

MiR-137 and its target TGFA modulate cell growth and tumorigenesis of non-small cell lung cancer

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Abstract. – **OBJECTIVE:** MiR-137 has been reported to serve as a tumor suppressor in non-small cell lung cancer (NSCLC). However, the potential mechanism remains largely unclear. The present study aimed to explore the potential molecular mechanisms by which miR-137 regulated NSCLC.

MATERIALS AND METHODS: Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to quantify the expression levels of miR-137 in NSCLC tissues and cell lines. Dual-luciferase reporter assay was employed to confirm the specificity of miR-137 target genes. An MTT assay and flow cytometry were used to determine the rates of cell proliferation and cell cycle distribution. Furthermore, the effect of miR-137 up-regulation on TGFA expression was examined by western blot.

RESULTS: miR-137 expression levels in NSCLC cell lines or tissue were significantly lower than in a normal human lung cell line or adjacent normal tissues. We further found that upregulation of miR-137 inhibited the proliferation of NSCLC cells, whereas silencing of miR-137 promoted the proliferation of NSCLC. Moreover, we identified TGFA as a direct target gene of miR-137 in NSCLC cell. Finally, Similarly, knockdown of TGFA led to the suppression of NSCLC cell proliferation.

CONCLUSIONS: Overall, our findings indicated that miR-137 served as a tumor suppressor in NSCLC and its suppressive effect is mediated by repressing TGFA expression.

Key Words:

miR-137, NSCLC, Proliferation, TGFA.

ular-targeted therapy, the prognosis for NSCLC is still dismal, and the overall 5-year survival rate is about 15%². Thus, investigations on the molecular mechanisms involved in NSCLC are urgently demanding tasks to explore novel therapeutic target and biomarkers for its prognosis.

MicroRNAs (miRNAs) are a subclass of endogenous non-coding RNAs, which play an essential role in the negatively regulation of gene expression^{3,4}. MiRNAs play important roles in the regulation of gene expression for developmental timing, cell proliferation, and apoptosis^{5,6}. Increasing evidence showed that miRNAs function as tumor promoters or suppressors in all types of tumors^{7,8}. Recently, miR-137 has been reported to serve as a tumor suppressor in many different tumors, including melanoma⁹, lung cancer¹⁰, ovarian cancer¹¹, and glioblastoma¹². Given the complexity of its functionality, it would be of interest to explore the molecule mechanism of miR-137 in NSCLC development.

In the present work, we investigate whether miR-137 is detectable and altered in NSCLC tissues or cell lines compared with adjacent normal tissues or normal cell lines. We also performed an *in vitro* study to determine the role as well as the mechanism of action of miR-137 in NSCLC cell proliferation, involving TGFA. Our results revealed that miR-137 downregulation may be very important for NSCLC progression, highlighting it as a potential target for NSCLC therapy.

Materials and Methods

Tissue Sample

Sixty-two pairs of primary human NSCLC lung tissue samples and adjacent tumor-free tissue samples were obtained Chinese PLA General Hospital. All patients did not receive chemo-

Introduction

Non-small cell lung cancer (NSCLC) is one of the most common cancers and is the leading cause of death from cancer in men worldwide¹. Despite recent improvements in chemotherapy and molec-

therapy or radiotherapy before surgery. All tissue samples must be frozen in liquid nitrogen immediately and stored at -80°C for RNA extraction. This study was approved by the Ethics Committee of Chinese PLA General Hospital, and written informed consent was obtained from each participant.

Cell Culture and Transfection

Four NSCLC cancer cell lines (A549, SK-MES-1, H129, and H520) and a normal human bronchial epithelial cell line (BEAS-2B) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Xuhui, Shanghai, China). The cells were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% bovine calf serum (BCS) (Gibco) at 37°C in a humidified atmosphere containing 5% CO_2 .

miR-137 mimic, miR-137 inhibitor, and si-TGFA were synthesized by GenePharma (Pudong, Shanghai, China). Transient transfection was performed using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in antibiotic-free Opti-MEM medium (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Real-time PCR

The total RNA was extracted from the paraffin-embedded tissue samples using the Recover-All™ Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed with TransScript II Green One-Step qRT-PCR SuperMix (Transgen, Tianjin, China) with an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). The reaction was performed as follows: one cycle of 95°C for 5 min and 40 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec. Each sample was run in triplicates, and the mean value was used for analysis. Primers for miR-137 and U6 were purchased from GeneCopoeia (Carlsbad, CA, USA) and the expression of miR-137 was normalized with U6. The $\Delta\Delta\text{Ct}$ method was used for relative quantification.

Cell Proliferation Detection

A total of approximately 5.0×10^3 cervical cancer cells were plated in 96-well plates. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to determine cell viability after seeding. The culture plates were then shaken for 15 min and the optical absorbance values were read at 490 nm. Five replicate wells were set up in each group and

five independent experiments were performed repeatedly.

Cell Cycle Analysis

Cells were grown and transfected as mentioned above. Cell cycle phase was assessed by flow cytometry. Cells were harvested by trypsinization, washed twice using cold phosphate buffered saline (PBS) and fixed in 70% ethanol at 4°C , then treated with 25 lg/ml of DNase-free RNase A and stained with 50 lg/ml of propidium iodide at room temperature for 30 min.

Luciferase Reporter Assay

A fragment of TGFA 3'-UTR, which contained the putative miR-137 binding sites, was cloned into the downstream region of the luciferase gene in the pGL3-REPORT luciferase vector (Invitrogen, Carlsbad, CA, USA). Cells were cultured in triplicate in 24-well plates (1×10^5 /well) and co-transfected with pEZX-TGFA-3'UTR and 100 nM of miR-137 mimics or miR-137-mut or control mimics using Lipofectamine 2000 Reagent. Luciferase activity was detected after 36 hr using a dual-luciferase reporter assay system and normalized to Renilla activity.

Western Blot Analysis

Cells or mice tissues were lysed using RIPA buffer containing protease inhibitors cocktail according to the manufacturer's instruction. Proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked in Tris-buffered saline-Tween buffer containing 5% low-fat milk for 50 min with gentle shaking. Then, probed with rabbit polyclonal primary anti- β -actin (1:500, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or rabbit polyclonal primary anti-TGFA-C. And then the membrane strips were probed with a secondary antibody. Finally, ECL detection systems (Super-Signal West Femto, Pierce, Haidian, Beijing, China) were used for detection.

Statistical Analysis

Statistical analysis was performed using SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA). The results obtained from experiment assays are presented as mean $\pm$ SEM from five separate experiments in triplicates per experiment. Statistical analyses between the two groups were evaluated using two-tailed Student's *t*-test. $p < 0.05$ was considered statistical significant.

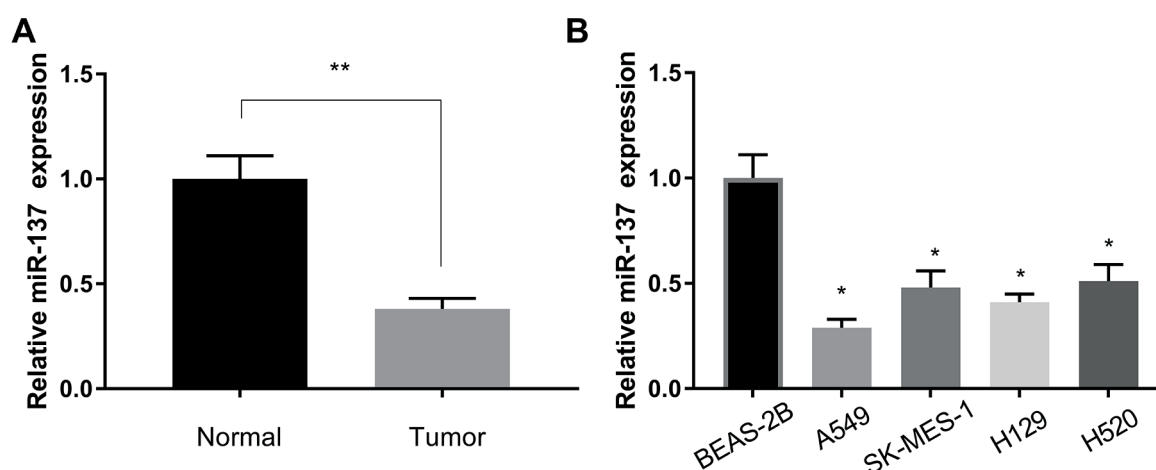


Figure 1. miR-137 is significantly down-regulated in NSCLC tissues and NSCLC cell lines. **(A)** The relative expression of miR-137 in 62 pairs of NSCLC. **(B)** The expression level of miR-137 in the normal lung bronchus epithelial cell line BEAS-2B and four NSCLC cell lines (A549, SK-MES-1, H129, and H520). * $p < 0.05$, ** $p < 0.01$.

Results

Aberrant Expression of miR-137 in Human NSCLC Cells and Tumors

To research potential effects about miR-137 in NSCLC, the miR-137 levels were estimated in tissue and cell specimens. As shown in Figure 1, a significant down-regulation of miR-137 was observed in NSCLC tissues compared to normal tissues ($p < 0.01$). Next, we explored the expression levels of miR-137 in four NSCLC cells lines and our results showed that the miR-137 expression levels were markedly reduced in NSCLC cells.

miR-137 Suppresses NSCLC Cell Proliferation

miR-137 was transfected into H129 cells to investigate its effect on NSCLC cell proliferation using MTT. The results of PCR showed that miR-137 mimics up-regulated the miR-137 levels in H129 cells, and anti-miR-137 inhibited the expression of miR-137 (Figure 2A, 2B). Forced miR-137 expression significantly decreased the proliferation abilities of H129 cells compared with the NC group (Figure 2C). On the contrary, depletion of miR-137 expression significantly increased the proliferation abilities of H129 cells compared with the NC group (Figure 2D). To check the influence of miR-137 on cell cycles, we applied the flow cytometry to detect the changes of the H129 cell cycle. Our findings revealed that over-expression of miR-137 markedly induced G1 phase arrest of esophageal cancer cells ($p < 0.05$, Figure 2E). Consistent with this result,

knockdown of miR-137 led to a distinct decrease in the cellular population in G0/G1 phase but an increase in S phase ($p < 0.05$, Figure 2F).

TGFA is a Direct Target of miR-137

To identify the potential target genes of miR-137, TargetScan and miRanda was used in combination. As shown in Figure 3A, our data showed that miR-137 was identified as a potential regulator of TGFA expression. We further used luciferase reporter assay to confirm the association between miR-137 and TGFA. The results showed that miR-137 significantly decreased the luciferase activity of the wild-type (Wt) but not the Mut 3'-UTR of TIMP2 in H129 cells (Figure 3B). Also, Western blot was performed to detect TGFA expression in miR-137 overexpressed or suppressed cells. The results indicated that over-expression of miR-137 suppressed the expression levels of TGFA (Figure 3C). Taken together, These findings indicated that miR-137 targeted TGFA directly.

Depletion of TGFA Inhibits the NSCLC Cell Proliferation

We next explore the effect of TGFA on NSCLC cell proliferation. The si-TGFA was transfected into the H129 cells using Lipofectamine 2000. As shown in Figure 4A, the expression of TGFA was reduced in H129 transfected si-TGFA. The MTT assay showed that H129 cells transfected with si-TGFA presented a slower speed of proliferation (Figure 4B). Furthermore, flow cytometry results also suggested that silence of TGFA significantly induce G1 phase arrest of H129 (Figure 4C). All

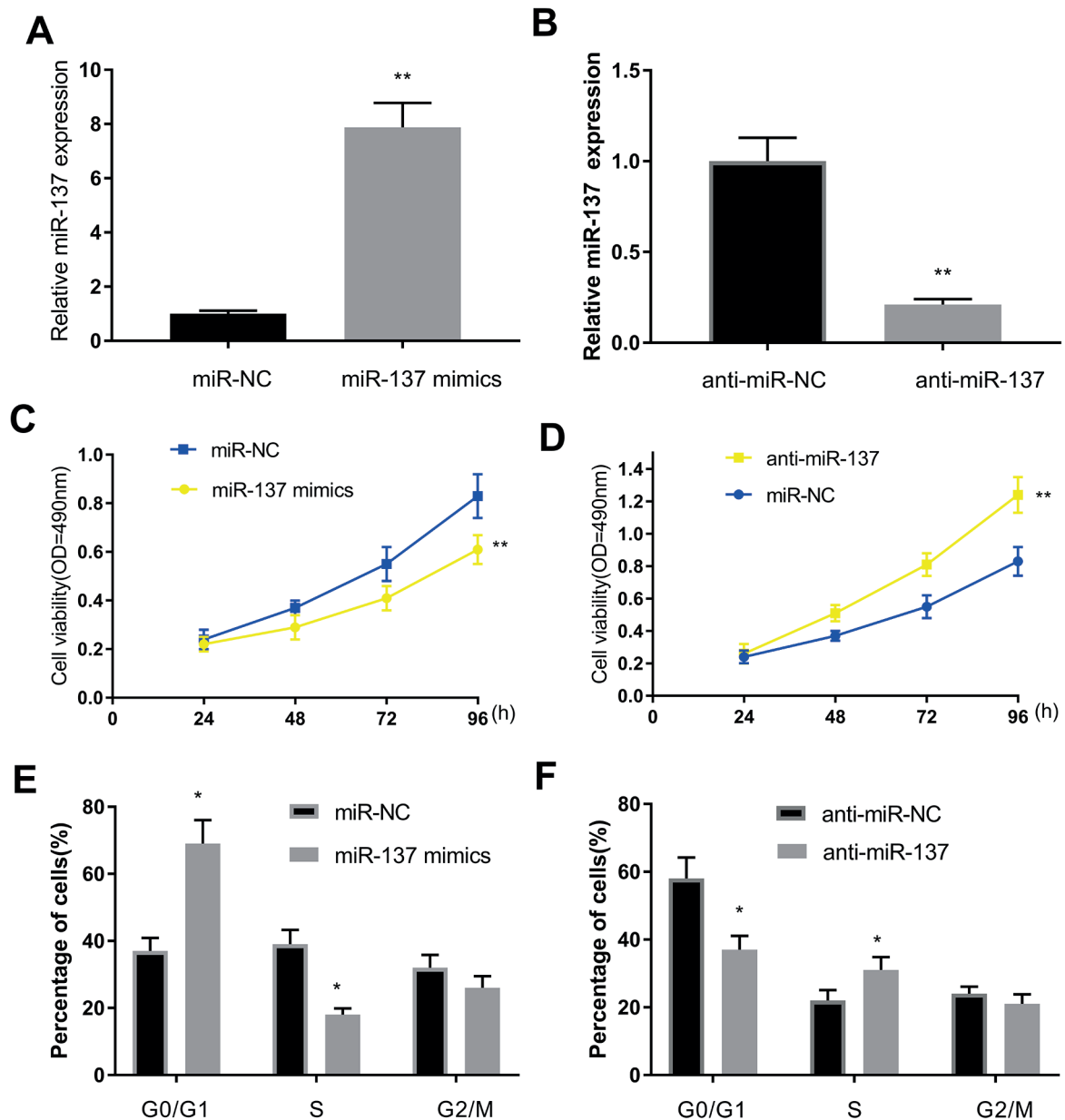


Figure 2. miR-137 suppressed NSCLC cell proliferation. (A,B) H129 cells were transfected with miR-137 mimics or miR-137 inhibitors (anti-miR-506), and the miR-137 level was detected using RT-PCR. (C,D) Effect of 137 on cell proliferation was tested by MTT assay. (E,F) Cell cycle distribution of transfected Eca109 cells assessed by flow cytometry. * $p < 0.05$, ** $p < 0.01$.

the results indicated that the potential role of miR-129 on NSCLC cell lines was dependent on TGFA.

Discussion

Mounting evidence suggests that miRNAs played essential roles in many aspects of tumorigenesis¹³. Hence, it would provide a vital clue for

diagnosis and therapy of patients with NSCLC by exploring specific miRNAs and their targets involved in the progression of NSCLC. In this study, we found that the expression of miR-137 is downregulated in NSCLC tissues and cell lines compared to normal lung tissue and human normal lung cells. Next, we investigated the effects of miR-137 on cell proliferation in H129. Our data showed that overexpression of miR-137 inhibited cell proliferation, while inhibition of miR-137 ac-

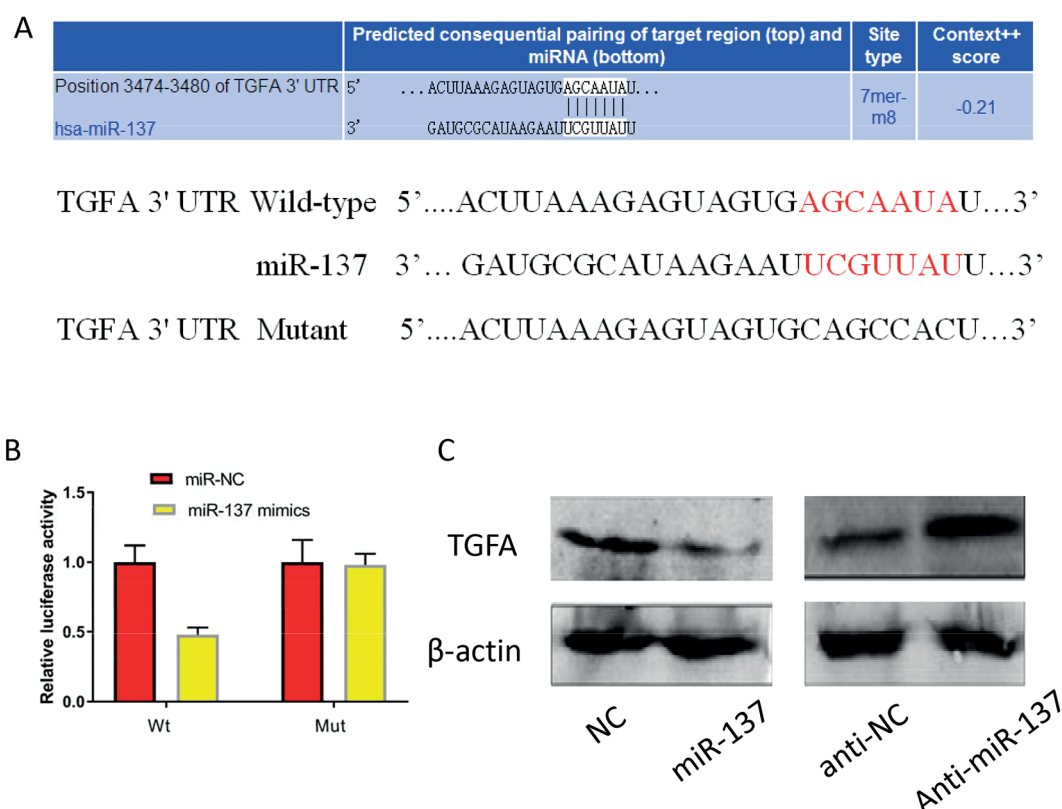


Figure 3. miR-137 negatively regulates TGFA by binding to the 3'UTR of TGFA. **(A)** The potential miR-137 binding sequence of TGFA 3'UTR and the mutant was shown. **(B)** H129 cells were co-transfected with miR-137 mimic or miR-NC and Wt or Mut TGFA 3'UTR report plasmid. Luciferase activity was measured. **(C)** Western blot was performed to detect protein level of TGFA in H129 cells transfected with miR-137 mimics or anti-miR-137. * $p < 0.05$, ** $p < 0.01$.

celerated these processes. We further proved by the application of the luciferase reporter gene assay that miR-137 directly targeted 3'-UTR of TGFA. To identify the effect of TGFA on cell proliferation in H129, we downregulated the expression of TGFA and found that knockdown of TGFA could inhibit cell proliferation. Taken together, these results indicate that miR-137 inhibited NSCLC cell proliferation through targeting TGFA.

Previous has reported the functional effect of miR-137 in various tumors. For instance, Shu et al observed that miR-137 may serve as a tumor promoter in conferring tumorigenic features such as growth and invasion on human glioblastoma¹⁴. Du et al¹⁵ showed that overexpression of miR-137 could inhibit the cell migration, proliferation, and promote cell cycle arrest in the G0/G1 stage in gastric cancer cells. Han et al¹⁶ revealed that overexpression of miR-137 in breast cancer cells drastically inhibited cell proliferation, colony formation, migration, and invasion through targeting CtBP1. Notably, Zhu

et al¹⁷ found that miR-137 inhibits the proliferation of lung cancer cells by targeting Cdc42 and Cdk6. Another finding by Bi et al¹⁸ showed that paxillin (PXN) was a target gene of miR-137 in NSCLC cells and restored expression of PXN abolished the miR-137-mediated suppression of cell migration and invasion. All those results showed that miR-137 served as an anti-oncogene in NSCLC. Our present study further identified a target gene of miR-137.

TGFA and its receptor epidermal growth factor receptor are crucial oncogenes in the development of different cancers¹⁹. TGFA makes an important contribution to cell proliferation and invasion in triple negative NSCLC²⁰. Previous studies showed that several miRNAs may negatively regulate TGFA gene expression at the posttranscriptional level. For instance, Jin et al²¹ revealed that MicroRNA-376c inhibits cell proliferation and invasion in osteosarcoma by targeting TGFA. Qin et al reported that miR-124 inhibits TGF- α -induced EMT in prostate cancer cells by

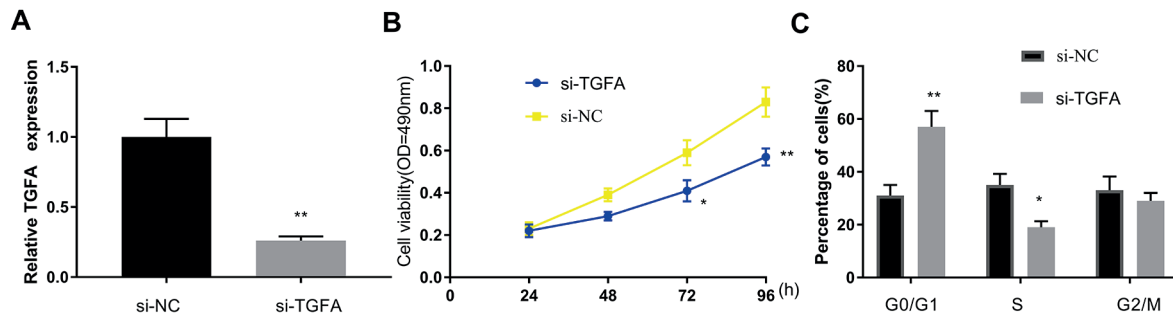


Figure 4. Reduction of TGFA by si-TGFA suppressed the growth of H129 cells. **(A)** The relative TGFA protein expression level was significantly decreased in si-TGFA-transfected cells. **(B)** Cell proliferation was measured by MTT after 96-h transient transfection. **(C)** Cell cycle distribution of transfected H129 cells assessed by flow cytometry. * $p < 0.05$, ** $p < 0.01$.

targeting Slug²². Most recently, Wu et al²³ found that overexpression of miR-374a led to inhibition of lung adenocarcinoma cell proliferation by targeting TGFA gene expression. Here, we also demonstrated that miR-137 was frequently down-regulated in NSCLC. We showed that TGFA was directly targeted by miR-137.

Conclusions

We provided evidence that overexpression of miR-137 suppressed cell growth by repressing TGFA expression. Our study suggests miR-137 may provide novel biomarker in improving the therapeutic care of NSCLC.

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Conflict of interest

The authors declare no conflicts of interest.

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