

The miR-224 promotes non-small cell lung cancer cell proliferation by directly targeting RASSF8

L. WANG¹, W. LIU², Y.-P. ZHANG¹, X.-R. HUANG¹

¹Department of Respiration Medicine, People's Hospital of Rizhao, Rizhao, Shandong Province, P.R. China

²Department of Pharmacy, People's Hospital of Rizhao, Rizhao, Shandong Province, P.R. China

Abstract. – OBJECTIVE: The purpose of this study was to explore the mechanism of microRNA-224 (miR-224) in NSCLC.

PATIENTS AND METHODS: Quantitative RT-PCR (qRT-PCR) was used to evaluate expression levels of miR-224. The association of miR-224 with the clinicopathologic features of NSCLC was evaluated in 56 patients. The roles of miR-224 in cell proliferation were analyzed *in vivo* and *in vitro* with pre-miR-224 transfected cells. Also, the regulation of RASSF8 by miR-224 was evaluated by qRT-PCR, Western blotting and luciferase reporter assays.

RESULTS: In this study, we identified miR-224 to be significantly up-regulated in NSCLC tissues and associated with tumor size. Increased miR-224 expression promotes NSCLC cell proliferation by down-regulating RASSF8 at the mRNA and protein levels. The AKT pathway was found aberrantly activated after over-expression of miR-224. RASSF8 was identified as a direct target of miR-224 by bioinformatics analysis and luciferase reporter assay.

CONCLUSIONS: The miR-224 played an oncogenic role in the proliferation of NSCLC by direct targeting RASSF8, and it is suggested that miR-224 may be a potential therapeutic target for NSCLC.

Key Words:

Nonsmall cell lung cancer, miRNA-224, RASSF8, Proliferation.

Instruction

Lung cancer is the most common cause of cancer-related death worldwide, and nonsmall cell lung cancer (NSCLC) accounts for 80% of all lung cancer¹. NSCLC is comprised of adenocarcinoma, squamous cell carcinoma and large cell undifferentiated carcinoma. Although advances have made in surgical and medical treatments, the 5-year survival rate of lung cancer patients

remains frustratingly poor¹, and the molecular pathogenesis of NSCLC remains poorly understood². Understanding the molecular mechanisms of NSCLC progression would help to identify potential therapeutic targets and optimize already existing therapies.

MicroRNAs (miRNAs) are a class of small, non-protein-coding RNAs that can post-transcriptionally regulate protein expression by negatively regulating mRNA stability or inhibiting them from translating into proteins³. It has been proven that miRNAs play essential role in various cellular progresses, such as growth, apoptosis, differentiation and development^{4,5}. Currently, miRNA expression profiling studies have shown that several miRNAs functions as protooncogenes or tumor suppressors in human cancers⁶, such as liver cancer⁷, lung cancer⁸. MiR-224 has been reported to be over-expressed in breast cancer⁹, hepatocellular carcinoma¹⁰ and NSCLC¹¹. It has been proven that miR-224 promotes cellular migratory, invasive, and proliferative capacity and tumor growth both *in vitro* and *in vivo* by targeting SMAD4 and TNFAIP1 in NSCLC¹¹.

RASSF8, a number of Ras-association domain family (RASSF), is a candidate tumor suppressor. It is not only found in the nucleus, but is also a membrane-associated at sites of cell-cell adhesion, co-localizing with the adherens junction (AJ) component b-catenin and binding to E-cadherin. It has proven that RASSF8 inhibits cell growth by regulating Wnt and NF- κ B signaling pathway in lung cancer¹². Besides, it was reported that the over-expression of RASSF8 leads to inhibition of anchorage-independent growth in soft agar in lung cancer cells (A549 cells)¹³. In esophageal squamous cell carcinoma, RASSF8 downregulation promotes lymphangiogenesis and metastasis¹⁴. The role of RASSF8 in tumor growth has attracted increasing interest.

In this study, we demonstrated that miR-224 was frequently upregulated in NSCLC, and the increased miR-224 can promote lung cancer cell proliferation *in vitro* and tumor growth *in vivo*. Furthermore, we identified RASSF8 as a functional target of miR-224. The over-expression of RASSF8 inhibited lung cancer cell proliferation. Our results suggested that miR-224/ RASSF8 axis partially elucidates the molecular mechanism of NSCLC growth and represents a new potential therapeutic target for NSCLC treatment.

Patients and Methods

Cell Culture

Two NSCLC cell lines (A549 and H460) and normal human bronchial epithelial cell line (16HBE) were purchased from Cell Bank, Chinese Academy of Sciences (Xuhui District, Shanghai, China). NSCLC cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA) medium containing 10% FBS3 (HyClone, South Logan, UT, USA) and 100 U/mL penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA). 16HBE cells were cultured in keratinocyte serum-free medium with 25 µg/mL bovine pituitary extract and 0.2 ng/mL recombinant epidermal growth factor (Invitrogen Life Technologies, Carlsbad, CA, USA). Cell cultures were incubated in a humidified atmosphere of 5 % CO₂ at 37°C.

Tissue Samples

A total of 56 pairs of human NSCLC tissues and their corresponding normal adjacent tissues (NATs) were collected from People's Hospital of Rizhao (Rizhao, Shandong Province, China) between 2005 and 2010. None of the patients had received radiotherapy or chemotherapy before surgery. Disease histology was determined in accordance to the criteria of the World Health Organization. The use of human tissues was approved by the People's Hospital of Rizhao Ethics Committee.

Plasmid Construction

The human pre-miRNA expression construct Lenti-miR-224 vector and human miRZip-224 anti-miR-224 miRNA construct were obtained from System Biosciences (Palo Alto, CA, USA). To generate RASSF8 expression construct, the ORFs were amplified by PCR and cloned into pC-

DH-RASSF8 vector. LightSwitch empty vector and RASSF8-3'-UTR were ordered from Active Motif (Carlsbad, CA, USA). Mutations were carried out using Stratagene QuikChange® Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA).

Transfection

The transfection of RASSF8 expression vector was performed using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA), and RNA-related vectors were transfected into cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA).

Quantitative Real-time PCR

Total RNA was extracted using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). For miR-224 detection, reverse-transcribed complementary DNA was synthesized with the PrimeScript RT reagent Kit (TaKaRa, Dalian, China), and quantitative RT-PCR (qRT-PCR) was performed with SYBR Premix ExTaq (TaKaRa, Dalian, China) with the Roche LightCycler 480 system (Roche, Basel, Switzerland). The relative expression ratio of miR-224 was calculated by 2^{-ΔΔCT} method. TaqMan gene expression assays for RASSF8 were purchased from Applied Biosystems (Carlsbad, CA, USA) to determine its expression. GAPDH was used as internal control for normalization of mRNA expression. All reactions were conducted in triplicates. All primers used in the study were presented in Table I.

Western Blot Analysis

The cells were lysed with RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology, Danvers, MA, USA), and separated in 10% SDS-PAGE gels and blotted on Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA, USA). Then, Membranes were blocked with 5% BSA in Tris-Buffered Saline with Tween 20 (TBST) buffer and incubated with primary antibody, followed by incubation with appropriate secondary antibody at room temperature. Specific proteins were detected using the enhanced chemiluminescence system (GE Healthcare, Chicago, IL, USA). Antibodies against p-AKT, AKT, RASSF8 and GAPDH were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). GAPDH was used as internal reference.

Table I. Primer pairs used in the study.

Primers for qRT-PCR	Primer pairs (5'-3')
MiR-224 for qRT-PCR F:	GAGCCAAGTCACTAGTGGT
MiR-224 for qRT-PCR R:	GTGCAGGGTCCGAGGT
RASSF8 for qRT-PCR F:	AGCAGTTCATCCAGCAGACA
RASSF8 for qRT-PCR R:	GAGATGAACCAGGTCGCTTC
Primers for RASSF8 cloning	
RASSF8 for cloning F:	AGCAGTTCATCCAGCAGACA
RASSF8 for cloning R:	GAGATGAACCAGGTCGCTTC
Sequence for knockdown of RASSF8	
siRNA	ATGACTCCATGGATGAAATGA

F indicates forward primer. R indicates reverse primer.

Cell Proliferation Assays

Cell proliferation was monitored using Cell Proliferation Reagent Kit I (MTT) (Roche, Basel, Switzerland). After transfection, cells were plated at 3000 cells/well in 96-well plates, and cell proliferation was measured every 24 hours following the manufacturer's protocol. On the indicated days, the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide reagent (AMRESCO, Solon, OH, USA) was added, and the cells were incubated for 4 h at 37°C. The supernatants were then removed, and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO) (150 μ L/well). The absorbance at 490 nm for each sample was measured using a multilabel plate reader (PerkinElmer, Waltham, MA, USA).

Luciferase Reporter Assay

To determine if miR-224 directly targets the 3'-UTRs of RASSF8, 5×10^4 16HBE cells were seeded in 24-well plates overnight and then transfected with miR-224 mimic (Thermo Scientific, Waltham, MA, USA) plus empty 3'-UTR vector or 3'-UTR vectors containing WT or MUT-3'-UTR. After 48 h, the cells were harvested and assayed using Dual Luciferase Assay (Promega, Fitchburg, WI, USA) according to the manufacturer's protocols. Transfections were performed in duplicate and repeated at least three times in independent experiments.

In Vivo Tumor Growth Analysis

Animal studies were performed according to Institutional Animal Care and Use Committee guidelines of China. The cells transfected with miR-224 were harvested by trypsin treatment, washed with PBS and resuspended in Matrigel/PBS (1:1). 10^6 cells were injected into the lung of nude mice (3 mice per group), followed by 20 days growth.

Statistical Analysis

All statistical analyses were performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Differences between variables were assessed using the χ^2 test. All data presented in this study have been repeated at least three times from three independent experiments and are presented as means \pm SD. Measurement data were analyzed using the Student's *t*-test. Differences were considered significant at *p*-values < 0.05.

Results

The Expression of miR-224 is Up-regulated in NSCLC Tissues

Previous studies suggested that miR-224 over-expression promotes tumor progression in NSCLC¹¹. We performed qRT-PCR analysis of miR-224 expression in three NSCLC cancer cell line (A549 and H460) and a normal human bronchial epithelial cell line (16HBE). The level of miR-224 was markedly up-regulated in NSCLC cell lines compared with the normal cell lines (*p* < 0.01, Figure 1A).

Furthermore, clinicopathological analysis of 56 patients showed that an increase in miR-224 was significantly correlated with larger tumor size (*p* = 0.003) (Table II).

We further investigated the expression of miR-224 in 30 pairs of NSCLC tissues and the corresponding NATs. qRT-PCR analysis showed that the level of miR-224 expression was significantly higher in NSCLC tissues compared with the NATs (*p* < 0.01, Figure 1B).

Over-expression of miR-224 Promotes Proliferation of Lung Cancer Cells in vitro

To examine the effect of miR-224 on the proliferation of lung cells, a vector containing miR-

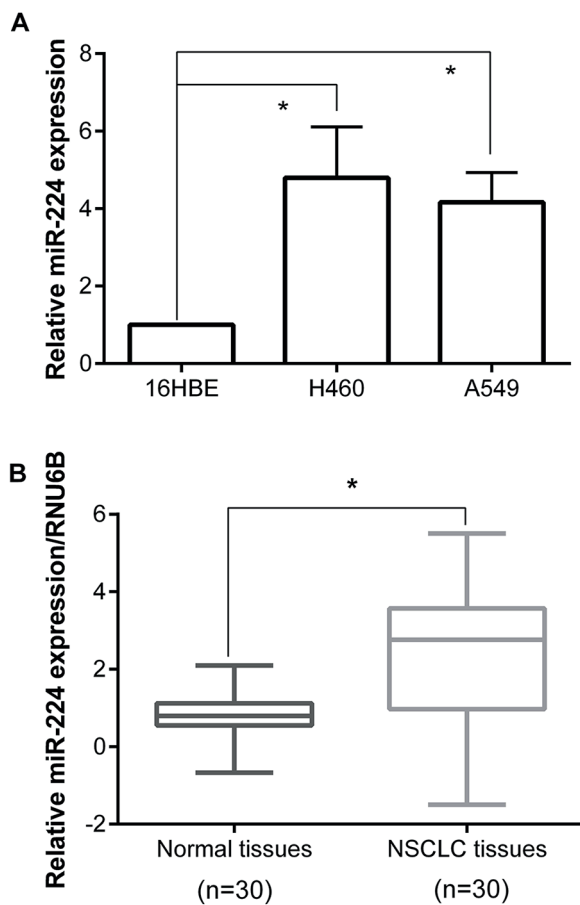


Figure 1. The miR-224 is significantly upregulated in NSCLC tissues. **(A)** The level of miR-224 was markedly up-regulated in NSCLC cell lines compared with the normal cell lines. Expression level was calculated by expression ratio ($2^{-\Delta\Delta Ct}$). **(B)** The level of miR-224 expression was significantly higher in NSCLC tissues compared with the NATs (n=30).

224 precursor was constructed and transduced into NSCLC cell lines (H460). To knockdown miR-224 in cells, a vector containing miRZip-224 anti-miR-224 miR was constructed and transfected into A549. The successful over-expression or knockdown of miR-224 was confirmed by qRT-PCR (Figure 2A). The proliferative abilities were significantly increased in miR-224-transfected cell lines H460 when compared with miR-NC transfected cell lines. In A549 cells, the knockdown of miR-224 significantly decreased proliferative abilities (Figure 2B).

Over-expression of miR-224 Induces Expression of AKT Signaling Pathway

AKT mediates some of cellular processes, including cell survival, migration, metabolism and autophagy¹⁵. It is aberrantly expressed in many cancers¹⁶. In this study, we found that the expression of p-AKT was dramatically increased when miR-224 was over-expressed in H460 cells compared with control cells. While in A549 containing miR-224 inhibitor, the expression of p-AKT was reduced when compared with control cells (Figure 2C).

MiR-224 Targets the 3'-UTR of RASSF8

To explore downstream targets of miR-224, we performed bioinformatics analyses, including TargetScan (<http://www.targetscan.org/>) and mi-Randa (<http://www.microrna.org/microrna/home.do>). RASSF8 was predicted a theoretical target gene. Therefore we performed further analysis to confirm the target.

Table II. MiR-224 expression and clinicopathological features in non-small cell lung cancer (NSCLC) patients.

	miR-224 expression		p-value*
	Low	High	
Age (year)			0.607
<50 (27)	13 (48.1%)	14 (51.9%)	
≥50 (29)	14 (48.3%)	15 (51.7%)	
Gender			0.570
Male (23)	10 (43.5%)	13 (56.5%)	
Female (33)	17 (51.5%)	16 (48.5%)	
Tumour size (cm)			0.003
<5 (18)	10 (55.6%)	8 (44.4%)	
≥5 (38)	6 (21.1%)	32 (78.9%)	
Site of tumor			0.590
Left lung (21)	9 (42.9%)	12 (57.1%)	
Right lung (35)	18 (51.4%)	17 (48.6%)	
Clinical stage			0.031
I + II (37)	15 (40.5%)	22 (59.5%)	
III + IV (19)	2 (10.5%)	17 (89.5%)	

* χ^2 test.

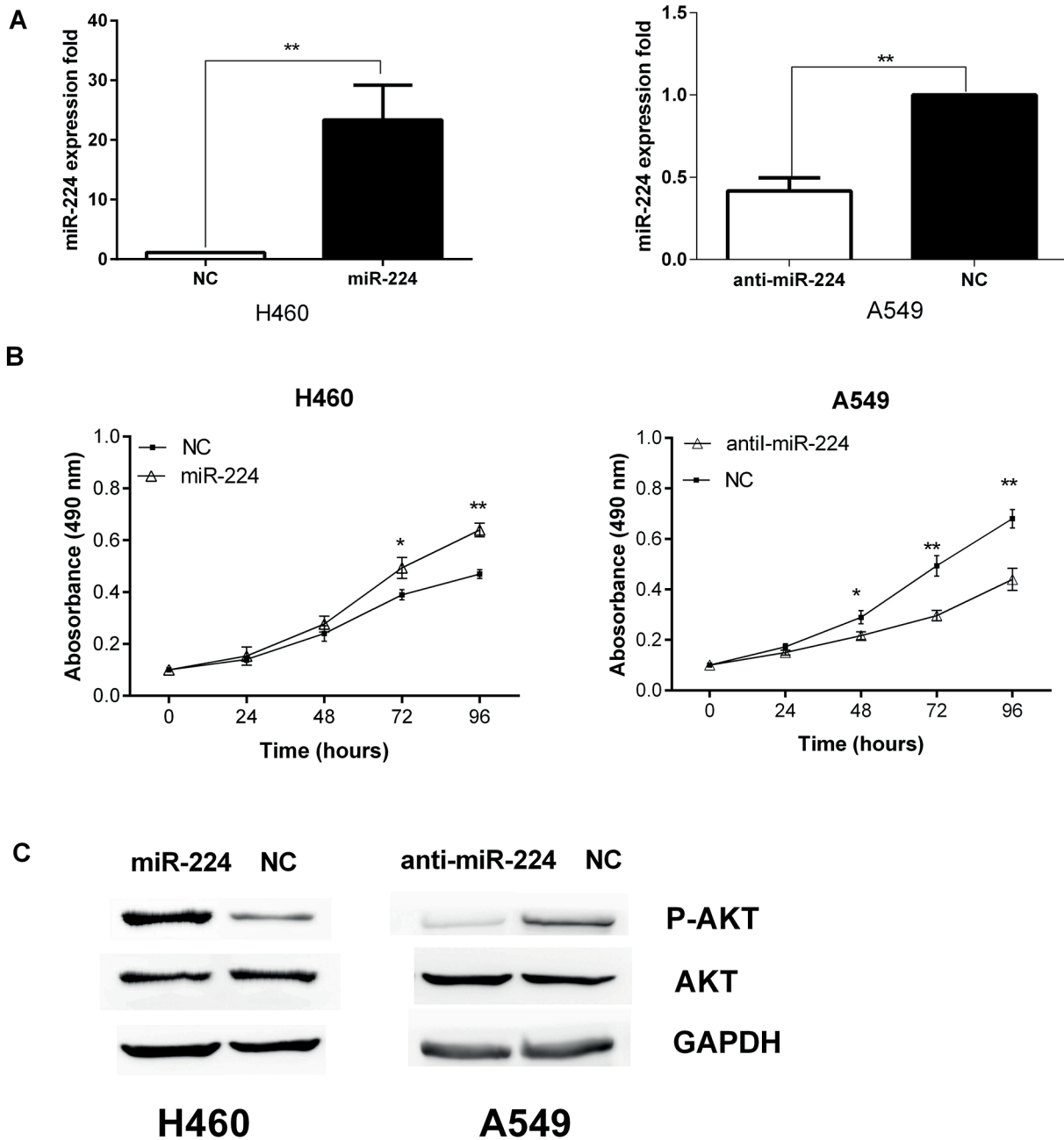


Figure 2. Over-expression of miR-224 promotes proliferation of lung cancer cells and AKT signaling pathway *in vitro*. (A) The successful over-expression in H460 or knockdown of miR-224 in A549 was confirmed by qRT-PCR. (B) Cell proliferation assay (MTT) for miR-224 tested at OD490. (C) Western blot analysis of p-AKT levels and AKT levels in lung cancer cells.

A luciferase reporter assay was performed in 16HBE cells to verify that miR-224 directly targets RASSF8. A mutation of 3'-UTR of RASSF8 was constructed using the QuikChange Mutagenesis Kit at the binding site, named as MUT (Figure 3A). The luciferase reporter constructs harboring MUT or control were constructed and then

transfected into 16HBE cells. Luciferase activities were then measured. The relative luciferase activity was significantly reduced in cells containing 3'-UTR-WT and miR-224 than in control cells. The reduction of luciferase activities was attenuated when cells were cotransfected with miR-224 and MUT 3'-UTR (Figure 3B).

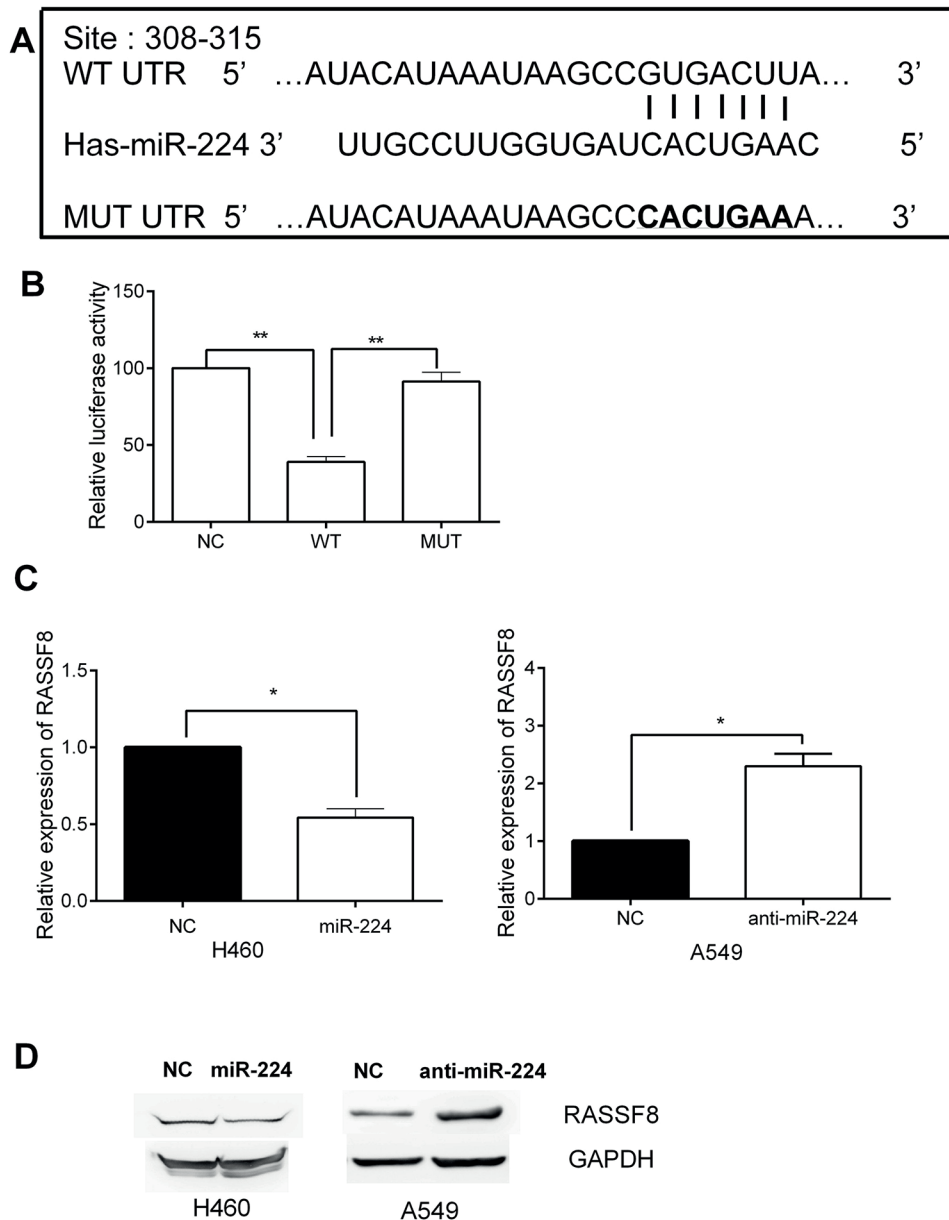


Figure 3. MiR-224 targets the 3'-UTR of RASSF8. **(A)** The putative miR-224 binding sequences in the 3'-UTRs of RASSF8. The mutation was generated in the RASSF8 3'-UTR sequence in the complementary site. **(B)** Analysis of luciferase activity. 16HBE cells were co-transfected with miR-224 and WT or mutant of RASSF8 3'-UTR. **(C)** qRT-PCR analysis of RASSF8 mRNA levels in H460 and A549 cells infected with miR-224 or anti-miR-224 compared with control vector. **(D)** Western blot assay of RASSF8 protein levels in H460 and A549 cells infected with miR-224 or anti-miR-224 compared with control vector. Each assay was conducted three times and Data are presented as means \pm SD.

Besides, the over-expression of miR-224 significantly reduced RASSF8 expression at mRNA and protein levels in H460 cells. The silence of miR-224 increased the expression of RASSF8 at the mRNA and protein levels in A549 (Figure 3C and D).

The Over-expression of miR-224 Promotes NSCLC Proliferation in vivo

Given that miR-224 remarkably promoted the proliferation of NSCLC cells *in vitro*, we further investigated whether miR-224 could affect tumor proliferation *in vivo*. H460 stably expressing miR-224

and control cells were injected into the lung of nude mice. After 20 days of inoculation, over-expression of miR-224 in H460 cells markedly increased tumor growth compared with H460/vector group *in vivo*, as shown in Figure 4 A and B.

Discussion

Recently, many studies have been focused on studying aberrant expression of miRNAs in cancer, indicating that miRNAs contributes to the pa-

thogenesis of most human malignancies⁸. A number of miRNAs functions as oncogenes or tumor suppressors in the majority of cancers. Strategies that interfere with miRNA function are considered to offer novel target for cancer treatment¹⁷. MiRNAs are important regulators of many biological processes^{18,19}. In NSCLC, miR-let-7c inhibits migration and invasion by targeting ITGB3 and MAP4K3²⁰, miR-21 promotes growth and metastasis by targeting PTEN², and MicroRNA-135b promotes lung cancer metastasis²¹. Besides, miR-224 enhances invasion and growth in NSCLC by

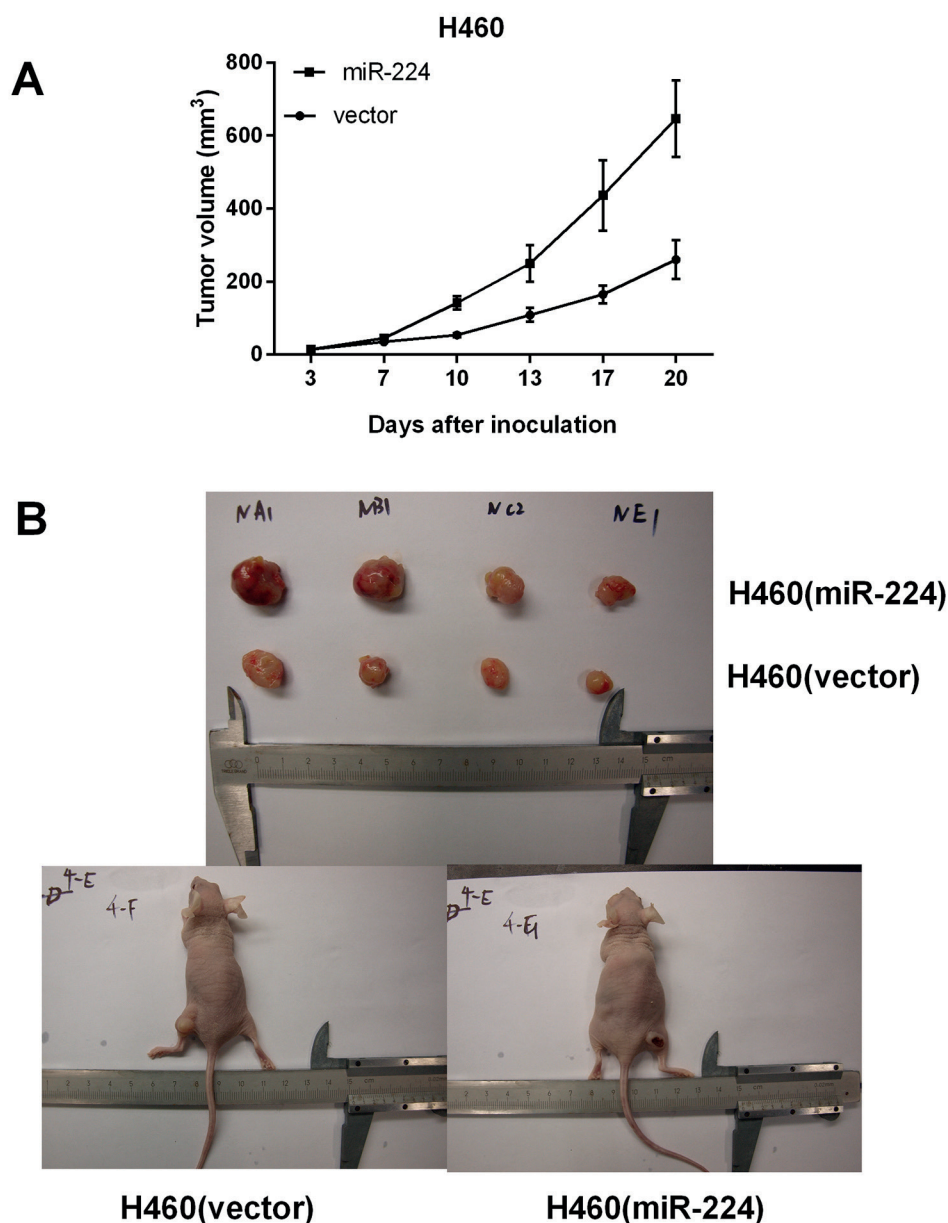


Figure 4. MiR-224 promotes NSCLC proliferation *in vivo*. (A) Tumor growth in nude mice (n = 5 per group). (B) Comparison of tumor size at day 20 after inoculation with H460 (miR-224) or H460 (vector) cells.

shifting the equilibrium of the partial antagonist functions of SMAD4 and TNFAIP1^{11,22}. Furthermore, miR-224 expression is frequently upregulated in various human tumor types, including colorectal cancer²³, hepatocellular cancer (HCC)²⁴, and renal cancer²⁵. These studies suggest that miR-224 is an onco-miR. In the current study, we demonstrated that miR-224 is commonly upregulated in NSCLC, and the re-expression of miR-224 can promote NSCLC cell growth *in vitro* and *in vivo*. These findings agree with the results of Cui et al¹¹ and Wang et al²⁶.

This study suggested that RASSF8 was a candidate miR-224 target. The luciferase reporter assay firstly demonstrated its downregulation was mediated by the direct binding of miR-224 to the RASSF8 3'-UTR. We found that over-expression of miR-224 promoted cell growth by silencing RASSF8. As over-expression of RASSF8 can often lead to toxicity¹², we aimed to investigate the effects of decreased levels of RASSF8 on cell growth by RASSF8 depletion. After decreasing expression of RASSF8, the promotion effects of miR-224 on growth of NSCLC cells are partially increased.

RASSF8 is ubiquitously expressed in all major organs and tissues, including the kidney, liver, brain, heart and lung. Endogenous RASSF8 is expressed in both the nucleus and cell membrane, and it promotes cell-cell adhesion by maintaining adherens junction (AJ) stability¹³. In previously studies^{12,13}, RASSF8 has been proposed as a candidate tumor suppressor in cancer. Lock et al¹² showed that RASSF8 expression might control cellular proliferation of NSCLC cells by regulating b-catenin localization and the canonical Wnt signaling pathway and, concomitantly, p65 localization and the NF- κ B signaling pathway^{13,27}. Falvella et al¹³ proposed that Expression of RASSF8 protein by transfected lung cancer cells led to inhibition of anchorage-independent growth in soft agar in A549 cells and reduction of clonogenic activity in NCIH520 cells. Besides, Korkola et al²⁸ found that RASSF8 expression was down-regulated in male germ cell tumors. These results provided further evidence for our work.

Besides the gene RASSF8, p-AKT signaling pathway was also mediated by miR-224. Over-expression of miR-224 promoted the expression of p-AKT, and deletion of miR-224 inhibited the expression of p-AKT. It has been demonstrated that in colorectal cancer miR-224 activates PI3K/Akt signaling and promotes cell survival through modulation of FOXO3a, p21Cip1, p27Kip1, and cyclin D1

expression^{29,30}. And the inhibition of AKT signaling pathway promotes apoptosis³¹. This pathway represents a new mechanism which may underlie the development of NSCLC, while the specific mechanism still needs further studies to verify.

Lastly, our *in vivo* research presented that mice with miR-224 over-expression showed significantly increasing in tumor size compared to control size. The results strongly support the results from *in vitro* analyses. Thus, our studies might provide a potential molecular mechanism of NSCLC proliferation.

Conclusions

The increased levels of miR-224 in NSCLC patients indicated that miR-224 was a potential biomarker in the proliferation. The results showed that miR-224 plays an oncogenic role in NSCLC as miR-224 promotes tumor cell proliferation by targeting RASSF8. It is suggested that miR-224 may be a potential therapeutic target for NSCLC.

Conflict of Interest

The authors declare no conflicts of interest.

References

- 1) SIEGEL RL, MILLER KD, JEMAL A. Cancer statistics, 2016. *CA Cancer J Clin* 2016; 66: 7-30.
- 2) LIU ZL, WANG H, LIU J, WANG ZX. MicroRNA-21 (miR-21) expression promotes growth, metastasis, and chemo- or radioresistance in non-small cell lung cancer cells by targeting PTEN. *Mol Cell Biochem* 2013; 372: 35-45.
- 3) WAHID F, SHEHZAD A, KHAN T, KIM YY. MicroRNAs: synthesis, mechanism, function, and recent clinical trials. *Biochim Biophys Acta* 2010; 1803: 1231-1243.
- 4) BANDRES E, AGIRRE X, RAMIREZ N, ZARATE R, GARCIA-FONCILLAS J. MicroRNAs as cancer players: potential clinical and biological effects. *DNA Cell Biol* 2007; 26: 273-282.
- 5) KROL J, LOEDIGE I, FILIPOWICZ W. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet* 2010; 11: 597-610.
- 6) DI LEVA G, CROCE CM. Roles of small RNAs in tumor formation. *Trends Mol Med* 2010; 16: 257-267
- 7) WEI R, HUANG GL, ZHANG MY, LI BK, ZHANG HZ, SHI M, CHEN XQ, HUANG L, ZHOU QM, JIA WH, ZHENG XF, YUAN YF, WANG HY. Clinical significance and prognostic value of microRNA expression signatures in hepatocellular carcinoma. *Clin Cancer Res* 2013; 19: 4780-4791.

- 8) HU Z, CHEN X, ZHAO Y, TIAN T, JIN G, SHU Y, CHEN Y, XU L, ZEN K, ZHANG C, SHEN H. Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. *J Clin Oncol* 2010; 28: 1721-1726.
- 9) HUANG L, DAI T, LIN X, ZHAO X, CHEN X, WANG C, LI X, SHEN H, WANG X. MicroRNA-224 targets RKIP to control cell invasion and expression of metastasis genes in human breast cancer cells. *Biochem Biophys Res Commun* 2012; 425: 127-133.
- 10) LI Q, WANG G, SHAN JL, YANG ZX, WANG HZ, FENG J, ZHEN JJ, CHEN C, ZHANG ZM, XU W, LUO XZ, WANG D. MicroRNA-224 is upregulated in HepG2 cells and involved in cellular migration and invasion. *J Gastroenterol Hepatol* 2010; 25: 164-171.
- 11) CUI R, MENG W, SUN HL, KIM T, YE Z, FASSAN M, JEON YJ, LI B, VICENTINI C, PENG Y, LEE TJ, LUO Z, LIU L, XU D, TILI E, JIN V, MIDDLETON J, CHAKRAVARTI A, LAUTENSCHLAGER T, CROCE CM. MicroRNA-224 promotes tumor progression in nonsmall cell lung cancer. *Proc Natl Acad Sci U S A* 2015; 112: E4288-4297.
- 12) LOCK FE, UNDERHILL-DAY N, DUNWELL T, MATALLANAS D, COOPER W, HESSON L, RECINO A, WARD A, PAVLOVA T, ZABAROVSKY E, GRANT MM, MAHER ER, CHALMERS AD, KOLCH W, LATIF F. The RASSF8 candidate tumor suppressor inhibits cell growth and regulates the Wnt and NF-kappaB signaling pathways. *Oncogene* 2010; 29: 4307-4316.
- 13) FALVELLA FS, MANENTI G, SPINOLA M, PIGNATIELLO C, CONTI B, PASTORINO U, DRAGANI TA. Identification of RASSF8 as a candidate lung tumor suppressor gene. *Oncogene* 2006; 25: 3934-3938.
- 14) ZHANG L, WANG JH, LIANG RX, HUANG ST, XU J, YUAN LJ, HUANG L, ZHOU Y, YU XJ, WU SY, LUO RZ, YUN JP, JIA WH, ZHENG M. RASSF8 downregulation promotes lymphangiogenesis and metastasis in esophageal squamous cell carcinoma. *Oncotarget* 2015; 6: 34510-34524.
- 15) PAN CC, SHAH N, KUMAR S, WHEELER SE, CINTI J, HOYT DG, BEATTIE CE, AN M, MYTHREYE K, RAKOTONDRAIBE LH, LEE NY. Angiostatic actions of capsicodendrin through selective inhibition of VEGFR2-mediated AKT signaling and dysregulated autophagy. *Oncotarget* 2017; 8: 12675-12685.
- 16) STEMKE-HALE K, GONZALEZ-ANGULO AM, LLUCH A, NEVE RM, KUO WL, DAVIES M, CAREY M, HU Z, GUAN Y, SAHIN A, SYMMANS WF, PUSZTAI L, NOLDEN LK, HORLINGS H, BERNS K, HUNG MC, VAN DE VIJVER MJ, VALEIRO V, GRAY JW, BERNARDS R, MILLS GB, HENNESSY BT. An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. *Cancer Res* 2008; 68: 6084-6091.
- 17) ZHAO L, CHEN X, CAO Y. New role of microRNA: carcinogenesis and clinical application in cancer. *Acta Biochim Biophys Sin (Shanghai)* 2011; 43: 831-839.
- 18) WANG Y, LEE CG. Role of miR-224 in hepatocellular carcinoma: a tool for possible therapeutic intervention? *Epigenomics* 2011; 3: 235-243.
- 19) BOGUSLAWSKA J, WOJCIKKA A, PIEKIELKO-WITKOWSKA A, MASTER A, NAUMAN A. MiR-224 targets the 3'UTR of type 1 5'-iodothyronine deiodinase possibly contributing to tissue hypothyroidism in renal cancer. *PLoS One* 2011; 6: e24541.
- 20) ZHAO B, HAN H, CHEN J, ZHANG Z, LI S, FANG F, ZHENG Q, MA Y, ZHANG J, WU N, YANG Y. MicroRNA let-7c inhibits migration and invasion of human non-small cell lung cancer by targeting ITGB3 and MAP4K3. *Cancer Lett* 2014; 342: 43-51.
- 21) LIN CW, CHANG YL, CHANG YC, LIN JC, CHEN CC, PAN SH, WU CT, CHEN HY, YANG SC, HONG TM, YANG PC. MicroRNA-135b promotes lung cancer metastasis by regulating multiple targets in the Hippo pathway and LZTS1. *Nat Commun* 2013; 4: 1877.
- 22) ZHU D, CHEN H, YANG X, CHEN W, WANG L, XU J, YU L. Decreased microRNA-224 and its clinical significance in non-small cell lung cancer patients. *Diagn Pathol* 2014; 9: 198.
- 23) MENCIA N, SELGA E, NOE V, CIUDAD CJ. Underexpression of miR-224 in methotrexate resistant human colon cancer cells. *Biochem Pharmacol* 2011; 82: 1572-1582.
- 24) MEES ST, MARDIN WA, SIELKER S, WILLSCHER E, SENNINGER N, SCHLEICHER C, COLOMBO-BENKMANN M, HAIER J. Involvement of CD40 targeting miR-224 and miR-486 on the progression of pancreatic ductal adenocarcinomas. *Ann Surg Oncol* 2009; 16: 2339-2350.
- 25) KANG Y, SIEGEL PM, SHU W, DROBNJAK M, KAKONEN SM, CORDON-CARDO C, GUISE TA, MASSAGUE J. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 2003; 3: 537-549.
- 26) WANG H, ZHU LJ, YANG YC, WANG ZX, WANG R. MiR-224 promotes the chemoresistance of human lung adenocarcinoma cells to cisplatin via regulating G(1)/S transition and apoptosis by targeting p21(WAF1/CIP1). *Br J Cancer* 2014; 111: 339-354.
- 27) LANGTON PF, COLOMBANI J, CHAN EH, WEPF A, GSTAIGER M, TAPON N. The dASPP-dRASSF8 complex regulates cell-cell adhesion during Drosophila retinal morphogenesis. *Curr Biol* 2009; 19: 1969-1978.
- 28) KORKOLA JE, HOULDSWORTH J, CHADALAVADA RS, OLSHEN AB, DOBRZYNSKI D, REUTER VE, BOSL GJ, CHAGANTI RS. Down-regulation of stem cell genes, including those in a 200-kb gene cluster at 12p13.31, is associated with in vivo differentiation of human male germ cell tumors. *Cancer Res* 2006; 66: 820-827.
- 29) LIAO WT, LI TT, WANG ZG, WANG SY, HE MR, YE YP, QI L, CUI YM, WU P, JIAO HL, ZHANG C, XIE YJ, WANG JX, DING YQ. microRNA-224 promotes cell proliferation and tumor growth in human colorectal cancer by repressing PHLPP1 and PHLPP2. *Clin Cancer Res* 2013; 19: 4662-4672.
- 30) ROY SK, SRIVASTAVA RK, SHANKAR S. Inhibition of PI3K/AKT and MAPK/ERK pathways causes activation of FOXO transcription factor, leading to cell cycle arrest and apoptosis in pancreatic cancer. *J Mol Signal* 2010; 5: 10.
- 31) BROGNARD J, SIERECKI E, GAO T, NEWTON AC. PHLPP and a second isoform, PHLPP2, differentially attenuate the amplitude of Akt signaling by regulating distinct Akt isoforms. *Mol Cell* 2007; 25: 917-931.