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Original Article

Serum microRNA-21 is a potential diagnostic marker for earlier lung squamous cell carcinoma detection

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Abstract: High expression of MicroRNA-21 (miR-21) in tumor tissues is associated with shortened survival in patients with lung squamous cell carcinoma (LSCC). This study was to investigate whether serum miR-21 can be used as potential biomarkers for early detection of LSCC. Quantitative reverse transcription-polymerase chain reaction (QRT-PCR) was used to measure the expression of miR-21 in serum of 50 patents with early-stage LSCC, 50 patients with late-stage LSCC (n=50), and 20 patients with pulmonary bullas. The correlation between serum miR-21 expression levels and clinicopathological characteristics of patients was performed. Receiver operating characteristic (ROC) curve was used to assess the serum miR-21 test sensitivity and specificity. Serum levels (RQ Value) of miR-21 were significantly higher in LSCC patients than in patients with pulmonary bulla (P<0.001). MiR-21 was related to TNM stage with higher in late-stage compared to early-stage, but not related age, sex, smoking status. The optimal cutoff values were 2.505 for early-stage LSCC versus pulmonary bulla, 2.570 for late-stage LSCC versus pulmonary bulla, and 4.575 for late-stage LSCC versus early-stage LSCC, and the corresponding sensitivity and specificity was 76.0% and 70.0%, 92.0% and 70.0%, and 78.0% and 78.0%, respectively. Measuring the expression levels of serum miR-21 can serve as a circulating biomarker for the early diagnosis of LSCC.

Keywords: microRNA-21, serum, lung squamous cell carcinoma, early diagnosis

Introduction

The incidence of lung cancer is the highest of all cancers in males and the second highest in females, and the mortality rate is the highest of all cancers worldwide [1]. The 5-year survival rate of patients with lung cancer has not substantially improved over the past 20 years (15-20%). Although those with stage I lung cancer, for which surgery is the major treatment method, the 5-year survival rate is as high as 60-70% [2-4]. However, approximately three-fourths of the lung cancer patients are already in the advanced stages of the disease when diagnosed. In recent decades, a variety of tumor markers for NSCLC have improved early diagnosis and patient care. In particular, these targeted drugs have greatly improved both survival times and quality of life in patients with lung adenocarcinoma. However, the efficacy of the new drugs used for the treatment of lung squamous cell carcinoma (LSCC), such as tyrosine kinase inhibitors, pemetrexed and bevacizumab, is significantly inferior. Therefore, identifying effective, noninvasive, radiationless and cost-effective methods by which to detect LSCC in the early stages is of great clinical and socioeconomic significance.

Micro RNAs (miRNAs) are a group of endogenous noncoding RNAs with the molecular weight of ~22 nucleotides that were first described in 1993. The miRNAs are involved in the regulation of cell growth and development, metabolism, and apoptosis and play important roles in the development, progression, and metastasis of tumors [5]. The miR-21 is located on chromosome 17 at q23.1, which could downregulate the transcription of tumor suppressor genes (such as PTEN and TPM1) and thus could promote the formation of tumors. Abnormal expression of miR-21 is associated with the metastasis, relapse, surgery efficacies, and resistance to radiochemotherapy of lung cancers [6-10]. Also high miR-21 expression is associated with shortened survival time in tissue

of lung squamous cell carcinoma (LSCC) patients [11]. Assessing serum miRNA expression, a noninvasive method by which to detect lung cancer, is of significance in the early diagnosis, prognosis, and treatment in managing the disease. In the present study, the value of serum miR-21 expression in early diagnosing of LSCC was investigated.

Materials and methods

Specimen collection

All blood samples were collected from the Department of Thoracic Surgery and the Department of Radiotherapy in Taizhou Hospital, Zhejiang, China, from August 2007 to August 2011. All lab specimens were collected on the second day after admission and before treatment. Written informed consent was obtained from each patient before specimen collection. This study was approved by the Ethics Committee of Taizhou Hospital, and blood specimen acquisition was carried out in accordance with institutional guidelines.

The inclusion criteria for the patients were as follows: 1) No previous tumor-related diseases; 2) Never been treated with radiotherapy or chemotherapy; 3) Postoperative pathological examinations or biopsy confirmed the diagnosis of LSCC; 4) In the early stages (I or II) or late stages (III or IV) of LSCC according to the TNM Classification of Malignant Tumors staging criteria (7th edition) issued by the American Joint Committee on Cancer; 5) Patients with pulmonary bulla were included as the control group.

The exclusion criteria were as follows: 1) Pregnant or having other tumors; 2) Complications such as infection, renal or liver dysfunction, diabetes, and cardiac-cerebral vascular diseases.

Specimen processing

Four milliliters of whole blood were added into an ethylenediaminetetraacetic acid coagulated tube and centrifuged at 4.0°C and 1200 g for 10 min to collect serum. The precipitate was centrifuged again at 4.0°C and 12000 g for 10 min to collect additional serum. The serum was placed in freezing tubes and stored at -80°C until use. For the experiments, the serum samples were put on ice and allowed to thaw; 200 μl serum were used for total RNA extraction using the miRNeasy Serum/Plasma kit (QIAGEN, Germany) according to the manufacturer's instructions, and RNase free water (QIAGEN, Germany) was used to elute the RNA from the column. The concentration and purity of the RNA were measured using a quantitative nucleic acid detector (BIO-RAD Smart Spec plus, USA). The RNA samples were stored at -80°C until use. The first quantitative reverse transcription (QRT)-PCR was performed the same day or within 24 hours after extraction of the RNA and repeated three times within one month.

The expression of miR-21 was measured with fluorescence QRT-PCR. The sequence of miR-21 was obtained from miRBase, and the primers of miR-21 and U6 (internal reference) were synthesized by Ruibo Biological Technology Co., Ltd (Guangzhou, China). Because the results of micro-spectrophotometer detection showed that total RNA concentration was relatively low, a fixed-volume model was used for the measurements in the reaction system of QRT and fluorescent PCR. For RT (reverse transcription), 2.0 μl (62.5 nM) RT products and 4.0 μl RNA sample were used, and RNase free water (QIAGEN, Germany) was added to obtain a volume of 11 μl. The mixture was placed in a 70°C water bath for 5.0 min and then immediately placed on ice to cool, after which 1.0 μl M-MLV reverse transcriptase, 5.0 μl QRT-PCR buffer, 2.0 μl (2.5 mM) dNTP mixture, 0.5 μl (40 U/μl) Rnasin Ribonuclease Inhibitor, and 0.5 μl (200 U/μl) RT enzyme were added. RNase free water (QIAGEN, Germany) was added to obtain a final volume of 25 μl. The solution was mixed and then PCR was performed (reaction conditions: 42°C 1.0 h, 70°C 10 min, and 4.0°C 10 min). The 20 μl reaction system for the fluorescent Q-PCR, 2 μl cDNA, 0.8 μl (5.0 μM) forward primer, 0.8 μl (5 μM) reverse primer, 10 μl 2×Go Taq qPCR Master Mix, and 0.2 c 100×CXR were added and Nuclease-Free water (Promega, Madison, USA) was added to obtain a total volume of 20 μl. The reaction conditions were 50°C for 2.0 min, 95°C for 10 min, 95°C for 15 sec, and 60°C for 1.0 min for 40 cycles. The conditions for the analysis of the solubility curve were 95°C for 10 sec, 60°C for 1.0 min, 95°C for 15 s, and 60°C for 15 sec. Each sample was measured in triplicate and a negative control was also used. All reactions were per-

Table 1. Clinicopathological characteristics

Characteristics		Number of patients	Pulmonary bulla	Early-stage LSCC	Late-stage LSCC	*P* value
Gender	Male	78	13	28	37	0.169
	Female	42	7	22	13	
Age	Median (min-Max) (years)	60 (38-78)	57 (38-72)	65 (42-78)	59 (48-75)	0.672
Smoking	No	46	8	23	15	0.255
	Yes	74	12	27	35	

LSCC: Lung squamous cell carcinoma; min: minimum; Max: Maximum.

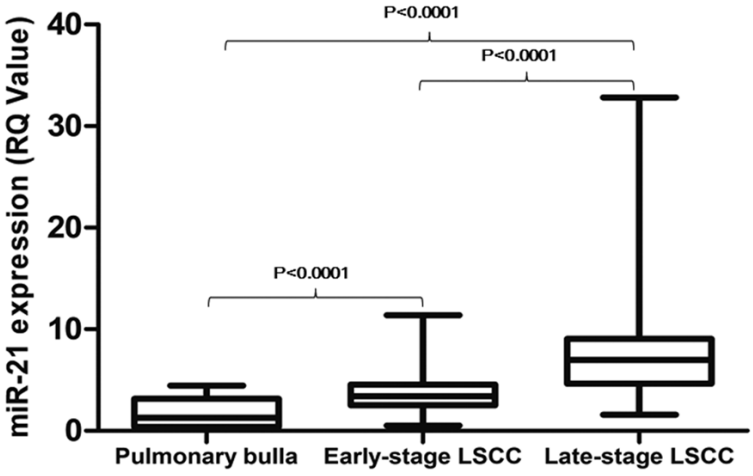

Figure 1. Difference of serum miR-21 levels in patients with pulmonary bulla and early- and late-stage LSCC. (Serum levels (RQ Value) of miR-21 were significantly higher in patients with early- and late-stage LSCC compared with pulmonary bulla (*P*<0.0001 and <0.0001, respectively). And serum miR-21 levels were significantly higher in patients with late-stage LSCC than early-stage LSCC (P<0.0001).

formed using the ABI 7500 Real-Time PCR System (Ambion Life Technologies, Grand Island, NY, USA). The kits used for the QRT-PCR were produced by Promega, Madison, USA. The $2^{-\Delta\Delta ct}$ method was used to calculate the relative expression levels of miR-21.

Statistical analyses

GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for the statistical analyses. Data for continuous variables were presented as mean value ± SD. Cateogorical variables are presented as frequencies and percentages. Comparisons between groups were performed by analysis of variance for normally distributed continuous variables and the non-parametric Mann-Whitney test for non-normally distributed variables as appropriate, while chi-square test was used for categorical variables. A receiver operating characteristic (ROC) curve was used to calculate the sensitivity and specificity. P< 0.05 was considered statistically significant.

Results

Patients

Twenty in the pulmonary bulla control group, 50 in the early-stage group (30 with stage I and 20 with stage II), and 50 in the late-stage group (24 with stage III and 26 with stage IV cancer) were enrolled in the study. The pulmonary bulla control group, median age was 57 years (38 to 72 years), 7 were females and 13 were males, respectively. The patients with early-stage LSCC, median age was 65 years (42 to 78 years), 22 were females and 28 were males, respectively. For the patients with late-stage LSCC, the median age was 59 years (48 to 75 years), 13 were females and 37 were males, respectively. Clinical features between the three groups were not statistically different (**Table 1**).

Serum miR-21 expression

Results of the real-time florescent QRT-PCR showed that the serum miR-21 RQ value levels in patients with early- or late-stage LSCC were significantly higher than in those with pulmonary bulla (P<0.0001 and <0.0001, respectively). In addition, the serum miR-21 RQ value levels in patients with late-stage LSCC were significantly higher than in those with early-stage LSCC (P<0.0001) (**Figure 1**).

Table 2. Correlation between serum microRNA-21 expression (RQ Value) and clinicopathological characteristics

Characteristics		Number of patients	microRNA-21 low expression (RQ≤3.77)*	microRNA-21 high expression (RQ>3.77)*	*P* value
Gender	Male	78	33	45	0.055
	Female	42	26	16	
Age	≤59 years	64	28	36	0.272
	>59 years	56	21	25	
Smoking	No	46	28	18	0.060
	Yes	74	21	43	
Groups	Pulmonary bulla	20	19	1	0.000
	Early-stage lung cancers	50	29	21	
	Late-stage lung cancers	50	11	39	

*RQ Value Median =3.77.

Table 3. Univariate analysis of serum microRNA-21 status (RQ Value) and clinicopathological characteristics

Variables	Exp (B)	95% CI	P
Gender	0.541	0.213~1.372	0.196
Age	0.600	0.247~1.457	0.259
Smoking	1.700	0.682~4.237	0.255
Stage	0.695	0.287~1.685	0.421
Groups	11.774	1.831~75.727	0.009

The correlation between serum miR-21 RQ value levels and clinicopathological characteristics of patients

Table 2 shows the correlation between serum miR-21 RQ value levels and clinicopathological characteristics of patients. Serum miR-21 levels were significantly correlated with three groups (P=0.000).

Univariate analysis of the correlation between serum microRNA-21 status (RQ Value) and clinicopathological characteristics

As shown in **Table 3**, Univariate analysis of serum microRNA-21 status (RQ Value) and three groups was statistically significant (P=0.009).

ROC curve analysis

The ROC curve showed that serum miR-21 could distinguish the patients with pulmonary bulla and early-stage and late-stage LSCC (**Figure 2A**, **2B**). Compared with patients with pulmonary bulla, the area under the ROC curve (AUC) of serum miR-21 levels was 0.8040 (95% CI: 0.6875-0.9205) and 0.9420 (95% CI: 0.8921-0.9919) for patients with early- and late-stage LSCC, respectively. In addition, AUC for patients with late-stage LSCC was 0.7764 (95% CI: 0.6844-0.8684) compared with those with early-stage LSCC (**Figure 2C**). The optimal cutoff values were 2.505 for early-stage LSCC versus pulmonary bulla, 2.570 for late-stage LSCC versus pulmonary bulla, and 4.575 for late-stage LSCC versus early-stage LSCC, and the corresponding sensitivity and specificity was 76.0 and 70.0%, 92.0 and 70.0%, and 78.0 and 78.0% respectively.

Discussion

The discovery of micro RNA (miRNA) has promoted the molecular diagnosis of tumors to a new level. Abnormal regulation of miRNA is closely related to the development and progression of tumors, cell apoptosis, and cell growth [12-15]. Similarly, many studies have reported that miRNA were abnormal expressed in NSCLC tissues compared with adjacent normal lung tissues [16-18]. The miR-21 have reported that was highly expressed in many cancers, such as breast, colon, lung, pancreas, prostate, and stomach. The miR-21 had been considered as a novel potential biomarker in the diagnosis, prognosis, and treatment of malignancies [14, 19]. Recent studies had shown that miR-21 drove tumorigenesis through inhibition of negative regulators of the Ras/MEK/ERK pathway and inhibition of apoptosis in NSCLC [20]. And Zhang J, et al. found that the miR-21 also targeted tumor suppressor-gene, phosphatase and tensin homolog (PTEN) in NSCLC [21]. Also Wen Gao et al. [11] found that high miR-21 expression is associated with

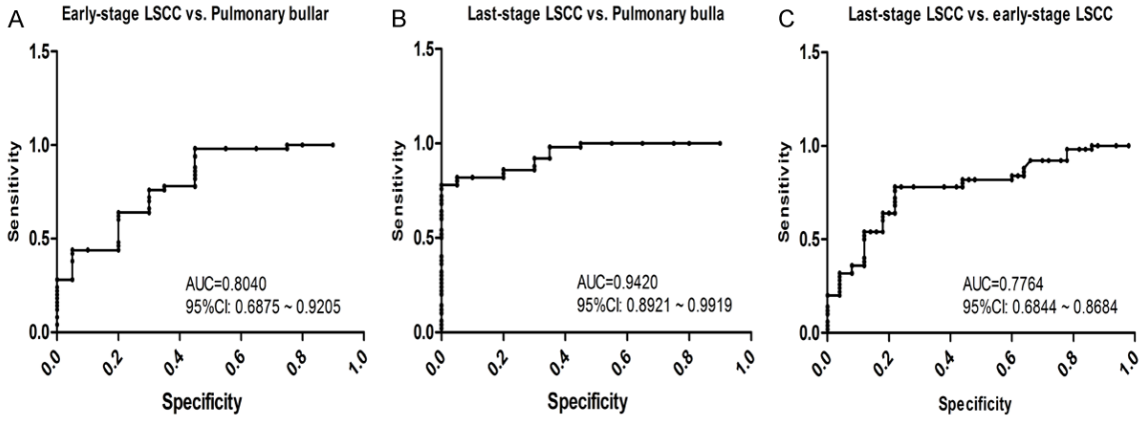

Figure 2. ROC curves of miR-21. A: Early-stage LSCC vs. pulmonary bulla (The corresponding sensitivity and specificity was 76.0% and 70.0%); B: Late-stage LSCC vs. pulmonary bulla (The corresponding sensitivity and specificity was 92.0% and 70.0%); and C: Late-stage LSCC vs. early-stage LSCC (The corresponding sensitivity and specificity was 78.0% and 78.0%).

shortened survival time, indicating that miR-21 may serve as a molecular diagnostic and prognostic marker for patients with squamous cell lung carcinoma. The potential prognostic value of miR-21 may also be able to help physicians identify and select the patients who are most likely to benefit from therapy, in order to improve the treatment outcome of squamous cell lung carcinoma.

Mitchell et al. [11, 22-24] showed that there are great numbers of stable miRNAs in human serum. miRNAs in circulating blood come from tumor tissues and circulating tumor cells, and thus different tumors can induce an abnormal expression of the corresponding miRNA which makes it possible to diagnose diseases using serum levels of miRNAs. Liu et al. [25] found that high expression of serum miR-21 was associated with a poor survival in NSCLC patients. In the present study, we found that the serum miR-21 expression levels were significantly higher in patients with early- or late-stage LSCC than in those with pulmonary bulla, and the levels were also higher in those with late-stage LSCC than in those with early-stage LSCC (all P values <0.001). The sensitivity and specificity of serum miR-21 in distinguishing with pulmonary bulla were 76.0 and 70.0% for the diagnosis of early-stage LSCC, and 92.0 and 70.0% for the diagnosis of late-stage LSCC respectively. The sensitivity and specificity of serum miR-21 in distinguishing early- and late-stage LSCC were 78.0% and 78.0% respectively. We speculate that the reasons for the increased serum miR-21 levels in late-stage LSCC patients could be from the increased tumor cells that enter the circulatory system with the progression of the tumors. Previous studies found also that serum miR-21 levels were higher in patients with head and neck squamocellular carcinomas than in healthy controls [26]. Therefore, in further studies, the sample size will be increased to investigate the differences in the expression of miR-21 in serum before and after surgery as well as before and after radiochemotherapy. The relationships between miR-21 levels and the survival of patients will also be explored to help identify noninvasive, cost-effective examination methods by which to facilitate individual treatments for LSCC patients. In summary, the findings of the present study confirmed that serum miR-21 levels were higher in LSCC patients, even in those with early-stage LSCC, compared with patient with pulmonary bulla. In addition, the serum miR-21 levels were different between patients with early- and late-stage LSCC. Determining the miR-21 levels in human serum could help in the early diagnosis and staging of LSCC.

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Disclosure of conflict of interest

None.

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