miR-4782-3p Inhibited Non-Small Cell Lung Cancer growth via USP14

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Key Words
miR-4782-3p • NSCLC • USP14 • ZEB2 • XIAP

Abstract

Background: Lung cancer is the leading cause of cancer-related mortality worldwide, with near 1.4 million deaths each year. NSCLC accounts for nearly 85% of all case of lung cancer. MiRNAs play important roles in regulation of gene expression at the post-transcriptional level. MiRNAs profiles may predict prognosis and disease recurrence in early-stage NSCLC. Our previous study proved that over-expression of ubiquitin specific peptidase 14 (USP14), a deubiquitinating enzyme, was associated with favorable prognosis in NSCLC patients and promoted tumor cells proliferation. Here, we tried to identify which miRNAs targeted USP14, and the roles of these miRNAs in NSCLC. Methods: MiR-4782-3p and its potential targeted genes were identified by bioinformatics algorithm. Dual luciferase reporter assay system was used to analyze the interaction between miR-4782-3p and targeted genes. Cell proliferation was assayed by MTT and BdU assay. MiRNAs and mRNA expression were assayed by qRT-PCR. USP14 protein level was assayed by Western blot. The role of miR-4782-3p in patients survival was revealed by Kaplan-Meier plot of overall survival. Results: Up-expression of miR-4782-3p in NSCLC cells decreased the USP14 expression. Down-expression of miR-4782-3p increased USP14 expression. In NSCLC specimen, Negative correlation between USP14 mRNA level and miR-4782-3p level was identified. Higher miR-4782-3p expression is associated with longer survival. USP14, ZEB2, XIAP overexpression reversed the inhibitory effect of miR-4782-3p. Conclusions: High expression of miR-4782-3p was associated with favorable prognosis in NSCLC patients. MiR-4782-3p inhibited cell proliferation in NSCLC by targeting USP14, ZEB2 and XIAP.
Introduction

Approximately 1.6 million new cases of lung cancer are diagnosed each year throughout the world, the mortality related to lung cancer continues to rise [1]. It is the leading cause of cancer-related mortality worldwide, with near 1.4 million deaths each year. Lung cancer has an extremely poor prognosis, with an overall 5 year survival of 16% in the USA and less than 10% in the UK [2]. In China, mortality of lung cancer has increased by 465% during the past 30 years, and such cancer has become the main cause of death in urbanites and the second leading cause of death in rural population after liver cancer [3]. Non-small cell lung cancer (NSCLC) is any type of epithelial lung cancer other than small cell lung carcinoma (SCLC). NSCLC includes adenocarcinoma, large cell carcinoma, bronchioloalveolar carcinoma, and squamous cell carcinoma, and accounts for nearly 85% of all case of lung cancer [4, 5].

MicroRNAs (miRNAs) are a class of endogenous non-coding, single-stranded small regulatory RNA molecules, which are approximately 22 nucleotides in length [6]. MiRNAs inhibit translation and cleave mRNA by base-pairing to the 3' untranslated region of the target genes [7-9]. MiRNAs play important roles in regulation of gene expression at the post-transcriptional level. It is estimated that miRNAs can regulate at least 20%-30% of all human genes [10], and that an average miRNA has more than 100 targets [11]. Half of all miRNAs genes are found within or near chromosomal fragile sites, common breakpoints, or minimal regions of loss-of-heterozygosity or amplification [12]. It is known that miRNAs are grossly in NSCLC, and may serve as oncogenes or tumor suppressors [13]. Importantly, recent data showed that specific miRNA profiles may predict prognosis and disease recurrence in early-stage NSCLC [14-18].

Here, we studied the role of miR-4782-3p in NSCLC, and found miR-4782-3p inhibited cell proliferation in NSCLC. Our study may provide a new therapy target of NSCLC.

Materials and Methods

Patients

Surgical specimens from 22 NSCLC patients and matched normal control adjacent lung tissues were obtained postoperatively in 2008 from the Department of Respiratory Medicine, Changhai Hospital, Second Military Medical University (Shanghai, China). All patients gave signed, informed consent for their tissues to be used for scientific research. Ethical approval for the study was obtained from Changhai Hospital, Second Military Medical University (Shanghai, China). All diagnoses were based on pathological and/or cytological evidence. The histological features of the specimens were evaluated by senior pathologists according to the World Health Organization classification criteria. Tissues were obtained before chemotherapy and radiotherapy and were immediately frozen and stored at −80 °C prior to qRT-PCR assay. 20 patients had been followed-up for 3 years and complete clinical data were electronically recorded.

Cell culture

HEK293, human normal lung fibroblast cell line MRC-5, and human NSCLC cell lines (A549, H1299, SPC-A-1, LTED-A-2 and SK-MES-1) were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China) and cultured in DMEM medium (Hyclone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine and 100 μg/mL penicillin/streptomycin (Bio Light, Shanghai, China) as described in our previous studies [19].

EdU cell proliferation assay and MTT assay

The cell proliferation assay were assayed by EdU Flow Cytometry Assay Kits (invitrogen). EdU (5-ethyl-2'-deoxyuridine) is a nucleoside analog to thymidine and is incorporated into DNA during active DNA synthesis. Detection is based on a click reaction [20], a copper catalyzed covalent reaction between an azide and an alkyne. Flow Cytometry Assay was performed by using CellQuest software (Becton Dickinson, Franklin Lakes, Nj, USA) as described previously [21, 22]. For MTT assay, 500 cells per well were seeded in triplicate in a 96-well plate with complete growth medium. Cells were counted over 5 days using the
MTT assay (Promega, Fitchburg, WI, USA) as described previously [22, 23]. The data were measured by Microtiter plate reader 570-nm filters (Promega, USA).

**RNA extraction and Real time q-PCR**

RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The cDNA synthesis and real-time qPCR were subsequently performed using the Qiagen system as described in our previous studies [19, 24]. Real-time quantitative PCR analysis was performed using standard protocols on an Applied Biosystem’s 7500 HT sequence Detection System. MiR-4782-3p expression was assessed using a mirVana™ qRT-PCR miRNA Detection Kit (Ambion, USA). The primer design is based on standard protocols [25], and synthesized by Shengong Company (Shanghai).

Relative miRNA levels of USP14 were normalized to levels of the housekeeping gene GAPDH and calculated by the 2-

**MiRNAs mimics, miRNAs antisense oligonucleotides and overexpression plasmids**

MiRNAs mimics and miRNAs antisense oligonucleotides (ASO) were obtained from GenePharma (GenePharma, China). MiRNAs mimics, negative control (NC) were transfected into cells at a concentration of 50nM using Lipofectamine 2000 (Invitrogen, Canada) transfection reagent according to the manufacturer's instructions. 48h or 72h later cells were collected for further experiments. The overexpression plasmid (pcDNA3.1-USP14, pcDNA3.1-ZEB2, pcDNA3.1-XIAP) were constructed and confirmed by Shengong Company (Shanghai).

**USP14 3′UTR reporter analysis**

The USP14 3′UTR reporter plasmid (pRL-USP14) was constructed by Shengong Company (Shanghai). Mutation in the miR-4782-3p seed regions of the USP14 3′UTR were generated using QuikChang Multi site-directed mutagenesis kit (Stratagene). RL reporter plasmids (3.6fmol) and pGL3-control (500ng for normalization; Promega) were transfected with Lipofectamine 2000 (Invitrogen) into HEK293 cells (6×10^4 cells per well). Cells were collected after 48h for assay using the Dual Luciferase reporter assay system (Promega) [26].

**Western blot and antibodies**

Cells or tumor tissues were harvested, lysed, and blotted as described previously [27]. Membranes were blocked with blocking solution (5% skim milk in TBST) and incubated with primary antibody, followed by the incubation with appropriate HRP-conjugated secondary antibody. The USP14 antibody (anti-USP14) was purchased from Santa Cruz Biotechnology, Inc. The densitometry of Western blot results was measured using ImageJ software.

**MicroRNAs targets prediction**

To identify which miRNAs binding the 3′UTR of USP14 or Targets of miRNAs, TargetScanHuman (http://www.targetscan.org/vert_61/) [10, 28-30] is applied.

**Apoptosis assay**

Cells were labeled with Annexin V-FITC and propidium iodide (PI) using an apoptosis detecting kit (Invitrogen, Canada) as described previously [31]. Samples were determined by FACS assays and the results were analyzed using CellQuest software (Becton Dickinson, San Jose, CA) [32].

**Statistical Analysis**

Data were presented as the mean ± s.e.m. from at least three independent experiments. The difference between groups were analyzed using two-tailed Student’s t test when only two groups were compared. The difference between groups were analyzed using ANOVA when three or more than three groups were compared. Correlation analysis was performed by two-tailed Person’s correlation coefficient analysis. Patients survival was determined by Kaplan-Meier analysis. Statistical analyses were performed using SPSS software (version 17.0). P<0.05 was considered significantly different.
Results

miR-4782-3p inhibited USP14 expression

Our previous study proved that over-expression of USP14 was associated with poor prognosis in NSCLC patients and promoted tumor cell proliferation [19]. To identified which miRNAs binding the 3'UTR of USP14, bioinformatics algorithm is applied. We found that 9 potential miRNAs may probably bind the 3'UTR of USP14 (Fig. 1A). Of these 9 miRNAs, the role of miR-4782-3p has not been investigated. To confirm whether miR-4782-3p could target USP14, the putative miR-4782-3p binding site in 3' UTR USP14 was mutated (Fig. 1B). WT or mutated USP14 3' UTR were cloned into luciferase reporter plasmid. MiR-4782-3p mimics and reporter plasmid were co-transfected into HEK293 cells. We found that miR-4782-3p inhibited renilla luciferase (RL) reporter genes, and mutation of miR-4782-3p binding sites in USP14 prevented down-regulation of reporters by miR-4782-3p mimics (Fig. 1C). Next, we transfected HEK293 cells with miR-4782-3p mimics, 48h later, we found that the USP14 protein expression were inhibited (Fig. 1D). So, our data indicated that miR-4782-3p inhibited USP14 expression.

Correlations between miR-4782-3p, USP14 and overall survival in NSCLC patients

To confirm the correlations between miR-4782-3p and USP14, the miR-4782-3p expression and USP14 in human normal lung tissue, human normal lung fibroblast cell line (MRC-5), 7 human NSCLC cell lines (A549, H1299, SPC-A-1, LTEP-A-2 and SK-MES-1) were analyzed. We found that miR-4782-3p expression in NSCLC cell lines was lower than in human normal lung tissue and MRC-5 (Fig. 2A), and there was a negative correlation between USP14 mRNA level and miR-4782-3p level (Fig. 2B). 22 pairs of NSCLC tumor tissues and matched normal adjacent tissue analysis revealed that in 17 pairs, the miR-4782-3p in NSCLC tumor tissues expression was lower than matched normal adjacent tissue, and in 3 pairs, miR-
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4782-3p in NSCLC tumor tissues expression was higher than matched normal adjacent tissue (Fig. 2C). Then the general expression of miR-4782-3p in 22 NSCLC tumor tissues was compared with matched normal adjacent tissue, the expression in normal tissue were arbitrary defined as 100%. Data are mean ± s.e.m. of three separate experiments (D). Kaplan-Meier plot of overall survival in NSCLC patient post-operation according the expression of miR-4782-3p (E). *P<0.05.

The role of miR-4782-3p in cell proliferation and apoptosis

Data above indicated that miR-4782-3p may play an important role in NSCLC. We over-expressed the miR-4782-3p in A549, SPC-A-1 by miRNAs mimics transfection. After 48h transfection, miR-4782-3p in A549 and SPC-A-1 reached the maximum value (Fig. 3A), and the USP14 level were inhibited (Fig. 3B). MTT assay showed that up-regulation of miR-4782-3p in A549, SPC-A-1 inhibited cells proliferation (Fig. 3C). EdU assay also showed that miR-4782-3p inhibited A549, SPC-A-1 proliferation. The percent of EdU positive cells in miR-4782-3p pretreated group was lower than in miR-NC pretreated group (Fig. 3D,E). MiR-4782-3p showed more inhibitory effect on cells proliferation in A549 than in SPC-A-1 (Fig. 3E). Cell apoptosis assay revealed that miR-4782-3p induced more cell apoptosis in A549 and SPC-A-1 than miR-NC (Fig. 3F).
Down-regulation of miR-4782-3p promoted H1299 and MRC-5 proliferation

Data above showed that H1299 and MRC-5 have a high miR-4782-3p level (Fig. 1A), thus we down-regulated the miR-4782-3p level by miR-4782-3p ASO transfection (Fig. 4A). 48h later, qRT-PCR were performed to assay the USP14 mRNA level, and MTT assay was performed to assay cells proliferation. We found that miR-4782-3p ASO increased the USP14 expression (Fig. 4B) and promoted cell proliferation in H1299 and MRC-5 (Fig. 4C). Then, the role of miR-4782-3p ASO in H1299 and MRC-5 were confirmed by EdU test, we found that the miR-4782-3p ASO increased the percent of EdU positive cells in H1299 and MRC-5 (Fig. 4D).
UPS14 played a part role in the inhibitory effect of miR-4782-3p

Our previous data proved that over-expression of USP14 promoted NSCLC proliferation [19]. Data above indicated that miR-4782-3p inhibited USP14 expression (Fig 1C,D), and
inhibited cell proliferation and induced cell apoptosis (Fig. 3B,C,D). So, we guessed that miR-4782-3p exerted its role in NSCLC by interacting with USP14. To confirm the hypothesis, we over-expressed the USP14 after the miR-4782-3p transfection, we found that USP over-expression could partly reduced the inhibitory effect of miR-4782-3p (Fig.5 A,B). So, we guessed that another target genes of miR-4782-3p existed.

Another target genes of miR-4782-3p

To identified the potential target genes of miR-4782-3p, bioinformatics algorithm methods were used, and hundreds of genes were predicted (Table S1). Based on our interest, we chose 10 putative genes (ZEB2, XIAP, RAB35, LEF1, ELK1, SOX14, KLF13, KLF16, STAB1, FBXW7) for further study (Fig. 6A). Then 3’UTR of these genes were cloned into luciferase reporter plasmid. We co-transfected miR-4782-3p with these reporter genes into HEK293 cells, and found that there are five gene which is significantly inhibited by miR-4782-3p besides USP14 (Fig. 6B).

USP14, ZEB2, XIAP overexpression reversed the inhibitory effect of miR-4782-3p

As USP14, ZEB2, XIAP showed the most effect in reporter gene assay, we chose the three genes for further analysis. Data above showed miR-4782-3p inhibited the NSCLC proliferation (Fig. 3B,C). We found co-transfection of USP14, ZEB2, XIAP overexpression plasmid reduced the inhibitory effect of miR-4782-3p, and more importantly, co-transfection of the three USP14, ZEB2, XIAP overexpression plasmid together totally reversed the inhibitory effect of miR-4782-3p (Fig. 7A). Unsurprisingly, EdU assay also showed that overexpression of USP14, ZEB2, XIAP could restore the cell proliferation to the same level of negative control in SPC-A-1 cells, and MTT assay also showed the similar result in both cell lines (Fig. 7B).

Discussion

To the best of our knowledge, this was perhaps the first report demonstrating the role of miR-4782-3p in NSCLC. Previous data showed that a four-microRNA signature (hsa-let-7a, hsa-miR-221, hsa-miR-372, and hsa-miR-182) that is associated with survival and cancer
relapse in NSCLC patients. More importantly, the five microRNAs signature can predict patient survival within cancer stages and histological subgroups of NSCLC patients [15]. Our data showed that patient with high miR-4782-3p level (10 case) had a significantly longer overall survival than those with low miR-4782-3p level (10 case) (P<0.05). Our finding may added a potential miRNA predictor for clinical practice.

Here, we proved that miR-4782-3p inhibited NSCLC proliferation by interacting with USP14, ZEB2, XIAP. Our previous data proved that over-expression of USP14 promoted tumor cell proliferation [19]. ZEB2 is a key mediator of epithelial to mesenchymal transition (EMT). It is known that the miR-200 family (miR-200a, miR200b, miR-200c, miR-141 and miR-429) and miR-205 regulated EMT by targeting ZEB1 and SIP1(ZEB2), inhibition of microRNAs was sufficient to induce EMT in a process requiring up-regulation of ZEB1 and/or SIP1(ZEB2) [33]. Our data showed that ZEB2 played a part role in the inhibitory effect of miR-4782-3p on cell proliferation. We guessed that may miR-4782-3p could regulate epithelial to mesenchymal transition via ZEB2, and we wondered whether miR-200 family and miR-4782-3p have collaboration or interaction during epithelial to mesenchymal, or in the regulation of cell proliferation. These questions remained further investigation. The X-linked inhibitor of apoptosis (XIAP) is the only cellular protein that has evolved to potently inhibit the enzymatic activity of mammalian caspases and promotes resistance to apoptosis [34]. Previous reports demonstrated that miR-23a or miR-200bc/429 were associated in altered conditions such as cerebral ischemia [35] or chemotherapy resistance in highly selected cancer cell clones [36], respectively. To the best of our knowledge, this is the first

**Fig. 7.** USP14, ZEB2, XIAP overexpression plasmid together totally reversed the inhibitory effect of miR-4782-3p. A549 cells (6×10^5 cells/well) were co-transfected with miR-4782-3p and overexpression plasmid (pcDNA3.1-USP14, pcDNA3.1-ZEB2, pcDNA3.1-XIAP) together or separately. After 48h transfection, cells were treated with 10μM of EdU for 1 hour as protocol indicated. Then, the pretreated cells were assayed by flow cytometry. The percent of positive cells were calculated. Data are mean ± s.e.m. of three separate experiments (A). A549 and SPC-A-1 cells (6×10^5 cells/well) were co-transfected with miR-4782-3p and overexpression plasmid (pcDNA3.1-USP14, pcDNA3.1-ZEB2, pcDNA3.1-XIAP) together. After 48h transfection, cells were treated with 10μM of EdU for 1 hour, then, were assayed by flow cytometry. The percent of positive cells were calculated (C). Or these cells were assayed by MTT test. All of these data are mean ± s.e.m. of three separate experiments. *P<0.05.
report demonstrating a novel role for miR-4782-3p in determining proliferation in cancer cells. Whether miR-4782-3p could regulate apoptotic cell death resistance via XIAP needed further investigation.

In conclusion, we proved that miR-4782-3p inhibited cell proliferation in NSCLC by targeting USP14, ZEB2 and XIAP. Our study may provide a potential target for NSCLC therapy.

Conflict of Interest

The authors have declared that no competing interests exist.

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Reference


