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MicroRNA-148a regulates the MAPK/ERK signaling pathway and suppresses the development of esophagus squamous cell carcinoma via targeting MAP3K9

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Abstract. – OBJECTIVE: Esophagus squamous cell carcinoma (ESCC) was a dominant histological type of esophagus cancer, which has a very high incidence due to distant metastasis and local invasion. MicroRNA-148a (miR-148a) functioned as a tumor suppressor in a variety of cancers. The purpose of our study was to explore the vital role of miR-148a in esophagus squamous cell carcinoma.

PATIENTS AND METHODS: The Kaplan-Meier method was applied to calculate the 5-year overall survival of esophagus squamous cell carcinoma patients. Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) and Western blot were conducted to calculate the mRNA levels of miR-148a and genes. The cell counting kit-8 (CCK-8) and transwell assays were performed to measure the proliferative and invasive ability.

RESULTS: MiR-148a was observed to be significantly downregulated and the downregulation of miR-148 predicted poor prognosis of esophagus squamous cell carcinoma patients. MAP3K9 was a target gene of miR-148a and its expression was mediated by miR-148a through directly binding to the 3'-untranslated region (3'-UTR) of its mRNA in the esophagus squamous cell carcinoma. Moreover, miR-148a remarkably inhibited the proliferation and invasion through directly targeting to MAP3K9 *via* extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway and epithelial-mesenchymal transition (EMT) in the ESCC cells. In addition, overexpression of miR-148a inhibited the growth of ESCC *in vivo*.

CONCLUSIONS: MiR-148a inhibited the proliferation and invasion through directly targeting to MAP3K9 by ERK/MAPK pathway and EMT in ESCC cells. The newly identified miR-148a/MAP3K9 axis provides a novel insight into the pathogenesis of the esophagus squamous cell carcinoma.

Key Words:
Esophagus Squamous cell carcinoma, MiR-148a, MAP3K9, Proliferation, Invasion.

Introduction

Esophagus cancer was the fourth leading cause of cancer-related death in China, including two main subtypes: esophagus squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC)[1,2]. Although the treatment of ESCC has made great advances, great progress has been made in diagnosis and treatment in recent years, but the prognosis of ESCC was still poor due to distant metastasis and local invasion[3]. Therefore, it is urgent to explore novel biomarkers for ESCC metastasis and treatment. MicroRNAs (miRNA), a class of conserved endogenous non-coding RNAs that are approximately 19-25 nucleotides in length, could regulate the gene expression *via* directly binding to the 3'-untranslated region (3'-UTR) of target mRNA at post-transcriptional level[4]. In recent years, evidence has illuminated that miRNAs may act as oncogenes and tumor suppressors in some tumors[5-7]. In the esophagus squamous cell carcinoma, multiple miRNAs have been reported[8-11] to act as tumor suppressors including miR-107, miR-127, miR-498, and miR-148a. MiR-148a has been reported[12] to function as a tumor suppressor by inhibiting proliferative and migratory capacities in gastric cancer. Similarly, Wang et al[13] has elucidated that miR-148a suppressed the proliferation and invasion in epithelial ovarian cancer cells. Ma et al[14] have revealed that miR-148a inhibited estrogen-induced viability and migration in breast cancer. Thus, we

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strongly believe that miR-148a may play a vital role in esophagus squamous cell carcinoma.

Mitogen-activated protein kinase kinase kinase 9 (MAP3K9), also known as MLK1, is a member of the mitogen-activated protein kinase (MAPK) pathway and could interact with body mass index, alcohol consumption, and cigarette smoking[15]. In the intervertebral disc degeneration, MAP3K9 has found to be a target gene of miR-15a and a knockdown of MAP3K9 inhibited the proliferation and promoted apoptosis of NP cells[16]. Moreover, Nie et al[17] have illuminated that MAP3K9 was a direct target gene of miR-148a and miR-148 inhibited the proliferation and enhanced cell apoptosis in human renal cancer. In our study, miR-148a was found to be downregulated in esophagus squamous cell carcinoma tissues and cell lines in comparison with corresponding peritumoral normal tissues and normal epithelial cell line. Downregulation of miR-148a was connected with poor prognosis in esophagus squamous cell carcinoma. MiR-148a inhibited the proliferation and invasion by directly targeting to the 3'-UTR of MAP3K9 mRNA through the regulation of the MAPK/extracellular-signal-regulated kinase (ERK) signaling pathway and epithelial-to-mesenchymal transition in esophagus squamous cell carcinoma cells. In addition, miR-148a suppressed the growth of esophagus squamous cell carcinoma *in vivo*.

Patients and Methods

Patients and Tissue Samples

ESCC and the corresponding peritumoral normal tissues were obtained from 49 patients who underwent the operation at Beijing Tongren Hospital from June 2015 to June 2017. None of the patients received radiotherapy or chemotherapy before the operation. All the specimens were immediately frozen in liquid nitrogen and followed stored at −80°C. This investigation was content informed of the patients and approved by the local Human Research Ethics Committee in Beijing Tongren Hospital.

Cell Lines and Culture Conditions

Two human ESCC cell lines (TE-2 and TE-8) and a normal epithelial cell Het-1A were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10%. of fetal bovine serum (FBS) were applied to culture all the cells at 37°C in a humidified atmosphere containing 5% of CO_2. The RPMI-1640 medium and FBS were purchased from Gibco-BRL (Rockville, MD, USA) and Haoyang Biological Manufacture (Tianjin, China), respectively.

Plasmid Construction and Transfection

To upregulate or downregulate miR-148a, the miR-148a mimic and the miR-148a inhibitor oligo fragments were obtained from Gene-Pharma (Shanghai, China) and were transfected in TE-2 cells. Referring to the manufacturer's protocol, the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) reagent and the oligo fragments were diluted with the Opti-MEM/Reduced serum medium (Thermo Fisher Scientific, Waltham, MA, USA) and then mixed the two solutions, and then added in the cells. For the transient transfected cells, the cells were harvested after 48h, while for the stable transfected cells were selected by Geneticin (G418; Thermo Fisher Scientific, Waltham, MA, USA).

RNA Extraction and Real Time-quantitative Polymerase Chain Reaction (RT-qPCR)

The TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the mirVana miRNA Isolation Kit (Thermo Fisher, Waltham, MA, USA) were employed to extract total RNAs and total miRNAs, respectively. The first cDNA chain of mRNA and miRNA were synthesized by the Prime Script RT Reagent Kit (TaKaRa Biotechnology Co. Ltd., Dalian, China) and the miRNA Reverse Transcription Kit (Life Technologies, Foster City, CA, USA). The SYBR Premix Ex Taq (TaKaRa, Dalian, China) and the MystiCq microRNA qPCR Assay Primer (Sigma-Aldrich, St. Louis, MO, USA) was performed the qPCR using an Applied Biosystems Step One Plus™ Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The endogenous control of miR-148a and MAP3K9 were U6 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. The relative folds changes mirroring gene expression levels were evaluated by the $2^{-\Delta\Delta Ct}$ method. The primers for RT-qPCR were as follows: miR-148a F: 5'-GAGATGCATTCTGCCCTGCAGCAGCTT-3'; R: 5'-CATGCTCGAGTCAAAAGACCAAACGTGCTGTC-3'; U6: F: CTCGCTTCGGCAGCACA, R: AACGCTTCACGAATTTGCGT; MAP3K9 F: 5'-CTCGCCCTTCCTATTCTACGT-3'; R:

5'-CCCGGGAGAAGAGGAGGAGGAG; F: 5'-CCCGGGAGAAGAGGAGGAGGAG-3'; GAPDH F: CAGGTGAAGACGGGCGGA, R: GAGTTAAAAGCAGCCCTGGTG.

Western Blot Analysis

Cells were lysed by using the Radioimmunoprecipitation assay (RIPA) Lysis Buffer (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% of phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, St. Louis, MO, USA) on ice. The protein lysate was centrifuged at 12,000×g speed for 20 min at 4°C and then, we collected the supernatants. Equal amount of proteins was separated by 10% of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) through electrophoresis and then transferred on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking in 5% fat-free milk for 1 h at ream temperature, the blots were incubated the primary antibodies at 4°C overnight. The primary antibodies were against MAP3K9 (1:1000; Abcam, Cambridge, MA, USA), E-cadherin (1:1000; Abcam, Cambridge, MA, USA), N-cadherin (1:1000; Abcam, Cambridge, MA, USA), Vimentin (1:1000; Abcam, Cambridge, MA, USA), p-MAPK (Sigma-Aldrich, St. Louis, MO, USA), MAPK (Sigma-Aldrich, St. Louis, MO, USA), p-ERK (Sigma-Aldrich, St. Louis, MO, USA), ERK (Sigma-Aldrich, St. Louis, MO, USA) and GAPDH (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, the membranes were incubated by secondary horse radish peroxidase (HRP)-conjugated antibody at room temperature for 2 h. Finally, signals were visualized using enhanced chemiluminescence (ECL; Pharmacia Biotech, Arlington, IL, USA).

Cell Counting Kit-8 (CCK-8) Assay

The cell proliferative ability was evaluated by the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). TE-2 cells were seeded in 96-well plate and cultured at 37°C for 24 h, 48 h, 72 h or 96 h. Subsequently, 10 μL of CCK-8 solutions were added into each well and cultured for 1 h at 37°C; followed assessed the absorbance at 450 nm using a microplate reader (BioTek, Winooski, VT, USA). This experiment was repeated three times.

Transwell Assays

According to the manufacturer's instructions, the transwell insert (8 μm membrane; Corning, Corning, NY, USA) covered with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), was employed to evaluate the cell invasive ability. The inserts were put in a 24-well plate and formed the upper and lower two chambers. The upper chamber was filled by 200 μL TE-2 cells suspension, which were suspended by RPMI-1640 serum-free medium. Meanwhile, the lower chamber was filled with 500 μL normal RPMI-1640 medium containing 15% of FBS. After incubated 24 h at 37°C, the non-invasive cells on the upper surface were removed by a cotton swab. The invasive cells were fixed and then stained by 4% of paraformaldehyde and 10% of crystal violet, respectively. Next, the number of invaded cells was counted in five fields under a microscope (Olympus Corporation, Tokyo, Japan).

Dual-Luciferase Reporter Assay

MAP3K9 was predicted to be a potential target gene of miR-148a by TargetScan (http://www.targetscan.org/vert_71/), and the binding site was located at 109−116 on 3'-UTR of MAP3K9 mRNA. To verify whether miR-148a was directly binding to the 3'-UTR of MAP3K9 mRNA, the binding sequences was mutated from UGCACUG to ACGUGAC, and then, both the wild type and the mutant sequences were inserted in the pmirGlo luciferase reporter vector. The miR-148a mimic and the wild type 3'-UTR or the mutant 3'-UTR of MAP3K9 mRNA were co-transfected in the TE-2 cells. The luciferase activity was calculated by a Dual-Luciferase Reporter assay system (Promega, Madison, WI, USA) using *Renilla* luciferase activity as the internal reference, followed by comparing the relative luciferase activities.

Tumor Xenograft Model in Nude Mice

We purchased 4-week-old nude mice from Charles River Laboratories (Beijing, China) and cultured for one week to adjust to the environment. To investigate the effect of miR-148a on the growth of ESCC *in vivo*, we injected TE-2 cells stably expressing miR-148a subcutaneously into nude mice. Tumor volume was assessed every 3 days, mice were sacrificed, and xenografts were removed 26 days after the implantation of cells. All animal experiments were performed at the Beijing Tongren Hospital, Capital Medical University Animal Laboratory Center and approved by the Beijing Tongren Hospital, Capital Medical University Animal Care and Use Committee.

Statistical Analysis

Experimental results were obtained from at least three independent experiments. All the data were presented as the mean ± standard deviation (SD), which were analyzed by Statistical Product and Service Solutions (SPSS) statistical software version 16.0 (SPSS, Inc., Chicago, IL, USA). The significance between the two groups was compared by two-tailed Student's *t*-test. Comparison between groups was done using the One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). *p*-value <0.05 was found to be statistically significant.

Results

Downregulation of MiR-148a Predicted Poor Prognosis of Esophagus Squamous Cell Carcinoma

We evaluated the level of miR-148a in 49 pairs of esophagus squamous cell carcinoma and corresponding non-tumor tissues. As expected, miR-148a was lowly expressed in esophagus squamous cell carcinoma tissues *versus* the corresponding non-tumor tissues ($p<0.05$) (Figure 1A). What's more, the downregulation of miR-148a predicted the poor prognosis of esophagus squamous cell carcinoma patients ($p<0.05$) (Figure 1B).

MiR-148a Suppressed The Proliferation and Invasion in Esophagus Squamous Cell Carcinoma Cells

We calculated the expression of miR-148a in two esophagus squamous cell carcinoma cell lines (TE-2 and TE-8) and a normal epithelial cell line Het-1A. Similarly with tissues, the expression of miR-148a was higher in Het-1A cells than that in TE-2 ($p<0.01$) and MGC-803 ($p<0.05$) cells (Figure 2A). To explore the great roles of miR-148a, the miR-148a mimic and the miR-148a inhibitor were conducted to up- ($p<0.01$) or down-regulate ($p<0.05$) miR-148a in TE-2 cells, which were evaluated by RT-qPCR (Figure 2B).

The CCK-8 assay elucidated that the proliferative ability was suppressed by the miR-148a mimic ($p<0.05$), while it was promoted by miR-148a inhibitor ($p<0.05$) in TE-2 cells (Figure 2C). In addition, the transwell assay indicated that overexpression of miR-148a suppressed ($p<0.05$) the invasive ability, while miR-148a inhibitor presented the opposite results ($p<0.05$) (Figure 2D). All the findings elucidated that miR-148a suppressed the proliferative and invasive abilities in esophagus squamous cell carcinoma cell TE-2.

MiR-148a Regulated The Expression of MAP3K9 Through Directly Binding to The 3'-UTR of MAP3K9 mRNA

TargetScan predicted that MAP3K9 was one target gene of miR-148a, and the binding site was located at 109−116 on the 3'-UTR of MAP3K9 mRNA. The binding sequences of miR-148a on 3'-UTR of MAP3K9 mRNA were mutated from UGCACUG to ACGUGAC, and then calculated the luciferase activity (Figure 3A). The luciferase reporter assay indicated that miR-148a reduced ($p<0.05$) the luciferase activity of TE-2

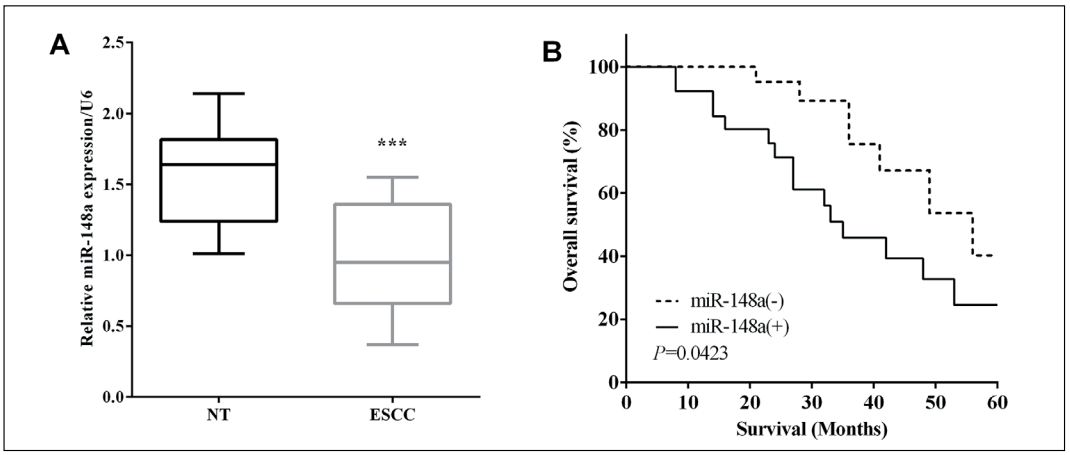

Figure 1. Downregulation of miR-148a predicted poor prognosis of esophagus squamous cell carcinoma. ***A,*** Expression of miR-148a was lower in esophagus squamous cell carcinoma tissues than that in corresponding non-tumor tissues. ***B,*** Downregulation of miR-148a predicted poor prognosis in esophagus squamous cell carcinoma.

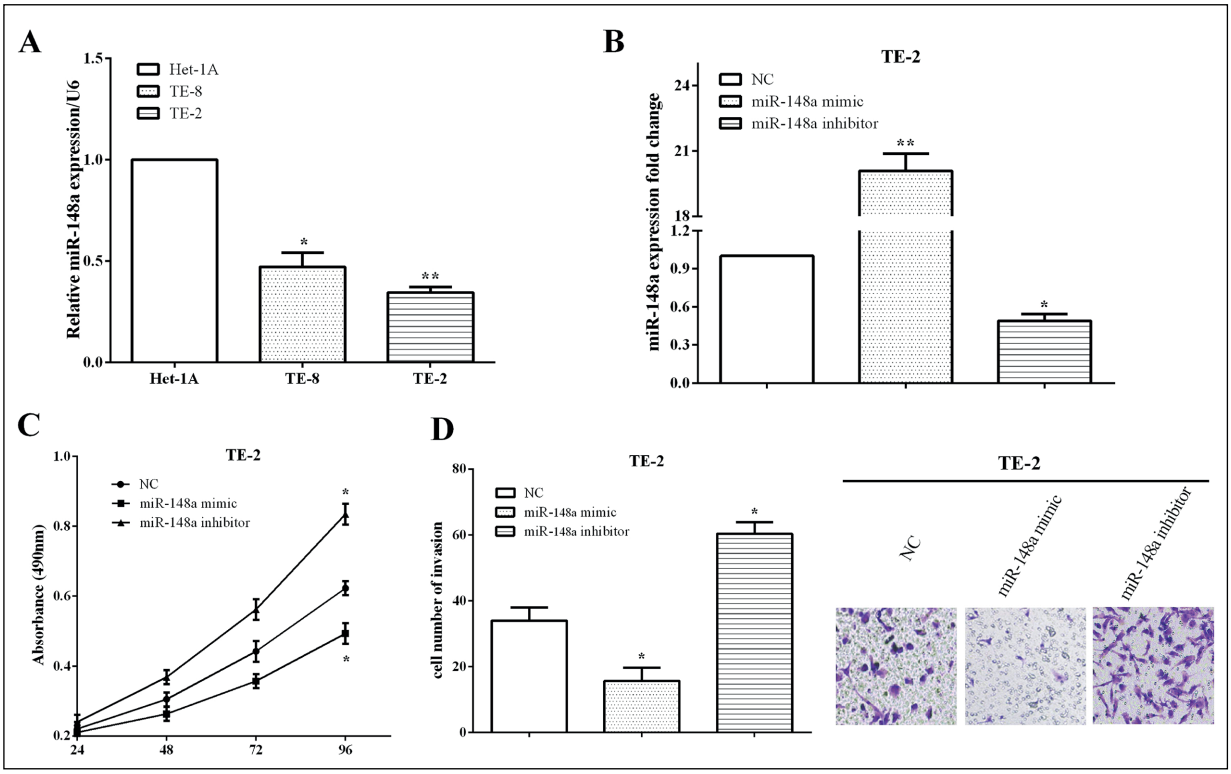

Figure 2. MiR-148a suppressed the proliferation and invasion in esophagus squamous cell carcinoma cells. ***A,*** Expression of miR-148a was higher in Het-1A cells than that in TE-2 and MGC-803 cells. **B,** MiR-148a mimic and the miR-148a inhibitor were conducted to up- or down-regulate miR-148a in TE-2 cells. ***C,*** CCK-8 assay elucidated that the proliferative ability was suppressed by miR-148a mimic, while it was improved by the miR-148a inhibitor in TE-2 cells. ***D,*** Transwell assay indicated that miR-148a invasive abilities in esophagus squamous cell carcinoma cell TE-2 (The magnification was 200×).

cells which transfected the wild type 3'-UTR of MAP3K9 mRNA, while did not alter (p<0.05) the mutant 3'-UTR of MAP3K9 mRNA (Figure 3B). Moreover, the mRNA levels of MAP3K9 we calculated after transfected the miR-148a mimic or the miR-148a inhibitor in TE-2 cells. As expected, overexpression of miR-148a suppressed (p<0.05) the mRNA level of MAP3K9, while the knockdown of miR-148a enhanced (p<0.05) the expression of MAP3K9 in TE-2 cells (Figure 3C). All the results indicated that miR-148a regulated the expression of MAP3K9 through directly binding to its mRNA 3'-UTR in ESCC cell line TE-2.

MiR-148a Inhibited the Invasion-Mediated Epithelial-Mesenchymal Transition (EMT) and Suppressed The Proliferation Through MAP3K9/ERK Signal Pathway

The expression of MAP3K9 was evaluated in tissues and cell lines using RT-qPCR. Not unfortunately, MAP3K9 was upregulated in ESCC tissues *versus* corresponding non-tumor tissues (p<0.05) (Figure 4A). Similar results with those in tissues, the expression of MAP3K9 was also assessed to be higher in TE-2 (p<0.01) and TE-8 (p<0.05) than that in normal epithelial cell line Het-1A (Figure 4B). What's more, the proteins expression associated with EMT and ERK pathway were assessed by Western blot in TE-2 cells. We discovered that the miR-148a mimic impaired the expression of MAP3K9 and E-cadherin, while enhanced the expression of N-cadherin and Vimentin in TE-2 cells (Figure 4C), which suggested that miR-148a inhibited the EMT through targeting to MAP3K9. Meanwhile, overexpression of miR-148a inhibited the expression of p-MAPK and p-ERK in TE-2 cells (Figure 4D), which elucidated that miR-148a inhibited the proliferation through the MAP3K9/MAPK/ERK pathway. All the results revealed that miR-148a inhibited the proliferative and invasive abilities by directly targeting to the 3'-UTR of MAP3K9 mRNA through regulation of the MAPK/ERK signaling pathway and epithelial-to-mesenchymal transition in ESCC cells.

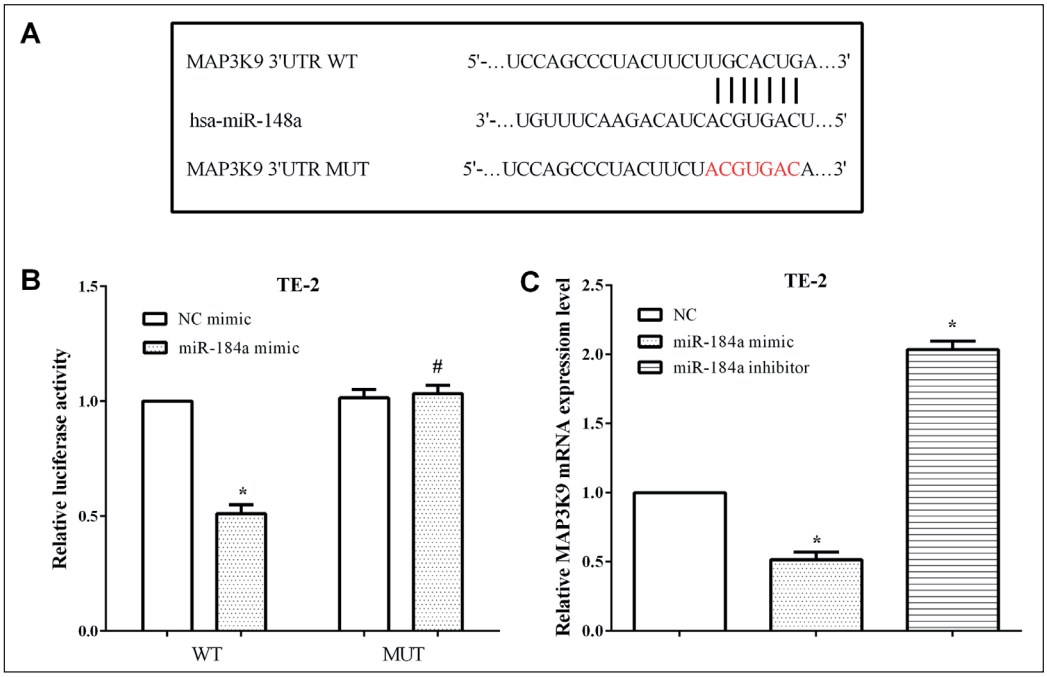

Figure 3. MiR-148a regulated the expression of MAP3K9 through directly binding to the 3'-UTR of MAP3K9 mRNA. ***A,*** TargetScan predicted MAP3K9 was one of the target genes of miR-148a, and the binding site was located at MAP3K9 mRNA 3'-UTR. ***B,*** Luciferase reporter assay indicated that miR-148a binding to the 3'-UTR of MAP3K9 mRNA in TE-2. ***C,*** MiR-148a regulated the expression of MAP3K9 through directly binding to mRNA 3'-UTR in ESCC cells TE-2.

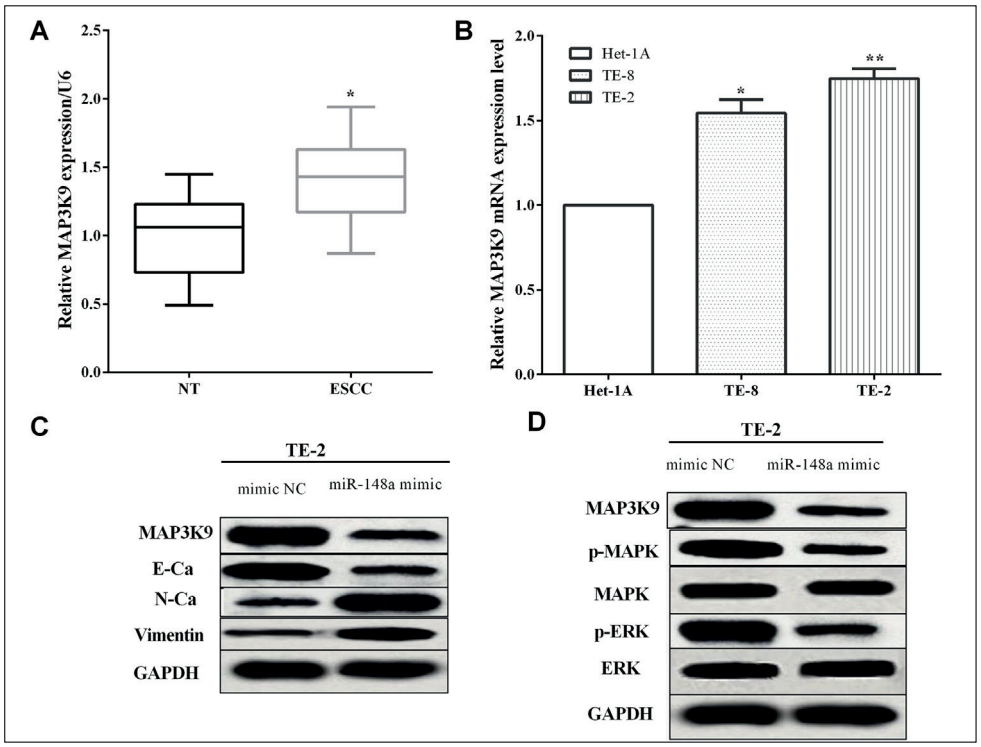

Figure 4. MiR-148a inhibited the invasion-mediated EMT and proliferation through MAP3K9/ERK signal pathway. ***A,*** MAP3K9 was upregulated in ESCC tissues *versus* corresponding non-tumor tissues. ***B,*** Expression of ING5 was higher in ESCC cells than that in normal epithelial cell line. ***C,*** MiR-148a inhibited the EMT through directly targeting to the 3'-UTR of MAP3K9 mRNA. ***D,*** MiR-148a inhibited the proliferation through the MAPK/ERK pathway.

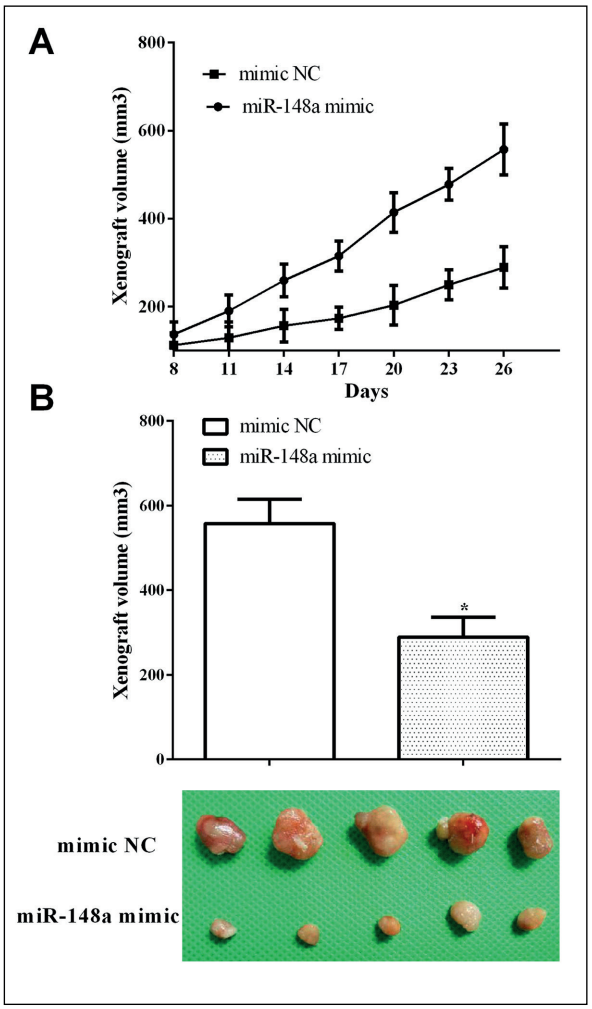

Figure 5. MiR-148a suppressed the xenograft growth *in vivo*. ***A,*** Overexpression of miR-148a inhibited the growth of ESCC xenograft. ***B,*** Tumor volume of cells that overexpressed miR-148a was smaller than the control group.

MiR-148a Suppressed The Growth of ESCC In Vivo

TE-2 cells that stably transfected with the miR-148a mimic or control plasmid were injected subcutaneously into nude mice. Tumor volume was calculated every 3 days and we found that the growth rate of the transfected with miR-148a mimic group was slower than control group, indicating that overexpression of miR-148a inhibited the growth of ESCC (Figure 5A). After the nude mice were sacrificed and the tumor was removed and the tumor volume was calculated, we found that the tumor volume of cells overexpressing miR-148a was smaller than that of control group ($p<0.05$) (Figure 5B).

Discussion

Esophagus squamous cell carcinoma is a subtype of esophagus cancer that has very high incidence and is the fourth leading cause of cancer-related death in China[2,18]. Recently, the prognosis of ESCC was poor due to distant metastasis and local invasion[3], therefore, it is urgent to explore novel biomarkers for ESCC metastasis and treatment.

MiRNA regulate gene expression *via* directly binding to the mRNA 3'-UTR at post-transcriptional level[4]. In recent years, evidence illuminated that miRNAs may act as oncogenes and tumor suppressors in tumors[5-7]. MiR-148a has been reported to act as a tumor suppressor and inhibited the proliferative, migratory, and invasive capacities in osteosarcoma[19]. Shi et al[20] has revealed that miR-148a suppressed the migration and invasion in gastric cancer cells. Consistent with all the findings, miR-148a was found to be lowly expressed in esophagus squamous cell carcinoma and the downregulation of miR-148a predicted poor 4-year survival. Moreover, we also found that miR-148a inhibited the proliferation and invasion in esophagus squamous cell carcinoma, which was the first time to propose the association between miR-148a and metastasis in ESCC. Overexpression of MiR-148a inhibited the growth of ESCC cells *in vivo*. Peng et al[21] has indicated that miR-148a suppressed the EMT in pancreatic cancer; consistent with Peng et al[21], we discovered that miR-148a inhibited the EMT through directly binding to the 3'-UTR of MAP3K9 mRNA in ESCC cells.

MAP3K9 has been reported to be a target gene of multiple miRNAs that including miR-34a, miR-1247, miR-1247 and miR-148a[22-24]. Our results were consistent with the findings of Nie et al[17] in human renal cancer, MAP3K9 was found to be a target of miR-148a and its expression was mediated by miR-148a in esophagus squamous cell carcinoma. MAP3K9 was upregulated in ESCC tissues and cell lines compared to the corresponding non-tumor tissues and normal cell line. What's more, Luo et al[24] has revealed that miR-148a regulated the proliferation and metastasis through the ERK/MAPK pathway in cutaneous squamous cell carcinoma. Our results were consistent with the findings of Luo et al[24] that miR-148a inhibited the proliferation and invasion through directly targeting to MAP3K9 by the ERK/MAPK pathway and EMT in ESCC cell.

Conclusions

We showed that MiR-148a was downregulated and the downregulation of miR-148a predicted poor prognosis in esophagus squamous cell carcinoma. MAP3K9 was discovered to be a target gene of miR-148a and its expression was mediated by miR-148a in esophagus squamous cell carcinoma. Moreover, miR-148a inhibited the proliferation and invasion through directly targeting to MAP3K9 by the ERK/MAPK pathway and EMT in ESCC cell. In addition, the overexpression of miR-148a inhibited the growth of TE-2 cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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