Clinical Value of Serum and Exhaled Breath Condensate miR-186 and IL-1β Levels in Non-Small Cell Lung Cancer

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Abstract
Objective: Our study aimed to investigate the expression level and clinical significance of serum and exhaled breath condensate miR-186 and IL-1β in non-small cell lung cancer patients. Methods: The serum and exhaled breath condensate specimens of 62 non-small cell lung cancer patients and 60 healthy controls were collected to detect miR-186 expression levels by real-time fluorescent quantitative PCR. Enzyme linked immunosorbent assay was applied to examine IL-1β concentration. Statistical analyses were used to evaluate the correlation between miR-186 and IL-1β in serum and clinicopathological features, traditional serum tumor markers, and inflammatory markers. The diagnostic efficacy of miR-186 and IL-1β for non-small cell lung cancer was evaluated by receiver operating characteristic curve analysis. The correlation between miR-186 and IL-1β was determined. Results: The relative expression level of miR-186 was greatly reduced in the serum and EBC of patients with non-small cell lung cancer, and the miR-186 expression level was reduced in different TNM stages of non-small cell lung cancer, from the early to later stages. The IL-1β concentration in serum and exhaled breath condensate of patients with non-small cell lung cancer was increased. Serum miR-186 and IL-1β levels were closely related to lymph node metastasis, and the low expression of serum miR-186 and the high concentration of IL-1β were associated with higher serum carcinoembryonic antigen, C-reactive protein, and erythrocyte sedimentation rate levels. ROC curve analysis showed that exhaled breath condensate miR-186 had higher area under the curve than serum miR-186, and the combined detection showed higher diagnostic efficacy than the separate detection. In addition, the combined detection of IL-1β and miR-186 has a larger AUC than the separate detection of both. The correlation between serum miR-186 and IL-1β was negative. Conclusion: miR-186 and IL-1β are expected to be potential diagnostic biomarkers for non-small cell lung cancer.

Keywords
miR-186, interleukin-1β, non-small cell lung cancer, diagnosis, exhaled breath condensate

Abbreviations
CEA, carcinoembryonic antigen; CRP, C-reactive protein; EBC, exhaled breath condensate; ELISA, enzyme linked immunosorbent assay; ESR, erythrocyte sedimentation rate; IL-1β, interleukin-1β; NSCLC, non-small cell lung cancer; PCT, procalcitonin; qRT-PCR, real-time fluorescent quantitative PCR; ROC, receiver operating characteristic

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Introduction

According to global cancer statistics in 2018, Lung cancer is ranked as the most common tumor in males and females and the leading cause of cancer death (18.4% of total cancer deaths) worldwide.¹ Non-small cell lung cancer (NSCLC) is the most common subtype of lung cancer, accounting for nearly 80% of lung cancer. Despite advances in NSCLC prognosis with new pharmaceutical products and technological improvements, finding convenient and effective biomarkers remains a significant challenge.²³ Fortunately, an increasing number of new biomarkers have been discovered in traditional matrices, such as serum, and new non-invasively collected body fluids. Exhaled breath condensate (EBC) is obtained by introducing exhaled breath into a cryogenic system and condensing into a liquid. Various proteins, cell debris, DNA, microRNAs, and microbiota (bacteria and viruses) are successively detected in EBC.⁴ Studies have shown that miRNA expression is deregulated in EBC from patients with NSCLC.

MicroRNAs, a class of 18-25 nt noncoding small RNAs, play a key role in regulating cancer development through various biological processes (angiogenesis, cell proliferation, migration, invasion, differentiation, and apoptosis).⁵⁷ Among tumor-associated miRNAs, miR-186 located in the chromosome band 1q31.1 has attracted considerable attention. miR-186 serves a dual function in a variety of cancers. A reduction in miR-186 was investigated in stomach cancer, NSCLC, liver cancer, oral squamous cell carcinoma, pancreatic cancer and breast cancer. However, it plays the opposite role in endometrial cancer, lymphoma, and peritoneal cancer.⁸¹³ miR-186 plays an essential role in the genetics of human NSCLC. A downregulation in miR-186 was found in NSCLC tissues and cells. By targeting different genes, miR-186 can inhibit the cell cycle progression, proliferation, migration, and invasion of NSCLC.¹⁴⁻²⁰ However, no study has established the expression of NSCLC miR-186 in serum and EBC. Moreover, the mechanism of miR-186 in NSCLC is not fully understood.

Chronic inflammation is a key factor in tumorigenesis, and the critical role of inflammation in cancer is well accepted worldwide today.²¹²² Recently, increasing evidence shows that miRNAs are a new class of inflammation-related molecules involved in multiple stages of tumor development, such as initiation, tumor progression, invasion, and metastasis. Tumor-associated miRNAs can regulate a range of inflammatory factors. Studies have shown that miRNA186 can inhibit Interleukin-1β (IL-1β) expression by regulating NLRP3 inflammasome, suggesting that miRNA186 may participate in tumorigenesis by regulating the tumor microenvironment.²³ IL-1β plays a pro-inflammatory cytokine role involved in inflammatory responses, both acute and chronic. Multiple experiments in vitro and vivo have shown that IL-1β has a tumor-promoting effect and can promote lung cancer metastasis through adhesion, invasion, and tumor angiogenesis of lung cancer cells.²⁴

In the present experiment, we examined the levels of miR-186 and IL-1β in serum and EBC and explored the clinical significance of miR-186 and IL-1β levels in NSCLC. The correlation between serum miR-186 and IL-1β was performed.

Materials and Methods

Patients

A total of 62 NSCLC patients who were treated in the Department of Thoracic Surgery and Respiratory Medicine of Affiliated Hospital 2 of Nantong University between September 2017 and September 2019 were enrolled in our study. All patients were eligible according to the following inclusion criteria: (a) NSCLC was diagnosed by pathological examination; (b) the subjects did not undergo chemotherapy, immunotherapy, or surgery before collection; and (c) patients had no lung inflammatory diseases. At the same time, a total of 60 healthy individuals were selected as the control group, and the control group had no history of malignant tumors and no systemic organ diseases. Tumor staging was assessed using the TNM staging of lung cancer published by the 2017 International Union Against Cancer. The clinical characteristics of the study participants are expressed in Table 1. The collection of all samples was approved by the Medical Ethics Committee of the Affiliated Hospital 2 of Nantong University (approval no. 2019KY139), and patients’ written informed consent was obtained.

Specimen Collection

The blood and EBC specimens of the study participants were collected on the first day of admission. Serum specimen: 3 ml of fasting blood was collected and placed in a coagulation tube. The supernatant was collected after centrifugation at 3500 rpm for 5 min. EBC specimens: EBC specimens were collected using an EcoScreen condenser manufactured by Eric Jaeger Company. The subjects were asked to breathe into the collecting system for 15 min at −20°C.²⁵ All specimens were stored in a −70°C low-temperature refrigerator for the following inspection.

RNA Extraction and Real-Time Fluorescent Quantitative PCR (qRT-PCR)

Total RNA was extracted from serum and EBC using the miRNA extraction kit (Tiangen DP503, China) in accordance with the manufacturer’s protocol. Then, total RNA containing miRNA was reverse transcribed to cDNA by using the miRNA cDNA first strand synthesis kit (Tiangen KR211, China). The qRT-PCR was performed on a StepOnePlus real-time PCR instrument by using the SYBR Green I fluorescent dye method. miR-39 was selected as the external reference gene because of its stable presence in the serum according to previous studies.²⁶ Three replicate wells were provided for each sample. The relative expression of miR-186 was calculated by using the 2⁻∆∆Ct method, where ∆∆Ct = NSCLC (Ct target gene — Ct reference gene) — healthy controls (Ct target gene — Ct reference gene).
Table 1. Characteristics of the Study Participants.

|                     | NSCLC (n = 62) | Control (n = 60) | P value |
|---------------------|----------------|-----------------|---------|
| Age (years)         | 62.05 ± 9.24   | 59.48 ± 7.56    | >0.05   |
| Gender              |                |                 |         |
| Male                | 32             | 29              | >0.05   |
| Female              | 30             | 31              |         |
| Smoking             |                |                 |         |
| Yes                 | 27             | 35              | >0.05   |
| No                  | 35             | 25              |         |
| BMI                 |                |                 |         |
| <25                 | 48             | 45              | >0.05   |
| ≥25                 | 14             | 15              |         |
| Histological type   |                |                 |         |
| Adenocarcinoma      | 45             | -               |         |
| Squamous cell carcinoma | 17       | -               |         |
| Lymph node metastasis|              |                 |         |
| Yes                 | 28             | -               |         |
| No                  | 34             | -               |         |
| TNM stage           |                |                 |         |
| I-II                | 33             | -               |         |
| III-IV              | 29             | -               |         |

Abbreviations: BMI: body mass index.
p-values were determined using Student’s t-test, or the chi-square test as appropriate.

Enzyme-Linked Immunosorbent Assay (ELISA)

IL-1β concentration was determined using the Proteintech ELISA kit (catalog number: KE00021, USA), and the procedure was carried out in strict accordance with the kit’s instructions.

Statistical Analysis

Statistical analysis was performed by IBM SPSS Statistics, and graphs were generated using GraphPad Prism 7.0. The data distribution of each group was assessed by the Kolmogorov–Smirnov test. For the normal distribution data, 2 group comparisons were conducted using Student’s t-test when the variance is equal. When the variance is not uniform, t-test (Satterthwaite method) was used, and continuous variables were summarized as the mean ± standard deviation. Non-normally distributed data were analyzed using the Mann–Whitney U, and Continuous variables were presented as median (interquartile range). The chi-square (χ²) test was used to compare the distribution of categorical variables between groups. Pearson correlation was used to determine the association between variables. The diagnostic value of miR-186 and IL-1β to differentiate NSCLC patients and controls was assessed using receiver operating characteristic (ROC) curve analysis. P < 0.05 was considered statistically significant.

Results

Decreased Expression of Serum and EBC miRNA186 in NSCLC

To explore the clinical value of miR-186 in NSCLC, we first measured the relative expression of miR-186 in serum and EBC of 62 patients with NSCLC and 60 age- and sex-matched healthy controls by qRT-PCR. As shown in Figure 1, the relative expression of miR-186 was significantly decreased in patients with NSCLC than in controls in serum and EBC (P < 0.05). In addition, a low expression of miR-186 was observed in patients with stage I–II NSCLC when compared with the stage III–IV group (P < 0.05). The difference in miR-186 expression between patients with I–II NSCLC and healthy controls was significant (P < 0.05).

Correlation Analysis of miR-186 in Serum and EBC

Then, the correlation between the expression levels of miR-186 in serum and EBC was analyzed by Pearson correlation. In Figure 2, the level of miR-186 in serum is positively related to that in EBC (r = 0.480, P < 0.05).

Increased IL-1β Concentration in Serum and EBC of NSCLC

IL-1β concentrations in the serum and EBC of patients with NSCLC and healthy controls were detected using ELISA. The serum IL-1β level in patients with NSCLC was significantly higher than that in healthy controls. Statistics showed a progressive upregulation of IL-1β with increasing NSCLC severity, represented by TNM staging (P < 0.05, Figures 3A and B). Similarly, Figure 3C and D showed higher concentration of IL-1β in EBC from NSCLC patients when compared with healthy controls. IL-1β concentration was related to TNM staging (P < 0.05).

Association Between miRNA186 and IL-1β and Clinical Features in Patients With NSCLC

The correlation between the expression level of miR-186 and IL-1β and clinicopathological characteristics of NSCLC was investigated (Tables 2 and 3). The serum levels of miR-186 in the lymph node metastasis group were significantly lower than those in the non-metastasis group, and higher concentration of IL-1β was observed in lymph node metastasis group (P < 0.05, Table 2). The serum levels of miR-186 and IL-1β were not significantly associated with age, sex, and smoking history (P > 0.05, Table 2). The squamous cell carcinoma group had a higher serum IL-1β level compared with the adenocarcinoma group (P < 0.05, Table 2). However, there was no significant association between serum miR-186 level and histological type of NSCLC (P > 0.05, Table 2).

Table 3 shows that the expression level of miR-186 in EBC was lower in adenocarcinoma than in squamous cell carcinoma (P < 0.05). The correlation between miR-186 and IL-1β expression level in EBC and other clinicopathological factors, including age, sex, smoking history, and lymph node metastasis, was found to be non-significant (P > 0.05).
Correlation Between Serum miRNA186 and IL-1β Level and Traditional Tumor Markers and Inflammatory Markers

The correlation between serum levels of miR-186, IL-1β and traditional tumor markers in NSCLC patients was explored. The expression of serum miR-186 was significantly lower in NSCLC with higher carcinoembryonic antigen (CEA) level ($P < 0.05$, Table 4), and no statistically significant associations were found between miR-186 expression and other abovementioned tumor markers, such as cytokeratin-19-fragment, squamous cell carcinoma associated antigen, and neuron-specific enolase ($P > 0.05$, Table 4). The correlation between IL-1β levels and traditional tumor markers were found to be non-significant ($P > 0.05$, Table 4).

Recently, increasing evidence exhibits that miRNAs are a new class of inflammation-related molecules. Thus, we analyzed the association between the serum level of miR-186, IL-1β and procalcitonin (PCT), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) in patients with NSCLC. The results are shown in Table 4. The low expression level of serum miR-186 and the high concentration of IL-1β were associated with high serum CRP and ESR levels ($P < 0.05$). The correlation between miR-186 and IL-1β level and PCT were found to be non-significant ($P > 0.05$).

Diagnosis Efficacy of miR-186 and IL-1β Expression in NSCLC

The ROC curve was drawn in accordance with the expression levels of miR-186 and IL-1β in serum and EBC of NSCLC.
patients and healthy controls. The Youden index (sensitivity + specificity − 1) was used to determine the Cutoff value. ROC curve analysis revealed that serum miR-186 could distinguish NSCLC patients from controls with area under the curve (AUC) value of 0.801, and the sensitivity and specificity were 80.6% and 73.3%, respectively. Similarly, serum IL-1β could

### Table 2. Correlation Between miRNA186, IL-1β Level in Serum and Clinical Characteristics in NSCLC.

| Characteristics          | N   | miR-186  | IL-1β (pg/ml) |
|--------------------------|-----|----------|---------------|
| Age (years)              |     |          |               |
| ≤ 60                     | 25  | 0.637 ± 0.209 | 131.400 ± 27.720 |
| >60                      | 37  | 0.661 ± 0.332 | 138.700 ± 31.140 |
| P value                  |     | 0.727    | 0.348         |
| Gender                   |     |          |               |
| Male                     | 32  | 0.702 ± 0.311 | 134.100 ± 27.410 |
| Female                   | 30  | 0.598 ± 0.253 | 137.400 ± 35.530 |
| P value                  |     | 0.159    | 0.671         |
| Smoking                  |     |          |               |
| Yes                      | 27  | 0.500 (0.380-0.780) | 129.600 ± 27.990 |
| No                       | 35  | 0.670 (0.460-0.820) | 143.600 ± 30.720 |
| P value                  |     | 0.214    | 0.067         |
| Histological type        |     |          |               |
| Adenocarcinoma           | 45  | 0.653 ± 0.283 | 130.700 ± 28.250 |
| Squamous cell carcinoma  | 17  | 0.647 ± 0.306 | 148.900 ± 30.550 |
| P value                  |     | 0.940    | 0.031         |
| Lymph node metastasis    |     |          |               |
| Yes                      | 28  | 0.495 (0.393-0.693) | 149.600 ± 30.760 |
| No                       | 34  | 0.710 (0.515-0.863) | 124.200 ± 23.790 |
| P value                  |     | 0.013    | 0.001         |

### Table 3. Correlation Between miRNA186, IL-1β Level in EBC and Clinical Characteristics in NSCLC.

| Characteristics          | N   | miR-186  | IL-1β (pg/ml) |
|--------------------------|-----|----------|---------------|
| Age (years)              |     |          |               |
| ≤ 60                     | 25  | 0.464 ± 0.210 | 20.660 (19.820-25.880) |
| >60                      | 37  | 0.524 ± 0.285 | 21.070 (19.820-26.200) |
| P value                  |     | 0.368    | 0.929         |
| Gender                   |     |          |               |
| Male                     | 32  | 0.459 ± 0.213 | 21.600 (20.080-25.880) |
| Female                   | 30  | 0.538 ± 0.291 | 20.660 (19.350-25.930) |
| P value                  |     | 0.235    | 0.188         |
| Smoking                  |     |          |               |
| Yes                      | 27  | 0.546 ± 0.313 | 21.070 (20.030-26.510) |
| No                       | 35  | 0.464 ± 0.201 | 20.660 (19.610-23.370) |
| P value                  |     | 0.243    | 0.158         |
| Histological type        |     |          |               |
| Adenocarcinoma           | 45  | 0.459 ± 0.242 | 21.070 (19.510-25.880) |
| Squamous cell carcinoma  | 17  | 0.607 ± 0.272 | 21.070 (20.240-26.410) |
| P value                  |     | 0.042    | 0.524         |
| Lymph node metastasis    |     |          |               |
| Yes                      | 28  | 0.538 ± 0.265 | 21.600 (20.240-26.350) |
| No                       | 34  | 0.468 ± 0.250 | 20.870 (19.400-25.410) |
| P value                  |     | 0.288    | 0.340         |

**Figure 3.** Concentrations of IL-1β in serum (A and B) and EBC (C and D) in controls and NSCLC patients.
miR-186 expression among patients with stage III-IV NSCLC was remarkably lower than that of patients with stage I-II NSCLC. These results agreed with numerous studies that reported reduced miR-186 expression in NSCLC tissues and cells. It meant that miR-186 plays a tumor suppressor gene role in NSCLC and is associated with the extent of disease progression. Our work reported for the first time that miR-186 is down-expressed in serum and EBC samples collected from NSCLC patients.

By binding the 3'-untranslated region (3'-UTR) of cyclin-dependent kinase 1 (CDK1), miR-186 reduces CDK1 expression in NSCLC cells, at the same time, it inhibits lung cancer cells growth.27 Cell division cycle 42 (cdc42) serves a key role in NSCLC and is associated with the extent of disease progression. Our work reported for the first time that miR-186 is down-expressed in serum and EBC samples collected from NSCLC patients.

The pathogenesis of lung cancer is complex, and immune inflammatory reactions are involved. In addition to participating in the body’s inflammatory response and its own anti-infective defense, IL-1β participates in the occurrence and metastasis of some malignant tumors. IL-1β is involved in NSCLC progression and may be involved in immunosuppression and in promoting NSCLC development.29 A study proposed the possibility that anti-inflammatory therapy by targeting IL-1β may reduce the prevalence of lung cancer among patients who have higher CRP.30 Recently, a phase 3 clinical trial has reported an interesting result, anakinumab, an inhibitory antibody targeting IL-1β, could significantly reduce the incidence and mortality of lung cancer.31

### Table 4. Correlation Between Serum miRNA186, IL-1β Level and Traditional Tumor Biomarker, Inflammatory Factor.

| Factors        | N  | miRNA186   | IL-1β (pg/ml) |
|---------------|----|------------|---------------|
| CEA (ng/ml)   |    |            |               |
| ≤2.90         | 32 | 0.72 ± 0.30| 128.70 ± 30.86|
| >2.90         | 30 | 0.58 ± 0.25| 143.20 ± 27.14|
| P value       |    | 0.048      | 0.055         |
| CA211 (ng/ml) |    |            |               |
| ≤3.27         | 31 | 0.67 (0.49-0.86)| 129.90 ± 28.27|
| >3.27         | 31 | 0.50 (0.40-0.78)| 141.50 ± 30.60|
| P value       |    | 0.205      | 0.128         |
| SCC (ng/ml)   |    |            |               |
| ≤0.77         | 35 | 0.62 (0.40-0.80)| 131.30 ± 26.34|
| >0.77         | 27 | 0.62 (0.48-0.82)| 141.40 ± 33.41|
| P value       |    | 0.670      | 0.191         |
| NSE (ng/ml)   |    |            |               |
| ≤16.38        | 32 | 0.71 ± 0.33| 131.60 ± 34.48|
| >16.38        | 30 | 0.59 ± 0.22| 140.10 ± 23.61|
| P value       |    | 0.102      | 0.260         |
| PCT (ng/ml)   |    |            |               |
| ≤0.034        | 32 | 0.66 ± 0.31| 132.20 ± 28.54|
| >0.034        | 30 | 0.64 ± 0.26| 139.40 ± 31.12|
| P value       |    | 0.781      | 0.346         |
| ESR (mm/h)    |    |            |               |
| ≤10           | 34 | 0.72 ± 0.30| 126.60 ± 26.39|
| >10           | 28 | 0.57 ± 0.25| 146.80 ± 30.37|
| P value       |    | 0.031      | 0.007         |
| CRP (mg/L)    |    |            |               |
| ≤5.395        | 31 | 0.80 ± 0.35| 126.70 ± 26.44|
| >5.395        | 31 | 0.57 ± 0.28| 144.70 ± 30.65|
| P value       |    | 0.001      | 0.017         |

CEA: carcinoembryonic antigen; CA21: Cytokeratin-19-fragment; SCC: squamous cell carcinoma associated antigen; NSE: neuron-specific enolase; PCT: procalcitonin; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

Serve as a reliable biomarker for differentiating NSCLC patients from controls with 80.0% specificity, 83.9% sensitivity, and an AUC value of 0.829. Interestingly, the AUC of combined detection was 0.850, which was significantly better than that of miR-186 and IL-1β, and the sensitivity and specificity were 72.6% and 91.7%, respectively (Figure 4, Table 5).

ROC curve analysis was used to evaluate the capability of serum miRNA186, IL-1β to differentiate NSCLC patients and healthy controls.

### Correlation Between miR-186 and IL-1β in Serum

We evaluated the relationship between miR-186 and IL-1β in serum and found a negative correlation between IL-1β and miR-186 ($r = -0.640$, $P < 0.05$, Figure 6).

### Discussion

In our study, serum and EBC miR-186 expression levels can discriminate NSCLC patients from healthy controls. Moreover,
Similar to previous studies, our experiments indicated that IL-1β concentration in serum and EBC from patients with NSCLC is enhanced compared with that of healthy controls, and IL-1β level is related to disease stage. Meanwhile, serum IL-1β concentration is negatively correlated with miR-186, both of them were associated with NSCLC.

This study indicates that the expression of miR-186 level in serum is negatively associated with lymph node metastasis. We did not find any correlation between the expression level of serum miR-186 and age, sex, smoking history, and histological type. Yet, EBC miR-186 level was correlated with histological type but not lymph node metastasis. The reasons for these results may be as follows: first, the sample size of our experiment was very small; second, miR-186 and pathological types might not be related; finally, EBC samples were obtained from a location close to the lesion.

Few studies have compared miRNAs with traditional tumor markers. We studied the relationship between serum miR-186 level and traditional tumor biomarkers and inflammatory factors. Interestingly, we found that serum miR-186 expression was associated with CEA, ESR, and CRP.

The ROC curve was performed to estimate the diagnostic efficacy of miR-186, and the AUC of EBC miR-186 was higher than that in the serum. EBC miR-186 has better NSCLC diagnostic efficacy and higher specificity, indicating that EBC miR-186 is more effective than serum in NSCLC diagnosis. The sensitivity of the combined detection was 75.8%, the specificity was 83.3%, and the AUC of combined detection was significantly better than that in separate detection.

This work still has some shortcomings. First, the sample size is small and needs to be further expanded. Second, experimental results suggest the link between miR-186 and IL-1β, which may be related to the development of NSCLC. This finding provides new perspective for the promotion of tumorigenesis by inflammation. However, the specific mechanism of miR-186 and IL-1β on NSCLC remains unclear, and relevant experiments are needed.

In summary, the expression levels of miR-186 and IL-1β in serum and EBC contribute to the diagnosis and severity assessment of NSCLC. Thus, miR-186 and IL-1β are expected to be potential diagnostic markers for NSCLC.

**Authors’ Note**

Haiqin Xie and Jinliang Chen contributed equally and are joint first authors.

**Declaration of Conflicting Interests**

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