

MiR-140-5p Targeted YES1 Inhibits Proliferation of Lung Cancer Cell A549

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Abstract

Objective: To test the expression difference of mir-140-5p between lung cancer tissues and lung tissues, and to observe whether miR-140-5p can regulate the expression of YES1, a proto-oncogene, so as to affect cell proliferation and apoptosis. This study provides research direction for the accumulation of data for targeted treatment of lung adenocarcinoma.

Methods: The clinical information related to 45 NSCLC cancer cases was collected, and the expression differences of MiR-140-5p between tumor and para-carcinoma tissues was tested by qRT-PCR. After transfection for 48h, A549 cells were divided into a MiR-140-5p mimic group and a control group. Apoptosis of cells in each group was detected by flow cytometry. According to the predicted action sites of MiR-140-5p in YES1 from bioinformatics, whole-gene synthesis of 3'utr sequences containing target sites and mutant 3'utr sequences was performed, and dual-fluorescent vector detection was performed to investigate whether YES1 was the target gene of MiR-140-5p.

Results: By comparing the expression of MiR-140-5p in tumor and para-carcinoma tissues by fluorescence PCR, it was found that the expression of MiR-140-5p in para-carcinoma tissues was much higher than that in tumor tissues ($P < 0.05$). Flow cytometry was used to detect the apoptosis of lung adenocarcinoma cells transfected with MiR-140-5p, and it was confirmed that MiR-140-5p could promote the apoptosis of lung cancer cells. According to the predicted action sites of MiR-140-5p in YES1 from bioinformatics, the 3'utr sequence containing the target sites and the mutant 3'utr sequence were synthesized, and the fluorescence reporter vector detection confirmed that YES1 was the target gene of MiR-140-5p.

Keywords

MiR-140-5p; Lung adenocarcinoma. A549 cell line; Cell apoptosis; Micrnas. YES1 genes

With the development of the world health level, the part of the lung cancer incidence of a disease of developed countries began to slow down, but because of its relatively high malignant degree, illness development is rapid, treatment difficulty, morbidity and mortality is still the cause of death in the world the first place, some developing countries, including China, the mortality of lung cancer increased significantly, including non-small cell lung cancer accounts for 80% of total lung cancer [1], and adenocarcinoma is the most common pathological types of small cell lung cancer. Although the research on the treatment of lung cancer has made great progress at the present stage, there is still no good monitoring means to monitor its occurrence and development. In general, the 5-year survival rate of lung cancer patients has not been greatly improved. At present, more and more experts and scholars have turned their attention to the study of the mechanism of action of miRNA, and have made corresponding achievements in the diagnosis, treatment, monitoring and prognosis of tumor treated with miRNA. These experimental studies have pointed out a new direction for the development of lung cancer monitoring and treatment.

MiRNA is a general term for a group of non-coding RNA molecules, mostly small RNA with 17 to 25 undisputed nuclei. It regulates gene expression at the level of mRNA transcription and translation, and is mostly shown to inhibit the activity of corresponding target genes of mRNA. MiRNA is expressed in most types of cancer and lung cancer [2]. As a unique class of post-transcriptional regulators, miRNAs can participate in most biological processes, such as cell apoptosis, differentiation, proliferation, and immune response. In addition, miRNAs can exist as tumor suppressors or carcinogenic factors, and play an important role in disease progression, including cancer [3].

Existing studies [4] have shown that the expression of miRNA-140-5p in human peripheral circulating plasma is helpful in monitoring the development and changes of pulmonary malignant tumors and whether distant metastasis occurs. Consider time and economic factors, this research selected A549 cell experiments, discusses from the miR-140-5p in normal lung cells and lung adenocarcinoma whether there was a significant difference in cancer cells, as well as proto-oncogene YES genes can be miR-140-5p control and other aspects, hope the miR-140-5p can help to provide some feasible basis lung cancer diagnosis and treatment.

Chapter One Materials and methods

1.1. Experimental materials

Human lung adenocarcinoma A549 cell line was preserved by the central laboratory of Qinhuangdao first hospital. Double luciferase reporter gene detection kit was purchased from Beijing huaxia ocean technology co., LTD. Australian fetal bovine serum, DMEM high glucose medium, 0.25% trypsin, 0.02% EDTA solution, DMSO and PBS buffer were purchased from ThermoFisher Scientific. Bought PrimeScriptOR micromnas RT-PCR Kit from dalian Takara co., LTD.

1.2. Patients and tissues

The clinical information related to 45 NSCLC cancer cases was collected from July 2016 to July 2017 at the Qinhuangdao First People's Hospital (Qinhuangdao, China). A total of 45 patients were recruited in the current study (age, 31-75 years; mean age, 53±21.8 years; gender distribution (male : female), 26:19). All patients were diagnosed with stage III or II. Tumor and para-carcinoma tissues were removed and immediately placed in liquid nitrogen or 10% formalin. All samples were confirmed by pathological examination, and no radiotherapy or chemotherapy was performed prior to surgery. The present study was approved by the Ethics Committee of Qinhuangdao First People's Hospital (approval no. QDPH160503) and written informed consent was obtained from each participant prior to the study.

1.3. Experimental methods

1.3.1. Real-time quantitative PCR was used to analyze the expression difference of mir-140-5p in 45 pairs of lung cancer tissues and adjacent normal tissues

(1). MiRNAs from tissue samples were collected:

Sample tissues stored in liquid nitrogen were selected for grinding treatment, and liquid nitrogen was added to freeze at any time until the tissues were ground into extremely fine powder. An appropriate amount of Trizol(1ml for every 100mg of tissue, or slightly more lysate) was added to the mortar. Careful grinding allowed the tissues and lysate to fully mix and interact. Percussion homogenate until transparent carefully, and then transferred to new EP 1.5 ml tube, each 1 ml Trizol added 200 μ l precooling chloroform, fully blending, upside down on the ice placed 5 min, cracking liquid in 4 °C for 15 min, centrifuge speed is set to 12000 RPM, centrifugal after careful drain on clear liquid, and go to the new EP in the tube. Join the precooling of anhydrous ethanol 1 ml (1:5), blending, placed in - 80 °C above 30 min in the fridge. Changing the centrifuges to 4 °C 12000 RPM, 30 min, the centrifugal sedimentation RNA. Then joined the precooling 1 ml of 75% ethanol, and precipitation will bounce gently, then in 4 °C environment centrifuge for 10 min, RPM to 12000 RPM. The supernatant was absorbed and discarded. The supernatant was allowed to stand at room temperature for 5min, and 20 l DEPC was added to each tube to dissolve for 15 min. Dissolve RNA liquid blending fully, in addition to return 1 (including 1 used to quantitative detection, to save the rest of the RNA solution to - 80 °C in the fridge.

(2). Preparation of cDNA by RT:

U6 - RT: 5 'GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATGGAAC3'

MiR-140-5p -RT: 5 'CGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACCTACCAT3'

Reflected in the following reaction system:

Total RNA 3.0 μ L

RT primer (100 pmol/ μ L) 1.0 μ L

DEPC 9.5 μ L

Total: 13.5 μ L

After 65 °C for 10 min, turn 25 °C for 5 min, and then the ice bath for 2 min. Then add the following reagents:

5×buffer 4.0 μL
 dNTPs (10 mM) 1.0 μL
 RNasin (40 U/μL) 0.5 μL
 M-MLV (200 U/μL) 1.0 μL
 Total: 20 μL

42 °C after 30 min, adjust the 70 °C inactivated 10 min, product stored in - 20 °C. The original data were processed and analyzed, and the scatter plot was drawn.

1.3.2. Cell culture

In DMEM containing 10% fetal bovine serum sugar medium culture A549 cells, culture conditions for 37 °C and 5% CO₂, saturated humidity, 48 h after training batches.

1.3.3. Passage

Observe the growth of A549 cells every day. Passage was conducted when the cells grew to 90% confluence rate. The third to fifth generation cells were selected for the experiment

1.3.4. Cell apoptosis was detected by flow cytometry

(1). The cells were divided into three groups:

- A. A549 + mimics NC
- B. A549 + mimics miR-140-5p
- C. The NC group

(2). Cell transfection: After 80% fusing, the Lipofectamine 3000 was transfected with a concentration of 60 nmol/L for MiR-140-5p mimic and control.

(3) Apoptosis detection by flow cytometry: cells transfected for 48h (about $1 \times 10^5 \sim 5 \times 10^5$ cells in each group) were collected. After washing with PBS, 10 μL Annexin V-FITC reagents were added. The cells were isolated from light at room temperature for 15 min, centrifuged at 1500 r/min for 5 min, and the supernatant was discarded. The apoptosis of cells in each group was detected by flow cytometry with precooled PBS resuspension and the addition of 10 mg/L PI

1.3.3 The dual luciferase vector assay was used to detect whether mir-140-5p ACTS on YES1 gene:

(1). Target gene prediction: the target genes of MiR-140-5p were predicted by retrieval of targetscan, pictar and MICROCOSM databases. The intersection of target genes predicted by the three databases was confirmed that multiple regions of the 3' utr of YES1 mrna could be completely complementary to the seed sequences of MiR-140-5p, and the possible target genes of MiR-140-5p were considered.

(2). Carrier synthesis: according to the target gene prediction database role in Mir-140-5p YES1 gene loci, will include targeted the 3' UTR sequences and the mutation type 3' UTR sequence for the gene synthesis, PCR amplification, PCR amplification procedure: 94°C for 3 min. 94°C for 15 s; 62°C for 40 s; 40 cycles. See the following figure for specific action sites and sequence design:

Position 143-149 of YES1 3' UTR	5' ... GAUAUGGUUGCACAAAACCACUU...
hsa-miR-140-5p	3' GAUGGUAUCCCAUUUUGGUGAC
Mutant position of YES1 3' UTR	5' ... GAUAUGGUUGCACAACCAACACU...

Figure 1: PCR action sites and sequence design

(3). Target genes fluorescence detection: will be at logarithmic phase of A549 cells inoculated in 35 mm petri dishes, at 37°C, saturated humidity 5% CO₂ incubator culture for the night. Cells such as convergence rate is 70% with Luciferase reporter gene plasmid and lacz expression plasmid transfection cells, the team set up YES1 wild type transfection group, the mutant gene transfection group and wild type controls, mutation model control group, 100 nmol/L end transfection concentration, operation by Lipofectamine transfection Reagent manual for 2000, 24-36h after transfection, absorb nutrient solution, washing with precooling PBS buffer cells. In each petri dish to join 350 μl precooling harvest buffer, in 4°C or placed 10 min cracking cells on the ice. A sufficient amount of 1.5ml microcentrifuge tube was prepared. ATP buffer and luciferin buffer were mixed in a ratio of 1:3.6 to form the reaction solution and then separated into separate containers, 100 μl per tube. The cell lysate of equal volume (100 μl) was taken to the centrifuge tube and mixed rapidly. The experiment was repeated for 3 times. Finally, the fluorescence values measured in each group were summarized and analyzed. The experimental groups are shown in the figure below:

	Wild type YES1 3'utr gene test group	YES1 3'UTR gene mutant test group
MiR-140-5p mimics	Wild-type transfection group	Mutant transfection group
Control mimics	Wild type control group	Mutant control group

Figure 2: Experimental grouping of fluorescent reporter vectors

Chapter Two Experimental results

2.1. MiR-140-5p expression was significantly decreased in tumor tissues

The results of fluorescence quantitative qRT-PCR showed that the expression level of MiR-140-5p in tumor tissues was lower than that in para-carcinoma tissues, and the low expression level of MiR-140-5p in lung adenocarcinoma group might be closely related to the occurrence and development of lung cancer.

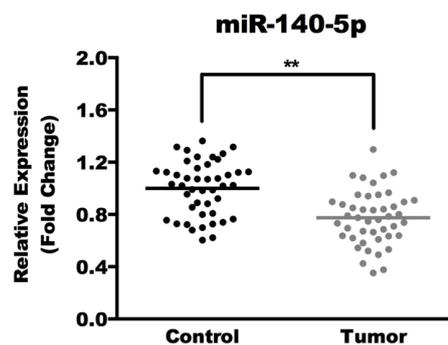


Figure 3: MiR-140-5p content between tumor and para-carcinoma tissues

2.2. MiR-140-5p can promote apoptosis

Flow cytometry assay showed that after 48 h transfection with MiR-140-5p mimic, the apoptosis rate of A549 cells increased significantly compared with the control group, while the apoptosis rate of NC group and blank control group was not significantly different, considering that MiR-140-5p might be related to apoptosis of A549 cells.

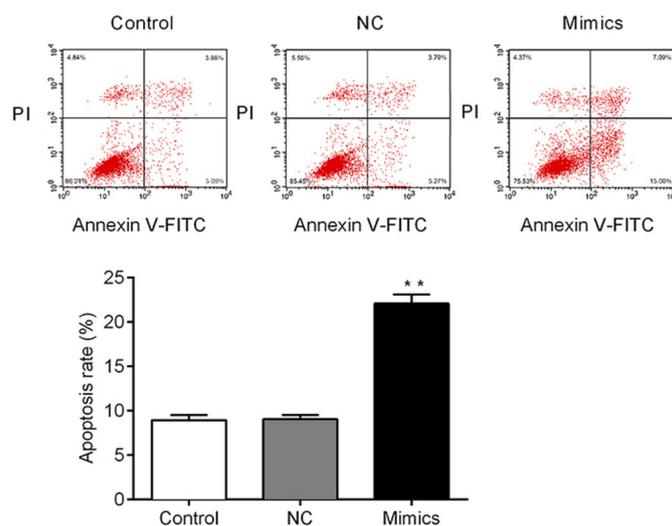


Figure 4: Apoptosis rates in control group, NC group and MiR-140-5p mimic group

2.3. MiR-140-5p can target YES1 gene

Fluorescent reporter vector assay was used to verify that MiR-140-5p can indeed act on the target gene YES1. In the fluorescence reporter vector experiment, YES1 wild-type transfection group, mutant transfection group, wild-type control group and mutant control group were set respectively, and the expression level of fluorescent protein was detected. The results showed that the expression level of fluorescent protein in YES1 3'utr wild-type control group was significantly higher than that in YES1 3'utr wild-type transfection group. The expression level of fluorescent protein in YES1 3'UTR mutant control group was similar to that in YES1 3'UTR mutant transfection group. Meanwhile YES1 3'UTR with wild type controls YES1 3'UTR mutant fluorescent protein expression level in the control group were similar, show that mir - 140-5 p can with YES1 gene mrna 3'UTR specificity, exert its micrnas inhibition, greatly reduce the fluorescent protein expression level, and when YES1 gene mrna 3'UTR mutations, mir - 140-5 p lost its binding site, can't combine with YES1 mrna specificity, cannot play its inhibitory function of biology, The results showed that MiR-140-5p could target YES1 gene.

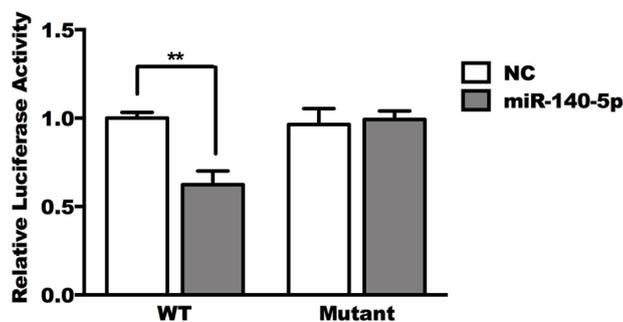


Figure 5: Fluorescence protein expression levels after YES1 wild-type and mutant co-transfection with MiR-140-5p, respectively

Chapter Three Discussion

The treatment of lung cancer progress by leaps and bounds, many experts and scholars both at home and abroad work together, the treatment of lung cancer has been transformed from traditional medicine to accurate medicine, based on the type and molecular biology gene characteristics of patients with molecular targeted therapy and immunotherapy gradually popularization, make option for patients with more accurate, more targeted, medicine has become a lung cancer is now the main direction of research accurately.

MiRNA is a small molecule RNA, which can form rna-induced silencing complex together with other proteins. MiRNA and the 3'utr of mrna recognize each other. If completely or partially complementary, the rna-induced silencing complex can be activated to degrade or inhibit the translation of mrna, thereby affecting the transcription and translation of the target genes of mrna and regulating the expression of target genes. The 5' -terminal seed sequence of miRNA can form incomplete complementation with the 3' -utr of multiple mRNAs, so one miRNA may bind to hundreds of mRNAs and regulate the expression of hundreds of target genes. At the same time, MiR-140 is different from most miRNAs, and there are two forms of MiR-140-5p and MiR-140-3p, the expression levels of which are basically the same, but there is no significant difference, and the underlying molecular mechanism is still unclear.

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has-miR-140-5P 3' GAUGGUAUCCCAUUUUGGUGAC
has-miR-140-3P 3' CUACCUAUGGGUAAAACCACUG

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Figure 6: Gene sequences of MiR-140-5p and MiR-140-3p

In recent years, many researchers at home and abroad have conducted a series of studies on the correlation between MiR-140 and lung malignant tumors. Yanaiharu [5] et al. Compared the miRNA expression differences between lung cancer tissues and normal lung tissues, and found that MiR-140-5p was down-regulated in lung cancer tissues compared with normal tissues. Tan [6] et al. Further compared the miRNA expression differences between lung tissues and lung squamous cell carcinoma, and found 5 miRNAs with differential expression characteristics. MiR-140-3p was one of them, and its expression was decreased in SCC. Kong [7] et al. And Dong [8] et al. Also found that MiR-140-3p was low expressed in lung cancer tissues, and was closely related to the proliferation, invasion and migration of lung cancer cells, and further proved that its target genes were ATP6AP2 and ATP8A1. Yuan [9] et al. Found that MiR-140-5p presented low expression in non-small cell lung cancer tissues and cell lines, and was closely related to the high expression of IGF1R, further promoting the proliferation, invasion and metastasis of tumor cells. Ling Hu [10] et al. Study on delete three duplicate data and conducted a meta-analysis after 11 invalid data, put forward the mir - 140 can significantly reduce the MMD protein levels in non-small cell lung cancer, and by

adjusting the erk1/2 signaling inhibition of cell proliferation, mir - 140 is put forward as a combination with other 13 kinds of micromas, have a more accurate forecast value, at a higher sensitivity, higher rate of specialized and statistically significant way to distinguish between cancer cases. Zhang [11] et al. Found that Smad3 is the target gene of MiR-140-5p, and the low expression of MiR-140-5p leads to the weakened inhibition of psmad3, resulting in the enhanced activity of psmad3 and promoting the invasion and metastasis of tumor cells.

These studies indicated that MiR-140-5p exists in human body as a cancer suppressor, which is negatively correlated with the occurrence and development of lung cancer, and this experiment also confirmed that the expression of MiR-140-5p in normal cells is much higher than that in lung adenocarcinoma cells. Meanwhile, this study also showed that MiR-140-5p can promote apoptosis of lung cancer cells, and the mechanism of MiR-140-5p on lung cancer may be related to its targeted action on YES1 gene.

YES proto-oncogene belongs to the family of Src non-receptor protein tyrosine kinase, and the overexpression of Src signal is found in most human tumors. Experimental studies have confirmed that the overexpression of Src signal greatly promotes the progress of tumor TNM staging. YES1, as a member of Src family, can regulate cell proliferation, adhesion and differentiation, which is a key factor worthy of attention. Data showed that YES1 expression was associated with a variety of malignant tumors, including malignant mesothelioma, rhabdomyosarcoma and pancreatic tumors. Considering that high expression of YES1 in tumor can promote tumor cell proliferation and metastasis, YES1 is of great significance in the clinical diagnosis of tumors [12].

Studies have shown that found YES1 genes associated with lung cancer development, this research through the retrieval targets can and pictar and MICROCOSM, and other target gene prediction database, get the conclusion after the intersection between each database, predict YES1 likely to target genes of mir - 140-5 p, and the 3'UTR of YES1 gene mRNA in multiple regions with mir - 140-5 p seed sequence fully complementary, physi CAL consider mir - 140-5 p as a cancer suppressor, The seed sequences were also completely complementary to the 3'UTR of YES1 mRNA, which may regulate the expression of proto-oncogene YES1. Physi CAL by dual luciferase experiments confirm this measurement, proved YES1 target genes of mir - 140-5 p, mir - 140-5 p may through regulating YES1 gene, which affects the expression of its downstream related proteins, and the impact on cell proliferation and apoptosis, physi CAL follow-up experiments will dig deep mir - 140-5 p in lung cancer development role in the process of the molecular mechanism and signal pathway, and mir - 140-5 p regulation YES1 gene expression of the specific biological process, and thus provide a new experimental basis for the diagnosis and treatment of lung cancer.

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