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MicroRNA-200c suppressed cervical cancer cell metastasis and growth via targeting MAP4K4

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Abstract. – OBJECTIVE: To dissect the functioning mode of miR-200c on cervical cancer cell metastasis and growth and provide therapeutic targets for cervical cancer.

PATIENTS AND METHODS: By quantitative Real-time polymerase chain reaction, the miR-200c expression level in 42 pairs of cervical cancer tissue samples and six cervical cancer-derived cell lines were examined. Using miR-200c mimics, we analyzed the effects of miR-200c over-expression on cell proliferation, invasion, and migration. Dual-luciferase activity assay was recruited to examine the potential target gene MAP4K4 that predicted by several databases. Protein level was studied using Western blot.

RESULTS: miR-200c expressed significantly lower in cervical cancer tissue samples and cell lines. And over-expression of miR-200c in cervical cancer cells significantly decreased the cell invasion, migration and proliferation abilities. Dual-luciferase and Western blot confirmed MAP4K4 as a target gene of miR-200c. Furthermore, up-regulation of MAP4K4 counteracted the suppressive effect of miR-200c over-expression on cell growth and metastasis.

CONCLUSIONS: miR-200c could suppress cervical cancer cell proliferation and progression via regulating MAP4K4, which might provide a new target for cervical cancer diagnosis and therapy.

Key Words:
Cervical cancer, miR-200c, Proliferation and metastasis, MAP4K4.

Introduction

In recent years, with the progress in the development of the human life, the incidence rate of tumor is increased year by year. Among female patients, breast cancer and cervical cancer display the highest incidence rates, which seriously affect patient's quality of life and survival time[1]. Meanwhile, the incidence rate of cervical cancer ranks seventh among all cancers, which ranks third in female suffering cancer, and mortality rate ranks fourteenth[2]. Cervical cancer frequently occurs in China and even Asia, the incidence rate of which is much higher than that in North America, southern Europe and other regions[3]. Compared with that in the last century, the average age of onset of cervical cancer in women in China has fallen at least 13 years old, which is about 45 years old nowadays[4]. The average age of cervical cancer in China is 10 years earlier than 55 years old in the foreign countries during the same period. Squamous cell carcinoma and adenocarcinoma are the most common cervical cancers[5]. In order to treat these tumors and improve the survival time and quality of life of patients, the characteristics and related pathogenesis of these tumors and the key regulatory molecules or influencing factors in the progression of tumors are needed to be understood, so that the scientific study on female tumor is also increasing. It is expected that the effective way to prevent, early diagnose and targeted treat tumor diseases by human intervention can be found through research.

Tumor is affected by multiple regulating factors, the incidence of which is closely related to unhealthy living habits, inadequate nutrition intake, environmental pollution and infection[6]. With the development of molecular biology, people's knowledge of genes gradually deepens. Through revealing the pathogenesis of tumor at the molecular level, it is considered that tumor is a complex genetic disease, the molecular biology bases of which include oncogene activation, inactivation or loss of tumor-suppressor genes, dysfunction of apoptosis-regulatory genes and deoxyribonucleic acid (DNA) repair genes, etc[7]. The change of gene causes imbalance of hormone level, weakening of immune mechanism and disorder of various growth factors in the body, thus leading to the significantly abnormal growth and proliferation of cells. The abnormal changes in genes include the changes in structure and expression of encoding and noncoding genes[8]. Therein, the regulation of micro-ribonucleic acid (miRNA) on target gene

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is of importance for regulating cell growth and proliferation.

MiRNA is also a class of biological small-molecule substance that plays an indispensable role in the tumor. It has a targeted regulating effect on many key regulatory molecules and biological signaling molecules in cell cycle and apoptosis[9]. A lot of previous studies have demonstrated some classical miRNA target genes and their specific regulatory functions. For example, the correlated effects and target genes of miR-196, miR-143, miR-375, miR-1, miR-133 and miR-175 in the mammalian, miR2-a and miR2-b in the drosophila, lsy-6, miR-273, let-7 and lin-41in the nematode are identified. Due to its regulatory effect in the tumor, it can be seen that miRNA is abnormally expressed in many tumors[10]. Although the kinds of miRNA in different types of tumors may be various, the abnormal expression is common. In order to investigate the role of miRNA in tumor, the important molecules related to tumor is often combined with miRNA to determine the effect of miRNA in the certain tumor by exploring its targeting regulatory function.

At present, the key role of MAP4K4 in tumor has been widely recognized, but the regulation of miRNA on MAP4K4 in tumor is not very extensive. Now only hepatocarcinoma, colon cancer and pancreatic cancer have been explored to investigate the effect of miR-141 and miR-194 on the regulation of MAP4K4, while there are no relevant studies in cervical cancer. So we decided to explore the relationship between miRNA and MAP4K4 regulation and function in cervical cancer.

Patients and Methods

Tissue Samples and Cell Lines

Tissue samples: collect 42 pairs of cervical cancer tissues and adjacent tissues (from The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University), age 45±10.2 years old. All tissues were confirmed by clinical pathological biopsy. The staging criteria for cervical cancer refers to the 2014 FIGO staging criteria. All the tissues were sampled during the operation and obtained informed consent. The Ethics Committee was granted by the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University. Samples were cut with scissors and grinded into tissue homogenate. The tissues RNA were extracted and stored at -80°C for storage.

Human cervical cancer cell lines (C33A, HeLa, HeLa229, HCC94, HT-3 and ME-180) and primary normal cervical squamous cells (NCSC) were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), and were maintained at temperature of 37°C with 5% CO_2 in the humid air. The method of cell cryopreservation was similar to that of tissue cryopreservation.

Cells Transfection

MiR-200c mimics and mimics-NC were synthesized and purified by Shanghai Tamar Company (Shanghai, China). One day before transfection, 4-5*10^4 cells were inoculated on the 6-pore plate and 2 mL contained FBS basal medium. Cells with confluence of 70% were transfected with miR-200c mimics or mimics-NC using lipofectamine 3000 vectors and, then, incubated at 37°C with 5% CO_2 for 24 hours. The cells' morphology and also the transfection efficiency were observed by fluorescence microscopy. The changes of miR-200c expression in each group were detected by qRT-PCR.

RNA Extraction and Quantitative Real-time PCR

Total RNA of tissue samples and cells were isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reversed using a miRNA Reverse Kit (TaKaRa, Dalian, China). The miR-200c expression level was detected using SYBR Premix kits (TaKaRa, Dalian, China) with ABI Step One (ABI, Waltham, MA, USA) and U6 was used as internal control. Each experiment was confirmed three times. All the relative expression levels were measured by using the $2^{-\Delta\Delta CT}$ method.

CCK8 Assay

Cervical cancer cell line was inoculated into a 96-well plate. When it grew up to about 70%, cells were treated as follows: blank control group was treated with lipofectamine 3000 diluent, with no plasmid; in the mitogen-activated protein kinase k 4 (MAP4K4) overexpression group, MAP4K4 overexpression vector was transfected; in the miR-200c regulation group, MAP4K4 overexpression vector and miR-200c mimics were transfected at the same time. 10 μL Cell Counting Kit-8 (CCK-8) reagents were respectively added into each well. After the loading of sample, the

cell line was incubated for 2 h. Subsequently, the absorbance values at 12 h, 24 h, 48 h and 72 h were read at 450 nm, respectively.

Colony Formation Assay

Cervical cancer cells were plated in 6-well plates at a density of 600 per well and maintained in normal medium for 10 days. The colonies were fixed in 70% methanol for 20 min and then stained with 0.5% crystal violet for 10 min on ice, washing each well 3 times with phosphate-buffered saline (PBS).

Wound-Healing Assay

70% of cells were transfected. The cell fluid was changed 6 h after transfection. Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS) was used as the fresh medium. After the culture for 6 h, the dead cell was washed away by phosphate-buffered saline (PBS). 100 μL pipette tip was used to draw a "+" in the well of 6-well plate. Subsequently, it was slightly rinsed by PBS again, and serum-free RPMI-1640 culture medium was used, with 2 mL into each well. After the line was drawn, the photo was taken immediately to record the scratches. The cell fluid was changed every 24 h, and the changes in scratches were photographed for record at the 48 h. The migration force and invasion in cells among groups were judged according to the variation of width of scratches among different groups.

Transwell Assay

The upper chamber surface of the bottom membrane of the transwell chamber was dried with 50 mg/L matrigel with 1:8 dilution, and dried at 4°C. A diluted matrigel (3.9 ug/uL) 60-80 uL was added to the polycarbonate film on the upper chamber, and the matrigel was polymerized into a gel at 37°C for 30 min. After 48 h of transfection, cells were digested by trypsin and were washed by PBS about 2 times; then, they were suspended with serum containing bovine serum albumin (BSA) medium. The cell density was adjusted to $5x10^4$/mL. 6 mL FBS containing medium were added into the lower chamber. After 24 h of normal culture, the basement membrane of the lower chamber was removed.

Dual-Luciferase Assay

The Dual-Luciferase reporter system (Promega, Madison, WI, USA) was employed to test the activity of luciferase. The MAP4K4 3'-UTR cDNA fragment containing the wild type or mutant miR-200c binding site was amplified and cloned into pGL3 luciferase vector (Promega, Madison, WI, USA). Next, cervical cancer cells were transfected with miR-200c mimics and the conducted PGL3 vector using lipofectamine 3000. The activity of luciferase was determined using luminometer (Promega, Madison, WI, USA) and measured as the fold-change to the basic pGL3 vector relatively.

Western Blot

Reagent RIPA (Beyotime, Shanghai, China) was utilized to extract protein from cells. BCA protein assay kit (TaKaRa, Dalian, China) was chosen for quantifying protein concentrations. The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, they were replaced to the polyvinylidene difluoride (PVDF) membrane, which was then incubated with antibodies. Cell Signaling Technology (CST, Danvers, MA, USA) provided us rabbit anti-MAP4K4 and rabbit anti-β-actin, as well as goat anti-rabbit secondary antibody. Chemiluminescent film was applied for assessment of protein expression with Image J software.

Statistical Analysis

IBM statistical product and service solutions (SPSS) 22 statistical software (Armonk, NY, USA) was conducted for data processing and analysis. Statistical quantitative data of normal distribution was described by the mean and standard deviation of the method; quantitative data consistent with homogeneity of variance was conducted by Levene test. $p<0.05$ was considered as the standard of significance difference.

Results

MiR-200c Expressed Lower in Cervical Cancer Tissues and Cell Lines

To detect the relationship between the miR-200c expression and cervical cancer, we examined miR-200c levels in 42 pairs of cervical cancer tissues and adjacent normal tissues. Cervical cancer tissues expressed significantly lower miR-200c level than adjacent normal tissues (Figure 1A).

We selected three different cervical cancer cell lines: C33A, HeLa, HeLa229, HCC94, HT-3, ME-180, and a primary normal cervical squamous cell line: NCSC. We measured the amount of

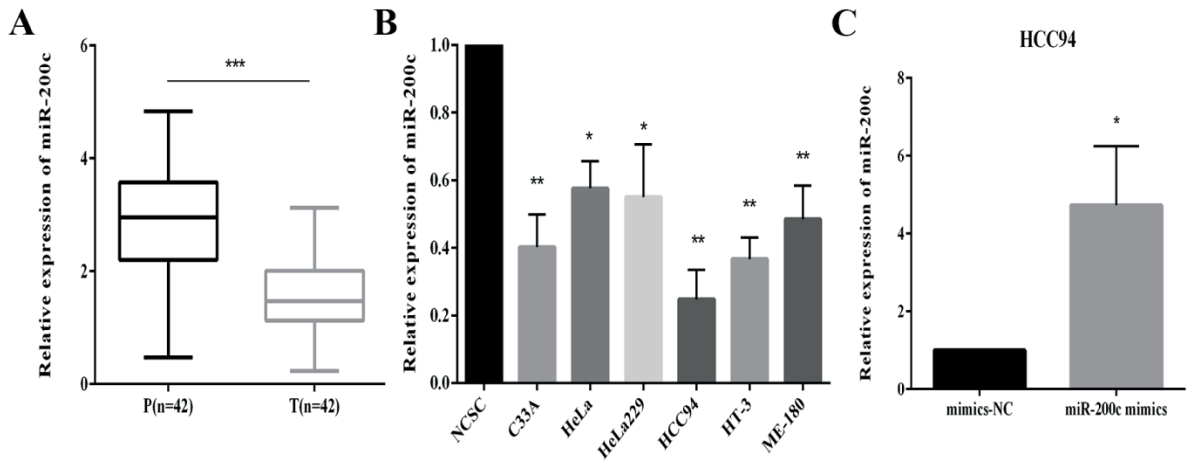

Figure 1. MiR-200c was down-regulated in cervical cancer tissues and cell lines. ***A,*** Analysis of the expression level of miR-200c in 42 pairs of cervical cancer tumor and adjacent tissues. ***B,*** Expression of miR-200c in cervical cancer cells. **C,** Expression of miR-200c in miR-200c mimics treated cervical cancer cells. MiR-200c was detected by qRT-PCR and U6 was used as an internal control. Data are presented as the mean ± SD of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

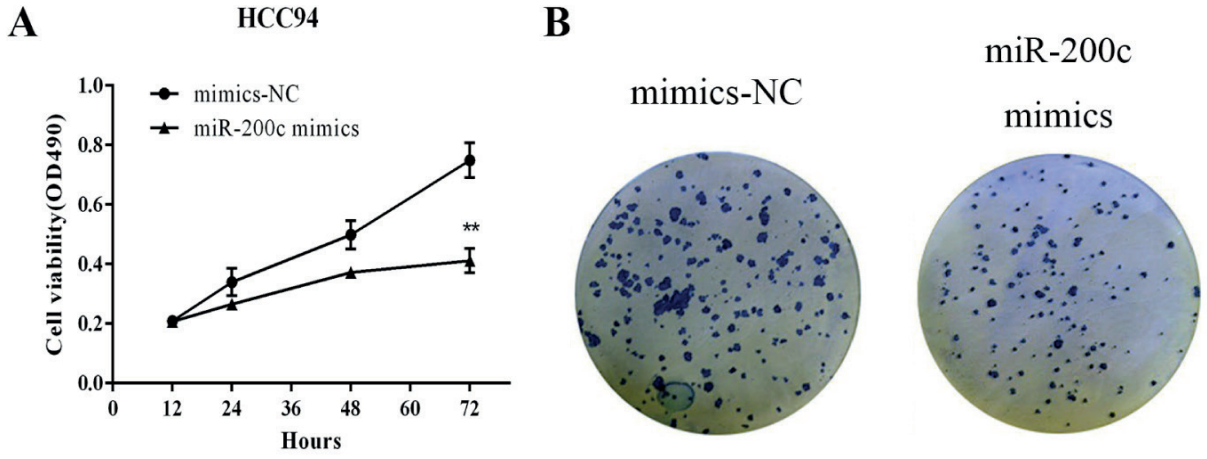

Figure 2. MiR-200c effected the proliferation of cervical cancer cells. ***A,*** CCK8 assay was performed to determine proliferation of cervical cancer cells treating with miR-200c mimics compared to negative control. ***B,*** Colony formation assay was performed to determine the growth of cervical cancer cells transfected with mimics. Data are presented as the mean ± SD of three independent experiments. $^{**}p<0.01$.

miR-200c in each cell line by qRT-PCR, and this was repeated 3 times in each group. The results showed that the relative expression of miR-200c in cervical cancer cell lines was lower than that in NCSC. The results also showed that the expression of miR-200c inHCC94 was lowest, and there was statistical difference (Figure 1B). To further investigate the role of miR-200c in cervical cancer, cells were transfected with miR-200c mimics. The transfection efficiency was shown in Figure 1C.

MiR-200c Over-Expression Inhibited the Proliferation of Cervical Cancer Cells

CCK8 assay was used to detect the effect of miR-200c on proliferation capacity of cervical cancer cells in each group. The results showed that within 12 h after transfection, the number of viable cells in the three groups was not significantly different. After 12 h, the proliferation capacity of cells transfected with miR-200c mimics was significantly inhibited compared to mimics-NC (Figure 2A). The results showed that

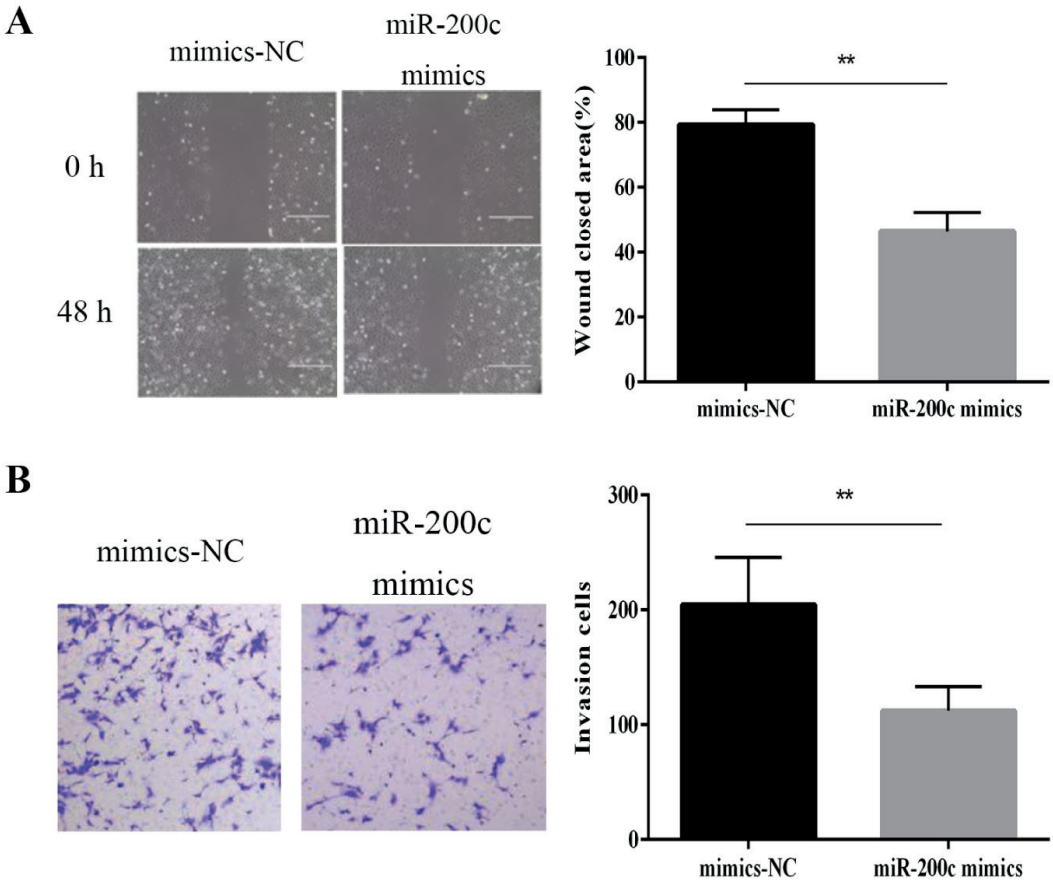

Figure 3. MiR-200c effected the migration and invasion of cervical cancer cells. ***A,*** Wound-healing assay was performed to determine proliferation of cervical cancer cells treating with miR-200c mimics compared to negative control. ***B,*** Transwell invasion assay was used to detect the invasion ability of miR-200c mimics treated cervical cancer cells. Data are presented as the mean ± SD of three independent experiments. $^{**}p<0.01$.

upregulation of miR-200c expression could inhibit the proliferation of cervical cancer cells. Also, cervical cancer cells formed lesser colonies after miR-200c mimics-treatment compared to control group (Figure 2B). All these data suggested that miR-200c could inhibit the proliferation of cervical cancer cells.

MiR-200c Over-Expression Inhibited Cervical Cancer Cell Migration and Invasion

Cell migration was detected by wound-healing assay. We used the pictures of 48 h after scratch and compared the relative moving velocity of cells in each group by the ratio of cell migration area. As a result, the migration rate of the experimental group was 40% (Figure 3A) of the negative control group. It can be seen that the high expression of miR-200c could inhibit the migration capacity of cervical cancer cells.

The invasion capacity of the cells in each group was detected by transwell assay. The experimental findings showed that the invasive ability of the experimental group was significantly lower than that of the negative control group, only about 40% of the NC group (Figure 3B). The results showed that upregulation of miR-200c could significantly inhibit the invasion capacity of cervical cancer cells.

MAP4K4 was a Target Gene of miR-200c

To further explore the molecular mechanism of miR-200c involved in cervical cancer, we next searched for two databases: Targetscan and miRwalk. After comprehensive analysis, we found MAP4K4 as a candidate target gene of miR-200c. To confirm the prediction, we employed dual-luciferase assay using conducted wild-type or mutant MAP4K4 3'-UTR vector (Figure 4A). The result of dual-luciferase as-

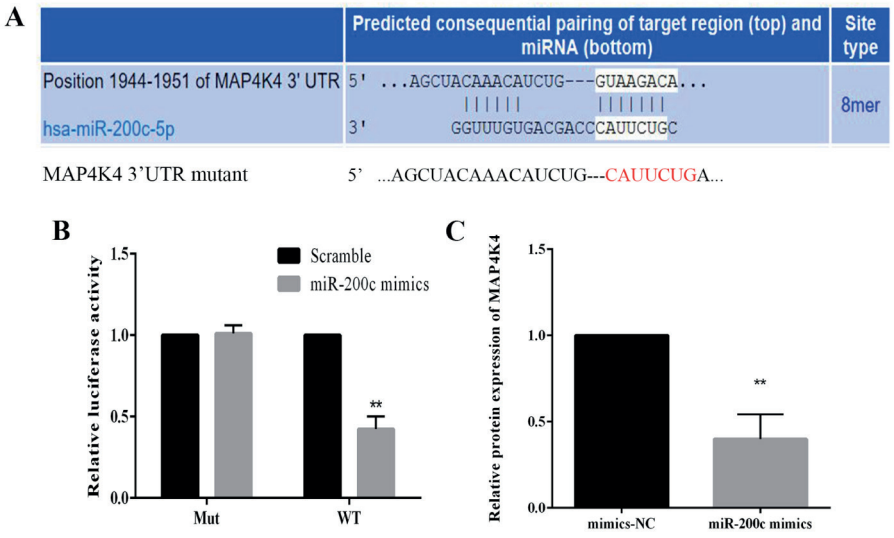

Figure 4. MAP4K4 was a direct target of miR-200c. ***A,*** The predicted binding sites of miR-200c in the 3'-UTR of MAP4K4. ***B,*** Dual-luciferase reporter assay was used to determine the binding site. Cervical cancer cells treated by mimics or NC were transfected with pGL3 construct containing the WT or mutant MAP4K4 3'-UTR site. ***C,*** Levels of MAP4K4 protein measured by Western-blot in miR-200c overexpression cervical cancer cells. The protein levels were normalized to that of β-actin. Data are presented as the mean ± SD of three independent experiments. $^{**}p<0.01$.

say displayed a significant activity decrease in the WT group but no difference in mutant group (Figure 4B). The protein expression of MAP4K4 in miR-200c mimics treated cells was measured by Western blot analysis. Upregulation of miR-200c reduced the MAP4K4 level in cervical cancer cells (Figure 4C). These above results suggested that cervical cancer was a direct target of miR-200c.

MAP4K4 Over-Expression Counteracted the Effect of miR-200c Up-Regulation

As we speculated miR-200c suppressed cervical cancer cells proliferation, migration and invasion via down-regulating MAP4K4, we established plasmid pcDNA3.1-MAP4K4 to reverse the effect of miR-200c mimics to further confirm these results. As shown in Figure 5A, CCK8 assay showed that MAP4K4 over-expression in miR-200c mimics treated cervical cancer cells significantly rescued the cell proliferation ability. Furthermore, the decrease of invasion activity caused by miR-200c mimics was reversed by MAP4K4 up-regulation (Figure 5B). Then, Western blot assay showed MAP4K4 level was rescued by MAP4K4 up-regulation (Figure 5C). These data indicated that miR-200c suppressed cervical cancer cells proliferation and metastasis via targeting MAP4K4.

Discussion

Cervical cancer is one of the malignant tumors that seriously endanger women's lives and health in the world. It ranks second among women with malignant tumors, and more than 85% of them are in developing countries. The recurrence and metastasis of cervical cancer are the main reasons for the failure of cervical cancer treatment[11]. The number of patients with cervical cancer in China is close to 1/4 of that in the world, and 0.13 million of people die of cervical cancer every year[12]. Therefore, cervical cancer has seriously affected lives and health of women in China and even around the world. The prevention and treatment of cervical cancer are of great significance for the development of China's health undertakings and the maintenance of China's and even the world's women's life and health. The data and experimental results of miRNA expression microarray revealed that multiple miRNAs are abnormally expressed in cervical cancer tissues and cell lines, and the occurrence and progression of cervical cancer are affected by these abnormal expressions of miRNAs. For example, miR-34a can be directly regulated by protein p53 to play the role of tumor-suppressor gene[13]; miR-125b plays an inhibitory role in immune response and apoptosis[13]; miR-203 and miR-125b can regulate the deoxyribonucleic acid

(DNA) replication process of human papillomavirus (HPV)[14]. MiR-34a and miR-125b can also regulate the expression of HPV, thus promoting the infection and progression of cervical cancer[14]. MiR-200a can also affect the metastasis of cervical cancer. The survival of patients can be predicted by detecting the expression levels of miR-200a and miR-9 in cervical cancer[15]. MiR-200b is lowly expressed in the invasive cervical cancer[16]. The experiment showed that miR-200b inhibits the invasion and metastasis of cervical cancer through inhibiting the epithelial-mesenchymal transition (EMT) of cervical cancer. The inhibition of miR-21 expression or enhancement of miR-143 expression in tissues may be a strategy for the treatment of cervical cancer[17]. This study aims to identify a potential miRNA therapy target for the diagnosis and treatment of cervical cancer. Recent studies have shown that as an important member of miRNA, the significant role of miR-200c in development of tumor has drawn people's attention. A large number of studies have proved that miR-200c is involved in many biological processes such as growth, invasion and migration of tumor cells. In bladder cancer, miR-200c can inhibit proliferation, invasion and migration of tumor, indicating that miR-200c may exert a significant role in the occurrence and development of human tumors[18]. Of note, the detection of tissue in colon cancer revealed that the expression of miR-200c was inhibited, suggesting that miR-200c plays an important role in the cancer[19]. This study aimed to elucidate the relationship between miR-200c and the occurrence and development of cervical cancer. In order to further study the relationship between miR-200c and metastasis of cervical cancer, we detected the expression of miR-200c in 42 cases of cervical cancer and adjacent tissues. The results showed that the expression of miR-200c in cervical cancer was lower than that in the adjacent tissues. These data indicate that miR-200c is low expressed in cervical cancer tissues, which may play an important role in inhibiting tumorigenesis, development, and inhibiting invasion and metastasis. Furthermore, we conducted miR-200c-overexpressed cell lines and employed several cell function experiments, which demonstrated that upregulation of miR-200c inhibited cell proliferation in cervical cancer. As a conclusion, we considered miR-200c functioned as a suppress-factor in cervical cancer.

MiRNAs could bind to the 3'-UTR region of target genes and influence their expression to change cellular processes. In order to detect the molecular mechanism of miR-200c, we speculated MAP4K4 as a potential target gene due to several databases. MAP4K4 belongs to an upstream kinase of mitogen-activated protein kinase (MAPK) signaling system. MAPK signaling pathway is involved in multiple biological behaviors

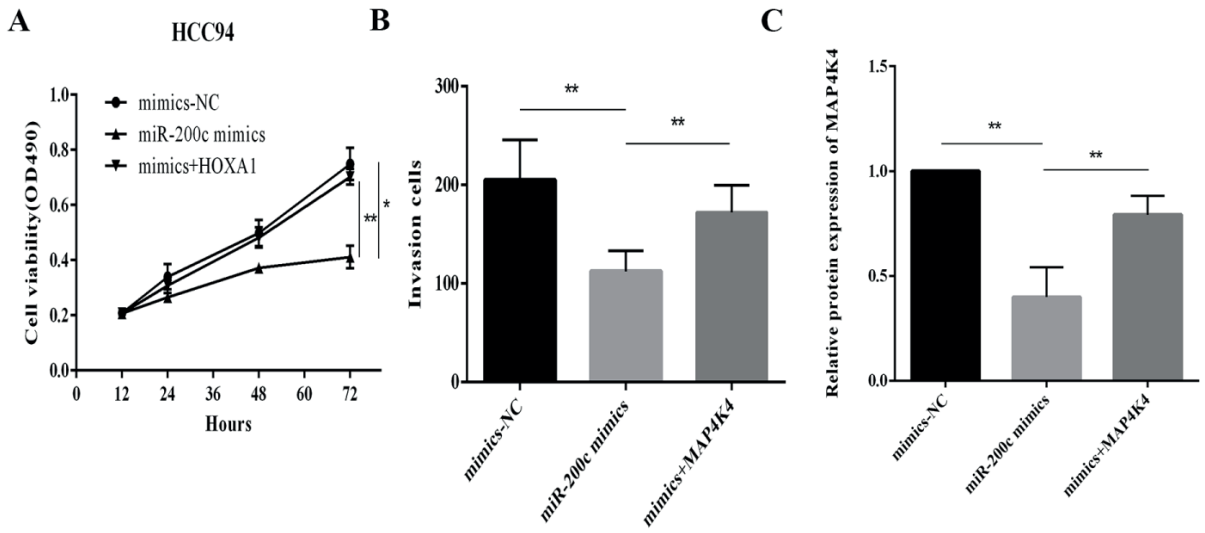

Figure 5. MAP4K4 rescued the effects of miR-200c mimics in cervical cancer cells. ***A,*** Analysis of the cell proliferation ability by MTT assay in miR-200c NC, mimics, or mimics + MAP4K4 treated cervical cancer cells; ***B,*** Cell invasion ability was measured by transwell assay; ***C,*** Western-blot analyses of MAP4K4 expression level. β-actin was used as an internal control. Data are represented as the mean ± SD of three replicates. *$p<0.05$, **$p<0.01$.

of cells, including apoptosis, differentiation and proliferation, cell cycle regulation, maintenance of cell survival and malignant transformation of cells. The study displayed that MAP4K4 is highly expressed in many tumor cells, which can accelerate cell transformation, promote cell invasion and reduce the adhesion to tissue culture cells, and affects the prognosis of tumor. Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 signaling pathways may be involved in the effect of MAP4K4 in the tumor[20]. In recent years, the significant progress has been made in the study on the activation of MAP4K4 and occurrence and development of tumors. MAP4K4 can promote cell growth and proliferation, inhibit apoptosis, advance cell invasion and metastasis, and promote angiogenesis. However, the underlying upstream mechanism of MAP4K4 in cervical cancer has not been well identified and reported yet. In our present paper, we initially revealed that MAP4K4 was directly targeted by miR-200c, and MAP4K4 expression was negatively correlated with miR-200c in cervical cancer tissues and cell lines. Moreover, restoration of MAP4K4 could rescue tumor suppression role by downregulated miR-200c on cervical cancer cell growth. The evidence indicated that miR-200c might be the upstream of MAP4K4 involved in cervical cancer tumorigenesis.

Conclusions

Overall, we first demonstrated miR-200c expression was decreased in cervical cancer and its over-expression inhibited cervical cancer cell proliferation, migration and invasion. Further, we elucidated miR-200c function as a tumor suppressor via targeting MAP4K4. These results could provide a potential target for cervical cancer diagnosis and therapy in the future.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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