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Original Article

MiR-199b-5p suppresses proliferation and invasion of non-small cell lung cancer (NSCLC) via HER2/PI3K/Akt pathway

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Abstract: The aberrant expression of microRNAs (miRNAs) has been found in various types of cancer. miR-199b-5p was reported to be aberrantly expressed in human cancers, however, the expression and function of miR-199b-5p in non-small cell lung cancer (NSCLC) remains poorly understood. The objective of this study was to investigate the expression of miR-199b-5p in NSCLC and to explore its molecular mechanism in NSCLC carcinogenesis. In this study, miR-199b-5p was down-regulated in human NSCLC specimens compared to that in NC specimens. Overexpression of miR-199b-5p in H460 cells inhibited NSCLC proliferation and invasion. Next, luciferase reporter assay identified human epidermal growth factor receptor 2 (HER-2) as a novel direct target of miR-199b-5p. In addition, miR-199b-5p inhibited HER2 downstream signaling by PI3K/AKT pathways in NSCLC cells. The inhibitory effect on NSCLC cell proliferation and invasion was mediated by miR-199b-5p inhibition of the translation of a proto-oncogene, HER2. These results suggest that miR-199b-5p may be a potential to be a novel therapeutic target for NSCLC by regulating HER2/PI3K/Akt pathway.

Keywords: MiR-199b-5p, non-small cell lung cancer (NSCLC), HER2, PI3K/Akt pathway

Introduction

Lung cancer is one of the most common aggressive malignancies and non-small-cell lung carcinoma (NSCLC) is responsible for almost 80% of lung cancer-related deaths [1, 2]. Although there has been great improvement in chemotherapy and molecular-targeted therapy, the outcome of lung cancer remains poor. The invasiveness and metastasis of tumor cells are critical challenges in the clinical management of NSCLC. Therefore, an improved understanding of the molecular mechanisms regulated by microRNAs involved in NSCLC development is required as a basis to identify novel strategies for the treatment of lung cancer.

MicroRNAs (miRNAs), an abundant class of ~22 nucleotide small noncoding RNAs, post-transcriptionally regulate gene expression through binding to multiple target mRNAs (mRNAs) [3-5]. Accumulated miRNAs are reported as important regulators involving in the development of human diseases. Recent studies showed that the dysregulated miRNAs is closely associated with carcinogenesis and cancer progression [6, 7]. For example, expression levels of microRNA miR-126, miR-200c and miR-125a-5p are associated with progression and prognosis of non-small cell lung cancer [8, 9]. miR125b, as oncogenic miRNA, is up-regulated in lung cancer and reported to promote cancer progression [10], whereas miR-338-3p, miR-27b, miR-216a, miR-30 and miR-99a, as tumor-suppressor, are downregulated in breast cancer [11-16]. Recent studies have shown that miRNAs play an important role in regulating tumorigenesis and metastasis [17-20], thus suggesting the potential application of miRNA-based therapeutics in cancer therapy.

Recently, an increasing number of studies have demonstrated that the expression of miR-199b-5p is deregulated in various cancers including prostate cancer, medulloblastoma, choriocarcinoma, follicular thyroid carcinoma, ovarian cancer, renal cell carcinoma and papillary thyroid carcinoma [21-25]. For example, miRNA micro-

array analysis revealed that miR-199b-5p expression in osteosarcoma tissue samples had increased 10-fold compared with normal controls [26]. miR-199b-5p was significantly differentially expressed between nodular goiter and papillary thyroid carcinoma and between highly invasive and low invasive papillary thyroid carcinoma [24]. However, the accurate expression and mechanistic function of miR-199b-5p in NSCLC remain unclear.

Her2 (ErbB2) is a member of the epidermal growth factor receptor (EGFR) family of transmembrane tyrosine kinase type receptors, it activate its downstream signaling cascades, including the PI3K/AKT pathway, which could promote cell proliferation, metastasis and angiogenesis in tumors [27, 28]. Recent studies demonstrated that Her2 expression is relation to survival of patients with non-small cell lung cancers [29-31]. Laboratory methods for assessment of HER2 positivity in NSCLC include immunohistochemistry (IHC) for protein overexpression, fluorescent in situ hybridization (FISH) for gene amplification, and next generation sequencing (NGS) for gene mutations [29, 31]. These studies emphasize the important functional role of HER2 in the progression of NSCLC and highlight its potential as a therapeutic target. However, the molecular mechanism of HER2 in NSCLC remains unclear.

In this study, we examined miRNA-199b-5p expressions in NSCLC tissue and cells via real-time quantitative PCR analysis. The proliferation and invasion ability of miR-199b-5p in NSCLC and mechanism were investigated by in vitro studies. We demonstrated, for the first time, that miR-199b-5p was expressed at a lower level in NSCLC tissue and cells compared with nontumor human lung tissues and the normal lung bronchus epithelial cell line (16HBE), respectively. We discovered, for the first time, that miR-199b-5p inhibited HER2 gene expression by direct targeting its 3'-UTR and inhibited the activation of the HER2 downstream PI3K/AKT signal pathway in NSCLC. We also found overexpression of miR-199b-5p inhibited NSCLC cell proliferation and invasion by suppressing HER2 expression. In conclusion, our novel findings suggest that aberrant expression of miR-199b-5p is critical for the invasion of human NSCLC and targeting miR-199b-5p may aid to the development of novel therapeutic strategies against NSCLC.

Materials and methods

Tissue samples and cell lines

Paired NSCLC and normal adjacent lung tissues were obtained, with informed consent, from 25 patients who underwent primary surgical resection of NSCLC between 2013 and 2014 at the Yantaishan Hospital (Yantai, Shandong, China). The NSCLC cell lines A549, SK-MES-1, H460 and H520 and the normal lung bronchus epithelial cell line 16HBE were obtained from Yantaishan Hospital and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. All cells were incubated in a humidified (37°C, 5% CO_2) incubator.

Transfection

MiR-199b-5p mimics (dsRNA oligonucleotides), negative control mimics (NC) (sense: 5'-UUC UCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3'), miR-199b-5p inhibitors (anti-miR-199b-5p), inhibitor negative control (anti-NC, CAGUACUUUUGUGUAGUACAA) were purchased from GenePharma (Shanghai, China), and added to culture media at a final concentration of 100 nM and transfected into cells using Lipofectamine™ 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. Western blot and qRT-PCR were performed at 48 h posttransfection.

qRT-PCR

The PCR amplification for the quantification of the miR-199b-5p and U6 was performed using TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and TaqMan Human MiRNA Assay Kit (Applied Biosystems, Foster City, CA, USA). The relative expression of miR-199b-5p was shown as fold difference relative to U6. The PCR amplification for the quantification of the SSRP1 and GAPDH mRNAs was performed using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and a SYBR® Premix Ex Taq™ ii (Perfect Real Time) Kit (Takara Bio, Shiga, Japan). The primers were as follows: Homo-HER-2, sense: 5'-CCATCTGCACCATTGATGTC-3'; antisense: 5'-ATGCGGGAGAATTCAGACAC-3'; β-actin, sense: 5'-CATTAAGGAGAAGCTGTGCT-3' and antisense: 5'-GTTGAAGGTAGTTTCGTGGA-3'.

Western blot

Whole cell extracts were prepared with a cell lysis reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manual, and then, the protein was quantified by a BCA assay (Pierce, Rockford, IL, USA). Then, the protein samples were separated by SDS-PAGE (10%) and detected by Western blot using with following specific antibodies: HER2 (1:1,000, cs-2242, Cell Signaling Technology), PI3K (1:1,000, Cell Signaling Technology), phospho-AKT (Ser 473, 1:1,000, Cell Signaling Technology), total AKT (1:1,000, Cell Signaling), in 5% bovine serum albumin (BSA) or β-actin (1:5,000; Sigma). Goat anti-rabbit IgG (Pierce, Rockford, IL, USA) secondary antibody conjugated to horseradish peroxidase and ECL detection systems (Super Signal West Femto, Pierce) were used for detection.

Luciferase reporter assay

The 3'-UTR sequence of SSRP1 was amplified from normal human genomic DNA and subcloned into the pmirGLO luciferase reporter vector (Promega). HEK293T cells (3.5×10^4) were seeded in triplicate in 24-well plates and cotransfected with wild-type (WT) or mutant (Mut) 3'-UTR vectors and miR-miR-199b-5p mimics using Lipofectamine 2000. After 48 h, the cells were assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) by following the manufacturer's instructions. The luciferase activities were normalized to Renilla luciferase activity. The luciferase activity of the cells that were transfected with miRNA mimics or inhibitors is represented as the percentage of activity relative to that of cells that were transfected with negative controls. All experiments were performed in triplicate.

Cell proliferation assay and invasion assay

The 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to estimate cell viability [32]. Briefly, cells were plated at a density of 1×10^4 cells per well in 96-well plates. After exposure to specific treatment, the cells were incubated with MTT at a final concentration of 0.5 mg/ml for 4 h at 37°C. After the removal of the medium, 150 mM DMSO solutions were added to dissolve the formazan crystals. The absorbance was read at 570 nm using a multi-well scanning spectrophotometer reader. Cells in the control group were considered 100% viable.

The capability of cell invasion was examined by transwell invasion assay. Cells were cultivated to 80% confluence on the 12-well plates. Then, we observed the procedures of cellular growth at 72 h. All the experiments were repeated in triplicate. The transwell migration chambers were used to evaluate cell invasion. Then cells invading cells across the membrane were counted under a light microscope.

Statistical analysis

Each experiment was repeated at least three times. Data were shown as mean ± s.d. and analyzed using SPSS 18.0. Statistical comparisons between groups were analyzed using Student's t-test and a two-tailed $P < 0.05$ was considered to indicate statistical significance.

Results

miR-199b-5p is downregulated in NSCLC tissue and cells

Here, we performed qRT-PCR to investigate miR-199b-5p expression in NSCLC. As shown in **Figure 1A**, the expression of miR-199b-5p was significantly decreased in human primary NSCLC tissues compared with normal adjacent lung tissues. **Figure 1B** showed that the expression of miR-199b-5p was significantly decreased in human NSCLC cell lines (A549, SK-MES-1, H460 and H520) compared with the normal lung bronchus epithelial cell line 16HBE. Taken together, these results suggest that miR-199b-5p may play an important role in NSCLC progression.

miR-199b-5p directly targeted HER2 in NSCLC

To elucidate whether HER2 is a potential downstream target gene of miR-199b-5p in NPC cells, we constructed luciferase reporter vectors containing the wild-type (Wt) or mutant (Mut) miR-199b-5p target sequences of the HER2 3'-UTR (**Figure 2A**). Overexpression of miR-199b-5p significantly inhibited the luciferase activity of the Wt HER2 3'-UTR reporter gene but not the Mt reporter gene (**Figure 2A**).

Next, we transfected HER2 siRNAs in H460 cells (**Figure 2B**). As shown in **Figure 2**, overex-

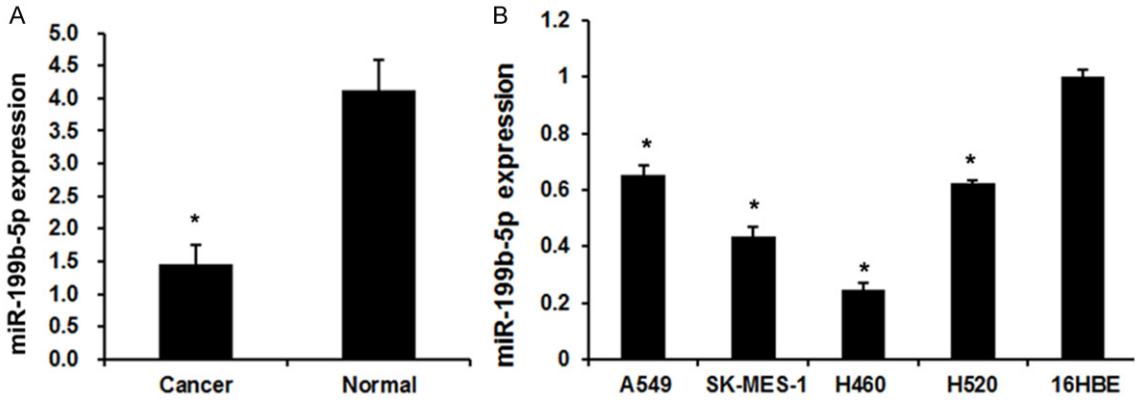

Figure 1. miR-199b-5p expression in NSCLC tissues and cell lines. A. qRT-PCR assay revealed miR-199b-5p expression in NSCLC tissues and adjacent normal tissues. B. qRT-PCR assay revealed miR-199b-5p expression in human NSCLC cell lines (A549, SK-MES-1, H460 and H520) compared with the normal lung bronchus epithelial cell line 16HBE. Error bars represent ± S.E. and $^{*}P < 0.01$ versus normal tissues and 16HBE cell.

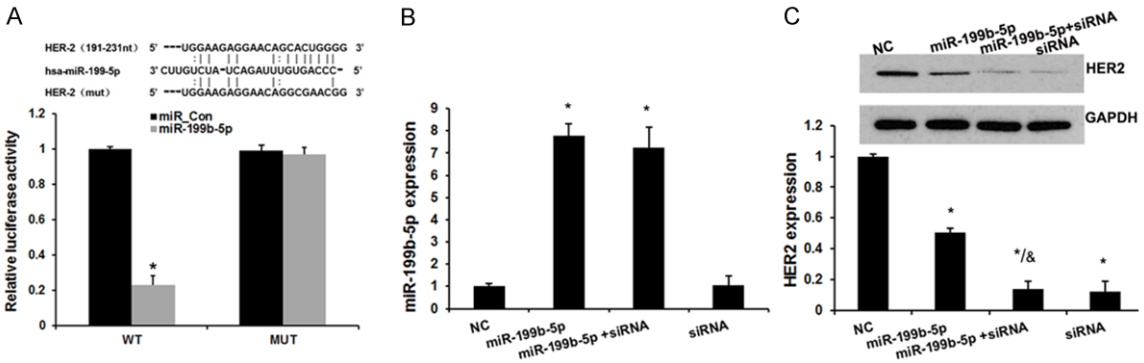

Figure 2. HER2 was a direct target of miR-199b-5p. A. Representative diagram of the predicted wild-type (WT) or mutant (Mut) binding site of miR-199b-5p in the 3'-untranslated region (UTR) of HER2 mRNA. The luciferase reporter plasmid containing the WT or Mut HER2 3'-UTR was cotransfected into HEK293T cells with miR-199b-5p mimics. Luciferase activity of the cells was assayed at 48 h after transfection, and the values were normalized to the normal control values. $^{*}P < 0.01$ (compared with the control). B. qRT-PCR analysis revealed the effects of HER2 siRNA and miR-199b-5p mimics on the expression level of miR-199b-5p. C. Western blot analysis revealed the effects of HER2 siRNA and miR-199b-5p mimics on the expression level of HER2. Error bars represent ± S.E. $^{*}P < 0.01$ versus NC, $^{\&}P < 0.01$ versus miR-199b-5p.

pression of miR-199b-5p markedly reduced the expression of HER2 (**Figures 2C**), but silenced HER2 did not affect miR-199b-5p expression (**Figure 2B**). These results demonstrated that HER2 is a direct target of miR-199b-5p in NPC cells.

miR-199b-5p inhibited HER2 downstream signaling by PI3K/AKT pathways in NSCLC cells

The downstream signaling induced by HER2 includes PI3K/AKT pathway. Because HER2 was direct targeted by miR-199b-5p, we investigated whether miR-199b-5p was able to interfere with these pathways using Western blotting. As shown in **Figure 3**, PI3K, Akt and phospho-AKT (p-AKT) levels relative to HER2 expression were significantly reduced in the H460 cells transfected with HER2 siRNA. A similar result was also observed when miR-199b-5p was overexpressed in H460 cells transfected with miR-199b-5p mimics compared to negative control (NC).

miR-199b-5p regulated NSCLC cell proliferation and invasion by targeting HER2

To determine the role of NSCLC and miR-199b-5p in the NSCLC cell growth and metastasis, H460 cells were transiently transfected with

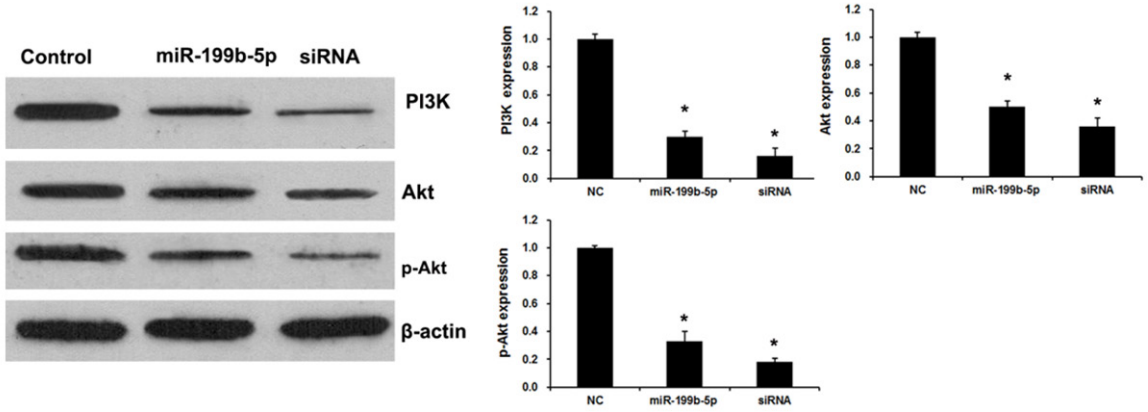

Figure 3. Western blot analysis revealed effects of miR-199b-5p and HER2 on expression of PI3K/AKT pathway in NSCLC.

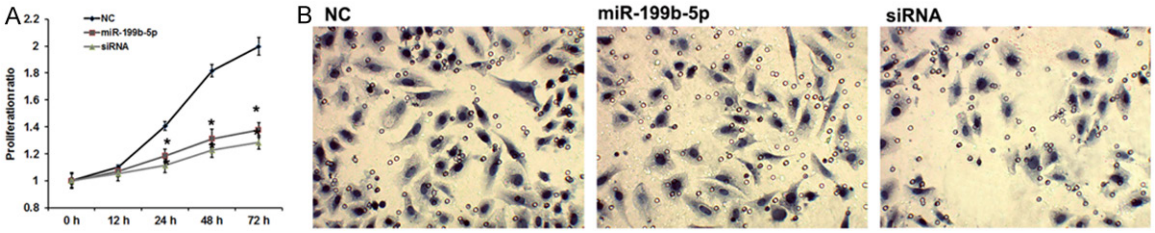

Figure 4. miR-199b-5p promotes cell proliferation and invasion by suppressing HER2 expression in NSCLC. A. MTT assay revealed the effects of miR-199b-5p and HER2 on NSCLC cancer proliferation. B. Transwell invasion assay revealed the effects of miR-199b-5p and HER2 on NSCLC cancer invasion. Error bars represent ± S.E. $*P < 0.01$ versus control and NC.

HER2 siRNA (siRNA) or miR-199b-5p mimic (miR-199b-5p). Consistent with the effects induced by overexpression of miR-199b-5p, knockdown of HER2 significantly suppressed the cell viability and invasion (**Figure 4A** and **4B**).

Discussion

Recently, attentions have focused on the role of miRNA in tumorigenesis. In this study, we focused on miR-199b-5p which was deregulated in several tumor types. Our results showed that miR-199b-5p was downregulated in NSCLC tissue and cells, and HER2 was one direct functional targets of miR-199b-5p in NSCLC cells. miR-199b-5p regulates NSCLC cells proliferation and invasion via HER2/PI3K/ATK pathway.

The aberrant expression of miRNAs is associated with cancer progression including proliferation, migration, invasion and apoptosis. Deregulation of miRNAs such as miR-221, miR-222, miR-143, miR-204, miR-10b, miR-99a, miR-206 and miR-181a in NSCLC is a key factor underlying tumorigenesis [6, 7, 33]. miR-199b-5p is deregulated in various cancers [21-25], however, the expression of miR-199b-5p is less well known in human cancers especially in NSCLC. In this study, we detected downregulated miR-199b-5p in NSCLC tissue and cells, which suggests miR-199b-5p is a potential tumor suppressor in NSCLC. These findings prompted us to investigate the regulation of miR-199b-5p in NSCLC cells.

HER2 is a member of the epidermal growth factor receptor (EGFR) family of transmembrane tyrosine kinase type receptors, and emerging evidences have revealed the association between dysfunction of the HER2 and multiple human diseases including cancer [34, 35]. It had been reported that Her2 expression is relation to survival of patients with non-small cell lung cancers [29-31]. Moreover, HER2 acted as a novel tumor antigen in NSCLC [29, 31]. To investigate the underlying mechanism of up-regulation of HER2 protein levels in NSCLC.

miRNA-binding sites analysis revealed that HER2 as one direct functional target of miR-199b-5p in NSCLC cells. Transfection of miR-199b-5p mimics into NSCLC cells led to a significant decrease of HER2 levels. PI3K/AKT pathway is the downstream signaling induced by HER2, which involves multiple biological progress including tumorigenesis and metastasis. Here, we analyze PI3K/AKT pathway in miR-199b-5p overexpressed and HER2 silenced NSCLC. We found that PI3K, Akt and phospho-AKT (p-AKT) levels relative to HER2 expression were significantly reduced in the H460 cells transfected with HER2 siRNA and miR-199b-5p mimics. These results indicated that miR-199b-5p regulates PI3K/AKT pathway via repressing HER2 expression. Furthermore, repressed HER2 expression by miR-199b-5p in NSCLC cells also led to inhibition of cellular proliferation and invasion.

In conclusion, our results indicated that miR-199b-5p, as tumor suppressor, was downregulated in NSCLC tissue and cells. HER2 was directly targeted and down-regulated by miR-199b-5p, and involved miR-199b-5p-regulated NSCLC cells proliferation and invasion via PI3K/AKT pathway.

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Disclosure of conflict of interest

None.

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