

Original Article

Serum miR-1826 expression serves as a diagnostic biomarker and inhibits cell proliferation, migration, and invasion of lung cancer

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Received March 12, 2019; Accepted June 5, 2019; Epub August 15, 2019; Published August 30, 2019

Abstract: Many studies have indicated that abnormal expression levels of microRNAs play an important role in the development of several cancer types. The current study investigated the roles of miR-1826 in lung cancer. Expression levels of miR-1826 were detected using quantitative real-time polymerase chain reaction (qRT-PCR) in the serum of lung cancer patients and lung cancer cell lines. Association of miR-1826 with clinicopathological parameters of patients was analyzed using χ^2 tests. Receiver operating characteristic curve (ROC) analysis was adopted to estimate the potential diagnostic value of miR-1826. Cell proliferation was measured using MTT assays. Cell migration and invasion were identified by Transwell assays. Moreover, miR-1826 expression levels were significantly decreased in the serum of lung cancer patients and cell lines, compared to controls (all $P < 0.05$). Expression of miR-1826 was significantly associated with lymph node metastasis ($P = 0.015$) and TNM stage ($P = 0.033$). ROC curve analysis results showed that miR-1826 possesses diagnostic value in differentiating lung cancer patients from healthy individuals, with an area under the ROC curve (AUC) of 0.873, showing promising sensitivity and specificity. Using miR-1826 mimics and inhibitors, cell proliferation, migration, and invasion were inhibited by miR-1826 overexpression, but promoted by a reduction of miR-1826 (all $P < 0.05$). Downregulation of miR-1826 may serve as a novel marker for diagnosis of lung cancer. It may also be involved in tumor progression of lung cancer.

Keywords: miR-1826, lung cancer, diagnosis, progression

Introduction

Lung cancer, a common primary malignant tumor, is a leading cause of cancer-related deaths, worldwide, with high morbidity and mortality rates [1, 2]. Of all types of lung cancer, non-small cell cancer (NSCLC) accounts for approximately 85% [3]. Many studies have demonstrated that cigarette smoking is one of the leading high-risk factors in the development of lung cancer [4-6]. Lung cancer is a severe health burden in China, with incidence and mortality rates growing rapidly [7]. Although treatment strategies for lung cancer have greatly improved, prognosis of patients in the advanced stage remains unsatisfactory. Therefore, it is crucial to identify effective diagnostic biomarkers and novel therapies.

MicroRNAs (miRNAs) are a large class of small, non-coding, and endogenous RNA molecules that regulate gene expression through interac-

tion with the 3' untranslated regions (3'UTRs) of target mRNAs [8]. Abnormal expression of miRNAs has been strongly associated with tumor development and involved in the regulation of diverse physiological and pathological functions, including regulating cell proliferation, differentiation, migration, invasion, apoptosis, and tumor metastasis [9-11]. Many studies have demonstrated that miRNAs function as either oncogenes or tumor suppressors in the tumorigenesis process [12-16]. Other studies have indicated that miRNAs are associated with lung cancer [17, 18]. Accumulated evidence has suggested that miRNAs might be diagnostic or prognostic biomarkers for tumor detection and treatment therapies in cancers. Moreover, miR-1826 was found to be abnormally expressed and could serve as a non-invasive biomarker for diagnosis and prognosis in a number of cancers, including colorectal cancer and renal cell carcinoma [19, 20]. Abnormal

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Table 1. Association between miR-1826 expression and clinicopathological variables of lung cancer patients

Parameters	Cases No. (n = 124)	miR-1826 expression		P
		Low (n = 74)	High (n = 50)	
Age				0.338
< 60	58	32	26	
≥ 60	66	42	24	
Gender				0.702
Male	72	44	28	
Female	52	30	22	
Tumor size				0.709
< 5 cm	67	41	26	
≥ 5 cm	57	33	24	
Smoking status				0.201
No	73	47	26	
Yes	51	27	24	
Differentiation				0.107
Well + Moderate	63	42	21	
Poor	61	32	29	
Lymph node metastasis				0.015
Negative	68	34	34	
Positive	56	40	16	
TNM stage				0.033
I-II	70	36	34	
III-IV	54	38	16	

expression of miR-1826 was also found to be involved in the progression of various cancers, such as bladder cancer and renal cancer [21, 22]. However, the roles of miR-1826 in lung cancer remain elusive.

The present study detected expression levels of miR-1826 in the serum of lung cancer patients and healthy controls, as well as in cell lines. The effects of miR-1826 on biological behaviors of cancer cells were also assessed. The aim of this study was to identify the roles of miR-1826 in lung cancer.

Materials and methods

Patients and specimens

A total of 124 patients with lung cancer and 115 healthy controls, hospitalized or physically examined, were collected at People's Hospital of Wenshang Country, from May 2010 to April 2017. The patients had not received chemotherapy, radiotherapy, or surgical resections before sampling. Whole blood specimens were

collected from each participant during physical examinations. The serum was reserved by centrifugation. Serum samples were put into blood collection tubes of EDTA and stored at -80°C until further use. Detailed clinicopathological characteristics of the patients are collected and summarized in **Table 1**. This study was approved by the Ethics Committee of People's Hospital of Wenshang Country. Written informed consent was obtained from all participants in advance.

Cell lines and transfection

Human lung cancer cell lines (A549, H1299, H460, and H520) and normal bronchial epithelial 16HBE cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were grown in RPMI-1640 medium with 10% fetal bovine serum (FBS). They were incubated at 37°C in a humidified incubator with 5% CO₂. They were seeded into 6-well plates at a density of 1 × 10⁵ cells per well for 24 hours, then transfected with

miR-1826 mimics, miR-1826 inhibitors, or respective negative controls (NC) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to manufacturer's instructions. Untreated cells were used as blank controls. After 48 hours of transfection, the transfection efficiency of cells was detected using quantitative real-time PCR (qRT-PCR). The cells were then used for further analysis.

RNA isolation and qRT-PCR analysis

Total RNA was extracted from serum samples of lung cancer patients and healthy controls, as well as from cell lines, using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. The RNA was purified to an OD A260/A280 ratio close to 2.0. Complementary DNA (cDNA) synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Vilvoord, Brussel, Belgium). Afterward, the qRT-PCR reaction was carried out with the SYBR Green PCR master mix (Applied Biosystems, USA) in an ABI PRISM 7900 HT system (Applied Biosystems), accord-

ing to manufacturer's instructions. U6 was used as an endogenous control. Relative quantification of miR-1826 expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation assay

Cell viability was detected using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Cells were seeded in 96-well culture plates after transfection (5×10^4 cells per well). After incubation for 0, 24, 48, 72, and 96 hours, 20 μ l MTT solution (Sigma-Aldrich, 5 mg/mL) was added to each well for 4 hours at 37°C. Subsequently, the medium with MTT was removed and 100 μ l dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to each well, dissolving formazan crystals. Absorbance was measured at 490 nm using a spectrophotometer (Multiskan MK3, Thermo, Waltham, MA).

Cell migration and invasion assays

Cell migration and invasion assays were performed using Transwell chambers (Corning, Tewksbury, MA, USA). Eight nm pore size culture inserts were placed into the 24-well plates to separate the top and the lower chambers. For migration assays, 1×10^5 cells were plated in the top chamber in 100 μ l FBS-free medium. For invasion assays, 2×10^5 cells were plated in the top chamber precoated with Matrigel (Bedford, MA). The lower chambers were filled with 600 μ l RPMI 1640 medium containing 20% FBS as a chemoattractant. Cells were incubated at 37°C for 24 hours. Next, non-migrated/non-invaded cells were removed using a cotton swab. Migrated/invading cells on the lower side of the inserts were stained with formaldehyde and 0.1% crystal violet. Cells were then counted under a light microscope (Olympus, Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using SPSS 21.0 software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0 software (GraphPad Software, Inc., Chicago, USA). Data are presented as mean \pm standard deviation (SD). Data for group comparisons were analyzed with paired Student's t-tests or one-way analysis of variance (ANOVA). The relationship between miR-1826 expression and clinicopathological

parameters of patients was analyzed using χ^2 tests. The diagnostic value of serum miR-1826 was evaluated through the establishment of a receiver operating characteristic curve (ROC), calculating the area under the ROC curve (AUC). $P < 0.05$ indicates statistical significance. All experiments were repeated at least three times.

Results

Expression of miR-1826 in the serum of lung cancer patients and cell lines

This study detected expression levels of miR-1826 in the serum samples of lung cancer patients and healthy controls, as well as in the cell lines, employing qRT-PCR. As shown in **Figure 1A**, serum miR-1826 expression was significantly downregulated in lung cancer patients, compared with healthy controls ($P < 0.001$). Similarly, compared to normal bronchial epithelial 16HBE cell lines, miR-1826 showed lower expression levels in lung cancer cell lines (all $P < 0.01$, **Figure 1B**).

Correlation of circulating miR-1826 with clinicopathological characteristics in lung cancer patients

Analyzing the association between miR-1826 expression and clinicopathological parameters of lung cancer patients, the lung cancer patients were divided into a high-expression group ($n = 50$) and low-expression group ($n = 74$), according to the mean value of miR-1826 expression levels (1.396). As shown in **Table 1**, expression of miR-1826 was associated with lymph node metastasis ($P = 0.015$) and TNM stage ($P = 0.030$). However, no positive correlation was observed between miR-1826 expression and other parameters, such as age, gender, tumor size, smoking status, and differentiation (all $P > 0.05$, **Table 1**).

Diagnostic value of miR-1826 for lung cancer

The ROC curve was established to estimate the diagnostic value of miR-1826 using serum miR-1826 expression levels of lung cancer patients and healthy individuals. Corresponding healthy individuals were used as controls to produce the ROC curve (**Figure 2**). As shown in **Figure 2**, the AUC value was 0.873, yielding sensitivity of 70.2% and specificity of 90.4%, with a cutoff

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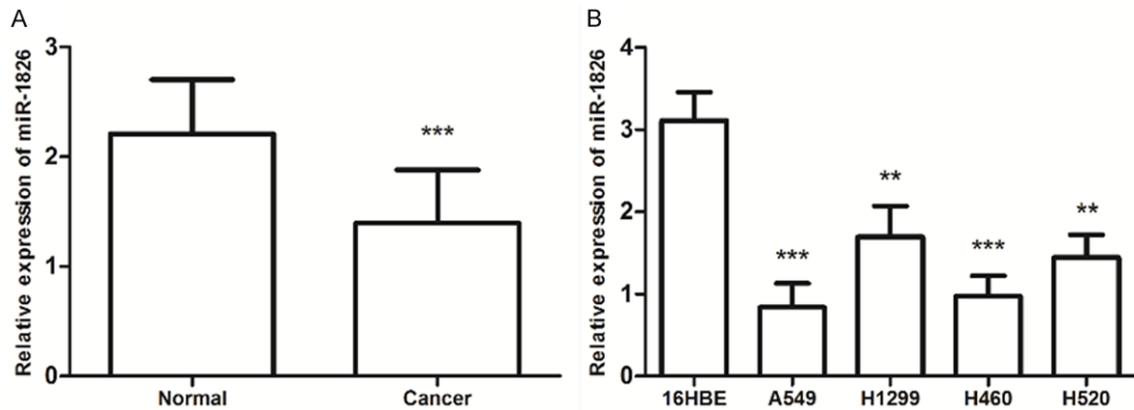


Figure 1. Expression of miR-1826, measured by qRT-PCR, in the serum of lung cancer patients and cell lines. A. Serum expression of miR-1826 was decreased in lung cancer patients, compared to that in healthy controls ($***P < 0.001$); B. Expression of miR-1826 was lower in lung cancer cell lines than in normal lung cancer cells ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

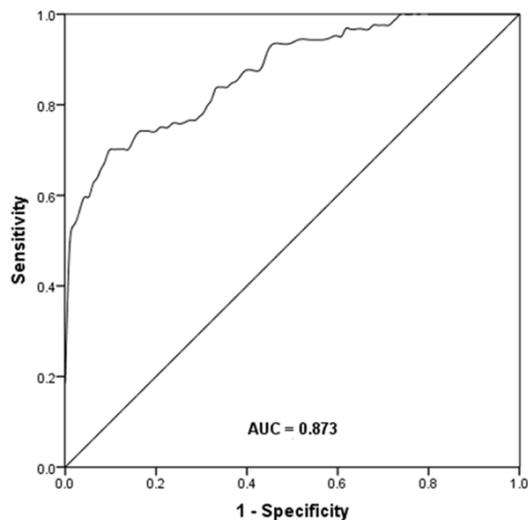


Figure 2. Diagnostic value of miR-1826 via ROC. It showed an AUC of 0.873, combined with a sensitivity of 70.2% and a specificity of 90.4%.

value of 1.570. Results showed that miR-1826 provided relative accuracy in differentiating lung cancer patients from healthy individuals.

Effects of miR-1826 on lung cancer cell proliferation, migration, and invasion

Investigating the roles of miR-1826 in lung cancer cells, the effects of miR-1826 on lung cancer cell proliferation were examined *in vitro*. Moreover, miR-1826 mimics or mimic NC and miR-1826 inhibitors or inhibitor NC were transfected into A549 and H460 cells, which showed extremely low miR-1826 expression. Subse-

quent to transfection, expression of miR-1826 in these cells was verified by qRT-PCR. Results showed that expression levels of miR-1826 were significantly increased in the cells transfected with the miR-1826 mimics, but expression levels of miR-1826 were decreased in cells transfected with miR-1826 inhibitors, compared with blank controls (all $P < 0.01$, **Figure 3A**). MTT assays were performed to evaluate the effects of miR-1826 on lung cancer cell proliferation. Results showed that cell proliferation abilities were markedly inhibited by miR-1826 mimics, while promoted by miR-1826 inhibitors (all $P < 0.05$, **Figure 3B**). In addition to cell proliferation, the effects of miR-1826 on cell migration and invasion were examined using Transwell assays. As shown in **Figure 4**, overexpression of miR-1826 by miR-1826 mimics decreased cell migration and invasion abilities. Silencing of miR-1826 by miR-1826 inhibitors promoted the migration and invasion abilities in both A549 and H460 cells, compared with blank controls (all $P < 0.05$).

Discussion

Lung cancer continues to be the most common type of cancer and a major oncologic public health burden worldwide. Despite advances in standard treatments, such as surgery, radiotherapy, and chemotherapy, 5-year survival rates of patients at the advanced stage remain unsatisfactory [23-25]. Most patients are initially diagnosed at an advanced stage due to the lack of effective early diagnosis methods. Early detection and treatments are essential to

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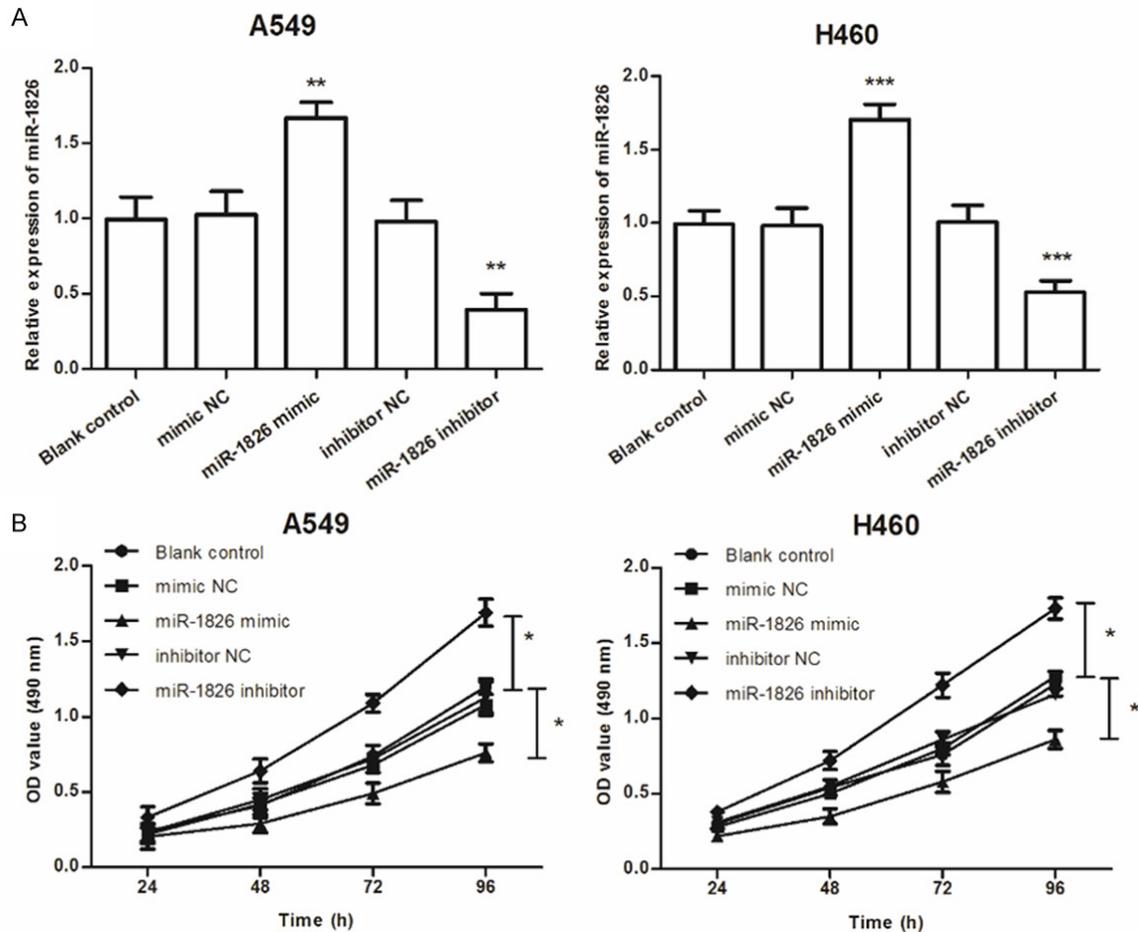


Figure 3. Effects of miR-1826 on cell proliferation in A549 and H460 cells. A. qRT-PCR analysis of miR-1826 in A549 and H460 cells. Expression of miR-1826 was decreased in cells transfected with miR-1826 mimics, while increased in cells transfected with miR-1826 inhibitors; B. Cell proliferation was suppressed by overexpression of miR-1826 but was enhanced by knockdown of miR-1826 in both A549 and H460 cells. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

prolonging the overall survival of lung cancer patients. Thus, accurate noninvasive sensitive markers for lung cancer detection or treatments are urgently needed.

Substantial evidence has demonstrated that miRNAs have diagnostic or prognostic significance in a variety of human malignancies [26-30]. In lung cancer, some miRNAs have also been identified. Expression of serum and tissue miR-421 has been found to be upregulated in NSCLC patients, serving as a prognostic biomarker in NSCLC. Knockdown of miR-421 has been shown to suppress proliferation, migration, and invasion of NSCLC cells [31]. Serum miR-411 has been considered to have clinical potential as a non-invasive diagnostic biomarker for NSCLC patients [32]. In the present study, expression patterns of miR-1826 in the serum

of lung cancer patients were investigated. This study showed that miR-1826 was significantly downregulated in the serum of lung cancer patients, compared with healthy controls, indicating that miR-1826 may act as a suppressor gene in lung cancer. Moreover, miR-1826 may be related to the development of lung cancer. Analysis results of the correlation between miR-1826 expression and patient clinicopathological characteristics showed that miR-1826 expression was significantly associated with lymph node metastasis and TNM stage. This suggests that miR-1826 expression was essential for the progression and metastasis of lung cancer. Based on altered miR-1826 expression in lung cancer patients and healthy controls, this study further analyzed whether miR-1826 has diagnostic value to distinguish lung cancer patients from healthy individuals. Based on

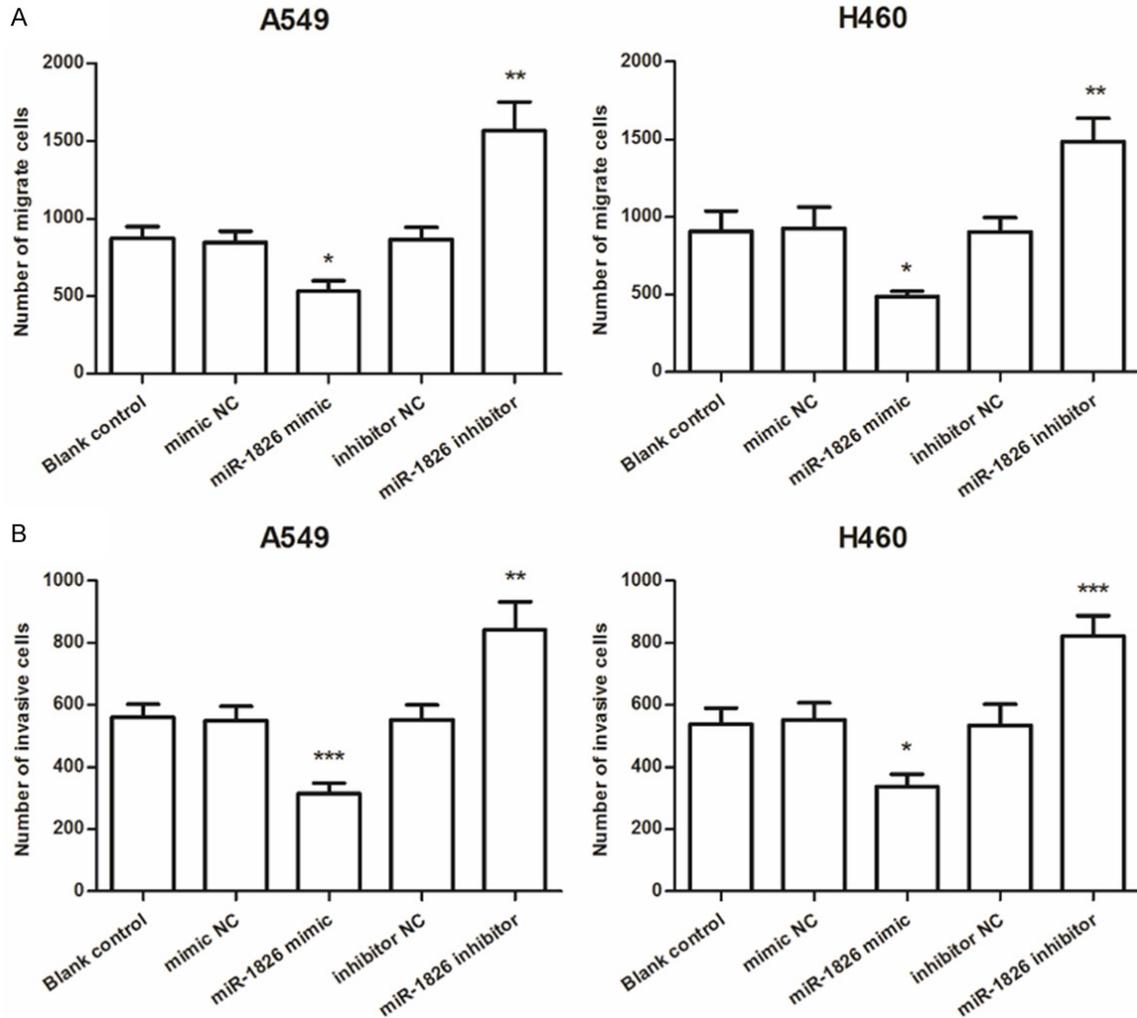


Figure 4. Results of migration and invasion assays for A549 and H460 cells. A, B. Overexpression of miR-1826 by miR-1826 mimics could inhibit cell migration and invasion, but downregulation of miR-1826 expression could promote cell migration and invasion (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

ROC curve analysis results with a high AUC value, as well as sensitivity and specificity, serum miR-1826 showed the ability to discriminate lung cancer patients from healthy individuals, with a relatively high diagnostic performance.

In addition, miRNAs have been shown to be involved in numerous aspects of cancer biology, including cell proliferation, apoptosis, migration, and invasion [33, 34]. For example, downregulation of miR-486-5p has been found in NSCLC. This could inhibit the proliferation and invasion of NSCLC cells [17]. Previous studies have indicated that identification of cancer-related miRNAs and corresponding direct target genes is essential for lung carcinogenesis and progression, as well as improvement of

lung cancer treatments. The current study explored the functional roles of miR-1826 in progression of lung cancer. The effects of miR-1826 on lung cancer cell proliferation, migration, and invasion were explored by regulating miR-1826 expression using miR-1826 mimics and inhibitors. Results showed that overexpression of miR-1826 by miR-1826 mimics could inhibit cell proliferation, migration, and invasion. Conversely, reduction of miR-1826 by miR-1826 inhibitors promoted cell proliferation, migration, and invasion in both A549 and H460 cell lines.

In recent years, the roles of miR-1826 have been reported in other malignancies. A systematic review study by Gu and co-workers [20] revealed that miR-1826 expression was down-

regulated in renal cell carcinoma and associated with poor prognosis. A study by Hu and his colleagues [35] indicated that miR-1826 was upregulated in colorectal cancer and miR-1826 inhibitors could suppress cell proliferation, migration, and invasion, as well as promote apoptosis. These studies have suggested that miR-1826 may act as either an oncogene or a tumor suppressor, depending on the cancer type. In the studies by Hiroshi Hirata and co-workers, they searched for miRNAs targeting beta-catenin, MEK-ERK pathway genes, and several growth factors (VEGFs). They observed that miR-1826 played an important role as a tumor suppressor by downregulating beta-catenin and MEK1 in renal cancers and by CTNNB1/MEK1/VEGFC downregulation in BC [21, 22]. It was speculated that miR-1826 may also play a tumor suppressor role by targeting beta-catenin and MEK1 expression in lung cancer. However, potential molecular mechanisms of miR-1826 in lung cancer were not examined in the present study. Future studies should confirm the roles of miR-1826 in progression of lung cancer.

In conclusion, decreased expression of miR-1826 was shown to be tightly associated with cancer cell proliferation, migration, and invasion of lung cancer. Thus, it may be a potential predictive diagnostic biomarker for early detection of lung cancer.

Disclosure of conflict of interest

None.

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