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Original Article

Clinical significance of miRNA21 in exhaled breath condensate of non-small-cell lung cancer

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Abstract: Background: Lung cancer has the highest morbidity and mortality rates among all malignant tumours; as such, tumour markers associated with the development and progression of lung cancer must be investigated. This study aims to investigate the clinical significance of miRNA21 in exhaled breath condensate (EBC) of patients with non-small cell lung cancer (NSCLC). Methods: EBC samples were collected from 30 patients with NSCLC and 30 healthy volunteers by using an EBC collector. The expression levels of EBC and serum miRNA21 were detected. Results: The miRNA21 levels in the serum and EBC in the NSCLC group were higher than those in the healthy groups. The miRNA21 levels in the serum and EBC in stage III NSCLC group were considerably higher than those in stages I and II. In addition, EBC and serum miRNA-21 levels in patients with NSCLC and lymph node metastasis were significantly higher ($P < 0.01$) than those without lymph node metastasis. Analysis of receiver operating characteristic curve showed that the area under the curve (AUC) for EBC was 0.851, with 76.7% sensitivity and 83.3% specificity. Moreover, the AUC for serum was 0.858, with 86.7% sensitivity and 73.3% specificity. Conclusion: Detection of miRNA21 in EBC, instead in serum, can improve the diagnosis, progression monitoring and prognosis of NSCLC.

Keywords: Non-small cell lung cancer, exhaled breath condensate, serum, miRNA21, tumour marker

Introduction

Lung cancer is one of the most common malignant tumours with high morbidity and mortality rates [1]. Non-small cell lung cancer (NSCLC) accounts for almost 85% of all lung cancer cases. The 5-year survival rate of patients with NSCLC is less than 20% [2]. Hence, tumour markers associated with the development and progression of lung cancer must be investigated.

Exhaled breath condensate (EBC), which is directly obtained from the respiratory tract and lungs, contains liquids secreted by mucous membranes as well as mixtures of volatile molecules [3], which may provide information on cancers in the airways. EBC features simple operation, good repeatability, non-invasive properties and wide application range. Thus, numerous studies have focused on tumour markers in EBC of patients with lung cancer [4-6].

MicroRNAs (miRNAs) are small, single-stranded and non-encoding RNAs that play important roles in the regulation of gene expression and in the occurrence and development of tumours. Previous studies showed the presence of miRNA expression patterns in lung cancer tissues and serum as well as in non-invasively collected body fluids, such as EBC [7]; hence, EBC miRNA exhibits potential as a novel non-invasive molecular marker for NSCLC. miRNA-21 is a cancer gene related to the development of NSCLC and participates in the regulation of proliferation, apoptosis, invasion and metastasis of lung cancer cells [8]. In this study, we assessed the feasibility of using EBC miRNA-21 as a non-invasive tool to diagnose and predict prognosis for NSCLC.

Materials and methods

Research object

We selected 30 patients pathologically diagnosed with NSCLC from the Second Affiliated

Table 1. Characteristics of the study subjects

	NSCLC	Healthy	*P value*
N	30	30	
Age (years)	64.03 ± 8.04	63.70 ± 8.17	0.847
Male/female	19/11	17/13	0.598
Smoking (yes/no)	15/15	16/14	0.796

Hospital of Nantong University from May 2014 to August 2015. Thirty healthy volunteers were also enrolled in this study as controls. Staging was based on the TNM classification system of the Union for International Cancer Control 2009 [9]. No statistical differences in age and gender were found between the two groups, and the general characteristics of the participants are summarised in **Table 1**. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Nantong University. All participants signed the informed consent form.

Specimen collection

We collected EBC by using an EBC collector (HAAK EK20 EcoScreen). The subjects were asked to clean their mouths while the collector was pre-cooled for about 20 min. The subjects were then asked to breathe normally by biting mouthparts and while wearing nose clips for 20 min [10]. The exhaled air turned into a snow-like substance. Subsequently, 1-3 ml of melted EBC was collected. Fasting venous blood samples were also collected from all subjects and then centrifuged for serum extraction. All samples were stored at -70°C until analysis.

Experimental method

Reagents and instruments: The instruments used included LightCycler 480 Real-time PCR amplification (Roche); miRNeasy Serum/Plasma Kit (Qiagen); Taqman MicroRNA Reverse Transcription Kit (Life Technology); Taqman MicroRNA Assay, which includes the primer of cel-miRNA39 and hsa-miRNA21 (Life Technology); and Taqman Universal Mix No AmpErase® UNG (Life Technology).

Total RNA extraction: The miRNeasy Serum/Plasma Kit was used in accordance with the manufacturer's instructions.

Reverse transcription reaction system with miRNA39/microRNA21: The reverse transcription reaction system (15 μl) was prepared with the following: 0.15 μl of 100 mM dNTPs (with dTTP), 1 μl of MultiScribe™ Reverse Transcriptase (50 U/μl), 1.5 μl of 10× Reverse Transcription Buffer, 0.19 μl of RNase inhibitor (20 U/μl) and 4.16 μl of nuclease-free water. The system was mixed gently and centrifuged to bring the solution to the bottom of the tube. Finally, 3 μl of 5× RT primer and 5 μl of the total RNA were transferred into the corresponding RT reaction tube. Reverse transcription conditions included 16°C for 30 min, 42°C for 30 min, 85°C for 5 min and finally maintained at 4°C.

Real-time PCR reaction system of MicroRNAs: Real-time PCR reaction system (20 μl) required the following equipment and conditions: 1 μl of TaqMan® MicroRNA Assay (20×), 1.33 μl of product from RT reaction, 10 μl of TaqMan® Universal PCR Master Mix II (2×) and 7.67 l of nuclease-free water. Real-time PCR conditions included 50°C for 2 min and 95°C for 10 min. Denaturation required 50 cycles of 95°C for 15 s and 60°C for 60 s.

Data analysis: The expression levels of miRNA-21 were normalised to that of miR-39 and calculated using $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = \Delta Ct_{(CtmiRNA\text{-}21\text{-}CtmiR\text{-}39)NSCLC} - \Delta Ct_{(CtmiRNA\text{-}21\text{-}CtmiR\text{-}39)Mean\ normal}$.

Statistical analysis

SPSS21.0 statistical software was used for statistical analysis, and graphs were generated using GraphPad Prism5.0. All data were presented for the normal distribution test by Kolmogorov-Smirnov Z test. Mann-Whitney's U test was used to compare data between the two groups because variables were not normally distributed, and Kruskal-Wallis H (K) test was used for multi-group numerical comparison. If data were consistent with normal distribution, the measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). T test was used to compare two samples, and chi-square test was used to compare count data. Specificity and sensitivity for diagnosis of lung cancer were analysed using receiver operating characteristic (ROC) curves. The reported parameters included area under the curve (AUC) with its 95% confidence interval, and the cut-off point at which the sum of sensitivity and specificity was the highest. Statistical significance was defined as $P < 0.05$.

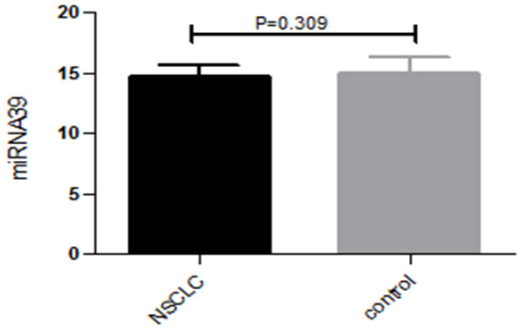

Figure 1. The mean Ct value of miR-39 of serum in NSCLC group and that in the healthy group.

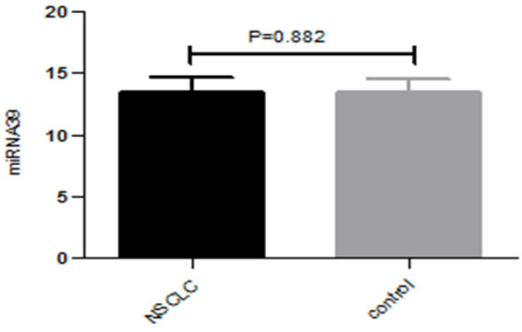

Figure 2. The mean Ct value of miR-39 of EBC in NSCLC group and that in the healthy group.

Results

External reference

In this study, miR-39 was selected as the external reference. The mean Ct values of the miR-39 in the serum in the NSCLC and healthy groups were 14.67 ± 0.93 and 14.97 ± 1.29, respectively; no statistical significance was observed between the two groups (**Figure 1**). Similarly, no statistical significance was found in the expression levels of miR-39 in EBC between NSCLC (13.46 ± 1.15) and healthy groups (13.50 ± 1.06) (**Figure 2**). Therefore, miR-39 served as external control.

Relative miRNA-21 expression levels in the EBC and serum between NSCLC and healthy groups

The relative expression level of serum miRNA-21 in the NSCLC group (6.02 ± 4.28) was higher than that in the healthy group ($P < 0.01$, **Figure 3**). The EBC miRNA-21 level in the NSCLC

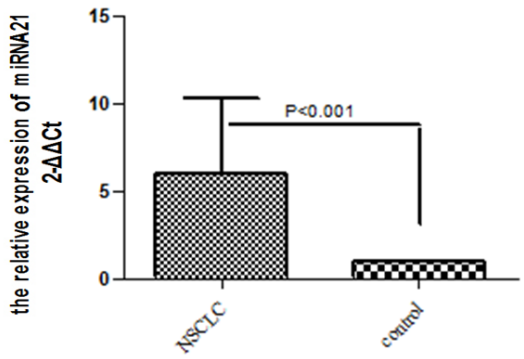

Figure 3. The level of serum miRNA-21 in NSCLC group and healthy group.

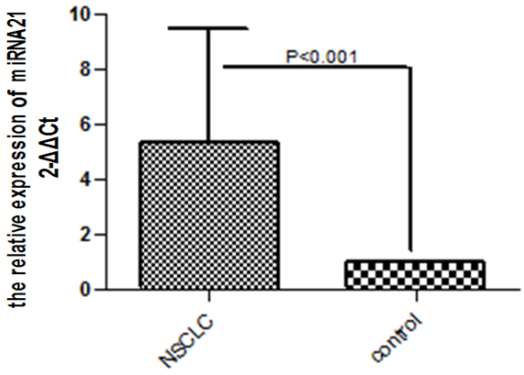

Figure 4. The level of EBC miRNA-21 in NSCLC group and healthy group.

group (5.36 ± 4.13) was higher than that in the healthy group ($P < 0.01$, **Figure 4**).

EBC and serum miRNA-21 levels in patients with NSCLC

The EBC miRNA-21 level in stage III NSCLC group (8.59 ± 4.33) was significantly higher than that in stages I + II (3.98 ± 3.25; $P < 0.01$). The EBC miRNA-21 level in patients with NSCLC and lymph node metastasis (7.00 ± 4.18) was significantly higher than that in patients without lymph node metastasis (3.93 ± 3.63, $P < 0.01$). For other clinical features, no significant difference was observed [sex ($P = 0.921$), age ($P = 0.406$), smoking condition ($P = 0.282$) and histological type ($P = 0.217$); **Figure 5**].

The serum miRNA-21 level in stage III NSCLC group (10.95 ± 3.54) was higher than that in stages I + II (3.91 ± 2.44, $P < 0.01$). The serum miRNA-21 level in patients with NSCLC and

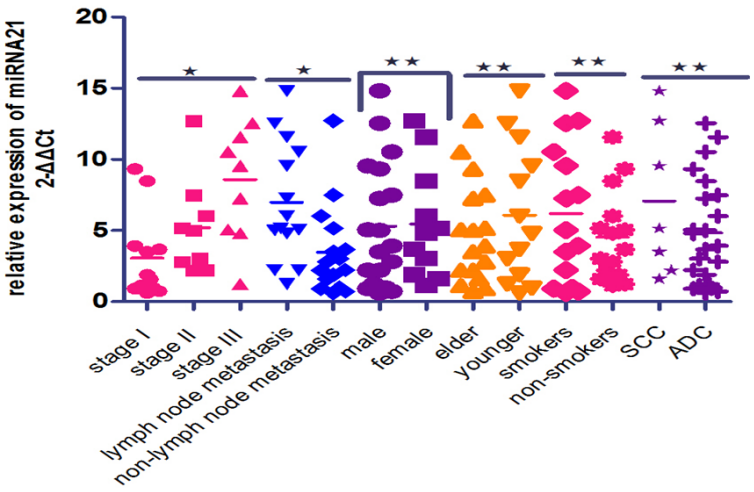

Figure 5. Relationship between EBC miRNA-21 level and patient clinical characteristics.

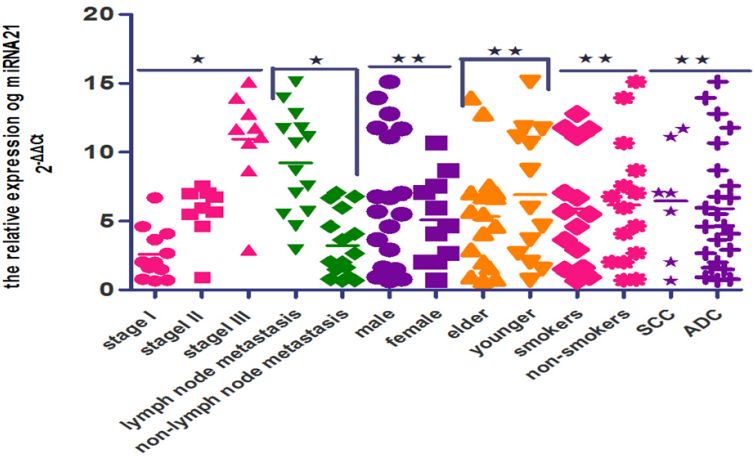

Figure 6. Relationship between serum miRNA-21 level and patient clinical characteristics.

lymph node metastasis (9.22 ± 3.74) was higher than that in patients without lymph node metastasis (3.23 ± 2.35, $P < 0.01$). For the other clinical features, no significant difference was observed [sex ($P = 0.375$), age ($P = 0.319$), smoking condition ($P = 0.855$) and histological type ($P = 0.591$; **Figure 6**].

Analysis of the sensitivity and specificity of miRNA-21 levels for diagnosis of lung cancer

Analysis of the ROC curve showed that the AUC for EBC was 0.851 (95%: 0.755-0.947). When the cut-off was 1.73, the sensitivity was 76.7% and the specificity was 83.3%. The AUC for serum was 0.858 (95%: 0.765-0.951). When the cut-off was 1.31, the sensitivity was 86.7% and the specificity was 73.3% (**Figure 7**).

The correlation between EBC and serum miRNA-21 levels

Figure 8 shows, miRNA-21 level in serum and EBC was linear positive correlation, correlation coefficient was 0.768, $P < 0.01$ (**Figure 7**).

Discussion

MicroRNAs are synthesised by long primary transcripts of the miRNA genes (pre-miRNAs) in the nucleus and cytoplasm with the help of various enzymes. MiRNAs are expressed mainly by base-pairing to the 3'-UTR of the target mRNA and play an important role in the regulation of post-transcriptional gene expression [11]. MiRNAs include cancer and tumour-suppressor genes; in particular, the miRNA-21 gene is located at the 10th intron of the coding region for TMEM49 at chromosome 17 [12]. At the cellular level, miRNAs play critical roles in differentiation, proliferation, apoptosis and metabolism [13, 14].

EBC is a promising source of biomarkers for lung disease [15]. EBC contains substances including DNA, proteins, peptides, cytokines, oxidation stress markers, volatile substances and non-volatile substances [16], which are involved in various respiratory diseases. EBC biomarkers are ideal mainly because they are safe, non-invasive, can be repeatedly measured and represent the airway milieu. Microsatellite change, P16 gene mutation and P53 gene mutation can be detected in the EBC of patients with lung cancer and has certain value for the diagnosis and prognosis of lung cancer [17-19]. From this study, There was a good correlation between EBC miRNA-21 and serum miRNA-21, the correlation coefficient was 0.7683. So we can detect miRNA-21 levels for diagnosis in NSCLC patients by collecting EBC.

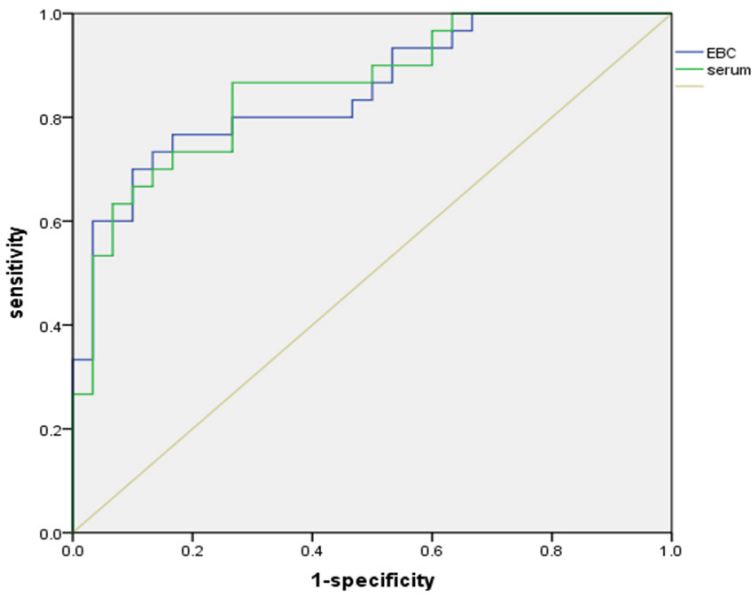

Figure 7. The ROC curves of miRNA-21 in the diagnosis of lung cancer.

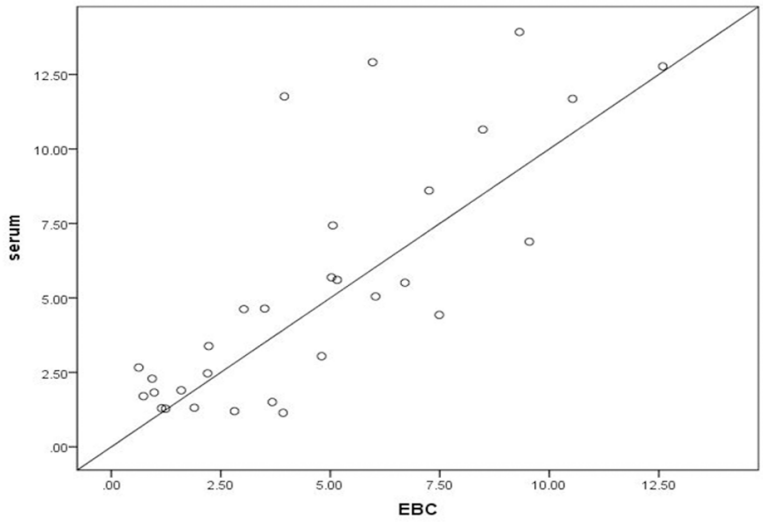

Figure 8. Correlation scatter diagram of EBC and serum levels of miRNA-21.

In this study, the miRNA-21 levels in EBC and serum in the NSCLC group were higher than those in the healthy group, which is consistent with the findings of Mozzoni [7]. Analysis of the ROC curve showed that the AUC for EBC was 0.851 (95%: 0.755-0.947), the sensitivity was 76.7% and the specificity was 83.3%. The AUC for serum was 0.858 (95%: 0.765-0.951), the sensitivity was 86.7% and the specificity was 73.3%. Thus, the miRNA-21 in EBC can be used as a tumour biomarker for diagnosis of lung cancer.

The EBC and serum miRNA-21 levels in stage III of the NSCLC group were higher than those in stages I and II. Moreover, the EBC and serum miRNA-21 levels in patients with NSCLC and lymph node metastasis were higher than those in patients without lymph node metastasis ($P < 0.01$). This finding indicated that the progress of the disease could be evaluated according to the miRNA-21 levels in the EBC of patients with NSCLC. We believe that high miRNA-21 levels in the EBC of patients with NSCLC indicate later tumour stage, wide range of infiltration and distant metastasis.

Previous studies showed that miRNA-21 expression levels are higher in non-smoking patients with NSCLC than those in patients who smoke [20]. However, no significant difference was observed between the serum and EBC miR21 levels in smokers and non-smokers in our study. Basing on these findings, we proposed that miRNA-21 is not related to smoking. No significant difference was observed for other clinical features as well, such as sex, age and histological type. Nevertheless, some problems should be considered when explaining the results of this study. Because of the small sample size, larger cohort studies are necessary. Moreover, the molecular mechanism of miRNA-21 in NSCLC development and progression remains unclear, confirming the need for further research.

In conclusion, we confirmed that miRNA-21 detection in the EBC in patients with NSCLC is feasible. miRNA-21 levels in EBC proved to be important in the diagnosis, progression monitoring and prognosis of NSCLC. Research on EBC will expand understanding of pathophysio-

logical mechanisms underlying lung cancer. Further studies are necessary in this field.

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Disclosure of conflict of interest

None.

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