contains type I collagen and type IV collagen. The degradation of type IV collagen is a crucial step in cell invasion and migration. Therefore, type IV collagenase (MMP-2 and MMP-9) are studied most frequently. Yang et al[29] have reported that loss of THBS2 expression may directly lead to increased MMP-2 protein level, resulting in the cell-Matrigel defect and further speeding up the invasion and migration of tumor cells. According to the study of Hirose et al[30], reduced protein expression of THBS2 directly affects the expressions of MMP-2 and MMP-9 among all MMPs. Some studies[31,32] also have revealed that the expression of THBS2 protein can repress angiogenesis and metastasis, which exerts negative correlations with the prognosis of breast cancer and colon cancer. Nakamura et al[33] have discovered that the overexpression of THBS2 significantly decreases the expression of MMP-9. Consequently, the invasiveness of pancreatic cancer cells cultured *in vitro* is markedly inhibited. This research finding is consistent with the experimental result of Calabro et al[34]. They have constructed plasmids with overexpressed THBS2 and conducted xenograft in nude mice. In our research, miR-93-5p was highly expressed in both CC tissues and cells. In subsequent experiments, we selected one of the downstream targets of miR-93-5p, THBS2, for further studies. We found that miR-93-5p could significantly inhibit the expression of THBS2 in siHa cells. Meanwhile, miR-93-5p had a valuable promotion effect on the proliferation, invasion and migration of siHa cells. At the same time, it was observed that in siHa cells with stably up-regulated miR-93-5p expression, the protein expressions of MMP-2 and MMP-9 expressions were markedly up-regulated. However, ECM expression was remarkably decreased. The changes in these protein molecules might be the reason for the enhanced vicious capacity of siHa cells with up-regulated miR-93-5p expression in the previous experimental research.

Conclusions

We indicated that miR-93-5p expression was significantly up-regulated in CC tissues. The regulation of THBS2 expression promoted the proliferation, invasion and migration of CC cells. This might be achieved by regulating the THBS2/MMPS signaling pathway. As such, miR-93-5p acted as an oncogene in CC. Our findings might provide experimental evidence for searching new molecular therapeutic targets for CC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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