

## Original Article

# Up-regulation of miR-182-5p predicts poor prognosis in patients with lung cancer and associates with tumor cell growth and migration

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**Abstract:** Oncogenic microRNA-182-5p (miR-182-5p) has been shown to be associated with tumorigenesis in various types of human cancers. However, the clinical significance and roles of miR-182-5p in lung cancer have not been illustrated. The aim of this study was to assess the effects of miR-182-5p in lung cancer and its potential relevance to clinicopathological characteristics and survival. Here, we found that miR-182-5p was markedly up-regulated in lung cancer tissues and cell lines compared with adjacent non-tumor tissues and normal bronchial epithelial cell. The high expression of miR-182-5p was found to be significantly associated with lymph nodes metastasis. The lung cancer patients with high miR-182-5p expression had a notably shorter overall survival than those with low miR-182-5p expression. Furthermore, multivariate cox proportional hazards model showed that expression of miR-182-5p was independent prognostic factors for overall survival of patients with lung cancer. In addition, by 3-(4,5-dimethylthi-azol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and wound scratch assays, we found that knock-down of miR-182-5p resulted in retarded cell proliferation and migration in lung cancer. In summary, our findings indicate that miR-182-5p silencing suppresses the growth and migration of lung cancer and its up-regulation expression is associated with unfavorable prognosis, suggesting that miR-182-5p may be a promising biomarker for further risk stratification in the treatment of this cancer.

**Keywords:** MiR-182-5p, lung cancer, prognosis, proliferation, migration

## Introduction

Lung carcinoma is currently the highest incidence and mortality malignant cancer all over the world [1]. Due to the lack of obvious symptoms for early diagnosis and highly malignant metastasis in advanced stage, the 5-year survival rate of lung cancer is less than 15% [2, 3]. Thus, it is necessary to identify new biomarkers to provide early diagnosis and efficient therapy for patients with lung cancer.

MicroRNAs (miRNAs) are endogenous RNAs with 19~25 nucleotides in length, which can play important roles in numerous biological processes, including cell proliferation, differentiation, apoptosis, metastasis and immunity and metabolism [4, 5]. MiRNAs can bind to 3'-untranslational regions (3'-UTRs) of their target mRNAs, leading to mRNAs cleavage or translational repression [6, 7]. During past decades, more than 1000 miRNAs were identified

in eukaryote. Accumulating evidences have shown that aberrant expression of miRNAs has been observed in various human cancers, including bladder cancer [8], colorectal cancer [9, 10], hepatocellular carcinoma [11] and gastric cancer [12]. Recently, several studies have reported that miRNAs can function as tumor suppressors or oncogenes in many human cancers by regulating tumor cell growth, metastasis, apoptosis [13, 14].

MiR-182-5p has been reported to be over-expressed in prostate cancer and promoted cell invasion and proliferation [15, 16]. However, the clinical significance and roles of miR-182-5p in lung cancer remain unknown. In this study, we hypothesized that miR-182-5p may associate with prognosis in patients with lung cancer and act as an oncogene in lung cancer. In order to test this hypothesis, we initially detected miR-182-5p expression in lung cancer

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**Table 1.** Correlation between miR-182-5p expression and different clinicopathological characteristics in patients with lungcancer

Characteristics	Cases	MiR-182-5p expression		P value
		Low (n=16)	High (n=31)	
Gender				0.358
Male	25	10	15	
Female	22	6	16	
Age (years)				0.252
<50	17	4	13	
≥50	30	12	18	
Smoking history				0.122
Yes	28	12	16	
No	19	4	15	
Tumor size				0.069
≤3 cm	29	7	22	
>3 cm	18	9	9	
Histological type				0.458
Adenocarcinoma	27	8	19	
Squamous carcinoma	20	8	12	
TNM stage				0.761
I+II	10	3	7	
III+IV	37	13	24	
Lymph node metastasis				0.000*
Yes	24	15	9	
No	23	1	22	

MiR: microRNAs; TNM: Tumor Node Metastasis. \*P<0.05.

tissues and cell lines. Additionally, the relationships between clinical features of patients with lung cancer and miR-182-5p expression were analyzed and the prognostic value of miR-182-5p was also evaluated. Finally, we performed in vitro assays to observe potential tumor cell promotion effects of miR-182-5p on lung cancer cells.

### Material and methods

#### Ethics statement

Each participant provided written informed consent in compliance with ethics of the World Medical Association (Declaration of Helsinki). The study was approved by the Tissue Committee and Research Ethics Board of Qingdao Municipal Hospital (Qingdao, China).

#### Clinical specimens

Forty seven paired lung tissues and adjacent non-tumor tissues were obtained from NSCLC patients underwent lung resection at the Department of Respiratory Diseases, Qingdao

Municipal Hospital (Qingdao, China) between January 2009 to January 2014. None of patient received radiotherapy or chemotherapy prior to surgery. The samples were snap-frozen in liquid nitrogen immediately, and then stored at -80°C until use. Detailed information of patients was presented in **Table 1**. All of the patients were enrolled in the 5-year follow-up investigation.

#### Cell lines and cell culture

A normal human bronchial epithelial cell line (HBE) and human lung cancer cell lines (HTB-182, A549, SPC-A-1, H596 and H1299) were obtained from the Chinese Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 µg/ml streptomycin and 100 U/ml penicillin. The cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

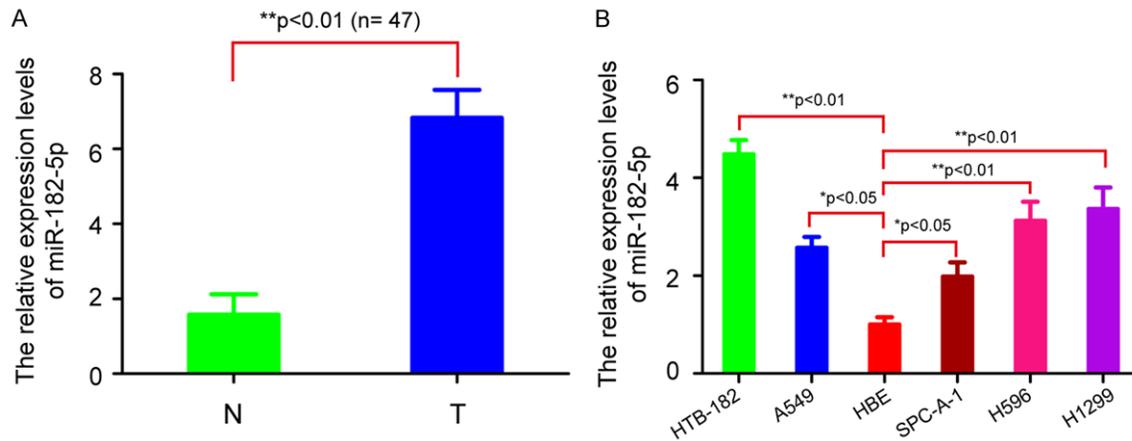
#### Cell transfection

MiR-182-5p inhibitors and inhibitors negative control (inhibitors-NC) were purchased from GenePharma Co., Ltd (Shanghai, China). The A549 or H596 cells (6×10<sup>6</sup> per well) were seeded in 6-well culture plates. 100 mM miR-182-5p inhibitors or inhibitors-NC was transfected into A549 or H596 cells by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. 48 h after transfection, the expression levels of miR-182-5p was determined by quantitative real-time polymerase chain reaction.

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

Total RNAs of lung tissues and cells were isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. The complementary DNA (cDNA) was synthesized by using a PrimeScript™ One Step RT-PCR kit (Takara, Japan) according to the manufacturer's protocols. The qRT-PCR reaction was conducted by using a SYBR Green PCR

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**Figure 1.** MiR-182-5p was markedly up-regulated in lung cancer tissues and cell lines. A. Relative expression of miR-182-5p in 47 paired of lung cancer tissues and adjacent non-tumor tissues. B. MiR-182-5p expression in lung cancer cell lines and a normal human bronchial epithelial cell line (HBE). U6 was used as an internal control. MiR: microRNAs; U6: small nuclear RNA U6; T: lung cancer tissues; N: adjacent non-tumor tissues. \* $P < 0.05$  and \*\* $P < 0.01$ .

kit (Takara, Japan) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The condition of qRT-PCR was shown as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec and 72°C for 30 sec. Small nuclear RNA U6 (U6) was used as an internal control. The primers used for the amplification were as follows: miR-182-5p: 5'-TTTGGCAATGGTAGAACRCACACT-3' (sense), universal primer (antisense); U6: 5'-CTCGCTTCGGCAGCACCA-3' (sense), 5'-ACGCTTCACGAATTGCGT-3' (antisense). The miR-182-5p expression was calculated and normalized to those of U6 by using the  $\Delta\Delta C_t$  method [17].

### Cell proliferation assay

Cell proliferation was analyzed by using MMT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Briefly, A549 and H596 cells were seeded into 96-well plates at a density of 6,000 cells/well. After cultured for 24 h, the cells transfected with miR-182-5p inhibitors or inhibitors-NC according to the recommended concentration. 30  $\mu$ l of MTT reagents (Sigma, USA) (5 mg/ml) was added to each well and incubated for 4 h at 37°C after 0, 24, 48 or 72 h transfection. The results were measured at a wavelength of 480 nm (with 630 nm as the reference wavelength) with a microplate reader (Model 354; Thermo Fisher Scientific; Waltham, MA, USA).

### Cell migration assay

The migratory ability of lung cancer cells was assessed by wound scratch assay in vitro.

Approximately  $6 \times 10^6$  cells/well were seed into each 6-well plates and transfected with miR-182-5p inhibitors or inhibitors-NC by using lipofectamine 2000 reagent. After 6 h of transfection, the cells were rinsed with PBS (phosphate buffered saline) and vertical horizontal wounds were made with 10  $\mu$ l sterile pipette tips. Images of the wounds were observed and calculated in five randomly fields under light microscope (Olympus, Tokyo, Japan) at 0 and 16 h after transfection.

### Statistical analysis

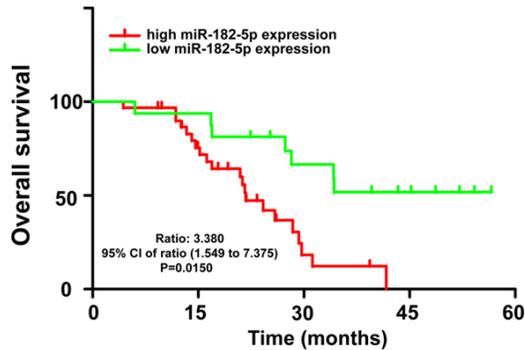
The statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Data was presented as mean  $\pm$  SD (standard deviation) from three independent experiments with each measured in triplicate. The expression differences between lung cancer tissues and adjacent non-tumor tissues were analyzed using paired student's t-test. Chi-square ( $\chi^2$ ) test was used for expression correlation analysis. One-way ANOVA was performed to analyze CCK-8 data. A value of  $P < 0.05$  was considered to be a statistically significant difference.

## Results

### MiR-182-5p was markedly up-regulated in lung cancer tissues and cell lines

Previous study found that miR-182-5p was increased in prostate cancer compared with matched tumor-adjacent normal prostate tissues [16]. In order to identify the clinical significance and roles of miR-182-5p in lung cancer,

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**Figure 2.** The association between miR-182-5p expression with overall survival of lung cancer patients was analyzed using Kaplan-Meier survival curves. Patients with high expression of miR-182-5p were associated with shorter overall survival time compared with the low expression miR-182-5p group. \* $P < 0.05$ .

we initially detected miR-182-5p expression in lung cancer tissues and cell lines. As shown in **Figure 1A**, the miR-182-5p expression is significantly up-regulated in lung cancer tissues compared to non-tumor tissues ( $P < 0.01$ ). We then analyzed the expression levels of miR-182-5p in lung cancer cell lines (HTB-182, A549, SPC-A-1, H596 and H1299) and a normal bronchial epithelial cell (HBE). Consistent with our speculation, miR-182-5p expression was also increased in the five lung cancer cells compared with the HBE (**Figure 1B**;  $P < 0.05$ ).

### *Up-regulation of miR-182-5p was associated with lymph nodes metastasis of patients with lung cancer*

The 47 cases of lung cancer patients were divided into two groups based on the average value of relative miR-182-5p expression level, including low ( $n=16$ ) and high ( $n=31$ ) miR-182-5p expression groups. Chi-square ( $\chi^2$ ) test was applied to evaluate the correlations between miR-182-5p expression and clinicopathologic variables. As shown in **Table 1**, the high level of miR-182-5p expression was correlated with lymph nodes metastasis ( $P=0.000$ ). But not correlated with patient's gender ( $P=0.358$ ), age ( $P=0.252$ ), smoking status ( $P=0.122$ ), histological type ( $P=0.458$ ), TNM stage ( $P=0.761$ ) and tumor size ( $P=0.069$ ).

### *The relationship between miR-182-5p expression and the overall survival of patients with lung cancer*

Kaplan-Meier survival analysis showed a clear negative correlation between miR-182-5p ex-

pression and survival time of lung cancer patients (**Figure 2**). As shown in **Figure 2**, the patients with high levels of miR-182-5p expression had a significantly shorter overall survival time compared with those with the low miR-182-5p expression group ( $P < 0.05$ ).

Univariate and multivariate Cox regression analyses were used to assess the prognostic value of miR-182-5p and other factors in lung cancer patients. From the **Table 2**, we found that miR-148b expression ( $P=0.002$ ) was a significant independent predictor of survival in patients with lung cancer.

### *Inhibition of miR-182-5p suppresses lung cancer cells growth*

To investigate the effect of miR-182-5p on lung cancer cells proliferation, A549 and H596 cells were transfected with miR-182-5p inhibitors or inhibitors-NC. After effective transfection, miR-182-5p expression levels was confirmed by qRT-PCR (**Figure 3A**;  $P < 0.01$ ). Results of MTT assay showed that inhibition of miR-182-5p significantly suppressed proliferation of A549 and H596 cells (**Figure 3B**;  $P < 0.05$ ). The results suggest that inhibition of miR-182-5p inhibits lung cancer cells proliferation in vitro.

### *Knock-down of miR-182-5p retarded lung cancer cells migration*

A wound scratch assay was used to test whether miR-182-5p down-regulation could affect the migration of lung cancer cells. As shown in **Figure 4**, the data showed that miR-182-5p inhibitors treatment was able to repress the migration of A549 or H596 cells ( $P < 0.05$ ). The data demonstrate that miR-182-5p silencing decreases lung cancer cell migration.

## Discussion

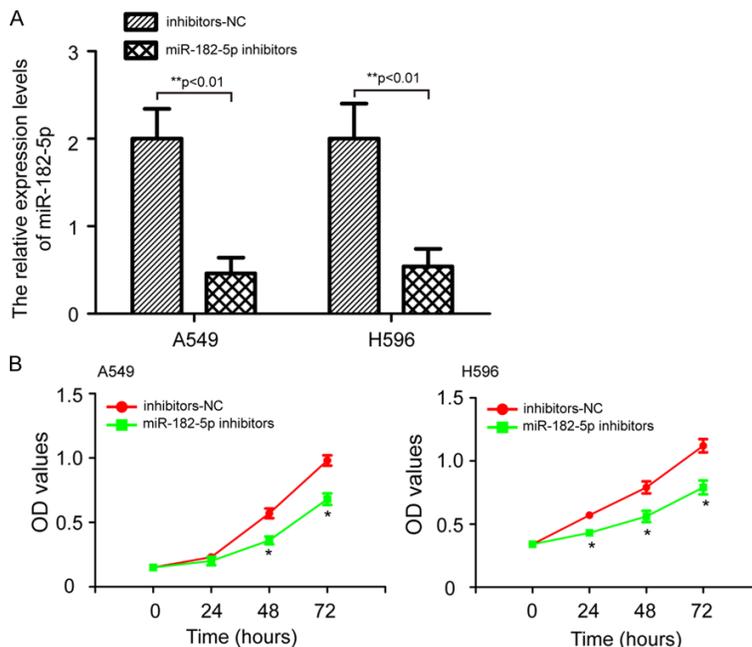
Ample evidences have shown that altered miRNAs expression resulted in the development and progression of lung cancer. For example, Mizuno et al reported that tumor-suppressive miR-29 family inhibited tumor cell migration and invasion by directly targeting LOXL2 (lysyl oxidase-like 2) in lung squamous cell carcinoma [18]. Edmonds et al showed that miR-31 initiated lung tumorigenesis and promoted mutant KRAS-driven lung cancer [19]. Jin et al found that expression of miR-375 was associated with carcinogenesis in three subtypes of

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**Table 2.** Univariate and multivariate analysis of overall survival in lung cancer patients

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Gender (male vs. female)	1.109 (0.719-1.527)	0.758	-	-
Age (<50 vs. ≥50)	0.786 (0.336-1.216)	0.332	-	-
Smoking history (yes vs. no)				
Tumor size (≤3 cm vs. >3 cm)	1.437 (0.566-2.138)	0.570	-	-
Histological type (adenocarcinoma vs. squamous carcinoma)	1.047 (0.446-1.358)	0.224	-	-
TNM stage (I+II vs. III+IV)	1.362 (0.626-2.013)	0.420	-	-
Lymph node metastasis (yes vs. no)	2.112 (1.324-4.438)	0.018*	-	-
MiR-182-5p (low vs. high)	2.663 (1.823-7.524)	0.002*	2.217 (1.409-5.117)	0.027*

\*P<0.05.



**Figure 3.** Inhibition of miR-182-5p suppresses lung cancer cells growth. A. The transfection efficiency of A549 and H596 cells were determined 48 h after incubation with miR-182-5p inhibitors or inhibitors-NC. B. MTT assay was used to assess the growth ability of A549 and H596 cells after treatment with miR-182-5p inhibitors or inhibitors-NC. NC: negative control; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. \*P<0.05 and \*\*P<0.01.

lung cancer [20]. Yang et al suggested that miR-137 inhibited cell migration and invasion by targeting bone morphogenetic protein-7 (BMP7) in non-small cell lung cancer [21]. Therefore, determination of clinical importance and functional of miRNAs in lung cancer may provide novel diagnostic and prognostic biomarkers of the disease.

Up-regulation of oncogenic miR-182-5p has been reported in prostate cancer, suggesting miR-182-5p may play important roles in tumori-

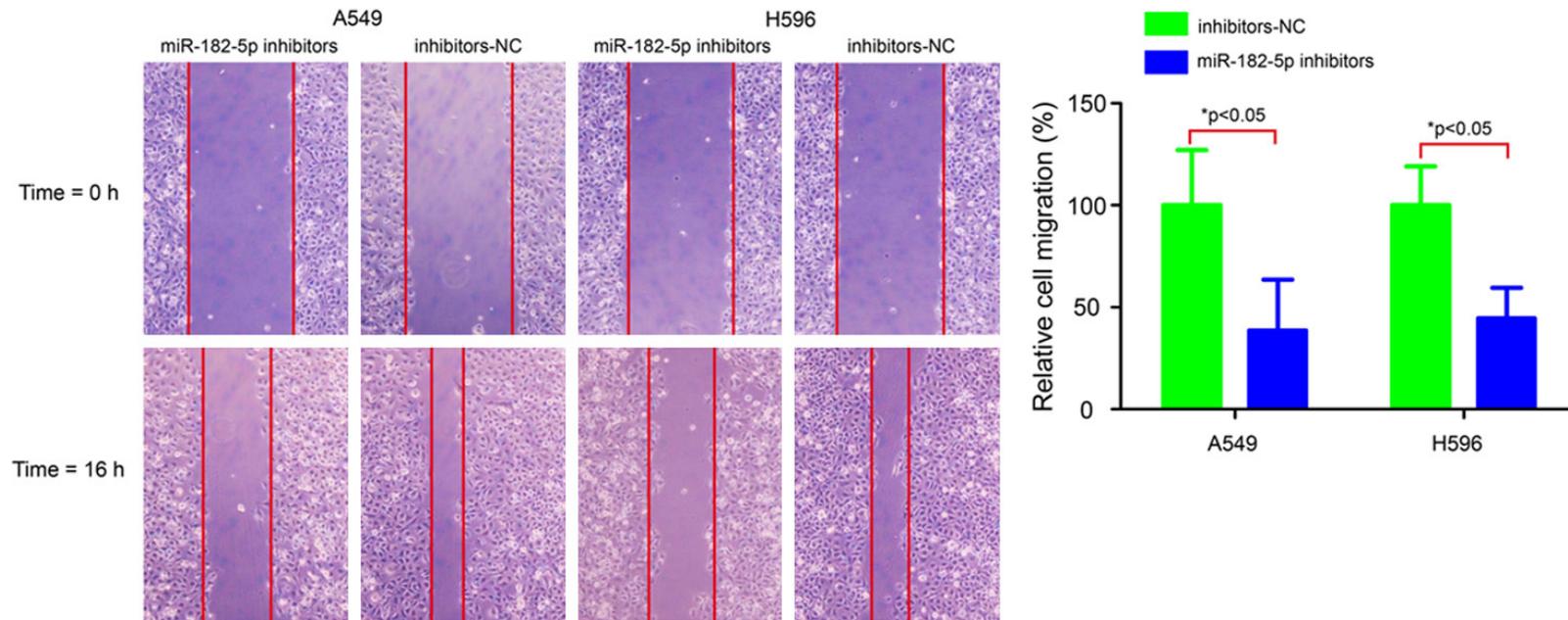
genesis and progression of lung cancer. Here, we investigated the function of miR-182-5p in lung cancer and estimated its clinical significance. qRT-PCR analysis showed that miR-182-5p was significantly up-regulated in lung cancer tissues and cell lines compared with adjacent non-tumor tissues and normal bronchial epithelial cell. We then determined the relationship of miR-182-5p expression with clinicopathological features and survival in patients with lung cancer. Our finding showed that high expression of miR-182-5p was related to lymph nodes metastasis. These results suggest that increased expression of miR-182-5p may be responsible for the progression of lung cancer.

To illustrate the biological roles of miR-182-5p in lung cancer, the impacts of miR-

182-5p on cell proliferation and migration was analyzed by using MTT assay and wound scratch assays. Interesting, we found that knock-down of miR-182-5p suppressed A549 and H596 cells proliferation. Meanwhile, miR-182-5p down-regulation inhibited A549 and H596 cells migration. These findings confirm that miR-182-5p acts important roles in the progression of lung cancer.

Previous studies have shown that miRNAs can serve as valuable prognostic factor in lung can-

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**Figure 4.** Knock-down of miR-182-5p retarded lung cancer cells migration. A549 cells were transfected with miR-182-5p inhibitors or inhibitors-NC, cell migration was determined by wound scratch assay. MiR-182-5p inhibitors treatment was able to repress the migration of H596 cells. The red lines indicate the migration front. Representative photographs were shown (magnification, 200 $\times$ ). \*P<0.05.

cers. For instance, decreased expression of miRNA-124 was associated with poor prognosis in lung cancer [22]. Up-regulation of miRNA-221 was associated with poor prognosis in non-small cell lung cancer patients [23]. Down-regulated miRNA-148b as predictive biomarker and its prognostic significance associated with clinicopathological features in patients with non-small-cell lung cancer [24]. Here, the overall survival of lung cancer patients at different expression levels of miR-182-5p was also analyzed and the results showed that there was significant association between them. Patients with high miR-182-5p expression revealed a shorter overall survival time compared with those with low miR-182-5p expression. Cox regression analysis was performed to assess the prognostic value of miR-182-5p expression and the results demonstrated that miR-182-5p was a potential biomarker for lung cancer prognosis.

In conclusion, our results demonstrate that increased expression of miR-182-5p is associated with poor overall survival of patients with lung cancer. Inhibition of miR-182-5p suppresses cell proliferation and migration in lung cancer. Therefore, oncogenic miR-182-5p, as a potential prognostic biomarker, may provide effective therapy target for lung cancer patients in the future.

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### Disclosure of conflict of interest

None.

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