

miR-584-5p regulates migration and invasion in non-small cell lung cancer cell lines through regulation of MMP-14

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Abstract. An increasing number of studies have demonstrated that microRNAs (miRNAs/miRs) are involved in cancer progression. In 2010, an estimated 1,500,000 patients suffered mortality from lung cancer (LC) worldwide, and ~80% of LC patients were diagnosed with non-small-cell lung cancer (NSCLC). miR-584-5p was reported to be a potential biomarker in the diagnosis of LC; in addition, miR-584 was recently observed to suppress the progression of thyroid carcinoma, glioma and gastric cancer. However, the specific function of miR-584-5p in NSCLC remains unclear. In the present study, miR-584-5p was decreased in the tumor tissues of NSCLC patients. Furthermore, miR-584-5p markedly inhibited the migration and invasion of NSCLC cells. The direct regulatory association between miR-584-5p and matrix metalloproteinase (MMP)-14 was verified by a luciferase reporter gene assay. Furthermore, the results indicated that miR-584-5p inhibited the expression of MMP-14 at the protein and mRNA levels. miR-584-5p also inhibited the expression of MMP-4 and Slug, which are involved in tumor invasion and metastasis. Taken together, these results indicated that the miR-584-5p/MMP-14 axis may serve as an anticancer target in the treatment of NSCLC patients.

Introduction

In 2010, an estimated 1.5 million individuals died from lung cancer (LC) worldwide, accounting for 19% of all cancer deaths (1). In 2008, statistics from the International Agency for Research on Cancer (IARC) reported that ~520,000 new cases of LC were diagnosed in China (2,3). There are two major types of LC, i.e., non-small-cell lung cancer (NSCLC) and

small-cell lung cancer (SCLC), and the disease is the leading cause of cancer-related mortality worldwide (4,5). NSCLC accounts for ~80% of LC cases (6). Despite recent advances in the treatment of patients with NSCLC, its high mortality rate has not declined significantly (7), and the 5-year survival rate does not exceed 15% (8). Thus, effective treatment strategies for NSCLC patients are urgently required.

MicroRNAs (miRNAs) are a type of endogenous short and highly conserved non-coding RNA that participate in numerous developmental processes. miRNAs repress gene expression by base-pairing with the 3' untranslated region (UTR) of mRNA (9). Deregulation of miRNAs is associated with cancer initiation and progression, indicating their roles as oncogenes or tumor suppressor genes (10). Numerous miRNAs involved in LC pathogenesis have emerged, and research into miRNA-targeted therapy has attracted significant attention (7,11,12). Recently, miR-584 has attracted the attention of researchers; for example, in 2015, miR-584 was found to suppress cell invasion and cell migration in thyroid carcinoma through targeting Rho-associated coiled-coil containing protein kinase 1 (ROCK1) (13); in 2016, microRNA-584-3p was reported to reduce cell migration and cell invasion in glioma through targeting ROCK1 (14); and in 2017, miR-584-5p overexpression was found to inhibit cell proliferation and induce cell apoptosis in gastric cancer through targeting WW domain-containing E3 ubiquitin protein ligase 1 (15). Interestingly, miR-584-5p was verified as a potential biomarker for the diagnosis of LC (16). However, how dose miR-584-5p affect the progression of NSCLC has not been reported to date. The aim of the present study was to investigate the action of miR-584-5p in NSCLC.

Materials and methods

Cell culture. A549 cells were incubated in DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotics (penicillin-streptomycin; Gibco; Thermo Fisher Scientific, Inc.) in an incubator with 95% humidity and 5% CO₂ at 37°C.

Cell transfection. A549 cells (2x10⁵ cells/well) were seeded in 6-well plates and cultured until reaching 90% confluence,

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after which time cell transfection was performed. In brief, 2.5 μ l hsa-miR-584-5p mimics and 5 μ l Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) were added to 250 μ l DMEM. The mixture was thoroughly mixed and added to the 6-well plates to a concentration of 20 nmol/l.

Prediction of promoter-targeting miRNA. The binding sites between miR-584-5p and mRNAs were analyzed by algorithm miRWalk (17) and genome-wide Argonaute-chromosome interaction profiling data (GSE40536).

Luciferase reporter assay. Cells transfected with miR-584-5p mimics or miR-NC mimics were seeded into 96-well plates (1×10^3 cells/well). The cells were cultured in DMEM supplemented with 10% FBS for 24 h. MMP-14 3'UTR was amplified from cDNA of A549 cells and inserted into p LightSwitch Prom (Switchgear Genomics, Menlo Park, CA, USA). Subsequently, pLightMMP-14-WT-3'UTR reporter plasmid or pLightMMP-14-MUT-3'UTR was transfected into the cells by FuGENE transfection reagent (Roche Diagnostics, Basel, Switzerland) in accordance with the manufacturer's protocol. At 24 h after cell transfection, luciferase activity was examined by LightSwitch Luciferase Assay (Switchgear Genomics) in accordance with the manufacturer's protocol.

Patients. A total of 30 pairs of tumor tissues and paired normal tissues from NSCLC patients used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were acquired from Weifang Second People's Hospital after obtaining written informed consent from all the subjects. The present study was approved by the Ethical Committee of Weifang Second People's Hospital (Shandong, China). The patients included 22 men and 8 women, 17 of whom were aged ≥ 60 years and 13 were aged < 60 years. A total of 10 patients had never smoked and 20 patients had a history of smoking; in addition, 22 patients had lymph node metastasis and 8 patients had no metastatic lymph nodes; there was no gender bias in the lymph node metastasis.

Western blot analysis. At 48 h after transfection, A549 cells were rinsed by cold phosphate-buffered saline followed by the addition of protein lysis buffer. Total protein was extracted through centrifugation of A549 cells at 14,000 rpm at 4°C for 10 min. Proteins were first separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the proteins were transferred onto nitrocellulose membranes. Subsequently, the nitrocellulose membranes were incubated with primary antibodies at 4°C overnight followed by treatment by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 2 h. Finally, the blots were treated with ECL (Thermo Fisher Scientific, Inc.).

RT-qPCR analysis. Total RNA from A549 cells and patient tissue samples was extracted by TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. Reverse transcription and the TaqMan microRNA assay were performed using the Hairpin-it™ miRNA qPCR Quantitation kit (Shanghai GenePharma Co., Ltd., Shanghai, China) in accordance with the manufacturer's

protocol. Thereafter, RT-qPCR was performed with a SYBR Green kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the reaction was performed with the ABI 7500 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec. Relative expression levels were determined by the $2^{-\Delta\Delta C_q}$ method (18). The expression of miR-584-5p was normalized to U6 and the expression of matrix metalloproteinase (MMP)-14 was normalized to GAPDH.

Wound healing assay. A549 cells (1×10^5 cells/well) transfected with miR-584-5p mimics or miR-NC mimics were inoculated into 6-well plates and cultured in DMEM for 6 h. A wound was created using a 10- μ l Eppendorf pipette tip. After washing with serum-free medium 3 times to remove the detached cells, A549 cells were cultured in DMEM in an incubator with 5% CO₂ for 24 h at 37°C. The distance between the edges of the gap was measured using Image-Pro Plus v6.0 software.

Transwell assay. Cell invasion was evaluated by Transwell assay (Corning Incorporated, Corning, NY, USA) with 10 μ l Matrigel (BD Biosciences, San Jose, CA, USA). A549 cells (5×10^5 cells per 200 μ l) transfected with miR-584-5p mimics or miR-NC mimics were inoculated into the upper chambers, while DMEM was added into the lower chambers. Following incubation at 37°C for 24 h, the invasive cells were fixed with 4% paraformaldehyde and stained with crystal violet solution (0.1%). Five random areas from each chamber were examined under a microscope.

Statistical analysis. Statistical analyses were performed with SPSS v13.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as mean \pm standard deviation. Differences between two groups were analyzed using two-tailed Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Evaluation of miR-584-5p levels in tissue samples in vivo and cells in vitro. First, we examined the miR-584-5p level in tissue samples. As shown in Fig. 1A, the level of miR-584-5p was significantly lower in tumor samples compared with that in normal tissues ($P < 0.05$). This result was consistent with a recent study published in 2017, which indicated decreased miR-584-5p levels in the arterial plasma as well as tumor tissues of LC patients and identified miR-584-5p as a biomarker in the diagnosis of LC (16).

We were eager to know the effects of miR-584-5p over-expression in tumor progression. Thereafter, we tested the expression change of miR-584-5p in A549 cells after the transfection of miR-584-5p mimics. As showed in Fig. 1B, compared with the miR-NC group, miR-584-5p mimics significantly elevated the expression of miR-584-5p *in vitro*, which provide the evidence that the transfections were successful.

As shown in Table I, there was no significant difference in miR-584-5p expression by sex ($P = 0.896$), age ($P = 0.552$) or smoking history ($P = 0.676$). However, there was a significant

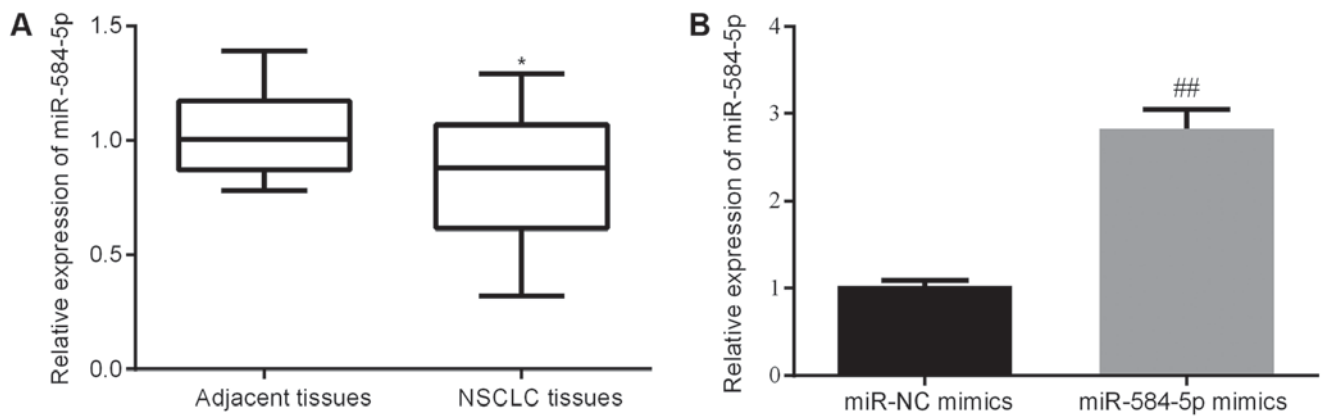


Figure 1. Level of miR-584-5p is decreased in tissue samples. (A) The results of RT-qPCR analysis revealed that the miR-584-5p level was significantly lower in tumor samples when compared with adjacent normal tissues. (B) The results of RT-qPCR analysis revealed that when compared with the miR-NC group, miR-584-5p mimics significantly elevated the expression of miR-584-5p *in vitro*. * $P < 0.05$ vs. adjacent normal tissues; ## $P < 0.01$ vs. miR-NC mimics. RT-qPCR, reverse transcription-polymerase chain reaction; NC, negative control; NSCLC, non-small-cell lung cancer; miR, microRNA.

difference by lymph node metastasis, as 22 patients with lymph node metastasis expressed significantly lower miR-584-5p levels compared with the remaining 8 patients who had no metastatic lymph nodes ($P = 0.009$); and a significant difference in TNM stage, 13 patients at stage I + II expressed higher miR-584-5p levels compared with the remaining 17 patients who were at stage III+IV ($P = 0.038$).

Identification of the mRNA target of miR-584-5p. miR-584-5p could target multiple mRNAs, including WWP1 (15), ROCK1 (19), and KLRG1 (20). The binding site between miR-584-5p and MMP-14 is shown in Fig. 2A. As is well known, MMP-14 plays a key role in tumor invasion and metastasis (21-23). Overexpression of MMP-14 has been correlated with poor prognosis in NSCLC patients (23), and the MMP-14 protein may be helpful in predicting the prognosis of NSCLC (24,25).

Dual luciferase assay in A549 cells revealed that miR-584-5p mimics resulted in decreased MMP-14 promoter activity ($P < 0.01$; Fig. 2B).

Evaluation of the effects of miR-584-5p on cell migration and cell invasion. In the wound healing assay, overexpression of miR-584-5p inhibited the migration of A549 cells compared with that in the miR-NC mimics group ($P < 0.01$, Fig. 3A and B).

In the Transwell assay, miR-584-5p mimics reduced A549 cell invasion compared with that in the miR-NC mimics group ($P < 0.01$; Fig. 3C and D).

These results are consistent with those of a previous study on the tumor suppressive role of miR-584-5p in human neuroblastoma (26).

Evaluation of the effects of miR-584-5p on MMP-14 level. Afterwards, we examined the changes in the expression of MMP-14 at the protein and mRNA level in A549 cells. The expression of MMP-14 was found to be reduced by miR-584-5p compared with that in the miR-NC mimics group ($P < 0.01$; Fig. 4A-C).

Evaluation of the effects of miR-584-5p on MMP4 and Slug level. Afterwards, we examined the changes in the expression

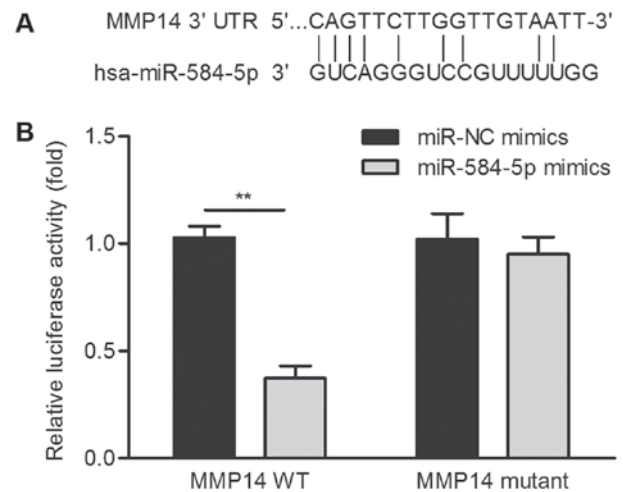


Figure 2. MMP-14 is a target of miR-584-5p. (A) The binding site between MMP-14 and miR-584-5p was identified. (B) Dual luciferase assay revealed that miR-584-5p mimics decreased the MMP-14 promoter activity in A549 cells. ** $P < 0.01$, as indicated. MMP, matrix metalloproteinase; NC, negative control; miR, microRNA; WT, wild-type; UTR, untranslated region.

of MMP-4 and Slug at the protein and mRNA levels in A549 cells. The expression levels of MMP-4 and Slug were reduced by miR-584-5p compared with that in the miR-NC mimics group ($P < 0.01$; Fig. 5A-C).

Discussion

In breast cancer, miR-584 downregulation is a prerequisite for transforming growth factor β -induced cell migration and motility, its downstream target being phosphatase and actin regulator 1 (27); in renal cell carcinoma, miR-584 downregulation is indispensable for decreasing cancer cell invasion by targeting ROCK1 (28). In conclusion, these results demonstrated that miR-584 potentially acts as a tumor suppressor. In accordance with the aforementioned reports, the present study also demonstrated that miR-584-5p was decreased in the tumor tissues of NSCLC patients.

Due to the elevation of MMP-14 and its role in predicting poor overall survival in glioma (29,30), we suggested that

Table I. Characteristics of non-small cell liver cancer patients.

| Characteristics | Cases (n) | miR-584-5p (mean \pm SD) | P-value |
|-----------------------|-----------|----------------------------|---------|
| Gender | | | 0.896 |
| Male | 22 | 0.830 \pm 0.063 | |
| Female | 8 | 0.815 \pm 0.071 | |
| Age (years) | | | 0.552 |
| ≥ 60 | 17 | 0.852 \pm 0.077 | |
| < 60 | 13 | 0.792 \pm 0.056 | |
| Smoking history | | | 0.676 |
| No | 10 | 0.796 \pm 0.107 | |
| Yes | 20 | 0.841 \pm 0.054 | |
| Lymph node metastasis | | | 0.009 |
| No | 8 | 1.033 \pm 0.047 | |
| Yes | 22 | 0.781 \pm 0.058 | |
| TNM stage | | | 0.038 |
| I+II | 13 | 0.942 \pm 0.080 | |
| III+IV | 17 | 0.737 \pm 0.054 | |

TNM, tumor-node-metastasis; miR, microRNA; SD, standard deviation.

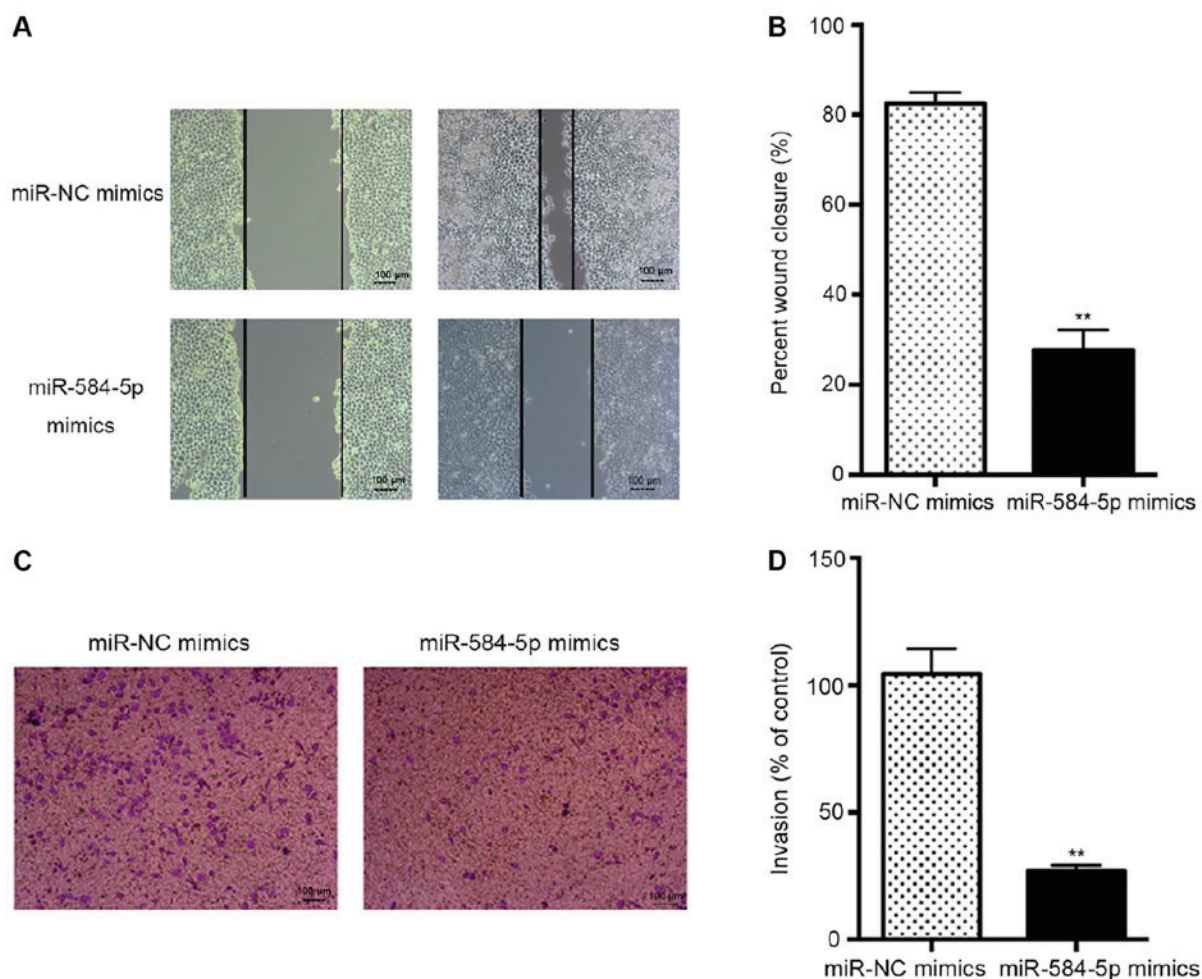


Figure 3. miR-584-5p inhibits cell migration and cell invasion. (A and B) miR-584-5p mimics attenuated the migration of A549 cells when compared with cells transfected with miR-NC mimics at 24 h following scratching. (C and D) miR-584-5p mimics impaired the invasion of A549 cells when compared with cells transfected with miR-NC mimics at 24 h following incubation in Transwell chambers (scale bars, 100 μ m). ** $P < 0.01$ vs. miR-NC mimics. NC, negative control; miR, microRNA.

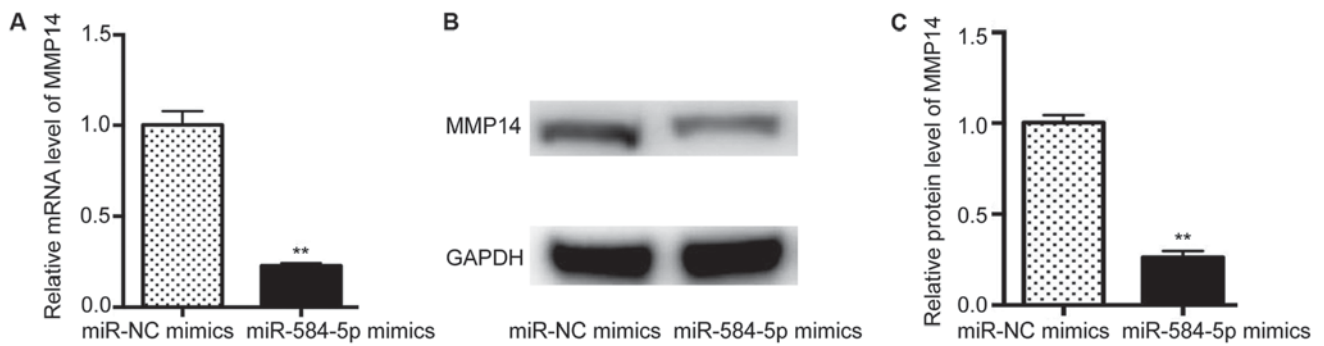


Figure 4. miR-584-5p decreases MMP-14 level. The expression of MMP-14 at the (A) mRNA and (B and C) protein level in A549 cells was reduced by miR-584-5p mimics at 48 h following cell transfection. ** $P < 0.01$ vs. miR-NC mimics. MMP, matrix metalloproteinase; NC, negative control; miR, microRNA.

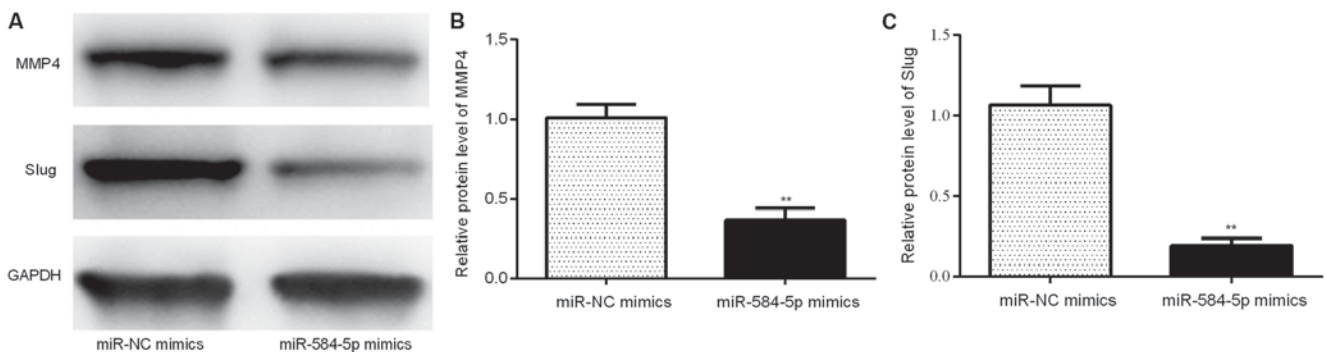


Figure 5. miR-584-5p decreases MMP4 and Slug levels. (A) The protein expression levels of (B) MMP-4 and (C) Slug were reduced by miR-584-5p when compared with the miR-NC mimics group. ** $P < 0.01$ vs. miR-NC mimics. MMP, matrix metalloproteinase; NC, negative control; miR, microRNA.

miR-584-5p downregulation may induce a decrease in the MMP-14 level in NSCLC. We demonstrated that, compared with the miR-NC group, miR-584-5p mimics significantly elevated the expression of miR-584-5p *in vitro*, which provide the evidence that the transfections were successful; and compared with the miR-NC mimics group, miR-584-5p mimics significantly inhibited MMP-14 expression by targeting the promoter, as shown by a luciferase reporter gene assay, and that miR-584-5p inhibited the expression of MMP-14 at the protein and mRNA level.

LC is characterized by a high propensity for tumor invasion and migration, which is the main cause of LC-related death (31,32). We next investigated the effects of miR-584-5p on the migration and invasion of the NSCLC cell line A549. Consistent with previous reports demonstrating the tumor suppressive role of miR-584-5p in breast cancer and renal cell carcinoma (27,28), our results also revealed that miR-584-5p acted as a tumor suppressive factor in NSCLC, as evidenced by miR-584-5p overexpression decreasing the migration and invasion of NSCLC cells *in vitro*.

Changes in proteins involved in tumor invasion and metastasis, such as MMP4 (33) and Slug (34) were also detected by western blot. Results demonstrated that the expression levels of MMP-4 and Slug were reduced by miR-584-5p compared with that in the miR-NC mimics group.

In summary, the findings of the present study demonstrated the tumor suppressive role of miR-584-5p in NSCLC, and indicated that miR-584-5p/MMP-14 axis may be a novel target for the treatment of NSCLC patients.

However, there are several limitations in the present study as listed: 1. We mainly focus on the effects of miR-584-5p on cell migration and cell invasion, there was no data about the effect of miR-584-5p on LC cell proliferation which we will complement in our further study; 2. MMP-14 plays a key role in tumor invasion and metastasis, we mainly checked the expression changes of MMP-14 in the present study. stretching, MMP4, Slug are involved in tumor invasion and metastasis, which will be detected in the future work; 3. there was no data about the role of miR-584-5p *in vivo*, and the effects of miR-584-5p on clinical cases related to the clinical staging of LC, which will be complemented in our further study; 4. It would be necessary to use either primary cell lines from a few patients or use aggressive and non-aggressive NSCLC cell lines in the future study.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

TG, CZ and ZW performed the experiments. TG, CZ and XZ analyzed the data. XZ designed the project and prepared the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of Weifang Second People's Hospital (Shandong, China). Written informed consent was obtained from all the subjects.

Patient consent for publication

Written informed consent was obtained from all the subjects.

Competing interests

The authors declare that they have no competing interests.

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