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MicroRNA-204 targets SOX4 to inhibit metastasis of lung adenocarcinoma

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Abstract. – OBJECTIVE: The dysregulation of microRNAs (miRNAs) has been found in human cancers. In this study, the functions of miR-204 and SOX4 (sex-determining region Y-box 4) and their interaction on lung adenocarcinoma cell metastasis and epithelial-mesenchymal transition (EMT) were investigated.

PATIENTS AND METHODS: MiR-204 and SOX4 expressions were examined via quantitative Real-time polymerase chain reaction (qRT-PCR) in lung adenocarcinoma. Western blot was used to detect the expressions of SOX4 and EMT markers. The relationship between miR-204 and SOX4 was verified by a dual-luciferase reporter assay. Transwell assay was utilized to explore the functions of miR-204 and SOX4 associated with lung adenocarcinoma metastasis.

RESULTS: First, downregulation of miR-204 was examined in lung adenocarcinoma tissues. Moreover, overexpression of miR-204 inhibited metastasis and EMT of lung adenocarcinoma cells. In addition, SOX4 has been shown to be a direct target of miR-204 in lung adenocarcinoma. SOX4 silencing suppressed cell metastasis and EMT in lung adenocarcinoma. And the upregulation of SOX4 impaired the inhibitory effect of miR-204 on lung adenocarcinoma metastasis.

CONCLUSIONS: MiR-204 inhibited cell metastasis and EMT in lung adenocarcinoma through targeting SOX4.

Key Words:
Lung adenocarcinoma, miR-204, Metastasis, Epithelial-to-mesenchymal transition, SOX4.

Introduction

Lung cancer is the most malignant tumor which is further the most harmful to human health and life[1]. In many countries (including China), the incidence of lung adenocarcinoma has exceeded that of lung squamous cell carcinoma, accounting for almost half of all lung cancer[2]. Air pollution is becoming more and more serious, and the occurrence of lung adenocarcinoma has gradually attracted people's attention. Although modern medical technology has improved, the prognosis of lung adenocarcinoma is not optimistic[3]. Moreover, it is associated with some factors, such as gender, smoking history, tumor size, histological differentiation, and size of invasive cancer[4]. Early detection of lung adenocarcinoma infiltration, invasion and metastasis, combined with comprehensive surgery based on timely surgery, is of great significance for improving the survival rate of lung adenocarcinoma. Recently, microRNAs (miRNAs) have been widely recognized as potential biomarkers for the diagnosis and prognosis of human cancers. MiRNAs play an important role in tumorigenesis and development through regulating the expressions of their target genes[5]. In lung adenocarcinoma, many miRNAs have been reported to be abnormally expressed and show different effects. For example, overexpression of miR-361-5p played an oncogenic role in human lung adenocarcinoma through modulating SMAD2[6]. In contrast, miR-29c was a suppressive miRNA that targets vascular endothelial growth factor A (VEGFA) in lung adenocarcinoma[7]. Today, the dysregulation of miR-204 in various cancers has caught our attention. Upregulation of miR-204 has been identified in oral cavity squamous cell carcinoma[8]. And miR-204 promoted fracture healing through enhancing the cell viability of osteoblasts[9]. On the contrary, miR-204-5p exerted anti-tumor effects on melanoma cells[10]. Shuai et al[11] also reported that miR-204 inhibited tumor growth and cell motility by regulating CXCL8 in colorectal cancer. However, the effect of miR-204 on cell metastasis remains unclear in lung adenocarcinoma. As a member of the sox family, SOX4 (sex-determining region Y-box 4) is closely related to proliferation, metastasis and tumor progression. For instance, SOX4 was upregulated

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in urothelial bladder carcinoma[12] and upregulation of SOX4 promoted hepatocellular carcinoma metastasis[13]. Moreover, the carcinogenic effect of SOX4 has been observed in human breast cancer[14]. In addition, SOX4 promoted epithelial-mesenchymal transition (EMT) in prostate cancer and was associated with poor prognosis[15]. EMT is well known to be involved in cancer metastasis. The main reasons of treatment failure are metastasis and recurrence. In addition, the EMT process involves the disintegration of cell-cell junctions, resulting in lower adhesion of mesenchymal phenotype cells, as well as higher cell migration and invasion[16]. In the present study, expression of miR-204 was detected in lung adenocarcinoma. Moreover, the function of miR-204 associated with cell metastasis and EMT has been explored in lung adenocarcinoma. These findings will provide novel potential biomarkers for the diagnosis of lung adenocarcinoma.

Patients and Methods

Patients and Tissue Specimens

The lung adenocarcinoma specimens of 77 patients and adjacent normal lung tissues were obtained from the Faculty of Clinical Medicine, Jilin University. All patients received no treatment except surgery. This study was approved by the Faculty of Clinical Medicine, Jilin University Ethics Committee. All patients were provided with written informed consent. These lung adenocarcinoma tissues were frozen in liquid nitrogen and stored at -80°C for subsequent experiments.

Cell Culture and Transfection

The human lung adenocarcinoma cell lines NCI-H1975, NCI-H2228 and BEAS-2B cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). These cells were cultured in Dulbecco's modified Eagle' medium (DMEM) medium (Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). All cell lines were supplemented with penicillin and streptomycin, and were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The miR-204 mimic and inhibitor, SOX4 siRNA (si-SOX4) were purchased from GenePharma (Shanghai, China). They were then transferred to NCI-H1975 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNAs were extracted from lung adenocarcinoma tissues and cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The complementary deoxyribose nucleic acid (cDNA) was reverse transcribed by the First-Strand cDNA Synthesis kit (Promega, Madison, WI, USA) under the following conditions: 37˚C for 30 min, 85˚C for 5 sec and kept at 4˚C until use. Then, qRT-PCR was performed using SYBR Green Master Mix kit (TaKaRa, Otsu, Shiga, Japan) on ABI Prizm 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as the following thermo cycling conditions: 95°C for 3 min, 95°C for 15 s and 60°C for 15 s for a total of 40 cycles. MiR-204 and SOX4 were normalized by U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression and quantified by the $2^{\Delta\Delta ct}$ method. The primer sequences were as follows: miR-204 Forward, 5'-GCGGCGCAAAGAATTCTC-CT-3', reverse, 5'-GTGCAGGGTCCG AGGT-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3', reverse, 5'-AACGCTTCACGA ATTTGCGT-3'; SOX4 forward, 5'-ACCGGGACCTGGATTTTAAC-3'; and SOX4 reverse, 5'-AAA CCAGGTTGGAGAT-GCTG-3'; GAPDH forward, 5'-CGGAGTCAACG-GATTTGG TCG TAT-3', reverse, 5'-AGCCTTCTC-CATGGTGGTGAAGAC-3'.

Transwell Assays

Migration and invasion of NCI-H1975 cells were examined *via* transwell assay. First, 5×10^3 NCI-H1975 cells with miR-204 mimics or inhibitor were placed in the upper chamber for 24 h at 37°C, while lower chamber filled with 10% (fetal bovine serum) FBS to induce NCI-H1975 cells to migrate through the membrane. Following incubation for 24 h, cells remaining in the upper chamber were removed. The migrated cells were fixed in methanol and then stained with crystal violet for 30 min at room temperature. After washing three times with phosphate-buffered saline (PBS), cells were counted under an inverted microscope (Olympus Corporation, Tokyo, Japan). In addition, cells were placed in the upper chamber along with Matrigel (BD, Franklin Lakes, NJ, USA) for the invasion assay. The other steps are the same as the migration assay.

Dual Luciferase Assay

For reporter assays, cells were seeded into 24-well plates at 50-60% confluence. The pGL3-SOX4-wt or pGL3-SOX4-mut vectors (Promega Corporation,

Madison, WI, USA) was transfected into NCI-H1975 cells with miR-204 mimics. Cells were harvested and lysed for luciferase assays after transfection for 48 h. The luciferase activities were determined by performing a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Western Blot Analysis

Transfected NCI-H1975 cells were harvested using cell scrapers washed with PBS and lysed using Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The protein concentration was determined using a bicinchoninic acid assay protein kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the same amount of protein (20 μg) was separated by a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Next, the membranes were blocked with Tris-buffered saline and Tween-20 (TBST) supplemented with 5% skimmed milk for 2 h at room temperature. The membranes were then incubated with SOX4, EMT markers (E-cadherin, N-cadherin and Vimentin) and GAPDH primary antibodies overnight at 4°C, followed by incubation with the corresponding secondary antibodies. Protein expression levels were then measured by electrochemiluminescence (ECL, Pierce, Rockford, IL, USA).

Statistical Analysis

Statistical analysis was performed by Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) and Graphpad Prism 6 (La Jolla, CA, USA). The correlation between miR-204 and clinical features of lung adenocarcinoma patients was analyzed by Chi-squared test. The overall survival rates and survival differences were tested through the Kaplan-Meier method with log-rank test. Data are expressed as mean ± SD (Standard Deviation). When $p<0.05$, the difference was considered significant.

Results

Downregulation of miR-204 was Detected in Lung Adenocarcinoma

First, miR-204 expression was examined in lung adenocarcinoma tissues and cell lines. And miR-204 expression was identified lower in the lung adenocarcinoma tissues than the control (Figure 1A). Similarly, miR-204 was downregulated in NCI-H1975, NCI-H2228 cells compared to BEAS-2B cells (Figure 1B). The correlation between clinical characteristics of lung adenocarcinoma patients and miR-204 was analyzed. As shown in Table I, abnormal miR-204 expression was associated with lymph node metastasis ($p=0.011$) and TNM stage ($p=0.001$). Besides that, the Kaplan-

Table I. Correlation between the clinicopathological characteristics and miR-204 in lung adenocarcinoma.

Characteristics	Number of Cases (n=77)	miR-204		p-value
		High (n=29)	Low (n=48)	
Age (years)				0.311
≥ 60	35	12	23	
< 60	42	17	25	
Gender				0.731
Male	43	18	25	
Female	34	11	23	
Tumor size (cm)				0.392
≥ 3	47	19	28	
< 3	30	10	20	
Lymph node metastasis				0.011*
Absent	51	19	32	
Present	26	10	16	
TNM stage				0.001*
I + II	29	11	18	
III + IV	48	18	30	
Differentiation				0.074
Well	33	13	20	
Moderate-poor	44	16	28	

Statistical analyses were performed by the χ^2 test.TNM, tumor-node-metastasis. *$p<0.05$ was considered significant.

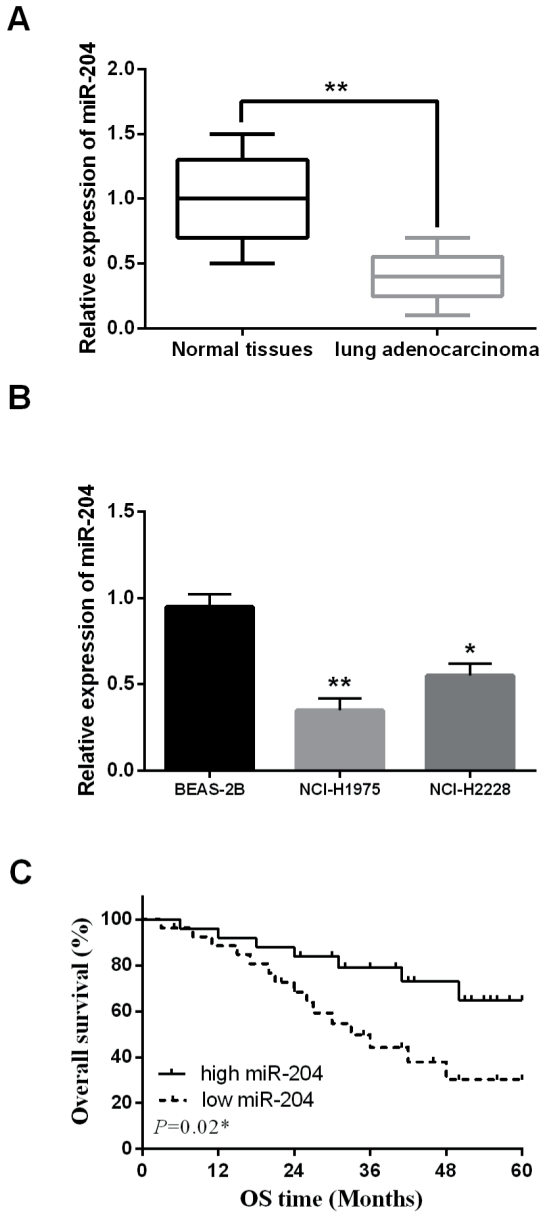

Figure 1. Downregulation of miR-204 was identified in lung adenocarcinoma. ***A,*** MiR-204 expressions in lung adenocarcinoma tissues and normal tissues were detected *via* qRT-PCR. ***B,*** The miR-204 expression was observed in NCI-H1975, NCI-H2228 and BEAS-2B cells. ***C,*** Lung adenocarcinoma patients with high expression of miR-204 showed longer overall survival. *$p<0.05$, **$p<0.01$.

Meier survival curves indicated that shorter overall survival was associated with low miR-204 expression (p=0.02, Figure 1C) in lung adenocarcinoma patients. Therefore, downregulation of miR-204 predicted poorer clinical outcomes in lung adenocarcinoma patients and can be used as an indicator of prognosis in lung adenocarcinoma.

MiR-204 Suppressed Cell Metastasis and EMT in Lung Adenocarcinoma

Next, we transfected miR-204 mimics or inhibitors into NCI-H1975 cells to explore their function. The miR-204 mimics were found to enhance the expression level of miR-204, which was inhibited by the miR-204 inhibitor (Figure 2A). Moreover, overexpression of miR-204 significantly suppressed cell migration, which was promoted by knockdown of miR-204 in NCI-H1975 cells (Figure 2B). Cell invasion regulated by miR-204 mimics or inhibitor was similar to the above results (Figure 2C). At the same time, the effect of miR-204 on EMT was analyzed. Overexpression of miR-204 inhibited the expressions of N-cadherin and Vimentin and promoted the expression of E-cadherin. Inversely, knockdown of miR-204 was found to have an opposite effect on the three markers in NCI-H1975 cells (Figure 2D). Taken together, miR-204 suppressed cell metastasis by blocking EMT in lung adenocarcinoma.

SOX4 was a Direct Target of miR-204

Further, many target genes for miR-204 were searched from Targetscan (http://www.targetscan.org) to illustrate the molecular mechanisms involved in lung adenocarcinoma metastasis in. It was predicted that SOX4 showed binding sites to miR-204 (Figure 3A). Next, the dual-luciferase reporter assay suggested that miR-204 inhibited the luciferase activity of SOX4-wt, but had no effect on SOX4-mut (Figure 3B). Moreover, miR-204 was found to reversely regulate SOX4 expression in lung adenocarcinoma tissues ($p<0.0001$, $R^2=0.6258$; Figure 3C). Additionally, the expression of SOX4 regulated by miR-204 mimics and inhibitor was observed in NCI-H1975 cells. Consistent with the above results, the miR-204 mimics significantly reduced SOX4 expression, while miR-204 inhibitor promoted its expression (Figure 3D, 3E). Briefly, miR-204 directly targets SOX4, which reverses the regulation of SOX4 expression in lung adenocarcinoma cells.

The Knockdown of SOX4 Inhibited Cell Metastasis and EMT in Lung Adenocarcinoma

Subsequently, upregulation of SOX4 was detected in lung adenocarcinoma tissues and cell lines (Figure 4A, 4B). SOX4 siRNA was transfected into NCI-H1975 cells to investigate the effect of SOX4 on cell metastasis. SOX4

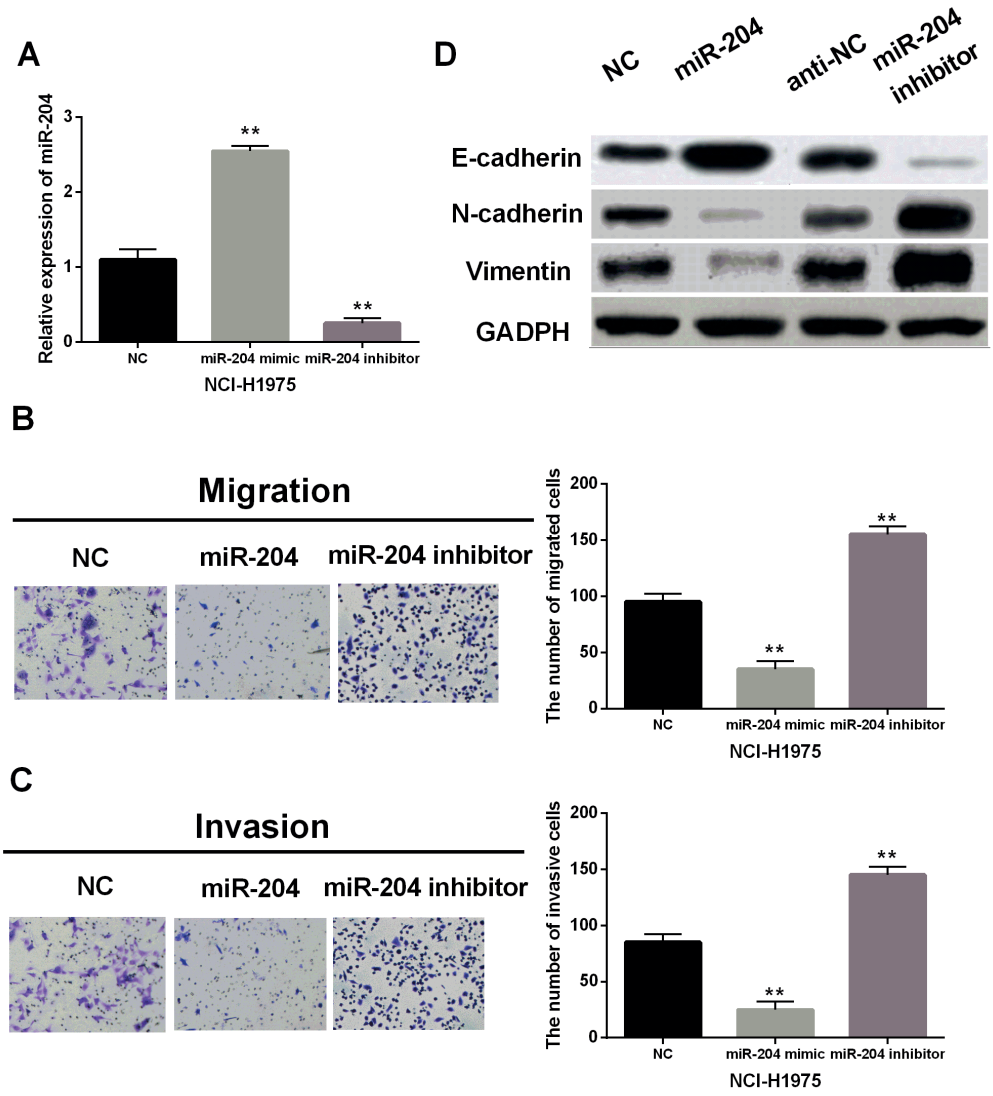

Figure 2. MiR-204 suppressed cell metastasis and EMT in lung adenocarcinoma. ***A,*** MiR-204 expression was observed in NCI-H1975 cells with miR-204 mimics or inhibitor. ***B-C,*** The cell migration and invasion were observed in transfected NCI-H1975 cells. ***D,*** Western blot analysis was performed for E-cadherin, N-cadherin and Vimentin in transfected NCI-H1975 cells. $^{**}p<0.01$.

siRNA significantly reduced SOX4 expression in NCI-H1975 cells (Figure 4C). Functionally, SOX4 silencing has the same effect as miR-204 overexpression on cell migration and invasion in NCI-H1975 cells (Figure 4D, 4E). Furthermore, the effect of SOX4 on EMT was also investigated in NCI-H1975 cells. SOX4 silencing was found to enhance E-cadherin expression level and reduce N-cadherin and Vimentin expressions (Figure 4F). These results implied that SOX4 promoted cell metastasis and EMT in lung adenocarcinoma cells.

Overexpression of SOX4 Impaired the Inhibitory Effect of miR-204 in Lung Adenocarcinoma

Finally, the miR-204 mimic and SOX4 vector were transfected into NCI-H1975 cells to further verify their interaction. We found that upregulation of SOX4 restored a decrease in SOX4 expression induced by miR-204 mimic (Figure 5A, 5B). In NCI-H1975 cells, overexpression of SOX4 attenuated the inhibitory effect of miR-204 on cell migration and invasion (Figure 5C, 5D). Overall, overexpression of SOX4 impaired the

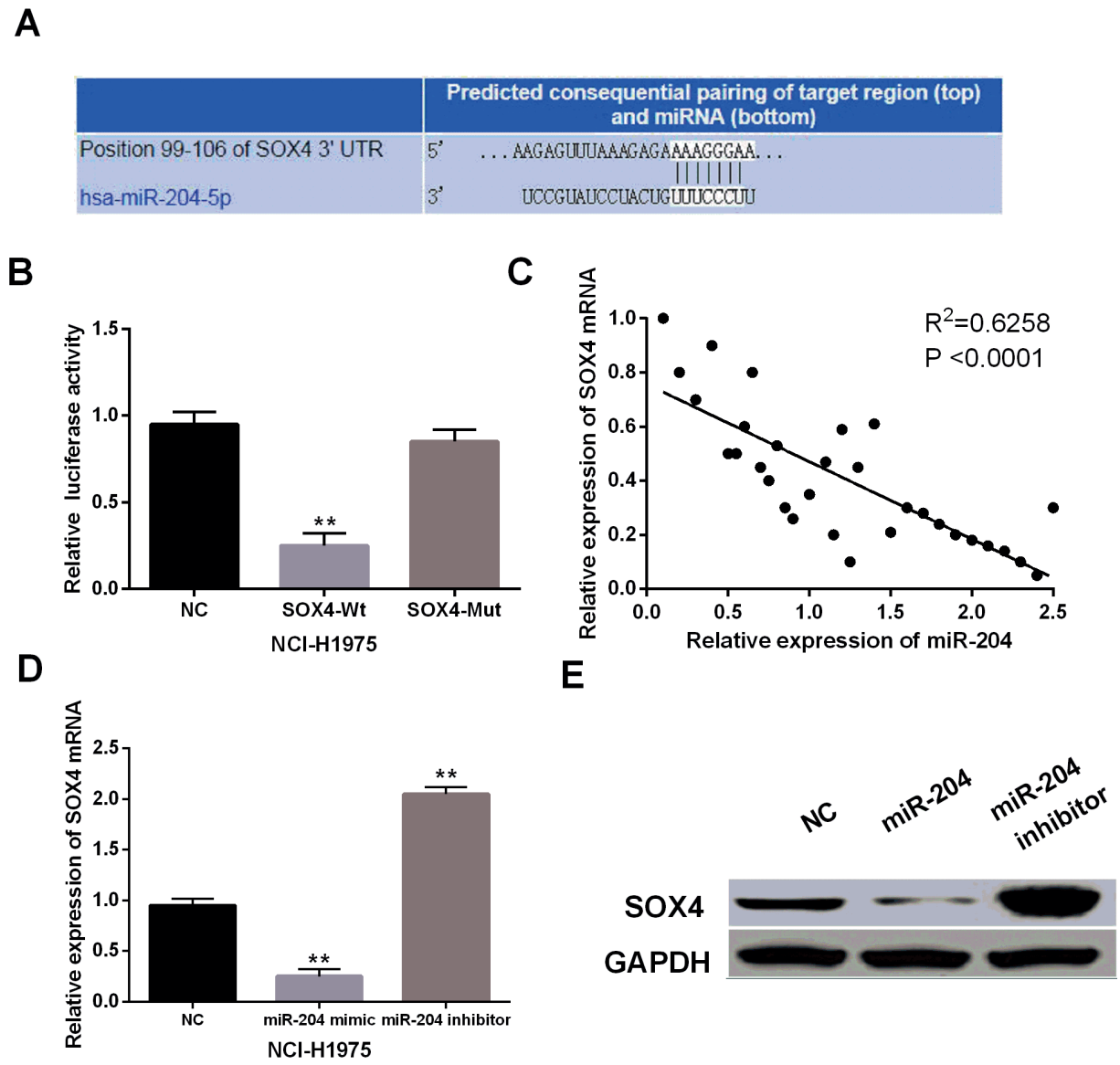

Figure 3. SOX4 was a direct target of miR-204. ***A,*** MiR-204 has binding sites with SOX4. ***B,*** Luciferase reporter assay. ***C,*** The negative association between miR-204 and SOX4 was detected in lung adenocarcinoma tissues. ***D-E,*** The SOX4 expressions were analyzed in NCI-H1975 cells containing miR-204 mimics or inhibitor **$p<0.01$.

inhibitory effect of miR-204, indicating that miR-204 inhibited cell metastasis and EMT by regulating SOX4 expression in lung adenocarcinoma.

Discussion

MiRNAs have been reported to be involved in the pathogenesis and progression of human cancers[17]. It is important to determine the role of miRNAs and their target genes in tumor progression. Many miRNAs are abnormally expressed in lung adenocarcinoma, including miR-145, miR-21 and miR-486[18-20]. Downregulation of miR-204 has been identified in non-small-cell lung carcinoma (NSCLC) and overexpression of miR-204 suppressed invasion and migration of NSCLC cells through regulating JAK2[21]. Similarly, miR-204 was also downregulated in lung adenocarcinoma. Besides that, we also found that low expression of miR-204 was associated with a shorter overall survival time in lung adenocarci-

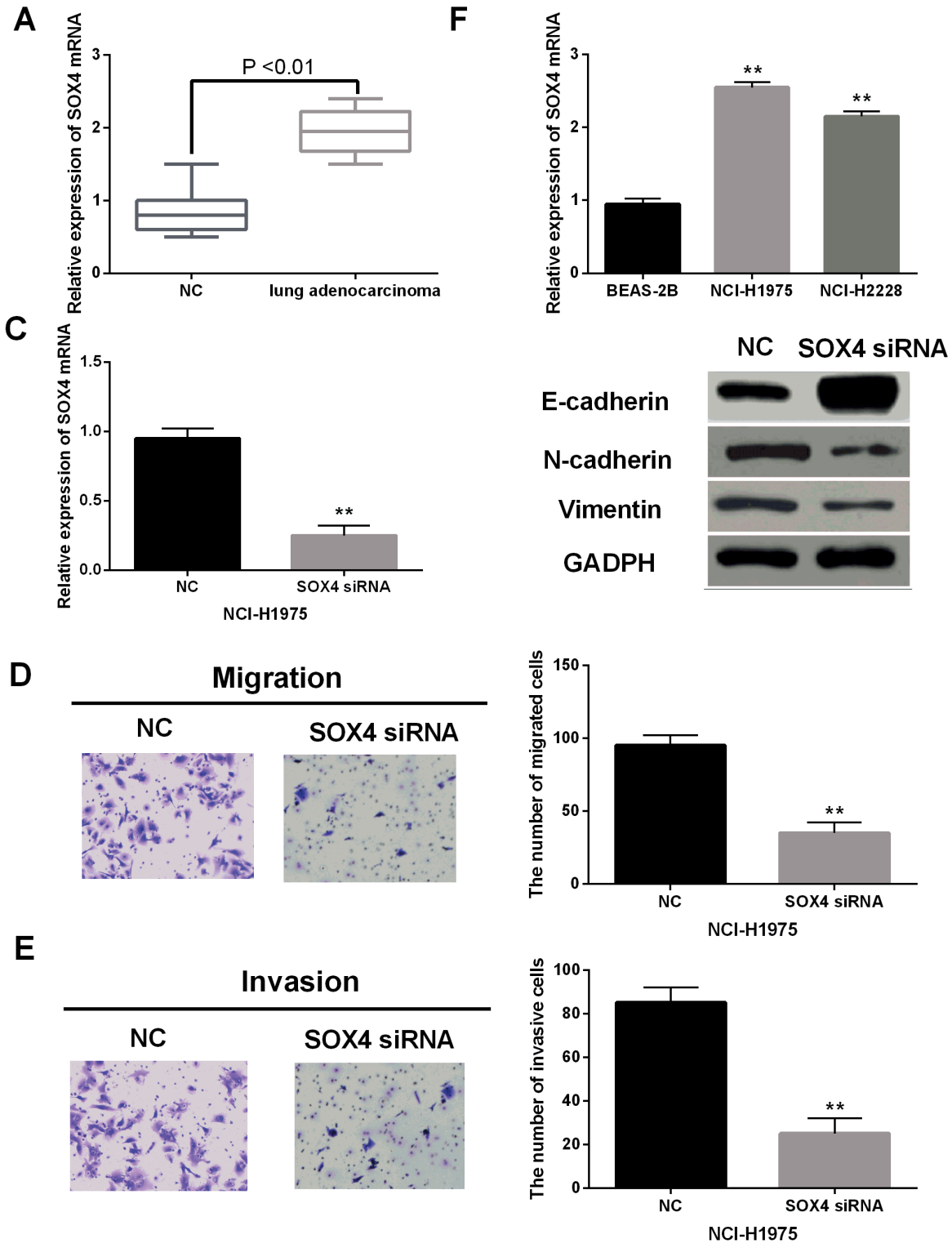

Figure 4. The knockdown of SOX4 inhibited the metastasis and EMT in lung adenocarcinoma. ***A,*** The SOX4 expressions were identified in lung adenocarcinoma tissues. ***B,*** The SOX4 expression was examined in NCI-H1975, NCI-H2228 and BE-AS-2B cells. ***C,*** The SOX4 expression was observed in NCI-H1975 cells with SOX4 siRNA. ***D-E,*** Cell migration and invasion analysis in NCI-H1975 cells with SOX4 siRNA was detected by Transwell assay. ***F,*** The expression of EMT markers was identified in NCI-H1975 cells with SOX4 siRNA. $^{**}p<0.01$.

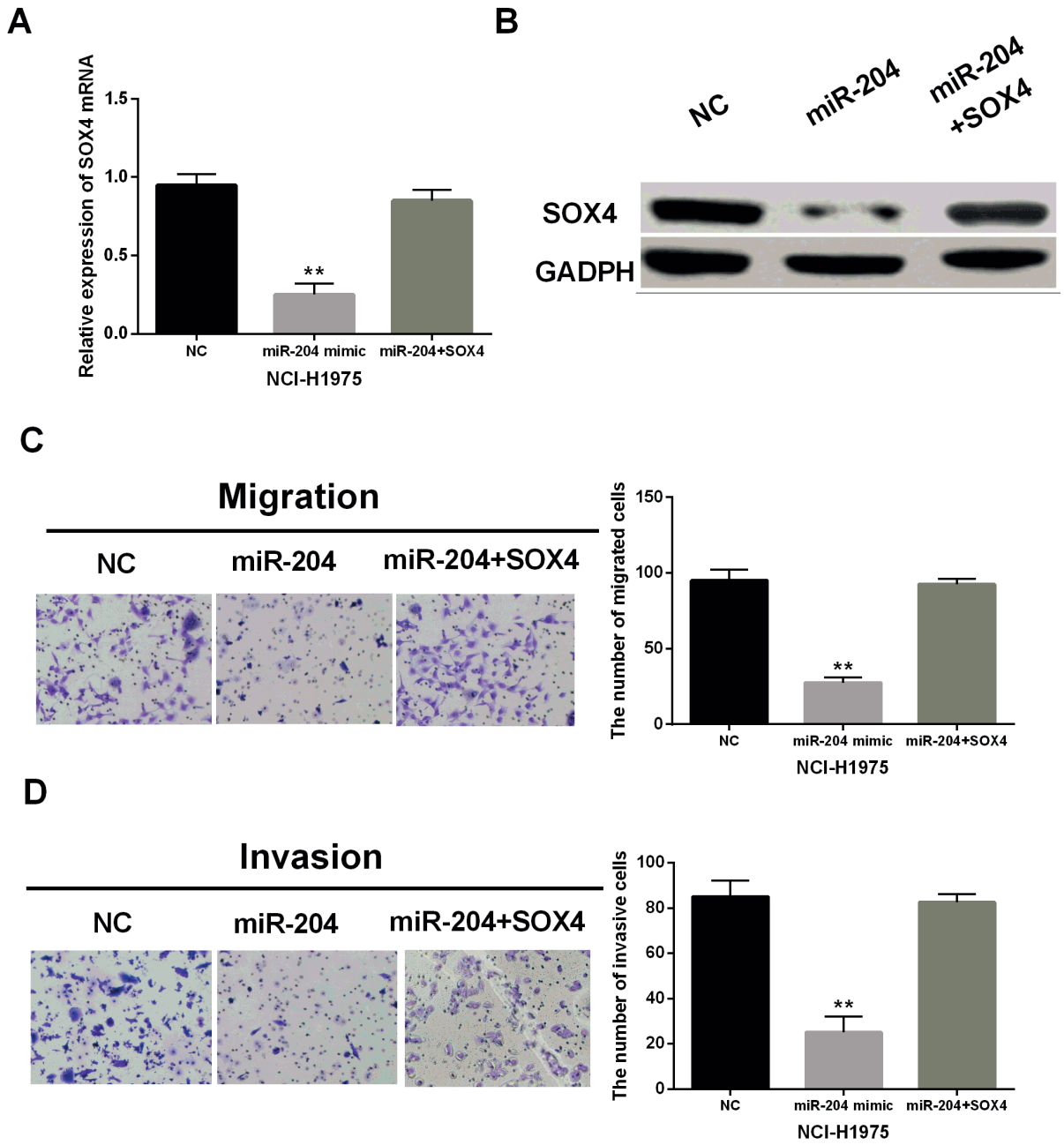

Figure 5. Overexpression of SOX4 impaired the inhibitory effect of miR-204. ***A-B,*** SOX4 expressions were measured in NCI-H1975 cells containing SOX4 vector and miR-204. ***C-D,*** The cell migration and invasion were examined in NCI-H1975 cells containing SOX4 vector and miR-204. $^{**}p<0.01$.

noma patients. However, the regulatory mechanism between miR-204 and SOX4 is unclear. The current study once showed the effect of miR-204 on EMT regulation and metastasis in lung adenocarcinoma. The function of miR-204 has been investigated in several human cancers. Similarly, downregulation of miR-204 has been identified in melanoma, gastric cancer and endometrial cancer[22-24]. In addition, Gao et al[25] demonstrated that miR-204 overexpression suppressed invasion and metastasis of laryngeal squamous cell carcinoma. In lung adenocarcinoma, miR-204 also has the same effect. Besides that, lung adenocarcinoma patients with high expression of miR-204 had a

longer overall survival. Luan et al[26] also implied that low expression level of miR-204-5p predicted a lower overall survival rate in melanoma patients. Further, we found that miR-204 suppressed EMT in lung adenocarcinoma. Consistently, miR-204 inhibited EMT in esophageal cancer[27]. Additionally, the miR-204/ZEB2 axis played a key regulatory role in breast cancer through regulating EMT[28]. This study demonstrated that SOX4 is a direct target gene of miR-204. The interaction of miR-204/SOX4 was also investigated in lung adenocarcinoma. It has been observed that SOX4 expression was increased in many human cancers, such as liver cancer, gastric cancer and breast cancer[29-31]. Upregulation of SOX4 was also found in lung adenocarcinoma. Knockdown of SOX4 inhibited cell metastasis and EMT in lung adenocarcinoma. Castillo et al[32] revealed that SOX4 was involved in the development of small cell lung cancer. SOX4 has been detected to participate in the EMT process *via* regulating EZH2[33]. SOX4 induced EMT and contributed to breast cancer progression[34]. More importantly, we found that miR-204 targeted SOX4 to inhibit metastasis of lung adenocarcinoma. Analogously, miR-338-3p suppressed cell metastasis through blocking EMT and targeting SOX4 in lung cancer[35]. In lung adenocarcinoma, the suppressive effect of miR-204 is similar to miR-338. These findings prove that miR-204 has an inhibitory effect on cell metastasis through targeting SOX4 and regulating EMT in lung adenocarcinoma.

Conclusions

We revealed a downregulation of miR-204 in lung adenocarcinoma tissues, which predicted worse clinical outcomes in patients with lung adenocarcinoma. Furthermore, miR-204 inhibited the metastasis of lung adenocarcinoma by suppressing EMT and targeting SOX4. This study will help us better understand the pathogenesis of lung adenocarcinoma.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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