miR-21-5p/SMAD7 axis promotes the progress of lung cancer

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Abstract
Background: Lung cancer is one of the most common malignant tumors threatening human health. The aim of this study was to investigate the function of miR-21-5p in lung cancer progression.

Methods: We analyzed the expression levels of miR-21-5p in lung cancer tissues and cell lines. The qRT-PCR and MTT assays were performed after transfection with miR-21-5p mimic, inhibitor and negative control into lung cancer cells.

Results: Luciferase reporter assays showed miR-21-5p directly target SMAD7. The miR-21-5p inhibitor significantly suppressed lung cancer cell proliferation, invasion and migration. We found that SMAD7 was upregulated in lung cancer tissue. In addition, we found that SMAD7 inhibited lung cancer cell proliferation and miR-21-5p mimic damaged the inhibitory effect of SMAD7.

Conclusions: miRNA-21-5p may promote cell proliferation, migration and invasion by spoiling SMAD7 expression in lung cancer cells.

KEYWORDS
lung cancer, microRNA, miR-21-5p, SMAD7

INTRODUCTION

Lung cancer is one of the most common malignant tumors threatening human health. Although the treatment of lung cancer has made great progress, the mortality rate is increasing year by year. So far, little is known about the mechanism of lung cancer occurrence and development. The occurrence of lung cancer is a multifactor, multistage, multigene synergistic process which is related to the dysregulation expression of oncogenes and tumor suppressor genes.

Recent studies have revealed that some microRNAs (miRNAs) are involved in tumor formation. MicroRNAs are a class of non-coding single-stranded small molecule RNA with a length of 19 to 22 nucleotides that have been discovered in recent years. Mature miRNAs are retained in a functional complex and regulate gene expression by binding to the 3'-untranslated region of target mRNA. MicroRNAs play a crucial role in many biological processes from embryonic development, differentiation, immune regulation, disease cause (including oncogenes activation and tumor suppressor genes inactivation) and senescence. In recent years, more and more studies have shown that many miRNAs can be used as proto-oncogenes or tumor suppressor genes, and play an important role in the occurrence and development of cancer. Among them, miRNA-21 is the most interesting.

The coding gene of miRNA-21 is located at 17q23.2, vacuolemembraneprotein-1 (VMP1) coding region, the tenth intron of VMP1 gene. Studies have shown that some tumors have genomic 17q23.2 amplification mutations, such as breast and prostate cancer, and Hodgkin's lymphoma. Overexpression of miRNA-21-5p has been confirmed in various cancers, such as breast, prostate, and gastric cancer. The clinical significance of miRNA-21-5p expression in human cancer has been previously reported. miRNA-21-5p is involved in cell proliferation, differentiation, and apoptosis, and is therefore closely related to tumor growth, invasion, and metastasis. Yan et al. reported that miRNA-21-5p overexpression is associated with specific clinical pathological characteristics of breast cancer, advanced tumor stage, lymph node metastasis, and patient survival. Although many studies have reported that the expression level of miRNA-21-5p is significantly increased in tumor cells and has oncogenic activity, because microRNA has several or even dozens of target genes, the mechanism of cell biological behavior changes caused by
miRNA-21 has not yet been fully elucidated. The relationship
between miRNA-21-5p expression and lung cancer has not
been fully elucidated, and more detailed mechanism studies
are needed to elucidate its complex biological role.

Studies have shown abnormal expression of SMAD7 in
various types of tumors such as breast, gastric, pancreatic and
prostate cancers. In colorectal cancer, SMAD7 protein expres-
sion is increased and its overexpression is negatively correlated
with the survival rate of colorectal cancer patients.14 Kleeff
et al. report that the SMAD7 expression level in pancreatic
cancer is higher by comparison with that of the normal pan-
creas.15 The pancreatic cancer cells transfected with SMAD7
have been found to have increased malignancy and enhanced
tumorigenic ability in nude mice, suggesting that abnormal
expression of SMAD7 will contribute to tumor development.16
However, some studies have shown different results. It has
been reported that transfection of SMAD7 antisense gene
could block the growth promotion of TGFbeta.17 At present,
there are few studies on the role of SMAD7 in the occurrence,
development, invasion and metastasis of lung cancer.

METHODS

Cells and reagents

The human lung cancer cell lines A549, H460, H1299 and
normal human bronchial epithelial cell line (HBE) were pur-
chased from the Shanghai Cell Bank of Chinese Academy of
Sciences. All cells were cultured in RPMI1640 cell culture
medium containing 100 ml/L fetal bovine serum, 100 U/ml
penicillin, 100 g/ml streptomycin and 2 mmol/L glutamine
at 37°C with 5% CO2 in full humidity.

Transient transfection

Cells were counted and seeded on a 24-well plate one day
before transfection. RPMI1640 culture solution with no
serum or antibiotics was diluted for transfection. miRNA-
21-5p inhibitor, mimic and negative control were designed
and synthesized by Biomic Biotechnology (USA). The
sequence of primers are as follows: miRNA-21-5p sense chain
5'-UAGCUUAUCAGACUGAGUGUA-3', antisense chain: 5'-AA
CAUCAGUCUAUCAUU-3'; miRNA-21-5p sense chain of inhibitor: 5'-UCA
ACAGUCUGUACUA-3'. The miRNA-21-5p inhibitor, the mimic
and the negative control were mixed with the Lip-
oolfectamineTM 2000 reagent, left at room temperature for
20 min, then added to the corresponding well cells, and incu-
bated at 37°C in a 5% CO2 incubator for 4–6 h.

Quantitative real-time (qRT-PCR)

Total RNA was extracted from lung cancer cells using TRI-
zol reagent (Life Technologies). The cell growth medium
was removed and 1 ml of TRIzol reagent was added to each
105 cells to lyse the cells. Then, 200 μl of chloroform was
added for every 1 ml of TRIzol and incubated for 2 min.
The sample was centrifuged at 12 000 × g at 4°C for
15 min. The aqueous phase containing RNA was collected,
and the RNA was precipitated, washed, and dissolved. The
miScript reverse transcription kit (Qiagen) was used for
reverse transcription (RT) of microRNA, and the TaqMan
universal PCR master mix was used to detect the expression
of mature miRNA-21-5p.

MTT

Cells were seeded in 96-well plates with a density of 2000
cells/well in 100 μl medium. The plate was incubated for
the appropriate period and then 10 μl of MTT (5 g/L) solution
was added to each well of the plate. Incubation continued
for 4 h, and the culture medium was carefully aspirated from
the well. Then, 150 μl DMSO was added to each well and
and the plate was shaken for 10 min to fully dissolve the
crystals. The optical density value (OD) at 450 nm was
detected using a microplate spectrophotometer.

Transwell assay

The cell culture inserts were placed into the wells of the cul-
ture plate. The polycarbonate filter was either precoated
with 50 μl of Matrigel for the invasion assay or uncoated for
the migration assay. The cells were seeded into the upper
chamber with a medium without FBS. The lower chambers
were filled with culture medium with 10% FBS. After incu-
bation for 48 h, the cells (noninvasive cells) on the upper
surface of the insert were removed with a cotton swab. The
invaded cells were stained with 0.1% crystal violet. The
number of cells passing through the membrane in five
different fields of view under a 100x light microscope were
selected, and the average value calculated.

Luciferase reporter assay

The wild-type (wt) or mutated (mut) mir-21-5p binding
sequences from SMAD7 3'UTR were cloned into the pGL3
basic vector. Cells seeded at a density of 50% were placed
into a 12-well culture plate. After 16 h of continuous culture,
the luciferase reporter gene plasmid and pRL-TK plasmid
were transfected when the cell density was about 70%.
According to the manufacturer’s protocol, Lipofectamine
2000 was used to cotransfect the cells with mir-21-5p
mimics, SMAD7 (wt) and SMAD7 (mut). The renilla lucif-
erase was used as a loading control. The cells were
harvested. Luciferase activity was analyzed using a dual luciferase reporting system (Promega). In brief, after
cotransfection of the plasmid for 48 h, the medium was dis-
carded and the cells were washed with PBS. The 12-well
plate was tilted and the remaining PBS aspirated. Then, 50 μl PLB was added to each well and shaken for 30 min on a shaker to ensure complete cell lysis. In a white opaque 96-well plate, 10 μl supernatant and 100 μl luciferase assay reagent II was added to each well, and the luciferase intensity measured. After the test had been completed, we added 100 μl Stop&Glo reagent to each well, and measured the renilla luciferase activity.

Statistical analysis
All experiments were performed independently at least three times. Statistical analysis was performed using a Student’s t-test or a one way ANOVA. All data is shown as mean ±/− SEM.

RESULTS
miR-21-5p is upregulated in lung cancer and correlated with prognosis
We performed a pan-cancer analysis of miR-21-5p expression using dbDEMC 2.0 database (https://www.biosino.org/dbDEMC/index). A heat map showed that the expression level of miR-21-5p was significantly upregulated in lung cancer tissues (LUCA) (Figure 1(a)). In addition, the expression of miR-21-5p between lung cancer and normal lung tissue was initially analyzed using the starBase v2.0 database (http://starbase.sysu.edu.cn/) (Figure 1(b)). In addition, the relationship between miR-21-5p expression and the prognosis of lung cancer patients was analyzed. The starBase database analysis results showed the survival rate of lung cancer patients with low expression of miR-21-5p was better than that of the patients with high expression of miR-21-5p (Figure 1(c)).

Function of miR-21-5p in lung cancer cells
RT-qPCR results showed that miR-21-5p expression in lung cancer cell lines (A549, H460, H1299) was significantly higher than that in normal bronchial epithelial cells (HBE) (p < 0.05). As shown in Figure 2(a), the expression level of miR-21-5p was the highest in H1299 cells. The miR-21-5p inhibitor was transfected into H1299 cells. The results of RT-qPCR showed that miR-21-5p inhibitor significantly reduced the expression level of miR-21-5p (Figure 2(b)). The MTT assay showed that miR-21-5p inhibitor inhibited the proliferation of lung cancer cells (Figure 2(c)). In addition, a transwell assay was used to compare the effect of miR-21-5p expression on the migration and invasion ability of lung cancer cells. The data showed that miR-21-5p inhibitor inhibited the invasion and migration of lung cancer cells (Figure 2(d)).

miR-21-5p targets SMAD7 in lung cancer cells
To identify miR-21-5p target genes involved in lung cancer cell behavior, we searched the Targetscan database. The
FIGURE 2  The function of miR-21-5p in lung cancer cells. (a) The expression level of miR-21-5p in lung cancer cell lines. (b) miR-21-5p expression in H1299 cells was measured after transfection with miR-21-5p inhibitor. (c) Cell proliferation was detected in H1299 cells after transfection with miR-21-5p inhibitor. (d) Transwell assay was performed in H1299 cells after transfection with miR-21-5p inhibitor. **p < 0.01, *p < 0.05, one-way ANOVA for multiple comparisons in a Student’s t-test was used in (b) and (c).

FIGURE 3  The miR-21-5p targets SMAD7 in lung cancer cells. (a) Predicted binding site between miR-21-5p and 3′-UTR of SMAD7. (b) Luciferase activity in H460 cells transfected with wild-type or mutation SMAD7 and miR-21-5p mimic or NC-miRNA. (c) SMAD7 mRNA expression was measured in H460 cells. (d) Cell proliferation was measured in H460. **p < 0.01, one-way ANOVA for multiple comparisons in (b), (c) and (d).
results showed that there was a complementary sequence with miR-21-5p on the 3'UTR end of SMAD7 mRNA (Figure 3 (a)). Renilla-luciferase reporter assay was performed to determine whether SMAD7 was a direct target of miR-21-5p. We cloned the full length 3'UTR sequence of SMAD7 mRNA containing wild-type or mutant miR-21-5p binding sequence in the dual luciferase vector. Dual-luciferase reporter assay showed that miR-21-5p mimic inhibited luciferase activity of the SMAD7 reporter gene (Figure 3(b)). In addition, we found that miR-21-5p mimic could reduce the expression level of SMAD7 mRNA, while miR-21-5p inhibitor could increase the expression level of SMAD7 mRNA (Figure 3(c)). These results suggest that SMAD7 is a direct target of miR-21-5p in lung cancer cells. The miR-21-5p mimic could
abrogate the inhibition of proliferation by ectopically expressing SMAD7 in SMAD7-overexpressed CRC cells (Figure 3(d)).

**Expression level of SMAD7 in lung cancer tissues**

We analyzed SMAD7 expression in lung cancer tissues using the starBase and GEPIA databases. Statistical analysis of SMAD7 expression showed a reduction in lung cancer tissues compared to normal lung tissues (Figure 4(a) and (b)). In addition, according to the survival curves defined by high and low expression of SMAD7 in lung cancer patients, it was found that a high expression level of SMAD7 had an important effect on the overall survival of patients. The starBase and GEPIA databases showed that high levels of SMAD7 expression had a good effect on overall survival of patients (Figures 4(c) and (d)).

**DISCUSSION**

Abnormal cell proliferation is an important feature of tumor biology, and miRNAs play an important role in regulating cell proliferation, differentiation, and apoptosis. Many studies have reported that miRNAs play a role of carcinogenic or tumor suppressor genes. Some miRNAs with carcinogenic effects can promote the occurrence of tumors by inhibiting the expression of tumor suppressor genes. Compared with normal tissues, the expression level of miRNAs in tumor tissues is significantly different from normal tissues, so these abnormally expressed miRNAs are expected to be important markers for tumor diagnosis, prognosis and treatment. Studies have shown that miRNA-21-5p expression in various tumor tissues is increased, such as in gastric, prostate, breast, pancreatic, cervical, and colon cancers. Inhibiting the expression of miRNA-21-5p can reduce the proliferation of breast cancer cell lines and promote apoptosis of breast cancer cells by indirectly downregulating the antiapoptotic factor Bcl-2. After inhibiting miRNA-21-5p expression with antisense oligonucleotides, activity of Caspase-3 and Caspase-7 increased, and apoptosis of glioblastoma cells increased significantly, which shows that miRNA-21-5p has an antiapoptotic effect and its abnormal expression inhibits expression of death-related genes causing tumorigenesis. Seike et al. treated H3255 cell lines with EGFR mutation and H441 cell lines with wild-type EGFR mutation with EGFR-TKIs, and the miRNA-21-5p expression in these two cell lines was downregulated, indicating that the activated EGFR signaling pathway has a positive regulatory effect on miRNA-21-5p. In addition, several studies have confirmed that miRNA-21-5p can regulate tumor cell proliferation, migration and sensitivity to chemotherapy by acting on the tumor suppressor genes PTEN, MARCKS, PDCD4, and Cdc25A. In summary, miRNA-21-5p is closely related to the occurrence and development of various tumors, and can be used for tumor diagnosis, treatment and prognosis, and has important clinical significance. However, due to the lack of in-depth understanding of the transcriptional regulation mechanism of miRNA-21-5p target genes, the clinical application of miRNA-21-5p is limited. It can be expected that with further research, mir_21 will have broad application prospects in the diagnosis and treatment of tumors.

It has been previously reported that SMAD7 expression is reduced in cisplatin-resistant lung cancer A549 cell lines. In this study, miRNA-21-5p mimics and inhibitors were transfected into H460 cells. The results of the qRT-PCR assay showed that SMAD7 expression increased significantly in cells after transfecting miRNA-21-5p inhibitor; the expression of SMAD7 in cells was significantly reduced after transfection with miRNA-21-5p mimic.

In the MTT experiment, the cells transfected with SMAD7 had a significantly decreased OD 450 nm value compared to the scramble transfected cells. At the same time, there was no difference in the cells cotransfected with miRNA-21-5p mimic and SMAD7 with scramble. The above results suggest that miRNA-21-5p mimic may promote the proliferation of H460 cells by inhibiting SMAD7 expression.

In summary, miRNA-21-5p affects the migration and proliferation of lung cancer H1299 cells through targeted regulation of SMAD7 expression in lung cancer cells, suggesting that miRNA-21-5p is a potential target, which can be used for the intervention and prevention of the lung cancer process.

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**CONFLICT OF INTEREST**

No conflict of interest is reported.

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