Non-small lung cancer cells-derived exosome miR-3157-3p primes pre-metastatic niche by inducing angiogenesis and vascular permeability

Zijian Ma

Jiangsu Province Hospital and Nanjing Medical University First Affiliated Hospital

Ke Wei

Jiangsu Province Hospital and Nanjing Medical University First Affiliated Hospital

Fengming Yang

Jiangsu Province Hospital and Nanjing Medical University First Affiliated Hospital

Zizhang Guo

Jiangsu Province Hospital and Nanjing Medical University First Affiliated Hospital

Chunfeng Pan

Jiangsu Province Hospital and Nanjing Medical University First Affiliated Hospital

Yaozhou He

Jiangsu Province Hospital and Nanjing Medical University First Affiliated Hospital

Jun Wang

Jiangsu Province Hospital and Nanjing Medical University First Affiliated Hospital

Zhihua Li

Jiangsu Province Hospital and Nanjing Medical University First Affiliated Hospital

Liang Chen

Jiangsu Province Hospital and Nanjing Medical University First Affiliated Hospital

Yang Xia

Jiangsu Province Hospital and Nanjing Medical University First Affiliated Hospital

Yijiang Chen (✉ chenyjnmu@163.com)

The First Affiliated Hospital of Nanjing Medical University

Research

Keywords: Non-small cell lung cancer, miR-3157-3p, TIMP2, KLF2, pre-metastatic niche

Posted Date: August 20th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-61544/v1>

Abstract

Background: Cancer-derived exosomes are considered to be the main driving force for cancer-induced metastasis niche formation at foreign sites, but the mechanism is unclear.

Methods: Identification of differentially expressed miRNAs in exosomes by miRNA sequencing. Through a series of in vitro and in vivo experiments, it was studied that the tumor-derived exosome miR-3157-3p can be taken up by the endothelium and exert biological functions. The molecular mechanism of miR-3157-3p was confirmed by Western blot, luciferase test and rescue experiment. Finally, real-time fluorescence quantitative PCR and RNA in situ hybridization were used to evaluate the clinical relevance of miR-3157-3p expression.

Result: In metastatic NSCLC patients, the expression level of miR-3157-3p in circulating exosomes was significantly higher than that of non-metastatic NSCLC patients. Here, we found that miR-3157-3p can be transferred from NSCLC cells to vascular endothelial cells through exosomes. Exosome miR-3157-3p has regulated the expression of VEGF / MMP2 / MMP9 and occludin in endothelial cells by targeting TIMP / KLF2, thereby promoted angiogenesis and increased vascular permeability. In addition, exosome miR-3157-3p from NSCLC cells enhances NSCLC metastasis in mouse lungs.

Conclusion: Our work indicates that exosome miR-3157-3p is involved in the formation of pre-metastatic niche and may be used as a blood-based biomarker for NSCLC metastasis.

Introduction

The pre-metastatic niche is a pre-formed microenvironment formed by exosomes secreted by the primary tumor site before extensive metastasis[1-3]. It is characterized by angiogenesis, immunosuppression, inflammation, lymphangiogenesis, vascular permeability, organotropism, and reprogramming[4-7]. In addition, targeted therapy before metastasis niche may be a potential strategy for the treatment of cancer metastasis[8, 9]. It has been found that several pre-metastatic niche biomarkers are important for the diagnosis and prognosis prediction of cancer[10, 11].

Exosomes are extracellular vesicles with a diameter of 30–150 nm[12-14]. As a key participant in cell-to-cell communication, exosomes convey information through their cargo levels, including proteins, lipids, DNA, messenger RNA, and microRNA[15-20]. Recently, it has been found that the pre-metastatic niche depends on tumor-derived exosomes[5]. The function of exosomes depends on the cell type from which they are derived[21, 22]. Studies have shown that tumor-derived exosomes participate in the exchange of genetic information between tumor cells and basal cells, resulting in the generation of a large number of new blood vessels, thereby promoting tumor growth and invasion[23, 24]. Various researches have told us that exosomes have played a key role in cancer tumorigenesis, growth, apoptosis, immune response and chemotherapy resistance[25-29]. We have known that the NSCLC-derived exosome miR-3157-3p can be transferred to vascular endothelial cells to target TIMP2 / KLF2, by which to promote the angiogenesis and increase the permeability. In addition, we have demonstrated that exosome miR-

3157-3p mediates the pre-metastatic niche before transfer in nude mice by inducing angiogenesis, thereby promoting NSCLC transfer.

Methods

Cell lines and human tissue samples

We have collected tumor and paraneoplastic specimens of 47 patients who underwent radical surgery between 2015 and 2019. All patients did not undergo radiotherapy and chemotherapy. Fresh biopsy tissue is frozen and stored in liquid nitrogen until being used. All plasma samples were collected from NSCLC patients (40 without metastasis and 40 with metastasis) in our hospital between April 2013 and June 2019, and age-matched healthy human plasma samples (n = 30) were selected from the Physical Examination Center of our hospital. The blood sample was then centrifuged at 2500g for 10 minutes to extract the serum and stored at -80 ° C. Each patient has signed an informed consent form and this was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University.

This study involved 4 human NSCLC cell lines (H1299, SPCA1, PC9, A549), 1 human bronchial epithelioid cell line (16HBE) and HUVEC (human umbilical vein endothelial cells). All cell lines were purchased from Shanghai Academy of Sciences. (H1299, SPCA1, PC9, A549 were cultured in DMEM medium (GIBCO, Gaithersburg, USA) supplemented with 10% fetal bovine serum. HUVEC was cultured in F12-K medium (GIBCO with 10% fetal bovine serum, Gaithersburg, USA). 16HBE was cultured in Defined Keratinocyte SFM medium (GIBCO, Gaithersburg, USA) containing 10% fetal bovine serum (HyClone, Logan, USA).

Microarray analysis

The miRNA 4.0 Array of Affymetrix was used to analyze the miRNA expression in serum exosomes (normal) of healthy people and in NSCLC patients with lymph node metastasis. What we have done has obtained the approval from the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). Informed consent was signed by each patient on the day of admission. Three clinical samples were collected from each group. The miRNA array experiment was conducted in the microarray core laboratory of Beijing Boao Jingdian Biotechnology Co., Ltd. (Beijing, China).

Cell transfection

Lentiviral vectors that over-express miR-3157-3p and repress miR-3157-3p were constructed and generated. Mimics and inhibitor were purchased from GiKai GENE (Shanghai, China) and transfected according to the manufacturer's instructions. Plasmid TIMP2 / KLF2, empty vector, si-TIMP2 / si-KLF2 were purchased from Gene Pharma (Shanghai, China). Transfection was performed using elipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instructions.

PCR

To determine the expression level of miR-3157-3p, total RNA was isolated and extracted from tissues and cells by TRIzol reagent (Invitrogen, CA, USA) according to the instructions. CDNA was generated by reverse transcription using total RNA and PrimeScriptRT reagent (Takara, Kusatsu, Japan), and detected using SYBR Green (Takara) at ABI StepOnePlus real time quantitative PCR instrument (StepOnePlus, ABI Company, Oyster Bay, NY, USA). Taking GAPDH and U6 as endogenous controls, mRNA and miRNA were normalized. The $2^{-\Delta\Delta CT}$ method was used to quantify the relative levels of mir-3157-3p and TIMP2/KLF2. Each sample is in triplicate. miRNA quantification: Bulge-loopTM miRNA qRT-PCR Primer Sets (one RT primer and a pair of qPCR primers for each set) specific for miR-3157-3p is designed by RiboBio (Guangzhou, China). The oligonucleotides used in this study: U6 F:CTCGCTTCGGCAGCACATATACT.U6 R:ACGCTTCACGAATTTGCGTGTC. GAPDH F: AAGGTGAAGGTCTGGAGTCA.GAPDH R:GGAAGATGGTGATGGGATTT

Isolation and identification of exosomes

Exosomes were purified from NSCLC cell supernatant or NSCLC patient serum by ultracentrifugation. NSCLC cells were cultured in DMEM medium supplemented with 10% fetal bovine serum without exosomes. Total Exosome Isolation Reagent (from cell culture media, Invitrogen, USA) is used to extract exosomes from cell supernatant, and the amount of exosomes was measured by BCA protein assay kit (KeyGEN BioTECH). For transmission electron microscopy (TEM), 5-10 μ l extracted exosomes are placed on the copper carrier grid. Add 10-20 μ l of EM solvent dropwise, and carefully suck up with clean filter paper after 1min, then observe the exosomes with Philips CM120 biological dual transmission electron microscope (FEI Company, USA). The method of Nanoparticle-tracking analysis is used to analyze the size of exosomes. The exosomes (10-20 mg) in 1 mL PBS and vortex were dissolved for 1min to distribute the exosomes evenly. Then, the NanoSight Nanoparticle Tracking Analyzer (NTA, Malvern Analysis, UK) was used to measure and observe the scale distribution of the exosomes. For exosome labeling, PKH67 membrane dye (Sigma) was used to fluorescently label exosomes according to the instructions.

Dual luciferase report

The 3'UTR fragments of KLF2 and KLF4 genes were amplified and inserted into the vector. Then complete co-transfection of TIMP2 and KLF2 3'UTR plasmids with miR-3157-3p lentiviral vector into cells by using Lipofectamine 2000 (Invitrogen USA). 48 hours after the transfection, Dual-Lumi[™] dual luciferase reporter gene detection kit (Biyuntian: RG088S) detects dual luciferase. The Renilla activity was taken as a normalization of luciferase activity. All measurements were performed in triplicate, and each experiment was repeated three times.

Western blotting

The total protein was extracted from the cells with the RIPA reagent (Beyotime, Shanghai, China) who contains 10 μ g / mL Phosphatase inhibitor and 100 μ g / mL PMSF (Beyotime, Shanghai, China). The protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and

then transferred onto polyvinylidene difluoride (PVDF) membrane. After sealing for 1 hour, the membrane were diluted with primary antibody diluted, TIMP2 (SAB, # 41500, 1: 500 dilution) vascular endothelial growth factor (VEGF) (SAB, # 320217, 1: 500 dilution) (MMP2) (Abcam, ab92536, 1: 1000 dilution), MMP-9 (Abcam, ab76003, 1: 1000 dilution), ZO-1(Abcam, ab96587, 1: 500 dilution), occludin(Abcam, ab216327, 1: 1000 dilution), claudin 5(GeneTex, GTX00796, 1: 500 dilution) and GAPDH (Abcam, ab181602, 1: 10000 dilution) overnight at 4 ° C. The TBST was washed 5 minutes for three times and then incubated for 2h in the corresponding secondary antibody. After which, it was developed by enhanced chemiluminescence (ECL).

EDU assay, migration assay, and angiogenesis assay.

From EDU we know that spread the transfected cells evenly in a 24-well plate with 3×10^4 cells per well, then use EdU Apollo567 extracorporeal flow cytometry kit (Guangzhou RiboBio) to perform EdU according to the instructions, by which we can acquire images with a fluorescence microscope. From recruitment assay, we get the following result: Approximately 2×10^4 cells in serum-free medium(400 μ l) were seeded into the upper Transwell chamber for migration assay, who use 8.0 μ m Transwell Permeable Supports (Corning, New York, USA). The lower chamber was supplemented with 600 μ l DMEM medium containing 10% FBS, and was incubated at 37 ° C for 24 hours. Remaining cells in the upper membrane were cleared with Cotton swabs, and they were stained with paraformaldehyde and 0.1% crystal violet (Beyotime, Shanghai, China) for 20 minutes at 37°C.

Tube formation: Spread 250u Matrigel (BD Bioscience, USA) on a 24-well plate and place in an incubator for half an hour. Approximately 1×10^5 treated HUVEC cells were resuspended in each well of a 24-well plate. Tube formation was induced at 37 ° C for 8 h. Then we observe the tube length with a microscope. For the measurement of the aortic ring, the thoracic aorta cut from Sprague Dawley (SD) rats of 10 to 12 weeks old was cut into a cross section of 1 to 2 mm in length. Place the ring on the wells coated with Matrigel, using F12-K medium (GIBCO, Gaithersburg, Maryland, USA), containing 10% fetal bovine serum, supplemented with 10ng / ml vascular endothelial growth factor and 25 μ g / ml Heparin per well. After 24 hours, the aortic ring was incubated with conditioned medium derived from NSCLC cells or exosomes transfected with miR-3157 mimics, miR-3157 inhibitors. After 5 days, observe the number of buds with a microscope, and quantify blood vessel growth by counting all buds from one ring [30]. From CAM, we get the result: Incubate 20 fertilized eggs at 37.5 ° C and 70% humidity for 8 days, and an artificial airbag was created, also a small window was cut in the shell above the artificial airbag. We use exo-miR-3157-3p-mimics, exo-NC mixed with equal volume of Matrigel (BD Biosciences, Bedford, MA) to inject into CAM. The area around the implanted Matrigel was photographed, and the number of blood vessels was obtained by counting the branches of the blood vessels[31]. Each experiment was repeated three times. From the in vitro permeability measurement, we get the following result: Rhodamine B isothiocyanate-dextran (Sigma) was added to the top well of the transwell filter, and HUVEC (10^5 cells per well) was treated on it for 3 days. Then collect the culture medium in the bottom well after 30 minutes and monitor the appearance of fluorescence under 544 nm excitation and 590 nm emission.

Animal experiment

The six-week-old nude mouse was purchased from the Animal Center of Nanjing Medical University (Nanjing, China) and what we have done was approved by the Animal Ethics Committee of Nanjing Medical University. Nude mice were randomly divided into 4 groups, 4 in each group. The 3×10^6 A549 cell samples were injected subcutaneously into the groin area of BALB / c nude mice. After 9 days, 5 μ g of exosomes were injected into xenografts every other day. Three days after the last injection, the tumor tissue was dissected, fixed in 10% formalin, and embedded in paraffin for further study. For the determination of tail vein metastasis, 6-week-old nude mice were injected with 5 μ g exosomes through the tail vein every other day for 2 weeks. The 2×10^6 A549 cells were injected into the tail vein of nude mice treated with exosomes. After 60 days, the mice were sacrificed, and the lungs were removed for examination and HE staining.

Statistical analysis

All statistical analyses were performed by spss 19.0 and GraphPad software 8.0. The *P*-values were analysed with Student's *t* test, one-way ANOVA. We select for statistical significance when *P* is less than 0.05.

Results

Exosomal miR-3157-3p is abundant in NSCLC patients.

To identify miRNAs potentially associated with metastasis in NSCLC, serum exosomes were extracted from NSCLC patients or healthy controls and identified. First, the exosomes are separated by differential ultracentrifugation. The morphology of the exosome was examined by TEM (Figure 1A). Nanosight particle tracking shows that the size of these exosomes is about 100 nm (Figure 1B). In addition, Western blot analysis confirmed the expression of specific exosome markers such as CD63 and TSG101(Figure 1C). Next, exosome RNA sequencing (RNA-seq) was performed, miRNA profiles were compared and shown in Figure 1D. Among miRNAs with different expressions, compared with normal controls, 44 miRNAs with abnormal expression in metastatic NSCLC tissues, including 3 miRNAs (miR-3157-3p, miR-3613-5p, miR-3921)(Figure S1A) increased . We performed GO and KEGG enrichment analysis on the differential miRNAs and found that the differential miRNAs were mainly enriched in the four categories of "Angiogenesis", "cell junction", "cancer" and "positive regulation biological process" (Figure 1E-F, Figure S1B-C, Figure S2A-B). We further conducted GO-Standard analysis on the expression of differential miRNA in "biological process", "cellar component" and "Molecular function" (Figure S2C). Because angiogenesis and tight junctions between cells are related to the pre-metastatic microenvironment, we suspect that abnormally expressed miRNAs may be associated with metastasis. RT-PCR analysis confirmed that three of these mRNAs (miR-3157-3p, miR-3613-5p, and miR-3921) showed higher levels of serum exosomes in NSCLC patients than healthy people (10 NSCLC patients and 10 healthy people) (Figure 1G). We found that miR-3157-3p has the highest expression,so we selected miR-3157-3p for

further study. The results of qRT-PCR showed that circulating exosomal-miR-3157-3p in NSCLC patients with metastasis was higher than without metastasis (Figure 1H). What's more interesting is that we found that miR-3157-3p in the tissues of patients with metastasis was significantly higher than that of patients without metastasis (Figure 1I). So we predict that exo-miR-3157-3p is closely associated with metastasis.

NSCLC transfer miR-3157-3p to HUVECs through exosomes

To study the molecular mechanism of NSCLC cells regulating HUVEC, we first verified the level of miR-3157-3p in NSCLC cell lines and 16HBE cells (Figure 2A). The exosomes were successfully isolated from A549 and H1299 serum, and by transmission electron microscopy (TEM) there showed a teacup-like double-sided structure of 30-100nm exosomes, and the next step is the NTA verification of exosomes (Figure 2B). In addition, CD63 and TSG101 are exosome-specific markers which were used to quantify exosomes, and calnexin is not expressed in exosomes, which was determined by Western blotting. The results showed that CD63 and TSG101 were abundant in the isolated exosome components (Figure 2C). The extracted exosomes were co-cultured with HUVECs. And RT-qPCR was used to measure the expression of miR-3157-3p. RT-PCR results showed that the expression of miR-3157-3p in HUVEC increased significantly in a time-dependent manner (Figure 2D). A549 and H1299 transfected with Lv-miR-3157-3p and miR-3157-3p from A549/H1299 exosomes were significantly up-regulated (Figure 2E-F). HUVEC was incubated with PKH67-labeled exosomes derived from A549/H1299 cells, which were transfected with Cy3-labeled miR-3157-3p mimics. Cy3 fluorescence and PKH67 lipids were observed under a confocal microscope in the incubated HUVEC. The dye is distributed around the nucleus (Figure 2G). Therefore, these findings concluded that NSCLC transferred miR-3157-3p to HUVEC through exosomes.

Up-regulation of miR-3157-3 promotes proliferation, migration, vascular leakiness and angiogenesis of HUVEC

To determine whether miR-3157-3p can affect the proliferation, migration and angiogenesis of HUVEC in vitro, Lv-miR-3157-3p or Lv-miR-3157-3p-inhibitor was transfected with HUVEC. The expression efficiency of miR-3157-3p was detected by qRT-PCR. (Figure S3A) Overexpression of miR-3157-3p enhances HUVEC proliferation, migration of vessel penetration and tube formation. In contrast, inhibiting the expression of miR-3157-3p leads to a reduction in HUVEC proliferation, migration, vascular permeability, and tube formation (Figure 3A, 3B, 3C, 3D). VEGF has played a powerful role in promoting angiogenesis, and MMP-2 and MMP-9 are the two main enzymes that degrade type IV collagen and promote the invasion and migration of malignant tumors. ZO-1, occludin and Claudin5 are essential for the integrity of the endothelial barrier because they can enhance tight junction-related proteins. Western blot analysis showed that the up-regulation of miR-3157-3p increased the protein levels of VEGF, MMP-2 and MMP-9, while decreased the protein levels of ZO-1, occludin and Claudin5. Inhibiting the expression of miR-3157-3p played the opposite role (Figure 3E, 3F). The expression of ZO-1, occludin and Claudin5 in transfected HUVEC was further verified in the IF experiment (Figure 3G). These findings indicated that elevated miR-3157-3p promotes HUVEC proliferation, migration, vascular penetration, and angiogenesis in vitro.

TIMP2 and KLF2 are functional targets of miR-3157-3p in HUVECs.

To explore how miR-3157-3p regulates angiogenesis and vascular permeability, three mRNA target prediction algorithms (miRDB, miRWalk, and Targetscan) were used to identify potential downstream targets of miR-3157-3p. Among potential targets, TIMP2 overlaps in all databases, and KLF2 overlaps in miRWalk and Targetscan (Figure 4A, 4E). According to reports, TIMP2 is a natural inhibitor of MMP, inhibiting angiogenesis and invasion and metastasis. KLF2 is considered to be essential to the integrity of the endothelial barrier. Some scholars have found that KLF2 can regulate occludin. In addition, KLF2 inhibits angiogenesis by inhibiting the promoter activity of VEGF2[32]. We found that overexpressed KLF2 has an inhibitory effect on angiogenesis, but can maintain vascular permeability.(Figure S3 D-E). Overexpressed TIMP2 has an inhibitory effect on angiogenesis, but not on vascular permeability (Figure S3 F-G). Therefore, we believe that the angiogenesis and vascular permeability of TIMP2 and KLF2 in the niche before transfer are crucial. TIMP2 and KLF2 expression is reduced in lung cancer, and patients with increased expression have a poorer prognosis (Figure 4B,4C, 4D, 4F,4G,4H). In patients with elevated miR-3157-3p, TIMP2 / KLF2 expression decreased(Figure S3 B-C). To determine whether TIMP2 and KLF2 are the targets of miR-3157-3p, we conducted a luciferase reporter gene experiment in HUVECs. As shown, TIMP2 and KLF2 3'UTR contain potential miR-3157-3p binding sites. In addition, dual luciferase reporter gene assays were performed with TIMP2 / KLF2-Wt and TIMP2 / KLF2-Mut co-transfected into cells with miR-3157-3p mimics or NC. Compared with the NC mimics, the luciferase activity of TIMP2-Wt and KLF2-Wt is inhibited ($p < 0.05$) in the presence of miR-3157-3p mimics, which indicates that miR-3157-3p can specifically bind TIMP2 and KLF2 (Figure 4I, 4J). Western blot analysis showed that overexpressed miR-3157-3p can inhibit the expression of TIMP2 and KLF2, while inhibiting the expression of miR-3157-3p played the opposite role (Figure 4K). In order to further verify that whether TIMP2 and KLF2 are the target genes of miR-3157-3p in HUVEC, we conducted a reversion experiment. Stably transfecting HUVEC cells with TIMP2 / KLF2 plasmid and siRNA-TIMP2 / KLF2. The high expression of TIMP2 has reversed the effect of miR-3157-3p up-regulation on HUVEC cell proliferation, migration and tube formation. Similarly, the down-regulation of TIMP2 effectively counteracted the inhibitory effect of miR-3157-3p inhibitors (Figure S4A, S4B,S4D). In addition, it was found that the up-regulation of KLF2 can inhibit the vascular permeability of miR-3157-3p, while the low expression of KLF2 had the opposite effect (Figure S4C).However, TIMP2 overexpression or si-TIMP2 had no effect on vascular permeability compared with the NC group (Figure S4E). So we found that cells transfected with plasmid TIMP2 / KLF2 showed up-regulation of TIMP2 / KLF2 compared with the control group, which can inhibit the effect of Lv-miR-3157-3p. However, TIMP2 / KLF2 was down-regulated in si-TIMP2 / KLF2 transfected cells, inhibiting the effect of Lv-miR-3157-3p-inhibitor (Figure S4F).

NSCLC-secreted miR-3157-3p primes pre-metastatic niche

We speculate that exosome miR-3157-3p can significantly promote the proliferation, migration, angiogenesis and increased permeability in HUVECs in a manner similar to endogenous miR-3157-3p, and the formation of pre-metastatic niche. Then, we conducted a series of experiments to further confirm whether exosome miR-3157-3p induces angiogenesis and vascular permeability. The exosomes isolated

from transfected NSCLCs were co-cultured with HUVECs in order to determine whether exosomal miR-3157-3p mediates angiogenesis and permeability of HUVECs. Compared with the NC-Exos, miR-3157-3p-Exos can significantly increase HUVEC angiogenesis and vascular permeability. Related processes include HUVEC proliferation (Figure 5A), tube formation (Figure 5 B-C), permeability (Figure 5D), and migration (Figure 5E). The expressions of ZO-1, Occludin, and Claudin5 were further studied in the IF experiment (Figure 5F). To further determine that miRNAs in exosomes play a role in promoting metastasis, we used PCR to study the expression level of miR-3157-3p in each group (Figure 6A). In addition, treatment with miR-3692-3p inhibitor and GW4869 (an inhibitor of exosome internalization), A549 / miR-3157-3p exosomes fail to induce angiogenesis and migration of HUVECs (Figure 6 B-C). We have confirmed in WB and found that in HUVEC cells with high miR-3157-3p expression, TIMP2, KLF2, ZO-1, Occludin, Claudin5 expression decreased, while VEGF, MMP2, MMP9 expression increased (Figure 6D). In order to further study whether TIMP2 and KLF2 are Exo-miR-3157-3p target genes in HUVEC, we conducted a rescue experiment. We stably transfected HUVEC cells with TIMP2 / KLF2 plasmid and siRNA-TIMP2 / KLF2. The high expression of TIMP2 reversed the effect of miR-3157-3p-Exo-regulation on HUVEC cell tube formation and migration. Similarly, the down-regulation of TIMP2 effectively counteracted the inhibitory effect of miR-3157-3p inhibitors-Exo (Figure 6E, 6G). The expression of TIMP2 has no effect on vascular permeability (Figure 6F). In addition, the up-regulation of KLF2 inhibited miR-3157-3p-Exo vascular penetration, while the low expression of KLF2 had an opposite effect (Figure 6H).

Exo-miR-3157-3p promotes angiogenesis and vascular leakage in vivo

We have established a mouse xenograft model to assess angiogenesis in vivo (Figure 7A). Consistently, miR-3157-3p-Exos-treated mouse xenografts resulted in higher microvessel density (MVD) and larger tumor size compared to NC-Exos treatment (Figure 7B, 7C, 7D). IHC staining found that compared to the control group, TIMP2 decreased in the miR-3157-3p-Exos treatment group, while the expression of EGFR / MMP2 / KLF2 increased (Figure 7 F-H). To further confirm that NSCLC cells affect angiogenesis in vivo, a chicken chorioallantoic membrane (CAM) model was established to study the effect of miR-3157-3p on angiogenesis in vivo. The overexpression of miR-3157-3p enhanced angiogenesis, and the number of blood vessel branches (5 mm) around the CAM vehicle increased significantly (Figure 7I). These results then showed that exosome miR-3157-3p significantly enhanced angiogenesis in vitro and in vivo. To study whether miR-3157-3p-induced vascular permeability can promote NSCLC metastasis, A549 cells were injected into the tail vein of nude mice pretreated with exosomes. As expected, mice injected with A549 / miR-3157-3p-Exo had more lung metastatic colonies than mice derived from A549 / NC-Exo (Figure 7J). Taken together, these results clearly show that cancer-derived exosome miR-3157-3p can induce the formation of a niche before metastasis, thereby promoting the metastasis of NSCLC.

Exosomal miR-3157-3p is associated with metastatic progression and microvessel density in NSCLC patients.

In previous experiments we have confirmed that circulating exosomes are significantly elevated in the blood of metastatic patients compared to non-metastatic patients. To assess whether exosome miR-3157-

3p acts as a biomarker, we performed receiver operating characteristic curves. The area under the curve (AUC) was 0.7181 in exosome miR-3157-3p (Figure 8A). Through ISH experiments, we found that miR-3157-3p in metastatic lung cancer tissues was higher than non-metastatic lung cancer tissues (Figure 8B). Interestingly, we found that in tissues with high miR-3157-3p expression, CD34 expression also increased. We suspect that miR-3157-3p will promote angiogenesis in cancer tissue (Figure 8C). Therefore, our clinical data indicate that high levels of miR-3157-3p in circulating exosomes are associated with NSCLC metastasis.

Discussion

Lung cancer is the malignant tumor that causes the most deaths among cancers, and metastasis is the main cause of death of patients[33]. The niche before metastasis is the microenvironment prepared for colonizing circulating tumor cells in specific organs, including inflammation, immune response, angiogenesis, organic matter, matrix remodeling, and biomarker expression. The microenvironment before metastasis can increase angiogenesis and vascular permeability, thereby promote the metastasis. Studies have showed that exosomes released by hypoxic tumors are more likely to cause angiogenesis and vascular leakage[33, 34]. Cancer-induced vascular permeability and angiogenesis have played a key role in cancer metastasis. Exosomes from NSCLC can be used as a communication medium between cells. MiR-3157-3p secreted from NSCLC can induce a niche before metastasis by promoting angiogenesis and destroying the tight junction of venous endothelial cells.

Exosomes are extracellular vesicles with a diameter of 30–150 extranm. The contained miRNA can regulate tumor immunity and microenvironment, which may promote tumor invasion, metastasis and angiogenesis. GSC-derived exosomes such as overexpressing miR-26a can promote HBMEC proliferation and angiogenesis in vitro by inhibiting PTEN[35]. Exosome miR-25-3p is involved in niche formation before the transfer and may be used as a blood-based biomarker for CRC transfer[30]. Therefore, exosome miRNA plays an important role in regulating cancer progression [36].

miR-3157-3p is abnormally expressed in NSCLC patients, especially those with metastases. We found that miR-3157-3p in vascular endothelial cells can induce vascular permeability and angiogenesis by down-regulating KLF2 and TIMP2. Kruppel-like factors (KLFs) are a subclass of the zinc finger family of DNA-binding transcription factors. KLF2 can regulate the key tight junction protein occludin in endothelial cells to maintain the integrity of the endothelial barrier function[37]. TIMP2 is a natural inhibitor of MMP activity and inhibits angiogenesis. In NSCLC, both KLF2 and TIMP2 are down-regulated. Our results showed that miR-3157-3p in HUVEC can absorb exosomes downregulates KLF2 and TIMP2, and subsequently reduce its downstream target, occludin, and increase the levels of VEGF / MMP2 / MMP9. Our data indicate that the exosome miR-3157-3p secreted by NSCLC promotes vascular permeability and angiogenesis by silencing KLF2 and TIMP2(Figure 8D).

Tumor-derived exosomes are considered to be the main driving force of the pre-metastatic niche. Exosomes participated in angiogenesis and increased vascular permeability, thereby promote the

formation of niche before transfer. Before metastasis, a microenvironment suitable for tumor metastasis has been created for tumor metastasis. In this study, we explored that whether exosome miR-3157-3p can regulate niche formation before metastasis. Cancer-induced vascular permeability / angiogenesis is one of the characteristics of the pre-metastatic niche. In prostate cancer, miR-21-5p and miR-139-5p, which are microRNAs derived from exosomes, could coordinately regulate the ecological niche before metastasis, and was highly relevant to differentiate prostate cancer (PCa) carcinogenesis, fibroblast proliferation, differentiation, migration and angiogenesis[38]. In vitro permeability measurements showed that the exosome miR-25-3p of NSCLC cells significantly promoted HUVEC permeability and angiogenesis, reduced the levels of KLF2, ZO-1, occludin, Claudin5, and increased VEGF / MMP2 / MMP9 level. We also found that the upregulation of miR-3157-3p in NSCLC cells promoted proliferation, angiogenesis and metastasis. In summary, we have proposed a new role of TIMP2 / KLF2 in the formation of cancer-induced pre-metastatic niche, and provided new insights into the formation of the metastasis niche. Also we have prevented the transport of exosomes to recipient cells may be an effective strategy to prevent tumor metastasis. There are many targets that can be used to inhibit the formation of pre-metastatic niche, for example, the prevent of the production of pro-inflammatory factors, the inhibition of the recruitment of BMDC, the prevent of angiogenesis and vascular penetration, and the destroy of local matrix and reactivating anti-tumor immune responses). These goals may develop potential methods for preventing and controlling cancer metastasis in the future. Therefore, we believe that blocking exosome miR-3157-3p from NSCLC cells can maintain the tight junction between vascular endothelium, reduce pulmonary vascular permeability and subsequent NSCLC metastasis. It shows that miR-3157-3p can be used as a therapeutic target for the intervention in NSCLC metastasis.

Conclusion

In conclusion, we found that the level of serum exosome miR-3157-3p is related to NSCLC metastasis. However, the molecular mechanism of miR-3157-3p regulating NSCLC metastasis has not been reported. It was confirmed by experiments that exosomes secreted by NSCLC cells can transfer miR-3157-3p to HUVEC, thereby promoting angiogenesis and destroying the integrity of vascular endothelium, and resulting in an increase in vascular permeability.

Abbreviations

NSCLC: non-small cell lung cancer; qRT-PCR: Quantitative real-timePCR; miRNAs: MicroRNAs; KLF2: Kruppel-like factor 2 VEGF: Vascular endothelial growth factor; FBS: Fetal bovine serum; PVDF: polyvinylidene difluoride; ECL: enhanced chemiluminescence; CCK8: Cell counting kit-8; EdU: 5-Ethynyl-2'-deoxyuridine; IHC: Immunohistochemistry; IF: Immunofluorescence; ISH: RNA insitu hybridization; 3'UTR: 3'untranslatedregion; TEM: Transmission electron microscope; NTA: Nanoparticle Tracking Analyzer; MVD: microvessel density; CAM: chicken chorioallantoic membrane; PCa: prostate cancer;

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Animal Protection and Use Committee of Nanjing Medical University. Human tissue research was approved by the Medical Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. All participants have obtained written informed consent.

Consent for publication

All patients obtained written informed consent.

Data availability statement

All datasets and materials generated and / or analyzed during the current studies are available.

Conflict of interests

The authors declare that there are no conflict of interests.

Funding information

Major Program of Science and Technology Foundation of Jiangsu Province, Grant/Award Numbers: BE2016790, BE2018746; National Natural Science Foundation of China Youth , Fund Number: 81902328/81702262.

Author contributions

Y.X and Y.C have conceived the experiment, L.C, K.W, and C.P have designed the experimental procedure, Z.M, Y.H, and Z.G have performed the experiment and analyzed the data, F.Y has provided bioinformatics help and guided the experiment. Z.M has wrote the manuscript, and Y.C has deployed and arranged the overall work.

Acknowledgments

The authors would like to give thanks to all patients, researchers, students who have participated in this study, and the animals sacrificed.

References

1. Li, K., et al., *Exosomes play roles in sequential processes of tumor metastasis*. International journal of cancer, 2019. **144**(7): p. 1486-1495.
2. Mashouri, L., et al., *Exosomes: composition, biogenesis, and mechanisms in cancer metastasis and drug resistance*. Molecular cancer, 2019. **18**(1): p. 75.
3. Yu, Z., et al., *Pancreatic cancer-derived exosomes promote tumor metastasis and liver pre-metastatic niche formation*. Oncotarget, 2017. **8**(38): p. 63461-63483.

4. Peinado, H., et al., *Pre-metastatic niches: organ-specific homes for metastases*. Nature reviews. Cancer, 2017. **17**(5): p. 302-317.
5. Kaplan, R.N., et al., *VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche*. Nature, 2005. **438**(7069): p. 820-827.
6. Sceneay, J., M.J. Smyth, and A. Möller, *The pre-metastatic niche: finding common ground*. Cancer metastasis reviews, 2013. **32**(3-4): p. 449-464.
7. Liu, Y. and X. Cao, *Characteristics and Significance of the Pre-metastatic Niche*. Cancer cell, 2016. **30**(5): p. 668-681.
8. Chafe, S.C., et al., *Carbonic anhydrase IX promotes myeloid-derived suppressor cell mobilization and establishment of a metastatic niche by stimulating G-CSF production*. Cancer research, 2015. **75**(6).
9. Zhang, W., et al., *CD8+ T-cell immunosurveillance constrains lymphoid premetastatic myeloid cell accumulation*. European journal of immunology, 2015. **45**(1): p. 71-81.
10. Ell, B., et al., *Tumor-induced osteoclast miRNA changes as regulators and biomarkers of osteolytic bone metastasis*. Cancer cell, 2013. **24**(4): p. 542-556.
11. Zhou, W., et al., *Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis*. Cancer cell, 2014. **25**(4): p. 501-515.
12. Arscott, W.T. and K.A. Camphausen, *Exosome characterization from ascitic fluid holds promise for identifying markers of colorectal cancer*. Biomarkers in medicine, 2011. **5**(6): p. 821-822.
13. El Andaloussi, S., et al., *Extracellular vesicles: biology and emerging therapeutic opportunities*. Nature reviews. Drug discovery, 2013. **12**(5): p. 347-357.
14. Record, M., et al., *Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiology*. Biochimica et biophysica acta, 2014. **1841**(1): p. 108-120.
15. Choi, D.-S., et al., *Proteomics, transcriptomics and lipidomics of exosomes and ectosomes*. Proteomics, 2013. **13**(10-11): p. 1554-1571.
16. Martins, V.R., M.S. Dias, and P. Hainaut, *Tumor-cell-derived microvesicles as carriers of molecular information in cancer*. Current opinion in oncology, 2013. **25**(1): p. 66-75.
17. Peinado, H., S. Lavotshkin, and D. Lyden, *The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts*. Seminars in cancer biology, 2011. **21**(2): p. 139-146.
18. Tetta, C., et al., *Extracellular vesicles as an emerging mechanism of cell-to-cell communication*. Endocrine, 2013. **44**(1): p. 11-19.
19. Valadi, H., et al., *Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells*. Nature cell biology, 2007. **9**(6): p. 654-659.
20. Zöller, M., *Pancreatic cancer diagnosis by free and exosomal miRNA*. World journal of gastrointestinal pathophysiology, 2013. **4**(4): p. 74-90.
21. van Niel, G., G. D'Angelo, and G. Raposo, *Shedding light on the cell biology of extracellular vesicles*. Nature reviews. Molecular cell biology, 2018. **19**(4): p. 213-228.

22. Xu, J., K. Liao, and W. Zhou, *Exosomes Regulate the Transformation of Cancer Cells in Cancer Stem Cell Homeostasis*. Stem cells international, 2018. **2018**: p. 4837370.
23. Hurley, J.H., *ESCRT complexes and the biogenesis of multivesicular bodies*. Current opinion in cell biology, 2008. **20**(1).
24. Pegtel, D.M. and S.J. Gould, *Exosomes*. Annual review of biochemistry, 2019. **88**: p. 487-514.
25. Rivoltini, L., et al., *TNF-Related Apoptosis-Inducing Ligand (TRAIL)-Armed Exosomes Deliver Proapoptotic Signals to Tumor Site*. Clinical cancer research : an official journal of the American Association for Cancer Research, 2016. **22**(14): p. 3499-3512.
26. Ye, S.-B., et al., *Tumor-derived exosomes promote tumor progression and T-cell dysfunction through the regulation of enriched exosomal microRNAs in human nasopharyngeal carcinoma*. Oncotarget, 2014. **5**(14): p. 5439-5452.
27. Au Yeung, C.L., et al., *Exosomal transfer of stroma-derived miR21 confers paclitaxel resistance in ovarian cancer cells through targeting APAF1*. Nature communications, 2016. **7**: p. 11150.
28. Lobb, R.J., L.G. Lima, and A. Möller, *Exosomes: Key mediators of metastasis and pre-metastatic niche formation*. Seminars in cell & developmental biology, 2017. **67**.
29. Melo, S.A., et al., *Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis*. Cancer cell, 2014. **26**(5): p. 707-721.
30. Zeng, Z., et al., *Cancer-derived exosomal miR-25-3p promotes pre-metastatic niche formation by inducing vascular permeability and angiogenesis*. Nature communications, 2018. **9**(1): p. 5395.
31. Liu, L.-Z., et al., *MiR-21 induced angiogenesis through AKT and ERK activation and HIF-1 α expression*. PloS one, 2011. **6**(4): p. e19139.
32. Bhattacharya, R., et al., *Inhibition of vascular permeability factor/vascular endothelial growth factor-mediated angiogenesis by the Kruppel-like factor KLF2*. The Journal of biological chemistry, 2005. **280**(32): p. 28848-28851.
33. Kucharczywska, P., et al., *Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development*. Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(18): p. 7312-7317.
34. King, H.W., M.Z. Michael, and J.M. Gleadle, *Hypoxic enhancement of exosome release by breast cancer cells*. BMC cancer, 2012. **12**: p. 421.
35. Wang, Z.-F., et al., *Glioma stem cells-derived exosomal miR-26a promotes angiogenesis of microvessel endothelial cells in glioma*. Journal of experimental & clinical cancer research : CR, 2019. **38**(1): p. 201.
36. Sun, Z., et al., *Effect of exosomal miRNA on cancer biology and clinical applications*. Molecular cancer, 2018. **17**(1): p. 147.
37. Lin, Z., et al., *Kruppel-like factor 2 regulates endothelial barrier function*. Arteriosclerosis, thrombosis, and vascular biology, 2010. **30**(10): p. 1952-1959.

38. Sánchez, C.A., et al., *Exosomes from bulk and stem cells from human prostate cancer have a differential microRNA content that contributes cooperatively over local and pre-metastatic niche*. Oncotarget, 2016. 7(4): p. 3993-4008.

Tables

Exo-miR-3157-3p expression			
Factor	High	Low	P Value
Gender			
Male	19	22	0.173
Female	24	15	
Age(y)			
≥60	18	16	0.651
<60	22	24	
Histology			
LAC	21	28	0.185
LSC	18	13	
Smoker			
Yes	22	19	0.269
No	17	24	
TNM stage			
T1-T2	25	15	0.010*
T3	35	5	
Lymph node metastasis			
Yes	32	8	0.017*
No	22	18	

LAC: lung adenocarcinoma; LSC: lung squamous cell carcinoma.

* $P < .05$ (Chi-square test).

Figures

Figure 1

Exosomal miR-3157-3p is abundant in NSCLC patients. A The morphology of exosomes was examined by TEM. B The exosomes were quantified by nanoparticle tracking analysis. C CD63 and TSG101 were determined by western blot. D A heatmap representing microarray data for the indicated NSCLC patient plasma with metastasis and corresponding normal plasma. Three clinical samples were pooled in each group. E-F GO enrichment analysis of DEGs: Two histograms refer to the cellular component (CC) and panther enrichment. G RT-PCR analysis of three miRNA (miR-3157-3p, miR-3613-5p and miR-3921) levels in 20 cases of serum exosomes from healthy controls or NSCLC patients (10 cases normal, 10 cases with NSCLC). H The expression levels of miR-3157-3p in serum exosomes from NSCLC patients (40 without metastasis and 40 with metastasis). I RT-PCR analysis of miR-3157-3p expression in 47 primary NSCLC

tissues with or without metastasis (29 without metastasis and 18 with metastasis).The data are shown as the mean $\pm$ SD(*P < 0.05; **P < 0.01, ***P < 0.001).

Figure 2

NSCLC-secreted miR-3157-3p is transferred to HUVECS.A NSCLC cell lines miR-3157-3p levels were analyzed using qRT-PCR.B Representative electron microscopy micrographs and NTA of A549 conditioning medium secreted exosomes, as well as H1299 conditioning medium secreted exosomes. Scale bar, 100 nm.C CD63, TSG101, and calnexin were determined by western blot.D RT-PCR analysis of miR-3157-3p expression in HUVECs incubated with exosomes derived from A549/NC for 2h, 12h, 24h, and 48h.E-F miR-3157-3p expression in A549 and H1299 transfected with Lv-miR-3157-3p and their derived exosomes using RT-qpcr.G Cy3 fluorescence and PKH67 lipid dye in HUVECs after adding PKH67-

labeled exosomes derived from A549 and H1299 cells for 48 h. The data are shown as the mean $\pm$ SD (* P < 0.05; ** P < 0.01, *** P < 0.001).

Figure 3

Restored miR-3157-3p enhances HUVEC proliferation, migration, tube formation, angiogenesis and permeability. A proliferation of HUVECs transfected with Lv-miR-3157-3p or Lv-miR-3157-3p-inhibitor detected using EdU assay ($\times 400$); B migration of HUVEC transfected with Lv-miR-3157-3p or Lv-miR-3157-3p-inhibitor assessed using transwell assay ($\times 100$). C Effect of Lv-miR-3157-3p or Lv-miR-3157-3p-inhibitor on permeability of HUVEC monolayers. D tube formation of HUVECs transfected with Lv-miR-3157-3p or Lv-miR-3157-3p-inhibitor ($\times 100$). E-F protein levels of TIMP2, VEGF, MMP2, MMP9, KLF2, ZO-1, Occludin, and Claudin5 of HUVEC transfected with Lv-miR-3157-3p or Lv-miR-3157-3p-inhibitor

measured using Western blot analysis;G Immunofluorescence staining analysis of ZO-1, occludin, Claudin5 expression in HUVECs ransfected with Lv-miR-3157-3p or Lv-miR-3157-3p-inhibitor(x400). The data are shown as the mean $\pm$ SD (*P < 0.05; **P < 0.01, ***P < 0.001).

Figure 4

TIMP2 and KLF2 are functional targets of miR-3157-3p.A The candidate gene targets were predicted by intersecting outputs from four distinct prediction algorithms (TargetScan, miRDB, and miRWalk).B TIMP2 expression was found to be statistically significant in NSCLC tissue.C The relationship between TIMP2and the survival of NSCLC patients (n = 963 in the TIMP2-low group and n = 962 in the TIMP2-high group).D The TIMP2 expression level in 40 NSCLC tissues and adjacent tissues was detected by qRT-PCR.E The candidate gene targets were predicted by intersecting outputs from four distinct prediction

algorithms (TargetScan and miRWalk).F KLF2 expression was found to be statistically significant in NSCLC tissue.G The relationship between KLF2 and the survival of NSCLC patients (n = 962 in the TIMP2-low group and n =963 in the KLF2-high group).H The KLF2 expression level in 40 NSCLC tissues and adjacent tissues was detected by qRT-PCR.I the miR-3157-3p binding site in TIMP2 3'UTR and the detection of luciferase activity.J the miR-3157-3p binding site in KLF2 3'UTR and the detection of luciferase activity.K A negative regulatory effect of miR-3157-3p on TIMP2/KLF2 was tested by western blot.The data are shown as the mean $\pm$ SD (*P < 0.05; **P < 0.01, ***P < 0.001).

Figure 5

NSCLC-secreted -miR-3157-3p promotes angiogenesis and vascular leakiness in vitro.A proliferation of HUVECs co-cultured with A549/miR-3157-3p exosomes, A549/NC exosomes,H1299/miR-3157-3p

exosomes, H1299/NC exosomes (× 400).B Effects of HUVECs co-cultured with A549/miR-3157-3p exosomes, A549/NC exosomes,H1299/miR-3157-3p exosomes, H1299/NC exosomes on vascular outgrowth of rat aortic rings.(× 40).C Effects of HUVECs co-cultured with A549/miR-3157-3p exosomes, A549/NC exosomes,H1299/miR-3157-3p exosomes, H1299/NC exosomes on tube formation(x100).D Effect of HUVECs co-cultured with A549/miR-3157-3p exosomes, A549/NC exosomes,H1299/miR-3157-3p exosomes, H1299/NC exosomes on permeability of HUVEC monolayers.E Effects of HUVECs co-cultured with A549/miR-3157-3p exosomes, A549/NC exosomes,H1299/miR-3157-3p exosomes, H1299/NC exosomes on migration (× 100).F Immunofluorescence staining analysis of ZO-1, occludin, Claudin5 expression in HUVECs co-cultured with A549/miR-3157-3p exosomes, A549/NC exosomes,H1299/miR-3157-3p exosomes, H1299/NC exosomes (× 400). The data are shown as the mean ± SD (*P < 0.05; **P < 0.01, ***P < 0.001).

Figure 6

TIMP2 and KLF2 are functional targets of Exo-miR-3157-3p in HUVECs. A Effect of A549/miR-3157-3p exosomes, A549/NC exosomes, A549/miR-3157-3p exosomes+ miR-3157-3p inhibitor, A549/miR-3157-3p exosomes+GW4869 treatments on miR-3157-3 expression in HUVECs. B Effect of A549/miR-3157-3p exosomes, A549/NC exosomes, A549/miR-3157-3p exosomes+ miR-3157-3p inhibitor, A549/miR-3157-3p exosomes+GW4869 treatments on tube formation(x100). C Effect of A549/miR-3157-3p exosomes, A549/NC exosomes, A549/miR-3157-3p exosomes+ miR-3157-3p inhibitor, A549/miR-3157-3p exosomes+GW4869 treatments on migration ($\times 100$). D protein levels of TIMP2, VEGF, MMP2, MMP9, KLF2, ZO-1, Occludin, and Claudin5 of HUVEC treated with A549/ miR-3157-3p exosomes, A549/NC exosomes, A549/miR-3157-3p exosomes+miR-3157-3p inhibitor, A549/miR-3157-3p exosomes+GW4869 measured using Western blot analysis E, G The effect of promoting tube formation and migration of Exo-miR-3157-3p was reversed by high expression of TIMP2, while knockdown of TIMP2 enhanced the role of Exo-miR-3157-3p. F. TIMP2 cannot reverse the role of Exo-miR-3157-3p on permeability. I The effect of permeability of Exo-miR-3157-3p was reversed by high expression of KLF2, while knockdown of KLF2 enhanced the role of Exo-miR-3157-3p. The data are shown as the mean $\pm$ SD (*P < 0.05; **P < 0.01, ***P < 0.001).

Figure 7

exosomal miR-3157-3p promotes angiogenesis and vascular leakiness in vivo. A, B After 7-9 days, subcutaneous mouse xenografts were intratumorally injected with miR-3157-3p-Exo or NC-Exo (n=4 each). C tumor volume. D-H CD34, TIMP2, VEGF, MMP2 and KLF2 expression levels the samples collected from nude mice were analyzed by IHC. I. the number of branches of blood vessels (5 mm) around the CAM vehicle; J The mice were injected with A549 cells via tail vein after exposure to miR-3157-3p-Exo or NC-Exo treatments. The number of lung metastatic sites was counted under the microscope. The data are shown as the mean ± SD (*P < 0.05; **P < 0.01, ***P < 0.001).

Figure 8

exosomal miR-3157-3p is associated with NSCLC metastasis. A ROC curves were used to evaluate whether exosomal-miR-3157-3p acts as a biomarker. B Representative images of in situ hybridization for miR-3157-3p in the tissues with or without metastasis. C Representative images of miR-3157-3p IHC for CD34 with high or low expression levels of miR-3157-3p. The scale bar in 200× images represents 100 μm. The scale bar in 400× images represents 50 μm. D Schematic diagram of the role of NSCLC-secreted miR-3157-3p in pre-metastatic niche formation. The data are shown as the mean ± SD (*P < 0.05; **P < 0.01, ***P < 0.001).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [AdditionalfileFigureS4.tif](#)
- [AdditionalfileFigureS4.tif](#)
- [AdditionalfileFigureS3.tif](#)
- [AdditionalfileFigureS3.tif](#)
- [AdditionalfileFigureS2.tif](#)
- [AdditionalfileFigureS2.tif](#)
- [AdditionalfileFigureS1.tif](#)
- [AdditionalfileFigureS1.tif](#)