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Original Article

Serum miR-10a-5p and miR-196a-5p as non-invasive biomarkers in non-small cell lung cancer

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Abstract: Background: Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for about 85% of all cases. MicroRNAs are stable molecules in the blood and can be used as biomarkers for early diagnosis of various malignancies. The aim of this study was to evaluate expression of miR-10a-5p and miR-196a-5p in tissue and serum of patients with NSCLC and to explore its relationship with clinicopathological characteristics. Methods: A total of 20 pairs of tissues and 80 serum samples were obtained from NSCLC patients. Seventy-five serum samples from healthy individuals of the same age and gender were also collected. The expression level of miR-10a-5p and miR-196a-5p was detected by quantitative real-time PCR. The relationship between miR-10a-5p and miR-196a-5p expression level in NSCLC tissues and serum and clinicopathological characteristics was estimated respectively. The diagnostic value of miRNA-10a-5p and miR-196a-5p in NSCLC was assessed by the Receiver-operating characteristic (ROC) curve method. Results: We found that miRNA-10a-5p and miR-196a-5p expression levels were increased significantly in NSCLC tissues compared with non-tumor adjacent normal tissues. Serum miR-10a-5p and miR-196-5p were over-expressed in NSCLC patients compared with healthy controls. The higher miR-10a-5p or miR-196-5p expression levels were positively correlated with advanced tumor stage and positive lymph node metastasis. The area under the curve (AUC) of serum miR-10a-5p and miR-196-5p to diagnose NSCLC were 0.709 and 0.785. Optimal sensitivity and specificity were 65.98% and 72.71%, 67.86% and 77.57%, respectively in differentiating NSCLC patients from healthy controls. The combination of these two miRNAs with carcinoembryonic antigen (CEA) further increased the diagnostic value, with an area under the curve (AUC) of 0.801 (sensitivity, 76.34%; specificity, 79.26%) using logistic regression model analysis. Conclusions: Serum miR-10a-5p and miR-196a-5p may be useful noninvasive biomarkers for the clinical diagnosis of NSCLC.

Keywords: microRNAs, non-small cell lung cancer, serum, diagnosis

Introduction

Lung cancer is one of the most common malignancies, ranking first in the global number of cancer deaths. This is largely due to the lack of effective early diagnosis and treatment [1]. It is divided into 2 broad classes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is the predominant type of lung cancer, accounting for about 85% of all lung cancer cases. It has a very high mortality rate as well as a low 5-year survival rate of less than 15% after initial diagnosis [2-4]. Identifying lung cancer by observing early molecular changes contributes to early diagnosis and treatment, may reduce lung cancer mortality. Thus, it is necessary to explore more stable, sensitive, and non-invasive biomarkers for NSCLC screening.

MicroRNAs (miRNAs) are generally 18-25-nucleotides, non-coding-RNAs that negatively regulate gene expression by binding to target messenger RNAs (mRNAs) at their 3'-untranslated region, leading to mRNA degradation or translation suppression [5]. Abnormal expression of miRNAs is associated with many human diseases, including cancer [6]. It has been demonstrated that miRNAs arestable molecules that can be found and measured in peripheral blood, showing the potential to serve as biomarkers in the detection, classification, prognosis, and

Table 1. Clinical characteristics of 80 NSCLC patients and 75 healthy individuals

	Patient	Healthy control	*P* value
Age (years)			0.492
≤60	46	39	
>60	34	36	
Gender			0.874
Male	49	45	
Female	31	30	
Smoking			0.066
No	33	42	
Yes	47	33	

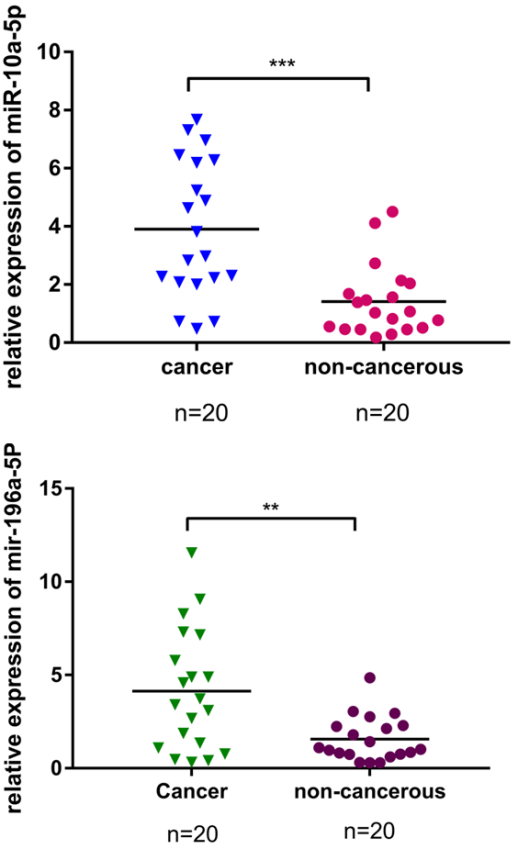

Figure 1. Expression of miR-10a-5p and miR-196a-5p in NSCLC tissues. U6 was used normalization. *P* values of miR-10a-5p and miR-196a-5p were 0.0001 and 0.0027, respectively. ***P<0.001, **P<0.01.

therapy of human malignancies [7]. Some previous studies have shown that the levels of circulating miRNAs are altered in patients with NSCLC compared with those in healthy individuals [8, 9]. Our study found that miRNA-10a-5p and miR-196a-5p expression in NSCLC cancer tissues were significantly higher than that in non-cancerous tissues. However, the technique of detecting miRNAs from tissue is limited, owing to its complication and need of invasive method to obtain. The diagnostic value of serum miRNA-10a-5p and miR-196a-5p in NSCLC patients remains unknown. In this study, we validated these two miRNAs as being dysregulated in the serum of NSCLC patients and explored their value in the diagnosis of the disease.

Materials and methods

Tissues sample

The study was approved by the Yangzhou University Ethics Committee (Yangzhou, China) and received informed consent from all patients. The 20 paired patient samples of primary lung cancer tissues and matched adjacent non-cancerous tissues were obtained from Affiliated Hospital of Yangzhou University (Yangzhou, China) between January 2015 and December 2016. Tissue samples were acquired from the routine therapeutic surgery of patients who did not receive anti-tumor treatment, including chemotherapy and radiotherapy. After surgical resection, human surgical specimens were immediately frozen in liquid nitrogen and stored at -80°C refrigerator until use.

Serum sample

Eighty cases were collected from primary NSCLC patients who were diagnosed by histology or cytology from May 2015 to August 2016 in Affiliated Hospital of Yangzhou University (Yangzhou, China). Histological diagnoses were independently formulated by two pathologists. Disease stage classifications were determined according to the pTNM requirements from the *8th Edition, AJCC Staging Manual*. Peripheral blood samples were collected before patients received any anticancer treatment, including surgery, chemotherapy, radiotherapy and hormonal treatment, etc. Blood samples (3.5 ml) were drawn from subjects using tubes without anticoagulant. These samples were processed by centrifugation at 3000 g for 5 minutes at room temperature. Serum was transferred into RNA-free EP tube every 500 μl aliquot and stored at -80°C before RNA extraction. As part of the routine clinical assessment, data pertaining to carcinoembryonic antigen (CEA) levels was ascertained from the patients' medical records from Affiliated Hospital of Yangzhou

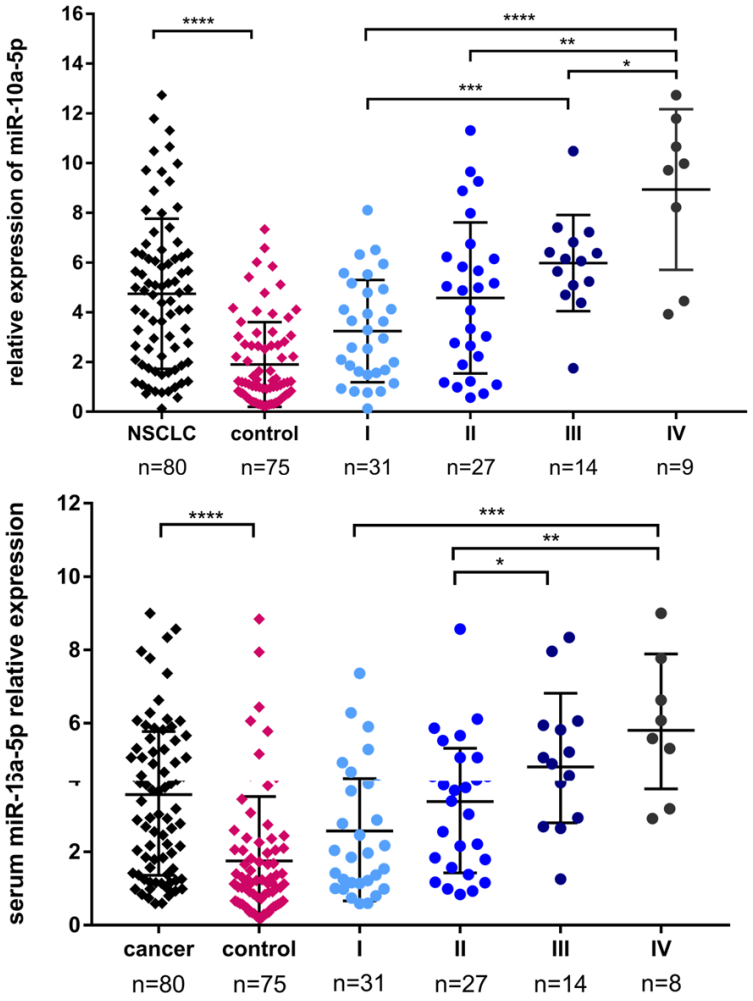

Figure 2. Serum expression levels of miR-10a-5p and miR-196a-5p in healthy controls and NSCLC patients. Cel-miR-39 was used for normalization. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

University. The characteristics with respect to age, gender, and smoking status of NSCLC patients and control subjects are summarized in **Table 1**.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from human tissue samples using TRIzol reagent (Invitrogen, CA) according to the manufacturer's protocol. Total RNAs of serum samples were extracted with Qiagen miRNeasy Mini Kit (Qiagen, Hilden, Germany). According to the manufacturer's protocol, RNA concentration was measured using Nanodrop 2100 (Thermo, Japan). Then the RNA samples were preserved at -80°C until ready for use. Repeated freeze-thawing was avoided to ensure the quality of the samples during storage. Complementary cDNA synthesis was performed using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan). Quantitative Real-time polymerase chain reaction was performed using SYBR® Premix Ex Taq™ II (Tli-RNaseH Plus) (TaKaRa, China). U6 was used for miRNA template normalization. The relative expression level of target RNAs was calculated by $2^{-\Delta\Delta Ct}$ method. As for serum samples, 100 fmol/ml of synthesized cel-miR-39 (Qiagen, Hilden, Germany) was added to equal volume of serum to serve as a normalizer before RNA extraction. The relative levels of miR-196a-5p and mir-10a-5p in the serum were expressed as $2^{-\Delta\Delta Ct}$ method. Quantitative real-time PCR was performed by using the ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, USA). All samples were performed in triplicate and independently repeated three times.

Statistical analysis

Statistical analyses were performed with the SPSS 23 software (IBM SPSS Statistics, Armonk, NY, USA) and graphs were generated using Graph Pad Prism 7.0 software (Graph Pad Software Inc., San Diego, CA). Mann-Whitney U test was used to compare miRNAs expression in paired tumor tissue and non-cancerous tissue. Two-tailed unpaired t-test was used to evaluate the differential expression of serum miRNA levels between NSCLC patients and controls. The Pearson's chi-squared test was used to assess correlation between the expression level of serum miRNAs and clinic pathological factors of NSCLC patients. A receiver operating characteristic (ROC) curve based on the relative expression ($2^{-\Delta\Delta Ct}$) of miRNA expression in samples was plotted and the area under the curve (AUC) was calculated to evaluate the diagnostic performance of miRNAs.

Table 2. Correlation between serum miRNAs status and clinicopathological characteristics

Characteristics	Number of patients	miR-10a-5p		*P* value	miR-196a-5p		*P* value
		Low	High		Low	High	
Number	80	40	40		40	40	
Age (years)				0.366			0.651
≤60	46	25	21		24	22	
>60	34	15	19		16	18	
Gender				0.084			0.491
Male	49	28	21		23	26	
Female	31	12	19		17	14	
Smoking				0.496			0.112
No	33	15	18		13	20	
Yes	47	25	22		27	20	
Histological type				0.737			0.875
Adenocarcinoma	50	22	28		26	24	
Squamous cell carcinoma	23	13	10		11	12	
Larger cell carcinoma	7	5	2		3	4	
Tumor stage				0.007*			0.016*
I	31	22	9		22	9	
II	27	13	14		12	15	
III	14	3	11		4	10	
IV	8	2	6		2	6	
Lymph node metastasis				0.006*			0.021*
No	30	21	9		20	10	
Yes	50	19	31		20	30	

*$p<0.05$.

Results

miR-10a-5p and miR-196a-5p expression in NSCLC tissues

To analyze the expression of miR-196a-5p and miR-10a-5p in patients with NSCLC, we measured the levels of two miRNAs in 20 pairs of NSCLC tissues and the non-cancerous tissues. As shown in **Figure 1**, the expression of two miRNAs were significantly higher in tumor tissues than that in non-cancerous tissues (*P*=0.0001, *P*=0.0027 respectively).

Serum miRNAs expression in NSCLC patients and healthy controls

The serum level of miR-10a-5p and miR-196a-5p of the two groups was compared. As shown in **Figure 2**, the expression of two miRNAs was significantly higher in NSCLC patients than that in healthy controls (*P*<0.0001). In addition, the gradual increase of two miRNAs expression levels wasclearly discernible when all NSCLC serum samples werebased on TNM staging (**Figure 2**).

Correlation of serum miRNAs with clinicopathological factors

The correlation of serum miR-10a-5p and miR-196a-5p expression levels with clinicopathological factors of NSCLC patients was statistically analyzed in **Table 2**. Based on miR-10a-5p and miR-196a-5p median expression, the serum miRNAs were categorized as low and high expression level. High levels of serum miR-196a-5p and miR-10a-5p expression were significantly correlated with high TNM stage (*P*=0.007, 0.016 respectively) and lymph node metastasis (*P*=0.006, 0.021 respectively). However, there were no obvious changes between the two miRNAs expression levels and other factors including age, gender, smoking history, and histological type.

Serum miRNAs as diagnosis signatures for NSCLCs

The data show that the levels of miR-10a-5p and miR-196a-5p were significantly distinct between NSCLC patients and healthy individu-

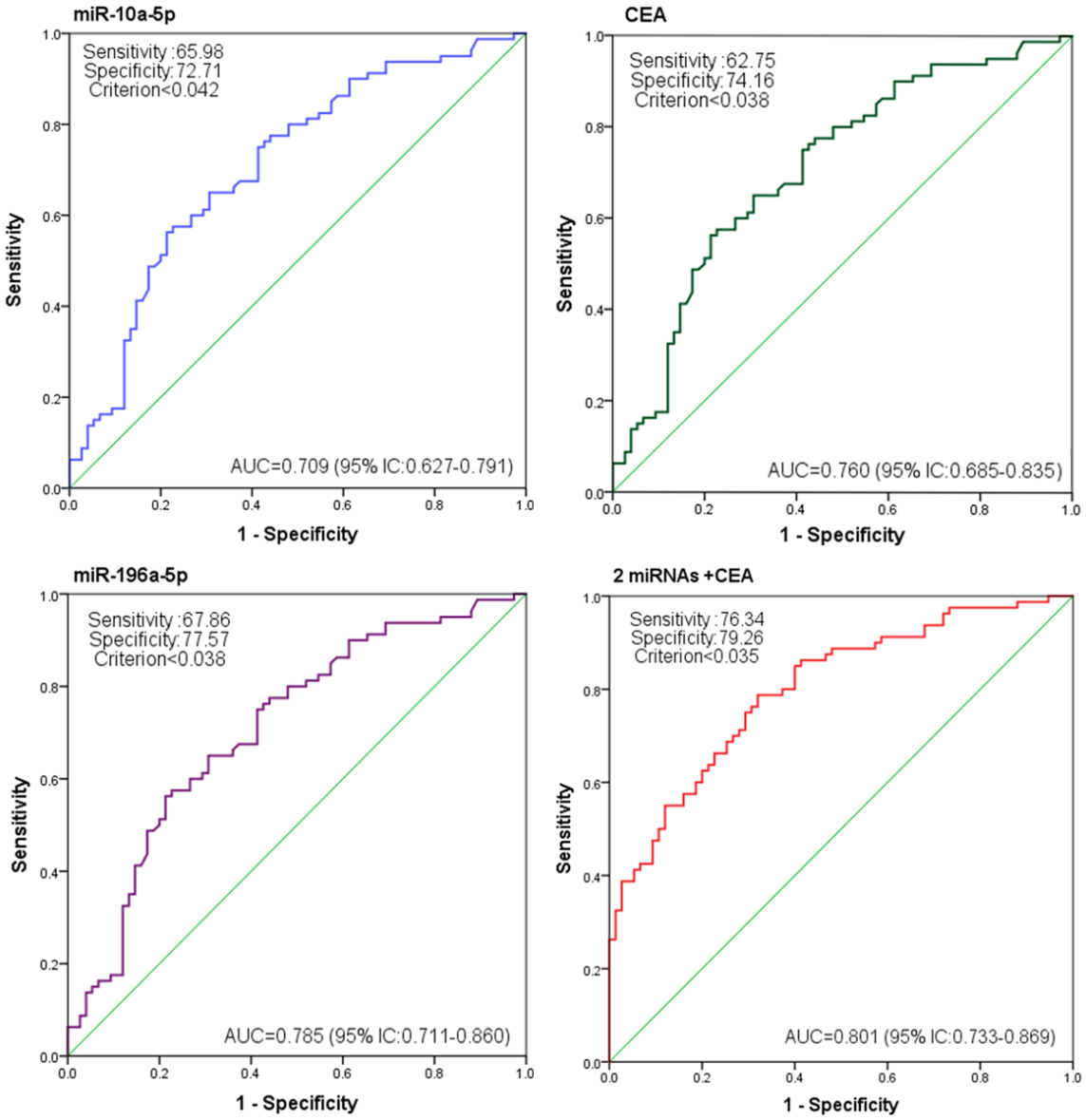

Figure 3. ROC curve analysis of the serum miR-10a-5p and miR-196a-5p to detect NSCLC. The *P* values of serum miR-10a-5p, miR-196a-5p, and CEA as well as the predictive value of logistic regression were <0.0001, 0.0018, 0.0029, and <0.0001 respectively. (CI, confidence interval).

als, the diagnostic accuracy of miR-10a-5p, miR-196a-5p, and CEA, as measured by the AUC, were 0.709, 0.785, and 0.760, respectively (**Figure 3**). Based on a logit model from the combination of these two miRNAs. Moreover, the predicted values of logistic regression analysis showed that the combined ROC analysis of two miRNAs and CEA revealed an increased AUC value of 0.801; the results are shown in **Figure 3**.

Discussion

Early diagnosis of NSCLC can significantly improve the survival rate. Many imaging and cytology-based strategies or puncture biopsy have been employed to augment early detection. However, because of traumatic and low sensitivity or supererogatory cost, these have not yet achieved high efficiency. Serum miRNAs are useful as novel tumor biomarkers for various types of human cancer. miRNAs are released from the primary tumor into peripheral circulation as stable molecules that are resistant to RNase digestion and other harsh conditions in serum. Thus, miRNAs have the advantage of convenience compared with those in the lung tissue. Deregulation of miRNAs is a factor in the initiation and progression of cancer. A mush-

rooming number of studies have shown that they may be potential biomarkers for several human cancers, including NSCLC [10-13]. In the past few years, the role of miRNA in the development of lung cancer has gradually been recognized; miR-125, miR-1290, miR-21, and miR-210 are all significantly elevated in lung cancer [14-17]. In contrast, miR-126a miR-146, miR-148, and miR-152 expression was significantly decreased in patients with lung cancer [18-21].

In the current study, we compared the expression differences of two miRNAs in serum from 75 healthy controls with 80 NSCLCs, and demonstrated that the serum levels of miR-10a-5p and miR-196a-5p were strongly higher in NSCLC patients than those from the healthy control. Our results are consistent with previous findings that miR-10a and miR-196a are oncogenic factors and are up-regulated in various malignant tumor tissues [22-24]. Zhi et al. reported that miR-10a-5p may serve as a biomarker useful to improving the management of acute myeloid leukemia patients [25], and similar results were reported in the following tumor tissues: kidney [26], cervical [27, 28], and thyroid [29]. In patients with breast cancer, serum miRNA-10a has a higher sensitivity than the conventional tumor marker CEA [30, 31]. However, the mechanisms are still largely unknown. Liu et al. found that miR-196a was significantly up-regulated in NSCLC tissues, and regulated NSCLC cell proliferation, migration and invasion, partially via down-regulation of HOXA5 [32]. Yu et al. reported that miRNA-10a is up-regulated in human NSCLC tissues and is associated with NSCLC progression, enhancing the growth and metastasis of NSCLC by activating the PTEN/AKT/ERK signaling pathway [33]. Some studies have demonstrated that miRNAs promote proliferation, invasion and migration at cellular level [34-36].

Furthermore, in our study the higher serum levels of miRNA-10a-5p and miR-196a-5p were associated with advanced clinical stage, suggesting that the two miRNAs have an oncogenic role during the development of cancer. Meanwhile, no obvious correlation was found between the two miRNAs level and certain clinicopathologic features, including age, gender, smoking history, histological type. The ROC curve analyses revealed that miR-10a-5p, miR-196a-5p alone was useful as a tumor biomarker for the early detection of NSCLC, with a high sensitivity and specificity. Furthermore, the combination of these two miRNAs with CEA further increased the diagnostic value, with an AUC of 0.801. These results suggest that serum miRNA-10a-5p and miR-196a-5p may serve as useful diagnostic biomarkers for NSCLC. In addition, our current study has some limitations, such as the size of the sample and the lack of comparative analysis with other malignancies. Therefore, a larger sample study is needed in the future to confirm the benefits of combining these two miRNAs in the early detection of subtypes of non-small cell lung cancer.

In conclusion, the high stability of serum miRNA-10a-5p and miR-196a-5p makes them idealnon-invasive biomarkers for NSCLC detection. The combination of two miRNAs with CEA may enable clinicians to distinguish NSCLC patients from healthy controls. Their roles and possible targets in NSCLC require further investigation.

Conclusions

In summary, the level of plasma miRNA-10a-5p and miR-196a-5p was upregulated in NSCLC patient serums and tissues. The expression level of these two specific miRNAs was associated with aggressive clinicopathological characteristics. Serum miRNA-10a-5p and miR-196a-5p measurement may thus be a novel and non-invasive method for NSCLC screening and differential diagnosis.

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Disclosure of conflict of interest

None.

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