Exosomal miR-660-5p promotes tumor growth and metastasis in non-small cell lung cancer

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Summary

Purpose: Non-small cell lung cancer (NSCLC) is still the commonest fatal malignancy worldwide. The relationship between miR-660-5p and progress of NSCLC has not been well confirmed in recent studies. This manuscript focused to the function of miR-660-5p during the appearance and progression of NSCLC.

Methods: To identify the expression level of miR-660-5p in NSCLC, patient plasma and exosomes, quantitative real-time polymerase chain reaction (qRT-PCR) assay was performed. Cell proliferation and colony formation abilities were examined by Cell Counting Kit-8 (CCK-8) assay and colony formation assay. Then, the influence of miR-660-5p on migration and invasion was analyzed by transwell assay. Bioinformatics and Luciferase report assay were used to find potential target genes. Western blot was chosen to assess the expression level of KLF9. Stably transfected NSCLC cells (A549 and H1299) were injected into nude mice to identify the function of miR-660-5p in tumorigenesis in vivo.

Results: Compared with healthy controls, the release of miR-660-5p in plasma and exosomes was increased in patients with NSCLC (n=80). Knockdown of miR-660-5p significantly suppressed proliferation, migration, and invasion, whereas overexpression of miR-660-5p had the opposite effect. KLF9 might be a potential target of miR-660-5p. In addition, up-regulation of miR-660-5p promoted tumorigenesis in vivo, and the protein level of KLF9 also decreased in xenografts.

Conclusions: Our current study suggests that miR-660-5p may control NSCLC proliferation, viability, and metastasis by targeting KLF9, which provides a potential therapeutic target for NSCLC.

Key words: exosomal miR-660-5p, KLF9, metastasis, NSCLC, proliferation

Introduction

Lung cancer is still the main cause of cancer-related deaths in both developed and less-developed countries [1]. As the main subtype of lung cancer, non-small cell lung cancer (NSCLC) can be further divided into the following subtypes: lung adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma [2]. More accurate biological therapies for different gene profiles are currently the most effective methods for treating NSCLC [3,4]. However, early diagnosis rate of NSCLC is not high, which emphasizes the need for early detection and therapeutic targets.

Intercellular communication including vesicles through cells is a very important process for both normal cells and tumor cells [5]. Exosomes, as one of the mediators in cell communication, have...
been shown to carry lipids, proteins, mRNA, non-coding MicroRNA, and even extracellular DNA [6]. Exosomes may play a role in immune surveillance by acting as an origin of tumor antigens, presenting antigens to T cells, and promoting spread of infectious agents [7]. Previous studies also found that exosomes might affect tumor progression including angiogenesis, extracellular matrix degradation and metastasis [8-10]. In the next few years, due to the diagnostic and prognostic properties of exosomes, they are expected to become members of liquid biopsies in NSCLC [11].

MicroRNAs (miRNAs) are small RNAs of 21-25 nucleotides that bind to partially complementary sequences in the 3'-untranslated region (3'-UTR) of messenger RNA (mRNA) and negatively affect post-transcriptional regulation [12,13]. Previous studies had found that miR-1247-3p was secreted from highly metastatic HCC cells, an exosome that directly down-regulated B4GALT3, bringing about activation of NF-κB signaling, thereby promoted tumor progression [14]. Exosomal miR-146a-5p inhibited autophagy by targeting ATG12, thereby increasing chemotherapeutic sensitivity of NSCLC to cisplatin [15]. For miR-660-5p, studies had found that it regulated the malignant performance of breast cancer cells by down-regulation of TFCP2, and was a novel therapeutic target for clinical treatment and potential prognostic indicator [16,17]. Yet, it remains to be seen what role miR-660-5p plays in the progression of NSCLC, especially in exosomes.

KLF9 is one of the earliest identified genes in the KLF (Krüppel-like factors) family and is essential for different physiological processes including proliferation, differentiation, and apoptosis [18,19]. In addition, KLF9 expression is remarkably reduced and shows negative correlation with the expression of matrix metalloproteinase 9 (MMP9) in breast cancer, and inhibits breast cancer metastasis [20]. In lung cancer, miR-570 controlled the progression of lung cancer through the KLF9 molecular network [21]. KLF9 bound to TCF4 and inhibited the expression of β-catenin/TCF signaling and its target gene Cyr61, thereby inhibited the progression of esophageal squamous cell carcinoma (ESCC) [22].

In this manuscript, we first discovered that miR-660-5p was expressed at high levels in the blood plasma and exosomes of NSCLC patients. In vitro experiments revealed that miR-660-5p promoted cell proliferation, migration, and invasion. Bioinformatics predictions, luciferase report assay, and western blot analysis were used to show that KLF9 was a possible downstream target of miR-660-5p. Finally, in vivo experiments demonstrated that miR-660-5p promoted tumor growth.

### Methods

#### Blood sample collection

Forty NSCLC patients having no preoperative radiotherapy or chemotherapy and 40 healthy controls who were hospitalized in Jiangsu Province Hospital of Traditional Chinese Medicine (TCM) were selected for study enrollment since 2018. This study was approved by the Ethics committee of Jiangsu Province Hospital of TCM. Patients with previous history of cancer were excluded from the study. Blood samples from all patients and healthy controls were taken at the hospital on a fasting basis. All samples were immediately centrifuged and placed in liquid nitrogen.

#### Exosome isolation and characterization

Blood samples were centrifuged to extract exosomes. For tumor cell samples, supernatants were collected after logarithmically growing cells (2×10⁵/plate) for 48 h in 10 cm plates. The supernatant was centrifuged at 10,000 g for 30 min to remove subcellular components and the concentrated solution was then filtered. The pellets were resuspended in 50 mL phosphate buffered saline (PBS) solution (Gibco, Rockville, MD, USA), mixed and centrifuged at 10,000 g for 60 min. One mL of PBS solution was used to suspend the purified exosome solution into the tube (Corning, Corning, NY, USA) and stored at -80°C.

**Transmission electron microscopy (TEM)**

The exosomes obtained were resuspended by adding 1 mL of PBS solution, followed by addition of 1 mL of 40% formaldehyde solution (Gibco, Rockville, MD, USA) to fix exosomes. Fifty μL of the fixative solution (Gibco, Rockville, MD, USA) was dropped on the carbon film of the microgrid. Then 1% glutaraldehyde solution (Invitrogen, Carlsbad, CA, USA) was added to fix the exosomes once. TEM was used to observe the morphology of exosomes.

#### Cell culture

NSCLC lines (H1299, H460, A549, H358) and normal human bronchial epithelial cells 16HBE were purchased from Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco’s modified Eagle medium (DMEM) (Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin (Gibco, Rockville, MD, USA) and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in an incubator containing 5% CO₂.

#### Cell transfection

Lentivirus (LV-hsa-miR-660-5p-mimics, LV-hsa-miR-660-5p-inhibitor, LV-NC) was purchased from Genechem (Shanghai, China). The lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used for transfection according to the manufacturer’s instructions.

#### Total RNA extraction

An appropriate number of exosomes was lysed each time by adding 1 mL TRizol solution (Invitrogen,
Carlsbad, CA, USA). Transfected cells in the logarithmic growth phase were digested with trypsin, washed once with PBS solution (Hyclone, South Logan, UT, USA) and lysed by adding 1 mL of TRIzol solution. Total RNA was then extracted with phenol chloroform. The purity of the extracted RNA was measured by UV spectrophotometry and labeled. Finally, the RNA samples were stored in a -80°C freezer.

*Quantitative real-time polymerase chain reaction (qRT-PCR)*

Reverse transcription was performed according to the instructions of the PrimeScript RT reagent (TaKaRa, Kusatsu, Japan). QRT-PCR was performed by choosing the SYBR Green Master Mix I (TaKaRa, Kusatsu, Japan) on the ABI 7500 Fast Real Time PCR System (ABI, Foster City, CA, USA). All miRNA samples were calibrated with U6 and all mRNA samples were calibrated with glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative quantification was performed using the 2^{\Delta\Delta C_t} method. For miRNAs, primer probes were designed and synthesized by Gemma (Shanghai, China). For mRNA, primer 5.0 was used to design gene primers and the specific gene primer sequence was as follows: KLF9 F: 5’-ACAGTGGCTGTGGGAAAGTC-3’, R: 5’-TCACAAGCGTTGGCCAGCG-3’, β-actin F: 5’-GATCATTGCTCCTCCTGAGC-3’, R: 5’-ACTCCTGCTTGCTGATCCAC-3’. Each sample was run in triplicate.

*Colonies formation*

Approximately 1.5×10^3 transfected NSCLC cells were seeded on 35 mm plates (Corning, Corning, NY, USA). Ten days later, crystal violet staining solution (Beyotime, Shanghai, China) was used to stain the plates, and colonies of ≥ 20 cells were chosen for counting. Each sample was run in triplicate.

*Cell Counting Kit-8 (CCK-8) assay*

The stably transfected NSCLC cells were seeded in 96-well plates (Corning, Corning, NY, USA) and then stained with 10 μL of CCK-8 solution (Dojindo, Tokyo, Japan) at 37°C for 3 hrs. Each absorbance was measured at 450 nm (A450) by using a spectrophotometer. Each sample was run in triplicate.

*Transwell assay*

A suitable amount of transfected cell suspension (FBS free) was seeded in the top chamber of pre-coated...
or uncoated insert Matrigel (BD, Franklin Lakes, NJ, USA). In the lower chamber, a medium supplemented with 10% FBS (Gibco, Rockville, MD, USA) was added. After 36 hrs of incubation, cells that migrated to the lower chamber were stained with crystal violet and counted at 10 random fields per well (magnification×100). Each sample was performed in triplicate.

**Luciferase report assay**

The wild sequence and mutated sequence of KLF9 were inserted into the pLG3 promoter vector (Promega, Madison, WI, USA), respectively and were named pLG3-KLF9-WT and pLG3-KLF9-MuT. HEK293 cells were paven in 6-well plates (Corning, Corning, NY, USA) and transfected with 100 ng of pLG3-KLF9-WT or pLG3-KLF9-MuT, miR-660-5p mimics and NC with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After 48 hrs, transfected cells were obtained for evaluation. Relative luciferase activity was conducted by using the Luciferase Assay Kit (Promega, Madison, WI, USA).

**In vivo tumor xenograft model**

The study was approved by the Animal Ethics Committee of Nanjing University of TCM Animal Center. Six 5-week-old female BALB/c nude mice were randomly divided into two groups. Then, a total of 1×10⁶ A549 cells (transfected with LV-660-5p or LV-NC) were injected subcutaneously. Two groups were monitored every 4 days and finally euthanized after 20 days.

**Western blot**

The tumor tissues of the nude mice were put in liquid nitrogen and radioimmunoprecipitation assay (RIPA) lysate was added (Beyotime, Shanghai, China). The following steps were followed by adding EDTA (Ethylene Diamine Tetraacetic Acid) (Beyotime, Shanghai, China): Protease inhibitors (Beyotime, Shanghai, China) and phosphatase inhibitors according to the manufacturer’s instructions (Beyotime, Shanghai, China). Protein samples were added to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (Beyotime, Shanghai, China) in

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**Figure 2.** Effects of miR-660-5p on NSCLC cell proliferation. (A): Transfection efficiency of lentivirus LV-miR-660-5p, LV-NC, LV-miR-660-5p-inhibitor. Total RNA was detected by qRT-PCR and U6 was used as an internal control. The Figure shows that the expression level of miR-660-5p in H1299 cells transfected with LV-HSA-miR-660-5p-mimics was significantly higher than in the negative control. The expression level of miR-660-5p in A549 cells transfected with LV-HSA-mir-660-5p-inhibitor was significantly lower than negative control. (B): CCK-8 assay was performed to determine the viability of A549 and H1299 cells. The Figure shows that overexpression of miR-660-5p promoted proliferation compared to LV-NC group. On the contrary, inhibition of miR-660-5p expression suppressed proliferation compared to anti-NC group; (C) and (D): Colony formation assay was performed to determine the proliferation of A549 and H1299 cells. Figure C shows that overexpression of miR-660-5p promoted colony formation and Figure D shows that knockdown miR-660-5p inhibited colony formation. Data are presented as mean±SD of three independent experiments. *p<0.05 **p<0.01, ***p<0.001.
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a 1:4 volume ratio and mixed well. The samples were then placed in boiling water for 5 min. Proteins extracted from BV2 cells and tissues were separated by 10% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat dry milk (BD, Franklin Lakes, NJ, USA), the membrane was incubated at 4°C with 5% non-fat dry milk (BD, Franklin Lakes, NJ, USA) for 2 hrs with the following antibodies: KLF9 (ab227920) (1:1000) and β-actin (ab8226) (1:2000; Abcam, Cambridge, UK). The membranes were then incubated with HRP-conjugated anti-mouse IgG (1:2000) for 2 hrs at room temperature and then incubated thrice with tris buffered saline-tween (TBST) (Beyotime, Shanghai, China). Immunoreactive bands were visualized by an enhanced chemiluminescence (ECL) system (BD, Franklin Lakes, NJ, USA). The experiments were performed in triplicate.

Statistics

All experimental data were analyzed by GraphPad software 6.0 (La Jolla, CA, USA) and SPSS 18.0 (SPSS Inc., Chicago, IL, USA). The p values were evaluated using Student’s t-test, one-way ANOVA and Spearman’s test. Comparison between groups was done using one-way ANOVA test followed by Post Hoc test (least significant difference). P<0.05 was considered statistically significant.

Results

MiR-660-5p was overexpressed in NSCLC exosomes

First, exosomes from patients with NSCLC and exosomes from NSCLC cell lines were extracted and photographed (Figure 1A and 1B). As shown in Figure 1C, the expression level of miR-660-5p was higher in patient’s plasma than in corresponding healthy controls. Similarly, miR-660-5p was significantly higher in exosomes of NSCLC cell lines than in healthy controls (Figure 1D). To further verify the changes in expression of miR-660-5p, qRT-PCR was performed on exosomes of NSCLC cell lines and 16HBE cells. We found that the expression of miR-660-5p in exosomes of NSCLC cell lines was significantly higher than that of 16HBE cells (Figure 1E). The above results indicate that miR-660-5p exhibits a trend for overexpression in exosomes of patients with NSCLC and cells.

Figure 3. Effects of miR-660-5p on NSCLC cell migration and invasion. (A): Migration assay was performed to detect migration ability of A549 and H1299 cells. The Figure shows that overexpression of miR-660-5p promoted the migration of A549 cells, while knockdown of miR-660-5p in H1299 cells attenuated the ability of migration. (magnification ×100); (B): Invasion assay was performed to detect invasion ability of A549 and H1299 cells. The Figure shows that overexpression of miR-660-5p promoted the invasion ability, while inhibition of miR-660-5p suppressed the invasion ability (magnification ×100). Data are presented as mean±SD of three independent experiments. *p<0.05, **p<0.01.
MiR-660-5p accelerated cell proliferation of NSCLC cells

Based on the expression level of miR-660-5p in exosomes of the NSCLC cell lines, H1299 cells were chosen to knock down the expression of miR-660-5p, and A549 cells were selected for transfection with lentivirus to achieve overexpression of miR-660-5p. As shown in Figure 2A, transfection efficiency of miR-660-5p was determined by qRT-PCR. Then, we performed CCK-8 assay and colony formation assay to determine the function of miR-660-5p on NSCLC cell proliferation. In Figure 2B, overexpression of miR-660-5p promoted proliferation compared to LV-NC group. On the contrary, inhibition of miR-660-5p expression showed the opposite performance. miR-660-5p promoted colony formation, whereas the knockdown group showed the opposite result (Figure 2C and 2D). These observations indicated that overexpressed miR-660-5p might promote cell growth of NSCLC in vitro.

Overexpression of miR-660-5p promoted cell migration and invasion of NSCLC cells

In order to explore the influence of miR-660-5p on migration and invasion, transwell assay were applied. As shown in Figure 3A, overexpressed miR-660-5p promoted the migration of A549 cells, while knockdown of miR-660-5p attenuated the ability of migration. As shown in Figure 3B, overexpression of miR-660-5p promoted the invasion ability, while inhibition of miR-660-5p showed the opposite result. In summary, miR-660-5p enhanced the ability of migration and invasion in vitro.

KLF9 is a potential target of miR-660-5p

Previous studies have shown that miRNAs interact with the target genes via the 3’ UTR [23]. To explore the direct targets for miR-660-5p, MicroRNA.org, MiRWalk and TargetScan were applied. The predicted binding site for miR-660-5p and KLF9 is shown in Figure 4A. In NSCLC cell

Figure 4. KLF9 is a direct target of miR-660-5p. (A): Luciferase reporter assay was conducted to verify that miR-660-5p directly binds to the 3’-UTR regions of KLF9; (B): Relative expression of KLF9 mRNA in NSCLC cell lines and 16HBE. The Figure shows that the mRNA expression of KLF9 in NSCLC cell lines was significantly lower than that in 16HBE; (C): Relative expression of KLF9 mRNA in stably transfected cells showing that overexpression of miR-660-5p in A549 cells significantly decreased the KLF9 mRNA expression. Downregulation of mir-660-5p in H1299 cells increased the expression of KLF9; (D): Luciferase activity was analyzed in cells co-transfected with miR-660-5p-mimics or negative control with pGL3-KLF9-WT or pGL3-KLF9-MuT. Total RNA was detected by qRT-PCR and β-actin was used as internal control. The Figure shows the luciferase activity of WT group was significantly lower than that of MUT group after 48 h. Data are presented as mean±SD of three independent experiments. *p<0.05, **p<0.01.
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lines, the expression level of KLF9 was clearly reduced (Figure 4B). After overexpression of miR-660-5p in A549 cells, the KLF9 mRNA expression was significantly decreased. On the contrary, the expression of KLF9 was increased in H1299 cells after downregulation of miR-660-5p (Figure 4C). To further verify the binding of miR-660-5p and KLF9, Luciferase activity assay was performed. The wild type and mutation site of KLF9 were inserted into the luciferase reporter vector. Then, the pGL3 luciferase reporter vector and miR-660-5p mimics or NC were co-transfected into HEK-293 cells. As shown in Figure 4D, the luciferase activity of WT group was meaningfully lower than that of Mut group after 48 hrs. The above results indicated that KLF9 might be a potential target of miR-660-5p.

**MiR-660-5p promoted tumorigenesis in vivo**

In order to further verify the role of miR-660-5p, A549 cells (stably upregulated miR-660-5p) and H1299 cells (stably downregulated miR-660-5p) were inoculated into each nude mouse in vivo. After 20 days, the tumors were removed and photographed (Figure 5A). In situ tumor growth was measured every 4 days over 20 days (Figure 5B). qRT-PCR showed that the expression miR-660-5p was remarkably increased in LV-miR-660-5p group (Figure 5C). Similarly, as shown in Figure 5D and 5E, the upregulation of miR-660-5p lowered KLF9 protein and mRNA expression. These results revealed that miR-660-5p promoted tumorigenesis of NSCLC.

**Discussion**

Lung cancer is still one of the most common cancers in the world. In developing countries, lung cancer is also the commonest cause of cancer-related deaths [24]. In recent years, it has been discovered that exosomes are a type of extracellular vesicles secreted by cells, which transfer a variety of biomolecules (proteins, mRNAs, microRNAs), including intercellular communication and regulate tumor-host interactions [6]. Previous studies have found that exo-miR-126 inhibited cell proliferation and induced apoptosis of NSCLC cells,

Figure 5. miR-660-5p promotes NSCLC growth in vivo. (A): Photographs of tumors obtained from the different groups of nude mice transfected with LV-660-5p and LV-NC, respectively. The Figure shows that the appearances of tumors in LV-miR-660-5p group were significantly larger than those in NC group. Scale, 1 cm; (B): Growth curves of tumor volumes. Tumor volume was evaluated using the following formula: tumor volume=(width^2 x length)/2. The Figure shows that the volumes of tumors in LV-miR-660-5p group were significantly larger than those in NC group. (C): The expression levels of miR-660-5p in the implanted tumors transfected with LV-660-5p and LV-NC were explored by miR qRT-PCR. Total RNA was detected by qRT-PCR and U6 was used as internal control. The Figure shows that the expression of miR-660-5p was significantly increased in LV-miR-660-5p group; (D): The expression levels of KLF9 in the implanted tumors that were transfected with LV-660-5p and LV-NC were explored by Western blot. The Figure shows that upregulation of miR-660-5p lowered KLF9 protein expression. (E): Total RNA was detected by qRT-PCR and β-actin was used as internal control. The Figure shows that upregulation of miR-660-5p lowered KLF9 mRNA expression. Data are presented as mean±SD of three independent experiments. **p<0.01, ***p<0.001.
which played a major role in regulating the niche of NSCLC microenvironment [25].

In this manuscript, miR-660-5p was first found to be highly expressed in the plasma and exosomes of cancer patients. To investigate the function of miR-660-5p in NSCLC, CCK-8 assay and colony formation assay revealed that miR-660-5p might promote the growth of A549 and H1299 cells. The role of miR-660-5p in promoting migration and invasion was also clarified via transwell assay. KLF9 was subsequently proven as a possible target of miR-660-5p. Previous reports have shown that overexpressed miR-378 binds directly to the 3'-UTR region of KLF9 mRNA and inhibited the expression of KLF9, thereby significantly promoting cell proliferation of osteosarcoma cells [26]. The decreased KLF9 expression promoted the proliferation of glioma cells in vitro and impanted tumors growth in vivo. KLF9 also interacted with the promoter of miR-21, resulting in inhibition of miR-21 expression and arrest of cell cycle [27]. In lung cancer, miR-570 directly downregulated KLF9 expression, promoted NSCLC cells proliferation and functioned as an oncogene in NSCLC pathogenesis [21]. Through in vivo experiments, we confirmed the role of miR-660-5p in promoting tumor progression. However, there are still some flaws in this study. KLF9 is just one of many potential targets of miR-660-5p. In this experiment, we only confirmed that miR-660-5p accelerates cell proliferation, migration, and invasion by targeting KLF9. Whether there are other mechanisms still needs further investigations. This study partially supports that miR-660-5p may promote tumor growth and metastasis by reducing the expression of KLF9, and act as a novel target for the diagnosis and treatment of NSCLC.

Conclusions

Our current study indicates that miR-660-5p has a tumor-promoting effect on the tumorigenesis and metastasis of NSCLC in vitro and in vivo. Moreover, miR-660-5p partially inhibits KLF9 to achieve its cancer-promoting effect. Our results suggest that miR-660-5p could serve as an innovative and prospective therapeutic target for NSCLC.

Conflict of interests

The authors declare no conflict of interests.

References


