Exosomal miR-3180-3p Inhibits Proliferation and Metastasis of Non-Small Cell Lung Cancer by Downregulating FOXP4

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Abstract

Background: Non-small cell lung cancer (NSCLC) is the most malignant cancers worldwide, but the pathogenesis is not completely known. In this study, we explored the function and mechanism of exosomes transferring miR-3180-3p in NSCLC progression.

Method: The expression of miR-3180-3p of NSCLC tissues and para-carcinoma tissues was from the GEO database (GEO: GSE53882). The exosomes derived from A549 cells were identified. The proliferation, migration and invasion were measured after treatment with exosomal miR-3180-3p or transfected by miR-3180-3p mimics. The relationship between miR-3180-3p and forkhead box P4 (FOXP4) was predicted by bioinformatics tool and measured dual-luciferase reporter gene assay and western blotting. At last, mouse xenograft model of NSCLC cells was established to verify the function of exosomal miR-3180-3p in vivo.

Results: We found that miR-3180-3p decreased in both NSCLC cell lines and patient tissues. Overexpression of miR-3180-3p or treatment with exosomal miR-3180-3p significantly repressed the cell proliferation and metastasis in NSCLC cell lines. Subsequently, we found miR-3180-3p performed function by downregulating FOXP4 protein expression. Furthermore, the volume and weight of nude mice tumor which expressed exosomal miR-3180-3p was significantly reduced.

Conclusion: Exosomal miR-3180-3p suppresses NSCLC progression by downregulating FOXP4 expression.

Introduction

Lung cancer is one of the most common tumors with highest mortality rate. Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancer^[1]. Despite progress in treatments for NSCLC, the 5-year survival rate is less than 15%^[2–3]. To better understand the mechanism of NSCLC and find the potential prognostic biomarkers is still a priority.

MicroRNAs (miRNAs) are a kind of single-stranded non-coding RNAs with 19–24 nt in length, which can regulate gene expression at post-transcriptional level by targeting 3'-untranslated region (3'-UTR) of mRNA^[4]. The aberrant expression of miRNAs is closely related to cancers and tumor microenvironment^[5]. MiR-421 was reported to contribute to NSCLC cell migration, invasion and inhibit cell apoptosis by mediating β -catenin-miR-421-KEAP1 axis^[6]. MiR-330-3p promotes brain metastasis and epithelial-mesenchymal transition (EMT) by mediating GRIA3-TGF- β 1 interaction^[7].

Exosomes are extracellular vesicles with lipid bilayer-enclosed structure which are smaller than 150 nm. They contain proteins and nucleic acids that can be released from many cell types^[8–9]. Exosomes often contain RNA molecules or proteins which provide a mechanism for these bioactive compounds to transfer into neighboring cells, thus mediating intercellular communication^[10–11].

The RNAs concentrated in exosomes include miRNAs, mRNAs, lncRNAs (long noncoding RNAs) and circRNAs (circular RNAs)^[12–14]. Increasing evidence has shown that miRNA enriched in exosomes can be shuttled to neighboring cells and regulate their biological functions^[15, 16]. Exosomes come from tumor cells which have been found relative to tumor angiogenesis, invasion and chemoresistance^[17–19]. For example, Human mesenchymal stem cells (MSCs) derived exosomes enriched miR-143 transfer to prostate cancer cells and inhibit PC3 cell proliferation and metastasis by suppressing TFF3 protein expression^[20]. Exosomes with low miR-34c-3p expression promote migration and invasion of NSCLC by upregulating integrin $\alpha 2\beta 1$ ^[21]. With increasing attention of the function of miRNA enriched in exosomes, in this study we focus on the mechanism of miR-3180-3p in exosomes.

Materials

Cell culture

All NSCLC cell lines were obtained from the Chinese Academy of Sciences (Shanghai, China). Cell lines were cultured in RPMI-1640 (Sigma) with 10% FBS (Thermo Fisher Scientific), 100 units/mL penicillin and 100 μ g/mL streptomycin at 37 °C in 5% CO₂. To remove exosomes, FBS for cell culture was ultracentrifuged at 100,000 g for 10 hours.

Isolation and characterization of exosomes

After culturing cells for 48 h in exosome-free FBS, medium was collected, filtrated with 0.22 μ m filter. Exosomes were got from ultracentrifugation-based isolation techniques^[22]. Briefly, the medium was centrifuged at 300 $\times$ g for 10 min, 2,000 $\times$ g for 15 min, 5,000 $\times$ g at 15 min and 12,000 $\times$ g for 30 min, then the supernatant was ultra-centrifuged at 100,000 $\times$ g for 70 min. Discarded the supernatant and used PBS to wash the bottom exosomes. Pelleted the exosomes by ultracentrifugation at 100,000 $\times$ g for 70 min. All ultracentrifugation steps were performed at 4 °C in Beckman ultracentrifuge (TL-100). The exosomes were assessed via nanoparticle tracking analysis (NTA; ZetaView, Particle Metrix, DE), western blotting and transmission electron microscopy (TEM; JEM-1400, JEOL, Japan).

For the exosome-tracking experiments, exosomes from A549 cells were labeled by PKH67 membrane dye (Sigma-Aldrich), labeled exosomes were washed twice by medium without FBS. A549 cells were seeded into 24-well chamber, labeled exosomes were added and incubated 3 h. Cells were fixed with 4% paraformaldehyde for 10 min. Cells were washed thrice with PBS, and blocked with 0.1% Triton-X 100 for 1 h, then incubated with Actin antibody at 4 °C overnight. Next day, cells were stained 20 μ g/mL Alexa Fluor 594 IgG (H + L) (Life Technologies, Grand Island, NY) for 10 min at room temperature. Cellular nuclei were stained with DAPI. The merge images were captured using the electron microscope (LSM 710, Carl Zeiss).

Dual-luciferase reporter assay

The FOXP4 3'UTR nucleotide sequence predicted miR-3180-3p target sites and the mutation sequence were synthesized (Genewiz, Beijing, China), digested with XhoI and NotI, and cloned into the psiCHECK-2 dual luciferase vector (Promega, Madison, WI, USA) to generate the FOXP4-3'UTR wt and FOXP4-3'UTR mut. Subsequently, NSCLC cells were plated in a 24-well plate and co-transfected with 50 ng FOXP4-3'UTR wt or FOXP4-3'UTR mut and 20 nM either miR-3180-3p mimics (5'-UGGGGCGGAGCUUCCGGAG-3') or miR-NC (5'-UUCUCCGAACGUGUCACGUTT-3'). After 48 h transfection, cells were collected, luciferase activities were measured by Dual-Luciferase Reporter assay kit (Promega) on a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA).

Western blotting

Cell or exosome lysates were acquired using RIPA lysis buffer (Beyotime, Shanghai, China) and separated by 10% SDS-PAGE. Proteins were transferred onto PVDF membranes and blocked with 5% solution of nonfat milk for 1 h. The membranes were treated with primary antibodies against FOXP4 (ab251688, Abcam) and GAPDH (#5174, CST) overnight at 4 °C. Subsequently, The membranes incubated with a secondary antibody for 1 hour at room temperature. Protein bands were detected by ECL Substrate (Tanon Science and Technology, Shanghai, China). GAPDH was used as an internal reference.

qRT-PCR

Total RNA was extracted by TRIzol (Invitrogen, CA, USA) according to the manufacture's instruction. The cDNA synthesis with reverse transcriptase (RT) was performed using a M-MLV First Strand kit (Life Technologies). MiRNA First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used for miRNA reverse transcriptase. The miScript SYBR Green PCR Kit (Qiagen) was used for RT-PCR reactions. Relative expression level of miRNAs or mRNA was calculated using the $\Delta\Delta C_t$ method. GAPDH and U6 small nuclear RNA (U6 snRNA) were used for normalization of mRNA and miRNA. The primer sequences are presented in Table 1.

Table 1
Primer Sequence for qRT-PCR

Gene	Primer Sequence (5' – 3')
miR-3180-3p	F: GCGTGGGGCGGAGCTT
	R: AGTGCAGGGTCCGAGGTATT
U6	F: AGAGCCTGTGGTGTCCG
	R: CATCTTCAAAGCACTTCCCT
GAPDH	F: CCTTCCGTGTCCCCACT
	R: GCCTGCTTCACCACCTTC
FOXP4	F: AGGACACGGAGGGTTCAG
	R: TGTGGGGAGAGCTGGTG
FOXP4, forkhead box P4; miR-3180-3p, microRNA-3180-3p; F, forward; R, reverse	

Cell proliferation and transwell assay

Transwell assay was performed using 24-well chambers containing membrane filter inserts. After 48 h of transfection, cell lines A549 and H460 were collected and resuspended in serum-free RPMI1640 medium at the cell density of 1×10^5 cells/mL. The chambers were used for migration assay and coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were used for invasion assay. The upper chambers were added to 200 μ L cell suspension. The under layers were added 600 μ L RPMI1640 medium with 20% FBS. After 24 h chambers were fixed with 4% paraformaldehyde for 30 min, stained with 0.5% crystal violet solution for 10 min, and washed by PBS for three times. The cells on the upper chambers were removed with a cotton swab. The transmembrane cells were counted over three random fields. The experiment was repeated 3 times.

Tumor xenograft and tail vein injection

The 24 4-week-old nude mice were divided into 2 groups with 5 mice in each group at random for tumor growth observation. 1×10^7 A549 cells were resuspended with 50% Matrigel. A total of 0.2 mL cells was subcutaneously injected into mice. To observe the effect of exosomes on tumor growth in vivo, 100 μ g exosomes from transfected A549 cells which transfected with miR-3180-3p mimics or negative control in 100 μ L PBS were injected into mice by tail vein every 3 days according to previous reports^[23]. The tumor size was measured every 7 days. After 5 weeks all nude mice were sacrificed, the tumors were removed and weighed. This study was approved and conducted by the Institutional Animal Care and Use Committee of the Soochow University.

Statistical analysis

Significance tests and correlation analyses were performed using GraphPad Prism 5.0 (GraphPad Software). Student's t test was used in the statistical analysis. *P*-value less than 0.05 was considered statistically significant.

Results

Low level expression of miR-3180-3p in NSCLC patients

We extracted non-small cell lung cancer expression profile data from the GEO database (GEO: GSE53882). We found miR-3180-3p was significantly lower than that in paired para-carcinoma tissues (Fig. 1a). Furthermore, we detected miR-3180-3p expression in NSCLC cell lines and found miR-3180-3p was significantly lower in A549 and H460 cell lines as compared to normal cell line HBE (Fig. 1b).

Overexpression of miR-3180-3p suppresses proliferation, migration, and invasion in NSCLC Cells

To explore the function of miR-3180-3p on the biological function of NSCLC cells, we transfected A549 and H460 cell lines with miR-NC and miR-3180-3p mimics. The proliferation, migration and invasion of NSCLC cells were examined. First, the transfection efficiency was determined by qRT-PCR. The results showed that the expression of miR-3180-3p was strikingly increased (Fig. 2c). The biological function showed that, compared with the control group, the proliferation, migration, and invasion abilities were significantly decreased in the miR-3180-3p mimic group ($p < 0.05$) (Figs. 2d-h).

FOXP4 is the target gene of miR-3180-3p

To determine the target gene of miR-3180-3p, we used the TargetScan software to identify FOXP4 as a possible target gene (Fig. 2a). To clarify its accuracy, a dual luciferase assay was performed. A549 and H460 cells were co-transfected with miR-NC or miR-3180-3p mimics and FOXP4-3'UTR wild type (WT) or FOXP4-3'UTR mutation type (MUT) luciferase reporter vector. As shown in Fig. 2b-c, compared with the control group, miR-3180-3p mimics significantly decreased the luciferase activity in the WT group ($p < 0.05$), but had no significant effect on the MUT group ($p > 0.05$).

To further demonstrate FOXP4 is a target gene of miR-3180-3p, we detected the FOXP4 mRNA and protein expression after transfecting by miR-3180-3p mimics. The results showed that both FOXP4 mRNA and protein expression were significantly decreased (Fig. 2d-e). The FOXP4 mRNA expression was verified by TCGA database (Fig. 2f). These results suggest that FOXP4 is the target gene of miR-3180-3p.

Knockdown of FOXP4 by siRNA shows the same phenotype as the effect of upregulating miR-3180-3p on NSCLC cells.

To explore the effects of FOXP4 on NSCLC, we used FOXP4-siRNA to knock down FOXP4 in A549 and H460 cells. The protein expression level of FOXP4 was significantly reduced in the two siRNA groups compared with the control group (Fig. 3a). In order to further clarify the effects of FOXP4-siRNA on cancer cell proliferation and metastasis, we used CCK-8 and transwell assay to determine the effects of FOXP4 on NSCLC cells. CCK-8 assay revealed that the growth rate of A549 and H460 cells transfected with

siRNA were significantly lower than control group cells (Fig. 3b). Transwell assay revealed that FOXP4 knockdown remarkably inhibited the migration and invasion of NSCLC cells (Fig. 3c-d).

Overexpression of FOXP4 reverses the effects of miR-3180-3p on NSCLC cells

Since our results suggested that miR-3180-3p inhibited cell growth and metastasis through the downregulation of FOXP4, we explored whether FOXP4 is a direct functional mediator of the inhibitory effects of miR-3180-3p. As expected, we observed that exogenous miR-3180-3p could significantly suppress the expression of FOXP4 and the FOXP4 expression was retarded after overexpression of FOXP4 (Fig. 4a-b). Functionally, CCK-8 assays showed that miR-3180-3p could suppress the proliferation of NSCLC cells and the suppression could be blocked by overexpressed FOXP4 (Fig. 4c-d). Furthermore, Transwell migration and invasion assays showed that miR-3180-3p could inhibit the metastasis of NSCLC cells and the inhibition could be blocked by increased FOXP4 (Fig. 4e-f). Collectively, these observations suggested that miR-3180-3p could inhibit the growth and metastasis of NSCLC, at least partially, through the miR-3180-3p-FOXP4 pathway.

Exosomal miR-3180-3p serves as a mediator in intercellular communication

It is well known that exosomes play an important role in cell-to-cell communication, and they can change the physiological function of recipient cells through biological active factors^[24]. From the website Exocarta we know that miR-3180-3p exist in exosomes. To further verify miR-3180-3p enriched in exosomes, we extract exosomes after transfecting miR-3180-3p mimics into A549 cells. The canonical morphology of exosomes was captured by transmission electron microscopy (TEM) which confirmed the presence of extracted exosomes (Fig. 5a). The sizes of exosomes were measured by particle size distribution. The sizes of exosomes was measured by particle size distribution with approximately 30–100 nm diameter (Fig. 5b). The expression of exosome markers such as Annexin V, Flotillin-1 and CD63 were examined by western blot which further proved the successful isolation of exosomes from the supernatants of NSCLC cells (Fig. 5c).

Exosomal miR-3180-3p inhibits proliferation, migration, and invasion in NSCLC Cells

To determine whether the purified exosomes could enter the cells and perform functions. Exosomes derived from A549 cells were labeled with a green fluorescent marker, PKH67. The result showed that PKH67 was localized in the cytoplasm of recipient cells (Fig. 5d). QPCR determined the effective functions of exosomal miR-3180-3p. The result showed that the expression of miR-3180-3p was significantly increased in recipient NSCLC cells when treating with Exo-miR-3180-3p (Fig. 5e).

Following the previous observation that miR-3180-3p is enriched in exosomes, we supposed that miR-3180-3p shuttled by exosomes could inhibit the proliferation, migration and invasion in NSCLC cells. To access the role of miR-3180-3p exosomes, NSCLC cells were treated with Exo-miR-NC or Exo-miR-3180-3p. The results showed that FOXP4 protein levels in A549 and H460 cells exposed to miR-3180-3p exosomes were significantly decreased compared to miR-NC exosomes (Fig. 5f). In addition, The

proliferation was inhibited by miR-3180-3p exosomes (Fig. 5g-h). And the migratory and invasive abilities obtained similar results (Fig. 5i-j).

Effect of exosomal miR-3180-3p on tumor in vivo

To further explore the role of exosomal miR-3180-3p in NSCLC in vivo, A549 cells were subcutaneously injected into nude mice. Exosomes extracted from A549 cells transfected with miR-3180-3p or miR-NC mimics were injected to mice by tail vein. In line with our analysis in vitro, tumor tissues of nude mice injected with miR-3180-3p exosomes attenuated tumor size and weight (Fig. 6a-d).

Discussion

With the rapid development of technology, many diseases can be cured in clinic. However, there is no effective treatment for cancer. Many studies have reports that miRNAs play important roles in cancers. Accumulating evidence indicates that miRNAs are involved in the progression of NSCLC. These provides miRNAs as a promising approach to treat cancer. However, the mechanism of miR-3180-3p in the regulation of NSCLC remains unclear.

In this study, we researched the roles of miR-3180-3p in NSCLC. We found downregulation of miR-3180-3p both in NSCLC cell lines and human samples. Upregulation of miR-3180-3p suppressed NSCLC cell proliferation and metastasis. We further determined that tumor oncogene FOXP4 was a direct target of miR-3180-3p.

FOXP4 belongs to Human Forkhead-box (FOX) gene family. Forkhead proteins have been proved to play key roles in cell cycle regulation and oncogenesis. Yin et al. reported microRNA-491-5p suppresses osteosarcoma cell proliferation and promotes apoptosis by targeting FOXP4^[25]. Restoration of FOXP4 expression significantly promotes breast cancer cell proliferation, migration and invasion^[26]. FOXP4 upregulates in NSCLC, especial in adenocarcinoma lung cancer. We found downregulated FOXP4 prominently suppressed NSCLC cell proliferation, migration and invasion.

Exosomes are secreted by cells, circulate in the blood and induce a series of cellular reactions when being captured by recipient cells. Recent studies have suggested that tumor-derived exosomes may play an important role in tumor progression^[27-29]. HCC cells release exosomes containing both SMAD3 protein and mRNA, these exosomes enhance the recipient HCC cells SMAD3 signaling and increase their adhesive ability^[30]. Feng Wei et al. showed miRNAs within exosomes play important roles in cancers. For example, exosomal miR-222-3p promotes the proliferation, gemcitabine resistance, migration and invasion of NSCLC cells by targeting the promoter of SOCS3^[31]. We isolated exosomes derived from two NSCLC cell lines - A549 and H460 under overexpression of miR-3180-3p. We found miR-3180-3p can be enriched in exosomes. The overexpression of exosomal miR-3180-3p and silencing of FOXP4 both suppressed NSCLC cell proliferation, migration and invasion.

In summary, we are the first time to demonstrate exosomal miR-3180-3p inhibited NSCLC proliferation and metastasis by targeting FOXP4. These results revealed a new regulatory network involving miR-3180-3p and FOXP4, which may be applied to the therapeutic treatment of NSCLC in the future.

Conclusions

We demonstrated that the miR-3180-3p carried by exosomes could suppress NSCLC cells proliferation and metastasis by downregulating FOXP4. These findings might provide a foundation for determination of innovative therapeutic strategies.

Abbreviations

NSCLC: Non-small cell lung cancer; FOXP4: forkhead box P4; EMT: epithelial-mesenchymal transition; NTA: nanoparticle tracking analysis; TEM: transmission electron microscopy; snRNA: small nuclear RNA; RT: reverse transcriptase; 3'-UTR: 3'-untranslated region; miRNAs: microRNAs.

Declarations

Ethics approval and consent to participate

This study was approved and conducted by the Institutional Animal Care and Use Committee of the Soochow University.

Consent for publication

Not applicable.

Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

No potential conflicts of interest were disclosed.

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Authors' contributions

Designed research and supervised: JZ; Performed research: TFC and YLL; Analysis and/or interpretation of data: CL, CD and JC; Wrote the manuscript: TFC; Revising the manuscript critically for important intellectual content: HFZ. All authors read and approved the final manuscript.

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Figures

Figure 1

Overexpression of miR-3180-3p inhibits proliferation, migration and invasion in NSCLC cells. (a) The expression of miR-3180-3p in non-small cell lung cancer and para-carcinoma tissues from the GEO database (GEO: GSE53882). (b) The expression of miR-3180-3p in 6 NSCLC cell lines and a normal cell

line. (c) The expression of miR-3180-3p after overexpression miR-3180-3p in A549 and H460 cells. (d-e) CCK-8 assay was performed in A549 and H460 cells transfected with miR-3180-3p and miR-NC. (f-g) The migratory and invasive ability of A549 and H460 cells transfected with miR-3180-3p and miR-NC was measured by transwell assay. *P < 0.05, **P < 0.01, ***P < 0.001, and n ≥ 3.

Figure 2

Identification of FOXP4 as a miR-3180-3p target gene. (a) The wild-type and a mutated type of binding site between miR-3180-3p and FOXP4. (b-c) A549 and H460 were co-transfected with miR-NC and miR-3180-3p with the WT and MUT 3'UTR. Luciferase activity was measured after 24 h co-transfection. (d-e) The mRNA and protein level of FOXP4 was analysed by qRT-PCR and western blotting in A549 and H460

cells. (f) The FOXP4 mRNA expression was extracted from The Cancer Genome Atlas (TCGA). *P < 0.05, **P < 0.01, and n ≥ 3.

Figure 3

Knockdown FOXP4 inhibits proliferation, migration and invasion in NSCLC cells. (a) The protein level of FOXP4 in A549 and H460 cells were analysed by western blotting after transfection with siRNA target to FOXP4. (b) CCK-8 assay was performed in A549 and H460 cells after knockdown FOXP4 by siRNA. (d-e) The migratory and invasive ability of A549 and H460 cells was measured by transwell assay after knockdown FOXP4 by siRNA. *P < 0.05, **P < 0.01, and n ≥ 3.

Figure 4

Partial reversal of the inhibitory effects of miR-3180-3p on the NSCLC cell phenotype by FOXP4. A549 and H460 cells were transfected with miR-NC, miR-3180-3p, miR-3180-3p+FOXP4 or FOXP4 respectively. (a-b) western blots were performed to detect FOXP4 protein expression. (c-d) CCK-8 assay was performed. (e-f) The migratory and invasive ability of A549 and H460 cells was measured by transwell assay. *P < 0.05, **P < 0.01, and n ≥ 3.

Figure 5

Exosomal miR-3180-3p derived from A549 and H460 cells suppresses proliferation and metastasis of NSCLC cells. (a) Observation of the morphology of exosomes under transmission electron microscope (scale bar, 50 nm). (b) The size distribution of exosomes. (c) The expression of exosomal marker Annexin V, Flotillin-1 and CD63. (d) Representative immunofluorescence image shows the internalization of PKH67-labeled A549-derived exosomes (green) by macrophages. Blue represents nuclear DNA staining by DAPI. Red represents cytoplasm incubation with β -actin antibody. (e) MiR-3180-3p levels in exo-A549

and exo-H460 were measured by RT-PCR. (f) western bolts were performed. (g-h) CCK-8 assay was performed. (i-j) The migratory and invasive ability of A549 and H460 cells was measured by transwell assay. *P < 0.05, **P < 0.01, ***P < 0.001 and n ≥ 3.

Figure 6

Exosomes enriched with miR-3180-3p supresses tumor growth in vivo. (a) Statistical analysis tumor volume growth curves of mice. (b-c) Photograph of tumor and curve of tumor weight. (d) Schematic model of exosomal miR-3180-3p inhibits NSCLC progression. **P < 0.01, ***P < 0.001 and n > 3.