

RETRACTED ARTICLE: MiR-625 Inhibits Tumor Cell Invasion, Migration and EMT by Negatively Regulating the Expression of Resistin in Non-Small Cell Lung

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Purpose: To investigate the role of miR-625 on the invasion, migration, and epithelial-mesenchymal transition (EMT) of non-small cell lung carcinoma (NSCLC) cells, and the related mechanisms.

Materials and Methods: The expression levels of miR-625 and Resistin mRNA in 80 pairs of NSCLC and para-cancerous lung tissues were analyzed by RT-PCR. The relationship between miR-625 and Resistin was predicted by bioinformatics and verified by a dual-luciferase gene reporter assay. NSCLC cells were transfected with Resistin plasmids, si-Resistin plasmids, miR-625 mimics, or miR-625 inhibitors, and proliferation, invasion, and migration were determined by CCK-8, Transwell, and wound scratch assays, respectively. EMT-related proteins were determined by Western blot assay. A xenograft model of NSCLC was established in nude mice to validate the in vitro findings.

Results: MiR-625 was significantly downregulated in NSCLC tissue compared to paired para-cancerous lung tissues, while Resistin was markedly increased in tumor tissue. The expression levels of miR-625 and Resistin were negatively correlated in NSCLC tissues, and high levels of Resistin correlated with greater tumor differentiation, more advanced clinical stage, and lymph node metastasis. Furthermore, Resistin was a target gene of miR-625, and the latter downregulated Resistin to inhibit the EMT, proliferation, invasion, and migration of NSCLC cells in vitro, likely via the PI3K/AKT/Snail signaling pathway. Finally, miR-625 also inhibited the tumorigenic effect of NSCLC cells in vivo by downregulating Resistin.

Conclusion: MiR-625 acts as a tumor suppressor in NSCLC and inhibits tumor cell invasion and metastasis by blocking the Resistin/PI3K/AKT/Snail pathway and by decreasing EMT.

Keywords: miR-625, resistin, EMT, invasion, migration, PI3K/AKT/snail

Introduction

Lung cancer is one of the most commonly diagnosed malignancies, and ranks high in terms of both incidence and mortality.¹ Nearly 85% of lung cancers cases are non-small cell lung cancer (NSCLC), which can be subdivided into adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and other types.² In recent years, the survival rate of NSCLC patients has improved significantly due to more advanced surgical techniques and targeted therapies. However, the 5-year survival is still only 20.6% since most patients already have regional or distant metastasis at the first visit due to late diagnosis, which precludes optimal treatment.^{1,3} Therefore, it is essential to identify novel biomarkers of NSCLC progression and metastasis in order to improve early diagnosis and predict patient prognosis.

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MicroRNAs (miRNAs) are endogenous single-stranded non-coding small RNAs (20–25 nucleotides long) that can bind the 3' untranslated region (UTR) of a target mRNA through complete or incomplete complementary base-pairing, and either degrade the transcripts or inhibit their translation.⁴ Although miRNAs comprise only 2% of the human genome, they regulate the expression of nearly one-third of all genes, especially those involved in embryonic development, cell proliferation, and apoptosis, immune response, and tumorigenesis. Several aberrantly expressed miRNAs have been identified in multiple cancers, and are highly promising diagnostic markers and therapeutic targets.⁵ MiR-625 is abnormally expressed in a number of solid malignancies. Zhou et al.⁶ reported a significant downregulation of miR-625 in breast cancer tissue that highly correlated with expression of the estrogen receptor (ER) and epidermal growth factor receptor 2 (EGFR2), as well as the clinical stage, and therefore was an independent factor for poor prognosis. In addition, miR-625 inhibited the proliferation and migration of breast cancer cells in vitro by downregulating the high mobility group (HMG)A1 protein. In liver cancer, miR-625 levels were significantly lower in tumor tissue when compared to adjacent normal tissue, which was associated with increased lymph node metastasis and poor overall survival. MiR-625 acted as a tumor suppressor in liver cancer by inhibiting the metastatic ability of hepatoma cells via downregulation of the IGF2BP1/PTEN signaling pathway.⁷ In addition, miR-625 expression was significantly decreased in colorectal cancer, gastric cancer, esophageal cancer, and other related tumors, and its low expression levels correlated with increased metastasis and poor prognosis.^{5,7,8} In another study, it was shown that miR-625 levels were significantly lower in the sera of NSCLC patients compared to those with benign lung disease and healthy controls, and as reported in other malignancies, correlated with the clinical stage.¹⁰ However, the underlying mechanisms remain to be explored further in NSCLC.

Resistin, a member of the Resistin-like molecules (RELMs) family of inflammo-regulatory proteins,¹⁰ is involved in the development of various chronic diseases and cancer.¹¹ Resistin levels were significantly higher in prostate tumor tissues compared to normal tissues, and correlated with tumor differentiation and pathological stage of prostate cancer. Mechanistically, Resistin promoted prostate cancer cell proliferation by activating the PI3K/Akt signaling pathway.¹² In addition, Resistin overexpression has been reported in lung adenocarcinoma tissues, where it promoted cancer cell invasion and infiltration in a concentration-dependent manner. Furthermore, Resistin also increases the

metastatic ability of adenocarcinoma cells through the TLR4/Src/EGFR/PI3K/NF- κ B pathway.¹³ Therefore, the question arises as to whether miR-625 and Resistin expression are correlated in NSCLC. In this study, we analyzed the expression of miR-625 and Resistin in NSCLC and normal lung tissue, and showed that it correlated with clinicopathological features of patients. The role of miR-625 and Resistin in NSCLC was further elucidated by in vitro and in vivo assays, and the underlying molecular mechanisms involved were determined to provide a basis for future clinical applications.

Materials and Methods

Tissue Samples

Eighty pairs of NSCLC and para-cancerous lung tissue that were resected from patients without prior radio-, chemo- or immunotherapy at Affiliated Hospital of North Sichuan Medical College from March 2018 to October 2019 were collected. Para-cancerous tissue was removed > 5 cm away from the tumor, and all tissues were confirmed pathologically. The study was approved by the ethics committee of Affiliated Hospital of North Sichuan Medical College (Ethical code number: 2018PHB206-01), and all subjects provided written informed consent, which was compliance with the Declaration of Helsinki. Tissue specimens were washed several times in chilled PBS to remove contaminating blood, and flash frozen in liquid nitrogen.

Reagents

RPMT-1640 and trypsin were obtained from Hyclone (Florida, USA), puromycin from Sigma (San Francisco, USA), and fetal bovine serum (FBS) and Opti-MEM from Gibco (New York, USA). Trizol and the reverse transcription kit were from Thermo (Massachusetts, USA), SYBR Green Real-time PCR kit from Guangzhou Ruibo Biotech, and Lipofectamine 3000 from Invitrogen (New York, USA). The miR-625 mimic/inhibitor and respective negative controls (NC) and si-resistin sequences, as well as the wild-type (WT) and mutant (MUT) resistin luciferase reporter plasmids were provided by Shanghai Gemma (Shanghai, China). The lentiviruses harboring these sequences were generated by Shanghai Jikai Gene Bios Inc. All primers were synthesized by Shanghai Sangon (Shanghai, China). Mouse anti-Resistin, anti-vimentin and anti-E-cadherin, and rat anti-p-AKT, anti-p-PI3K, anti-Snail, anti-Twist1 and anti- β -actin antibodies were purchased from Abcam (London, UK). Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse and anti-rat IgG secondary antibodies were obtained from Guangzhou

Jingcai, fluorescent enzyme detection kit from Promega (Beijing, China), and the IHC EnVision two-step kit and diazobenzidine (DAB) colorimetric kit from Beijing Zhongshan (Beijing, China). Other reagents were of analytical grade.

Cell Culture and Transfection

The normal human bronchial epithelial cell line BEAS-2B and human NSCLC cell lines A549, H322, GLC-82, and H226 were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Frozen stocks were routinely resuscitated and cultured in RPMI-1640 medium containing 10% FBS at 37°C under 5% CO₂. Cells were harvested using 0.25% trypsin at 70% to 80% confluency, and passaged further. The A549 and H226 cells were harvested in the logarithmic growth phase and seeded in 6-well plates at the density of 2×10^5 cells/well in Opti-MEM. Following overnight incubation, the cells were transfected with 100 nM miR-625 mimic, miR-625 inhibitor or NC, 50 ng si-Resistin or miR-625 inhibitor + si-Resistin plasmids using Lipofectamine 3000 as per the manufacturer's instructions. After 6 h of transfection, medium was replaced with complete RPMI-1640 and the cells for cultured for 48 h.

Immunohistochemistry (IHC)

Resected tissues were fixed in 4% formaldehyde for 24 h, dehydrated using an alcohol gradient, embedded in paraffin, and cut into 4 µm thick sections. In-situ expression of Resistin in human tissue was determined using the EnVision two-step method according to the manufacturer's instructions, using 1:500 dilution of the antibody. Immune-stained sections were scored using a modification of the method described by Sjöström.¹⁴ Briefly, the staining intensity was scored as 0 – colorless, 1 – light yellow, 2 – brownish yellow and 3 – tan, and the percentage of positive staining as 0 – negative, 1 – < 10%, 2 – 11–50%, 3 – 51–75% and 4 – > 75%. The final staining scores were calculated as the sum of the above; 0 to 3 indicated negative and 4 or above suggested positive Resistin expression. All specimens were independently evaluated by three pathologists in a double-blind manner.

Real-Time Quantitative PCR (RT-PCR)

Total RNA was extracted from tissues and cell lines using Trizol, and the purity and concentration of RNA were determined with a spectrophotometer. RNA was reverse transcribed into cDNA using the reverse transcription kit,

and real-time quantitative PCR was performed using SYBR Green Real-time PCR reagent as per the kit instructions. The reaction conditions were as follows: pre-denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 7 s, annealing at 60 °C for 30 s and 72 °C for 15 s. The primer sequences were as follows: miR-625-F - 5'-GGCTAGTTCACCTCCTCCTCC-3', miR-625-R - 5'-GTGCAGG GTCCGAGGT-3'; Resistin-F - 5'-CCAGCCATCAGCCATGAGGGT-3', Resistin-R - 5'-GGAGCCCTTTCTGAATCCGCA-3'; U6-F - 5'-GCTTCGGCAGCACATTAAT-3', U6-R - 5'-CGCTTCACGAATTTCTGTGCA-3'; GAPDH-F - 5'-AGAAGGCTGGGGCTCCTTGTG-3', GAPDH-R - 5'-AGGGGCCATCCACAGTCTTCT-3'. U6 and GAPDH were used as the internal reference for miR-625 or Resistin, and the relative expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method. Experiments were performed in triplicate.

Cell Proliferation Assay

Transfected A549 and H226 cells were harvested and seeded in a 96-well plate at a density of 2×10^3 cells/well. Following overnight incubation, 10 µL CCK-8 reagent was added to each well, and the absorbance at 570 nm was determined after culturing for 24 h, 48 h, and 72 h. Five replicates were tested for each condition, and percentage of proliferating cells was calculated from the growth curves.

Transwell Assay

The upper chambers of Transwell inserts were each coated with 40 µL Matrigel (diluted 1:5 in serum-free RPMI-1640 medium) for 4 h, and placed into 12-well plates. Transfected A549 and H226 cells were seeded into the upper chamber in 200 µL serum-free medium at the density of 4×10^4 cells/well. The lower chambers were filled with 600 µL complete RPMI-1640 medium. After 48 h of incubation, the upper chambers were removed and rinsed twice with PBS. The cells remaining on the filter were gently wiped with a wet cotton swab, and the migrated cells on the lower side of the filter were fixed in absolute water for 15 min and dried at room temperature. After staining with 0.1% crystal violet for 30 min at room temperature, cells were rinsed twice with PBS, and the number of migrated cells in 5 random fields were counted under an inverted microscope. Per condition, three replicates were tested.

Wound Scratch Test

Two lines spaced 5 mm apart were drawn transversely at the bottom of each well of a 6-well plate with a permanent marker pen. Subsequently, transfected A549 and H226 cells were seeded at a density of 2×10^5 cells/well in complete RPMI-1640 medium, and cultured until ~80% confluency. The monolayers were scratched perpendicular to the marker lines with a sterile 200 μ L pipette tip, and the dislodged cells were removed by washing three times with PBS. Fresh RPMI-1640 medium containing 2% FBS was then added to each well, and the cells were incubated further for 24 h. The migrated cells were observed under an inverted microscope, and the intercellular distance in the “wound” area was measured using Image J software.

Dual-Luciferase Gene Reporter Assay

The TargetScan program predicted Resistin as a putative target of miR-625, which was verified by the dual-luciferase gene reporter assay. Briefly, A549 and H226 cells were harvested in the logarithmic growth phase and seeded into 96-well plates at a density of 2×10^4 cells/well. Following overnight culture, 100 ng WT or MUT luciferase reporter plasmid was co-transfected with 50 ng miR-625 mimic or NC or miR-625 inhibitor using Lipofectamine 3000, and the cells were cultured for 48 h. The relative luciferase activity was determined according to the dual-luciferase assay kit instructions. Per condition, five replicates were tested.

Establishment of Xenograft Tumor Model

A549 cells in the logarithmic growth phase were inoculated into a 5 cm cell culture dish and grown until 80% confluency. The cells were harvested and seeded into 6-well plates at the density of 2×10^5 cells/well and transfected with miR-625 mimic, miR-625 inhibitor, NC + Resistin and miR-625 inhibitor + miR-625 inhibitor + Resistin lentiviruses at MOI of 50 in Opti-MEM. The medium was replaced with complete RPMI-1640 24 h later. Infected cells were harvested 48 h later, and resuspended in serum-free RPMI-1640 medium at the density of 4×10^7 cells/mL. Four-week old BALB/c nude mice (Beijing Weitong Lihua Biological Co. Ltd., Beijing, China) were each inoculated with 200 μ L of the respective cell suspensions ($n = 8$ per group) into their right upper extremity. The blank control group was inoculated with untreated A549 cells. Two weeks later, the tumor-bearing mice were euthanized and the tumors were weighed, and their volume was calculated as the shortest diameter of the

tumor² \times the longest diameter of the tumor/2. All animal experiments were approved by the Animal Ethics Committee of Affiliated Hospital of North Sichuan Medical College and conducted in accordance with the Guide for the Care and Use of Laboratory Animals and conformed to our institutional ethical guidelines.

Western Blot Assay

Tissues and harvested cells were washed three times with chilled PBS, and lysed with RIPA buffer supplemented with protease inhibitors. The extracted proteins were quantified using the BCA method, and the cell lysates were diluted in 5 \times loading buffer at a ratio of 1:4. After denaturing at 95 $^{\circ}$ C for 10 min, 30 μ g protein per sample was subjected to SDS-PAGE. Proteins were transferred to PVDF membranes and the latter were blocked with 5% skim milk at room temperature for 2 h. The membranes were incubated overnight with primary antibodies directed against Resistin (1:500), Vimentin (1:300), E-cadherin (1:500), p-AKT (1:500), p-PI3K (1:800), Snail (1:500), Twist1 (1:100), and β -actin (1:1000) at 4 $^{\circ}$ C on a shaker. After washing three times with TBST buffer, membranes were probed with a HRP-conjugated secondary antibody (1:5000) for 1 h at room temperature, followed by 3 washes with TBST. Positive bands were visualized using ECL reagent and exposed on a gel imager. Image J software was used to determine the gray value of each band, and the protein content was calculated relative to that of β -actin. The experiment was repeated thrice.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 and GraphPad Prism 5.0. Data were expressed as mean \pm standard deviation. Independent sample *t*-test was used to compare two groups, and one-way ANOVA along with Dunnett's or Bonferroni's test was used for multiple groups. Pearson's chi-square (χ^2) test was used to analyze the relationship between Resistin expression and the clinicopathological parameters. An α value of 0.05 and $p < 0.05$ were considered statistically significant.

Results

MiR-625 and Resistin Expression are Negatively Correlated in NSCLC

MiR-625 levels in 80 pairs of NSCLC tumors and corresponding para-cancerous tissues were determined by qRT-PCR. The results showed that the miR-625 expression in 44 of the NSCLC samples (55.0%) was at least 50% lower in tumor

tissues when compared to that in para-cancerous tissue (Figure 1A). Furthermore, the overall expression of miR-625 was significantly lower in tumor versus non-tumor tissue ($P<0.001$; Figure 1B). In contrast, Resistin mRNA levels in the NSCLC tissues were significantly higher than that in non-tumor tissues ($P<0.01$; Figure 1C). Correlation analysis further showed that miR-625 was significantly and negatively correlated with Resistin expression in the NSCLC and normal lung tissues ($r=-0.7183$, $P<0.01$; Figure 1D). Consistent with the mRNA levels, Resistin protein expression was also significantly higher in the NSCLC tissues (Figure 1E and F). In addition, high expression of Resistin was significantly correlated to greater tumor differentiation (advanced vs lower grade), advanced TNM stage (II/III vs I) and lymph node metastasis ($P<0.001$; Table 1). Consistent with the patient samples, the NSCLC cell lines showed significantly lower levels of miR-625 compared to BEAS-2B cells ($P<0.01$; Figure 1G), while Resistin mRNA and protein levels were higher in the former ($P<0.05$; Figure 1H and I). Accordingly, the lung adenocarcinoma cell line A549 and the lung squamous

carcinoma cell line H226 were selected for subsequent experiments.

Targeting Relationship Between Resistin and miR-625

The TargetScan database predicted that the 3'-UTR of the Resistin gene was complementary to miR-625, indicating that Resistin is a putative target of miR-625 (Figure 2A). The possible relationship was validated by dual-luciferase gene reporter assay which showed that the luciferase activity of NSCLC cells (A549 and H226 cell) was markedly reduced when co-transfected with a miR-625 mimic and WT Resistin, and co-transfected with the miR-625 inhibitor and WT Resistin markedly inhibited the luciferase activity, whereas co-transfection with a miR-625 mimic & MUT Resistin or miR-625 inhibitor & MUT Resistin had no significant effect on the luciferase activity (Figure 2B and C). Thus, Resistin is a target gene of miR-625 and is negatively regulated by the latter.

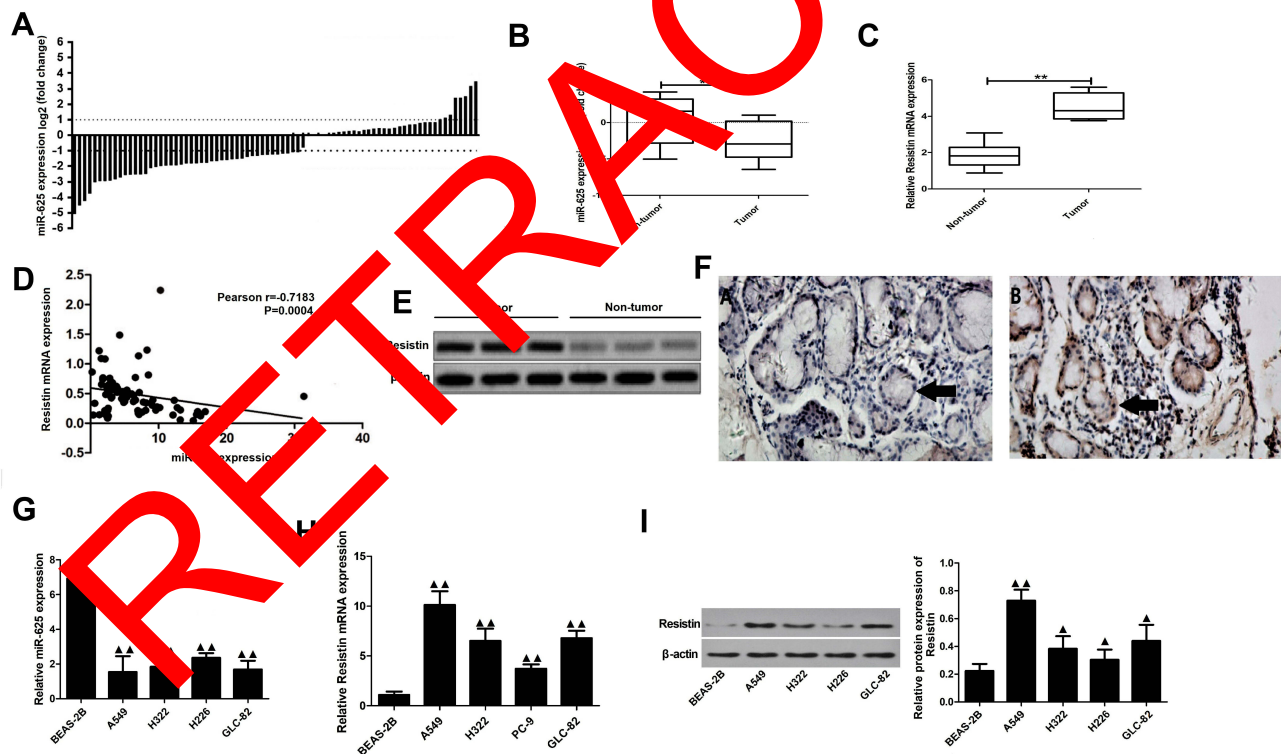


Figure 1 Expression of miR-625 and Resistin in NSCLC and para-cancerous tissues. (A) Proportion of NSCLC and para-cancerous tissues with positive miR-625 expression ($n=80$). (B and C) miR-625 and Resistin mRNA expression in 80 pairs of NSCLC and para-cancerous tissues ($n=80$). (D) Spearman correlation analysis of miR-625 and Resistin mRNA expression levels. (E) Immunoblot showing Resistin protein levels in NSCLC and para-cancerous tissues. (F) Representative immunohistochemistry (IHC) images showing in situ Resistin expression (black arrows) in NSCLC (A) and para-cancerous (B) tissues. (G) RT-PCR analysis of miR-625 expression in cell lines BEAS-2B, A549, H322, GLC-82, H226; (H, I) Resistin mRNA and protein expression in cell lines BEAS-2B, A549, H322, GLC-82, H226; Compared to non-tumor, $**P<0.01$ and $***P<0.001$; compared with BEAS-2B cells, $\Delta P<0.05$, $\Delta\Delta P<0.01$.

Table 1 Relationship Between Resistin Expression and Clinicopathological Features in 80 NSCLC Cases

Clinical Case Characteristics	n	Expression Rate of Resistin			χ^2	P
		Positive	Negative	Positive Rate (%)		
Gender						
Male	43	27	16	62.8	2.172	0.175
Female	37	25	12	37.2		
Age						
>60	39	26	13	66.7	0.986	0.271
≤60	41	26	15	63.4		
Histological type						
Adenocarcinoma	46	28	18	60.9	2.172	0.085
Squamous cell carcinoma	34	24	10	70.6		
Differentiation						
Low	14	4	10	28.6	0.112	0.003
Medium and high	66	48	18	52.7		
Lymph node metastasis						
Yes	58	46	12	79.3	11.267	0.001
No	22	6	16	27.3		
TNM staging						
I	19	4	15	21.1	11.185	0.001
II +	61	48	13	78.7		

Resistin Downregulation by miR-625 Inhibits Its Neoplastic Effects in NSCLC Cells

The biological relevance of the miR-625/Resistin axis in NSCLC cells was further analyzed by manipulating their expression levels through suitable constructs. As shown in (Figure 3A and E), the expression of miR-625 in the miR-625 mimic group and miR-625 inhibitor group was significantly increased and decreased respectively. Moreover, the miR-625 mimic significantly decreased the levels of Resistin mRNA ($P < 0.05$), whereas the miR-625 inhibitor had the opposite effect in A549 and H226 cells ($P < 0.05$). However, siRNA-mediated Resistin silencing did not influence miR-625 levels ($P > 0.05$); In miR-625 inhibitor

+si-Resistin group, the expression of miR-625 was significantly decreased ($P < 0.05$), and the expression of Resistin mRNA was not significantly changed ($P > 0.05$). In contrast to the miR-625 inhibitor group, the expression of Resistin mRNA was not significantly changed in the miR-625 inhibitor+si-Resistin group ($P > 0.05$). Overexpression of miR-625 or Resistin silencing significantly decreased the proliferation rate of both A549 and H226 cells when compared to un-transfected controls ($P < 0.05$), whereas inhibition of miR-625 enhanced the proliferative capacity of these cell types ($P < 0.05$). Resistin silencing abrogated the increased growth of NSCLC cells in the absence miR-625 ($P > 0.05$; Figure 3B and F). Similarly, in vitro migration and invasion of NSCLC cells were respectively inhibited and enhanced by the miR-625 mimic and inhibitor (P

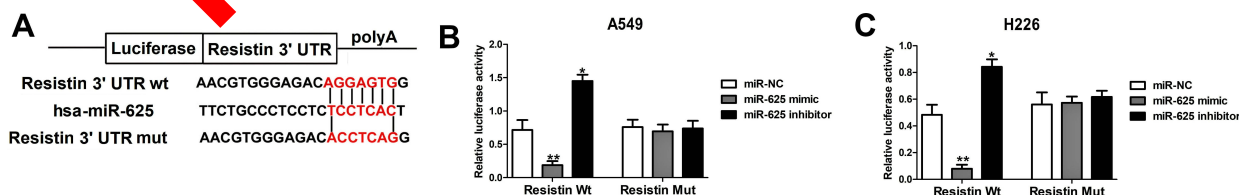


Figure 2 MiR-625 targeted regulation of Resistin expression. (A) TargetScan results showing the putative miR-625 binding sites on the 3'UTR of Resistin. (B and C) Dual-luciferase reporter assay validating that Resistin is the target gene of miR-625 in A549 and H226 cells. * $P < 0.05$, ** $P < 0.01$.

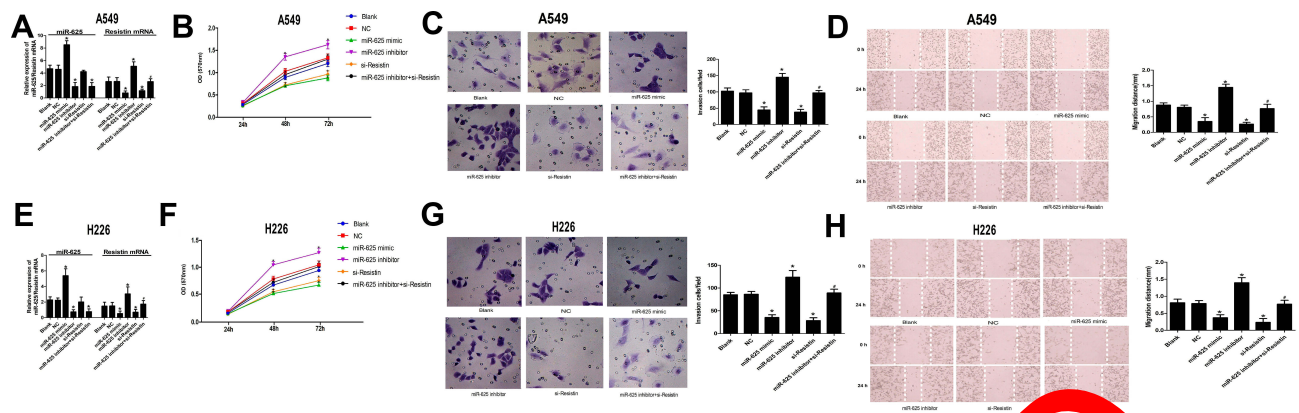


Figure 3 Effect of miR-625-mediated downregulation of Resistin in NSCLC cell lines. (A) RT-PCR results showing miR-625 and Resistin mRNA levels in A549 cells transfected with miR-625 mimic/inhibitor and/or si-Resistin; (B–D) Proliferation rates, invasiveness, and migration of A549 cells transfected with different constructs; (E) RT-PCR results showing miR-625 and Resistin mRNA levels in H226 cells transfected with miR-625 mimic/inhibitor and/or si-Resistin; (F–H) proliferation rates, invasiveness, and migration of A549 cells transfected with different constructs; * $P < 0.05$ compared to the Blank group, # $P < 0.05$ compared to the miR-625 inhibitor group.

< 0.05). Moreover, co-inhibition of miR-625 and Resistin significantly decreased the invasion and migration abilities of NSCLC cells when compared to miR-625 inhibition alone ($P < 0.05$; Figure 3C, D, G and H). Taken together, Resistin is necessary for the proliferation, migration, and invasion of NSCLC cells, and its downregulation by miR-625 abolishes its neoplastic effects.

MiR-625 Targets Resistin to Inhibit EMT of NSCLC Cells by Downregulating the PI3K/AKT/Snail Pathway

Ectopic expression of miR-625 blocked EMT in the NSCLC cells, as indicated by the significant decline in the expression of mesenchymal proteins like Snail, Twist1

and Vimentin and increased levels of the epithelial marker E-cadherin (Figure 4A and B; $P < 0.05$ for all). A similar anti-EMT effect was observed following Resistin silencing ($P < 0.05$ for all). In contrast, the miR-625 inhibitor skewed the balance towards the mesenchymal lineage, which was reversed by concomitant Resistin silencing (Figure 4A and B; $P < 0.05$ for all). In addition, when compared to the NC group, the protein expression of p-AKT and p-PI3K in the miR-625 mimic and si-Resistin groups was significantly reduced ($P < 0.05$), while the expression of p-AKT and p-PI3K in the miR-625 inhibitor group was significantly increased ($P < 0.05$). No significant changes in protein expression of p-AKT and p-PI3K were observed in miR-625 inhibitor + si-Resistin cells ($P > 0.05$). When compared with the miR-625 inhibitor group, the

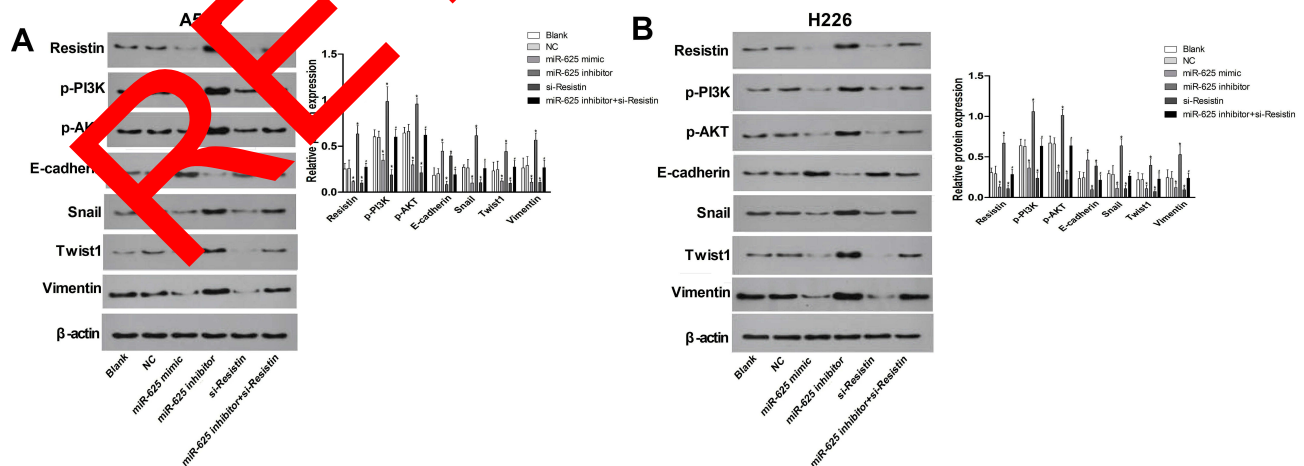


Figure 4 MiR-625 targets Resistin to inhibit EMT of NSCLC cells via downregulation of the PI3K/AKT/Snail pathway. (A and B) Immunoblots showing the expression levels of Resistin, p-AKT, p-PI3K, E-cadherin, Snail, Twist1, and Vimentin in A549 and H226 cells transfected with miR-625 mimic/inhibitor and/or si-Resistin. * $P < 0.05$ compared to the Blank group, # $P < 0.05$ compared to the miR-625 inhibitor group.

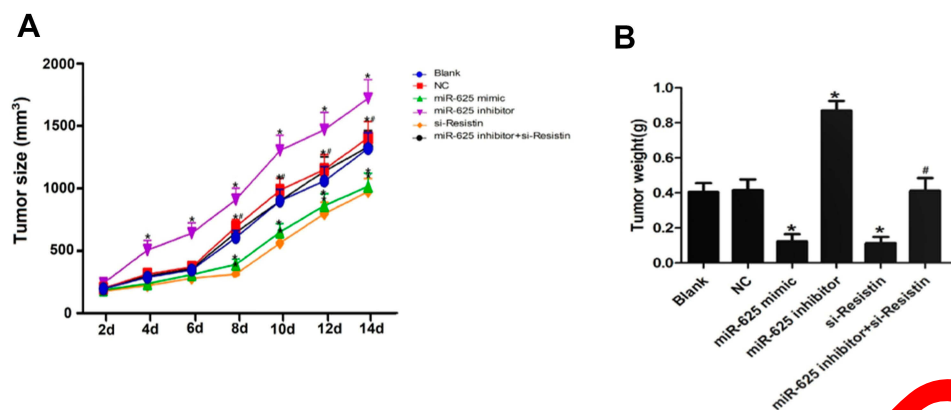


Figure 5 MiR-625 targeted Resistin to inhibit NSCLC growth in vivo. **(A)** Time-dependent tumor volume curve in Blank control, negative controls (NC), miR-625 mimic, miR-625 inhibitor, si-Resistin and miR-625 inhibitor + si-Resistin groups. **(B)** Tumor weight in the above groups. * $P < 0.05$ compared to Blank group, # $P < 0.05$ compared to the miR-625 inhibitor group.

expression of p-AKT and p-PI3K in the miR-625 inhibitor + si-Resistin group was significantly reduced ($P < 0.05$). Taken together, miR-625 may inhibit the EMT process of NSCLC cells by targeting Resistin, thereby down-regulating the PI3K/AKT/Snail pathway.

MiR-625 Targeted Resistin to Inhibit NSCLC Growth in vivo

Compared to the untreated A549 cells, those expressing miR-625 mimic or si-Resistin showed significantly reduced tumorigenic potency in nude mice ($P < 0.05$), whereas tumors in the miR-625 inhibitor group were significantly larger ($P < 0.05$). Consistent with the *in vivo* findings, NSCLC cells expressing both miR-625 inhibitor and si-Resistin showed less growth when compared to those expressing only miR-625 inhibitor ($P < 0.05$, Figure 5A and 5B).

Discussion

Lung cancer accounts for almost one-third of all cancer-related deaths worldwide, and despite the recent progress in therapeutic approaches, has unsatisfactory prognosis due to high recurrence and metastasis rates.¹ The incidence of lung cancer is increasing annually due to high levels of environmental pollution and a globally aging population. NSCLC is the predominant pathological type of lung cancer, and accounts for over 80% of all cases. The prognosis for most NSCLC patients is poor, with a only 20% 5-year survival rate even after early diagnosis.² Therefore, it is essential to identify novel biomarkers of NSCLC to develop more effective drugs and improve patient prognosis.

NSCLC initiation and progression are multi-step processes involving numerous genes and regulatory elements, and like all malignancies is characterized by upregulation of oncogenes and/or down-regulation of tumor suppressor genes. MiRNAs regulate 1/3rd of the human genome via translational inhibition or degradation of the cognate genes and form complex regulatory networks that have been implicated in malignant progression. Aberrant expression of miRNAs has been detected in multiple cancers, and are increasingly being considered as prognostic biomarkers and therapeutic targets.⁹ MiR-625 is significantly downregulated in gastric cancer, and its low levels are associated with a higher risk of lymph node metastasis. Mechanistically, it acts a tumor suppressor in gastric cancer by regulating ILK expression levels.⁵ Analysis of 158 pairs of esophageal cancer and para-cancerous tissues also revealed significantly lower levels of miR-625 in the tumor tissues. *In vitro* studies further showed that miR-625 inhibited the proliferation and invasion of tumor cells by targeting Sox2.⁸ In agreement with previous studies, we found that miR-625 expression was significantly lower in NSCLC tissues, while the expression of Resistin was significantly higher. Resistin levels were negatively correlated to miR-625, and significantly associated with tumor differentiation, clinical stage and lymph node metastasis in the NSCLC patients. Resistin is a cysteine-rich protein with a molecular weight of 12.5 kDa, and is primarily secreted by macrophages, dendritic cells and monocytes.¹⁰ In a previous study, significantly higher circulating Resistin levels were reported in NSCLC patients compared to healthy controls that was related to the weight loss in the former.¹⁵ Gong et al.¹³ also detected aberrantly

higher levels of Resistin in lung adenocarcinoma tissues, which promoted tumor cell invasion and metastasis by upregulating MMP2 and Twist1 via the TLR4/Src/NF- κ B/PI3K/Akt pathway. Bioinformatics analysis predicted Resistin as a target of miR-625, and the dual-luciferase gene reporter assay further confirmed that Resistin was negatively regulated by miR-625.

Consistent with the patient tissues, the human NSCLC cell lines also showed significantly lower levels of Resistin mRNA and protein compared to normal bronchial epithelial cells, along with lower miR-625 expression. Tumor invasion and metastasis are the basis of malignant progression, and hinge on tumor cell proliferation and migration.¹⁶ Upregulation of miR-625 using a mimic, as well as silencing of Resistin mRNA, significantly reduced the proliferation, invasion and migration of the NSCLC cells, while downregulation of miR-625 had a neoplastic effect. Metastasis involves multiple processes such as angiogenesis, loss of intercellular adhesion, and EMT.^{17,18} EMT plays a key role in the early stages of tumor metastasis wherein the epithelial cells gradually lose their attachment to the basement membrane and acquire mesenchymal characteristics such as minimal intercellular adhesion and increased motility, resulting in higher migration capacity, tissue invasion and metastasis.¹⁸ E-cadherin and vimentin typify the epithelial and mesenchymal phenotypes respectively. Studies have shown that AKT is a major regulator of the EMT process, and that TGF- β 1 induces EMT of lung cancer cells via the PI3K/AKT and MAPK/ERK1/2 pathways.¹⁹ AKT directly upregulates the mesenchymal Snail/Twist proteins and inhibits E-cadherin. Snail/Twist proteins also directly inhibit E-cadherin by binding to the latter's promoter region and drive the EMT.²⁰ We found that miR-625 upregulation or inhibition of Resistin downregulated mesenchymal markers as well as p-AKT and p-PI3K, and decreased E-cadherin levels. Finally, miR-625 and si-Resistin significantly impaired the tumorigenic ability of NSCLC in vivo, while the miR-625 inhibitor promoted tumorigenesis.

Conclusions

MiR-625 acts as tumor suppressor in NSCLC by targeting Resistin. It is frequently downregulated in NSCLC tissues, which likely promotes tumor progression and metastasis via increased EMT through the PI3K/AKT/Snail signaling pathway. Our findings provide novel insights into the roles of miR-625 and Resistin in NSCLC, and identify potential prognostic biomarkers and therapeutic targets.

Disclosure

The authors declare that they have no conflicts of interest in this work.

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