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

J.-G. ZOU1, L.-F. MA1, X. LI1, F.-L. XU1, X.-Z. FEI2, Q. LIU3, Q.-L. BAI4, Y.-L. DONG5

1Laboratory Medicine, People’s Hospital of Rizhao, Rizhao, China
2Department of Cardiology, People’s Hospital of Rizhao, Rizhao, China
3Department of endocrinology, People’s Hospital of Zhangqiu District, Jinan, China
4Operating Room, People’s Hospital of Zhangqiu District, Jinan, China
5Nursing Department, People’s Hospital of Rizhao, Rizhao, China

Introduction

Lung cancer, as one of the most common malignancy, is the leading cause of cancer-associated deaths all around the world1. 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 methods3. As a result, the survival rate of NSCLC patients has not improved significantly over the past few decades4. 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 effective5. However, there are still several noteworthy issues existed. For example, LDCT may result in harm to patients due to exposure to radiation6. LDCT may lead to an over-diagnosis of benign pulmonary lesions, eventually resulting in prominently increase in unnecessary surgical interventions7. In addition, as a screening tool, LDCT has a higher risk of false positivity8. 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
MicroRNA array for the diagnosis predictor of non-small cell lung cancer

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% CO₂ 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 $2^{-\Delta\Delta C_{\text{T}}}$ method. Primers used in this study were as follows: miR-182, F: 5’-CGTGAATGATAGTGAGGAAC-3’, R: 5’- GTGAACGATTTGCCACACA-3’; miR-200b, F: 5’-CCTTGTCCTATAGAAGCACAAC-3’, R: 5’-GTCATTTCCACAGCCCTGTGA-3’; and miR-205, F: 5’-CCACCAACGCTCTTGTCTACTG-3’, R: 5’-GGGCTACGGGCTTGTCACT-3’. U6: F: 5’-GCTTCGGCAGCACATATACTA-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.

Expression Levels of Circulating miRNA Array (miR-182, 200b and 205) 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 and 205) 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 controls.

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. **$p < 0.01$, ***$p < 0.001$. 

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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.

<table>
<thead>
<tr>
<th>Test</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(95% CI)</td>
<td>p</td>
<td>% (95% CI)</td>
</tr>
<tr>
<td>miR-182</td>
<td>0.601 (0.474 to 0.728)</td>
<td>0.110</td>
<td>53.0 (47.0 to 58.9)</td>
</tr>
<tr>
<td>miR-200b</td>
<td>0.673 (0.551 to 0.796)</td>
<td>0.006</td>
<td>55.1 (49.0 to 61.2)</td>
</tr>
<tr>
<td>miR-205</td>
<td>0.696 (0.578 to 0.814)</td>
<td>0.002</td>
<td>55.8 (49.7 to 61.8)</td>
</tr>
<tr>
<td>Combine</td>
<td>0.781 (0.674 to 0.888)</td>
<td>&lt; 0.001</td>
<td>58.3 (52.0 to 64.5)</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Test</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(95% CI)</td>
<td>p</td>
<td>% (95% CI)</td>
</tr>
<tr>
<td>miR-182</td>
<td>0.506 (0.354 to 0.659)</td>
<td>0.931</td>
<td>50.3 (41.7 to 58.9)</td>
</tr>
<tr>
<td>miR-200b</td>
<td>0.609 (0.457 to 0.761)</td>
<td>0.145</td>
<td>55.3 (46.3 to 64.2)</td>
</tr>
<tr>
<td>miR-205</td>
<td>0.584 (0.431 to 0.737)</td>
<td>0.260</td>
<td>54.1 (45.2 to 62.9)</td>
</tr>
<tr>
<td>Combine</td>
<td>0.649 (0.504 to 0.795)</td>
<td>0.045</td>
<td>57.2 (48.5 to 66.0)</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Test</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(95% CI)</td>
<td>% (95% CI)</td>
<td>% (95% CI)</td>
</tr>
<tr>
<td>miR-182</td>
<td>0.67 (0.545 to 0.804)</td>
<td>0.013</td>
<td>56.6 (49.3 to 63.8)</td>
</tr>
<tr>
<td>miR-200b</td>
<td>0.723 (0.604 to 0.843)</td>
<td>0.001</td>
<td>59.4 (52.3 to 66.6)</td>
</tr>
<tr>
<td>miR-205</td>
<td>0.782 (0.672 to 0.893)</td>
<td>&lt; 0.001</td>
<td>61.9 (54.6 to 69.3)</td>
</tr>
<tr>
<td>Combine</td>
<td>0.883 (0.792 to 0.975)</td>
<td>&lt; 0.001</td>
<td>66.2 (58.2 to 74.2)</td>
</tr>
</tbody>
</table>

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

**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.


Discussion

Lung cancer is one of the major causes of cancer deaths, in which NSCLC accounts for the majority\textsuperscript{17}. Importantly, late diagnosis of NSCLC is a common reason for high mortality rate\textsuperscript{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\textsuperscript{19,20}. Thus, the development of early detection markers is turning to novel minimally invasive techniques\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{24} have reported that declined circulating miR-375 in NSCLC patients can serve as a potential biomarker. Wiemer et al\textsuperscript{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\textsuperscript{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\textsuperscript{27}. In a recent work, miR-200b and miR-200c are confirmed as mediators and prognostic factors for gastric cancer\textsuperscript{30}. Furthermore, miR-200b can suppress nasopharyngeal carcinoma cell growth, migration and invasion through modulating Notch1\textsuperscript{29}. According to Duan et al\textsuperscript{30}, miR-205 functions as a biological marker in NSCLC. Moreover, miR-205 has been found to promote endometrial cancer cell epithelial-mesenchymal transition \textit{via} regulating AKT signaling\textsuperscript{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.

References


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27) Wang TH, Yeh CT, Ho JY, Ng KF, Chen TC. OncomiR miR-96 and miR-182 promote cell proliferation and invasion through targeting ephrinA5 in hepatocellular carcinoma. Mol Carcinog 2016; 55: 366-375.

