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Circulating microRNA array (miR-182, 200b and 205) for the early diagnosis and poor prognosis predictor of non-small cell lung cancer

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Abstract. – OBJECTIVE: Non-small cell lung cancer (NSCLC) is the most common cause for cancer-related mortality worldwide. Currently, early detection of NSCLC is one of the main available strategies for improving its prognosis. Due to the lack of non-invasive and convenient tools, early diagnosis of NSCLC remains poor. Recently, it has been reported that circulating microRNAs (miRNAs) can be stably detected in serum. Meanwhile, they play a powerful role as biomarkers in various tumors. Therefore, the aim of this study was to detect the expression levels of serum miR-182, 200b and 205 in NSCLC patients, and to investigate their diagnostic and prognostic values.

PATIENTS AND METHODS: Real-time quantitative polymerase chain reaction (RT-qPCR) was carried out to measure the expressions of miR-182, 200b and 205 in NSCLC tissues and normal controls. Receiver-operating characteristic (ROC) curve analysis was performed to assess the potential value of serum miRNAs for NSCLC diagnosis. Meanwhile, transwell assays were performed to observe the functional effects of miRNAs on the invasion and migration of NSCLC cells.

RESULTS: Compared with normal controls, serum levels of miR-182 and 205 in NSCLC patients were significantly upregulated, whereas miR-200b was remarkably downregulated. ROC analysis indicated that miRNA array (miR-182, 200b and 205) was useful biomarkers for early diagnosis of NSCLC. In addition, transwell assays demonstrated that miR-182 promoted the invasion and migration of NSCLC cells.

CONCLUSIONS: Our findings revealed that serum miR-182, 200b and 205 might serve as promising biomarkers for early detection and treatment of NSCLC.

Key Words:
Circulating microRNA array, Early diagnosis, Non-small cell lung cancer (NSCLC).

Introduction

Lung cancer, as one of the most common malignancy, is the leading cause of cancer-associated deaths all around the world[1]. Among all diagnosed lung cancers, NSCLC accounts for approximately 85%[2]. Currently, surgical resection is the main effective therapy for NSCLC patients. However, a high proportion of NSCLC patients are generally diagnosed at metastatic or advanced stages. Meanwhile, most NSCLC patients have lost the chances for surgery due to the lack of effective early diagnostic methods[3]. As a result, the survival rate of NSCLC patients has not improved significantly over the past few decades[4]. This may eventually contribute to the poor prognosis of NSCLC patients. Therefore, it is of great importance to develop early detection methods for NSCLC, thereby reducing NSCLC-related deaths. Nowadays, low-dose computed tomography (LDCT) screening test has been applied in prospective observational trials, which is proved to be effective[5]. However, there are still several noteworthy issues existed. For example, LDCT may result in harm to patients due to exposure to radiation[6]. LDCT may lead to an over-diagnosis of benign pulmonary lesions, eventually resulting in prominently increase in unnecessary surgical interventions[7]. In addition, as a screening tool, LDCT has a higher risk of false positivity[8]. These facts further strengthen the necessity and importance to search for novel sensitive tumor-specific biomarkers in the discrimination of tumors from benign pulmonary lesions as well as diagnosis of tumors' existence at earlier stages. Since blood tests are readily accessible, circulating biomarkers for earlier

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detection will be an integrated part of future NSCLC screening programs. MiRNAs are a type of non-coding RNA molecules, which can modulate gene expressions[9]. Scholars[10,11] have found that aberrant expressions of miRNAs are especially associated with various tumors. Currently, miRNAs are widely used as candidate biomarkers for multiple human malignancies. Previous investigations[12] have indicated that tumor cells release miRNAs into circulation, which can be detected in blood. It has been reported that stable circulating miRNAs can avoid degradation from RNases. In addition, circulating miRNAs can bind to proteins such as high-density lipoprotein (HDL) and be transported into target cells by serum, accompanied by alterations in gene expression[13]. Much effort has been made to find out circulating miRNAs for cancer prediction and therapy. For example, circulating miR-1290 has been found to be a novel diagnostic and prognostic biomarker for human colorectal cancer[14]. For human chronic lymphocytic leukemia, circulating miR-192 is demonstrated to be a diagnostic biomarker[15]. Meanwhile, circulating miR-132-3p is also confirmed as an effective diagnostic biomarker for malignant mesothelioma[16]. The accessibility makes circulating miRNAs attractive in the treatment of various cancers. However, the use of circulating miRNA array (miR-182, 200b and 205) as biomarkers in NSCLC remains relatively less explored.

Patients and Methods

Clinical Samples

A total of 50 NSCLC patients and 30 healthy controls were collected from People's Hospital of Rizhao from May 2015 to June 2017. All patients had no prior history of cancer-associated therapy, such as surgery, chemotherapy, radiotherapy, or antibiotic therapy. NSCLC patients and normal controls were free of malignancy, inflammatory condition or other complications, including diabetes, hepatitis, hypertension and so on. Tumor staging was performed according to the Sixth Edition of the American Joint Commission on Cancer tumor-node-metastasis (TNM) Staging System. This work was approved by the Ethics Review Committee of People's Hospital of Rizhao. Written informed consent was obtained from each subject before the study.

Serum Collection

Peripheral blood samples of NSCLC patients and normal controls were collected and stored at room temperature for a few minutes. Subsequently, collected samples were centrifuged at 3000 rpm, 4°C for 15 minutes and stored at -80°C for further experiments.

Cell Culture

NSCLC cell line A549 was obtained from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). A549 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) in a 37°C, 5% CO_2 incubator.

Cell Transfection

MiR-182 mimics, inhibitor and corresponding negative controls were purchased from Ribobio Co., Ltd. (Guangzhou, China). Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Calsbad, CA, USA).

Transwell Assays

For invasion assays, transfected A549 cells were cultured in serum-free medium and seeded into the upper chamber of transwell chamber inserts (8.0 μm pore size, Corning, Corning, NY, USA) coated with Matrigel (Sigma-Aldrich, St. Louis, MO, USA). For migration assays, transfected A549 cells were cultured in serum-free medium and seeded into the upper chamber of transwell chamber inserts without Matrigel. Subsequently, complete medium containing 10% FBS was added to the lower chamber. After incubation for 48 h, cells remaining on the upper chamber were removed. Meanwhile, cells that migrated or invaded into the lower chamber were fixed with methanol and stained with crystal violet. Finally, five fields were randomly selected for each sample, and the cells were imaged and counted under a microscope (Olympus, Tokyo, Japan).

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from 400 μL serum sample in strict accordance with the manufacturer's protocol of miRNeasy serum kit (Qiagen, Hilden, Germany). Expression levels of candidate

miRNA biomarkers in serum samples were detected by RT-qPCR assay. Firstly, extracted miRNAs were reverse transcribed to complementary DNA (cDNA) according to the instructions of TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Then, RT-qPCR assay was performed on a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). U6 was used as an internal control. Relative miRNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. Primers used in this study were as follows: miR-182, F: 5'-CGTGAATGATAGTGAGGAAC -3', R: 5'- GTGAACGATTTGCCACACACA -3'; miR-200b, F: 5'- CCTTGTCCTATAGAAGCACAAC -3', R: 5'-GTCATTTCCACAGCCCTGTGA -3'; and miR-205, F: 5'-CCACCACGCTCTTCTGTCTACTG -3', R: 5'- GGGCTACGGGCTTGTCACT-3'. U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3'.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 version (SPSS Inc. Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) were used for all statistical analysis. p-values < 0.05 were considered statistically significant. Receiver-operator characteristic (ROC) curve analysis and the area under the ROC curve (AUC) were used to determine the sensitivity and specificity of identified miRNAs for NSCLC diagnosis. Kaplan-Meier method and log-rank test were applied to estimate the survival rate and compare the survival curves, respectively.

Results

Expression Levels of Circulating miRNA Array (miR-182, 200b and205) in NSCLC Patients

To validate the reliability of identified circulating miRNAs as diagnostic biomarkers, we collected clinical serum samples from NSCLC patients and normal controls. Subsequently, RT-qPCR assay was performed to detect the expression levels of miR-182, 200b and 205. Results demonstrated that the expression of miR-182 in NSCLC samples was significantly upregulated when compared with that of normal controls (Figure 1A). However, the expression of miR-200b in NSCLC samples was significantly lower than that of normal controls (Figure 1B). Meanwhile, compared with normal controls, miR-205 expression level was also dramatically increased in NSCLC samples (Figure 1C).

Diagnostic Values of miRNA Array (miR-182, 200b and205) in NSCLC

Based on the above results, we further use ROC curve and the area under the ROC curve (AUC) to explore whether serum miRNAs alone or in combination could be useful for the diagnosis of NSCLC. For all NSCLC patients both in early stage and advanced stage, we found that serum levels of miR-182 (AUC = 0.601), miR-200b (AUC = 0.673) and miR-205 (AUC = 0.696) showed significantly worse diagnostic efficacies when compared with the combination of the three miRNAs (AUC = 0.781). This indicated that the combination of miR-182, miR-200b and miR-205 could distinguish NSCLC patients from healthy

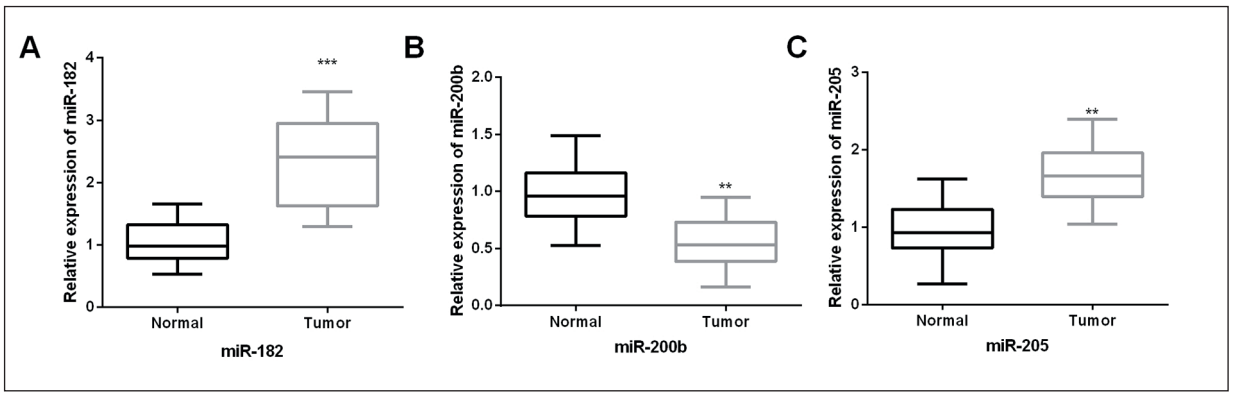

Figure 1. Expression levels of miRNA assays (miR-182, 200b and 205) in NSCLC samples and normal controls. ***A,*** MiR-182 expression was notably enhanced in NSCLC samples. ***B,*** MiR-200b was significantly downregulated in NSCLC samples. ***C,*** MiR-205 was dramatically upregulated in NSCLC samples. $^{**}p < 0.01$, $^{***}p < 0.001$.

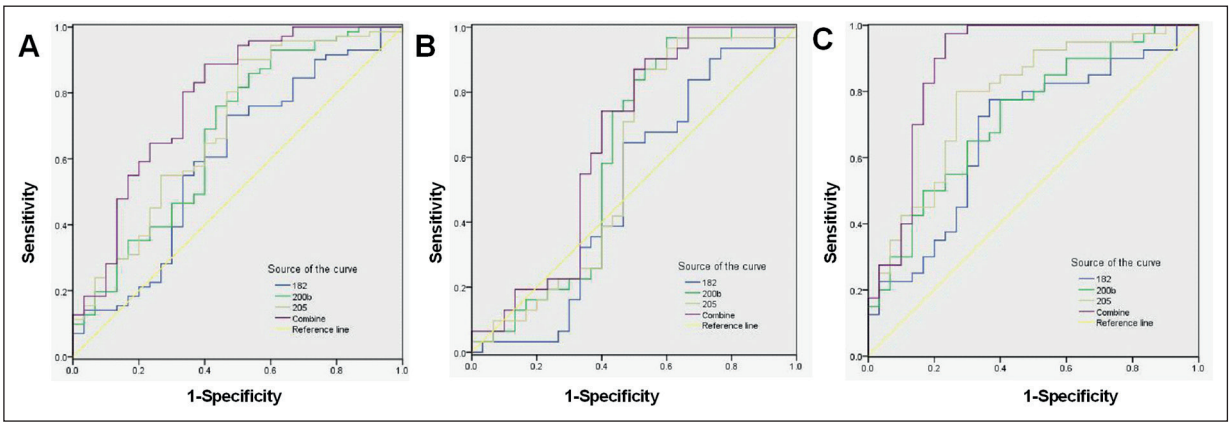

Figure 2. ROC curve was used to assess the diagnostic value of serum miRNAs in NSCLC. ***A,*** ROC curves of selected 3 miRNAs for NSCLC patients in all stages. ***B,*** ROC curves of selected 3 miRNAs for NSCLC patients only in advanced stages. ***C,*** ROC curves of selected 3 miRNAs for NSCLC patients only in early stages.

controls (Figure 2A, Table I). In the advanced stage, the diagnostic efficacies of miR-182 (AUC = 0.506), miR-200b (AUC = 0.609) and miR-205(AUC = 0.584) was significantly worse than that of early and advanced stage. In addition, although the combination of miR-182, miR-200b and miR-205 exhibited the highest AUC (0.649) in advanced stage, the efficacy was still remarkably worse than that of early and advanced stage (Figure 2B, Table II). Furthermore, in the early stage, the corresponding AUC for miR-182, miR-200b and miR-205 was 0.674, 0.723 and 0.782, respectively. This indicated that all the three miRNAs had the superiority of discriminating early stage NSCLC patients from healthy controls. Moreover, the combination of miR-182, miR-200b and miR-205 showed the best diagnostic utility with highest AUC value (AUC = 0.883) in early stage NSCLC patients (Figure 2C, Table III). These results illustrated that the combination of miR-182,

Table I. Comparison of ROC curves between miRNA detection for identifying early-stage and advanced-stage NSCLC.

Test	AUC		Sensitivity		Specificity	
	(95% CI)	*p*	% (95% CI)	*p*	% (95% CI)	*p*
miR-182	0.601 (0.474 to 0.728)	0.110	53.0 (47.0 to 58.9)	1.000	57.0 (51.6 to 62.4)	< 0.001
miR-200b	0.673 (0.551 to 0.796)	0.006	55.1 (49.0 to 61.2)	1.000	62.1 (56.9 to 67.2)	< 0.001
miR-205	0.696 (0.578 to 0.814)	0.002	55.8 (49.7 to 61.8)	0.999	63.6 (58.4 to 68.9)	< 0.001
Combine	0.781 (0.674 to 0.888)	< 0.001	58.3 (52.0 to 64.5)	0.093	69.6 (64.6 to 74.6)	< 0.001

ROC: Receiver-operator characteristic curve analysis; NSCLC: Non-small cell lung carcinoma; AUC: Area under ROC curve.

Table II. Comparison of ROC curves between miRNA detection for identifying advanced-stage NSCLC.

Test	AUC		Sensitivity		Specificity	
	(95% CI)	*p*	% (95% CI)	*p*	% (95% CI)	*p*
miR-182	0.506 (0.354 to 0.659)	0.931	50.3 (41.7 to 58.9)	0.125	50.3 (43.8 to 56.8)	0.016
miR-200b	0.609 (0.457 to 0.761)	0.145	55.3 (46.3 to 64.2)	0.208	55.4 (49.1 to 61.7)	0.002
miR-205	0.584 (0.431 to 0.737)	0.260	54.1 (45.2 to 62.9)	< 0.001	54.2 (47.7 to 60.7)	0.005
Combine	0.649 (0.504 to 0.795)	0.045	57.2 (48.5 to 66.0)	0.001	57.5 (51.0 to 64.0)	0.001

ROC: Receiver-operator characteristic curve analysis; NSCLC: Non-small cell lung carcinoma; AUC: Area under ROC curve.

Table III. Comparison of ROC curves between miRNA detection for identifying early-stage NSCLC.

Test	AUC		Sensitivity		Specificity	
	(95% CI)	*p*	% (95% CI)	*p*	% (95% CI)	*p*
miR-182	0.67 (0.545 to 0.804)	0.013	56.6 (49.3 to 63.8)	0.919	60.4 (53.4 to 67.4)	0.064
miR-200b	0.723 (0.604 to 0.843)	0.001	59.4 (52.3 to 66.6)	0.116	62.6 (55.6 to 69.5)	0.839
miR-205	0.782 (0.672 to 0.893)	< 0.001	61.9 (54.6 to 69.3)	0.554	65.9 (59.0 to 72.8)	0.015
Combine	0.883 (0.792 to 0.975)	< 0.001	66.2 (58.2 to 74.2)	< 0.001	71.6 (64.9 to 78.3)	< 0.001

ROC: Receiver-operator characteristic curve analysis; NSCLC: Non-small cell lung carcinoma; AUC: Area under ROC curve.

miR-200b and miR-205 was a distinctive marker for the diagnosis of NSCLC, especially for early stage patients.

MiR-182 Overexpression Promoted the Invasion and Migration of NSCLC Cells

According to the mean expression level of miR-182, NSCLC patients were divided into two groups. Kaplan-Meier analysis demonstrated the OS in NSCLC patients with higher miR-182 expression level was significantly shorter than those with lower level (Figure 3A). Cell invasion and migration are important aspects for tumor progression. We next performed transwell assays to determine whether miR-182 had direct functional roles in promoting NSCLC cell migration and

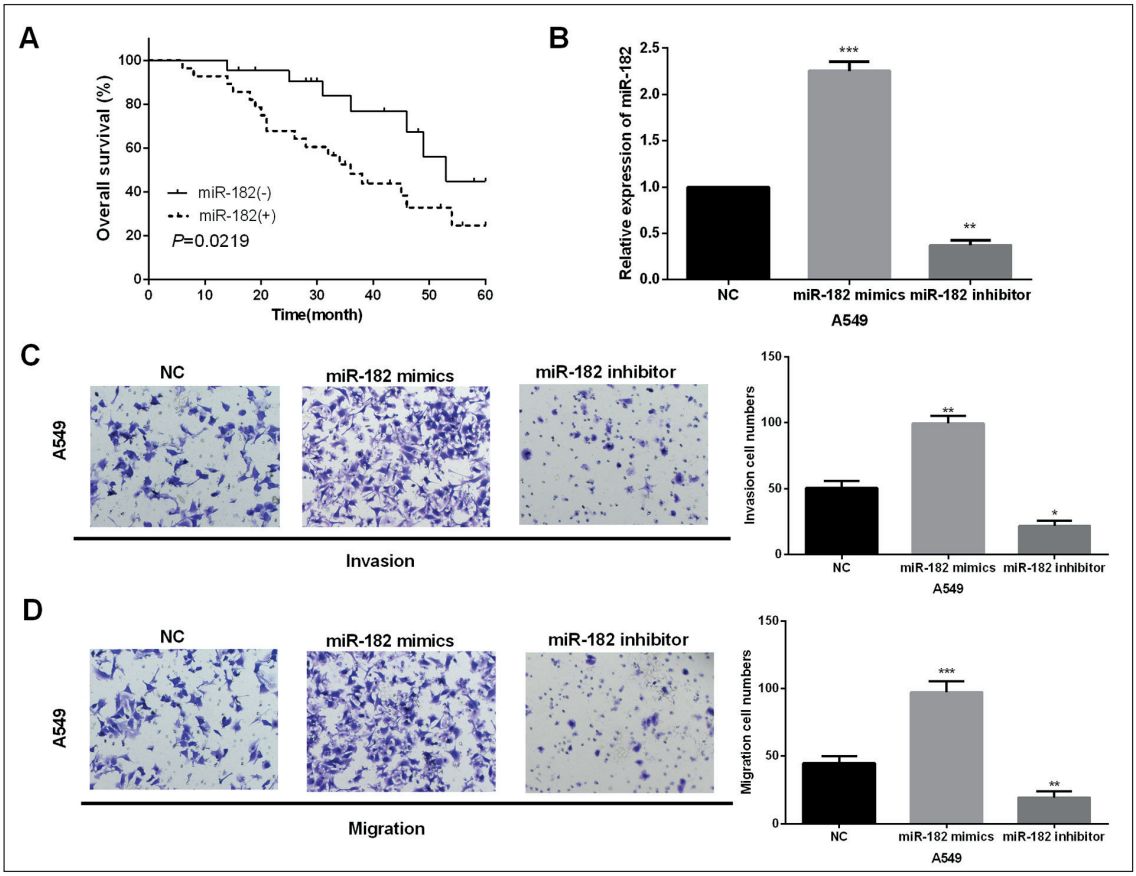

Figure 3. MiR-182 overexpression promoted the invasion and migration of NSCLC cells. ***A,*** Kaplan-Meier survival curve analysis showed that NSCLC patients with higher miR-182 expression had significantly shorter overall survival (OS). ***B,*** MiR-182 expressions in A549 cells transfected with miR-182 mimics or inhibitor. ***C,*** Cell invasion was measured by transwell assays in A549 cells transfected with miR-182 mimics or inhibitor. ***D,*** Cell migration was determined by transwell assays in A549 cells transfected with miR-182 mimics or inhibitor. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

invasion. A549 cells were transfected with miR-182 inhibitor or mimics to down- or up-regulate its expression, and the transfection efficiency was verified by RT-qPCR (Figure 3B). Moreover, transwell assays demonstrated that miR-182 overexpression significantly suppressed the invasion and migration abilities of A549 cells (Figure 3C and 3D).

Discussion

Lung cancer is one of the major causes of cancer deaths, in which NSCLC accounts for the majority[17]. Importantly, late diagnosis of NSCLC is a common reason for high mortality rate[18]. As a systematic disease, tumorigenesis needs to be judged from a comprehensive perspective. Early detection remains a major challenge for NSCLC. However, the risk of NSCLC patients with small pulmonary nodules cannot be accurately predicted by existing methods, including CT screening and chest X-ray. Meanwhile, additional follow-up examinations may enhance radiation exposure[19, 20]. Thus, the development of early detection markers is turning to novel minimally invasive techniques[21]. Blood-based tests, such as the determination of circulating miRNAs in plasma, can provide a crucial complement to existing diagnostic methods. It may also improve the screening and detection of NSCLC[22]. In the present study, we investigated the potential of circulating miRNAs as early detection markers by analyzing serum levels of miRNAs in NSCLC patients and normal controls. Up to now, increasing studies have suggested that circulating miRNAs may function as novel diagnostic and prognostic biomarkers for multiple tumors, including NSCLC. For example, Xu et al[23] have found that high expression levels of circulating miR-92a, miR-20a and miR-18a are correlated with poor prognosis of NSCLC patients. Yu et al[24] have reported that declined circulating miR-375 in NSCLC patients can serve as a potential biomarker. Wiemer et al[25] have indicated that circulating miR-150 may act as a prognostic biomarker for early-stage NSCLC. Moreover, the three miRNAs involved in the current study are also known to play important roles in tumor progression. Wang et al[26] have shown that miR-182 promotes the progression of prostate cancer via regulating Wnt/beta-catenin signal pathway. Additionally, miR-182 has been reported to promote the proliferation and invasion of hepatocellular carcinoma cells by regulating ephrin-A5[27]. In a recent work, miR-200b and miR-200c are confirmed as mediators and prognostic factors for gastric cancer[28]. Furthermore, miR-200b can suppress nasopharyngeal carcinoma cell growth, migration and invasion through modulating Notch1[29]. According to Duan et al[30], miR-205 functions as a biological marker in NSCLC. Moreover, miR-205 has been found to promote endometrial cancer cell epithelial-mesenchymal transition *via* regulating AKT signaling[31]. In the current study, we demonstrated that the expression levels of miR-182 and miR-205 were significantly upregulated in NSCLC patients, whereas miR-200b was significantly downregulated. This suggested that miR-182 and miR-205 acted as tumor suppressors, and miR-200b played as an oncogene in NSCLC. Blood-based screening test may be an important diagnostic method for early detection of NSCLC. ROC and AUC analysis indicated that the combination of circulating miR-182, miR-200b and miR-205 could discriminate NSCLC patients in early stage from healthy controls. Based on the above results, we further selected one appropriate miRNA (miR-182) for functional assays. Transwell assays demonstrated that miR-182 overexpression could remarkably promote the invasion and migration abilities of NSCLC cells. All these findings suggested that circulating miR-182, miR-200b and miR-205 could serve as diagnostic and therapeutic biomarkers for NSCLC treatment.

Conclusions

We showed that circulating miR-182, miR-200b and miR-205 might serve as reliable biomarkers for NSCLC early detection and therapy. However, the potential regulatory function and underlying molecular mechanism of miR-200b and miR-205 in NSCLC need to be further investigated.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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