

ORIGINAL ARTICLE

MiR-24 promotes migration and invasion of non-small cell lung cancer by targeting ZNF367

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Summary

Purpose: Non-small lung cancer (NSCLC) is one of most common cancers worldwide. microRNAs (miRNAs) play an important role in animal biological processes, such as cell growth, differentiation and apoptosis. The aim of this study was to investigate whether MiR-24 can regulate cell proliferation of NSCLC by targeting ZNF367.

Methods: Real time quantitative PCR (qRT-PCR) was used to detect the expression levels of MiR-24 and ZNF367. Western blot assay was employed to analyze the protein levels. Luciferase reporter assay was used to test the target gene of MiR-24. MTT assay was used to detect cell proliferation.

Results: MiR-24 was significantly up-regulated in NSCLC

tissues compared with their corresponding nontumorous tissues ($p < 0.05$). Over-expression of MiR-24 could significantly promoted cell migration and invasion ($p < 0.05$). ZNF367 was a downstream target of MiR-24 and down-regulated by MiR-24. Knockdown of ZNF367 remarkably promoted NSCLC cell proliferation ($p < 0.05$).

Conclusion: The novel identified MiR-24/ZNF367 axis offers new insights into tumorigenesis of NSCLC and a new biomarker for NSCLC treatment.

Key words: invasion, migration, MiR-24, proliferation, ZNF367

Introduction

It was reported that there were 117,920 new cases of lung cancer in men and 106,470 newly diagnosed lung cancer cases in women in United states during 2016 [1]. Lung cancers are divided into two main subtypes based on histology and etiology: NSCLC and small cell lung cancer (SCLC) [2,3]. NSCLC accounts for 85% of lung cancer cases [4] and can be divided into two main subtypes, including squamous cell carcinoma and nonsquamous cell carcinoma [5].

The etiology of NSCLC is complicated. Chemical, physical, environmental stimulators and living habits play an important role in tumorigenesis of NSCLC [6].

The purpose of this study was to explore the molecular mechanisms of NSCLC.

MicroRNA (miRNAs) are 22-26 nucleotides long and non-coding RNAs [7]. miRNAs bind to 3'-UTR of target genes in an inaccurate complementary manner [8]. miRNAs play an important role in animal biological processes, such as cell growth, differentiation and apoptosis [9,10]. In previous studies, many miRNAs have been reported to play a role in NSCLC, such as miR-338-3p [11], miR-187 [12], miR-221 [13], plasma miR-223 [14] and miR-9 [15]. These miRNAs played important roles in cell migration, invasion, cell cycle, cell proliferation and apoptosis. MiR-24 has not been reported in

NSCLC. Zhao et al. reported that miR-24 was highly up-regulated in NSCLC tissues and promoted NSCLC cell proliferation by targeting NAI1 [16]. It is reported that miR-24-3p was significantly up-regulated in hepatocellular carcinoma (HCC) tissues compared with nontumor tissues and could increase HCC cell growth [17]. Yanaihara et al. reported that MiR-24 was up-regulated in lung cancer tissues versus nontumor tissues by qRT-PCR [18].

Zinc finger proteins (ZFPs) recognize DNA sequences and bind the transcription factors to their appropriate promoters [19]. Zinc finger protein 367 (ZNF367) inhibited adrenocortical carcinoma cell proliferation, invasion and migration [20]. The ZNF185 gene was highly methylated in all of prostate cancer metastatic tissues and about half of localized tumor tissues [21]. The function of ZNF367 in NSCLC has not been studied.

In our study, we identified that MiR-24 was highly expressed in NSCLC tissues versus nontumor tissues by qRT-PCR. Over-expression of MiR-24 could significantly increase NSCLC cell migration and invasion. ZNF367 was confirmed as a target of MiR-24 verified by luciferase reporter assay. Knockdown of ZNF367 promoted cell proliferation of NSCLC.

Methods

Human samples and cell lines

A collection of 130 pairs of human NSCLC tissues and corresponding adjacent nontumor tissues were obtained from the Affiliated Hospital of Jining Medical University. These tissues were immediately frozen in liquid nitrogen and stored at -80°C . The experiments were approved by the ethics committee of the Affiliated Hospital of Jining Medical University and all patients provided signed informed consent.

Two human NSCLC cell lines, A549 and H460, were purchased from Tumor Cell Bank of Chinese Academy of Medical Science (Shanghai, China). The cell lines were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS).

Real time quantitative PCR

Total RNA was extracted from tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). miRNAs were extracted from tissues using MiRcute Extraction and Separation of miRNAs Kit (TIANGEN, Beijing, China). cDNAs were synthesized using PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China).

GAPDH was used as an internal control for ANF367. U6 snRNA expression level was used as normalizer for miRNA-24-2 detection. The qRT-PCR experiments were performed on Roche Light Cycler 480 instrument.

Western blot assay

Total proteins were extracted from tissues using RIPA Lysis Buffer (Strong). The protein concentration was detected by BCA reagent Kit (Solarbio, Beijing, China). The proteins were separated by electrophoresis in SDS-PAGE and transferred onto PVF membranes (Millipore, USA). The membranes were incubated with rabbit antibody against ZNF367 (Abcam, Cambridge, USA) at 4°C overnight, and then incubated with the secondary antibody conjugated with horse radish peroxidase (HRP) (Satata Cruz Biotechnology, TX, USA). The protein was developed using the Bio-Rad Gel Doc XR instrument (Bio-Rad, California, USA). Every experiment was performed in triplicate.

Luciferase reporter assay

The luciferase reporter assay was carried out using pmirGLO vector. The 3'-UTR sequence of ZNF367 was inserted into pmirGLO vector (pmirGLO-ZNF367-WT). The binding site of ZNF367 for MiR-24 was mutated and cloned into the pmirGLO vector (pmirGLO-ZNF367-MUT). miRNA-24-2 mimic or control and pmirGLO-ZNF367-WT or pmirGLO-ZNF367-MUT were co-transfected into A549 cells. The luciferase activities

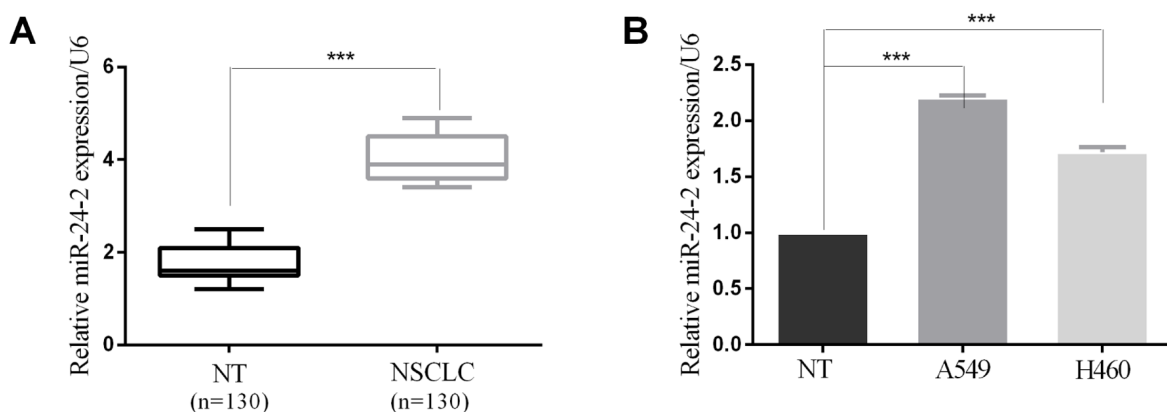


Figure 1. miR-24 is up-regulated in NSCLC tissues and cells. **(A):** The relative expression of miR-24 in 130 NSCLC tissues and normal tissues confirmed by qRT-PCR. **(B):** miR-24 expression levels of NSCLC cell A549, H460 and normal pneumonocytes. NT: normal tissues. *** $p < 0.0001$.

were tested using Dual-Luciferase Assay Kit (Promega, Wisconsin, USA).

Plasmid construction and transfection

The miR-24 mimic and miR-24 inhibitor were purchased from Thermo Fisher Scientific Corporation (Thermo Fisher Scientific, Massachusetts, USA). miR-24 inhibitor was used to suppress miR-24 expression. ZNF367-siRNA was used for interfering the expression of ZNF367. All the transfection experiments carry out using Lipofectamine 2000 (Invitrogen, California, USA).

Migration and invasion assays

The transwell inserts were used to measure the mi-

gration and invasion abilities with or without matrigel. 200 μ l of cell suspension was loaded into the Transwell chamber. The cell density was 5×10^5 /ml. Medium containing FBS (500 μ l) was loaded into the lower Transwell chamber. The cells were cultured for 36 hrs at 37°C and 5% CO₂. Crystal violet (0.1%) was used to stain cells and microscope was used to observe and count the cells.

Proliferation assay

3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) was used for the cell proliferation assay. The cells at the logarithmic growth phase with 80% confluence were suspended using trypsin (Transgene Biotech, Guangzhou, China). Each well of 96-well

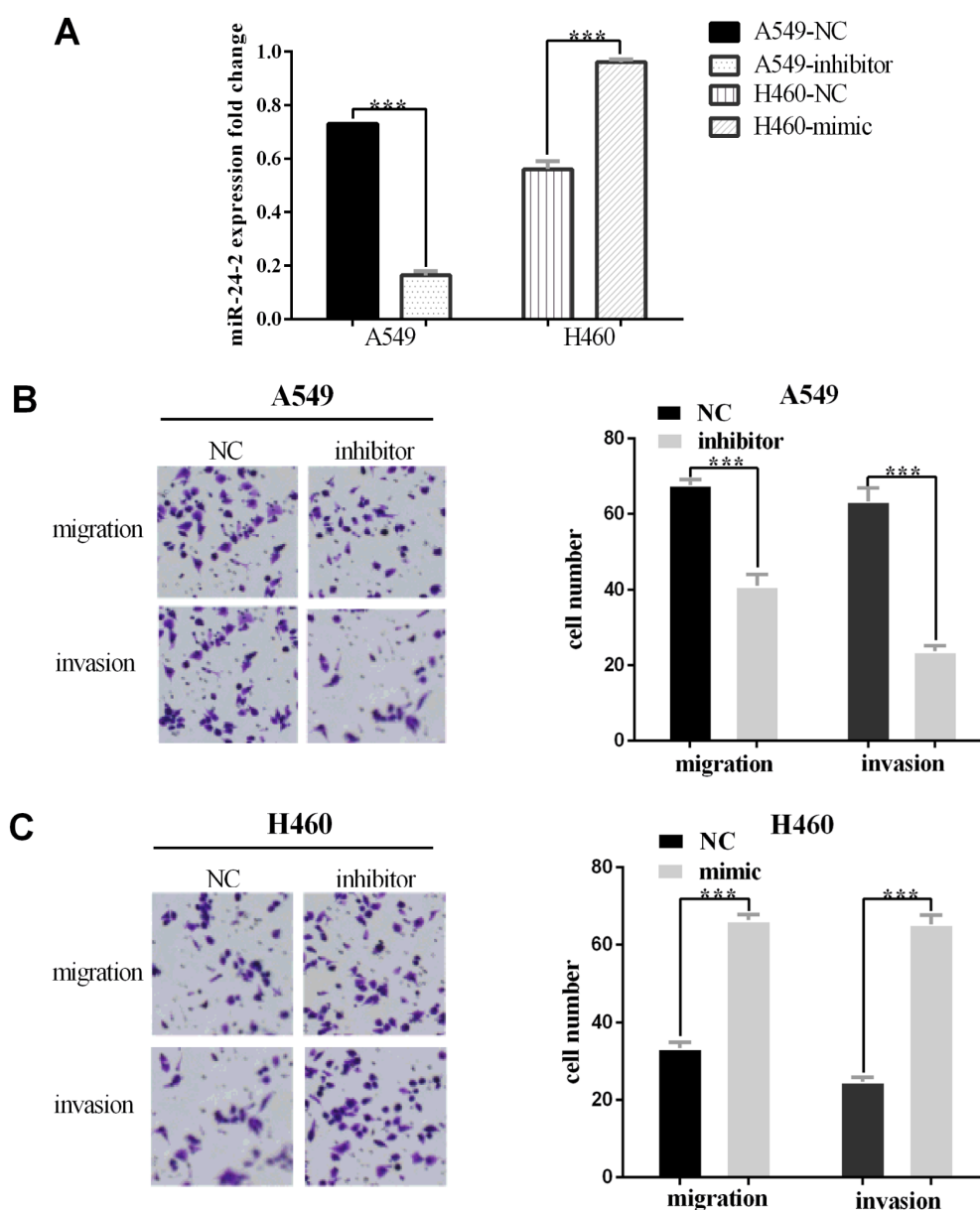


Figure 2. miR-24 promoted the migration and invasion of NSCLC cells in vitro. **(A):** Successful inhibition of the expression of miR-24 in A549 cells and over-expression of miR-24 in H460 cells respectively by miR-24 inhibitor and miR-24 mimic. **(B):** Reduced miR-24 ability on migration and invasion in A549 cells. **(C):** Improved expression level of miR-24 promoted the ability of migration and invasion in H460 cells. NC: negative control. The magnification of B and C was 200 \times and the cells were stained by crystal violet. * $p < 0.0001$.

plate was added with 100 μ l of cell suspension and the cells were cultured for 24 hrs at 5% CO₂ and 37°C. Afterwards, 10 μ l MTT (5mg/ml) were added into each well and cultured for another 4 hrs. Then RPMI 1640 medium was removed and 150 μ l DMSO (Sigma, NY, USA) was added into each well to dissolve formazan crystals. The absorbance at 490 nm was tested using Genios plus (Eppendorf, Hamburg, Germany).

Statistics

All statistical analyses were performed using SPSS 16.0 software. All data were presented as mean \pm SD.

Differences between groups were assessed using the Student's t-test unless otherwise noted and $p < 0.05$ was considered statistically significant.

Results

MiR-24 was significantly overexpressed in NSCLC tissues and A549 and H466 cells

qRT-PCR was used to investigate the miR-24 expression level. The results showed that miR-24 expression was significantly increased in NSCLC

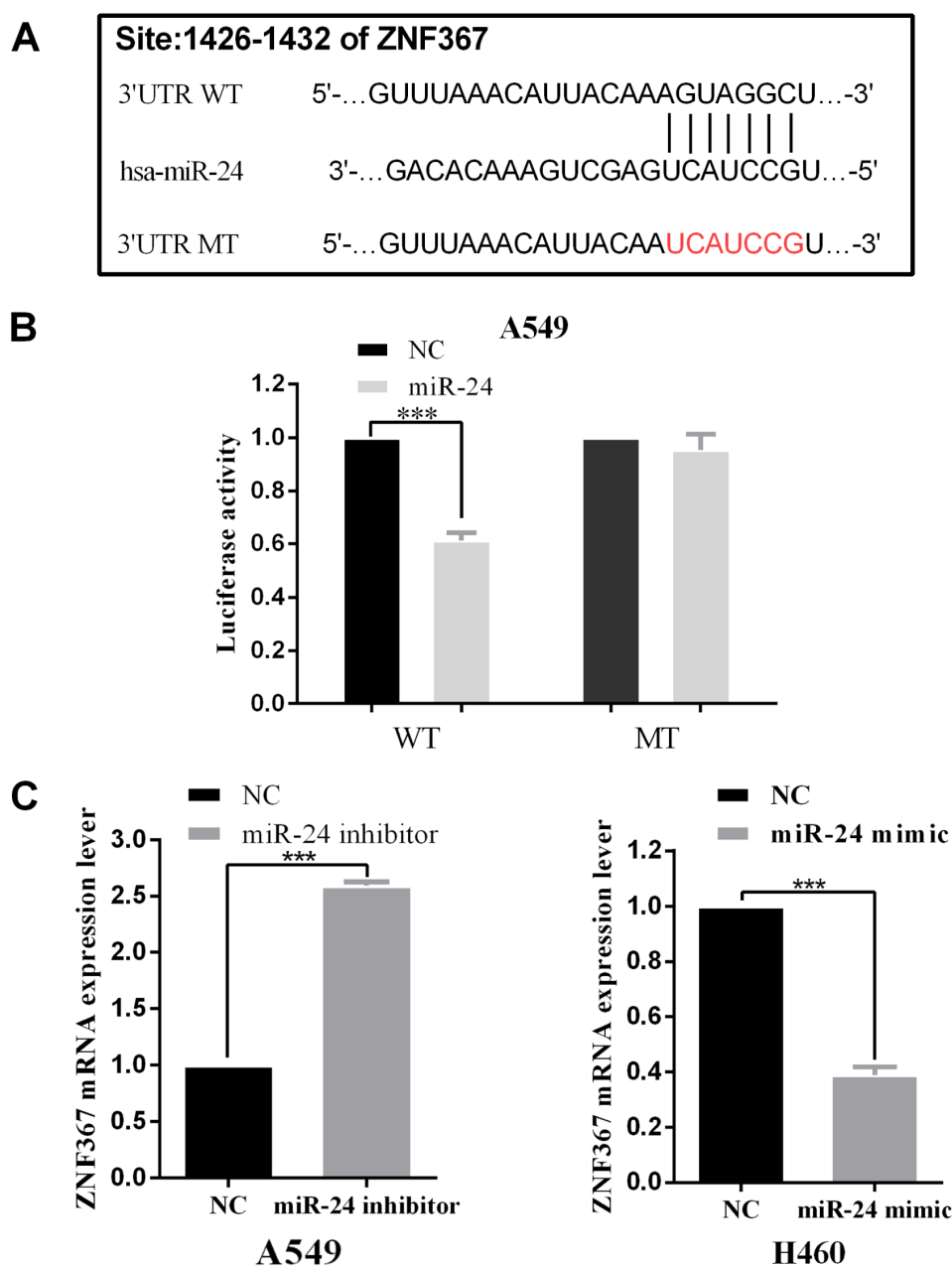


Figure 3. ZNF367 is a target of miR-24. **(A):** The putative binding site of miR-24 was at 1426-1432 of ZNF367 3'-UTR. **(B):** Firefly luciferase activity was decreased when co-transfected with miR-24 mimic and WT 3'-UTR reporter in A549 cells. The inhibition of miR-24 was abolished in cells co-transfected with miR-24 mimic and MT 3'-UTR reporter. **(C):** The expression level of ZNF367 decreased by miR-24 mimic and increased by miR-24 inhibitor. WT: wild type of ZNF367 3'-UTR, MT: mutant ZNF367 3'-UTR, NC: negative control. *** $p < 0.0001$.

tissues versus noncancer tissues ($p < 0.0001$; Figure 1A).

On the other hand, we determined the miR-24 expression levels of NSCLC A549 cells, H460 cell line and normal lung BEAS-2B cell line. The results showed that miR-24 was up-regulated about two-fold in NSCLC cells compared with normal cell line ($p < 0.0001$; Figure 1B).

miR-24 promoted migration and invasion of NSCLC A549 and H460 cells

MiR-24 mimic was used to overexpressed miR-24. The A549 cells were transfected with miR-24 inhibitor and negative control and H460 cells were transfected with MiR-24 mimic. The qRT-PCR results demonstrated that MiR-24 expression was highly increased by transfecting MiR-24 mimic and transfection of MiR-24 inhibitor significantly inhibited MiR-24 expression ($p < 0.0001$; Figure 2A).

Transwell assay was used to detect cell migration and invasion. Migration and invasion of H460 cells transfected with MiR-24 mimics increased significantly. On the other hand, migration and

invasion of A549 cells transfected with MiR-24 inhibitor decreased. The results showed that MiR-24 promoted cell migration and invasion (Figure 2B, 2C).

ZNF367 was a target of MiR-24 and mediated by MiR-24

TargetScan online tool (http://www.targetscan.org/vert_71/) was used to predict potential downstream targets of MiR-24. ZNF367 was identified as a target gene of MiR-24. The binding sites of ZNF367 for MiR-24 were located at 1426-1432 at 3'-UTR (Figure 3A).

Luciferase reporter assay was used to test the potential target of MiR-24. A549 cells were transfected with pmirGLO-ZNF367-WT or pmirGLO-ZNF367-MT and negative control. The luciferase activity was significantly reduced when cell lines were transfected with pmirGLO-ZNF367-WT compared with cell lines which were transfected with negative control ($p < 0.0001$). The reduced effect was attenuated when the cell lines were transfected with pmirGLO-ZNF367-MT ($p < 0.0001$; Figure 3B).

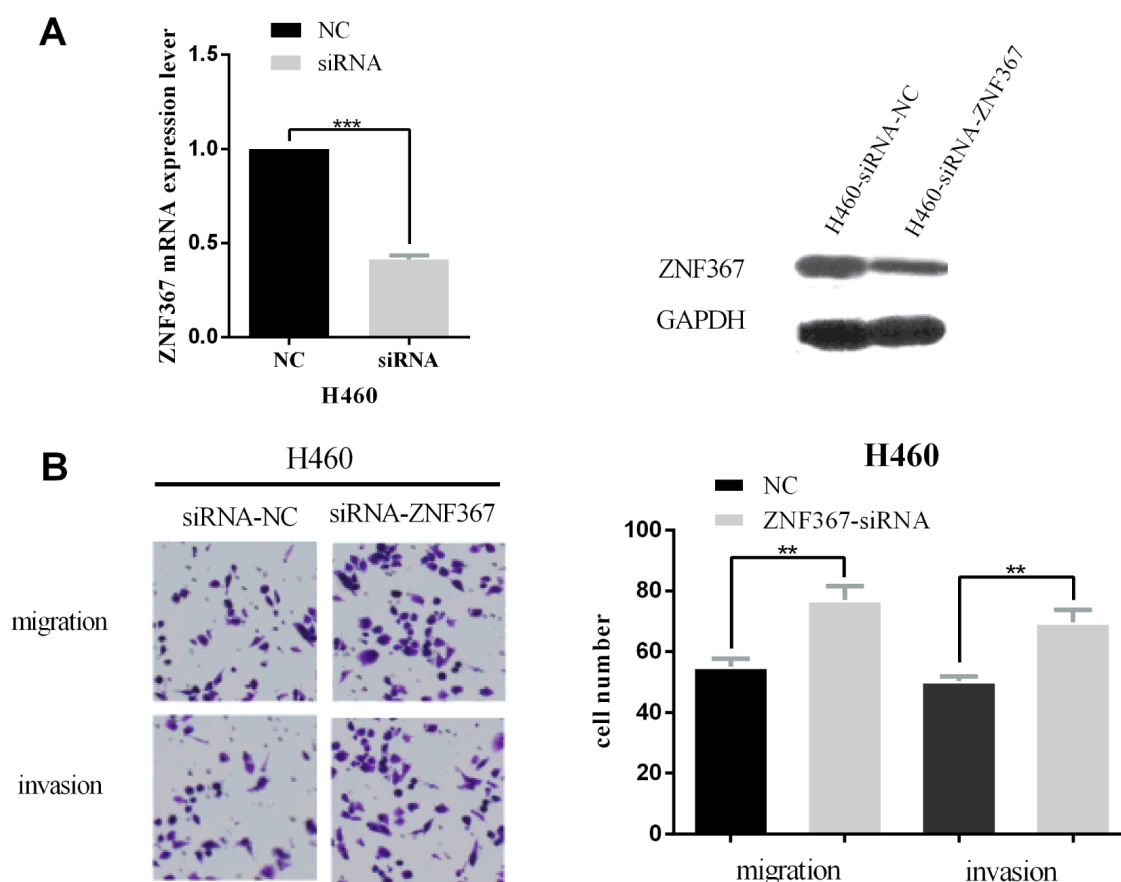


Figure 4. Knockdown of the expression of ZNF367 promoted the ability of migration and invasion. **(A):** ZNF367 expression change was detected by qRT-PCR and Western blot. **(B):** Transwell assay detected the ability of migration and invasion, which increased when interfered with ZNF367. Magnification of B and C was 200 \times and the cells were stained by crystal violet. ** $p < 0.01$; *** $p < 0.0001$.

On the other hand, we also investigate ZNF367 mRNA levels by qRT-PCR assay. The variation trend of mRNA levels was consistent with luciferase activity ($p < 0.0001$; Figure 3C).

Depletion of ZNF367 could partially reverse the function of MiR-24

siRNA for ZNF367 was used for interfering the expression of ZNF367 in A549 cells and Western blotting and qRT-PCR were used to test the effectiveness (Figure 4A). Migration and invasion were detected after interference by Transwell assay and the capacity of migration and invasion were significantly increased (Figure 4B).

Discussion

In this study it was shown that MiR-24 was up-regulated in NSCLC cell lines and tissues. The over-expression of MiR-24 significantly promoted NSCLC cell migration and invasion *in vitro*. Zhao et al. reported that miR-24 was up-regulated in NSCLC tissues with increased cell proliferation by targeting NAIF1 [16]. Consistent with this report, the MiR-24 was also up-regulated in NSCLC cell lines and tissues. However, the authors did not analyze the effect of miR-24 on NSCLC cell migration and invasion. On the other hand, ZNF367 was the downstream target of MiR-24 in our study. In colon cancer, TRIM11, a downstream target of miR-24-3p, promoted cell proliferation and suppressed apoptosis [22]. In the present study the effect of MiR-24 on cell apoptosis was not analyzed.

In breast cancer, the miR-24 could induce chemotherapy resistance and was also up-regulated in breast cancer cells [23]. Mishra et al. reported that miR-24 induced methotrexate resistance by binding to dihydrofolate reductase gene [24].

miR-24 was up-regulated in various types of cancer, but there are exceptions. Hoin Kang et al. [25] reported that miR-24-3p was down-regulated in metastatic cancers, such as breast cancer and HCC and inhibited cell migration and invasion. Reasonable explanations for the opposite results could be related to different experimental conditions, different mediums and culture conditions.

In our study, we demonstrated that ZNF367 was a downstream target of MiR-24. The knock-down of ZNF367 promoted NSCLC cell proliferation *in vitro*. ZNF367 suppressed adrenocortical carcinoma cell proliferation, invasion and migration *in vitro* and *in vivo* [20] and our results were consistent with this report. Compared with this report, one drawback of our study was that we didn't analyze ZNF367 expression level in NSCLC tissues and nontumor tissues.

These results suggested that ZNF367 was a tumor suppressor. Genes can be inactivated by DNA methylation, such as global genomic methylations [26] and promoter methylation of CpG islands [27]. Vanaja et al. reported that CpG islands of ZNF185 were methylated in all prostate cancer metastatic tissues and 44% of localized tumor tissues [21]. In our study, transfection of siRNA for ZNF367 into A459 cell line could significantly decrease ZNF367 expression level. In a future study, we will perform the ZNF367 methylation analysis in NSCLC cell lines, tissues and noncancer tissues.

In conclusion, we demonstrated a novel MiR-24/ZNF367 biomarker in NSCLC. The MiR-24/ZNF367 axis may provide a new therapeutic target for the treatment of NSCLC and deepen our understanding of NSCLC mechanisms.

Conflict of interests

The authors declare no conflict of interests.

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