MicroRNA-124 suppresses Slug-mediated lung cancer metastasis

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Abstract. – OBJECTIVE: Non-small cell lung cancer (NSCLC) is the most common type of lung cancer but its carcinogenesis remains not completely elucidated. Dysregulation of microRNAs (miRNAs) is involved in the development of various cancers, including NSCLC, whereas a role of miR-124 in the pathogenesis of NSCLC has not been reported. Here we addressed these questions.

PATIENTS AND METHODS: We examined the levels of Slug and miR-124 in NSCLC tissue, compared to the adjacent non-tumor tissue. We also examined the relationship between miR-124 and Slug. The levels of miR-124 were then modified in NSCLC cells, and then the effects on cell invasion were evaluated.

RESULTS: We detected significantly higher levels of Slug and significantly lower levels of miR-124 in NSCLC tissue, compared to the adjacent non-tumor tissue, and the values of miR-124 and Slug were inversely correlated. Moreover, the low miR-124 levels in NSCLC specimens were associated with poor survival of the patients. In vitro, overexpression of miR-124 significantly inhibited cell invasion, while depletion of miR-124 increased cell invasion in NSCLC cells. The effects of miR-124 on cell growth appeared to result from its regulation of Slug.

CONCLUSIONS: Our study highlights miR-124 as a previously unrecognized factor that controls NSCLC invasiveness, and the findings here may be important for developing the innovative therapeutic treatment for NSCLC.

Key Words: Non-small cell lung cancer (NSCLC), miR-124, microRNAs (miRNAs), Cancer cell invasion, Slug.

Introduction

Non-small cell lung cancer (NSCLC) contains three subtypes: squamous cell carcinoma, large cell carcinoma, and adenocarcinoma, and is often insensitive to chemotherapy and radiotherapy due to its invasiveness manner. Thus, understanding of the mechanisms underlying the invasiveness of NSCLC is extremely important for its therapy.

Epithelial-Mesenchymal Transition (EMT) is a critical biological event that triggers the modification of the cancer cell properties to allow the cancer cell to adapt to a phenotype favoring invasiveness and metastases. During EMT, cancer cells obtain the capability of secreting proteinases from neighbor stromal cells or inflammatory cells for breaking through collagenous protein barriers. Zinc finger protein Slug is a member of the Snail family of C4H2-type zinc finger transcription factors. Slug acts as a transcriptional repressor that binds to E-box motifs to repress E-cadherin transcription in some cancer cells. Thus, Slug is a potent trigger for EMT to allow cancer cells to invade and migrate. However, the signal pathways to control Slug activation in NSCLC has not been completely clarified.

MicroRNA (miRNA) is a group of non-coding small RNAs of about 22 nucleotides, and has been found to regulate more than an expression of one-third of the genes post-transcriptionally, through its base-pairing with the 3’-untranslated region (3’-UTR) of target mRNA. Cancer initiation and progression have been associated with aberrant expression of some miRNAs. Of note, the miRNAs have been found to play a critical role in the tumorigenesis of NSCLC. However, among all miRNAs, miR-124 has been rarely studied and reports on its involvement in lung cancer are not extensive.

Here, we analyzed the levels of Slug and miR-124 in NSCLC tissues, and studied the association of miR-124 levels with the prognosis of the patients. We further showed the relationship between miR-124 and Slug using bioinformatics analyses. We then overexpressed miR-124 or inhibited miR-124 in 2 commonly used NSCLC cell lines in vitro and studied their effects on Slug activation and NSCLC cell invasion.

Patients and Methods

Experimental Protocol Approval

All experimental protocols were approved by the Research Bureau of General Hospital of Beijing. All mouse experiments were approved by the European Review for Medical and Pharmacological Sciences 2016; 20: 3802-3811

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Institutional Animal Care and Use Committee at General Hospital of Beijing (Animal Welfare Assurance). The methods regarding animals and human specimens were carried out in “accordance” with the approved guidelines.

**Patient Specimens**

Surgical specimens from 30 NSCLC patients and matched adjacent non-tumor tissues (NT) were obtained postoperatively in General Hospital of Beijing from 2010 to 2015. All patients gave signed, informed consent for the tissue to be used for scientific research. Ethical approval for the study was obtained from the General Hospital of Beijing. All diagnoses were based on pathological and/or cytological evidence. The histological features of the specimens were evaluated by senior pathologists according to the World Health Organization classification criteria. All patients had been followed-up for 60 months. Complete clinical data was electronically recorded.

**Cell Line Culture and Transfection**

Two human NSCLC cell lines A549 and H460 were both purchased from APCC (American Type Culture Collection, Manassas, VA, USA), and have been widely used in NSCLC research. The A549 cell line was first developed in 1972 by Dr. Giard through the removal and culturing of cancerous lung tissue in the explanted tumor of a 58-year-old Caucasian male. H460 cell line was deposited at the ATCC by Dr. Adi F. Gazdar. Both cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) in a humidified chamber with 5% CO₂ at 37 °C. MiRNAs mimics (miR-124), miRNAs antisense oligonucleotides (as-miR-124), Slug, and short hairpin small interfering RNA for MMP (shSlug) were obtained from Origene (Beijing, China). A null sequence was used as a control (null). The plasmids were transfected into cells at a concentration of 50 nmol/l using Lipofectamine 2000, according to the manufacturer’s instructions. The transfection efficiency was more than 95%, based on expression of a GFP reporter.

**Transwell Cell Invasion Assay**

Cells (10⁴) were plated on the top side of polycarbonate transwell filter coated with Matrigel in the upper chamber of the BioCoat™ Invasion Chambers (Becton-Dickinson Biosciences, Bedford, MA, USA) and incubated at 37°C for 22 hours. The cells inside the upper chamber with cotton swabs were then removed. Migratory and invasive cells on the lower membrane surface were fixed, stained with hematoxylin, and counted for 10 random 100X fields per well. Cell counts are expressed as the mean number of cells per field of view. Five independent experiments were performed and the data are presented as mean ± standard deviation (SD).

**MiRNA Target Prediction and 3′-UTR Luciferase Reporter Assay**

MiRNAs targets were predicted as has been described before, using the algorithms TargetSan (https://www.targetscan.org). The Slug 3′-UTR reporter plasmid (pRL-Slug) and the Slug 3′-UTR reporter plasmid with a mutant at the miR-124 binding site (pRL-Slug-mut) were purchased from Creative Biogene (Shirley, NY, USA). NSCLC cells were co-transfected with pRL-Slug/pRL-Slug-mut and miR-124/as-miR-124/null by Lipofectamine 2000 (5×10⁴ cells per well). Cells were collected 36 hours after transfection for assay using the dual-luciferase reporter assay system gene assay kit (Promega, Fitchburg, WI, USA), according to the manufacturer’s instructions.

**Quantitative RT-PCR (RT-qPCR)**

Total RNA was extracted from resected specimens or cultured cells with the miRNeasy mini kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was randomly primed from 2 μg of total RNA using the Omniscript reverse transcription kit (Qiagen). Quantitative PCR (RT-qPCR) were performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed, using 2^{-ΔΔCt} method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α-tubulin, and then compared to the experimental controls.

**Western Blot**

Total Protein was extracted from resected specimens or cultured cells by RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). An equal amount of proteins was loaded in the gel. Primary antibodies for Western Blot are rabbit anti-Slug and anti-α-tubulin (all purchased from Cell Signaling, St. Jose, LA, USA). The secondary antibody is HRP-conjugated anti-rabbit
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The protein levels were first normalized to α-tubulin, and then normalized to control. Images shown in the figure were representatives from 3 repeats. Densitometry of Western blots was quantified with NIH ImageJ software (Bethesda, MA, USA).

Statistical Analysis

All statistical analyses were carried out using the SPSS 18.0 statistical software package (SPSS Inc., Chicago, IL, USA). All values in cell and animal studies are depicted as mean ± standard deviation and are considered significant if $p < 0.05$. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher’s Exact test for comparison of two groups. Patients’ survival was determined by Kaplan-Meier analysis.

Results

Low miR-124 Levels in NSCLC Specimens are Associated with Poor Prognosis

The levels of Slug and miR-124 in 30 NSCLC tissues and paired adjacent non-tumor tissues (NT) were measured by Western blot (A) and RT-qPCR (B), respectively. C, A correlation test was performed between Slug and miR-124, using the 30 NSCLC specimens. D, The 28 NSCLC patients were followed-up for 60 months. The median value of all 30 cases was chosen as the cutoff point for separating miR-124 high-expression cases (n=15) from miR-124 low-expression cases (n=15). Kaplan-Meier curves were performed. *$p<0.05$. **$p<0.01$. N=30.
followed-up for 60 months. The median value of all 30 cases was chosen as the cutoff point for separating miR-124 high-expression cases (n=15) from miR-124 low-expression cases (n=15). Kaplan-Meier curves indicated that NSCLC patients with low miR-124 levels had a significantly worse
5-year survival than those with high miR-124 levels (Figure 1E). These data suggest that low miR-124 levels in NSCLC specimens may associate with poor prognosis.

**MiR-124 Targets Slug to Inhibit its Protein Translation in NSCLC Cells**

Next, we examined miR-124 and Slug levels in several NSCLC cell lines. Among these cell lines, we found that H460 was a NSCLC cell line expressing relative high miR-124 and relative low Slug, while A549 was a NSCLC cell line expressing relative low miR-124 and relative high Slug (Figure 2A-B). Thus, we transfected A549 cells with miR-124 mimics (miR-124) (Figure 2C), or transfected H460 cells with antisense for miR-124 (as-miR-124) (Figure 2D). These cells were also transfected with a null sequence as a con-

![Figure 3](image-url) "Figure 3. MiR-124 decreases Slug protein but not mRNA in NSCLC cells. A-B. The Slug levels in miR-124-depleted (and Slug-depleted) H460 cells by RT-qPCR (A) and by Western blot (B). C-D. The Slug levels in miR-124-overexpressing (and Slug-overexpressing) A549 cells by RT-qPCR (C) and by Western blot (D). *p<0.05. NS: non-significant. N=5."
miR-124 inhibits NSCLC via Slug

trol (null). The levels of miR-124 in these modified NSCLC cells were assayed by RT-qPCR, 72 hours after transfection. The augment in miR-124 levels in A549-miR-124 cells and the knockdown of miR-124 levels in H460-as-miR-124 cells were confirmed (Figure 2C-D). Then we used these miR-124-modified NSCLC cells in the experiments that examine the functional binding of miR-124 to Slug mRNA, which was predicted by bioinformatics algorithms (Figure 2E). The intact 3’-UTR of Slug mRNA (Slug 3’-UTR), together with a 3’-UTR with mutant at the miR-124-binding site of Slug mRNA (Slug 3’-UTR mut), was then cloned into luciferase reporter plasmids, and used for co-transfection with miR-124-modified plasmids into NSCLC cells. First, H460 cells were co-transfected with 1 μg as-miR-124/null plasmids and 1 μg Slug 3’-UTR plasmids, showing that miR-124 depletion significantly increased luciferase activities (Figure 2F). Next, A549 cells were co-transfected with 1 μg miR-124/null plasmids and 1 μg Slug 3’-UTR or Slug 3’-UTR mut plasmids, showing that that Slug 3’-UTR plus miR-124 had the most repression for Slug, and the Slug 3’-UTR mut failed to decrease luciferase activities by miR-124 (Figure 2G). These data

![Graph and Images](image-url)

**Figure 4.** Depletion of miR-124 abolishes H460 cell invasion through Slug. (A-B) H460 cell invasion by miR-124 depletion (and Slug depletion) in a transwell cell invasion assay, shown by quantification (A), and by representative images (B). *p<0.05. N=5.
demonstrate that miR-124 specifically targets 3’-UTR of Slug mRNA to inhibit its translation in NSCLC cells.

**MiR-124 Decreases Slug Protein but not mRNA in NSCLC Cells**

We then evaluated the effects of miR-124 on Slug levels in NSCLC cells. We found that although the Slug mRNA did not alter by miR-124 depletion in H460 cells (Figure 3A), the protein levels of Slug in miR-124-depleted H460 cells was increased (Figure 3B). On the other hand, although the Slug mRNA did not alter by miR-124 overexpression in A549 cells (Figure 3C), the protein levels of Slug in miR-124-overexpressing A549 cells was significantly decreased (Figure 3D). Together, these data suggest that miR-124 may decrease Slug protein but not mRNA in NSCLC cells, consistent with above-mentioned results from luciferase reporter assay.

**Modification of miR-124 Regulates NSCLC Cell Invasion**

Next, the role of miR-124 in the invasion of cultured NSCLC cells was investigated. We found that miR-124 depletion in H460 cells significantly increased cell invasion in a transwell cell invasion assay, shown by quantification (Figure 4A), and by representative images (Figure 4B). Similarly, miR-124 overexpression in A549 cells significantly decreased cell invasion in a transwell cell invasion assay, shown by quantification (Figure 5A),

![Figure 5](image)

**Figure 5.** Overexpression of miR-124 suppresses A549 cell invasion through Slug. A-B, A549 cell invasion by miR-124 overexpression (and Slug overexpression) in a transwell cell invasion assay, shown by quantification (A), and by representative images (B). *p<0.05. N=5.
miR-124 inhibits NSCLC via Slug

and by representative images (Figure 5B). Thus, these data suggest that modification of miR-124 regulates NSCLC cell invasion.

**MiR-124 Regulates NSCLC Cell Invasion Through Slug**

In order to figure out whether miR-124 may regulate NSCLC cell invasion through Slug, we prepared plasmids for Slug overexpression (Slug) and depletion (shSlug). First, H460-as-miR-124 was further transfected with shSlug, which knockst downed Slug mRNA (Figure 3A) and protein (Figure 3B) in these cells. We found that Slug suppression abolished the effects of as-miR-124 expression on cell invasion in H460 cells (Figure 4A-B). Next, A549-miR-124 was further transfected with Slug, which increased Slug mRNA (Figure 3C) and protein (Figure 3D) in these cells. We found that Slug augmentation abolished the effects of miR-124 expression on cell invasion in A549 cells (Figure 5A-B). These data suggest that miR-124 may regulate NSCLC cell invasion through Slug (Figure 6).

**Discussion**

MiRNAs play demonstrative roles in the carcinogenesis in various cancers. In line with these notions, their participation in the cancer progression has been widely reported. These pioneering studies demonstrate microRNAs as an intriguing therapeutic target to prevent metastases of lung cancer cells.

NSCLC is a malignant lung cancer that affects millions of people worldwide. It is one of the most common causes of cancer-associated death. The poor outcome of NSCLC largely results from its fast growth and invasion. The role of Slug in NSCLC invasion and metastases has been well documented in the past studies. However, to the best of our knowledge, the current study is the first study that showed a direct regulation of Slug by miR-124 in NSCLC.

Here, miR-124 is a novel number of the miRNA family and has not been reported of any direct targets, including Slug. Here, we showed that Slug might be regulated by miR-124 in NSCLC cells. A low level of miR-124 in NSCLC tissues was associated with a poor survival rate in NSCLC patients. Moreover, the levels of miR-124 and Slug were inversely correlated. Then, we used a set of gain-of-function and loss-of-function experiments to show a regulatory relationship between miR-124 and Slug in NSCLC cells. In the promoter luciferase assay that showed that bindings of miR-124 to 3'-UTR of Slug mRNA inhibited protein translation, there seemed to be a significant repression of the mutant 3'-UTR in the presence of miR-124. Given that the mutant 3'-UTR should not bind miR-124 and yet did not have the same response as the wild-type 3'-UTR plus antisense, these data may suggest the presence of at least one cryptic miR-124 binding site. The large-scale changes in luciferase levels seemed unusual for miRNAs, which may be due to the importance of the binding site of miR-124 on the 3'-UTR of Slug mRNA to its translation. Besides regulation of

![Figure 6. Schematic of the model. MiR-124 regulates NSCLC cell invasion, through translational suppression of Slug.](image-url)
Slug by miRNAs, Slug protein levels may be also affected by modulation of its degradation, e.g. through protein ubiquitination. Also, an exact role of WNT signaling in the molecular regulation of Slug by all these mechanisms may be studied in future to fully understand the control of Slug in NSCLC cell invasion.

Conclusions

Our study here may provide evidence for using miR-124 as a novel target for treating NSCLC and contribute to the understanding of molecular regulation of Slug-mediated NSCLC cell invasiveness.

Conflicts of interest

The authors declare that no conflicts of interest exist.

References

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miR-124 inhibits NSCLC via Slug


