Early Detection of Lung Cancer in Serum by a Panel of MicroRNA Biomarkers

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Abstract

With the objective of identifying serum biomarkers for early detection of lung cancer, we used TaqMan probe–based real-time reverse transcription–quantitative polymerase chain reaction (RT-qPCR) to detect microRNA (miRNA) profiles in 118 early stage lung cancer patients. Validating the miRNA profiles in additional cases and controls suggested that miRNAs might be potential biomarkers for early detection of lung cancer.

Introduction: The objective of the study was to develop a panel of microRNAs (miRNAs) as highly sensitive and specific biomarkers for lung cancer early detection. **Materials and Methods:** The study contained 2 phases: first, preliminary marker selection based on previous reports on the serum of 24 early stage lung cancer patients and 24 healthy control subjects by TaqMan probe–based real-time reverse transcription–quantitative polymerase chain reaction (RT-qPCR); and second, validation of miRNA markers on 94 early stage lung cancer, 48 stage III to IV lung cancer, and 111 healthy control serum samples. **Results:** A total of 3 miRNAs (miR-125a-5p, miR-25, and miR-126) were selected for further analysis in this study. The combination of the 3 miRNAs could produce 0.936 area under the receiver operating characteristic curve value in distinguishing early stage lung cancer patients from control subjects with 87.5% sensitivity and 87.5% specificity, respectively. The diagnostic value of the miRNA panel in an independent set of lung cancer patients confirmed the sensitivity and specificity. **Conclusion:** The results demonstrated that the panel of miRNA biomarkers had the potential for the early detection of lung cancer.

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Introduction

Lung cancer is the leading cause of cancer mortality worldwide, and the 5-year survival rate has only slightly improved in the past 3 years. There are no clinically apparent symptoms for lung cancer until it has reached an advanced stage. More than 75% of lung cancers are diagnosed after the disease is already locally advanced or metastatic, and the late diagnosis of lung cancer in three quarters of patients has led to this poor survival rate.[1-3] The 5-year survival rate in early stage non–small-cell lung cancer (NSCLC) patients is approximately 50% to 70%. In contrast, the 5-year survival rate drops to 5% to 15% and less than 2% for patients with stage III and IV NSCLC.[4,5] The ability to diagnose lung cancer patients at early stage will effectively reduce the mortality. Chest X-ray and sputum cytology have been used to detect some lung cancers at early stage, but the sensitivity is low. Although computed tomography can noninvasively detect NSCLC earlier at small size, it has a high rate of false-positive results, which may lead to unnecessary surgery. Also, the exposure to computed tomographic scans may expose patients to harmful levels of radiation that could result in more cancers.[3,6,7] Several serum protein markers, such as cytokeratin 19 fragment (CYFRA21-1), carcinoembryonic antigen, neuron-specific enolase, tissue polypeptide specific antigen, cancer-associated antigen (CA) 125, CA 19-9 and chromogranin A,[8] have been used to

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diagnose lung cancer without a surgical procedure. However, these markers have limited sensitivity and specificity. Therefore, novel noninvasive, sensitive, and specific markers are needed for the early detection of lung cancer.

MicroRNAs (miRNAs) are a class of small, highly conversed noncoding RNAs involved in many developmental processes and act as posttranscriptional regulators of gene expression. They regulate many biologic processes, including differentiation, proliferation, and apoptosis.[9,10] Altered expression of miRNAs is associated with various human cancers, acting as oncogenes or tumor suppressor genes.[11] It has been reported that miRNAs are present in human serum in a highly stable form that is resistant to RNase digestion and harsh conditions, including boiling, multiple freeze–thaw cycles, changes in pH, extended storage, and so on.[12] Because serum is relatively easy to access, circulating biomarkers hold promise as noninvasive and accurate test for cancer. The development of noninvasive biomarkers plays an important role in the early detection of lung cancer. Lung tumor is a heterogeneous disease and develops from complex and multiple processes.[13] Therefore, we hypothesized that simultaneously assessing multiple tumor-related miRNAs in serum could provide a highly sensitive and specific diagnostic test for early stage lung cancer. To verify the hypothesis, we evaluated the expression of serum miRNAs obtained from early stage lung cancer patients and cancer-free subjects to identify miRNAs as biomarkers that are capable of differentiating between the 2 groups and assessed their diagnostic value for the early detection of lung cancer.

Materials and Methods

Patients and Clinical Specimens

Lung cancer samples used in this study were collected at Shanghai Zhongshan Hospital. Clinical information was also collected for each patient at the time of blood collection. Histologic typing of the tumors was performed according to World Health Organization criteria. Staging was determined according to the seventh edition of the tumor, node, metastasis classification system classification of malignant tumors.[14] For all cancer patients, blood was collected at the time of diagnosis but before tumor resection or treatment. Healthy control subjects were collected from Shanghai Ruijin Hospital on the basis of their negative results of blood test, chest X-ray, and abdominal ultrasound examinations. Informed consent was obtained from each participant. Blood samples from the individuals were drawn in the morning into serum separator tubes. Tubes were centrifuged at 3000 rpm at 4°C for 15 minutes, within 4 hours after collection, and then the serum samples were formed into aliquots into 1.5 mL RNase-free Eppendorf tubes and stored at −80°C until use. The study was approved by institutional review board of Shanghai Zhongshan Hospital and Shanghai Ruijin Hospital.

Serum RNA Isolation

Total RNA containing small RNA was extracted from 200 μL of serum using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's protocol. There were no verified housekeeping miRNAs that can be used for normalization in serum. However, spiked-in RNAs such as cel-miR-39 and cel-miR-238 are a class of heterogeneous RNAs that have been used. Adding the same amount of spiked-in RNAs with an equal volume of serum provides a stable reference control. In addition, U6 RNA and 5S rRNA are degraded in serum samples. We thus chose cel-miR-39 as the reference miRNA.[15,16] Cel-miRNA-39 at 10 pM was spiked into the samples during RNA isolation after serum incubation with the provided 2 × denaturing solution. An equal volume of serum was processed for each sample. The final elution volume was 100 μL in water. The low amounts of RNA extractable from 200 μL of serum were not reliably measured by spectrophotometry, so fixed volumes rather than fixed amounts of RNA were used for the reverse transcription (RT) reaction in accordance with previous studies.[15] Careful normalization is essential for the accurate quantification of miRNA levels. The inclusion of the spiked-in RNAs is an important part for adjusting for differences in efficiency of RNA recovery between samples. The global median normalization method, which was usually used in microarray analysis, could be used for endogenous variation normalization. Both methods are accepted protocols for circulating miRNA studies. In the present study, we used volume normalization method for analysis.

MiRNA Quantification by Reverse Transcription–Quantitative Polymerase Chain Reaction (RT-qPCR)

The RT reaction was carried out with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, 5 μL of total RNA was reverse transcribed to cDNA using 3 μL specific stem-loop RT primers, 0.15 μL 100 mM dNTPs, 1.5 μL 10 × reverse transcription buffer, 1 μL (50 U/μL) MultiScribe Reverse Transcriptase, 0.19 μL RNase inhibitor (20 U/μL), and made up to 15 μL with RNase-free water. The 15 μL reactions were incubated in a thermocycler at 16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. QPCR was done using a standard TaqMan PCR kit protocol on a LightCycler 480 (Roche Diagnostics, Penzberg, Germany). The 20 μL PCR reaction included 1.33 μL RT products, 1 μL TaqMan miRNA assay primer and probe mix, 10 μL TaqMan 2 × Universal PCR master mix (No Amperase UNG), and 7.76 μL water. The reactions were incubated in a 96-well plate at 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. All qPCR reactions were done in triplicate, and negative controls were included with every qPCR assay. No amplification of the signal was observed when water was added instead of cDNA sample. The crossing point (Cp) was defined as the number of cycles required for the fluorescent signal to pass above background baseline in PCR. The $2^{-\Delta\Delta Cp}$ method was used to evaluate the relative expression of miRNA.[17-19] ΔCp was calculated by subtracting the Cp values of cel-miR-39 from the Cp values of the miRNA of interest. ΔΔCp was calculated by subtracting ΔCp of the normal control serum sample from ΔCp of the lung cancer serum sample, and the fold change of miRNA gene was determined by the equation $2^{-\Delta\Delta Cp}$.

Selection and Validation of Serum miRNA Markers

First, a panel of 9 miRNAs (miR-20a, miR-25, miR-486-5p, miR-126, miR-125a-5p, miR-205, miR-200b, miR-21, and miR-155) were selected on the basis of previous reports of lung