European Review for Medical and Pharmacological Sciences 2018; 22: 1899-1906

MiR-17-5p promotes cervical cancer cell proliferation and metastasis by targeting transforming growth factor-β receptor 2

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) play critical roles in post-translational gene expression. The aim of the current study was to investigate the effects of miR-17-5p in cervical cancer.

PATIENTS AND METHODS: Fifteen clinical cervical cancer tissue samples, as well as their paired adjacent noncancerous tissues, were collected. The microarray was performed to identify differential miRNAs in cervical cancer. Luciferase reporter assay was conducted to identify the target gene of selected miRNA. SiHa was transfected with mimics, inhibitors as well as negative controls of miR-17-5p and Targeting Transforming Growth Factor-β Receptor 2 (TGFBR2) open reading frame or siRNA. Cell counting kit-8 (CCK-8) assay and transwell experiment were performed to detect the proliferation rate and metastasis, respectively. Western blotting and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis were used to analyze TGFBR2 expression. Balb/c nude mice were utilized to verify the effect of miR-17-5p *in vivo*.

RESULTS: Microarray analysis identified miR-17-5p as our interesting miRNA, and luciferase reporter assay identified TGFBR2 as its target gene. MiR-17-5p overexpression significantly enhanced cervical cancer cell proliferation and metastasis. In-vivo study also verified that miR-17-5p overexpression stimulated cervical cancer growth.

CONCLUSIONS: MiR-17-5p enhances cervical cancer proliferation and metastasis via targeting TGFBR2. It is proposed that targeting miR-17-5p may be a promising therapeutic approach for cervical cancer.

Key Words:
microRNAs, Cervical cancer, Microarray, TGFBR2, miR-17-5p.

Introduction

Cervical cancer is known as one of the most common and lethal gynecological cancers in the world[1,2]. Increasing researches revealed that continuous infection of human papillomavirus (HPV), especially high-risk HPV[3,4], was correlated with the initiation of cervical cancer. HPV16 is the most prevalent among more than 20 common subtypes of high-risk HPV[5]. However, not all cervical cancer patients are detected with HPV infection, which indicates that many other factors may exert critical influence on the initiation and progression of cervical cancer[6-8]. Although emerging studies have been published about the progression mechanisms of cervical cancer, its mechanisms are still unclear.

MicroRNAs (also known as miRNAs) are a cluster of small, noncoding and single-stranded RNAs, which were highly conserved in plants, animals and even some viruses. They regulate gene expression post-translationally[9-11]. MiRNAs are partially or completely combined with mRNA molecules as complementary sequences[12,13]. The dysregulation of miRNAs is associated with many diseases. It has been reported that the mutation of miRNAs can be found in some inherited diseases such as hereditary progressive hearing loss[14] and skeletal and growth defects[15]. In recent years, an increasing number of miRNAs have been found to be linked with cancers. For example, low levels of miR-324a can serve as an indicator for poor survival[16]. Furthermore, even some specific miRNAs are associated with certain histological subtypes of colorectal cancer[17]. In recent years, researchers realized miR-17-5p expresses

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abnormally high in cervical cancer[18] while its detailed role in the progression of cervical cancer remains unclear.

In our work, miR-17-5p expression level and functions in cervical cancer were analyzed. Results of this study indicated high miR-17-5p expression in cervical cancer tissue samples when comparing with adjacent noncancerous tissue. The overexpression of miR-17-5p enhances cervical cancer cell proliferation and metastasis via Targeting Transforming Growth Factor-β Receptor 2 (TGFBR2). The results indicated a promising therapy target for cervical cancer.

Patients and Methods

Human Tissue Samples Collection

Fifteen cervical cancer samples and their counterpart paired adjacent noncancerous tissues from patients who received surgeries in Northwest Women's and Children's Hospital were collected. All patients agreed and signed the consent. This research was fully approved by the Ethics Committee of Northwest Women's and Children's Hospital. The pathologic type of all samples was confirmed by two independent pathological experts. Tissues were frozen in liquid nitrogen immediately and stored at -80°C.

Cell Line Culture and Transfection

SiHa was cultured in DMEM (Dulbecco's Modified Eagle Medium) (Gibco, Rockville, MD, USA) at 37°C with 5% CO_2 containing fetal bovine serum (FBS) (10%) (Gibco, Rockville, MD, USA) and penicillin/streptomycin (1%) (Solarbio, Beijing, China). SiHa was plated in 6-well plates and was transfected with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) and Opti-MEM (Gibco, Rockville, MD, USA). Mimics, inhibitors and negative controls of miR-17-5p were transfected into SiHa, respectively. In order to overexpress or downregulate TGFBR2, TGFBR2 open reading frame was purchased, which did not contain the miR-17-5p-responsive 3'-UTR from FulenGen (Guangzhou, China). TGFBR2 siRNA was synthesized by RiboBio Company (Guangzhou, China). An empty plasmid was utilized as a negative control.

Cell Proliferation Assay

Transfected SiHa was seeded in 96-well plates at a density of 5×10^3 cells each well. 48 h later, 100 μL Roswell Park Memorial Institute-1640 (RPMI-1640) medium mixed with 10 μL cell counting kit-8 (CCK-8) solution was added to each well. The optical density of each well was detected at 450 nm after incubation at 37°C for 30 min. The analysis was repeated for at least three times.

Microarray and miRNA Target Site Prediction

Fifteen cervical cancer tissue samples and fifteen paired noncancerous tissues from surgeries were selected. Total RNA was isolated with TRIzol reagent and reversely transcribed with miScript II RT kit (Qiagen, Hilden, Germany). Then, labeling and hybridization were performed by GeneChem (Shanghai, China) according to the Agilent human 8×60 K miRNA microarray system. After being washed sequentially, slices were scanned with microarray scanner at 5 μm promptly. At last, the scanned images were quantified with Feature Extraction software and differentially expressed miRNAs were recognized. For the differentially expressed miRNAs, we utilized miRanda (http://www.microrna.org) and Target Scan (http://www.targetscan.org) to find out promising target genes.

Luciferase Reporter Assay

After cells reached about 90%, pEZX-MT01 vector was transfected to SiHa cells where wild or mutant type of 3'UTR of TGFBR2 were cloned in special medium (reduced serum as well as antibiotics-free OptiMEM) supplemented with oligofectamine 2000 for 6 h. Firefly luciferase and renilla luciferase were the reporter gene and tracking gene, respectively. In addition, mimics, inhibitors or negative controls of miR-17-5p were transfected to cells. 24 h later, luciferase and renilla strength were detected in cell lysates by a Dual Luciferase Reporter Assay kit (Promega, Madison, WI, USA).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Analysis

TaqMan miRNA reverse transcription kit was purchased from Applied Biosystems (Shanghai, China). Total RNAs were extracted from cells with TRIzol and reversely transcribed to get cDNAs. TaqMan Universal PCR Master Mix plugin with MicroRNA Assay Mix together with cDNAs were mixed together and synthesized for DNAs with the Step One Plus Real-time PCR systems. U6 was used as internal control.

Total RNAs were extracted from cells and reversed for cDNAs. The relative mRNA levels of TGFBR2 were evaluated with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal reference. The primer sequences for TGFBR2 and GAPDH were as follows: TGFBR2 sense: 5'-GTAGCTCTGATGAGTGCAATGAC-3', TGFBR2 antisense: 5'-CAGATATGGCAACTCCCAGTG-3', GAPDH sense: 5'-AGAAGGCTGGGGCTCATTTG-3', GAPDH antisense: 5'-AGGGGCCATCCACAGTCTTC-3'.

Western Blotting Assay

After washing with ice-cold phosphate-buffered saline (PBS), cells were lysed with RIPA (radioimmunoprecipitation assay) buffer (Beyotime, Shanghai, China) to get total protein. Protein concentration was determined with a protein assay kit. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protein. After that, it was shifted to polyvinylidene difluoride (PVDF) membranes purchased from Millipore (Billerica, MA, USA). 5% fat-free milk was used to block non-specific protein interactions in Tris-buffered saline and Tween 20 (TBST) buffer. The membranes loaded with proteins were incubated with primary antibodies at 4°C. After washed for three times, membranes were then incubated at room temperature with secondary antibody conjugated with horseradish peroxide (HRP) (2 h). After washing these membranes in TBST buffer, the membranes using chemiluminescence were developed to detect antibodies concentration with GAPDH as an internal control. The antibodies, anti-TGFBR2 and anti-GAPDH were purchased from Abcam (Cambridge, MA, USA).

Transwell Cell Migration Detection

Transfected cells suspended with 200 µL serum-free medium were seeded in the upper chamber of 24-well Boyden chambers. About 600 µL medium supplemented with 10% fetal bovine serum (FBS) were added to the lower chamber. 24 h later, the chambers were fixed in formaldehyde (4%) and stained with 1% crystal violet. 5 fields of view in each membrane were randomly selected and migrated cells were analyzed.

Animals Experiment

Balb/c female mice of five-week were purchased from the Slaccs Experimental Animals Center (Shanghai, China). SiHa transfected with inhibitors, mimics or negative control of miR-17-5p were injected subcutaneously in the flank back (n = 6). We measured and calculated the volume of tumor every three days (V=a*b*b/2, a: the longest diameter, b: the shortest diameter) until the 15th day. All experiments were fully approved by the Ethics Committee of Animal Experiments in our hospital.

Statistical Analysis

Statistical product and service solutions 17.0 (SPSS Inc., Chicago, IL, USA) was used to analyze our data. Quantitative data were expressed as mean ± standard deviation (SD). Non-paired *t*-test was utilized to analyze intergroup differences in data. $p < 0.05$ was determined as statistically significant.

Results

Selection of Differentially Expressed miRNAs in Cervical Cancer

Microarray analysis was performed to detect differentially expressed miRNAs in cervical cancer samples. Results from microarray showed that some miRNAs showed high-level expression while some showed low expression. In accordance with published studies, Figure 1 showed high miR-17-5p expression in cervical cancer tissues.

MiR-17-5p Overexpression Enhances SiHa Proliferation and Migration

SiHa was transfected with inhibitors, mimics and negative controls of the miR-17-5p and

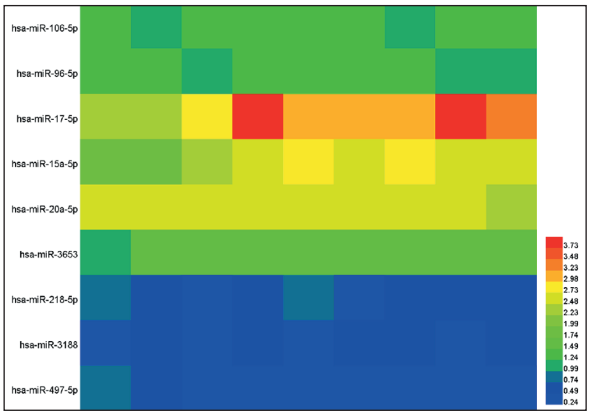

Figure 1. Selection of differential expressed miRNAs in cervical cancer. miRNA array expression profiling. Red denotes high expression and blue denotes low expression relative to the normal tissue. Only significantly upregulated and downregulated miRNAs are shown.

qRT-PCR were used to confirm the transfection effect (Figure 2A). CCK-8 assay and the transwell experiment were used to evaluate the proliferation and migration of SiHa cells, respectively. As shown in Figure 2, the miR-17-5p overexpression stimulated SiHa cell proliferation, whereas the miR-17-5p downregulation inhibited SiHa proliferation (Figure 2B). Results from the transwell experiment indicated miR-17-5p overexpression enhanced SiHa migration while miR-17-5p knock-down blocked SiHa cells migration significantly (Figure 2C). The results showed that the miR-17-5p up-regulation promoted SiHa proliferation and migration.

MiR-17-5p Targets TGFBR2 Directly

To illustrate the mechanisms of miR-17-5p promoting SiHa proliferation and migration, miRanda (http://www.microrna.org) and Target Scan (http://www.targetscan.org) were utilized to identify promising target genes of miR-17-5p. Finally, TGFBR2 was concerned among those potential targets. According to the prediction tools, 3'UTR of TGFBR2 was the target of miR-17-5p. Then a luciferase reporter construct involving the 3'UTR and mutations in the site of TGFBR2 was used to test whether miR-17-5p could directly exert influence on TGFBR2. The expression vectors of mutant and wild TGFBR2 were transfected with mimics of miR-17-5p in SiHa; then, the luciferase activity was detected. The reporter gene's luciferase activity was suppressed by upregulation of miR-17-5p, whereas this suppression effect was abolished by mutations in the miRNA binding site (Figure 3A). To further directly verify this target, SiHa cells were transfected with miR-17-5p mimics. Then, downregulated TGFBR2 levels were detected by qRT-PCR assay and Western blotting, indicating that TGFBR2 is the direct target of miR-17-5p (Figure 3B).

Silence of TGFBR2 Promotes SiHa Proliferation and Migration

In order to investigate the role of TGFBR2 in cervical cancer cell line SiHa proliferation and metastasis, we overexpressed or silenced TGFBR2 in SiHa, which was confirmed by qRT-PCR (Figure 4A). The data indicated that the silence of TGFBR2 enhanced the SiHa cells proliferation and migration while the upregulation of TGFBR2 inhibited this effect (Figure 4B-C).

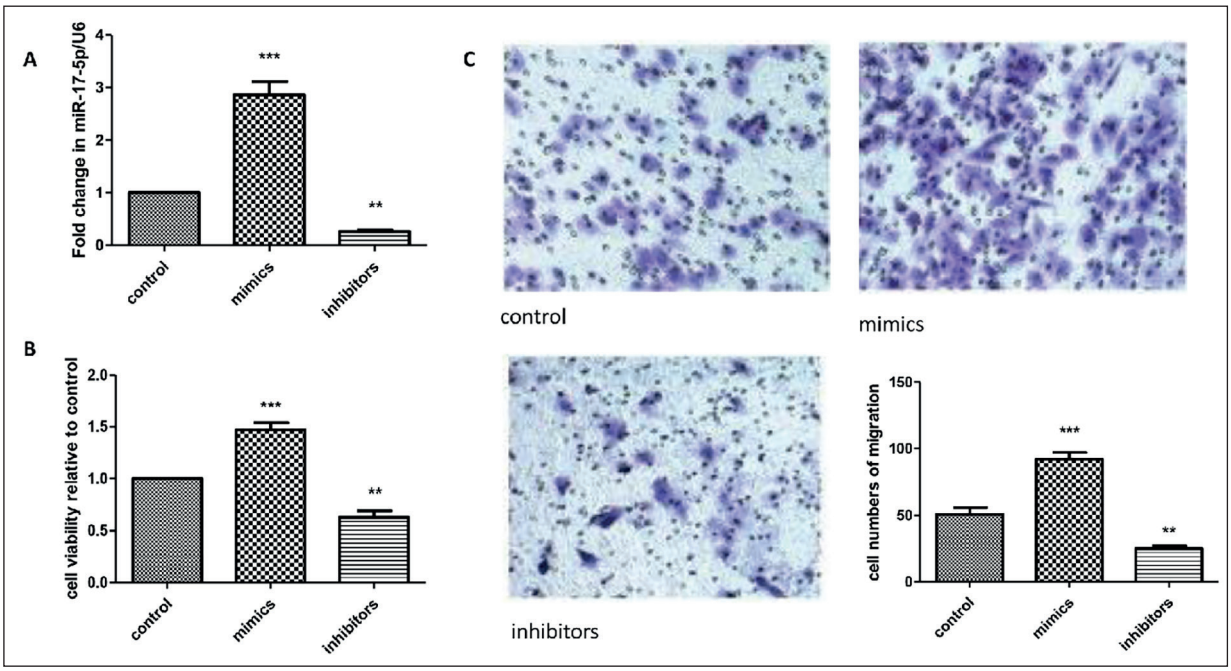

Figure 2. MiR-17-5p overexpression promotes SiHa proliferation and migration. ***A,*** SiHa cells are transfected with miR-17-5p mimics, inhibitors and negative control. QRT-PCR confirmed the corresponsive transfection effects. ***B,*** CCK-8 assay detected the cell viability of transfected cells in each group. ***C,*** Transwell assay detects the migrated cells in each group. U6 is used as the internal control. **, $p < 0.01$, ***, $p < 0.001$.

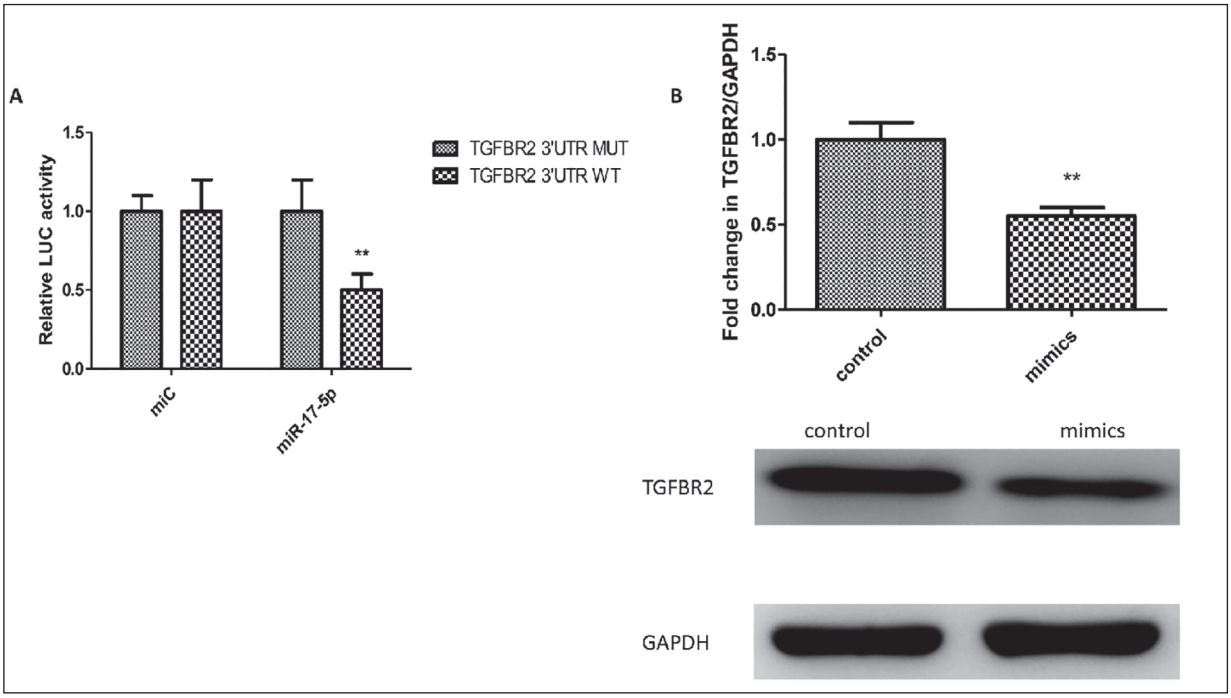

Figure 3. MiR-17-5p targets TGFBR2 directly. ***A,*** Effect of miR-17-5p over-expression on a dual luciferase reporter plasmid containing the TGFBR2-3' UTR is analyzed. Firefly and renilla luciferases are measured in cell lysate. ***B,*** QRT-PCR and Western blotting are used to measure the expression of TGFBR2 in miR-17-5p-overexpressed SiHa cells. GAPDH is used as an internal control. **, $p < 0.01$.

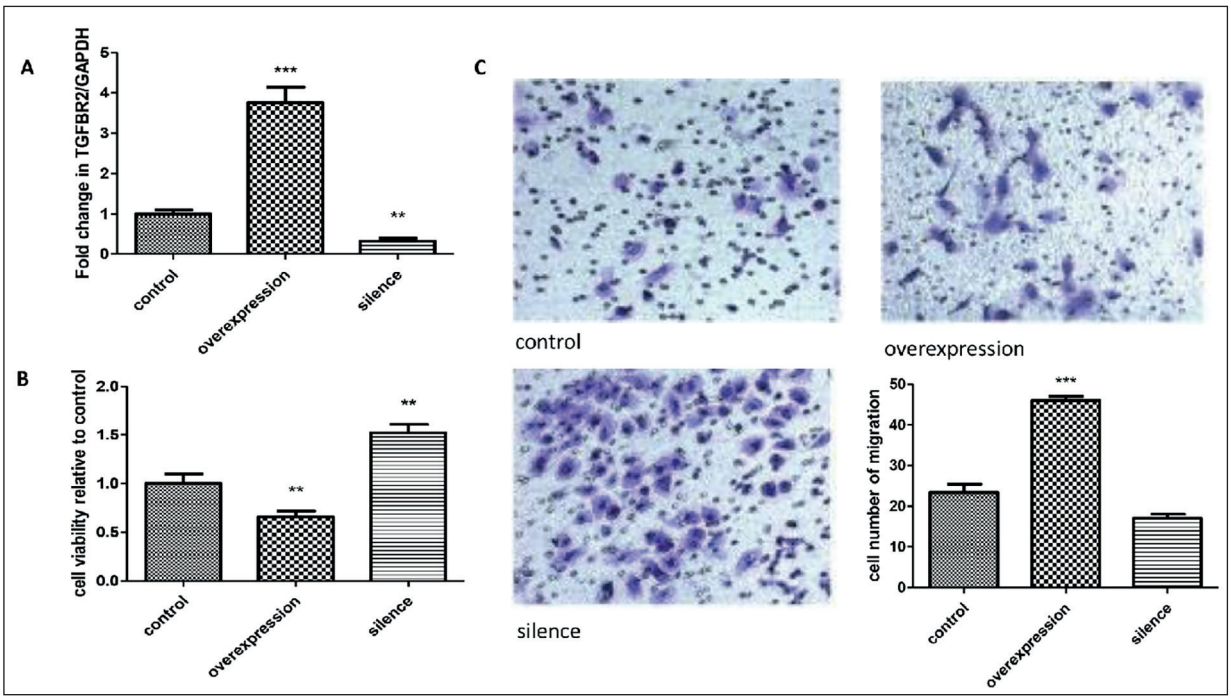

Figure 4. The silence of TGFBR2 promotes the proliferation and migration of SiHa. ***A,*** The overexpression and silence of TGFBR2 are verified by qRT-PCR analysis. GAPDH is an internal control. ***B,*** CCK-8 assay is performed to measure the cell viability of SiHa after TGFBR2 are overexpressed or inhibited. ***C,*** Transwell assay is used to detect the migration rate after TGFBR2 are overexpressed or inhibited. **, $p < 0.01$, ***, $p < 0.001$.

MiR-17-5p Overexpression Stimulates Cervical Cancer Proliferating In Vivo

In order to test the effects of miR-17-5p overexpression on cervical cancer *in vivo* further, SiHa transfected with inhibitors, mimics as well as negative controls of miR-17-5p were injected subcutaneously in the flank back (n = 6). By measuring the volume of tumors every three days in each group, it was found that the volume of tumors in miR-17-5p mimics group increased rapidly compared with that in control group (Figure 5A). Tumors were shown after all mice were sacrificed (Figure 5B). Compared with control group, tumors in miR-17-5p mimics group were larger in volume and heavier in weight.

Discussion

Currently, the role of miR-17-5p in cervical cancer was examined in this study. An inverse expression pattern of miR-17-5p and TGFBR2 was confirmed in clinical cervical cancer samples. Consisting with the prediction tools, Luciferase reporter assay verified that the TGFBR2 was the direct effective target of miR-17-5p. It was concluded that miR-17-5p overexpression promoted cervical cancer proliferation and migration while anti-miR-17-5p attenuated this effect from both *in-vitro* and *in-vivo* studies. The tumor-suppressive effect of TGFBR2 was confirmed further in cervical cancer cells by overexpressing and knocking down TGFBR2.

An emerging number of miRNAs are reported to be associated with tumor initiation, migration and invasion[19-22]. Other microarray investigations also revealed differential expressed miRNAs in different stages of cervical cancer including miR-21[23,24]. Even some studies reported that miRNAs may predict cancer patient's prognosis and responsiveness to chemotherapy[25-27]. MiR-17-5p is one of the members of miR-17-92 cluster, which is known as onco-miRNAs. MiR-17-5p was reported to be related with progression of ovarian cancers, prostate cancer and breast cancer[28]. We revealed that miR-17-5p showed high expression level in clinical cervical cancer tissues samples and promoted cervical cancer proliferation and migration.

TGF-β signaling pathway is involved in tumor inhibition and the suppression of this pathway promotes tumor progression[29]. It has been reported that TGFBR2 suppresses cancer progression by initiating the TGF-β signaling and TGFBR2 downregulation is tightly correlated with carcinogenesis as well as malignant tumor progression[30,31]. In our work, the knock down of TGFBR2 promoted the proliferation and metastasis of SiHa.

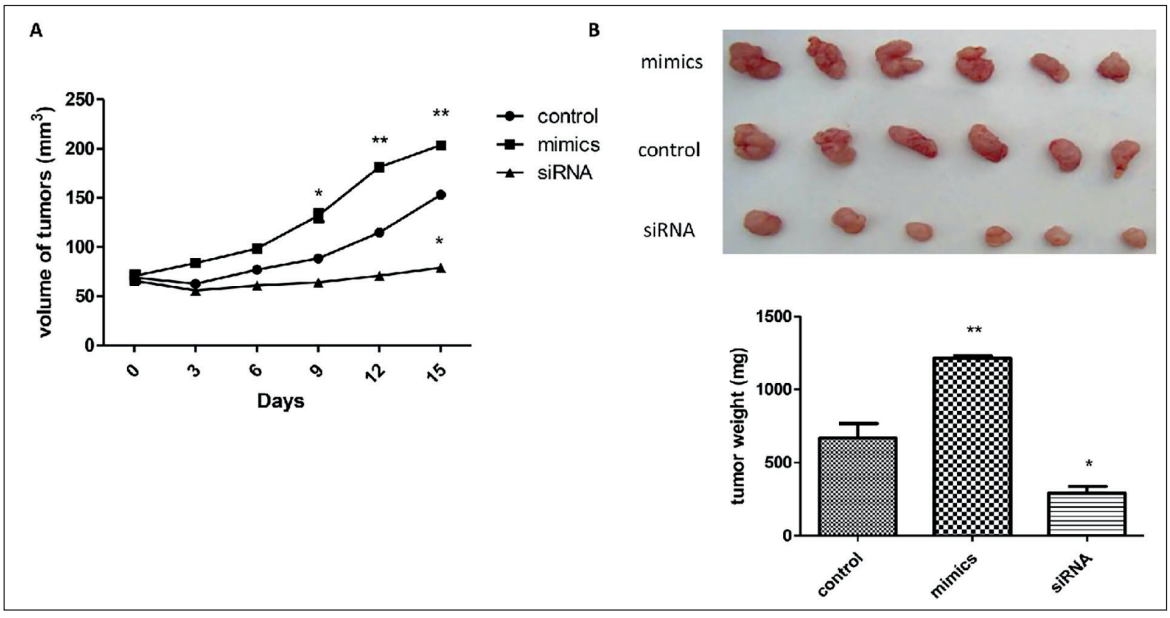

Figure 5. MiR-17-5p overexpression stimulates cervical cancer proliferating *in vivo*. ***A,*** SiHa cells transfected with miR-17-5p mimics, inhibitors and negative control are injected subcutaneously in the flank back of Balb/c mice. The volumes of tumors are measured every three days (V=a*b*b/2, a: the longest diameter, b: the shortest diameter). ***B,*** The tumors of all mice on the sacrifice day are shown. *, $p < 0.05$, **, $p < 0.01$.

Conclusions

We showed that miR-17-5p enhances cervical cancer proliferation as well as metastasis by targeting TGFBR2. What's more, the miR-17-5p overexpression functionally can stimulate tumor growth *in vivo*. Hence, it is proposed that targeting miR-17-5p might be a promising therapy for cervical cancer.

Acknowledgements

This work was supported by Young grant from the National Natural Science Foundation (No. 81301869).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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