miR-486-5p inhibits cell proliferation and invasion through repressing GAB2 in non-small cell lung cancer

SUNFENG YU*, SHUANG GENG* and YI HU

Department of Respiratory Medicine, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430014, P.R. China

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Abstract. Previous studies have reported that cell metastasis is the main reason for the high mortality of non-small cell lung cancer (NSCLC). Many miRNAs have been identified to be involved in the development of NSCLC. In this study, we explored the effect of miR-486-5p and GAB2 on cell proliferation and invasion in NSCLC. First, miR-486-5p and GAB2 expression levels were detected in NSCLC through quantitative RT-qPCR, and downregulation of miR-486-5p and upregulation of GAB2 were both identified in NSCLC. Then MTT and Transwell analysis were performed to confirm the functions of miR-486-5p and GAB2 for cell proliferation and invasion in NSCLC. Moreover, miR-486-5p overexpression was found to inhibit proliferation and invasion by suppressing GAB2 in NSCLC cells. Besides, miR-486-5p overexpression lessened GAB2 expression level in NSCLC, while miR-486-5p knockout enhanced GAB2 expression level. Additionally, miR-486-5p was identified to directly target GAB2 through dual luciferase reporter assay. The silence of GAB2 was found to inhibit proliferation and invasion of NSCLC cells. Collectively, miR-486-5p contributed to inhibiting proliferation and invasion of NSCLC cells through regulating GAB2, and miR-486-5p/GAB2 axis may provide a breakthrough for diagnosing NSCLC.

Introduction

The growth rate and metastasis of non-small cell lung cancer (NSCLC) have been reported to be slower and later than that of small cell carcinoma (1). Moreover, NSCLC accounts for 80-85% of the total lung cancer, and now chemotherapy is the main treatment for NSCLC (2). Moreover, low overall 5-year survival and high recurrence rate of NSCLC patients (3) make it urgent to develop novel treatments for NSCLC. Therefore, future direction is to further analyze potential therapeutic targets in the process of NSCLC occurrence.

Recently, microRNA has been paid increasing attention in cancer research in regulation of many biological processes including growth, proliferation, migration, invasion, and apoptosis (4-6). Especially in NSCLC, some downregulated miRNAs such as miR-134, miR-204, miR-34c, and miR-200b were reported to repress the development of NSCLC (7-10). In addition, many upregulated miRNAs such as miR-21, miR-205, miR-211, and miR-484 were reported to promote cell growth, cell proliferation, cell cycle and invasion (11-14). These previous studies elucidated that the alteration of miRNA expression could affect tumorigenesis of NSCLC.

Among these miRNAs, miR-486-5p has been identified in various human cancers and diseases. It has been reported that miR-486-5p was downregulated in hepatocellular carcinoma (15), breast cancer (16), duchenne muscular dystrophy (17) and chronic kidney disease (18). However, studies are rare on miR-486-5p in NSCLC. Based on the above research, we speculated that miR-486-5p expression level might be declined in regulation of the progression of NSCLC. Additionally, targeted genes such as NOB1 (19), RUNX3 (20) and FOXM1 (21) have been found to influence the tumorigenesis of NSCLC. Nonetheless, the role of GAB2 in NSCLC is rarely reported.

In the present study, we explored the effect of miR-486-5p on the NSCLC development. As predicted, miR-486-5p expression was decreased in NSCLC. Moreover, miR-486-5p repressed cell proliferation and invasion in NSCLC through suppressing GAB2. These findings provide a new way for treatment of NSCLC.

Materials and methods

Clinical tissues. Forty-six NSCLC and adjacent tissues were obtained from The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) after receiving written informed consent. None of the patients received treatment prior to the operation. Then the tissues were frozen in liquid nitrogen and stored at
miR-486-5p mimic or inhibitor were incubated for 0-72 h. The cells with cell proliferation. The cells (4x10^4 cells/well) were added onto 96-well plates in medium with 10% FBS. The cells with bovine serum (FBS) and cultured at 37°C with 5% CO₂.

The miR-486 mimic/mimic control (miR10004762-1-5/miR01201-1-5), the miR-486 inhibitor/inhibitor control (miR20004762-1-5/miR02201-1-5) and the GAB2 siRNA (si-GAB2, 5'-AAA CGC UUG UUU AUA CUG CGG-3'), purchased from RiboBio Co., Ltd. (Guangzhou, China), were transferred into NSCLC cells by Lipofectamine 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) based on the manufacturer's instructions.

**Quantitative RT-qPCR.** TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was applied for extracting total RNA containing miRNA to quantify miR-486-5p expression in NSCLC. To obtain the cDNA templates, 1 µg total RNA of each sample was used for reverse transcription using a miScript Reverse Transcription kit (Qiagen GmbH, Hilden, Germany). This reaction was performed at 37°C for 60 min, then 95°C for 5 min. Quantitative RT-qPCR was carried out through the SYBR-Green PCR kit (Tokara Bio, Inc., Osaka, Japan) on ABI 7500 Fast Real-Time PCR system (ABI; Thermo Fisher Scientific, Inc.). The cycling conditions for RT-qPCR were as follows: 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec and 60°C for 45 sec. U6 and GAPDH were used as control for miR-486-5p and GAB2. The primers were: GAPDH forward, 5'-CACAGTGGAGCTTGGCTCAT-3'; reverse, 5'-TGGTGGCTGTCCTGAGATG-3'; U6 forward, 5'-CCAGAGAAGATCTGGCCCTGTC-3'; reverse, 5'-GGGAGGAGAGTTGTGGTGG-3'; AGGAGAGGAGGGGTCGAT-3' miR-486-5p forward, 5'-ACACCTCACTGTGAGCTGAGG-3'; reverse, 5'-TATCTGAGTGAGGACTGAGG-3'; GAB2 forward, 5'-ACACTCCAGCTGTGAGCTGAGG-3'; reverse, 5'-TCTCACTGTGAGGACTGAGG-3'; GAPDH forward, 5'-TGCAGTGGAGCTTGGCTCAT-3'; reverse, 5'-GCAGAGAAGATCTGGCCCTGTC-3'; U6 forward, 5'-TCTCGCTTGCCAGACCA-3'; reverse, 5'-TACGCTTCACCGATATTGGCTG-3'. The expression was calculated using the 2^-ΔΔct method (22).

** Luciferase activity assay.** The wild or mutant type of 3'-UTR of GAB2 was inserted into the pGL3 promoter vector (Invitrogen; Thermo Fisher Scientific, Inc.) for luciferase reporter experiments. GAB2 vector (pCDNA3.1-GAB2) was purchased from RiboBio Co., Ltd. (Guangzhou, China). Then, we transfected GAB2 vector and miR-486-5p mimic into A549 cells. Subsequently, the dual luciferase reporter assay (Promega Corporation, Madison, WI, USA) was applied to perform luciferase assays.

**MTT assay.** The MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-di-phenyl-2H-tetrazolium bromide) assay was applied to measure cell proliferation. The cells (4x10^4 cells/well) were added onto 96-well plates in medium with 10% FBS. The cells with miR-486-5p mimic or inhibitor were incubated for 0-72 h. After incubation, the cells were added with MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and were cultured at 37°C for 4 h. The absorbance at 490 nm (OD=490 nm) was detected with a spectrophotometer (Model 680 microplate reader; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Cell invasion assay.** The cells were plated into the upper chambers (8 µm pore size; Corning Incorporated) and medium with 10% FBS was put into the lower chamber. Then these cells (5x10^4) were cultured for 24 h at 37°C in atmosphere with 5% CO₂. Then the invasive cells in the lower chamber were fixed with 70% ethanol and stained using crystal violet. Finally, light microscope (DM IRB; Leica Microsystems GmbH, Wetzlar, Germany) was used to measure the cell number.

**Western blot analysis.** The protein samples were obtained using RIPA lysis buffer. 10% SDS-PAGE was employed to separate proteins which were incubated with 5% non-fat milk in polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) at room temperature. Next, we incubated the membranes overnight at 4°C with rabbit monoclonal anti-GAB2 (1:1,000; catalog no. ab203478; Abcam, Shanghai, China), rabbit polyclonal anti-GAPDH (1:1,000; catalog no. ab70699; Abcam) and subsequently incubated with goat anti-rabbit IgG-H&L secondary antibody (1:1,000; catalog no. ab150077; Abcam). Then, protein expression levels were measured by an Enhanced Chemiluminescence Immunoblot Detection system (Pierce; Thermo Fisher Scientific, Inc.) and analyzed using Quantity One software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Statistical analysis.** The obtained data are shown as the mean ± standard deviation. The difference between the groups was calculated through Chi-square test or one-way ANOVA with Tukey's as a post hoc test. Statistical analysis was analyzed with GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 19.0 (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Expression of miR-486-5p and GAB2 mRNA was detected in NSCLC.** First, we detected miR-486-5p expressions in NSCLC via quantitative RT-qPCR. The results suggested that miR-486-5p expression was reduced in NSCLC tissues compared with normal tissues (Fig. 1A). Moreover, the down-regulation of miR-486-5p was also identified in SPC-A1 and A549 cell lines (Fig. 1B). Subsequently, the expression of GAB2 was analyzed in NSCLC as well. Interestingly, GAB2 mRNA expression was increased in NSCLC tissues and cell lines (Fig. 1C and D) which was contrary to miR-486-5p expression.

**miR-486-5p suppresses cell proliferation and invasion in NSCLC.** The function of miR-486-5p in NSCLC was verified by performing MTT and Transwell assay in A549 cells containing miR-486-5p mimic or inhibitor. Primarily, the miR-486-5p expression level was detected in cells with miR-486-5p mimic or inhibitor as shown in Fig. 2A and B. Importantly, MTT...
assay showed that miR-486-5p overexpression suppressed cell proliferation, while miR-486-5p downregulation exhibited the opposite result in NSCLC (Fig. 2C and D). Transwell assay suggested that the cell invasion was significantly repressed.
miR-486-5p inhibits cell proliferation and invasion in lung cancer by miR-486-5p overexpression. However, it was promoted by miR-486-5p inhibitor in NSCLC cells (Fig. 2E). These findings indicated that cell proliferation and invasion were inhibited in NSCLC by miR-486-5p overexpression.
GAB2 is a direct target gene of miR-486-5p in NSCLC. The target genes of miR-486-5p were searched through the database of TargetScan Human (http://www.targetscan.org/vert_71/), which indicated that it binds with the 3'-UTR of GAB2 (Fig. 3A). Moreover, we confirmed that miR-486-5p directly targeted GAB2 and had a binding site with the wild-type of GAB2 by dual luciferase reporter assay (Fig. 3B). Additionally, mRNA and protein expression of GAB2 were reduced by miR-486-5p mimics whereas increased by miR-486-5p inhibitor (Fig. 3C and D). Furthermore, si-GAB2 was transfected into A549 cells to further explore its role in NSCLC (Fig. 3E). Additionally, we found that si-GAB2 had the similar effect as the upregulation of miR-486-5p which suppressed cell proliferation and invasion in NSCLC (Fig. 3F and G). Therefore, it was inferred that miR-486-5p directly targeted GAB2 and knockout of GAB2 inhibited NSCLC cell proliferation and invasion.

miR-486-5p regulates cell proliferation and invasion through suppressing GAB2 in NSCLC. Finally, we transfected GAB2 vector and miR-486-5p mimics into NSCLC cells to verify their relationship, and we found that there was almost no change in expression of mRNA and protein of GAB2 in cells with miR-486-5p mimic and GAB2 vector in comparison with the control (Fig. 4A and B). Importantly, the suppression of miR-486-5p for proliferation of NSCLC cells was impaired by GAB2 vector (Fig. 4C). Moreover, the inhibitory action of miR-486-5p for cell invasion almost disappeared in GAB2 overexpression group (Fig. 4D). All these findings indicated that miR-486-5p inhibited cell proliferation and invasion to a certain extent through the regulation of GAB2 in NSCLC.

Discussion

A full understanding of the occurrence and development mechanisms of NSCLC is necessary for opening a new pathway for NSCLC patients to improve survival. We conducted this research to explore the alteration of miR-486-5p expression and its effect on the progression of NSCLC. Moreover, miR-486-5p expression levels were declined in NSCLC which promoted cell proliferation and invasion in NSCLC. Additionally, GAB2 was confirmed as a direct target gene of miR-486-5p in NSCLC. We considered that miR-486-5p would repress NSCLC development by affecting GAB2 expression, indicating that miR-486-5p had a suppressive effect on NSCLC progression.

In various human cancers, miR-486 is usually expressed aberrantly and has inhibitory action. Previous studies demonstrated that miR-486 could be used as a biomarker for early diagnosis and recurrence of NSCLC (23), and had an inhibitory action for the growth and development of NSCLC (24). Other researchers also found the downregulation of miR-486 in prostatic carcinoma (25) and esophageal cancer (26) which was similar to our results. Additionally, low miR-486-5p expression was identified in NSCLC and promoted tumor metastasis and development by regulating ARHGAP5 (27). Our findings also suggested miR-486-5p repressed cell proliferation and invasion by suppressing GAB2 in NSCLC. Although miR-486 was confirmed to associate with tumorigenesis and progression of NSCLC, its relationship with GAB2 in NSCLC is still not clear.

Grb2-associated binding protein 2 (GAB2) was frequently detected in many human malignancies (28). As an onco-gene, GAB2 has been identified in glioblastoma (29), gastric cancer (30) and renal cell carcinoma (31). Previously, GAB2 was identified to express highly in lung cancer (32). We found the upregulation of GAB2 in NSCLC as well. Functionally, GAB2 promoted cell proliferation and invasion in NSCLC. Similar to our results, GAB2 was also reported to have a positive impact on cell migration in NSCLC via Akt signaling pathway (33). Based on these results, we considered that GAB2 obviously promoted growth and metastasis of NSCLC. Hence, the low expression of GAB2 induced by miR-486-5p could help repress NSCLC development. However, due to the poor current laboratory conditions, there are still several limitations in this research such as the deficiency of in vivo experiments. We will further perform the experiments to validate our conclusion in future research.

In conclusion, we found that miR-486-5p was downregulated in NSCLC. Moreover, miR-486-5p inhibited cell proliferation and invasion through repressing GAB2 in NSCLC. This new pathway may help us better understand NSCLC pathogenesis and provide beneficial clues for the diagnosis and treatment of NSCLC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SY contributed significantly to the study design and data acquisition; SG performed the data analyses and wrote the manuscript. YH contributed to the conception of the study. All authors read and approved the final study.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Signed informed consents were obtained from the patients or the guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
miR-486 regulates metastasis and invasion in lung cancer.

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