Antitumor effect of miR-27b-3p on lung cancer cells via targeting Fzd7

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Abstract. – OBJECTIVE: Lung cancer is the most common malignancy with the highest mortality rate among cancers. microRNAs (miRNAs) have been confirmed to be closely related to the physiological disorder, especially the tumor process. This study aimed to investigate the effect of miR-27b-3p on lung tumor cells.

MATERIALS AND METHODS: The expressions of miR-27b-3p in lung tumors and adjacent non-tumors lung tissues were compared. We test the bonding effect of miR-27b-3p on the Fzd7 promoter, and miR-27b-3p effects on the Fzd7 expression in both NCI-H446 and A549 cells. Then, effects of miR-27b-3p and Fzd7 on these cells viability, survival and apoptosis were detected, respectively. In addition, the possible mechanism of miR-27b-3p affected these cells apoptosis was explored by analyzing the expression of apoptosis-related factors.

RESULTS: We found that miR-27b-3p was low expressed in lung tumors compared to adjacent non-tumorous lung tissues. miR-27b-3p directly targeted Fzd7 promoter and negatively regulated Fzd7 expression. Fzd7 promoted NCI-H446 and A549 cells viability and survival, inhibited cells apoptosis. However, miR-27b-3p effects on these cells were quite the opposite to Fzd7. The expressions of apoptosis-related factors were associated positively with miR-27b-3p and showed a negative correlation with Fzd7 expression.

CONCLUSIONS: The miR-27b-3p was lowly expressed in lung cancer tissues, and played the role of a tumor suppressor. It could promote cell apoptosis and suppress cancer cells viability and survival via down-regulating Fzd7. It suggested that miR-27b-3p might be a potential target for the prophylaxis and treatment of lung cancer.

Key Words: Tumor suppressor, microRNA27b-3p, Fzd7, Cell viability, Apoptosis.

Introduction

In recent decades, the incidence and mortality of lung cancer showed an upward trend in many countries and ranked as first among malignant neoplasms. The lung cancer has become a serious public health problem. The survival rate of patients with advanced lung cancer after been resected was only 8-12%, but the survival rate could reach 80% within 5 years if been early detected and resected. Therefore, early diagnosis and timely treatment are very important for patients with lung cancer. The studies of molecular mechanism and therapy resistance of lung cancer will help us to further understand its pathogenesis and progression, and offer more targets for effective therapy. Determining the genetic and epigenetic changes for cancers including lung cancer has great research evolve. The researches included genome-wide mRNA expression profiles, genome-wide DNA copy number variation changes, genome-wide DNA methylation changes, microRNA (miRNA) changes and mass spectroscopy proteomics analyses.

The miRNAs are small non-coding RNAs that are essential for maintaining cellular homeostasis and regulating cellular progress. The miRNAs regulate post transcription of target genes and dysregulation of miRNAs expressions has been proved to be associated with aberrant gene expression leading to pathological conditions. Due to the important role of miRNA in a wide array of biological processes, some miRNAs have been considered as biomarkers for disease diagnosis, treatment, and prognosis. It has also been demonstrated that miRNAs were particularly important in lung homeostasis and diseases including lung cancer. The unique growth suppressive of miRNAs in lung cancer has been reported, such as miR-145 and miR-142-5p. Some dysregulated miRNAs in lung cancer were also identified by bioinformatics analyses combined microarray or gene expression analysis of lung cancer, such as miR-339-5p, miR-200e, etc.

Recently, researches showed that miR-27b-3p, a potential biomarker, was participated in many cancers via regulating the expression of specific
target gene. For instance, miR-27b-3p suppressed cell proliferation in gastric cancer\textsuperscript{15}. It could regulate PPARγ in oocyte maturation\textsuperscript{16}, and targeted Bmal1 at the post-transcriptional level in liver\textsuperscript{17}. The miR-27b-3p exerted tumor-suppressive in cancers that suggested its therapeutic application in cancers\textsuperscript{18}. For lung cancer, even though miRNAs were reported to be associated with cancer development, the role of miR-27b-3p in lung cancer has not been comprehensively reported.

Thus, in this study, we assessed the expression of miR-27b-3p in lung tumor tissues compared with adjacent non-tumor tissue. Furthermore, the target gene of miR-27b-3p was verified and regulation effect of miR-27b-3p on the frizzled family encode 7-transmembrane domain protein (Fzd7) in NCI-H446, and A549 cells were also verified. Then, this study investigated the effect of miR-27b-3p on NCI-H446 and A549 cells viability, survival, and apoptosis as well as the possible mechanism. We hope this research would provide a potential target for prediction and treatment of lung cancer.

Materials and Methods

Patients and Cell Culture

The paired tumorous and adjacent non-tumorous human lung tissues were obtained from 15 patients underwent resection in the local hospital. All patients with lung cancer were diagnosed pathologically according to the criteria of the American Joint Committee on Cancer. The study was approved by the local Institutional Ethics Committee, and the written informed consents were obtained from all patients before specimen collection.

Human small cell lung cancer cell line NCI-H446 and human lung carcinoma cell line A549 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and penicillin (100 U/ml, Sigma-Aldrich, St. Louis, MO, USA). Cultures were maintained under an atmosphere containing 5% CO\textsubscript{2} at 37°C\textsuperscript{19}.

Cell Transfection

The recombinant vector pcDNA\textsuperscript{TM} 3.1(−)–Fzd7 with full-length of Fzd7 coding sequence or its negative control (Thermo Fisher, Scientific, Beijing, China) were transfected into NCI-H446 and A549 cells, respectively. The siRNAs directly against Fzd7 (sc-39990) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fzd7 Antibody (sc-31060, Santa Cruz Biotechnology, Santa Cruz, CA, USA) which was confirmed as nonsilencing double-stranded RNA, was used as a control for siRNA experiments. For miRNA transfection, cells were transfected with miR-27b-3p mimic, inhibitor, and negative miRNA control (Gene Pharma, Shanghai, China), respectively. All transfection were performed by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), according to the manual. At 48 h after transfection, the cells were collected for further investigation.

RNA Extraction and Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted by the Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and was reverse-transcribed by using Transcriptor First Strand cDNA Synthesis Kit (Roche Life Science, Shanghai, China), according to the manufacturer’s instructions. The qPCR was performed with SYBR Premix Ex Taq\textsuperscript{TM} (TaKaRa, Biotech Co., Ltd, Dalian, China) and run in a master cycle gradient (Eppendorf, Hamburg, Germany). U6 and GAPDH were served as internal controls for data normalization. All primers were synthesized by GenePharma (Shanghai, China).

Dual Luciferase Activity Assay

The potential target gene for miR-27b-3p was predicted by Targetscan, and Fzd7 was proposed. The Fzd7 3’UTR wild type, which contains the putative binding site of the miR-27b-3p (the sequences were shown in Figure 2A) or mutant, were cloned into the pGL2-basic vector (NEB, Beijing, China). Then, 100 ng of each recombinant construct was co-transfected with 50 nM miRNA mimic, inhibitor or scramble control into cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in the 24-well plate. After been screened, the cell extracts were harvested, and 48h after transfection the Firefly and Renilla luciferase activity were measured by using the Dual-Luciferase reporter system (Promega, Madison, WI, USA) according to the manufacturer’s instructions\textsuperscript{16}.

Cell Viability Assay

Cell viability was assessed using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay according to the standard methods. In brief, transfected cells were
seeded into 96-well plates at $1 \times 10^4$ cells/well. Cells viability was measured at 24 h, 48 h and 72 h after transfection. MTT (20 µl/well, Sigma-Aldrich, St. Louis, MO, USA) were added into each well and incubated for another 4 h at 37°C. Then cells were lysed in 150 µl dimethylsulfoxide (DMSO, Sigma, St. Louis, MO, USA) and UV-visible absorbance was read at 490 nm using 680 microplate enzyme-linked immunosorbent assay (ELISA) Reader (Bio-Rad Laboratories, Hercules, CA, USA).

**Cell Survival Assay**

To obtain quantitative information about the capability of cells, cells were cultured to 70% confluence and counted as usual for the control group. Treated cells ($1 \times 10^3$) were re-seeded into a new 60 mm culture dish (in duplicate) and incubated for 9 days (Fresh media was added at day 5). At day 9, the culture medium was carefully removed and added 1 ml Clonogenic Reagent (50% Ethanol, 0.25% 1, 9-dimethyl-methylene blue, Sigma-Aldrich, St. Louis, MO, USA), then cells were placed at room temperature for 45 min. At the end, cells were washed with PBS, and the blue colonies were counted. The data were expressed as percent survival relative to the control.

**Detection of Cell Early Apoptosis**

Cell apoptosis was assessed by using Annexin V-R-PE cell apoptosis detection kit (Southern Biotech, MI, USA) according to the instructions. The transfected cells were collected and suspended in 200 µl binging buffer, been incubated at room temperature for 30 min in the dark. Flow cytometry (BD Biosciences, San Jose, CA, USA) was used to detect the percentage of early apoptosis.

**Western Blotting**

The cellular proteins were extracted by lysis buffer (Beyotime Biotechnology, Nantong, Jiangsu, China) after transfection. The samples were separated on an 8% SDS-denatured polyacrylamide gel and then transferred onto nitrocellulose membrane. The membranes were incubated at 4°C overnight with primary antibodies (all 1:1000): Fzd7 (sc31001); Caspase-3 (sc271759); B-cell lymphoma (Bcl-2, sc509); Bcl-2-associated X (Bax, sc20288); c-Jun N-terminal kinase (JNK, sc7345); phosphorylated JNK (p-JNK, sc6254); GAPDH (sc365062) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and p51 (ab2505, Abcam, Eugene, OR, USA). The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Protein immunoreactivity of bands was developed by enhanced chemiluminescence reagent (GE, Healthcare, Little Chalfont, UK).

**Statistical Analysis**

Data were presented as mean ± standard deviation (SD), which were representative of at least three independent experiments and analyzed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). The two-tailed Student’s $t$-test was used to evaluate the significance of the differences between two groups; one-way analysis of variance (ANOVA) was used to evaluate the significance of the differences in mean values within and between multiple groups. The $p$ values < 0.05 were considered significant.

**Results**

miR-27b-3p was Low Expressed in Lung Tumors

Comparing the expression levels of miR-27b-3p between tumorous and adjacent non-tumorous human lung tissues, we found that miR-27b-3p was low expressed in tumors compared to adjacent non-tumor tissues ($p < 0.01$, Figure 1). It suggested that the miR-27b-3p expression level was downregulated in lung tumors.

![Figure 1](https://example.com/figure1.png)
miR-27b-3p Negatively Regulated the Expression of Fzd7 via Targeting its Promoter

According to the prediction of Targetscan, Fzd7 3’UTR region showed potential miR-27b-3p sequence alignment (Figure 2A). We verified the targeting effect of miR-27b-3p on Fzd7 3’UTR in both NCL-H446 (Figure 2B and 2C) and A549 cells (Figure 2D and 2E). The luciferase reporter assay results suggested that in miR-27b-3p mimic treated groups, fluorescence signal intensity was significantly decreased, suggesting the effective binding effect of miR-27b-3p to Fzd7 3’UTR ($p < 0.01$). While it showed the opposite trend in miR-27b-3p inhibitor groups ($p < 0.01$). Meanwhile, miR-27b-3p did not show obvious binding effect with Fzd7 3’UTR mutant. These results suggested that miR-27b-3p could efficiently binding to the promoter of Fzd7. Fzd7 was the target of miR-27b-3p in NCI-H446 and A549 cells.

In Figure 3A and 3B, the results showed that transfection of miR-27b-3p mimic significantly reduced Fzd7 expression level ($p < 0.01$), and Fzd7 mRNA expression levels were increased after miR-27b-3p inhibitor transfection in NCI-H446 and A549 cells.

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\text{miR-27b-3p, microRNA-27b-3p; Fzd7, the frizzled family encode 7-transmembrane domain protein; 3’-UTR, 3’-untranslated regions; ns, no significant influence; } **, p < 0.01
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Figure 2. Fzd7 was a direct target of miR-27b-3p. (A) Fzd7 3’UTR region showing potential miR-27b-3p alignment was shown. (B and C) The targeting effect of miR-27b-3p to Fzd7 in NCI-H446 cells was detected by luciferase reporter assay. (D and E) Luciferase reporter assay showed the effect of miR-27b-3p on Fzd7 in A549 cells. U6 acted as an internal control. miR-27b-3p, microRNA-27b-3p; Fzd7, the frizzled family encode 7-transmembrane domain protein; 3’-UTR, 3’-untranslated regions; ns, no significant influence; **, $p < 0.01$
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To further understand the role of Fzd7 in lung cancer cells, we overexpressed or inhibited the expression of Fzd7 in both NCI-H446 and A549 cells. The results in Figure 4B showed that the protein expression levels of Fzd7 were increased after been transfected with miR-27b-3p inhibitor. These results suggested that miR-27b-3p could effectively negatively regulate the expression of Fzd7 in NCI-H446 and A549 cells via targeting 3’UTR of Fzd7.

**Fzd7 Enhanced Cell Viability and Survival while Inhibited Apoptosis of NCI-H446 and A549 Cells**

The results in Figure 4A showed that Fzd7 mRNA level was higher in lung tumor than that in adjacent non-tumors tissues (p < 0.01). To further understand the role of Fzd7 in lung cancer cells, we overexpressed or inhibited the expression of Fzd7 in both NCI-H446 and A549 cells. The results in Figure 4B showed that the protein expression levels of Fzd7 were increased by overexpressing Fzd7, and Fzd7 silence effectively decreased the protein expression of Fzd7.

The effect of Fzd7 overexpression or silence on NCI-H446 and A549 cells viability, survival and apoptosis were also been explored. The results suggested that overexpression of Fzd7 significantly enhanced cell viability and survival of...
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On the contrary, NCI-H446 and A549 cell viability and survival were inhibited after being treated with Fzd7 siRNA (p < 0.05 or p < 0.01) (Figure 4C-4F). However, Figure 4G and 4H showed that
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Figure 5. Overexpression of miR-27b-3p suppressed cells proliferation while increased apoptosis of NCI-H446 and A549 cells. The miR-27b-3p mimic, inhibitor or scramble control were transfected into NCI-H446 and A549 cells, respectively. (A and B) The transfection efficiency in NCI-H446 and A549 cells was tested by qPCR. (C and D) The miR-transfected NCI-H446 and A549 cells were collected and cultured for 24-72 h, and then cell viability was determined by MTT assay. (E and F) The proliferation of miR-transfected NCI-H446 and A549 cells were measured by colony formation assay. (G and H) The apoptotic cells rate of NCI-H446 and A549 cells detected by flow cytometry.

miR-27b-3p, microRNA-27b-3p; qPCR, quantitative polymerase chain reaction; MTT, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide; NC, negative control for miR-27b-3p inhibitor; *, p < 0.05; **, p < 0.01.

cell apoptosis was promoted by Fzd7 overexpression and inhibited by Fzd7 silence (p < 0.05 or p < 0.01) in both NCI-H446 and A549 cells. Therefore, Fzd7 was up-regulated in lung tumor tissues.
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and high expression of Fzd7 enhanced cell viability and survival, while inhibited apoptosis of NCI-H446 and A549 cells. These results suggested that Fzd7 might be a risk factor for lung cancer cells.

**miR-27b-3p Inhibited NCI-H446 and A549 Cells Viability, Survival and Promoted Apoptosis**

At first, we verified the transfection efficiency of miR-27b-3p mimic and inhibitor. As shown in Figure 5A and 5B, in both NCI-H446 and A549 cells, the mRNA levels of miR-27b-3p were increased by miR-27b-3p mimic transfection and decreased after miR-27b-3p inhibitor transfection ($p < 0.01$). Then the MTT assay results (Figure 5C and 5D) suggested that both NCI-H446 and A549 cells viability were reduced after miR-27b-3p mimic transfection from 24h to 72 h, while kept at a higher level than the control group after miR-27b-3p inhibitor transfection with increasing trend over time ($p < 0.05$ or $p < 0.01$). The results of these two kinds of cells survival rates assay in Figure 5E and 5F, suggested that miR-27b-3p mimic transfection inhibited cell survival ($p < 0.05$) and transfection of miR-27b-3p inhibitor increased cell survival rate ($p < 0.01$). As shown in Figure 5G and 5H, NCI-H446 and A549 cells apoptosis was significantly promoted by miR-27b-3p mimic transfection ($p < 0.05$) and were inhibited by miR-27b-3p inhibitor ($p < 0.01$). We inferred that miR-27b-3p might have an antitumor effect on lung tumors.

**Potential Regulation Mechanism of miR-27b-3p on Lung Cancer Cells Apoptosis**

We examined the effect of miR-27b-3p and Fzd7 on the expression of apoptosis-related factor Caspase-3, Bcl-2, Bax, p51 and JNK by western blot. The results in Figure 6 showed that overexpression of miR-27b-3p could induce the increasing expression of Caspase-3, Bax, p-p51 and p-JNK, while reduced the expression of Bcl-2. After been treated with miR-27b-3p inhibitor, the completely opposite change trend of these proteins expressing was found compare with miR-27b-3p mimic transfection groups. In the other set of experiments, the abnormal expression of Fzd7 affect the expression of these factors and showed opposite results compare with miR-27b-3p transfection. Based on the results obtained, we inferred that miR-27b-3p, that been associated with Fzd7 expression, regulated the apoptosis-related factors expression to affect the bioactivity of lung cancer cells.

**Discussion**

In this study, we detected miR-27b-3p was downregulated in lung tumors compared with adjacent non-tumors tissues, suggesting that expression of miR-27b-3p was associated with lung cancer. Lung cancer is the main contributor of cancer mortality worldwide$^{21}$. In recent decades, with the rising incidence of lung cancer, mo-
modern medical methods of effective pre-diagnosis and treatment of lung cancer are more needed\textsuperscript{23}. The miRNAs have attracted much attention in recent years. It has been regarded as promising biomarkers, because of their extensive regulation in cell processes and the close relationship between its abnormal expression and disease\textsuperscript{24}. Some miRNAs were deregulated in lung cancers\textsuperscript{13,15}. Some suppressive or oncogenic miRNAs have been found in lung carcinogenesis. For example, miR-34c, miR-145, and miR142-5p were prominently repressed in lung cancer\textsuperscript{12,24,25}.

The aberrant miRNA expression is usually a hallmark of malignancies. As miR-27b-3p was down regulated in lung tumors, we explored the role of miR-27b-3p in lung cancer cells NCI-H446 and A549 and the underlying mechanism. By prediction, Fzd7 might be the target gene of miR27b-3p in lung cancer cells. We observed that miR-27b-3p could affect the expression of Fzd7 via binding to specific binding sites in the 3’UTR of Fzd7. Fzd7 as the Wnt signaling proteins receptor could mediate both canonical and non-canonical Wnt signals\textsuperscript{26,27}. It has been proved to affect cell proliferation in breast cancer\textsuperscript{28}. There were also studies showed that the expression of Fzd7 was lower in esophageal cancer cells and was up-regulated in human primary gastric cancer\textsuperscript{29}. And also Fzd7 might drive aggressiveness of Stem-A ovarian cancer via regulation of non-canonical Wnt/PCP pathway\textsuperscript{30}. In this study, the results suggested that the mRNA level of Fzd7 in lung tumor tissues was higher than adjacent non-tumor tissues. Overexpression of Fzd7 could promote cell viability, survival and inhibit apoptosis in both NCI-H446 and A549 cells. All these results suggested that Fzd7 might be a risk factor for lung cancer cells.

miRNAs could regulate their target genes which based on pairing to the target mRNA, resulting in mRNA degradation or the inhibition of translation\textsuperscript{31}. In our further study, we found that miR-27b-3p overexpression could inhibit the proliferation of lung cancer cells and promote apoptosis. While inhibition of miR-27b-3p expression could promote lung cancer cells proliferation and reduce apoptosis. In combination with the regulation of miR-27b-3p on Fzd7 expression, we inferred that the effect of miR-27b-3p on the biological activity of lung cancer cells might be done by binding to Fzd7 promoter and thus regulate the expression of Fzd7. Then miR-27b-3p could be speculated as an effective biomarker for the lung tumor.

miRNAs play important role in the lung cancer development and metastasis via inhibiting the expression of certain proteins\textsuperscript{32}. Meanwhile, miRNAs are also the promising anti-tumor agents\textsuperscript{33,34}. In our study, the expression of miR-27b-3p was positively correlated with the expression of apoptotic-related factors. For instance, Caspase-3, the executioner of cell apoptosis, is able to lead programmed death of cells\textsuperscript{35}. Bax, an apoptosis-promoting gene which overexpression could antagonize the protective effect of Bcl-2 on cells\textsuperscript{36}. And JNK, which belongs to the mitogen-activated protein kinase family, plays an important role in many physiological and pathological processes, including cell cycle, reproduction, apoptosis and cellular stress\textsuperscript{37}. On the contrary, the effect of Fzd7 as a miR-27b-3p target on these proteins was opposite. It was no doubt that miR-27b-3p might be a critical factor in lung cancer. This negative regulatory effect of miR-27b-3p on lung cancer cells involving the regulating of Fzd7 expression and also the apoptosis-related factors expression. We suggested that miR-27b-3p might be a tumor suppressor.

Conclusions

We showed that miR-27b-3p could affect Fzd7 expression via binding to the Fzd7 promoter. And Fzd7 might be a risk factor for lung cancer. The miR-27b-3p might be a tumor suppressor as its anti-tumor effect on Luger cancer cells that could suppress cell viability and survival, promote cancer cells apoptosis. We conjectured that miR-27b-3p might be a potential biomarker for lung cancer therapy. It is hoped that the further and accurate study about the exact mechanism of miR-27b-3p effect could contribute to the diagnosis and treatment of lung cancer.

Conflict of interest

The authors declare no conflicts of interest.

References


