Downregulation of miR-16 promotes growth and motility by targeting HDGF in non-small cell lung cancer cells

Yang Ke\textsuperscript{a}, Weiyong Zhao\textsuperscript{b}, Jie Xiong\textsuperscript{a}, Rubo Cao\textsuperscript{a,*}

\textsuperscript{a}Cancer Center, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China
\textsuperscript{b}Department of Oncology, The Second Affiliated Hospital of Nanjing Medical University, Nanjing 210011, China

ABSTRACT

MicroRNAs play important roles in the development and progression of non-small cell lung cancer (NSCLC). miR-16 functions as a tumor-suppressor and is inhibited in several malignancies. Herein, we validated that miR-16 is downregulated in NSCLC tissue samples and cell lines. Ectopic expression of miR-16 significantly inhibited cell proliferation and colony formation. Moreover, miR-16 suppressed cell migration and invasion in NSCLC cells. Hepatoma-derived growth factor (HDGF) was found to be a direct target of miR-16 in NSCLC cell lines. Rescue experiments showed that the suppressive effect of miR-16 on cell proliferation, colony formation, migration, and invasion is partially mediated by inhibiting HDGF expression. This study indicates that miR-16 might be associated with NSCLC progression, and suggests an essential role for miR-16 in NSCLC.

A R T I C L E I N F O

Article history:
Received 1 August 2013
Revised 6 August 2013
Accepted 6 August 2013
Available online 13 August 2013

Keywords:
imR-16
Non-small cell lung cancer
HDGF
Growth
Migration
Invasion

1. Introduction

Lung cancer, which results from uncontrolled proliferation of cells in the lung, is one of the most common human cancers which have etiologies of both genetic and environmental origins [1]. Most lung cancers are carcinomas, arising from epithelial cells, and non-small cell lung cancer (NSCLC) comprises over 80% of newly diagnosed lung cancer cases, and over 70% of NSCLC patients have advanced disorders. Although recent advances had been developed in surgery and chemotherapy, the 5-year survival rate of NSCLC is still only 16% [2]. Lung carcinogenesis is a multistep process, and systematic analyses of expression levels of both oncogenes and tumor suppressors involved in the pathogenesis of lung cancer have been performed, and numerous genes have been identified to be essential during carcinogenesis [3–5]. However, the underlying mechanisms of NSCLC remain poorly understood. Therefore, a better understanding of the detailed mechanisms might be helpful to find new therapeutic targets and strategies for the treatment of NSCLC.

MicroRNAs (miRNAs), which are small and non-coding RNAs, bind to the complimentary recognition sequences in the 3'-untranslated region (3'-UTR) of target mRNA, resulting in translational inhibition or target mRNA degradation [6]. miRNAs play essential roles in a variety of biological and pathological processes, including cell proliferation, migration, invasion, apoptosis, and metastasis [7]. The roles of miRNAs in NSCLC have been widely studied, and critical regulators of miRNA biogenesis, Dicer, DGCR8, and Drosha, have been reported to be disrupted in lung cancer [8,9]. miRNA microarrays studies have identified many miRNAs aberrantly expressed in lung cancer [10]. Among them, miR-16 was substantially decreased [11]. miR-16, which is located at chromosome 13q14, is a well-known tumor suppressor miRNA in multiple human cancers [12]. However, the detailed role of miR-16 in NSCLC is still poorly understood.

In the present study, we validated that miR-16 was significantly decreased and overexpression of miR-16 significantly inhibited the growth and motility of NSCLC cells. Furthermore, we found that miR-16 directly targeted HDGF, a potential oncogene.

2. Materials and methods

2.1. Tissue samples, cell lines and transfection

A total of 20 NSCLC tissue samples and matched non-tumor normal tissue samples were surgically obtained in our department. Informed consent was taken from all subjects and this work was approved by the Ethics Committee of Huazhong University of Science and Technology. Three NSCLC cell lines, A549, Calu3, and...
H1299, and human bronchial epithelial cell line (16HBE) were purchased from ATCC. Cells were grown in RPMI-1640 media containing 10% fetal bovine serum (FBS) at 37 °C with 5% CO2. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in Opti-MEM medium (Invitrogen, Carlsbad, CA, USA). Cells were incubated for 48 h prior to harvesting.

2.2. RNA extraction and quantitative real time PCR (qRT-PCR)

Total RNA was extracted by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). SYBR® Premix Ex Taq (Takara, Japan) was used to detect gene expression on ABI Stepone plus (Applied Biosystems, Foster City, CA, USA). miRNA was extracted using All-in-one microRNA extraction kit (GeneCopoeia, Carlsbad, CA, USA). miRNA was amplified from human cDNA using the following primers: sense, 5'-CTTTGACTTCTCTTGGAGTGTGGCTACAGG-3' and anti-sense, 5'-TTTCTATCAGGTGTCACCATC-3'. Primers for GAPDH: sense, 5'-AACGGATTGGTGCTATTTG-3' and anti-sense, 5'-GGAAAGATGGTGATGGGATT-3'. Primers for miR-16 and U6 were purchased from GeneCopoeia (Carlsbad, CA, USA). The expression of HDGF was normalized with GAPDH, and the expression of miR-16 was normalized with U6.

2.3. Plasmids

miR-16 mimic/inhibitor and the corresponding controls were obtained from Ribobio (Guangzhou, China). pcDNA3-HDGF was generated by using the following primers: sense, 5'-GGGTACCTCTCGAGAAACATTGGTGGCTACAGG-3' and anti-sense, 5'-CCCTCGAGAACATTTGGTGCTACTACGG-3'. The PCR fragment was inserted into pcDNA3.0 within KpnI and XhoI restriction sites (Invitrogen, Carlsbad, CA, USA). The 3'-UTR of HDGF mRNA was amplified from human cDNA using the following primers: sense, 5'-CTCTCGAGGACCTGGCTATAGAAGAAA-3' and anti-sense, 5'-TTGGCCGGCCACGACAGGAAACAGG-3'. The PCR fragment was inserted into psiCHECK2 vector within XhoI and NotI restriction sites (Promega, Madison, WI, USA). Mutation in the miR-16 binding-sites of HDGF was performed using whole-plasmid amplification in the seed region of miR-16 (NEB, Ipswich, Canada).

2.4. MTT cell proliferation assay

Cell proliferation was determined by MTT cell proliferation assay kit (Roche Applied Science, Foster City, CA, USA). Cells were seeded in 96-well plates with a density of 4 × 10^3 cells/well. 48 h after treatment, MTT was added to each well, and the optical density at 570 nm was measured under a microtiter plate reader.

2.5. Colony formation assay

Twenty four hour after treatment, 500 cells were seeded in a fresh 6-well plate and maintained in RPMI-1640 medium containing 10% FBS for 2 weeks. Colonies were fixed and stained with 0.1% crystal violet for 15 min. Colony numbers were quantified by Alpha Innotech imaging software (San Leandro, CA, USA), and percentage colony formation was analyzed by adjusting control cells to 100.

2.6. Cell migration and invasion assays

For migration assays, 24 h after transfection, 5 × 10^4 cells in serum-free medium were added into the upper chamber of an insert (8-μm pore size, Millipore, Billerica, MA, USA). For invasion assays, 1 × 10^5 cells in serum-free medium were placed into the upper chamber of an insert coated with Matrigel (Sigma–Aldrich, St. Louis, MO USA). Medium containing 10% FBS were added to the lower chamber as a chemoattractant. After 24 h of incubation, cells remaining on the upper membrane were removed, whereas cells which had migrated or invaded to the lower membrane were stained with 0.1% crystal violet, imaged, and counted using a microscope (Olympus, Tokyo, Japan). Experiments were performed independently three times.

2.7. Luciferase reporter assays

A549 cells were co-transfected with wild type HDGF 3'-UTR (WT) or the mutated 3'-UTR (Mut) and miR-16 mimic or the control mimic (miR-NC). Forty eight hour after transfection, cells were collected and luciferase activity was analyzed using a dual-luciferase reporter assay system (Promega, Wisconsin, WI, USA).

2.8. Western blotting

Cells were washed twice with PBS and proteins were extracted with SDS lysis buffer (Beyotime, Shanghai, China), and separated by 10% SDS–PAGE gel. Protein samples were transferred to polyvinylidine difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and were probed with primary antibodies against HDGF (Abcam, Cambridge, UK) or GAPDH (Abcam, Cambridge, UK). Membranes were incubated at 4 °C overnight, followed by incubation with AP-conjugated secondary antibodies and detected by ECL.

2.9. Statistical analysis

Two-tail Student’s t-test and One-way ANOVA were performed to analyze the data using SPSS 12.0. P < 0.05 was considered statistically significant.

3. Results

3.1. miR-16 was down-regulated in NSCLC tissues and cell lines

Expression of miR-16 in 20 NSCLC patient tissues and matched non-tumor normal tissue samples was detected by qRT-PCR. miR-16 was significantly down-regulated in NSCLC patient tissues compared with adjacent normal tissues (Fig. 1A). Similarly, expression of miR-16 in three NSCLC cell lines, A549, Calu3, and H1299 was significantly decreased compared with that in 16HBE cells (Fig. 1B).

3.2. miR-16 inhibited NSCLC cell growth in vitro

To examine the role of miR-16 in NSCLC cell growth, A549 and Calu3 cells were transfected with miR-16 mimic or miR-NC. Over-expression of miR-16 significantly inhibited the growth of A549 and Calu3 cells (Fig. 2A). Accordingly, miR-16 overexpression significantly inhibited colony formation in both NSCLC cell lines (Fig. 2B).

3.3. miR-16 suppressed NSCLC cell migration and invasion

To investigate the effect of miR-16 on the motility of NSCLC cells, miR-16 or miR-NC was transfected into A549 and Calu3 cells and migration and invasion assays were performed. miR-16 significantly suppressed the migration and invasion abilities of NSCLC cells (Fig. 3A, B).

3.4. HDGF was a direct target of miR-16

To explore the function of miR-16 in NSCLC, we used TargetScan 6.2 to screen the target gene of miR-16. HDGF was predicted to be a
target of miR-16 (Fig. 4A), an oncogene in several tumors including NSCLC. Luciferase activity assay showed that miR-16 significantly inhibited the luciferase activity of the WT 3′-UTR but not that of Mut 3′-UTR of HDGF in A549 cells (Fig. 4B). Furthermore, overexpression of miR-16 significantly suppressed HDGF mRNA and protein levels (Fig. 4C and D).

3.5. miR-16 inhibited NSCLC cell growth and motility by targeting HDGF

Further experiments were performed to explore whether overexpression of HDGF could attenuate the suppressive effect of miR-16. MTT assay (Fig. 5A), in vitro cell migration and invasion (Fig. 5B and C) all showed that supplementation of HDGF by pcDNA3-HDGF could significantly attenuate the suppressive effect of miR-16. The effect of HDGF plasmid was confirmed by qRT-PCR (Fig. 5D).

4. Discussion

In this study, we studied the expression of miR-16 in NSCLC patients and cell lines. We found that the expression levels of miR-16 were significantly decreased in NSCLC tissues. We also found that the ectopic expression of miR-16 significantly suppressed the
proliferation of NSCLC cells. Furthermore, overexpression of miR-16 remarkably inhibited the migration and invasion of NSCLC cell lines. By using luciferase activity assay and Western blotting, we found that HDGF was a direct target of miR-16. Overexpression of miR-16 suppressed cell growth and motility, whereas overexpression of HDGF antagonized this effect. Our data illustrate the possible role of miR-16 and HDGF in the pathogenesis of NSCLC.

Numerous miRNAs have been found to be decreased in NSCLC and many of them played essential roles in the regulation of oncogenesis of NSCLC. For instance, Decreased miR-148a was found to be associated with lymph node metastasis and poor clinical outcomes, which acted as a suppressor of tumor metastasis in NSCLC by target DNA (cytosine-5)-methyltransferase 1 (DNMT1) [13]. Our previous study found that miR-149 was down-regulated in NSCLC, and it inhibited epithelial-to-mesenchymal transition process of A549 cells by targeting Forkhead box M1 (FOXM1) [9]. Down-regulation of miR-181b was thought to be a potential prognostic marker of NSCLC. Low miR-181b expression was found to be tightly correlated with larger tumor size, positive lymph node metastasis, and higher p-TNM stage of NSCLC patients [14]. miR-449a, decreased in NSCLC, inhibited migration and invasion by targeting c-Met [15]. miR-16 has been reported to be decreased and to serve as a tumor suppressor in numerous cancer types. miR-16 has been also found to inhibit osteosarcoma cell proliferation by targeting...
insulin-like growth factor 1 receptor (IGF1R) and the Raf1-MEK1/2-ERK1/2 pathway [17]. miR-16 induced apoptosis of rat activated pancreatic stellate cells by inhibiting Bcl-2 expression in vitro [18]. miR-16 was down-regulated in NSCLC as well [11]. Herein we demonstrated that HDGF was a direct target of miR-16 in NSCLC cells and miR-16 acted as a tumor suppressor partially by inhibiting HDGF in NSCLC.

HDGF was highly expressed in different cancers and its expression was correlated with aggressive biological capabilities of cancer cells including proliferation and angiogenesis [19,20]. Knockdown of HDGF inhibited growth, colony formation, vessel formation in NSCLC tumors, which suggested that HDGF might be involved in the pathogenesis of NSCLC [21]. Our results showed that miR-16 inhibited growth, colony formation, migration and invasion by targeting HDGF in NSCLC cells. These data indicated that down-regulation of miR-16 in NSCLC might promote growth and motility through the elevation of HDGF.

In conclusion, this study showed that miR-16 was down-regulated in NSCLC tissues and cell lines, and ectopic expression of miR-16 suppressed cell proliferation and colony formation in vitro. HDGF was identified as a target of miR-16 in NSCLC, and overexpression of HDGF partially attenuated the suppressive effect of miR-16, indicating that miR-16 might be a useful marker and a potential therapeutic target for the treatment of NSCLC.

References