Original Article
Clinical relevance of serum miR-9 in resected non-small cell lung cancer patients

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Received April 25, 2016; Accepted July 30, 2016; Epub October 15, 2016; Published October 30, 2016

Abstract: Background: MiRNAs can be released from cancer cells to body fluids via secreting exosomes particles. MicroRNA-9 (MiR-9) was over expressed in non-small-cell lung cancer (NSCLC), and identified as promising prognostic markers in NSCLC. In the present study, we analyzed whether levels of serum miR-9 can be used as prognostic or predictive biomarkers in radically resected NSCLC patients. Methods: Serum samples from 161 radically resected NSCLC patients and 161 controls was collected. We used quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays to measure miR-9 and assess its association with clinicopathological parameters. Kaplan-Meier survival analysis and Cox proportional hazards models were used to estimate the serum miR-9 expression on survival. Results: The data showed that the serum levels of miR-9 was significantly upregulated in NSCLC patients compared with the healthy controls. MiR-9 expression was significantly correlated with the lymph node metastasis (P=0.0026), high II+III stage (P=0.0038) and recurrence (P=0.0001). Univariate analysis showed that advanced p-stage (P=0.002), an advanced regional lymph node metastasis status (P<0.001), lymphatic permeation (P<0.001), poor tumor differentiation (P=0.046), pleural involvement (P=0.012), vascular invasion (P=0.003) and high miR-9 expression (P=0.003) were significant prognostic factors. Multivariate analysis identified lymph node metastasis status (HR, 5.643; 95% CI, 2.315-13.76; P<0.001) and high miR-9 expression (HR, 4.08; 95% CI, 1.452-6.36; P=0.002) were as independent prognostic factors. Using univariate analysis, the low miR-9 expression patients had a significantly higher OS rate than did the low miR-9 expression patients (P=0.0016). Conclusions: Serum miR-9 was increased in NSCLC patients, and high serum miR-9 level was associated with poor outcomes in completely resected NSCLC patients.

Keywords: Non-small-cell lung cancer, MicroRNA-9, prognosis

Introduction

Lung cancer is a significant health issue and the leading cause of cancer death [1, 2]. The disease is divided into small cell lung cancer (SCLC) and nonsmall cell lung cancer (NSCLC). Approximately 85-90% of all cases of lung cancer are NSCLC. Most lung cancer patients have advanced stage disease at diagnosis, which is often associated with metastasis and a poor prognosis [3]. More information is needed to predict the prognosis of patients.

Despite the devastating problem of NSCLC and the estimated 51% increased numbers of cases of this disease since 1985 [4], a panel of reliable serum biomarkers has not yet been identified. Existing lung cancer protein biomarkers include tumor-liberated proteins such as CEA, NSE, TPA, chromogranin, CA125, CA19-9, and Cyfra 21-1. While these are the best options currently available in the clinic, they each have limitations as detailed by Tarro et al [5].

The interest in circulating RNAs as biomarkers is rapidly increasing as their potential is being realized. MicroRNAs (miRNAs) are a family of small, endogenous, non-coding functional RNA molecules of 18-25 nucleotides in length. These regulatory molecules function to modulate the activity of specific mRNA targets either by translational repression or by mRNA degra-
Serum miR-9 in resected non-small cell lung cancer patients

dation [6]. The sequences of miRNAs are evolutionarily conserved across species which suggests an important biological function [7]. miRNAs are key regulators of various biological processes including development, differentiation, proliferation, cell death and metabolism [8].

Recent studies have revealed that microRNA-9 (miR-9) is aberrantly expressed in many cancer types including breast cancer [9], oral and oropharyngeal squamous cell carcinomas [10], gastric cancer [11] and thyroid cancer [12] etc. The role of miR-9 in cancers remains controversial. It has been shown to be either an onco-

genic microRNA or a tumor suppressor depending on different tissue types and downstream targets [9, 13, 14]. It has found that miR-9 was overexpressed in lung cancer, and increased miR-9 expression promoted the growth and invasion of NSCLC cells [15].

miRNAs can be released from cancer cells to body fluids via secreting exosomes particles, which could protect them from RNase degradation in circulation. Therefore, miRNA might be a useful noninvasive biomarker for diagnosis and recurrent cancers. However, whether circulating miR-9 can be used as prognostic biomarkers in NSCLC remains unknown.

Here, we aimed to examine the miR-9 expression profiles in plasma samples of NSCLC patients to explore their clinical significance in disease development and progression, and provide information for personalised therapy.

Materials and methods

Patients and serum samples

After informed consent and approval by appropriate Institutional Review Board/Independent Ethical Committee of People’s hospital of Rizhao, China, serum was collected from 161 patients with histologically confirmed NSCLC (January 2005 and December 2010) and age-matched healthy controls with no symptomatic or chronic disease. No preoperative chemotherapy and/or radiotherapy case was included. Patients were excluded according to the following criteria: (1) radiotherapy or chemotherapy prior to surgery, (2) tumor tissue not available, (3) pathological stage IIIB or stage IV disease, (4) complete resection not achieved (not R0), and (5) postoperative survival <60 days. Clinical data were obtained from each patient’s medical records and summarized in Table 1. Clinical stage was assessed according to the seventh edition of the Lung Cancer Staging International Division, which was published by the Union for International Cancer Control (UICC) and the International Association for the Study of Lung Cancer (IASLC) in 2009. The selection criteria for healthy controls were as follows: good physical status, no acute or chronic disease, and not taking any medication. To use these serum samples for research purposes, prior informed consent from the patients and approval from the Institute Research Ethics Committee of people’s hospital of Rizhao were obtained.

Serum samples were collected and processed at Biobank in people’s hospital of Rizhao according to standard procedures. In brief, venous blood was drawn into serum tubes, clotted at room temperature for 1 h, and subsequently centrifuged at 2,500×g for 10 min. Serum was collected, distributed into 100 μl aliquots, and immediately stored at -80°C. Repeated freeze-thaw cycles were avoided for all serum samples.

Isolation of serum RNA

The total RNA, including microRNA, was extracted from serum using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany). Two hundred microliters of serum was mixed with denaturing buffer in the volumes described in the manufacturer’s protocols. The homogenate was incubated at room temperature for 5 min. Then 25 fmol of synthetic Cel-miR-39 (Ambion) was spiked into the mixture. Subsequently, the manufacturer’s protocols were followed for RNA extraction. Total RNA was eluted into 14 μl of nuclease-free water. The RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

qRT-PCR

Total RNA was extracted from serum samples of these patients or controls as described above. Real-time RT-PCR method was used to assess the expression levels of miR-9 with Express SYBR® GreenER qPCRs supermix Universal kit (Invitrogen) on a Rotor-gene 6000
system (Qiagen, Valencia, CA, USA). U6 RNA was used as an endogenous reference for normalizing the expression levels of miR-9. Initially, we calculated a \( \Delta \)Ct (target-reference), which is equal to the difference between threshold cycles for miR-9 (target) and those for U6 RNA (reference). The fold-change between cancer tissues and normal breast tissue control for miR-9 was calculated with the \( 2^{\Delta\Delta \text{Ct}} \) method, in which \( \Delta\Delta \text{Ct}=\Delta \text{Ct} \) (target-reference in tumor samples) - \( \Delta \text{Ct} \) (target-reference in normal samples). The relative expression levels of miRNAs in cancer compared to their non-tumorous controls were calculated using the method of \( 2^{\Delta\Delta \text{Ct}} \). The quantitative real-time PCR primers for miR-9 were designed as follows: forward: miR-9: 5' 5'-GTGCAAGGTCCGAGGT-3' and 5'-GGGCTTCTTTGTTATCGAC3'; U6: 5'-CTCGTTCGCACGACA-3' and 5'-AAGCCTCAGAATTGCTG-3'. The reaction parameters were: 56°C for 2 minutes, 95°C for 10 minutes, followed by 38 cycles of 95°C for 15 seconds and 55°C for 1 minute. PCR cycle threshold (Ct) values were recorded for each target gene and for normalization controls and were averaged across three independent runs. Primers for miR-9 was custom-ordered from Shanghai, China. In addition, each measurement was performed in triplicate.

### Statistical analysis

Differences in miR-9 expression and clinicopathologic variables were analyzed using the \( \chi^2 \) test. Age was dichotomized at the median value. Overall survival (OS) was defined as the time between surgery and death from any cause. Survival curves were calculated using the method of Kaplan-Meier and compared using the log-rank test. Factors shown to be of prognostic significance in the univariate models were evaluated using a multivariate Cox regression model. A \( p \) value less than 0.05 was considered statistically significant.

### Results

#### MiR-9 expression in serum of patients with NSCLC

The expression levels of serum miR-9 in 161 NSCLC patients and 161 controls were examined using qRT-PCR. Using U6 RNA as normalization control, the relative abundances of miR-9 was significantly upregulated in sera of cases compared with those of healthy controls (\( P<0.001 \), Mann Whitney test) (Figure 1A).

#### Diagnostic value of circulating miR-9

ROC curve analysis showed that miR-9 was useful marker for discriminating cases from

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**Figure 1.** Serum miR-9 levels in normal controls and NSCLC patients (n=161). A. The relative expression level of miR-9 was normalized to U6 RNA. The line represents the median value. Statistically significant difference was determined using Mann-Whitney tests. The result revealed a higher level of miR-9 in NSCLC patients (\( P<0.001 \)). B. Receiver operating characteristics (ROC) curve analysis for the diagnostic value of miR-9. The AUC (the areas under the ROC curve) was 0.878 (Ct: 0.743-0.913).
Serum miR-9 in resected non-small cell lung cancer patients

The 161 patients with NSCLC were divided into high and low miR-9 expression groups (92 and 69 patients, respectively) using the median miR-9 value as the cutoff point. As shown in Table 1, miR-9 expression was significantly increased in the groups of LN metastasis ($P=0.0026$), II+III stage ($P=0.0038$) and recurrence ($P=0.0001$). No relation was found between miR-9 expression and other characteristics of patients with NSCLC (Table 1). These results suggest that elevated miR-9 expression may be inversely correlated with NSCLC progression.

Overall survival analysis of NSCLC patients

The overall 5-year survival rate for resected NSCLC was 56.5%. Of the 161 patients, 83 (51.5%) were alive and 78 (48.5%) died during their follow-up. However, for 12 of those patients who died, it was difficult to determine whether the death was cancer-related due to the unavailability of a patient registry.

Univariate analysis of the clinicopathological factors that were relevant to patient survival showed that the following factors were statistically significant for the overall survival of patients: a more advanced p-stage ($P=0.002$), an advanced regional lymph node metastasis status ($P<0.001$), Lymphatic permeation ($P<0.001$), poor tumor differentiation ($P=0.046$), Pleural involvement ($P=0.012$), Vascular invasion ($P=0.003$) and high miR-9 expression ($P=0.003$) (Table 2). Using univariate analysis, the low miR-9 expression patients had a significantly higher OS rate than did the low miR-9 expression patients ($P=0.0016$, Figure 2).

In multivariate analysis, significant predictors were lymph node metastasis status (hazard
Serum miR-9 in resected non-small cell lung cancer patients

Table 2. Uni- and Multivariable analyses of the effect of miR-9 expression on survival

<table>
<thead>
<tr>
<th>Factors</th>
<th>Univariable analysis</th>
<th>Multivariable analysis</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Hazards</td>
<td>95% CI</td>
</tr>
<tr>
<td>MiR-9 expression High/Low</td>
<td>3.17</td>
<td>1.062-5.16</td>
</tr>
<tr>
<td>Age (Y) ≤65 vs &gt;65</td>
<td>1.14</td>
<td>0.69-1.93</td>
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<td>Gender Male vs Female</td>
<td>0.78</td>
<td>0.56-1.14</td>
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<tr>
<td>Tumour size (mm) ≤30 vs &gt;30</td>
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<td>0.94-2.16</td>
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<tr>
<td>Lymph node metastasis Positive vs Negative</td>
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<td>3.86-10.4</td>
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<tr>
<td>Disease stage (p-stage) I vs II+III</td>
<td>3.24</td>
<td>2.17-8.45</td>
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<tr>
<td>Lymphatic permeation Positive vs Negative</td>
<td>3.14</td>
<td>2.26-7.93</td>
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<tr>
<td>Vascular invasion Positive vs Negative</td>
<td>2.06</td>
<td>1.48-7.13</td>
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<tr>
<td>Pleural involvement Positive vs Negative</td>
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<tr>
<td>Tumor differentiation Well/Moderately vs poorly</td>
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<td>0.67-1.85</td>
</tr>
<tr>
<td>Smoking habit Current vs former+never</td>
<td>0.54</td>
<td>0.37-1.03</td>
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</table>

Discussion

Accumulated evidence has indicated that aberrant expression of miRNAs contributes to the pathogenesis of most human malignancies [16]. A number of miRNAs functions as onco-genes or tumor suppressors in the majority of cancers. miR-9 has dual functions: either as tumor suppressor or oncogene dependent on the specific cancer tissue type. It has been reported that miR-9 was up-regulated in a number of solid tumors, including NSCLC, and suggest a prognostic value of miR-9 in NSCLC [15].

Studies have found that elevated miRNAs malignancies could release to the serum, making them the ideal candidates for use as biomarkers for disease incidence and progression [17]. In patients of myeloma bone disease, high levels of serum miR-214 had a dismal survival with significantly shortened progression free survival (PFS) and overall survival (OS) [18]. In patients of melanoma, high serum circulating microRNA expression could distinguish the patients with metastasis from those without it [19]. Kur et al. has reported that high levels of serum miR-203 associate with poor survival and metastasis in patients with colorectal cancer [20]. Statistical analysis showed that patients of NSCLC with low serum miR-147 had much worse overall survival, and low serum miR-147 expression level was an independent prognostic factor for poor prognosis for NSCLC [21]. Li et al. has reported that higher serum miR-210 levels were significantly correlated with the clinical stage and the presence of regional lymph node metastasis in patients with NSCLC [22].

In the present study, we have examined the role of serum miR-9 as a prognostic factor in resected non-small cell lung cancer patients. In our series of patients, serum miR-9 expression was significantly upregulated in patients with tumor samples compared to the controls. Moreover, serum miR-9 was significantly associated with...
poorer differentiation, p-TNM stage, recurrence and lymph node metastasis of NSCLCs. In this study, although it was not statistically significant, there was a tendency for higher serum miR-9 in tumors with worse status for pleural involvement and vascular invasion. These results suggest that serum miR-9 alone is not only a useful diagnostic marker of NSCLCs, but also is a useful marker for more aggressive NSCLCs.

Further, serum miR-9 was significantly associated with poorer survival. High serum miR-9 expression groups has low 5-year overall survival rate than the high serum miR-9 expression groups. Multivariable analysis confirmed that high serum miR-9 expression increased the hazard of death after adjusting for other clinicopathological factors (HR 4.08, 1.452-6.36, P=0.002). Our results suggest that patients with NSCLC who have lower serum miR-9 expression are especially likely to have good outcomes after complete lung resection.

**Summary**

These findings suggest that serum miR-9 expression may be involved in more aggressive behavior of NSCLC.

High serum miR-9 expression seems to be an independent and significant predictor of poorer survival of resected non-small cell lung cancer patients. Further investigation in a prospective study is warranted to validate these findings and to examine potential miR-9-based therapeutic approaches.

**Disclosure of conflict of interest**

None.

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**References**


Serum miR-9 in resected non-small cell lung cancer patients


