MiR-214-3p suppresses cervical cancer cell metastasis by downregulating THBS2

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ABSTRACT

Cervical cancer (CC) is the most common human gynecological malignancy worldwide. Recently, accumulating evidences revealed the critical functions of miRNAs in the occurrence and development of cervical cancer. In our study, we aimed to demonstrate the function of miR-214-3p in regulating cell metastasis in cervical cancer. We showed low expression of miR-214-3p in cervical cancer cells and demonstrated downregulation of miR-214-3p promoted cervical cancer metastasis. Furthermore, THBS2 was identified as a novel target of miR-214-3p in cervical cancer cells. miR-214-3p suppressed THBS2 expression by directly targeting 3'UTR of THBS2, resulting in the inhibition of cell viability, invasion and migration. Taken together, the results implied inhibited effects of miR-214-3p on cervical cancer and provided new insight into potential ways for cervical cancer diagnosis and treatment.

Introduction

Cervical carcinoma, a highly prevalent gynecological malignancy, poses a significant threat to women's health worldwide (1). With over 500,000 new cases diagnosed each year and a high mortality rate (2), cervical cancer remains a major global health concern. While advancements in diagnostic and treatment techniques have contributed to a decline in the incidence and mortality of cervical cancer, its complex molecular regulation at the transcriptional and post-transcriptional levels continues to present challenges (3, 4).

In recent years, noncoding RNAs, particularly microRNAs (miRNAs/miRs), have emerged as a novel area of research that holds great promise for cancer diagnosis and understanding the underlying mechanisms of pathogenesis. MiRNAs are short, single-stranded noncoding RNAs approximately 22 nucleotides in length that function as post-transcriptional regulators by binding to specific mRNA targets. Numerous studies have implicated miRNAs in the development of cervical cancer, highlighting their roles in regulating cancer cell migration, invasion, epithelial-mesenchymal transition (EMT), and apoptosis (5-7).

One such miRNA of interest is miR-214, which has been implicated in various types of cancer and has shown both oncogenic and tumor-suppressive properties depending on the context. In human ovarian cancer, miR-214 downregulates PTEN levels by targeting its 3' untranslated region (3'UTR), leading to increased cell survival in the presence of cisplatin. Conversely, the downregulation of miR-214 results in PTEN accumulation and cell cycle arrest in the G1 phase in gastric cancer (8, 9). In breast cancer, miR-214 induces tumorigenesis by promoting the accumulation of Ezh2, thereby enhancing cell proliferation and invasion (10). Furthermore, miR-214 contributes to melanoma tumor progression through the suppression of TFAP2C (11).

In our study, we focused on miR-214-3p, a specific isoform of miR-214, and its potential involvement in cervical cancer. We observed downregulation of miR-214-3p in cervical cancer cells compared to normal human cervical cells. Through subsequent investigations, we identified thrombospondin-2 (THBS2) as a novel target of miR-214-3p. THBS2 is a multifunctional glycoprotein belonging to the thrombospondin (THBS) family and has been implicated in angiogenesis, apoptosis, cell adhesion, and cell motility through interactions with cell surface receptors, extracellular matrix proteins, and growth factors (12, 13). While previous studies have established THBS2 as an oncogene involved in the progression of melanoma, lung, and prostate tumors (14-19), limited evidence exists regarding its role in human cervical cancer (20-22).

Our study not only provides valuable insights into the dysregulation of miR-214-3p in cervical cancer but also uncovers its novel target, THBS2. By targeting the 3'UTR of THBS2, miR-214-3p effectively downregulates THBS2 expression in cervical cancer cells, leading to significant inhibition of cell viability, invasion, and migration. These findings collectively highlight the crucial role of miR-214-3p in cervical cancer.
3p in regulating cervical cancer metastasis.

The identification of miR-214-3p as a key player in cervical cancer metastasis adds to our understanding of the complex molecular mechanisms underlying this disease. Furthermore, our study offers new insights into potential diagnostic and therapeutic strategies for cervical cancer. By exploring the dysregulation of miRNAs, such as miR-214-3p, and their target genes, like THBS2, we can pave.

**Materials and Methods**

**Cell culture and cell transfection**

C33A, HEK293T cells were maintained in Dulbecco’s modified eagle medium (DMEM) (Gibco; Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco; Rockville, MD, USA) and penicillin/streptomycin (100 u/ml) at 37°C and 5% CO₂. Human primary cervical epithelial cells (HUM-iCell-F016) were purchased from iCell Bioscience Inc (Shanghai, China) and maintained in a human primary epithelial cell medium (PtiMed-iCell-001, iCell Bioscience Inc., Shanghai, China). miR-214-3p mimics (100 nM) and control were transfected into cells by using LipofectamineTM 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers’ instructions. The sequences of mimics and control: miR-214-3p mimics Sense 5'-ACAGCAGGCGACAGACGCGAGU-3’, Antisense 5'-UGCCUGUCUGGCUCCUU-3'; miRNA NC Sense 5'-UUCUCCGAGA- CGUUCGAAGATT-3’, Antisense 5'-ACGUGACAG- GUUCGAGAAATT-3’.

**RNA extraction and qPCR**

Total RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed to cDNA with an RT kit (TaKaRa Biotechnology, Tokyo, Japan). miR-214 -3p and THBS2 mRNA levels were measured by using real-time PCR. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal controls. Primer sequences used for the reactions were as follows: THBS2 (sense, 5'-CCCGCTGTTGCTACTTTTGCT-3’, antisense, 5'-CAGGGAGGTTAGAAGACCATC-3’), GAPDH (sense, 5’-CTCTCTGTCCCTCTGTTCGAC-3’, antisense, 5’-TGAGCGATGGTGTCCGCT-3’), miR-214-3p (sense, 5’-CTCTCTGTCCCTCTGTTCGCT-3’, antisense, 5’-TGAGCGATGGTGGCTCCGGT-3’), U6 (sense, 5’-TGAGCGATTGGTGGCTCCGGT-3’, antisense, 5’-GTATCCAGTGTGCTGGTGG-3’).

**Western blotting**

Total proteins were extracted and determined using a bicinchoninic acid kit (P0010; Beyotime Institute of Biotechnology, Shanghai, China). Proteins (50 µg) were separated using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After blocking with 5% non-fat milk at 37°C for 1 h, membranes were incubated with primary antibodies overnight at 4°C and HRP-conjugated secondary antibodies for 1 h at room temperature. Antibodies used were as followed: Anti-Thrombospondin 2 antibody (ab84469, Abcam, Cambridge, MA, USA), Anti-MMP2 antibody (ab92536, Abcam, Cambridge, MA, USA), Anti-MMP9 antibody (ab76003, Abcam, Cambridge, MA, USA), Anti-beta Actin antibody (ab8227, Abcam, Cambridge, MA, USA).

**Cell viability**

Cell viability was analysed by using a cell counting kit-8 (CCK-8) assay (Beyotime, Shanghai, China). In brief, cells were transfected with control and miR-214-3p mimics. 24 h after transfection, cells were re-seeded in a 96-well plate at a density of 1×10⁴ cells/well to maintain 48 h. Cells were incubated with CCK-8 solution at 37°C for 2 h and measured with a microplate absorbance reader (Thermo Fisher, Waltham, MA, USA) at a wavelength of 450 nm.

**Luciferase reporter assay.**

Wide type THBS2 3’ UTR (WT) and mutant THBS2 3’ UTR (Mut) were generated into pGL4.10 vector respectively (Promega Corporation, Madison, WI, USA). HEK293T cells were transfected with miR-214-3p mimics or control mimics together with WT THBS2 3’ UTR or mutant THBS2 3’ UTR using LipofectamineTM 2000 reagent (Invitrogen). Cells were lysed and luciferase activity was detected using a dual luciferase reporter assay kit (Promega Corporation, Madison, WI, USA) 48h after transfection.

**Scratched assay**

Cells were seeded in a 6-well plate at a density of 1×10⁴ cells/well. A scratch was performed using a sterile 200 µl pipette tip and cells were washed with PBS to remove detached cells. Images were taken at 0 h and 24 h after scratching by using a microscope.

**Transwell migration assay**

Transwell chambers were coated by Matrigel and rehydrated with serum-free DMEM in advance. Cells transfected with miRNAs as indicated were diluted and added to the upper chamber. DMEM with 10% FBS was added in the low chamber. After incubation for 6 h at 37°C, cells on the membranes were fixed and stained. Four random visual fields were counted for each sample.

**Statistical analysis**

All data were analysed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Student's t-test was used for comparing two groups. For comparing multiple groups, one-way analysis of variance (ANOVA) or two-way ANOVA was used. Results were expressed as mean ± SEM. *, P≤0.05; **, P≤0.01; ***, P≤0.001; ns, not significant.

**Results**

**Cervical cancer cells have low miR-214-3p expression.**

To determine the function of miR-214-3p in cervical cancer tumorigenicity, we analysed miR-214-3p levels both in human primary cervical epithelial cells (Control) and cervical cancer cells (C33A). Real-time PCR was performed to detect expression levels of miR-214-3p. As figure 1 showed, cervical cancer cells exhibited a decreased expression of miRNA-214 compared with human normal cervical cells.

**miR-214-3p directly targets THBS2 and downregulates THBS2 expression.**

To determine miR-214-3p target genes involved in cervical cancer, we employed two bioinformatic algorithms...
to predict the potential target candidates of miR-214-3p. Based on the prediction results, we found that THBS2 binds with miR-214-3p at 3’UTR of the gene (Figure 2A). For further confirmation, we performed a dual-luciferase reporter assay by evaluating the relative luciferase activities in HEK293T cells. We co-transfected reporter plasmid expressing the THBS2 3’UTR (WT) or THBS2 mutant 3’UTR (Mut) with miR-214 mimics. Dual-luciferase reporter assays demonstrated lower luciferase activities when we introduced mutation in THBS2 3’UTR (Figure 2B).

Next, we examined the protein levels of THBS2. We detected a high expression level of THBS2 in C33A compared with that in normal primary cervical cells (Figure 2C). In our study, western blotting also showed decreasing protein levels of MMP9 and MMP2 which are two key proteins that play critical functions in cancer cell metastasis regulated by THBS2 (22). The high expression level of THBS2 was greatly eliminated by overexpressing miR-214-3p mimics in C33A. Expressing levels of MMP9 and MMP2 were also reduced when miR-214-3p was overexpressed (Figure 3A). Meanwhile, we co-expressed exogenous THBS2 with miR-214-3p mimics to confirm the impact of miR-214-3p on THBS2 (Figure 3A). We found that overexpressing miR-214-3p mimics also significantly decreased exogenous THBS2 as shown in Figure 3A.

Taken together, these data indicated a direct interaction between miR-214-3p and the THBS2 3’UTR, which allows miR-214-3p to down-regulate THBS2 expression.

**miR-214-3p inhibits viability, migration and invasion of cervical cancer cells**

THBS2 was reported to promote cancer metastasis. Based on the previous studies and the above results, we next determined the role of miR-214-3p in regulating cervical cell viability, migration and invasion. As expected, miR-214-3p impaired C33A cell viability as that shown in Figure 4. Whereas the elimination of cell viability was obviously recovered with overexpression of THBS2. To confirm the result, we overexpressed THBS2 in C33A to get higher cell viability. However, elevated cell viability was also remarkably inhibited in the presence of miR-214-3p (Figure 4).

To study the effects of miR-214-3p on cervical cancer
cell migration and invasion, scratch wound-healing assay and transwell assay were performed in our study. C33A cells were transfected with miR-214-3p mimics only or co-transfected with miR-214-3p mimics and a plasmid carrying THBS2. Cells were applied to a scratch wound-healing assay. The results demonstrated that miR-214-3p inhibited the migration of C33A cells across the scratch wound even in cells overexpressing THBS2 (Figure 5). Meanwhile, C33A expressing miR-214-3p mimics exhibited a decreased invasion ability compared with control cells. In addition, the accelerated cell invasion caused by THBS2 overexpression was also abrogated in presence of miR-214-3p (Figure 6).

All of the above results suggested that miR-214-3p suppresses cervical cancer cell viability, invasion and migration. And the inhibition that miR-214-3p performed on cell viability, invasion and migration was based on its downregulation of THBS2.

**Discussion**

Dysregulation of miRNAs has been demonstrated in a diverse number of human cancers. Accumulating evidence have revealed the regulatory role of miRNAs in tumour metastasis, cancer cell migration and invasion (23-25).

Previously, numerous studies reported that miR-214-3p plays strong tumorigenic and tumour suppressive activities in many human cancers (25, 26). miR-214-3p has a low expression level in various human cancers. However, the function that miR-214 plays in cervical cancer is still not clear with controversial results from previous studies in Hela cells (27, 28). In our study, decreasing expression of miR-214-3p was clearly identified in human cervical cancer cell line C33A and suppressive function of miR-214-3p was also confirmed in our study. The transfection of miR-214-3p mimics into C33A cells led to a significant decrease in cell viability, invasion as well as cell migration. Together with the low expression of miR-214-3p in normal cervical cells we detected, our results revealed that miR-214-3p exhibits strong tumour suppressive functions in cervical cancers.

So far, a serious of genes were identified as targets of miR-214-3p, including ARL2, FOXM1, HMGA1 and EZH2 (28-31). To further understand miR-214-3p in cervical cancer and reveal the mechanism underlying, bioinformatics analysis detected THBS2 as a novel potential target of miR-214 in our study. In a previous study, THBS2 mediates cell-to-cell and cell-to-matrix interactions and promotes cancer metastasis (32-34). In addition, the downregulation of THBS2 reduced MMP2/MMP9 degradation and promoted VEGF production (21, 22, 35-37). In the current study, overexpression of miR-214-3p inhibited THBS2, subsequently down-regulated protein levels of MMP2 and MMP9, resulting in a decreased cell migration and invasion ability in the C33A cell line.

The current study sheds light on the role of miR-214-3p in cervical cancer and its potential implications for diagnosis and treatment. We observed that miR-214-3p expression was significantly reduced in the cervical cancer cell line (C33A), indicating its involvement in the development and progression of the disease. Through experimental overexpression of miR-214-3p, we demonstrated its inhibitory effects on cell viability, invasion, and migration. These findings suggest that miR-214-3p acts as a tumor suppressor in cervical cancer by limiting the aggressive behavior of cancer cells. Furthermore, we identified THBS2 as a novel target gene of miR-214-3p. THBS2, which was found to be highly expressed in cervical cancer cells, plays a crucial role in cancer progression and metastasis. By targeting the 3' untranslated region (3'UTR) of THBS2, miR-214-3p effectively downregulated the protein level of THBS2. This regulatory mechanism provides a molecular basis for the observed inhibitory effects of miR-214-3p on cervical cancer cell behavior. The identification of THBS2 as a downstream target of miR-214-3p expands our understanding of the molecular pathways involved in cervical cancer.

**Conclusion**

Overall, our findings suggest that manipulating miR-
214-3p levels or targeting THBS2 could be potential strategies for the diagnosis and treatment of cervical cancer. Further investigations and clinical studies are warranted to validate the clinical relevance of miR-214-3p and its downstream target THBS2 in cervical cancer patients. In summary, our study contributes to the growing body of knowledge on miRNA-mediated regulation in cervical cancer and provides valuable insights into the potential therapeutic avenues that can be explored in the future.

**Ethical Compliance**
Not applicable.

**Conflict of Interests**
The authors declared no conflict of interest.

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**Author contribution**
Xiaohua Wu and Xu Wang designed and directed the project; Xu Wang, Jing Xu, Fang Hua, Yujing Wang, Guiying Fang and Hongzhen Zhang performed the experiments. Xiaohua Wu and Xu Wang analyzed the data and wrote the manuscript.

**References**
presses the growth of cervical cancer cells by targeting EZH2.


