Pathology - Research and Practice xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

miR-486-5p functions as an oncogene by targeting *PTEN* in non-small cell lung cancer

Zhao-jia Gao[a,b], Wei-dong Yuan[a], Jun-qiang Yuan[a], Kai Yuan[a,b,*], Yong Wang[a,*]

[a] Division of Thoracic Surgery, The Affiliated Changzhou NO.2 People's Hospital of Nanjing Medical University, Changzhou, Jiangsu Province, China
[b] Heart and lung disease laboratory, The Affiliated Changzhou NO.2 People's Hospital of Nanjing Medical University, Changzhou, Jiangsu Province, China

ARTICLE INFO

Keywords:
miR-486-5p
Oncogene
Non-small cell lung cancer
PTEN
Therapy

ABSTRACT

Purpose: Lung cancer, the leading cause of cancer-related death worldwide, shows a poor 5-year overall survival rate. In our previous study, we demonstrated that *miR-486-5p* can be a potential blood-based biomarker for early diagnosis and recurrence prediction of non-small cell lung cancer (NSCLC). The aim of the present study was to investigate the possible roles and related target genes of *miR-486-5p* in NSCLC progression.

Methods: pcDNA3.1(+)/Pri-miR486 recombinant plasmid and *miR-486-5p* inhibitor were transfected into NSCLC cells and theirs effects were evaluated by qRT-PCR. Then, MTT assay and Colony formation assay were performed to determine the potential roles of *miR-486-5p* played on NSCLC cellular proliferation and cloning in vitro. We also initially investigated the target genes of *miR-486-5p* by using bioinformatic methods, qRT-PCR and western blot.

Results: pcDNA3.1(+)/Pri-miR486 recombinant plasmid significantly upregulated the expression of *miR-486-5p*, while *miR-486-5p* inhibitor significantly downregulated its expression. Upregulation of *miR-486-5p* promoted the cellular proliferation and cloning, while *miR-486-5p* silencing restrained the cellular proliferation and cloning. Furthermore, four potential target genes (*PIK3R1*, *PTEN*, *MAP3K7* and *FOXO1*) of *miR-486-5p* were screened out. Finally, we found that upregulation of *miR-486-5p* in NSCLC cells significantly reduced *PTEN* and increased *AKT* expression levels, whereas *miR-486-5p* silencing increased *PTEN* and reduced *AKT* expression. Therefore, we believe that miR-486-5p can regulate *PTEN-PI3 K/AKT* signaling.

Conclusions: *miR-486-5p* acts as an oncogene in the progression of NSCLC by influencing *PTEN-PI3 K/AKT* signaling. *miR-486-5p* may provide potential therapeutic targets for NSCLC.

1. Introduction

Lung cancer, which contains two histological types: non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC), is one of the most common malignancies and the leading cause of cancer-related death worldwide [6,23]. NSCLC accounts for approximately 80–85% of all patients with lung cancer. Due to the development of surgical techniques, strategies of chemoradiotherapy and target therapy, the survival of patients with lung cancer has been significantly improved in recent years [17,28]. Despite this, the prognosis of patients with locally advanced NSCLC is still unfavorable, with a 5-year overall survival rate of only 15% [22,25]. Therefore, further investigation of the molecular mechanisms involved in carcinogenesis and progression of NSCLC is essential for developing new effective therapeutic targets of NSCLC.

MicroRNAs (miRNAs) are a class of small, endogenous, noncoding RNA molecules (containing approximately 22 nucleotides) that function in RNA silencing and post-transcriptional regulation of gene expression [3,4]. It is reported that miRNAs were involved in various biological processes, such as differentiation, apoptosis, morphogenesis and tumorigenesis [8]. Accumulating evidences have shown that aberrant expression of miRNAs may play a role in tumorigenesis and tumor progression of various malignancies [2,7,13,19,29]. Up to date, a large number of miRNAs have been reported to involve in cellular growth, invasion, migration and metastasis of NSCLC [5,32,35,37]. In our previous study, we detected the expression of ten miRNAs in the plasma of patients with NSCLC and found that *miR-486-5p* is a potential blood-based biomarker for early diagnosis and recurrence prediction of NSCLC [10].

In the present study, we aimed to investigate the possible roles and related target genes of *miR-486-5p* in NSCLC progression. These results showed that *miR-486-5p* markedly modulated the cellular proliferation and cloning in NSCLC. Further investigations showed that *PTEN* played

* Corresponding authors at: Division of Thoracic Surgery, The Affiliated Changzhou NO.2 People's Hospital of Nanjing Medical University, 29 Xinglong Lane, Changzhou 213003, Jiangsu Province, China.
E-mail addresses: yuankai1978@163.com (K. Yuan), doctor_wang1960@163.com (Y. Wang).

https://doi.org/10.1016/j.prp.2018.03.013
Received 30 January 2018; Received in revised form 25 February 2018; Accepted 13 March 2018

as the functional target of *miR-486-5p* in NSCLC. Taken together, our study suggested that *miR-486-5p* modulated *PTEN* gene to regulate NSCLC and these findings might serve as a potential molecular target for NSCLC treatments.

2. Materials and methods

2.1. Cell line and cell culture

Human A549 cells were obtained from ScienCell Research Laboratories (ScienCell, USA) and cultured in F–12 K medium (Cell Signaling Technology, USA) supplemented with 10% fetal bovine serum (Gibco) and 100U/ml penicillin/streptomycin (Gibco) at 37 °C in a humidified incubator containing 5% CO2.

2.2. Upregulating or downregulating the expression of miR-486-5p in NSCLC cells

To upregulate the expression of *miR-486-5* in NSCLC cells, cells were transfected with pcDNA3.1(+)/Pri-miR486 recombinant plasmid (pri-miR-486 group) or pcDNA3.1(+) (Negative control group) by using lipofectamine 2000 Transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. To downregulate the expression of *miR-486-5p*, inhibitor of *miR-486* (sense: CUCGGGGCAGCUCAGUACAGGA) (miR-486 inhibitor group) or scrambled sequence (Negative control group) was transfected into NSCLC cells by using lipofectamine 2000 Transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. Before transfection, 1 μg recombinant plasmid DNA and 2 μl liposomes were separately dissolved in 50 μl serum-free opti-MEM medium and mixed after standing. Then the mixed solution was added into 1 ml serum-free opti-MEM medium, well-mixed and tiled on the rinsed A549 cells. After cultured for 6–8 h at 37 °C, the medium was aspirated and replaced with fresh medium and then continuously cultured for 24–72 h at 37 °C. Transfection efficiency was evaluated by qRT-PCR.

2.3. Bioinformatics

One software program, TargetScanHuman 7.1 (Release 7.1, June 2016; http://www.targetscan.org/vert_71/) was used to predict the potential target genes of *miR-486-5p* [1]. Then, the list of all potential target genes was submitted to PANTHER Classification System (Release 11.1, Oct 2016; http://www.pantherdb.org/) for pathway enrichment analysis [16].

2.4. RNA extraction and quantitative real-time PCR

Total RNAs from cultured cells was extracted using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA was reversely transcribed into cDNA using Taqman®MicroRNA Reverse Transcription Kit (Abcam, China) or High-Capacity cDNA Reverse Transcription Kit (ABI, USA). The cDNA was then used as the template for qRT-PCR amplification using a SYBR green master mix kit (ABI, USA). Furthermore, the qRT-PCR primer sequences for *FOXO1*, *MAP3K7*, *PTEN* and *PIK3R1* were shown in Table 1. To determine *miR-486-5p* transcript expression level, RNU48 served as the internal references. To determine the four genes (*FOXO1*, *MAP3K7*, *PTEN* and *PIK3R1*) transcript expression level, β-actin was used as an internal reference. The qRT-PCR reaction included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Each sample was run in triplicate and the relative gene expression was calculated and normalized using the $2^{-\Delta\Delta CT}$ method.

Table 1
Sequences of qRT-PCR primer.

Gene	sense	anti-sense
PIK3R1	GTTGCACCAGGTTCTTCGA	TGTCAGGAGGGGCAAACTG
PTEN	TCGTTAGCAGAAACAAAAGGAG	ACGCCTTCAAGTCTTTCTGC
MAP3K7	TGGATGGCACCTGAAGTT	CCAATCTCATCAAAGGGT
FOXO1	AGGATAAGGGTGACAGCAA	TGTAGGGACAGATTATGACG

2.5. Methyl thiazol tetrazolium (MTT) assay

For detection of cell growth, cells were seeded into a 96-well cell plate (1×10^5 cells per well) and subjected to MTT assay (Sigma). Briefly, cells were transfected with pcDNA3.1(+)/Pri-miR486 or *miR-486* inhibitor for 48 h. Then, MTT (4 mg/ml) was added to each well (20 μl per well) and incubated for 3 h. The formazan was then dissolved by dimethyl sulfoxide (200 μl per well) and the optical density (OD) value at 580 nm was quantified. The experiments were performed at least three times.

2.6. Colony formation assay

For colony formation assays, a total of 300 transfected cells were placed in a fresh six-well plate and maintained in media containing 10% FBS. After three weeks, cells were fixed with 1 ml 4% paraformaldehyde and stained with 0.1% crystal violet. Visible colonies were manually counted and each experiment was performed at least three times.

2.7. Protein preparation and western blot

All cells were homogenized and treated with lysis buffer on ice. The proteins were separated in SDS–PAGE gels and then transferred onto a PVDF membrane. After incubated with blocking buffer (5% BSA) for 1 h at room temperature, the membrane was probed with the primary antibodies diluted in blocking buffer (*PTEN* (Invitrogen, 1:1000), *p-AKT* (Invitrogen, 1:500), *t-AKT* (Invitrogen, 1:1000) or β-actin (1:1000)) overnight at 4 °C. After being washed, the membrane was incubated with anti-mouse IgG (7076) or anti-rabbit IgG (7074) (HRP-linked antibody, Cell signaling technology, 1:5000) for 1 h at room temperature. β-actin was used as an internal control and bands were visualized by ECL detection (Thermo Scientific). All experiments were repeated triple times.

2.8. Statistical analysis

All statistics are presented as means ± S.D. and statistical analysis was performed using Student's *t* test or Wilcoxon rank test (IBM, IL, USA). The data graphs were made with GraphPad Prism 6.02 software. $P < 0.05$ were considered statistically significant.

3. Results

3.1. Upregulation of miR-486-5p promotes cellular growth of NSCLC cells in vitro

In our previous study, we demonstrated that *miR-486-5p* is significantly high-expressed in plasma samples from NSCLC patients and its high-expression is correlated with recurrence of NSCLC. We hypothesize that high-expression of *miR-486-5p* in NSCLC can promote cellular growth. To validate the hypothesis, pcDNA3.1(+)/Pri-miR486 recombinant plasmid was constructed and transfected to NSCLC cells. Significantly upregulation of *miR-486-5p* in the cells was confirmed by qRT–PCR ($p < 0.01$) (Fig. 1a). MTT assay showed that compared with negative control group, upregulation of *miR-486-5p* significantly increased the proliferation rate of the NSCLC cells ($p < 0.01$) (Fig. 1b). Colony formation assay demonstrated that cellular cloning was significantly enhanced due to upregulation of *miR-486-5p* ($p < 0.01$)