miR-3120-5p acts as a diagnostic biomarker in non-small cell lung cancer and promotes cancer cell proliferation and invasion by targeting KLF4

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Received February 6, 2018; Accepted August 9, 2018

DOI: 10.3892/mmr.2018.9454

Abstract. Accumulating evidence indicates that microRNAs (miRs) are important regulators in a number of types of human cancer, including non-small cell lung cancer (NSCLC). The function of miR-3120-5p in NSCLC remains unclear. In the present study, it was demonstrated that miR-3120-5p was significantly upregulated in NSCLC tissues. Additionally, miR-3120-5p expression level was positively associated with NSCLC metastasis and tumor, node and metastasis stage. Furthermore, it was demonstrated that miR-3120-5p exhibited potential as an indicator of NSCLC for use in diagnosis. Through functional experiments, it was demonstrated that overexpression of miR-3120-5p promoted the proliferation, colony formation and invasion of NSCLC cells. miR-3120-5p overexpression significantly promoted cell cycle progression. Mechanistically, it was demonstrated that Krueppel-like factor 4 (KLF4) was a target of miR-3120-5p in NSCLC cells. Overexpression of miR-3120-5p repressed the expression of KLF4 in A549 and H460 cells. Furthermore, it was demonstrated that KLF4 was downregulated in NSCLC tissues and cell lines. Overexpression of KLF4 significantly reversed the effects of miR-3120-5p on NSCLC cell proliferation and invasion. In conclusion, the present study demonstrated that miR-3120-5p promoted NSCLC progression by directly targeting KLF4.

Introduction

Lung cancer is one of the most prevalent cancers and has the highest mortality rate worldwide (1). Currently, lung cancer has become a severe public health problem in humans (2). Of all lung cancer patients, non-small cell lung cancer (NSCLC) accounts for 80% cases and demonstrates a poor prognosis (3). The high potential of proliferation, invasion and metastasis remains a major obstacle for the treatment of NSCLC patients (4). Currently, it is very difficult to impede the development and progression of NSCLC, particularly at an advanced stage. Therefore, in order to identify effective biomarkers for diagnosis and therapeutic targets, it is necessary to investigate the mechanisms of NSCLC progression.

MicroRNAs (miRs) are a class of short noncoding RNAs (~20-22 nucleotides) and that have been demonstrated to regulate gene expression by binding to the complementary site in the 3'-untranslated region (UTR) of target mRNAs (5,6). An increasing number of studies indicate that miRs are involved in the regulation of a number of biological processes, including cell survival, division, migration and tumor angiogenesis (7,8). Due to their extensive biological functions, abnormal expression of miRs also results in occurrence of human malignances including NSCLC (8). For example, Yang et al (9) reported that miR-769-5p is downregulated in NSCLC and suppresses tumor cell proliferation, migration and invasion. Li et al (10) indicated that miR -9-5p promotes cell growth and metastasis in NSCLC through the repression of tumor growth factor b receptor 2. In addition, Wang et al (11) demonstrated that miR-124 inhibits growth and enhances radiation-induced apoptosis in NSCLC by inhibiting signal transducer and activator of transcription 3. In conclusion, several miRs have been demonstrated to be promising indicators for NSCLC diagnosis or prognosis (12,13). Therefore, identifying the function and mechanism of miRs in NSCLC is necessary.

Up to now, the knowledge regarding miR-3120-5p is limited. Only a recent report indicated that miR-3120-5p promotes colon cancer stem cell stemness and invasiveness (14). The function of miR-3120-5p in NSCLC remains unclear. In the present study, it was demonstrated that miR-3120-5p was significantly upregulated in NSCLC tissues and serves as a diagnostic biomarker. Furthermore, it was demonstrated that overexpression of miR-3120-5p remarkably enhanced the proliferation and invasion of NSCLC cells through directly targeting Krueppel-like factor 4 (KLF4).
results of the present study provided a novel mechanism that the miR-3120-5p/KLF4 axis regulates NSCLC progression and miR-3120-5p may be an indicator for NSCLC diagnosis.

Materials and methods

Clinical specimens. A total of 39 NSCLC tissues (28 males and 11 females) and adjacent normal tissues were obtained from patients who underwent surgery at The Second Affiliated Hospital of Harbin Medical University (Harbin, China) between January 2013 and December 2016. All patients were diagnosed as NSCLC by two professional pathologists. All patients had no preoperative adjuvant therapy. All tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA analysis. These samples were divided into two groups (miR-3120-5p high expression group and low expression group) using the median value of miR-3120-5p expression as the cutoff. Association of the expression of miR-3120-5p with clinicopathological features is listed in Table I. Written informed consent was obtained from all of the participants and the study was approved by the Institutional Review Board of The Second Affiliated Hospital of Harbin Medical University.

Cell culture and transfection. Four human NSCLC cell lines (A549, H1299, H1970 and H460) and one normal bronchial epithelial cell line BEAS-2B were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and supplemented with 10% fetal bovine serum (FBS; HyClone, GE Healthcare Life Sciences Logan, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in an environment containing 5% CO2.

The miR-3120-5p mimic (5'-CCUGUCUGUGCCUGU UGACA-3'), mimic scrambled control (miR-NC; 5'-UC A CA ACC UC C G A A G C A G G A G-3'), miR-3120-5p inhibitor (5'-UGUUCAGGACGCAGACAGG-3') were chemically synthesized by GenePharma Co., Ltd. (Shanghai, China). KLF4 coding sequence was constructed into pcDNA3.1 vector (Addgene, Inc., Cambridge, MA, USA) to obtain pcDNA3.1-KLF4 plasmid. A549 and H460 cells in logarithmic phase were transfected with 100 nM miRs/controls or 1 µg pcDNA3.1-KLF4 using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) coupled with BD Cell Quest Pro™ software according to the manufacturer's protocol. Further experiments were carried out at 48 h post-transfection.

Cell viability assay. Cell viability was determined using a Cell Counting Kit (CCK-8; Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's protocol. Briefly, the transfected cells (2x104 cells/ml) were seeded into a 96-well plate and then 10 µl CCK-8 solution was added into each well of the plate and the plates were cultured for 24, 48 and 72 h. The absorbance of each well at a wavelength of 450 nm was measured using a multidetection microplate reader (BMG Labtech, GmbH, Ortenberg, Germany).

Transwell invasion assay. Cell invasion was detected using Transwell chambers (8-µm pore size; BD Biosciences, Franklin Lakes, NJ, USA) coated with Matrigel (BD, Franklin Lakes, NJ, USA). The transected cells (5x104) suspended in 150 µl serum free RPMI-1640 medium were added into the upper chamber and the bottom compartment of the chamber were filled up with 500 µl RPMI-1640 medium with 20% (v/v) FBS as a chemoattractant. Following incubation for 48 h, the non-invading cells inside the upper chamber were scraped off by a cotton swab. The invaded cells on the lower chamber were then fixed with 4% formaldehyde at 25°C for 30 min and stained with 0.1% crystal violet at 25°C for 30 min. The cells were counted from five independent fields under a light microscope using a x200 magnification.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cultured cells using the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and cDNA was synthesized from total RNA by a PrimerScript RT Reagent kit (Takara Bio Inc. Tokyo, Japan). miR from total RNA was reverse transcribed using the Prime-Script miRNA cDNA Synthesis kit (Takara Bio Inc.). The RT reaction was performed at 37°C for 1 h and terminated at 85°C for 5 min. qPCR was performed with the SYBR green Premix Ex Taq II (Takara Bio Inc.) on an Applied Biosystems Step One Plus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 15 sec and elongation at 60°C for 1 min. GAPDH was used as the endogenous control for detection of mRNA expression level, while U6 was used as endogenous control for miR expression analysis. Relative expression fold was calculated according to the 2-ΔΔCT method (15). Primer details were as follows: U6 forward, 5'-AACGAGGCAGCAGACAG-3' and reverse, 5'-GCAAAT TCGTGAAGCGTCCATA-3'; GAPDH forward, 5'-ATG TTGCAACGGGAAAGGAA-3' and 5'-AGGAAAAACG TCACCAGGAG-3'; miR-3120-5p forward, 5'-AACGAGGCAC GAGCAGACAG-3' and reverse, 5'-CCTGTCTGTCCTGTC TGACA-3'; KLF4 forward, 5'-ATGCTCACCACCCACTTCT TC-3' and reverse, 5'-TTCTCACCTGTGTTGGTCTG-3'.

Flow cytometry analysis (FACS). FACS was used to analyze the cell cycle distributions. The A549 and H460 cells were fixed with 75% ethanol for 4 h at 4°C, washed with PBS, and stained with propidium iodide (PI) supplemented with RNaseA overnight at 4°C. Following staining, the cells were analyzed by flow cytometry using a BD FACSscan flow cytometer (BD Biosciences) coupled with BD Cell Quest Pro™ software (version 2; BD Biosciences).

Western blotting. A549 and H460 cells were lysed in cold radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.), and the protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Proteins (40 µg/lane) were separated via 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Thermo Fisher Scientific, Inc.). Following this, the membrane was blocked in 5% non-fat milk in PBS containing 0.1% Tween-20 (Sigma-Aldrich; Merck KGaA) at room temperature for 3 h. Subsequently, the PVDF membrane
miR-3120-5p expression is associated with cancer metastasis. A Transwell assay was performed to measure the effects of miR-3120-5p on NSCLC cell proliferation and invasion. It was demonstrated that overexpression of miR-3120-5p significantly promoted (P<0.05) the proliferation and colony formation whereas inhibition of miR-3120-5p expression significantly suppressed (P<0.05) cell proliferation in A549 and H460 cells (Fig. 2A). Then, CCK-8 and colony formation assays were performed to measure the effects of miR-3120-5p on NSCLC cell proliferation. It was demonstrated that overexpression of miR-3120-5p significantly enhanced the invasion of A549 and H460 cells and inhibition resulted in the opposite effect (P<0.05; Fig. 2D). The expression patterns of miR-3120-5p in NSCLC tissues and adjacent normal tissues were first analyzed by RT-qPCR. As presented in Fig. 1A, it was demonstrated that miR-3120-5p was significantly upregulated in NSCLC tissues compared with adjacent normal tissues (P<0.0001). To further check the association between miR-3120-5p expression and clinical characteristics, these samples were divided into two subgroups based on tumor metastasis. RT-qPCR analysis indicated that the NSCLC tissues with metastasis (n=23) displayed significantly increased levels of miR-3120-5p compared with non-metastatic tissues (n=16) (P<0.05; Fig. 1B). In addition, it was observed that increased expression of miR-3120-5p was associated with advanced clinical stage (stage III, n=21; Fig. 1C). Consistently, the relative miR-3120-5p expression was examined in four NSCLC cell lines including A549, H1975, H1299 and H460 cells and a normal lung epithelial cell line BEAS-2B. The results indicated that miR-3120-5p expression was higher in the NSCLC cell lines compared with the BEAS-2B cells (Fig. 1D). To determine whether miR-3120-5p could serve as a biomarker for NSCLC diagnosis, receiver operating characteristic analysis was performed. The results indicated that miR-3120-5p displayed a predictive signature, with an area under curve of 0.848 (P<0.01; Fig. 1E). Taken together, the aforementioned data suggested that miR-3120-5p was upregulated in NSCLC tissues and served as a predictor for NSCLC diagnosis.

miR-3120-5p promotes NSCLC cell proliferation and invasion. To investigate the function of miR-3120-5p in NSCLC, A549 and H460 cells were chosen for experiments, as miR-3120-5p expression was the highest in these two cell lines (Fig. 1D). A549 and H460 cells were transfected with miR-3120-5p mimic, inhibitor or negative controls (miR-NC). RT-qPCR analysis indicated that miR-3120-5p was significantly upregulated or downregulated when the mimic or inhibitor was used, respectively (P<0.05; Fig. 2A). Then, CCK-8 and colony formation assays were performed to measure the effects of miR-3120-5p on NSCLC cell proliferation. It was demonstrated that overexpression of miR-3120-5p significantly promoted (P<0.05) the proliferation and colony formation whereas inhibition of miR-3120-5p suppressed cellular proliferation in A549 and H460 cells (Fig. 2B and C). It was demonstrated miR-3120-5p expression is associated with cancer metastasis. A Transwell assay was then used to determine the effect of miR-3120-5p on cell invasion. As presented, overexpression of miR-3120-5p significantly enhanced the invasion of A549 and H460 cells and inhibition resulted in the opposite effect (P<0.05; Fig. 2D).

### Table I. Association of the expression of miR-3120-5p with clinicopathological features.

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<th>Clinicopathological parameters</th>
<th>miR-3120-5p</th>
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<tr>
<td></td>
<td>High (n=19)</td>
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<td>Age (years)</td>
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<td>≤50</td>
<td>13</td>
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<td>&gt;50</td>
<td>6</td>
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<td>Tumor size (cm)</td>
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<td>≤4</td>
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<td>&gt;4</td>
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<td>Lymph node metastasis</td>
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<td>Negative</td>
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χ² test was used to calculate P-values. miR, microRNA.
Effects of miR-3120-5p expression on cell cycle and epithelial mesenchymal transition (EMT) markers of NSCLC cells. Subsequently, the effects of miR-3120-5p on cell cycle were examined. By FACS, it was demonstrated that overexpression of miR-3120-5p significantly reduced the cells in G0/G1 phase, however increased the cell percentage in S phase and G2/M phase (P<0.05; Fig. 3A). Furthermore, the expression levels of proliferation-associated protein proliferating cell nuclear antigen (PCNA) and EMT-associated markers [epithelial (E)-cadherin and neural (N)-cadherin] was measured by western blotting. It was demonstrated that PCNA and N-cadherin levels were markedly upregulated following miR-3120-5p overexpression while E-cadherin expression was downregulated and the opposite effects were observed following miR-3120-5p inhibition (Fig. 3B). Therefore, these data indicated that miR-3120-5p promoted cell cycle progression and NSCLC malignant behaviors.

KLF4 is a target of miR-3120-5p in NSCLC cells. To further investigate the downstream mechanism of miR-3120-5p in NSCLC, the target genes of miR-3120-5p were searched for by TargetScan tool. Among all potential targets, KLF4 ranked top and has been demonstrated to suppress NSCLC progression. Therefore, KLF4 was chosen for the following investigation. First, a wild-type-KLF4-3'-UTR or mutant-KLF4-3'-UTR luciferase reporter vector was constructed (Fig. 4A). Using a luciferase reporter assay, it was demonstrated that overexpression of miR-3120-5p significantly inhibited the luciferase activity in A549 and H460 cells, and inhibition resulted in the opposite effects (P<0.05; Fig. 3B and C), which indicated miR-3120-5p directly targeted KLF4. Furthermore, RT-qPCR analysis and western blotting also demonstrated that miR-3120-5p overexpression significantly inhibited the mRNA and protein levels of KLF4 in A549 and H460 cells (P<0.05; Fig. 4D and E). Taken together, the present study proved KLF4 was a target gene of miR-3120-5p in NSCLC cells.

miR-3120-5p enhances proliferation and invasion by regulating KLF4 in NSCLC cells. To further determine whether KLF4 is involved in the regulation of miR-3120-5p-mediated promotion of NSCLC progression, a series of experiments were performed. Through RT-qPCR analysis and western blotting, it was demonstrated that the expression of KLF4 was significantly downregulated in NSCLC tissues compared with adjacent normal tissues (P<0.05; Fig. 5A and B). Similarly, KLF4 was also downregulated in NSCLC cell lines compared with normal cell line BEAS-2B (Fig. 5C). Then, KLF4 was overexpressed by transfection with pcDNA3.1-KLF4 plasmid in A549 and H460 cells (Fig. 5D). The CCK-8 assays demonstrated that miR-3120-5p overexpression enhanced cell proliferation and overexpression of KLF4 inhibited cell proliferation, compared with the control groups (Fig. 5E). However, cotransfection with miR-3120-5p mimic plus pcDNA3.1-KLF4 plasmid reversed the effects of miR-3120-5p mimic on cell proliferation in A549 and H460 cells (Fig. 5E). Consistently, the cellular invasion was also enhanced by miR-3120-5p mimic and inhibited by KLF4 overexpression (Fig. 5F). Restoration of KLF4 also abolished the effect of miR-3120-5p on NSCLC cell invasion (Fig. 5F). Taken together, these results suggested that miR-3120-5p...
promoted cell proliferation and invasion by regulating KLF4 expression in NSCLC cells.

Discussion

As the most common and prevalent type of cancer around the world, NSCLC gives rise to a large number of cancer-associated mortalities every year (1). There are still no effective therapeutic strategies for NSCLC intervention. Therefore, it is crucial to determine the underlying molecular mechanisms of NSCLC progression. miRs have been widely reported to participate in the progression of human cancer, including NSCLC (16). A number of studies indicate that miRs may be promising therapeutic targets for cancer treatment (17). Therefore, identifying the regulatory mechanism of miRs in tumorigenesis will contribute to the development of effective therapeutic methods. The present study investigated the expression pattern of miR-3120-5p in NSCLC tissues, investigated its effects on NSCLC cells and demonstrated its functional mechanism.

An increasing number of studies suggest that miRs could regulate the proliferation, migration, invasion and apoptosis of cancer cells (18). Quite a number of miRs were observed to be dysregulated in cancer, including in NSCLC (19). For example, miR-520e is downregulated in NSCLC tissues and suppresses cancer growth by targeting the Zbtb7a-mediated Wnt signaling pathway (20). Upregulation of miR-146a increases the sensitivity of NSCLC to cisplatin by downregulating cyclin J (21). Downregulation of miR-218 contributes to EMT and tumor metastasis in lung cancer by targeting Slug/ZEB2 signaling (22). As for miR-3120-5p, a recent study indicated that it regulates colon cancer stem cell stemness and invasiveness (14). Nevertheless, whether miR-3120-5p promotes NSCLC progression remains unknown. In the present study, it was demonstrated that miR-3120-5p was upregulated in NSCLC tissues. Furthermore, high miR-3120-5p expression

Figure 2. miR-3120-5p promotes NSCLC cell proliferation and invasion. (A) Relative expression of miR-3120-5p in A549 and H460 cells transfected with miR-3120-5p mimic, inhibitor or control. (B) Cell Counting Kit-8 assay indicated that miR-3120-5p promoted cellular proliferation. (C) Overexpression of miR-3120-5p enhanced the numbers of colony formation. (D) Transwell assays were utilized to check the invasion of A549 and H460 cells. *P<0.05 vs. the NC group. NSCLC, non-small cell lung cancer; miR, microRNA; OD, optical density; NC, negative control.

Figure 3. Effects of miR-3120-5p expression on cell cycle and epithelial mesenchymal transition markers of non-small cell lung cancer cells. (A) Overexpression of miR-3120-5p promoted cell cycle progression in A549 and H460 cells. (B) Protein levels of PCNA, E-cadherin and N-cadherin in A549 and H460 cells were measured by western blotting. *P<0.05 vs. the NC group. PCNA, proliferating cell nuclear antigen; E, epithelial; N, nuclear; miR, microRNA; NC, negative control.
miR-3120-5p promotes non-small cell lung cancer (NSCLC) proliferation/invasion

XU and WEN: miR-3120-5p PROMOTES NON-SMALL CELL LUNG CANCER PROLIFERATION/INVASION

was associated with NSCLC metastasis and tumor advanced stages. Furthermore, miR-3120-5p was identified as a diagnostic biomarker for NSCLC patients. Through functional experiments, it was demonstrated that miR-3120-5p promoted the proliferation and invasion of NSCLC cells.

KLF4 belongs to the Krueppel family of transcription factors and is thought to control the G1-to-S transition of the cell cycle. KLF4 has been demonstrated to serve the role of oncogene or tumor suppressor in several types of cancer. A previous study demonstrated that KLF4 serves as a tumor suppressor in lung cancer. Enhanced expression of KLF4 in lung cancer cells induces G1/S cell cycle arrest and inhibits tumor growth in vitro and in vivo. For example, a study indicated that KLF4 inhibits invasion of lung cancer cells via suppression of secreted protein acidic and rich in cysteine. Another study demonstrated that KLF4 is
significantly downregulated in lung adenocarcinoma compared with adjacent normal tissues (28). However, how KLF4 expression is regulated in lung cancer requires further investigation. In the present study, it was demonstrated that KLF4 was a direct target of miR-3120-5p in NSCLC cells through bioinformatics analysis. The results of the present study indicated that overexpression of miR-3120-5p suppressed the expression of KLF4 in A549 and H460 cells. Furthermore, it was also demonstrated that KLF4 was downregulated in NSCLC tissues compared with adjacent normal tissues. Furthermore, through CCK-8 and Transwell assays, it was demonstrated that overexpression of KLF reversed the effects of miR-3120-5p on NSCLC cell proliferation and invasion.

In conclusion, the results of the present study demonstrated that miR-3120-5p promoted the proliferation and invasion of NSCLC cells through regulating KLF4 expression. Furthermore, these results implied miR-3120-5p could serve as a diagnostic marker for NSCLC patients and may serve as a potential therapeutic target for NSCLC intervention.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HX initiated, designed this work, analyzed, interpreted the results and wrote the manuscript. QW performed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

For the use of human samples, the protocol for the present study was approved by the Institutional Ethics Committee of The Second Affiliated Hospital of Harbin Medical University and all enrolled patients signed a written informed consent document.

Patient consent for publication

All patients within this study provide consent for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

References


