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BRIEF REPORT

Tissue-specific and plasma microRNA profiles could be promising biomarkers of histological classification and TNM stage in non-small cell lung cancer

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Abstract

In a previous study, we determined that plasma miRNAs are potential biomarkers for cigarette smoking-related lung fibrosis. Herein, we determine whether tissue-specific and plasma miRNA profiles could be promising biomarkers for histological classification and TNM stage in non-small cell lung cancer (NSCLC). Plasma miRNA profiling preoperatively and seven days postoperatively, and cancer and normal tissue miRNA profiling were performed in NSCLC patients and matched healthy controls. There was a > twofold change for all signature miRNAs between the NSCLC patients and controls, with P values of < 0.05. We found that tissue-specific and plasma miR-211-3p, miR-3679-3p, and miR-4787-5p were promising biomarkers of different staging lung squamous cell carcinoma, and miR-3613-3p, miR-3675-3p, and miR-5571-5p were promising biomarkers of different staging lung adenocarcinoma. These results suggest that tissue-specific and plasma miRNAs could be potential biomarkers of histological classification and TNM stage in NSCLC.

Introduction

Lung cancer is the leading cause of cancer death worldwide and causes more cancer deaths annually than colorectal, breast, and prostate cancers combined.[1] Recent advances in the clinical management have only led to small improvements in overall survival of lung cancer patients, partly due to the majority of the cases are diagnosed as an advanced stage. Therefore, screening of individuals at a higher risk of developing lung cancer has the potential to improve clinical outcomes. Research from the National Lung Cancer Screening Trial show that annual low-dose computerized tomographic screening decreases mortality by approximate 20%.[2] However, 96% of the pulmonary abnormalities observed in the trial were benign lesions. Periodic radiological tests for screening may also expose individuals to a significant level of radiation, with possibly harmful consequences. In routine clinical practice, the incidence of pulmonary nodules detected in chest radiography ranges from 0.09–0.2% and is higher in more advanced radiological examinations.[3,4] The chance of such a nodule being malignant varies widely from 1–70%, and depends on a number of factors, such as the size of the nodule and the clinical setting.[3,5] So the detection of a lung nodule in a radiological examination not only causes patient anxiety, but also leads to further invasive and expensive tests, such as positron emission tomography and biopsy,

which are likely of no benefit to a large proportion of individuals. Therefore, developing non-invasive (e.g. blood-based) biomarker assay should be very useful to identify individuals that are most likely to have a malignant lesion in the lung.

Abnormal expressions of specific miRNAs are implicated in the pathogenesis of various human cancers, and some miRNA signatures identified are associated with clinical characteristics, such as disease progression and drug treatments. Recently, cell-free circulating miRNAs are reported to be detected in serum/plasma and levels of some tumor-derived miRNAs are significantly elevated in patients with lung cancer, suggesting that blood-based miRNAs could emerge as revolutionary biomarkers for lung cancer diagnosis and prognosis.[6,7] Therefore, we investigate whether specific plasma miRNA profiles could be promising biomarkers of histological classification and tumor node metastasis (TNM) stage in non-small cell lung cancer (NSCLC).

Materials and methods

Subjects

For this pilot study, we recruited NSCLC (n = 40) and pulmonary tuberculosis patients (n = 5) from West China Hospital who had received video-assisted thoracoscopic lobectomy from January 2012 to December 2013. Furthermore, 10 healthy people were included as controls. All NSCLC patients had primary lung cancer without any treatment, such as drugs, neo-radiochemotherapy or chemotherapy, and had no co-existing illness. All participating patients signed informed consent. The institutional ethics review committee of West China Hospital approved the study. All cases were reviewed by two pathologists, and diagnoses were confirmed according to the criteria recently established by the National Comprehensive Cancer Network (NCCN) (http://www.nccn.org/).

Sample collection

Blood samples, lung cancer tissues and their adjacent normal tissues were collected from lung cancer patients at the Department of Thoracic Surgery, West China Hospital, Sichuan University, China. The TNM stages of tumors were determined according to the standard TNM classification system of the International Union Against Cancer (7th edition) (http://www.uicc.org/). Blood samples were collected twice: a day before medical preparation for video-assisted thoracoscopic lobectomy and seven days postoperatively without any intervention. Blood samples were also collected in the healthy volunteers. Ethylenediaminetetraacetic acid (EDTA) anticoagulated peripheral blood was used to extract and purify total RNA. Lung cancer and their adjacent normal tissues were collected after video-assisted thoracoscopic lobectomy. The tissues were embedded in paraffin block (Leica CM3050, Leica Biosystems, Nussloch, Germany) after being kept in Bouin's fixative for 24 hours. For RNA extraction, 10 μm thick cross-sections of tissues were cut from the blocks.

Ribonucleic acid (RNA) extraction, purification, and micro (mi)RNA microarray analyses

Ribonucleic acid extraction, purification, and miRNA microarray analyses were conducted as previously described.[8] In short, total RNA was separated, isolated, and purified with a mirVana miRNA Isolation Kit (Cat#1928 or Cat# 1975 Ambion, Austin, TX, USA) following the manufacturer's instructions. The miRNA molecular in total RNA was labeled using the miRNA Complete Labeling and Hybridization Kit (Cat# 5190-0456, Agilent Technologies, Santa Clara, CA, USA) following the manufacture's instructions regarding labeling. Each slide was hybridized with 100 ng Cy3-labeled RNA using the miRAN Complete Labeling and Hybridization Kit in a hybridization oven (Cat#G2545A, Agilent Technologies) at 55°C, 20 rpm for 20 hours according to the manufacturer's instructions. After hybridization, slides were washed in stain dishes (Cat#121, Thermo Shandon, Waltham, MA, USA) with a Gene Expression Wash Buffer Kit (Cat#5188-5327, Agilent Technologies). Slides were scanned by Agilent Microarray Scanner (Cat#G2565BA) and Feature Extraction software 10.7 with default settings (Agilent Technologies). Raw data were normalized by quantile algorithm, Gene Spring Software 11.0 (Agilent Technologies).

Statistical analysis

Demographic and clinical characteristics of the study population are expressed as means and standard deviations. Statistical analysis was performed by SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Differences between groups were determined by Student's *t*-test. *P* values of less than 0.05 were considered significant.

Results

Characteristics of screening study population

Forty NSCLC patients were enrolled in our study, including 17 stage I, 14 stage II, and nine stage III, and 13 lung squamous cell carcinoma and 27 lung adenocarcinomas. Five pulmonary tuberculosis and 10 healthy controls were also included. Most of these participants lived outside the city and earned a middle to low income. Only one NSCLC patient had family history of lung cancer (Table 1). Nearly 92.3% of the

Table 1 Characteristics of subjects

Characteristics	NSCLC (N = 40)	TB (N = 5)	Healthy controls (N = 10)
Age	61.57 ± 10.81	59.43 ± 9.75	58.94 ± 12.5
Gender (male)	22 (55.0%)	4 (80.0%)	5 (50.0%)
Smoking history	13 (32.5%)	2 (40.0%)	4 (40.0%)
Family history	1 (2.5%)	0	
Income status			
Low	13 (32.5%)	2 (40.0%)	3 (30.0%)
Middle	21 (52.5%)	3 (60.0%)	5 (50.0%)
High	6 (15.0%)		2 (20.0%)
Residence			
City	8 (20.0%)	1 (20.0%)	4 (40.0%)
Suburbs	22 (55.0%)	2 (40.0%)	4 (40.0%)
Countryside	10 (25.0%)	2 (40.0%)	2 (20.0%)
Pathological type			
Adenocarcinoma	27 (67.5%)		
Squamous cell carcinoma	13 (32.5%)		
TNM stage of lung cancer			
I	17 (42.5%)		
II	14 (35.0%)		
III	9 (22.5%)		
Diameter of tumor (cm)	3.5 ± 1.6		

NSCLC, non-small cell lung cancer; TB, tuberculosis; TNM, tumor node metastasis.

squamous cell carcinoma patients had a smoking history, while 96.3% of adenocarcinoma patients were never smokers (Table 2).

Specific miRNAs were found in cancer tissues and plasma of non-small cell lung cancer (NSCLC) patients

It has been reported that miRNA expression patterns in human cancer appear to be tissue-specific.[9] Therefore, we analyzed the expression of miRNAs both in lung cancer tissues and plasma. We compared miRNA expression among NSCLC, pulmonary tuberculosis, and normal tissues using miRNA array. We found 31 different miRNA expressions in lung squamous cell carcinoma tissues and 15 in lung adenocarcinoma tissues. Meanwhile, we found 23 specific miRNAs were expressed in the plasma of lung squamous cell carcinoma patients and 13 in the plasma of lung adenocarcinoma patients, preoperatively and seven days postoperatively.

Table 2 Characteristics of lung cancer patients between smokers and never smokers

	Smokers, n = 13	Never smokers, n = 27
Age	61.00 ± 10.24	61.80 ± 11.56
Gender (male/female)	12/1	10/17
Family history	0	1 (3.7%)
Pathological type		
Adenocarcinoma	1 (7.7%)	26 (96.3%)
Squamous cell carcinoma	12 (92.3%)	1 (3.7%)
TNM stage of lung cancer		
I	7 (53.8%)	10 (37.0%)
II	3 (23.1%)	11 (40.7%)
III	3 (23.1%)	6 (22.2%)
Tumor diameter (cm)	3.8 ± 1.7	3.3 ± 1.6

TNM, tumor node metastasis.

Tissue-specific and plasma miRNAs were promising biomarkers for differentiating lung squamous cell carcinoma from lung adenocarcinoma

A number of patents regarding miRNA-based methods for the diagnosis, prognosis, and treatment of human cancers, such as lung and breast cancers, have been published.[10,11] Therefore, we analyzed the different expression of plasma miRNAs between lung squamous cell carcinoma and lung adenocarcinoma. We found that 15 specific plasma miRNAs, let-7d-3p, miR-106b-5p, miR-144-3p, miR-197-3p, miR-19b-3p, miR-211-3p, miR-30e-5p, miR-345-3p, miR-3679-3p, miR-423-5p, miR-451a, miR-4787-5p, miR-5001-5p, miR-5100 and miR-6068, could be promising biomarkers of lung squamous cell carcinoma, and nine specific plasma miRNAs, let-7d-3p, miR-223-3p, miR-21-5p, miR-33b-3p, miR-3613-3p, miR-3675-3p, miR-4728-3p, miR-5571-5p, and miR-575, could be promising biomarkers of lung adenocarcinoma. Furthermore, miR-211-3p, miR-3679-3p, and miR-4787-5p were constantly expressed in both tissues and plasma of different staging lung squamous cell carcinoma patients (Fig 1). miR-3613-3p, miR-3675-3p, and miR-5571-5p were

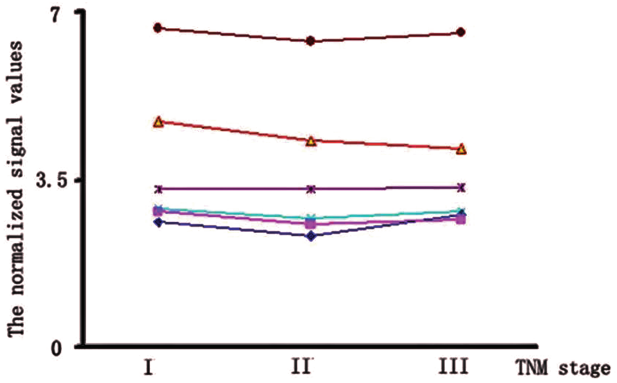

Figure 1 Constant expression level of miR-211-3p, miR-3679-3p, and miR-4787-5p in the tissues and plasma of different staging lung squamous cell carcinoma patients. →, plasma miR-211-3p; →, plasma miR-3679-3p; →, plasma miR-4787-5p; →, tissue miR-211-3p; →, tissue miR-3679-3p; →, tissue miR-4787-5p.

constantly expressed in both tissues and plasma of different staging lung adenocarcinoma patients (Fig 2).

Specific plasma miRNAs were promising biomarkers for TNM staging of NSCLC

Recent reports have revealed that the deregulation of miRNAs correlates with various human cancers and is involved in the initiation and progression of human cancers. Therefore, we sought to determine whether specific plasma miRNAs could be promising biomarkers for the TNM staging of NSCLC. We found let-7d-3p, miR-106b-5p, miR-197-3p, miR-19b-3p, miR-30e-5p, miR-423-5p, miR-451a, and miR-5100, were downregulated (Fig 3a), and miR-144-3p and miR-6068 were upregulated in all tissues and plasma of lung squamous cell carcinoma patients (Fig 3b), which correlated with TNM grade. Let-7d-3p, miR-233-3p, miR-21-5p, and miR-575 were upregulated and miR-33b-3p and miR-4728-3p were downregulated in all tissues and plasma of lung adenocarcinoma patients, which correlated with TNM grade (Fig 4).

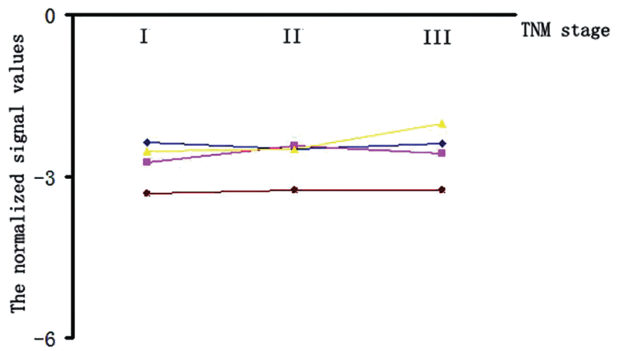

Figure 2 Constant expression level of miR-3613-3p, miR-3675-3p, and miR-5571-5p in the tissues and plasma of different staging lung adenocarcinoma patients. →, plasma miR-3613-3p; →, plasma miR-3675-3p; →, plasma miR-5571-5p; →, tissue miR-3631-3p; →, tissue miR-3675-3p; →, tissue miR-5571-5p.

Discussion

In recent years, changes in the expression levels of small, noncoding, single-strand RNAs, called miRNAs, have been detected and described. It is estimated that more than 1000 miRNAs are transcribed and that 30% of the human genome is modulated by miRNA, and one miRNA can modulate hundreds of downstream genes. More than half of the miRNAs genes are located in cancer-associated genomic regions or in fragile sites. Several miRNAs located in deleted regions demonstrate low expression levels in cancer tissues.[12] miRNAs can act as tumor suppressors, the dysfunction of miRNAs can initiate or contribute to the malignant transformation of a normal cell. The dysfunction of an miRNA could be a result of several mechanisms, including genomic deletion, mutation, epigenetic silencing, and/or miRNA processing alterations.[13]

Recently, several reports suggest that cell-free circulating miRNAs are detectable in serum/plasma and the levels of tumor-derived miRNAs are elevated in patients with lung cancer, suggesting that blood based miRNAs could emerge as revolutionary biomarkers for lung cancer diagnosis and prognosis.[7,14] It has been shown that the classification of multiple cancers based on miRNA expression signature is more accurate than mRNA-based signatures.[15] Therefore, we recruited NSCLC and pulmonary tuberculosis patients and healthy controls to determine whether tissue-specific and specific plasma miRNAs could be promising biomarkers of NSCLC. After comparing NSCLC with pulmonary tuberculosis and normal lung tissues, we found NSCLC tissue-specific miRNAs. We compared plasma miRNA expression between NSCLC and pulmonary tuberculosis patients and healthy controls, and found NSCLC plasma-specific miRNAs. Furthermore, as promising biomarkers, the expression levels of those plasma-specific miRNAs must gradually get to normal after the tumor was resected and without any other intervention. Our results determined 15 lung squamous cell carcinoma tissue-specific and plasma miRNAs: let-7d-3p, miR-106b-5p, miR-144-3p, miR-197-3p, miR-19b-3p, miR-211-3p, miR-30e-5p, miR-345-3p, miR-3679-3p, miR-423-5p, miR-451a, miR-4787-5p, miR-5001-5p, miR-5100, and miR-6068; and nine lung adenocarcinoma tissue-specific and plasma miRNAs: let-7d-3p, miR-223-3p, miR-21-5p, miR-33b-3p, miR-3613-3p, miR-3675-3p, miR-4728-3p, miR-5571-5p, and miR-575. However, the expression levels of specific miRNAs were different between tissues and plasma. As shown in Figure 1 and Figure 2, plasma miR-211-3p, miR-3679-3p, and miR-4787-5p are specific biomarkers of lung squamous cell carcinoma, and plasma miR-3613-3p,

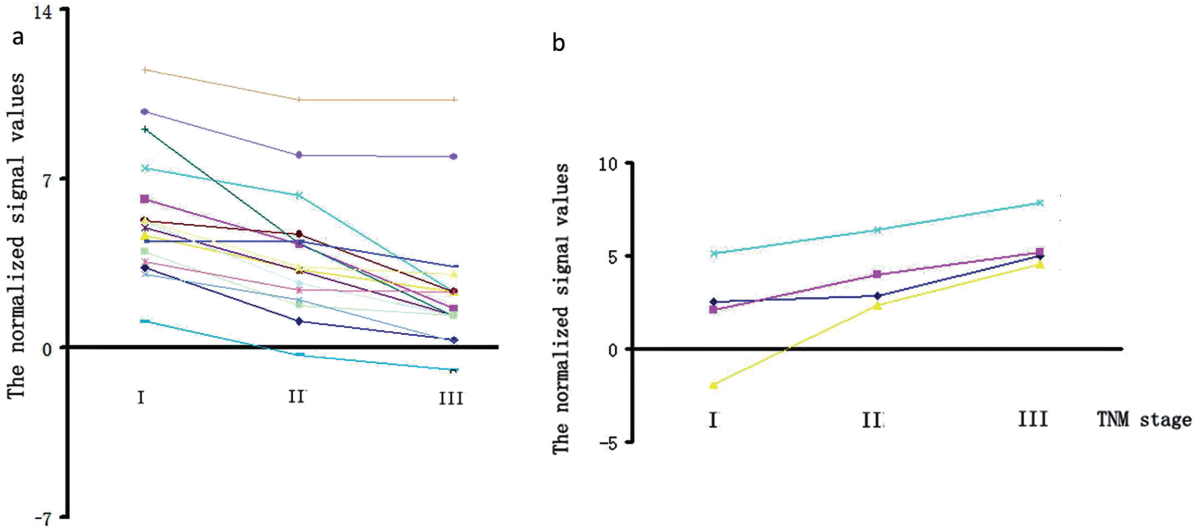

Figure 3 (a) Downregulation of tissue specific plasma micro ribonucleic acids in lung squamous cell carcinoma patients. —, plasma let-7d-3p; —, plasma miR-106b-5p; —, plasma miR-197-3p; —, plasma miR-19b-3p; —, plasma miR-30e-5p; —, plasma miR-423-5p; —, plasma miR-451a; —, plasma miR-5100; —, tissue let-7d-3p; —, tissue miR-106b-5p; —, tissue miR-197-3p; —, tissue miR-19b-3p; —, tissue miR-30e-5p; —, tissue miR-423-5p; —, tissue miR-451a; —, tissue miR-5100. (b) Upregulation of tissue specific plasma micro ribonucleic acids in lung squamous cell carcinoma patients. —, plasma miR-144-3p; —, plasma miR-6068; —, tissue miR-144-3p; —, tissue miR-6068.

miR-3675-3p, and miR-5571-5p are specific biomarkers of lung adenocarcinoma.

The mechanistic basis for alterations in serum or plasma miRNAs consequent to the presence of lung cancer is not clear. It could be that tumors themselves release miRNAs into circulation, as suggested by several studies.[16,17] However, it is unlikely that this is the cause for the majority of altered miRNAs.[18] It is believed that miRNAs are released into blood circulation by all cells of the body and not just tumors, which typically constitute only a very small fraction of the body's cellular mass.[19] There is no miRNA which is exclusively expressed by cancer cells, and compared with normal tissues, the changes in miRNA expression levels in cancer tissues are usually very modest.[20] It is, therefore, possible that the changes in miRNA expression seen in serum or plasma reflect the body's systemic response to the presence of cancer, including changes in miRNA expression in circulating blood cells.[21] Such a response may be exhibited in whole blood miRNA expression. Indeed, a number of recent studies have reported changes in the miRNA expression profiles of peripheral whole blood in patients with various malignancies, such as brain, breast, ovary, and pancreas cancers, as well as in non-malignant diseases.[22–27]

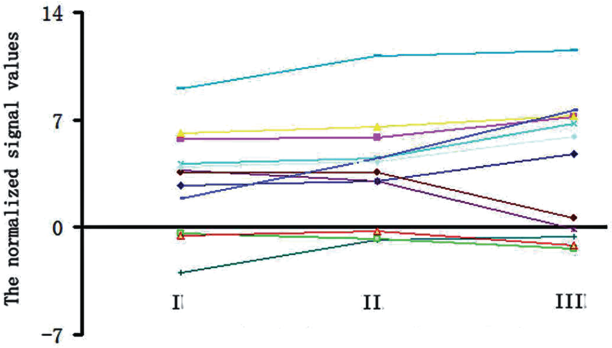

Figure 4 The expression of tissue specific plasma micro ribonucleic acids in lung adenocarcinoma patients. —, plasma let-7d-3p; —, plasma miR-233-3p; —, plasma miR-21-5p; —, plasma miR-575; —, plasma miR-33b-3p; —, plasma miR-4728-3p; —, tissue let-7d-3p; —, tissue miR-233-3p; —, tissue miR-21-5p; —, tissue miR-575; —, tissue miR-33b-3p; —, tissue miR-4728-3p.

Abnormal expressions of specific miRNAs are implicated in the pathogenesis of various human cancers, and miRNA expression profiling of human tumors has identified signatures associated with diagnosis, staging, progression, prognosis, and response to treatment. Promising diagnosing biomarkers should represent: (i) suitable biological matrices; (ii) suitable parameters, able to reflect internal exposure, biochemical or biological effects; (iii) suitable and reliable analytical methods; and (iv) reference and limit values which enable the interpretation of results.[28] Therefore, we sought to determine whether plasma specific miRNAs could be promising biomarkers of NSCLC. We examined the specific expression changes in plasma and tissues. Interestingly, in all tissues and plasma of lung squamous cell carcinoma, let-7d-3p, miR-106b-5p, miR-197-3p, miR-19b-3p, miR-30e-5p, miR-423-5p, miR-451a, and miR-5100 were down-regulated and

miR-144-3p and miR-6068 were up-regulated, which correlated with TNM stage (Fig 3a and Fig 3b). In all tissues and plasma of lung adenocarcinoma, let-7d-3p, miR-233-3p, miR-21-5p and miR-575 were up-regulated, and miR-33b-3p and miR-5771-5p were downregulated (Fig 4).

We compared our results with published studies which had reported an association in the changes in whole blood miRNA expression with lung cancer. There was minimal overlap between significant miRNAs. For example, we believed that let-7d-3p was a promising biomarker of NSCLC. However, Jeong, *et al.*'s study determined that let-7a, not let-7d-3p expression was reduced in the whole blood of lung cancer cases, which was not significantly different between cases and controls in our study and two other previous studies.[29–31] And miR-190b, miR-630, miR-942, and miR-1248, the most frequent constituents of the classifiers generated in a recent study, were not differentially expressed between cases and controls in our study, Keller *et al.*'s and Leidinger *et al.*'s studies.[9,31,32] The low consistency between these findings could be a result of the different miRNA quantificantion platforms used in the studies, or because of the significant variation of clinical and demographic profiles of the case and control cohorts between studies such as types of lung cancer, age, smoking history, and gender.

In addition, many of the controls in other studies did not undergo radiological investigations, such as computed tomography, whereas all the cases did in the present study. It is, therefore, possible that some of the changes in miRNA expression noted here are actually consequent to radiation exposure[33]. Finally, the recent discovery that miRNAs are present in the circulation has sparked interest in their use as potential biomarkers. While research on circulating miRNAs is still in its infancy, high analytical standards in statistics and study design are a prerequisite to obtain robust data and avoid repeating the mistakes of earlier genetic association studies. Studies may be published because of their novelty, despite low numbers, poorly matched cases and controls, and a lack of multivariate adjustment for conventional risk factors. Research on circulating miRNAs can only progress by bringing more statistical rigor to bear in this field and by evaluating changes of individual miRNAs in the context of the overall miRNA network. Such miRNA signatures may have better diagnostic and prognostic value.

Conclusion

Plasma miR-211-3p, miR-3679-3p, and miR-4787-5p were specific biomarkers of lung squamous cell carcinoma, and plasma miR-3613-3p, miR-3675-3p, and miR-5571-5p were specific biomarkers of lung adenocarcinoma. Tissue-specific and plasma miRNAs might be promising biomarkers of histological classification and TNM stage in NSCLC. And plasma microRNA assays show a significant process as diagnostic indicators in NSCLC. Additional studies with large sample sizes, and case and control cohorts matched for important variables, such as age, gender, smoking status, and blood cell counts, are required to confirm these results.

Acknowledgments

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Disclosure

No authors report any conflict of interest.

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