

# Effects of miR-218-1-3p and miR-149 on proliferation and apoptosis of non-small cell lung cancer cells

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**Abstract.** The aim of the present study was to explore the effects of miR-218-1-3p and miR-149 on the biological function of non-small cell lung cancer (NSCLC) cells A549. Paired NSCLC and adjacent tissues were obtained from 50 NSCLC patients admitted to Shandong Provincial Chest Hospital Affiliated to Shandong University (Jinan, China) from April 2015 to May 2018. The expression levels of miR-218-1-3p and miR-149 were detected by reverse transcription-quantitative PCR (RT-qPCR). The lung adenocarcinoma A549 cells were assigned into the blank group (without transfection), negative control (NC) group (transfected with miRNA NC), and the transfected groups miR-218-1-3p mimic and miR-149 mimic groups. Proliferation and cell growth were determined by CCK-8 assay and cell invasion ability *in vitro* was assessed by Transwell assay. Flow cytometry was carried out for the detection of cell apoptosis. RT-qPCR results showed that the expression levels of miR-218-1-3p and miR-149 in NSCLC tissues were significantly lower than those in adjacent tissues ( $P<0.001$ ). At 48 and 72 h, the cell growth of the A549 cells in the miR-218-1-3p mimic and miR-149 mimic groups was significantly lower than that in the NC and blank groups ( $P<0.05$ ). The number of invasive cells in the miR-218-1-3p mimic and miR-149 mimic groups was significantly lower than that in the NC and blank groups ( $P<0.05$ ). The apoptotic rate of A549 cells in the miR-218-1-3p mimic and miR-149 mimic groups was significantly higher than that in the NC and blank groups ( $P<0.05$ ). In conclusion, upregulation of miR-218-1-3p and miR-149 can inhibit the proliferation, invasion and migration of A549 cells in NSCLC, thereby promoting the apoptosis of A549 cells. Thus, miR-218-1-3p and miR-149 can be used as new molecular targets for the diagnosis and treatment of NSCLC.

## Introduction

Lung cancer (1) is one of the most frequently diagnosed cancers nowadays, with high morbidity and mortality (2), which poses a great threat to human health and life. There is small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), with the latter accounting for 80% of the total (3). Most lung cancer patients are diagnosed at moderate or even advanced stage, with low survival rate. The median survival of patients with untreated metastatic NSCLC is only 4-5 months and the 1-year survival rate is only 10% (4). In the treatment of advanced NSCLC, chemotherapy has very little effect on relieving the symptoms and improving survival (5). Although it has been reported (6) that the combined immune checkpoint blockade has promising benefits for lung cancer, the predictors of response to combined therapy are unclear, and combined immunotherapy cannot overcome the negative predictive impact of high tumor mutation burden. With the development of medical science and technology, targeted therapy has been widely accepted, which facilitates the diagnosis and treatment of NSCLC (7).

miRNA is a kind of small non-coding RNA of ~22 nucleotides (8,9). Studies have shown that abnormal expression of miRNAs can lead to the occurrence and development of human malignant tumors (10-12). In recent years, it has been reported that miR-218-1-3p, as an important member of miRNAs, can inhibit the proliferation of lung cancer cells, block their cell cycle and promote apoptosis (13). In addition, it has been demonstrated that the expression of miR-149 is commonly downregulated in various malignancies, including oral squamous cell carcinoma (14), prostate cancer (15) and colorectal cancer (16), which can inhibit the invasion and migration of related cancer cells (17-19). There are also studies that have shown that lower miR-218-1-3p expression in lung cancer cells (20) and the overexpression of miR-218 can inhibit the migration and invasion of NSCLC cells, but do not affect cell growth (21). Therefore, the effects of miR-218-1-3p and miR-149 on A549 cell apoptosis were explored in the present study.

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**Key words:** miR-218-1-3p, miR-149, non-small cell lung cancer, A549 cells, proliferation, apoptosis

## Patients and methods

**Patients.** Paired NSCLC and adjacent normal tissues were obtained from 50 patients who underwent NSCLC resection

in Shandong Provincial Chest Hospital Affiliated to Shandong University (Jinan, China) between April 2015 and May 2018. There were 33 males and 17 females, with an average age of  $56.28 \pm 7.32$  years. The inclusion criteria were as follows: Patients diagnosed with NSCLC, without other major diseases, with complete medical history and follow-up information available. The exclusion criteria were as follows: Patients who had undergone chemoradiotherapy before surgery, with blood diseases or severe liver and kidney dysfunction. The study was approved by the Medical Ethics Committee of the Shandong Provincial Chest Hospital Affiliated to Shandong University (SPCASU1903). Written informed consents were obtained from all patients or their legal guardians. The study is in line with the requirements for human studies (22).

#### *Main reagents, instruments and detection methods*

**Main reagents.** Human lung adenocarcinoma A549 cell line was purchased from Shanghai Huzhen Biological Technology Co., Ltd. Apoptosis detection kit was purchased from Shanghai Meilian Biotechnology Co., Ltd. DMEM was purchased from Shanghai Rhawn Chemical Technology Co., Ltd. Fetal bovine serum (10%; FBS) was purchased from Serana Europe GmbH. TRIzol<sup>®</sup> reagent was purchased from Shanghai Yuduo Biotechnology Co., Ltd., and Lipofectamine<sup>™</sup> 3000 was purchased from Tideradar Beijing Technology Co., Ltd. The primer sequences and transfected plasmid synthesis of miR-218-1-3p, miR-149 and internal reference U6 were purchased from Shanghai Xinghan Biotechnology Co., Ltd.

**miR-218-1-3p and miR-149 detection.** The expression levels of miR-218-1-3p and miR-149 in NSCLC and adjacent tissues were detected by reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted by TRIzol<sup>®</sup> reagent (Shanghai Yuduo Biotechnology Co., Ltd.) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA using TaqMan Reverse Transcription kit (4366596; Hangzhou Woosen Biotechnology Co., Ltd.) following the manufacturer's instructions. U6 was set as the internal control, and the expression levels of miR-218-1-3p and miR-149 were detected using specific primers of miR-218-1-3p and miR-149 in an ABI 7900HT fluorescence quantitative PCR instrument (Shanghai PuDi Biotechnology Co., Ltd.) according to the manufacturer's instructions. qPCR conditions were as follows: 94°C for 10 sec, 94°C for 5 sec, 52°C for 30 sec, 72°C for 15 sec, for a total of 40 cycles. Three replicates were set for each experiment, and the experiment was repeated three times. The experimental results were analyzed using a relative quantitative method, and the expression levels of miR-218-1-3p and miR-149 were quantified using the  $2^{-\Delta C_t}$  method (23). Primer sequences are shown in Table I.

**Cell culture and transfection.** High-glucose DMEM containing 10% FBS was used for routine passage culture in an incubator with 5% CO<sub>2</sub> at 37°C. The A549 cells were seeded into 6-well plates, and divided into blank group, negative control (NC) group (empty plasmid, 100 nM), miR-218-1-3p mimic group (miR-218-1-3p-shRNA, 100 nM), and miR-149 mimic group (miR-149-shRNA, 100 nM) before transfection. Transfection was then performed on the above four groups using Lipofectamine<sup>™</sup> 2000 kit, according to the manufacturer's

protocol, and the expression levels of miR-218-1-3p and miR-149 in the transfected A549 cells were detected. All plasmids were purchased from HedgehogBio Science and Technology, Ltd. The medium was changed 24 h after transfection and then the cells were further cultured for 48 h and collected.

**Cell growth detection.** Four groups of transfected A549 cells were inoculated into 96-well plates, respectively, and three multiple wells were set in each well. Cell proliferation colorimetric reagent (CCK-8) was added into the wells (20  $\mu$ l in each well) 2 h before the different time points of culture (24, 48, and 72 h), and then the plates were placed in a cell incubator with 5% CO<sub>2</sub> at 37°C. After the cell culture, proliferation was analyzed by measuring the absorbance (OD value) at a wavelength of 490 nm using a fully automated enzyme-labeling instrument (Image Trading Co., Ltd.).

**Detection of cell migration and invasion.** Cells were digested with trypsin, resuspended in serum-free medium, and then 200  $\mu$ l of the resuspended solution were used for the determination of cell migration. A total of  $5 \times 10^4$  cells were plated in the upper chamber of Transwell plates. Next, 200  $\mu$ l of the resuspended solution, containing  $\sim 5 \times 10^4$  cells, were collected for the migration experiment and medium containing 10% FBS was added to the subcompartment of the 6-well plate. Following incubation for 24 h at 37°C, the cells in the upper chamber of Transwell assay were wiped off with a cotton swab, whereas the cells that migrated to the lower chamber were stained with 4% paraformaldehyde and 0.1% crystal violet for 15 min. After the Transwell chamber was dried, the membrane was sealed and the penetrating cells were observed and counted under an optical microscope (x200). The experiment was repeated three times. The invasion assay was performed following the aforementioned steps using 8% Matrigel.

**Apoptosis detection.** Cell apoptosis was detected using an apoptosis detection kit in accordance with the manufacturer's instructions. The cells that had been transfected for 48 h in the 6-well plates and were stained with Annexin V and propidium iodide were detected by a BD flow cytometer (BD Biosciences), and the experiment was repeated three times. The apoptosis rate was analyzed using FlowJo v10 software (FlowJo LLC).

**Statistical analysis.** SPSS 22.0 software (IBM Corp.) was used for the statistical analysis of the experimental data. t-test was used for the comparison of the measurement data which were expressed as the mean  $\pm$  standard deviation (mean  $\pm$  SD). ANOVA (F analysis) was applied for multiple group comparisons, whereas repeated measures ANOVA was used for intra-group comparisons among different time points.  $P < 0.05$  was considered to indicate a statistically significant difference.

## **Results**

**Expression levels of miRNA-218-1-3p and miRNA-149 in NSCLC and adjacent tissues.** The expression levels of miR-218-1-3p in NSCLC and adjacent tissues were  $0.59 \pm 0.06$  and  $1.58 \pm 0.10$   $\mu$ g/ml, respectively, whereas the expression levels of miR-149 were  $2.88 \pm 0.80$  and  $4.98 \pm 1.34$   $\mu$ g/ml, respectively. miR-218-1-3p and miR-149 expression levels were

Table I. List of primer sequences.

Gene	Upstream primers	Downstream primers
miR-218-1-3p	5'-ACAUGGUUAGAUAAGCACA-3'	5'-UUGUACUACACAAAAGUACUG-3'
miR-149	5'-UCUGGCUCGUGUCUUCACUCCC-3'	5'-UUCUCCGAACGUGUCACGU-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

Table II. Expression levels of miR-218-1-3p and miR-149 in NSCLC and adjacent tissues.

Factor	NSCLC tissues (n=50)	Adjacent tissues (n=50)	t	P-value
miR-218-1-3p	0.59±0.06	1.58±0.10	60.03	<0.001
miR-149	2.88±0.80	4.98±1.34	9.515	<0.001

NSCLC, non-small cell lung cancer.

significantly lower in NSCLC tissues than those in adjacent tissues ( $P<0.001$ ), as shown in Table II.

*Relative expression levels of miR-218-1-3p and miR-149 in each group of cells after transfection with the mimic vector.* The expression levels of miR-218-1-3p in the miR-218-1-3p mimic, NC and blank groups were  $2.67\pm0.21$ ,  $0.58\pm0.05$  and  $0.58\pm0.06$   $\mu\text{g/ml}$ , respectively, and miR-218-1-3p expression in the miR-218-1-3p mimic group was significantly higher than that in the other two groups ( $P<0.05$ ) (Table III). In addition, the expression levels of miR-149 in the miRNA-149 mimic, NC and blank groups were  $5.03\pm0.20$ ,  $2.86\pm0.77$  and  $2.87\pm0.80$   $\mu\text{g/ml}$ , respectively, and the miR-149 expression in the miR-149 mimic group was significantly higher than that in the NC and blank groups ( $P<0.05$ ). There was no significant difference in the expression levels of miR-218-1-3p and miR-149 between the NC and blank groups ( $P>0.05$ ) (Table IV). These results indicated that miR-218-1-3p and miR-149 were successfully upregulated in A549 cells after transfection with mimic.

*Comparison of the cell growth of human adenocarcinoma cells A549 at different time points*

*A549 cell growth in the miR-218-1-3p mimic, NC and blank groups at different time points.* As shown in Table V and Fig. 1, there was no significant difference in the growth of A549 cells among the three groups at 24 h ( $P>0.05$ ), whereas at 48 and 72 h, the growth of A549 cells in the miR-218-1-3p mimic group was significantly lower than that in the NC and the blank groups ( $P<0.05$ ). Within each group, there were significant differences in the cell growth of A549 cells at 24, 48 and 72 h ( $P<0.05$ ).

*A549 cell growth in the miR-149 mimic, NC and blank groups at different time points.* The results demonstrated that the growth of A549 cells in the miR-149 mimic, NC and blank groups had no significant difference at 24 h ( $P>0.05$ ), whereas the growth of A549 cells in the miR-149 mimic group was significantly lower than that in NC and blank groups at 48 and 72 h ( $P<0.05$ ). Within each group, there were significant

differences in the cell growth of A549 cells at 24, 48 and 72 h ( $P<0.05$ ). Details are shown in Table VI and Fig. 2.

*Comparison of A549 cell invasion among groups.* The number of invasive cells in the miR-218-1-3p mimic, miR-149 mimic, NC and blank control groups were  $80.56\pm7.25$ ,  $81.34\pm6.98$ ,  $136.93\pm10.23$ , and  $139.78\pm11.98$ , respectively. The number of invasive cells in the miR-218-1-3p mimic and miR-149 mimic groups was significantly lower than that of the blank and NC groups, with statistically significant differences ( $P<0.05$ , as shown in Table VII.

*Comparison of A549 cell apoptosis after transfection among groups.* The apoptotic rates in miR-218-1-3p mimic and miR-149 mimic groups were  $26.34\pm2.12$  and  $25.74\pm3.95\%$ , respectively, which were significantly higher than those in the NC group ( $4.63\pm0.40\%$ ) and blank group ( $4.56\pm0.37\%$ ) ( $P<0.05$ ). The apoptotic rates of NC group and blank group showed no significant difference ( $P>0.05$ ). Details are shown in Table VIII.

## Discussion

As one of the most common malignant tumors, lung cancer can be divided into SCLC and NSCLC (24), with the latter presenting slower growth, slower proliferation and later metastasis, while characterized by high mortality and morbidity (25). Therefore, NSCLC has also been clinically treated by inhibiting the proliferation and migration of cancer cells.

MicroRNAs are non-coding small RNAs related to proto-oncogenes, whose abnormal expression results in continuous proliferation or spread and migration of cancer cells eventually leading to death. A previous study has demonstrated that miRNAs could inhibit the expression of important cancer-associated genes and therefore might prove to be useful in the diagnosis and treatment of cancer (26). On this basis, the effects of miR-218-1-3p and miR-149, as important members of the miRNA family, on the proliferation, invasion and apoptosis of NSCLC cells were explored in the present study.

Table III. miR-218-1-3p expression in each group after transfection.

Factor	miR-218-1-3p mimic group	NC group	Blank group	F	P-value
miR-218-1-3p	2.67±0.21	0.58±0.05 <sup>a</sup>	0.58±0.06 <sup>a</sup>	4,351.00	<0.001

<sup>a</sup>P<0.05, compared with the miR-218-1-3p mimic group. NC, negative control.

Table IV. miR-149 expression in each group after transfection.

Factor	miR-149 mimic group	NC group	Blank group	F	P-value
miR-149	5.03±0.20	2.86±0.77 <sup>a</sup>	2.87±0.80 <sup>a</sup>	183.30	<0.001

<sup>a</sup>P<0.05, compared with the miR-149 mimic group. NC, negative control.

Table V. Growth of A549 cells in the miR-218-1-3p mimic, NC and blank groups at different time points.

Time (h)	miR-218-1-3p mimic group	NC group	Blank group	F	P-value
24	0.58±0.05	0.59±0.07	0.58±0.06	0.454	0.635
48	0.97±0.10 <sup>a</sup>	1.56±0.11 <sup>a,c</sup>	1.56±0.10 <sup>a,c</sup>	542.2	<0.001
72	1.34±0.12 <sup>a,b</sup>	2.78±0.32 <sup>a-c</sup>	2.77±0.33 <sup>a-c</sup>	456.2	<0.001

<sup>a</sup>P<0.05, compared with the growth of A549 cells at 24 h; <sup>b</sup>P<0.05, compared with the growth of A549 cells at 48 h; <sup>c</sup>P<0.05, compared with the miR-218-1-3p mimic group. NC, negative control.

Table VI. Growth of A549 cells in the miR-149 mimic, NC and blank groups at different time points.

Time (h)	miR-149 mimic group	NC group	Blank group	F	P-value
24	2.86±0.60	2.87±0.80	2.88±0.80	0.009	0.991
48	3.23±0.62 <sup>a</sup>	3.72±0.83 <sup>a,c</sup>	3.72±0.82 <sup>a,c</sup>	6.943	<0.05
72	3.78±0.70 <sup>a,b</sup>	4.16±0.85 <sup>a-c</sup>	4.17±0.84 <sup>a-c</sup>	3.866	<0.05

<sup>a</sup>P<0.05, compared with the growth of A549 cells at 24 h; <sup>b</sup>P<0.05, compared with the growth of A549 cells at 48 h; <sup>c</sup>P<0.05, compared with the miR-149 mimic group. NC, negative control.

The expression levels of miR-218-1-3p and miR-149 in NSCLC and adjacent tissues were investigated. The results showed that the expression levels of miR-218-1-3p and miR-149 in NSCLC tissues were significantly lower than those in adjacent tissues, suggesting that miR-218-1-3p and miR-149 were downregulated in NSCLC. Ke *et al.* (27) also reported that deletion of miR-149 would lead to increased expression of oncogenes, in consistency with the results of the present study. The relative expression levels of miR-218-1-3p and miR-149 in each group after transfection were also determined. miR-218-1-3p expression in miR-218-1-3p mimic group was significantly higher than that of the NC and blank groups, and miR-149 expression in the miR-149 mimic group was also significantly higher than that in the NC and blank groups. These results suggest that miR-218-1-3p could be upregulated to inhibit the proliferation of cancer cells in

the treatment of NSCLC, in agreement with the results of a previous study (28). In addition, miR-149 could also inhibit the proliferation and migration of cancer cells through upregulation. In a preceding study (29), the downregulation of miR-149 was reported in NSCLC, which was roughly the same with the results of the present study. Furthermore, in the same study, the clinical significance of miR-149 in NSCLC was further investigated and the expression of miR-149 was shown to be negatively correlated with tumor size. Also, the 3-year survival rates of patients with different miR-149 expression levels were compared and the results indicated that the patients with poor survival had low miR-149 expression. All these results suggested that upregulation of miR-218-1-3p and miR-149 expression levels could inhibit the proliferation of cancer cells. In the present study, the growth, migration, invasion and apoptosis of human lung adenocarcinoma cells

Table VII. Invasion of A549 cells in each group.

Index	miR-218-1-3p mimic group (n=50)	miR-149 mimic group (n=50)	NC group (n=50)	Blank group (n=50)	F	P-value
No. of invasive cells	80.56±7.25	81.34±6.98	136.93±10.23 <sup>a,b</sup>	139.78±11.98 <sup>a,b</sup>	629.50	<0.001

<sup>a</sup>P<0.05, compared with the miR-218-1-3p mimic group; <sup>b</sup>P<0.05, compared with the miR-149 mimic group. NC, negative control.

Table VIII. Apoptosis of A549 cells after transfection in each group.

Index	miR-218-1-3p mimic group (n=50)	miR-149 mimic group (n=50)	NC group (n=50)	Blank group (n=50)	F	P-value
Apoptotic rate (%)	26.34±2.12	25.74±3.95	4.63±0.40 <sup>a,b</sup>	4.56±0.37 <sup>a,b</sup>	1,504.00	<0.001

<sup>a</sup>P<0.05, compared with the miR-218-1-3p mimic group; <sup>b</sup>P<0.05, compared with the miR-149 mimic group. NC, negative control.

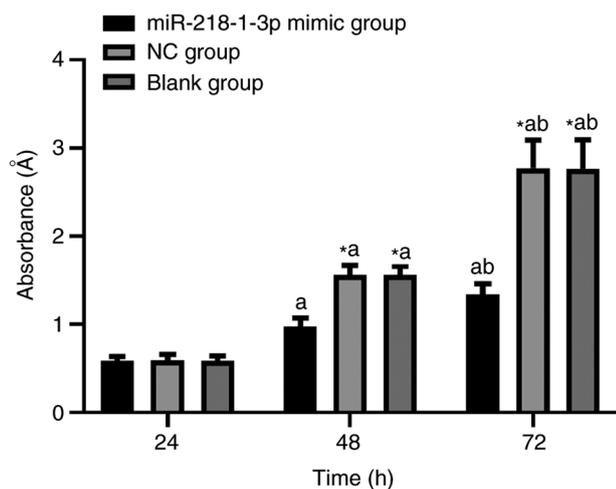


Figure 1. A549 cell growth after miR-218-1-3p upregulation. At 24 h, there was no significant difference in the growth of A549 cells among the miR-218-1-3p mimic, NC and blank groups ( $P>0.05$ ); whereas at 48 and 72 h, the growth of A549 cells in the miR-218-1-3p mimic group was significantly lower than that in the NC and blank groups ( $P<0.05$ ). Intra-group comparisons showed significant differences in the growth of A549 cells at 24, 48 and 72 h in the miR-218-1-3p mimic, NC and blank groups ( $P<0.05$ ). Inter-group comparisons at the same time point: <sup>a</sup>P<0.05, compared with the miR-218-1-3p mimic group. Intra-group comparisons between different time points: <sup>a</sup>P<0.05, compared with the growth of A549 cells at 24 h; <sup>b</sup>P<0.05, compared with the growth of A549 cells at 48 h. NC, negative control.

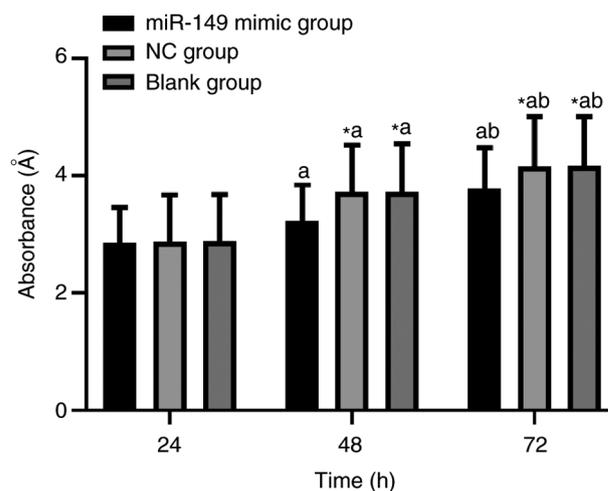


Figure 2. A549 cell growth after miR-149 upregulation. At 24 h, the growth of A549 cells among the miR-149 mimic, NC and blank groups showed no significant difference ( $P>0.05$ ); whereas, at 48 and 72 h, the growth of A549 cells in miR-149 mimic group was significantly lower than that in the NC and blank groups ( $P<0.05$ ). Intra-group comparisons showed significant differences in the growth of A549 cells at 24, 48 and 72 h in the miR-149 mimic, NC and blank groups ( $P<0.05$ ). Inter-group comparisons at the same time point: <sup>a</sup>P<0.05, compared with the miR-218-1-3p mimic group. Intra-group comparisons between different time points: <sup>a</sup>P<0.05, compared with the growth of A549 cells at 24 h; <sup>b</sup>P<0.05, compared with the growth of A549 cells at 48 h. NC, negative control.

A549 were also studied and compared among the different groups. The results revealed that the growth of A549 cells in the miR-218-1-3p mimic and miR-149 mimic groups was significantly lower than that in the NC and blank groups. Regarding cell invasion, the number of invasive cells in the miR-218-1-3p mimic and the miR-149 mimic groups was significantly lower than that in the blank and the NC groups. As to the apoptosis of human lung adenocarcinoma cells A549, the apoptotic rates in the miR-218-1-3p mimic and miR-149 mimic groups were significantly higher than those in the NC and blank groups, indicating that upregulation

of miR-218-1-3p and miR-149 could inhibit the growth and invasion of A549 cells and promote their apoptosis in NSCLC. Chen *et al* (30) used Transwell assay to detect the changes of cell migration and invasion after transfection. The results showed that inhibited expression of miRNA-218-1-3p could significantly enhance the migration and invasion of A549 cells, which was roughly in line with the findings of the present study. Chen *et al* (30) also used bioinformatics analysis and showed that miR-218 affected the migration and invasion of cancer cells through high expression, as the result of direct targeting of Robo1.

The present study has also some limitations. The correlation between miR-218-1-3p and miR-149 expression in the development of tumors was not investigated and no relevant research was conducted on drug resistance. These will be the aim of our future research.

In conclusion, miR-218-1-3p and miR-149 were both lowly expressed in NSCLC and upregulation of their expression could inhibit the proliferation and promote the apoptosis of A549 cells facilitating treatment. Thus, miR-218-1-3p and miR-149 could serve as potential markers for the diagnosis and prognosis of NSCLC, and as targeted sites for the treatment of NSCLC, providing a new direction for the clinical treatment and a theoretical basis for further research on new targets of gene therapy for NSCLC.

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### Availability of data and materials

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

### Authors' contributions

PG wrote the manuscript. PG and MS performed RT-qPCR and CCK-8 assay. HL and LJ were responsible for the Transwell assay and flow cytometry. NY and YS contributed to the statistical analysis of the data. All authors read and approved the final version of the manuscript.

### Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of the Shandong Provincial Chest Hospital Affiliated to Shandong University (SPCASU1903; Jinan, China). Patients who participated in this research had complete clinical data and written informed consents were obtained from all the patients or their legal guardians.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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