ABSTRACT: We have demonstrated that assessments of microRNA (miRNA) expressions in circulating peripheral blood mononucleated cell (PBMC) and sputum specimens, respectively, may help diagnose lung cancer. To assess the individual and combined analysis of the miRNAs across the different body fluids for lung cancer early detection, we analyse a panel of 3 sputum miRNAs (miRs-21, 31, and 210) and a panel of 2 PBMC miRNAs (miRs-19b-3p and 29b-3p) in a discovery cohort of 68 patients with lung cancer and 66 cancer-free smokers. We find that integrating 2 sputum miRNAs (miRs-31 and 210) and 1 PBMC miRNA (miR-19b-3p) has higher sensitivity (86.8%) and specificity (92.4%) compared with the individual panels. The synergistic value of the integrated panel of 3 biomarkers is confirmed in a validation cohort, independent of stage and histological type of lung cancer, and patients’ age, sex, and ethnicity. Integrating circulating immunological and sputum biomarkers could improve the early detection of lung cancer.

KEYWORDS: Lung cancer, early detection, biomarkers

Introduction

Lung cancer is the biggest cancer killer in both men and women. More than 85% lung cancers are non–small-cell lung cancers (NSCLCs), which mainly consist of adenocarcinoma (AC) and squamous cell carcinoma (SCC). Because the early detection of the disease can reduce the mortality, non-invasive biomarkers that can accurately and cost-effectively diagnose early-stage lung cancer are urgently needed.1

Cigarette smoking is the leading cause of NSCLC. Smoking can cause damages across the entire respiratory epithelial cells. The process is called field cancerization. The field cancerization could be observed in histologically normal–appearing lung epithelium, which may anchorize the same molecular aberrations as the ones in primary lung tumours.2,3 Examination of the exfoliated bronchial epithelia of airway in sputum can identify the lung tumour–related molecular alterations and hence provide a non-invasive and specific means for diagnosis of NSCLC.4–7 MicroRNAs (miRNAs) are small molecules and the aberrations contribute to carcinogenesis.8 We have found that aberrant miRNA expression detected in sputum biologically reflects those in primary lung cancer.9–14 We have also developed a 3-miRNA biomarker panel in sputum for diagnosis of NSCLC with 83% sensitivity and 88% specificity.15–17

Immune evasion occurs as a primary and important event in tumorigenesis.18–20 Peripheral blood mononuclear cells (PBMCs) are the front-line defence against tumorigenesis within immune system.21 Furthermore, miRNAs have important effects on the immune cells’ function, and the abnormalities contribute to the evasion proceedings of cancer cells in immunological surveillance.22–24 Therefore, miRNA changes of PBMCs would provide novel biomarkers for diagnosis of cancer.18,19,21,25 In addition, miRNA biomarkers of PBMCs may overwhelm the difficulties of cell-free (eg, plasma or serum)-based biomarkers because of the following reasons: (1) circulating PBMCs are a rich and readily accessible cell source in blood and thus produce large RNA quantity that has high quality for consistent analysis,19 and (2) miRNAs are purified from the PBMCs and thus do not have other sources of extracellular miRNAs (RBC-, protein-, exosome-, and vesicle-related miRNAs). We recently identified 2 PBMC miRNA (miRs-19b-3p and 29b-3p) as circulating immunologic biomarkers for NSCLC with 73% sensitivity and 83% specificity.26

Given that the miRNAs in sputum and peripheral PBMC have diverse functions in lung carcinogenesis through various cellular paths; here, we investigated whether integrating sputum and circulating immunological miRNA biomarkers would have a synergistic influence on the early detection of lung cancer.

Materials and Methods

Patients

The Institutional Review Boards at University of Maryland Baltimore and Veterans Affairs Maryland Health Care System reviewed and approved this study. Using the inclusion and/or exclusion criteria recommended by US Preventive Services Task Force for lung cancer screening in heavy smokers,27 we enrolled subjects between the ages of 55 to 80 years who had not less than a 30 pack-year smoking
history and were former smokers (quit within 15 years). Exclusion criteria included pregnancy, current pulmonary infection, surgery within 6 months, radiotherapy within 1 year, and life expectancy of <1 year. A total of 117 patients with NSCLC and 116 cancer-free smokers were recruited. No difference of age, race, sex, and smoking status was observed in the lung cancer cases vs cancer-free smokers. The cases and controls were randomly grouped into 2 cohorts: a discovery cohort and a validation cohort. The discovery cohort comprised 68 patients with lung cancer and 66 cancer-free smokers. The discovery cohort was used to simultaneously evaluate sputum miRNAs and PBMC miRNAs in the same set of clinical specimens and develop an integrated panel of miRNA biomarkers for NSCLC. The validation cohort included 49 patients with lung cancer and 50 cancer-free smokers and was used to confirm the synergistic effect of the integrated panel of miRNAs on the early detection of NSCLC. Detailed demographic and clinical characteristics of the 2 cohorts are shown in Tables 1 and 2.

### Table 1. Characteristics of patients with NSCLC and cancer-free smokers in a discovery cohort.

|                       | NSCLC CASES (N=68) | CONTROLS (N=66) | P VALUE |
|-----------------------|--------------------|----------------|---------|
| Age                   | 64.46 (SD: 11.37)  | 64.68 (SD: 11.32) | .31     |
| Sex                   |                    |                |         |
| Female                | 28                 | 28             | .32     |
| Male                  | 40                 | 38             |         |
| Race                  |                    |                | .40     |
| White                 | 51                 | 50             |         |
| African American      | 17                 | 16             |         |
| Pack-years (median)   | 34.56              | 33.27          | .47     |
| Stage                 |                    |                |         |
| Stage I               | 22                 |                |         |
| Stage II              | 20                 |                |         |
| Stage III-IV          | 26                 |                |         |
| Histological type     |                    |                |         |
| Adenocarcinoma        | 37                 |                |         |
| Squamous cell carcinoma | 31              |                |         |

Abbreviation: NSCLC, non–small-cell lung cancer.

### Sample collection and preparation

From the participants before they had any treatment, sputum and blood samples were collected as previously described.9–12,26–36 The preparation of airway bronchial epithelia from the sputum specimens was performed using a protocol developed in our previous studies.7,9–12,28–36 Peripheral blood was collected in BD Vacutainer spray-coated K$_2$EDTA Tubes (BD, Franklin Lakes, NJ, USA), and PBMCs were isolated using Ficoll gradient centrifugation as previously described.26

### Assessing expressions of the miRNAs using quantitative reverse transcription polymerase chain reaction

RNA was extracted from the samples using our established protocols.9–12,26,30,31 The expressions of 3 miRNAs (miRs-21, 31, and 210) in sputum, and 2 miRNAs (miRs-19b-3p and 29b-3p) in PBMC were determined by using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA).9–12,26,30,31 The levels of miRNAs were determined using a threshold cycle method.9–12,15,16 We included controls in each experiment: RNA extracted from a H358 NSCLC cell line (a positive control for RT [reverse transcription], preamplification, and ddPCR [Droplet Digital polymerase chain reaction]), genomic DNA (a positive control for genomic DNA detection), and nuclease-free water (control for contamination). All experiments were repeated 3 times.

### Statistical analysis

We used univariate analysis to identify the miRNAs whose expression levels being related to NSCLC. The significantly associated factors were then analysed using multivariate logistic regression models with stepwise regression based on receiver operator characteristic (ROC) curve to select an optimal prediction model for NSCLC. We also generated a 95% confidence
interval for the difference in the area under the ROCs (AUCs) using the bootstrap.\textsuperscript{37} The optimal cut-off value was generated using the Youden index.\textsuperscript{38,39} To compare different miRNA biomarker panels, we computed their AUCs to determine the sensitivity and specificity as previously described.\textsuperscript{40}

**Results**

*The individual sputum miRNAs and PBMC miRNAs could distinguish patients with lung cancer from cancer-free controls*

In the discovery cohort, the 3 sputum miRNAs (miRs-21, -31, and -210) and 2 PBMC miRNAs (miRs-19b-3p and 29b-3p) exhibited a different level in patients with NSCLC vs cancer-free smokers (all $P$s < .05). The analysis of the 3 sputum miRNAs created 0.923 AUC (Figure 1). Successively, the examination of the 3 sputum miRNAs produced 82.3% sensitivity and 87.9% specificity for diagnosis of lung cancer (Table 3). The level of miR-21 in sputum was related to AC ($P$ < .05); however, miR-210 was correlated to SCC ($P$ < .05). Overall, the 3 sputum miRNA panel did not have specific relationship with stage and histological type of lung cancer, and patients’ age, race, and sex (all $P$s > .05), but smoking history ($P$ < .05).

The analysis of the 2 PBMC miRNAs had an AUC of 0.837 (Figure 1), producing 72.1% sensitivity and 81.8% specificity for diagnosis of NSCLC (Table 3). Furthermore, the 2-PBMC miRNA biomarker panel created 80.7% sensitivity and 89.4% specificity for SCC, and 75.7% sensitivity and 68.2% specificity for AC. As a result, the 2-PBMC miRNA biomarker panel had a higher diagnostic value for SCC compared with AC. The 2 PBMC miRNA biomarkers did not have relationship with stage of NSCLC, and patients’ age, sex, and ethnicity of the participants (all $P$s $\geq$ .05), except the smoking history ($P$ = .03). Altogether, the results from

**Table 2. Characteristics of patients with NSCLC and cancer-free smokers in a validation cohort.**

|                | NSCLC CASES (N=49) | CONTROLS (N= 50) | $P$ VALUE |
|----------------|-------------------|-----------------|-----------|
| Age            | 65.23 (SD: 10.48) | 64.70 (SD: 11.15) | .30       |
| Sex            |                   |                 | .35       |
| Female         | 19                | 20              |           |
| Male           | 30                | 30              |           |
| Race           |                   |                 | .36       |
| White          | 37                | 38              |           |
| African American | 12               | 12              |           |
| Pack-years (median) | 35.52         | 35.16           | .43       |
| Stage          |                   |                 |           |
| Stage I        | 15                |                 |           |
| Stage II       | 15                |                 |           |
| Stage III-IV   | 19                |                 |           |
| Histological type |                |                 |           |
| Adenocarcinoma | 26                |                 |           |
| Squamous cell carcinoma | 23       |                 |           |

Abbreviation: NSCLC, non–small-cell lung cancer.

*Figure 1.* The comparison of integrated miRNA biomarkers with panels of sputum and PBMC miRNAs in a discovery cohort. A prediction model based on integrated use of 3 miRNA biomarkers (miRs-31, 210, and 19b-3p) across sputum and PBMC specimens was developed for distinguishing lung cancer patients from cancer-free smokers. The ROC curve of the integrated miRNA biomarkers produced a higher AUC (0.953), as compared with the panel of sputum miRNAs (0.923) and the panel of PBMC miRNAs (0.837) (all $P$s < .05). AUC indicates area under the ROC; miRNAs, microRNAs; PBMC, peripheral blood mononucleated cell.
In the discovery cohort, logistic regression model was applied to define performance of different patterns of the 3 sputum and 2 PBMC miRNA biomarkers. From the 5 genes, 2 sputum miRNAs (miRs-31 and 210) and 1 PBMC miRNA (miR-19b-3p) were selected as the best markers (all \(P_s < .001\)) and integrated into a logistic model. Integrated use of the 3 biomarkers created a greater AUC (0.953) than did the 3-sputum biomarker panel (0.923) or the 2-PBMC biomarker panel (0.837) \((P < .05)\) (Figure 1). Using Youden index, we set up optimal cut-off at 0.64. As a result, integrated use of the 2 sputum miRNAs and 1 PBMC miRNA produced higher sensitivity (86.8%) and specificity (92.4%) than did the 3-sputum biomarkers and the 2-PBMC biomarkers (all \(P_s < .05\)) (Table 3). Furthermore, combined use of all the 5 biomarkers (3 miRNA and 2 PBMC biomarkers) did not produce higher sensitivity and specificity compared with the integrated panel of the only 3 biomarkers \((P > .05)\). Moreover, integrated analysis of the 3 biomarkers across the different body fluids did not show special association with stage and histology of lung cancer, and patients’ age, race, and sex (all \(P_s > .05\)). Importantly, the estimated Pearson correlation among levels of the 3 miRNAs was very low (all \(P_s > .05\)), suggesting that the combination had complementary classification for diagnosis of lung cancer.

Validating the synergistic effect of the integrated panel of 3 miRNAs across the body fluids for detection of NSCLC

In the validation cohort, the integrated panel of 3 biomarkers was blindly confirmed in the specimens of 49 patients with lung cancer and 50 cancer-free smokers. The integration of the 3 biomarkers across sputum and blood specimens showed higher sensitivity (85.7% vs 81.6% and 71.4%) and specificity (92.0% vs 86.0% and 80.0%) than did the 3-sputum miRNA panel and 2-PBMC miRNA panel (all \(P_s < .05\)) (Table 3). Inconsistent with the findings in the discovery cohort, integrated analysis of the 3 biomarkers had no special relationship with stage and histological type of lung cancer, and patients’ age, race, and sex (all \(P_s > .05\)). Furthermore, the Pearson correlation between the 3 miRNAs’ expression levels was very low (all \(P_s > .05\)), supporting the synergistic value of this integrated panel of the 3 biomarkers for the early detection of NSCLC.

### Discussion

The development of sputum biomarkers is based on the concept that lung tumorigenesis developed in a field defect characterized by molecular aberrations in the airways. According to the filed defect concept, the alterations of miRNAs in bronchial airways reflect those in primary lung tumours in the distal lungs.\(^4\) The analysis of exfoliated airway bronchial epithelia in sputum for the altered miRNA expressions might indicate the lung tumour–related molecular alterations and hence identify NSCLC. On the contrary, the development of PBMC miRNAs as circulating tumour markers is based on the concept that PBMCs act as the primary defence line against cancer, and the abnormal miRNA expressions of PBMC contribute to lung tumorigenesis via the different mechanisms.\(^5,9,10,22,26\) Based on the different concepts, miRNA expression profile in PBMCs, unlike miRNAs detected in sputum cells, may not signify the abnormal miRNA changes in the lung tumours or mimic that in the primary cancer tissues.\(^41-43\) Therefore, there was no overlap between the PBMC miRNA vs sputum miRNA profiles of patients with lung cancer.\(^10,11,15,26,44\) Given that altered miRNAs in sputum and PBMCs contribute to lung tumorigenesis via the different mechanisms,\(^5,9,10,22,26\) we hypothesize that integrating the miRNAs across the different body fluids may have a synergistic effect and thus improve the diagnosis of lung cancer. Indeed, the combined analysis of the 2-sputum miRNA and 1-PBMC miRNA generated a better performance than did a single category of the markers. Also, there was no association among the miRNA aberrations in the 2 surrogate clinical materials, further supporting that the performances of the 2 categories of miRNA biomarkers might have a synergistic value. In addition, unlike PBMC miRNA markers that are

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**Table 3.** The comparison of integrated panel of 2 sputum and 1 PBMC miRNA biomarkers with individual panels of sputum and PBMC miRNA biomarkers in a discovery cohort and a validation cohort.

|                  | A DISCOVERY COHORT       | A VALIDATION COHORT       |
|------------------|--------------------------|---------------------------|
|                  | SENSITIVITY (95% CI)     | SPECIFICITY (95% CI)      | SENSITIVITY (95% CI)     | SPECIFICITY (95% CI)      |
| 3 sputum miRNAs  | 82.35 (71.20-90.53)      | 87.88 (77.51-94.62)       | 81.63 (67.98-91.24)      | 86.00 (73.26-94.18)       |
| 2 PBMC miRNAs    | 72.06 (59.85-82.27)      | 81.82 (70.39-90.24)       | 71.43 (56.74-83.42)      | 80.00 (66.28-89.97)       |
| Integrated 2 sputum and 1 PBMC miRNAs | 86.76 (76.36-93.77) | 92.42 (83.20-97.49) | 85.71 (72.76-94.06) | 92.00 (80.77-97.78) |

Abbreviations: CI, confidence interval; miRNAs, microRNAs; PBMC, peripheral blood mononucleated cell.
more specific to SCC, the integration of sputum and PBMC biomarkers was not related to histology of lung tumour and hence substantiates the utility for predicting lung cancer. Moreover, integrated use of the 3 miRNAs had a comparable diagnostic performance for lung tumour at the early vs late stages. Altogether, the findings would be an important feature if the assay is used for identifying early-stage lung cancer.

Disregurgulation of miR-31, 210, and 19b-3p have been found to associate with a variety of tumours. For example, miR-31 downregulation in breast cancer is related with tumour mettases, whereas elevated miR-31 expression in colorectal cancer may associate with late stage of the tumours. Abnormal miR-19b-3p expression has not been described in the immune system. Our ongoing project is to investigate the mechanisms, by which miR-19b-3p may involve in lung cancer cells’ evasion of immunological surveillance.

This study may have some limitations. (1) Clinically useful biomarkers should have high sensitivity and specificity. Integrated use of 3 miRNAs has a sensitivity of 88.2% and a specificity of 92.4%, which may not be sufficient in the laboratory settings. We will find new biomarkers that can be used together with the current ones to improve clinical performance of the assay. Furthermore, an ongoing project in our laboratory is to simultaneously evaluate cell-free miRNAs (eg, plasma- and serum-derived miRNAs) and PBMC miRNAs using the same clinical specimens to compare the diagnostic values of the 2 different miRNA biomarkers and investigate whether there is synergistic diagnosis between them. (2) The clinical samples were obtained from the patients who already have clinical diagnosis. The subjects might not represent heavy smokers in a lung cancer screening programme. We will conduct a large lung cancer screening trial to confirm the performance of the integrated panel of miRNA biomarkers. (3) Using low-dose computed tomography (LDCT) for the early detection of lung cancer can reduce the mortality. However, LDCT always produces over-diagnosis or a false-positive rate, presenting a major clinical obstacle for lung cancer early detection.

It would be interesting to evaluate whether the 3 miRNA biomarkers could complement LDCT for the early detection of lung cancer by specifically reducing its over-diagnosis.

Conclusions

We have for the first time demonstrated that integrating circulating and sputum markers has a synergistic value and presents a potential approach for the early detection of lung cancer. Nevertheless, a large multi-centre trial is required to confirm the integrated method before it could be translated in laboratory settings.

Author Contributions

JS, QL, YL, JM, FJ, and C-JL conducted the experiments and participated in data interpretation. HF and FJ participated in study design, coordination, and data analysis and interpretation, and prepared the manuscript. All authors read and approved the final manuscript.

Disclosures and Ethics

The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material.

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