

Received: 2020.03.31
Accepted: 2020.05.07
Available online: 2020.05.14
Published: 2020.05.23

Evaluation of Exosomal miRNA in Blood as a Potential Diagnostic Biomarker for Human Non-Small Cell Lung Cancer

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Source of support: This work was supported by the Jilin Provincial Science and Technology Department (grant no. 20160101111JC), the Jilin Provincial Science and Technology Department (grant no. 20160101114JC), and the Jilin Provincial Finance Department (grant no. SCZSY201728)

Background: Tumor-derived exosomes have been used as diagnostic biomarkers to discriminate between tumor patients and healthy people. This study explored the roles of exosomal miRNAs in lung adenocarcinoma metastasis by microarray and developed a novel method for diagnosis of lung adenocarcinoma.


Material/Methods: Four lung adenocarcinoma patients' peripheral blood, including 2 metastasis and 2 N-metastasis, were used for exosomes miRNA microarray analysis. Exosomes were extracted by ultracentrifugation and identified by transmission electron microscopy. All the raw data were normalized by R software with limma packet. qRT-PCR was used to validate the microarray results. A549 cells were used to identify the functions of miR-4448. Western blot, qRT-PCR, RNAi, CCK8, and transwell invasion assay were used to verify the metastasis and proliferation abilities.

Results: miR-4436a and miR-4687-5p were upregulated between the metastasis and N-metastasis group, while miR-22-3p, miR-3666, miR-4448, miR-4449, miR-6751-5p and miR-92a-3p were downregulated. miR-4448 was also downregulated between the metastasis and control group, whereas there was no significant difference between the N-metastasis group and control group. qRT-PCR confirmed the downregulation of miR-4448 in exosomes from lung adenocarcinoma patients compared with N-metastasis patients and healthy people. CCK8 and transwell invasion assay showed that A549 cells transfected with miR-4448 inhibitor had higher proliferation and metastasis ability. qRT-PCR and Western blot confirmed the high expression of MMP2 and MMP9 in A549 cells transfected with miR-4448 inhibitor.

Conclusions: miR-4448 can inhibit A549 cell proliferation and metastasis. miR-4448 in exosomes has the potential to serve as a diagnostic marker of patients with adenocarcinoma metastasis.

MeSH Keywords: **Exosomes • Gene Expression Profiling • MicroRNAs**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/924721>

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Background

Lung cancer is a public health issue that has become the leading cause of cancer-related deaths [1]. Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer [2]. Although the overall survival rate has improved in many types of cancer, the 5-year overall survival rate of NSCLC remains at 21% [3,4]. The early distant metastasis of NSCLC limits the treatment options. The ability to diagnose NSCLC before the advanced stage could improve survival for these patients. Unfortunately, the detail molecular mechanism of NSCLC metastasis remains unclear. It is well known that intercellular communication plays roles in tumor metastasis [5]. The main questions regarding tumor metastasis are how the local signaling of the primary tumor can escape immunological surveillance [6] and how distant metastasis occurs.

Recent research found that exosomes served as a necessary medium for cellular communication [7]. They were cup-shaped extracellular vesicles that transport bioactive molecules (DNA, RNA, and protein) from tumor cells to the microenvironment or distant cells [8]. Therefore, exosomes can act as an information transfer medium between the primary tumor and metastases sites. In addition, exosomes can be separated from blood, which makes them a novel blood biomarker for NSCLC [9].

miRNAs are small non-coding RNAs (nearly 22 nt in length) that are widely distributed in cells of the human body and regulate gene expression by degrading or repressing target mRNAs. miRNAs participate in many physiological and pathological processes, especially tumor invasion and metastasis [10–13]. Low expression of miR-144-3p was detected in NSCLC, which is believed to function as a latent tumor biomarker in the prediction of prognosis for NSCLC because it is correlated with tumor metastasis and vascular invasion [10]. High expression of miR-490-3p was found in NSCLC tissues and can enhance the invasion and migration ability of the A545 *in vitro*, suggesting that miR-490-3p is involved in NSCLC metastasis. Further confirmation experiments *in situ* showed that miR-490-3p can target poly r(C)-binding protein 1 to regulate NSCLC metastasis [11]. Some circulating miRNAs have shown potential as biomarkers for diagnosis and targeted treatments for NSCLC. For example, the upregulated miRNAs-141 in plasma could be used as a biomarker for early detection of NSCLC, with high specificity (98%) and sensitivity (82.7%) [12]. In addition, multivariate Cox regression analyses showed that high expression of plasma miR-18a, miR-20a, and miR-92a is associated with poor disease-free survival and overall survival, as well as lymphatic node metastasis [13].

Compared with circulating miRNAs, exosomal miRNAs have better stability and specificity [14]. Exosomal miRNAs play roles in invasion, metastasis, and prognosis of NSCLC, which could make them promising biomarkers of NSCLC. For example, upregulated

expression of miR-23b-3p, miR-10b-5p, and miR-21-5p in exosomes is associated with poor overall survival of NSCLC patients, and their use as biomarkers could improve accuracy of survival prediction from 0.88 to 0.91 [15]. In addition, upregulated expression of miR-330-3p promotes NSCLC cell migration, invasion, and metastasis *in vitro* as determined by transwell migration and invasion assays. Further experiments confirmed miR-330-3p promotes NSCLC cells invasion through activating the mitogen-activated protein kinase/extracellular-regulated protein kinases signaling pathway. Therefore, miR-330-3p could be used as a biomarker of NSCLC metastasis [16].

In this study, we explored the role of exosomal miRNAs in lung adenocarcinoma metastasis by use of microarray analysis, and found specific miRNAs in exosomes from NSCLC patients' blood that could be used as diagnostic biomarkers for NSCLC metastasis.

Material and Methods

Blood collection

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Second Hospital of Jilin University. We obtained blood samples from 2 patients with NSCLC metastasis, 2 patients with NSCLC N-metastasis, and 2 healthy people; therefore, there were 3 groups in this study: the metastasis group (n=2), the N-metastasis group (n=2), and the control group (n=2).

Isolation of exosomes from blood

Plasma was centrifuged at 300 g for 10 min, 2000 g for 10 min, 10,000 g for 30 min, and 100,000 g for 1 h, re-suspended, and centrifuged at 100,000 g for 1 h. Western blot analysis and electron microscopy were used to identify the exosomes. Exosome pellets (5 μ l) were transferred to carbon-coated 200-mesh copper electron microscopy grids (5 min), stained with uranyl acetate, and washed with PBS. Then, we observed them by transmission electron microscopy after drying.

RNA extraction and quality verification

miRNAs from exosomes were extracted using a miRNA extraction Kit (BioTek, Beijing, CHINA). NanoDrop ND-1000 was used to assess the concentration. All qualified samples were used for miRNA microarray analysis and subsequent experiments.

Exosome microarray analysis

miRNA was marked by cyanine 3-pCp with T4 ligase, followed by inspiation and desiccation of the marked cRNA, which was then redissolved. Cells were incubated for 30 min in 10 \times

Table 1. Primers of all genes in this study.

Gene name	Forward primer	Reverse primer
MMP-2	ACCCATTTACACCTACACCAAG	TGTTTGCAGATCTCAGGAGTG
MMP-9	CGAACTTTGACAGCGACAAG	CACTGAGGAATGATCTAAGCCC
miR-4448	GCGACGAGGCTCCTTGGT	TATGGTTGTTACAGACTCCTTAC
U6	GCGCGTCGTGAAGCGTTC	GTGCAGGGTCCGAGGT
GAPDH	CGGACCAATACGACCAATCCG	AGCCACATCGCTCAGACACC

blocking agent (11 μ l) plus 25 \times fragmentation buffer (2.2 μ l) with cRNA (1 μ l) at 60°C. GE hybridization buffer (55 μ l) were added to the mixture. Another hybridization solution (100 μ l) was dispensed onto the slide for 17 h at 65°C, followed by washing, fixing, and scanning.

Quantitative reverse transcription-PCR (qRT-PCR) and Western blot analysis

qRT-PCR was used to assess expression of miR-4448 from exosomes isolated from venous blood of 20 metastasis NSCLC patients, 20 N-metastasis patients, and 20 healthy volunteers. N-metastasis was defined as: 1) no sign of metastasis after preoperative examination including chest CT, abdominal ultrasound (liver metastasis) and bone scan; and 2) no sign of lymph node metastasis by postoperative histopathological examination. Metastasis was defined as: 1) preoperative examination including chest CT, abdominal ultrasound (liver metastasis) and bone scan found signs of tumor metastasis; and 2) postoperative histopathological examination confirmed the tumor cells in lymph nodes and pleura.

Primers used in this study were: forward GCGACGAGGCTCCTTGGT, reverse: TATGGTTGTTACAGACTCCTTAC. U6: forward: CTCGCTTCGGCAGCACA, reverse: AACGCTTCACGAATTTGCGT. Additional details are provided in Table 1. We normalized the results with U6 or GAPDH, and $2^{-\Delta\Delta Ct}$ method was used.

Antibodies used were: CD9 (1: 300, Abcam), CD63 (1: 300, Abcam), CD81 (1: 300, Abcam), MMP2 (1: 500, Abcam), and MMP9 (1: 500, Bioss). Total cell protein was isolated by SDS-PAGE, and transferred to nitrocellulose membranes. Blots were blocked with 5% nonfat milk with 0.1% Tween 20. We diluted the antibodies and continued with secondary antibodies and visualization.

Cell culture and RNA interference

A549 cells were cultured in RPMI-1640 with 10% fetal bovine serum. miRNA-4448 inhibitor and mimics with Lipofectamine 2000 were transfected into A549 cells. All the miRNAs and cell protein were extracted after 48 h.

Proliferation assay

The Cell Counting Kit (CCK) was used to assess the proliferation activity of the transfected A549. We cultured 3×10^3 cells with miR-4448 mimics, miR-4448 inhibitor, and normal A549 in a 96-well plate for 24 h. CCK solution was added and incubated. The absorbance at 450 nm was measured at 1, 2, 3, and 4 h.

Invasion assay

Transwell assay was used to verify the invasion ability. Cells were coated with 20 μ l matrigel and incubated at 37°C for 50 min. Nearly 10^5 cells were put into the upper chambers. Medium with 10% FBS was added into the lower chambers. Hematoxylin were used to stain the cells.

Statistical analysis

Data are expressed as mean \pm standard deviation, and compared by *t* test. Normally distributed continuous variables were compared using the *t* test. P value less than 0.05 was considered statistically significant. All statistical analyses were carried out using SPSS 20.0.

Results

Identification of exosomes

All the exosomes were extracted by ultracentrifugation. Transmission electron microscopy showed the extracellular vesicles were cup-shaped, with diameter of nearly 100 nm, consistent with exosomal morphology and size (Figure 1A). CD9, CD63, and CD81 were shown to be positive by Western blot (Figure 1B).

miRNA extracted from exosomes

The concentration of miRNA extracted from exosomes was 35.87 to 78.99 ng/ μ l, and the mean 260/280 rate was 1.68. Figure. 2A shows denaturing agarose gel electrophoresis of all RNA with good RNA integrity. The normalized intensity values are presented as box plots in Figure 2B, 2C.

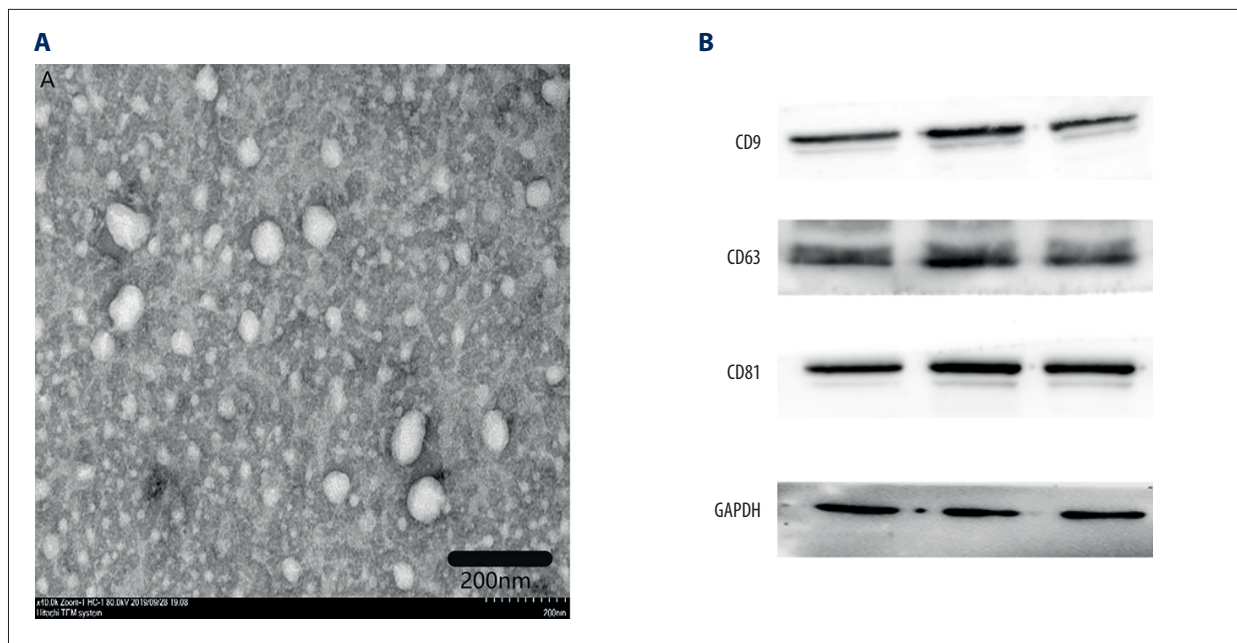


Figure 1. Identification of exosomes. (A) Transmission electron microscopy identified morphological characteristics of exosomes. All the extracellular vesicles were cup-shaped with diameters of nearly 100 nm. Scale bar: 100 nm. (B) Western blot shows the vesicles expressed CD9, CD63, and CD81.

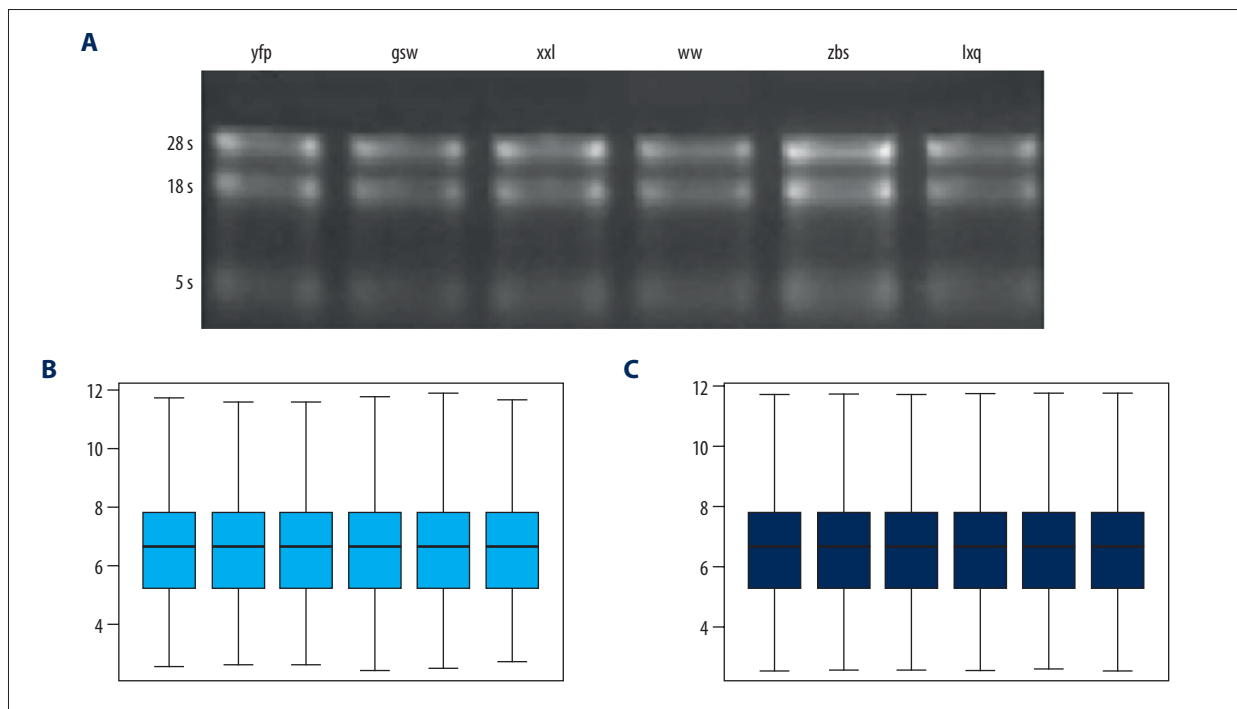


Figure 2. Integrity of RNA and normalized intensity values. (A) Denaturing agarose gel electrophoresis of all RNA. (B) Light blue represents intensity values. (C) Deep blue represents normalized intensity values.

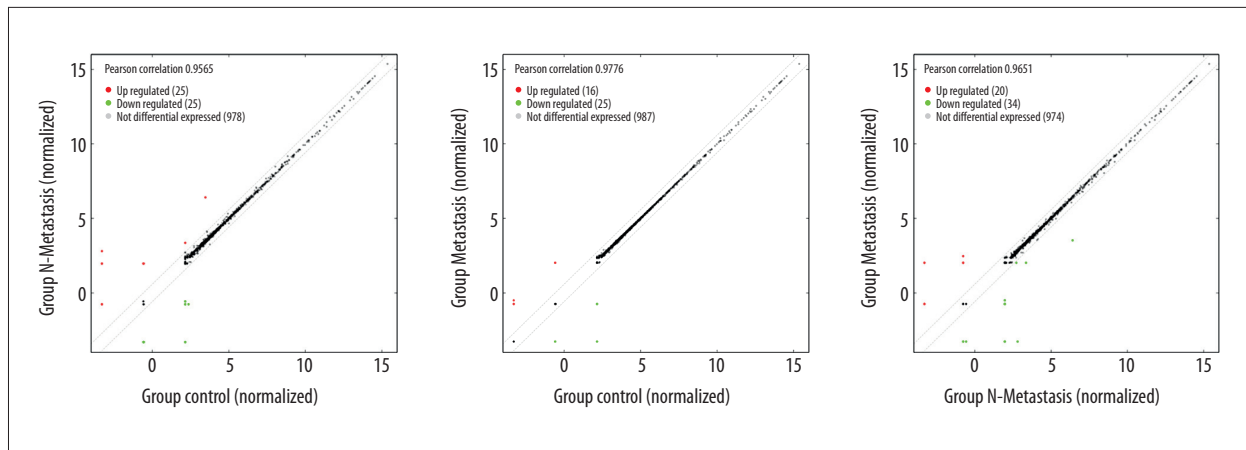


Figure 3. Volcano plot showed all the different expression miRNAs. The Pearson correlation was 0.9565 between N-metastasis and control group, 0.9776 between metastasis and control group, and 0.9651 metastasis and N-metastasis group. Red represents upregulated, green represents downregulated, and black means no significant change.

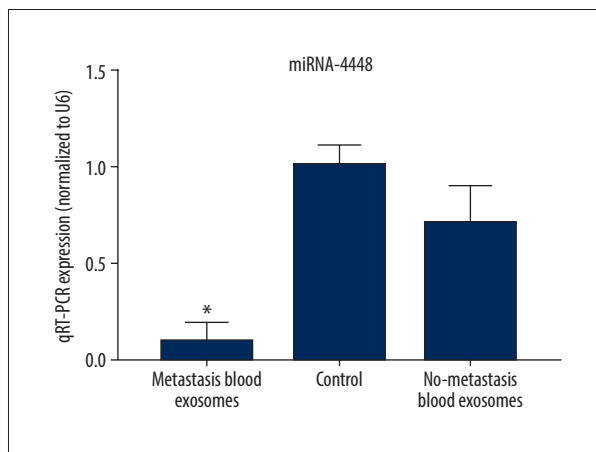


Figure 4. Expression of miR-4448 in exosomes among adenocarcinoma metastasis, no-Metastasis, and control. qRT-PCR showed that miR-4448 had significantly higher expression in N-metastasis (0.79 ± 0.34) and control exosomes (1.02 ± 0.36) compared with metastasis exosomes (0.18 ± 0.21), but there was no significant difference between N-metastasis and control. * $P < 0.05$.

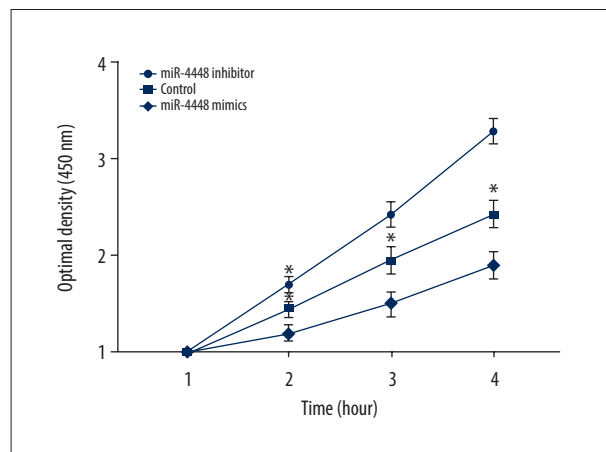


Figure 5. miR-4448 inhibits A549 proliferation. A549 transfected with miR-4448 mimics showed less proliferation activity than in the control group at 2 h (1.21 ± 0.11 vs. 1.45 ± 0.09 , $P = 0.043$), 3 h (1.51 ± 0.13 vs. 1.96 ± 0.14 , $P = 0.0151$), 4 h (1.91 ± 0.13 vs. 2.44 ± 0.142 , $P = 0.0086$). However, A549 transfected with miR-4448 inhibitor showed enhanced proliferation activity compared to the control group at 2 h (1.71 ± 0.12 vs. 1.45 ± 0.09 , $P = 0.0399$), 3 h (2.44 ± 0.13 vs. 1.96 ± 0.14 , $P = 0.0121$), and 4 h (3.32 ± 0.15 vs. 2.44 ± 0.142 , $P = 0.0018$).

Exosomes miRNA microarray

A total of 2549 human miRNAs were detected during miRNA microarray. Compared with the control group, miR-4324, 4675, 4687-5p, and 516b-5p were downregulated in the N-Metastasis group, but miR-1254, 22-3p, 3195, 3666, 4449, 4475, 4485-3p, 6751-5p, 8074, and 92a-3p were upregulated (Figure 3A). There were only 2 miRNAs (3160-5p and 4448) that were downregulated in the metastasis group compared with the control group (Figure 3B). Compared with the N-metastasis group, miR-4436 and miR-4687-5p were upregulated in the metastasis group, and miR-22-3p, 3666, 4448, 4449, 6751-5p, and 92a-3p were

downregulated (Figure 3C). Integration analysis showed that miR-4448 was the only miRNA that was downregulated in the metastasis group compared with the other 2 groups.

Verification of expression of miR-4448

We used blood from 20 Metastasis NSCLC, 20 N-Metastasis, and 20 healthy people to assess the expression of miR-4448. Exosomes were extracted as previously described. qRT-PCR showed that miR-4448 was significantly upregulated in N-metastasis (0.79 ± 0.34 vs. 0.18 ± 0.21 , $P < 0.001$) and control

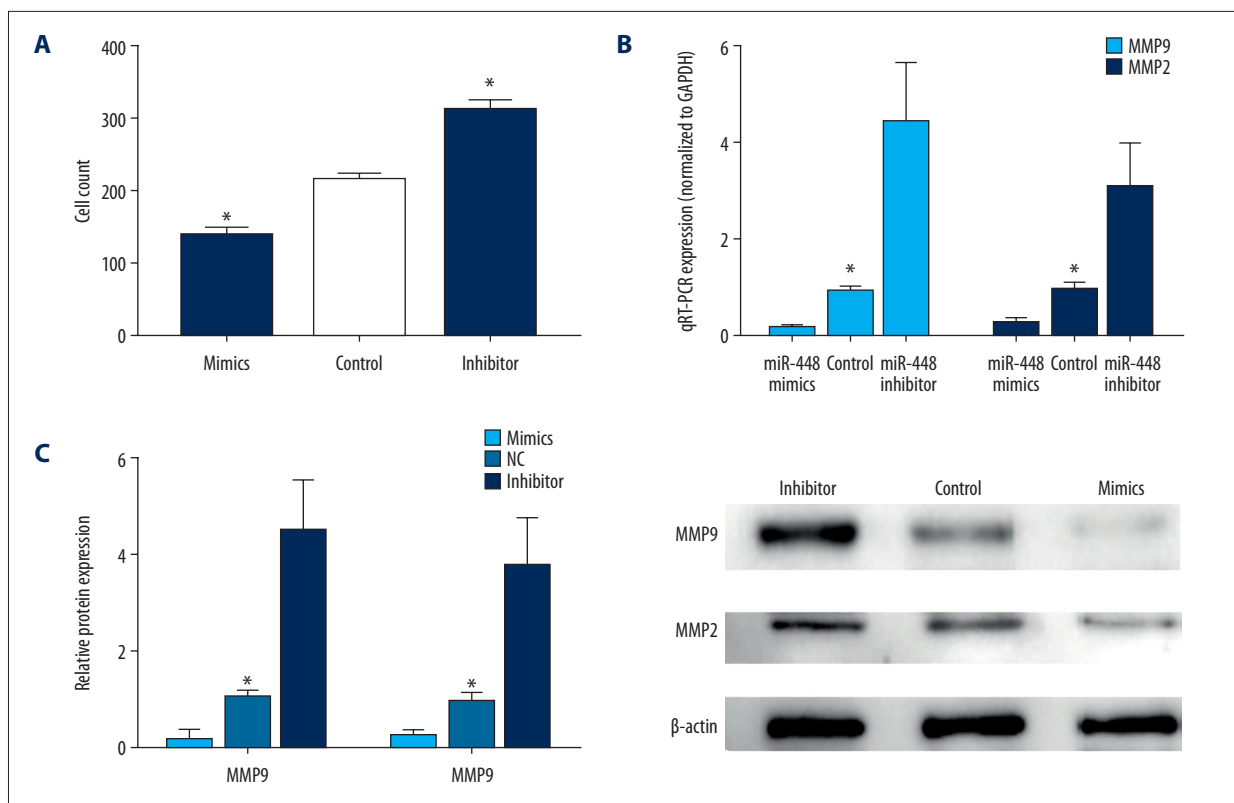


Figure 6. miR-4448 inhibits A549 migration. (A) Transwell invasion assay showed low expression of miR-4448 promoted migration. A549 transfected with miR-4448 inhibitor showed enhanced invasion activity (311.5 ± 11.66) compared to normal A549 (211.7 ± 10.51), $P=0.0004$. High expression of miR-4448 inhibited invasion (139.5 ± 10.77), $P=0.0011$. * $P < 0.05$. (B) qRT-PCR confirmed that high expression of MMP2 (3.57 ± 0.68 vs. 1.09 ± 0.43 , $P=0.0059$) and MMP9 (4.22 ± 0.89 vs. 1.11 ± 0.45 , $P=0.0057$) in A549 transfected with miR-4448 inhibitor. (C) Western blot analysis showed that high expression of MMP2 (3.82 ± 0.72 vs. 1.05 ± 0.23 , $P=0.0032$) and MMP9 (4.35 ± 0.79 vs. 1.06 ± 0.25 , $P=0.0023$) in A549 cells transfected with miR-4448 inhibitor.

exosome (1.02 ± 0.36 vs. 0.18 ± 0.21 , $P < 0.001$) compared with metastasis exosome (0.18 ± 0.21), but there was no significant difference between N-metastasis and control group (0.79 ± 0.34 vs. 1.02 ± 0.36 , $P=0.0655$) (Figure 4).

miR-4448 inhibited A549 proliferation

A549 cells transfected with miR-4448 inhibitor showed enhanced proliferation activity compared to normal A549 cells after co-incubation with CCK8 solution at 2 h (1.71 ± 0.12 vs. 1.45 ± 0.09 , $P=0.0399$), 3 h (2.44 ± 0.13 vs. 1.96 ± 0.14 , $P=0.0121$), and 4 h (3.32 ± 0.15 vs. 2.44 ± 0.142 , $P=0.0018$). However, A549 cells transfected with miR-4448 mimics showed weakened proliferation activity compared to the control group at 2 h (1.21 ± 0.11 vs. 1.45 ± 0.09 , $P=0.043$), 3 h (1.51 ± 0.13 vs. 1.96 ± 0.14 , $P=0.0151$), and 4 h (1.91 ± 0.13 vs. 2.44 ± 0.142 , $P=0.0086$) (Figure 5).

miR-4448 inhibited A549 migration

A549 cells transfected with miR-4448 inhibitor showed enhanced invasion activity (311.5 ± 11.66) compared to normal

A549 cells (211.7 ± 10.51) ($P=0.0004$), whereas high expression of miR-4448 inhibited invasion (139.5 ± 10.77) ($P=0.0011$) (Figure 6A). In addition, MMP2 (3.57 ± 0.68 vs. 1.09 ± 0.43 , $P=0.0059$) and MMP9 (4.22 ± 0.89 vs. 1.11 ± 0.45 , $P=0.0057$) showed high expression in A549 cells transfected with miR-4448 inhibitor, as determined using qRT-PCR (Figure 6B). Western blot analysis also confirmed the high expression of MMP2 (3.82 ± 0.72 vs. 1.05 ± 0.23 , $P=0.0032$) and MMP9 (4.35 ± 0.79 vs. 1.06 ± 0.25 , $P=0.0023$) after transfection with miR-4448 inhibitor (Figure 6C).

Discussion

In this study, we found that only miR-4448 was downregulated in the metastasis group, as shown by exosomes miRNA microarray analysis. qRT-PCR confirmed the downregulation of miR-4448 in blood exosomes from metastasis NSCLC patients. Up to now, there were only 9 studies reporting the different expression of miR-4448. Chaudhry et al. [17] first reported that miR-4448 was downregulated in TK6 cells after

4-h, 12-h, and 24-h irradiation and concluded that miR-4448 plays an important role in response to radiation exposure. They also inferred that low expression of miR-4448 might be useful for explaining the mechanisms of post-transcriptional gene regulation during conditions of stress. Rogerio et al. [18] reported that miR-4448 could inhibit osteoblast differentiation of hMSCs. They pointed that miR-4448 could directly target SMAD to control the osteoblast differentiation in an *in vitro* experiment. Su et al. [19] found the miR-4448 was upregulated during dengue infection in 2017. They used qRT-PCR to verify the upregulation of miR-4448 between dengue-infected and non-infected blood. Two years later, they confirmed the function of miR-4448 during dengue infection and found that miR-4448 could reduce dengue virus replication [20]. Thus, miR-4448 could be used as a diagnostic biomarker of dengue infection. In addition, miR-4448 also has different expression in ophthalmic diseases. Circulating miR-4448 was consistently highly expressing in glaucoma [21], whereas it presented low expression in diabetic retinopathy patients [22]. Therefore, detecting the miR-4448 in diabetes patients could suggest the degree of pathological changes in the retina in response to diabetes. High expression of circulating miR-4448 in patients with eye irritation might use useful in diagnosis of glaucoma.

However, only 3 reports of miR-4448 focused on cancers. Boo et al. [23] performed next-generation sequencing and found low expression of miR-4448 in the human breast MCF-7 cell line (spheroid MCF-7) and concluded that miR-4448 could be used as a biomarker for breast cancer stem cell and targeted therapy. The spheroid MCF-7 presented higher proliferation, migration, and invasion potential than parental cells, as shown by cell proliferation assay, motility assay, transwell assay, and wound-healing assay. Therefore, the authors inferred that miR-4448 is a tumor-suppressor gene that can inhibit spheroid MCF-7 proliferation and migration. Hibino et al. [24] had found that miR-4448 can inhibit cancer cell (gastric and liver cancer) proliferation and migration. EZH2, a well-known gene, can enhance tumorigenesis and is highly expressed in many tumor tissues. The authors used suberoylanilide hydroxamic acid (SAHA) and 3-deazaneplanocin A (DZNep) to restrain the expression of EZH2 in both AGS and HepG2 cells, and found that miR-4448 was markedly upregulated accompanied by cancer cell apoptosis, as determined using annexin V-FITC apoptosis assay. In addition, upregulated miR-4448 could promote cell cycle (G1/S) arrest after treatment with SAHA and DZNep. The width of the wound in wound-healing assay was increased with upregulated expression of miR-4448, which suggested that miR-4448 could reduce migration ability. Therefore, miR-4448/EZH2 could be used as a new diagnostic biomarker and drug target.

In a recent study of miR-4448 in glioblastoma extracellular vesicles [25] extracellular vesicles were harvested from culture

supernatants from the human glioblastoma cell line. Next-generation sequencing technique showed high expression of miR-4448 in glioblastoma extracellular vesicles. Although further experiments *in vitro* were not performed, the verification experiment by PCR showed the fold change of miR-4448 ranked third among all differential expression miRNA. Therefore, the authors inferred that miR-4448 in glioblastoma extracellular vesicles could serve as a biomarker for glioblastoma and novel therapeutic targets. However, the high expression of miR-4448 in glioblastoma was only assessed in 1 study, which showed miR-4448 acted as an oncogene, which disagreed with results of previous studies [23,24] and with our results. Therefore, the differential expression and function of miR-4448 in various tumors need to be further researched.

Detecting the miRNA in exosomes (miRNA 126, 2223-3p) is a new method of diagnosis NSCLC. Many tumor cells secrete exosomes to the microenvironment or distant cells. It has been known since 1980 that exosomes participate in cellular cross-talk [26]. The double lipid layer of exosomes carries various bioactive materials, including DNA, RNA, and proteins [27]. These bioactive materials are taken up by other recipient cells, which complete cell-cell communication through exosome mediators. In tumor metastasis, the exosomes are a key link between primary tumor cells and metastasis sites. Before metastasis, the primary tumor seemed to secrete exosomes to prepare a comfortable environment for circulating tumor cells [28]. Therefore, reduction of exosomes secretion from primary tumor cells or inhibition of exosomes uptake at metastases sites are 2 novel treatments. In our study, we confirmed that exosomes separated from NSCLC metastasis blood contained lower miR-4448 than in N-metastasis and normal people. *In vitro* experiments also confirmed low expression of miR-4448 in A549 cells, which promoted proliferation and migration. Therefore, miR-4448 in exosomes are key mediators during NSCLC metastasis. Further experiments with larger sample sizes were needed to verify the expression and function of miR-4448 in NSCLC, and research on mRNAs targeted by miR-4448 is also needed.

Conclusions

In summary, as the most common type of lung cancer, early diagnosis of NSCLC before advanced stage could give more treatment opportunities and significantly improved the overall survival rate. With the lack of diagnostic markers for NSCLC metastasis, the miR-4448 in exosomes from NSCLC patients' blood could be used as a diagnostic marker, especially for NSCLC metastasis. Detecting the miR-4448 in exosomes from blood is simpler and faster with modern molecular biology technology. In addition, inhibiting the miR-4448 in exosome secretions might be a new treatment for preventing NSCLC metastasis.

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