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Original article

Circulating microRNA-339-5p and -21 in plasma as an early detection predictors of lung adenocarcinoma

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ABSTRACT

Background: Many studies have shown that differentially expressed miRs in body fluids can serve as biomarkers in non-invasive detection of the cancers. However, the clinical significance of plasma miRs in the diagnosis of lung adenocarcinoma (LA) is still not clear. Therefore, we examined the LA-specific miRs in plasma, which could be utilized to diagnosis and monitor LA in routine clinical practice.

Methods: Twenty-eight LA cases and twenty-eight healthy controls were recruited to our study. MiRs differential expression in plasma was measured by miRNA Microarray assay and revalidated by using qRT-PCR based absolute quantification methods The diagnostic power of circulating miRs in LA was evaluated using the receiver operating characteristics (ROC) curves and the area under the ROC curves (AUC).

Results: Tumor tissues and plasma levels of miR-339-5p were significantly down-regulated in LA patients compared with those in the control group, whereas the levels of miR-21 in LA patients were significantly higher than control group. ROC analysis showed that miR-339-5p and miR-21 could distinguish LA patients from healthy controls with high AUC (0.900 and 0.880, respectively), sensitivity (0.821 and 0.821, respectively) and specificity (0.929 and 0.964, respectively). Importantly, the combination of miR-339-5p and miR-21 markedly improved AUC (0.963), sensitivity (0.929) and specificity (0.929).

Conclusion: Plasma miR-339-5p or miR-21 could serve as a potential biomarker for diagnosis of LA, however, the combination of miR-339-5p and miR-21 was more efficient for LA detection.

1. Introduction

Lung adenocarcinoma (LA) is the most frequent non-squamous cell carcinoma belonging to non-small cell lung cancer (NSCLC) and is accounted for approximately a half of lung cancer [21,24]. In recent years, the 5-year overall survival has no significant improvement in the clinical management of NSCLC, which may be at least partly in a lack of effective diagnostic methods, due to the majority of patients are diagnosed in later stages of the disease [22,23]. At present, the common clinical diagnosis includes low-dose computed tomography (CT) screening [1] and protein biomarkers [19], such as carcinoembryonic antigen (CEA) and CYFRA21-1, however, the side-effects of CT screening and the sufficient sensitivity and specificity of protein biomarkers have limited utility in the early detection of LA. Therefore, exploring novel and specific biomarkers for the non-invasive detection of LA should be emphasized.

A class of small non-coding RNA (known as microRNA) is composed of 18–25 nucleotides with lack of protein-coding capability and mediates post-translational regulatory mechanism, regulating a variety of physiological functions and pathological processes [26]. Previous studies have verified that deregulated expression of miRs is associated with the process of various kinds of tumors, including LA [6,29]. MiR-107, miR-21, miR-145 and miR-196a/b have been reported to serve as potential diagnostic or prognostic biomarkers for pancreatic cancer [9], osteosarcoma [17], NSCLC [33] and gastric cancer [28], respectively. Although miRs have shown great promise in routine clinical practice, it is difficult to obtain biopsies of tissue, thus they can not be executed for clinical screening purposes in patients with malignancies. Emerging evidence suggests that blood-based (non-cellular serum, plasma or whole blood) miRs expression profiles have shown promising results for non-invasive diagnostic applications in LA [7,22,35]. More importantly, even though large amounts of endogenous ribonucleases in the blood, miRs can stably exist in blood and can be isolated in human peripheral blood [27]. Recently, several clinical trials have already been approved by the Food and Drug Administration (FDA) to evaluate the clinical diagnosis value of serum miRs in cancer patients [34]. Therefore, the current findings provide strong evidence that the circulating miRs can be used as the diagnostic molecular predictors for lung cancer screening.

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In the present study, we examined the miRs expression profiles in plasma to investigate the clinical diagnostic significance in patients with LA. We selected two miRs (miR-339-5p and miR-21) in plasma through a comprehensive miRNA array-based approach. We finally validated that miR-339-5p and miR-21 in plasma samples represent the useful non-invasive biomarkers to screen LA.

2. Material and methods

2.1. Cell culture

Five lung cancer cell lines (A549, H322, SPC-A-1, H460 and H1299) and one normal human pulmonary epithelial cell line EBAS–2 B were purchased from the Cell Bank of China Academy of Sciences, Shanghai, China. Cell lines were cultured in RPMI medium 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Gibco, Grand Island, NY) and 1% penicillin-streptomycin at 37 °C in 5% CO_2 and were plated in 6-well plate at a density of 2×10^5 well. After incubation for 3 days, the cells were collected RNA isolation.

2.2. Patients and specimens

All the subjects (28 controls and 28 LA patients) were recruited at Guizhou Provincial People's Hospital (Guiyang, China) from January 2014 to December 2016. Written informed consent was obtained from all of the participants prior to blood and tumor samples collection. The study was approved by the Ethics Committee of the Guizhou Provincial People's Hospital (Guiyang, China). All subjects recruited in this study were not subjected to preoperative radiotherapy or chemotherapy and diagnosed with histopathological evaluation. The tumor stage was determined according to the International Union against Cancer's (UIAC) tumor-node-metastasis (TNM) system. Tumor tissues were acquired from LA patients by surgical operation. 5 mL of blood sample from preoperative and post-operative LA patients were collected with ethylenediaminetetraacetic acid (EDTA)-containing tubes (Becton, Dickinson and Company). The plasma was separated as described previously [35].

2.3. RNA isolation

RNA extraction from tumor tissues and cell lines was performed using Trizol reagent (Invitrogen, Carlsbad, CA, USA), whereas total RNA in plasma was isolated by using mirVana PARIS Kit (Ambion, USA), as described previously [27].

2.4. miRs expression profiling

Plasma miRs from LA patients and controls (n = 3) were labeled with Hy3 or Hy5 fluorescence using the miRCURY™ Array Power Labeling Kit (Exiqon) to obtain the fluorescent probe that can be hybridized with the chip. The labeled probe was hybridized with the miRCURY™ chip under the standard condition using the MAUI hybridization system. The fluorescence intensity of the chip was scanned with the Agilent chip scanner and analyzed using Agilent feature extraction software (version 12). The differentially expressed miRs were screened based on the fold change ≥ 2, $P < 0.05$ and FDR < 0.05, as described previously [22]. Finally, the differentially expressed miRs in plasma were displayed by hierarchical clustering analysis between the two groups. The figure was drawn by MeV software (version 4.8, Institute for Genomic Research, USA).

2.5. Real-time quantitative PCR (RT-qPCR) analysis

Reaction mixture (20 μL) containing 2 μg of total RNA was reversely transcribed to cDNA by using PrimeScript RT-polymerase (Takara, Dalian, China). Quantitative PCR was performed on the cDNA using specific primers (Sangon, Shanghai, China) as shown in Table 1. PCR reaction mixtures were contained 12.5 μL SYBR Green Supermix (Bio-Rad Laboratory, USA), 1 μL cDNA, 300 nM of each primer, and DEPC H2O to a final volume of 25 μL, and then RT-qPCR was performed using the Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific, Inc.). The Cq (quantification cycle fluorescence value) was calculated using SDS software, version 2.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.), and the relative expression levels of miRs were calculated using the $2^{-\Delta\Delta Cq}$ method [14] and normalized to the internal control U6.

Table 1
Primers were used to RT-qPCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
miR-339-5p	GGGTCCCTGTCCTCCA	TGCGTGTCGTGGAGTC
miR-21	GGGGTAGCTTATCAGACTGATG	TGTCGTGGAGCGGCAATTG
U6	CGCTTCGGCAGCACATATACTAA	TATGGAACGCTTCACGAATTTGC

2.6. Statistical analysis

Data were presented as the mean ± standard deviation (SD) for each group. All statistical analyses were performed using PRISM version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Student *t*-test was used to analyze two-group differences. Inter-group differences were analyzed by one-way analysis of variance, followed by a post hoc Tukey test for multiple comparisons. Pearson χ^2 tests were used to evaluate differences in the clinical characteristics between the two groups. Receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) were used to assess the ability of using plasma miRs as diagnostic tools for LA. The binary logistic regression analysis was used to combine the expression level of miR-339-5p and miR-21 as a combined diagnostic marker for LA. The maximum value of the Youden index was used as a criterion for selecting the optimum cut-off point. Spearman's rank analysis was used to identify the correlation of miRs levels between plasma and tumor tissues. A Bland–Altman plot (difference plot) was used to analyze the agreement of miRs expression between plasma and tumor tissues. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Clinical characteristics of control and patients with LA

Demographic features and pathological classification of 28 controls and 28 LA patients are summarized in Table 2. The pathological stage of LA patients was varying from IA to IIIB, and all of LA patients underwent surgical resection. The gender distribution had no obvious difference between control and LA patients. However, the mean age in LA patients group was 11.2 years higher than control group. In addition, the smoking history of ≥10 years in LA patients was significantly higher than that of subjects in the control group ($P = 0.031$).

3.2. MiRs expression profiles in plasma from LA patients and control

MiRs differential expression in plasma from LA patients and control was measured by miRNA Microarray assay. The average value for each miR was used for statistics after normalization. Based on the fold change ≥2, *p*-value < 0.05 and FDR ≤ 0.05, miRs were selected as candidate miRs. The findings indicated that 39 miRs were differentially expressed in plasma from LA patients and control, among which 17 miRs were significantly down-regulated, and 22 miRs were significantly up-regulated in the plasma from LA patients compared with that of control group. Based on the differential expression of miRs, a