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Original Article

Clinical significance of serum miR-31 as a predictive biomarker for lung adenocarcinoma

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Abstract: Emerging studies have demonstrated that microRNA-31 (miR-31) play a critical role in the tumorigenesis of lung adenocarcinoma (LUAD). Nonetheless, the diagnostic and prognostic value of serum miR-31 in LUAD was largely unknown. The purpose of this study was to investigate the association between serum miR-31 levels and prognosis of LUAD. We first analyzed miR-31 as a predictor of overall survival in the LUAD patients from The Cancer Genome Atlas (TCGA). Then miR-31 expression was measured in the serum samples from LUAD patients and healthy controls using qRT-PCR. LUAD patients with higher tissue miR-31 expression suffered a significantly shorter overall survival than those with lower tissue miR-31 expression. Serum miR-31 levels were upregulated in patients with LUAD and could distinguished LUAD patients from healthy control subjects. Increased serum miR-31 expression was significantly associated with lymph node metastasis, high pathological grade and advanced clinical stage. In addition, patients assigned to the high serum miR-31 group exhibited poorer overall survival and recurrence free survival compared with patients in the low serum miR-31 group. Furthermore, high serum miR-31 expression was an independent unfavorable prognostic factor. Our study suggests that serum miR-31 may have clinical value in the early detection and outcome prediction of LUAD.

Keywords: MicroRNA-31, biomarker, lung adenocarcinoma, prognosis, diagnosis

Introduction

Lung cancer is the leading cause of cancer-related death around the world and lung adenocarcinoma (LUAD) is the most common pathological type [1, 2]. Surgical resection remains the most effective form of therapy for LUAD. However, due to the lack of specific symptoms, most patients with LUAD are diagnosed at the advanced stage when surgery is not appropriate [3]. Therefore, identification of diagnostic and prognostic markers with high accuracy is imperative for improving the clinical outcome of LUAD.

MicroRNAs (miRNAs), which are endogenous, highly conserved, short 18-25 nucleotide-long, single stranded non-coding RNA molecules, have been demonstrated as critical players in regulating various biological function [4, 5].

They regulate gene expression by binding to the 3' untranslated regions (UTRs) of target message RNAs [6]. Given the important function of miRNAs, the dysregulation of miRNAs is closely associated with LUAD development. miRNA-432 was reduced in LUAD tissues and associated with worse clinicopathological parameters. In addition, ectopic expression of miR-432 inhibited cell proliferation and increased cisplatin sensitivity by directly targeting E2F3 and AXL, indicating miR-432 acted as a tumor suppressive miRNA [7]. MiR-297 levels were upregulated in lung adenocarcinoma tissues and cell lines. Overexpression of miR-297 promoted the proliferation, migration and invasion capacity of lung cancer cells by downregulating Glypican-5, suggesting miR-297 is an oncogenic miRNA in LUAD [8].

As miRNAs are resistance to endogenous RNase activity, they are very stable in the circulation system [9]. Thus detecting the changes of circulating miRNAs might contribute to the surveillance, diagnosis and prediction of prognosis of LUAD. The role of miR-31 in lung cancer

Table 1. Association of serum miR-31 expression with the clinical characteristics of LUAD

Variables	n 113 (100%)	Low miR-31 55 (48.7%)	High miR-31 58 (51.3%)	*P*
Gender				
Male	59 (52.2%)	26 (23.0%)	33 (29.2%)	0.306
Female	54 (47.8%)	29 (25.7%)	25 (22.1%)	
Age				
<60	50 (44.2%)	22 (19.5%)	28 (24.8%)	0.376
≥60	63 (55.8%)	33 (29.2%)	30 (26.5%)	
Tumor size (cm)				
<3	65 (57.5%)	34 (30.1%)	31 (27.4%)	0.368
≥3	48 (42.5%)	21 (18.6%)	27 (23.9%)	
Lymph node metastasis				
No	67 (59.3%)	38 (33.6%)	29 (25.7%)	0.039
Yes	46 (40.7%)	17 (15.0%)	29 (25.7%)	
TNM stage				
I-II	82 (72.6%)	46 (40.7%)	36 (31.9%)	0.010
III-IV	31 (27.4%)	9 (8.0%)	22 (19.5%)	
Pathological grade				
I-II	85 (75.2%)	47 (41.6%)	38 (33.6%)	0.014
III	28 (24.8%)	8 (7.1%)	20 (17.7%)	

remains controversial [10-12]. In addition, the clinical significance of serum miR-31 is unclear. The aim of current study was evaluate serum miR-31 levels in patients with LUAD and then determine its potential diagnostic and prognostic value in this malignancy.

Materials and methods

Study population

The current serum based study was approved by the Ethics Committee of Cangzhou Central Hospital. All patients and healthy volunteers gave written informed consent prior blood withdrawal. A total of 113 patients were diagnosed as LUAD in the Department of Cardiothoracic Surgery. None of these patients received chemotherapy, radiotherapy, and surgery before serum sample collection. The primary end points were overall survival (OS) time and recurrence free survival (RFS) time. Death and recurrence was recorded as events for OS and RFS respectively. OS and RFS were defined as the period between the date of diagnosis and recurrence/metastasis, death, or last day of follow-up. The detailed clinicopathological factors of the patients were reported in **Table 1**. Serum samples from thirty volunteers with no history of any cancer and in good state were used as controls.

Serum sampling

Five milliliters of venous blood was collected from each participant and processed within 1 hour of collection. The whole blood was centrifuged at 500 g for 10 min and then 10,000 g for 30 min at 4°C. The isolated serum samples were aliquoted in sterile tubes and stored at -80°C until further processing.

TCGA patient data and miR-31 expression

The miR-31 expression levels (RNAseq V2 level 3 data) of LUAD samples and corresponding clinical information were obtained from The Cancer Genome Atlas (TCGA) data portal. Then the association between miR-31 expression in LUAD with overall survival based on TCGA cohort was analyzed.

RNA isolation and qRT-PCR analysis

Small RNAs were enriched from 300 µl serum samples using the miRcute miRNA Isolation Kit (Tiangen Biotech Co., LTD.; Beijing, China) according to the manufacturer's instructions. cDNA was reverse transcribed from 5 µl of RNA with the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and then amplified by SYBR® Green PCR Master Mix (Applied Biosystems). qRT-PCR was performed on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) and carried out at the following conditions: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The relative expression levels of serum miR-31 were normalized by miR-16, and the $2^{-\Delta\Delta Ct}$ method was used to quantify miRNA expression. All reactions were run in triplicate.

Statistical analysis

The Mann-Whitney U-test was conducted to compare the differences in the serum miR-31

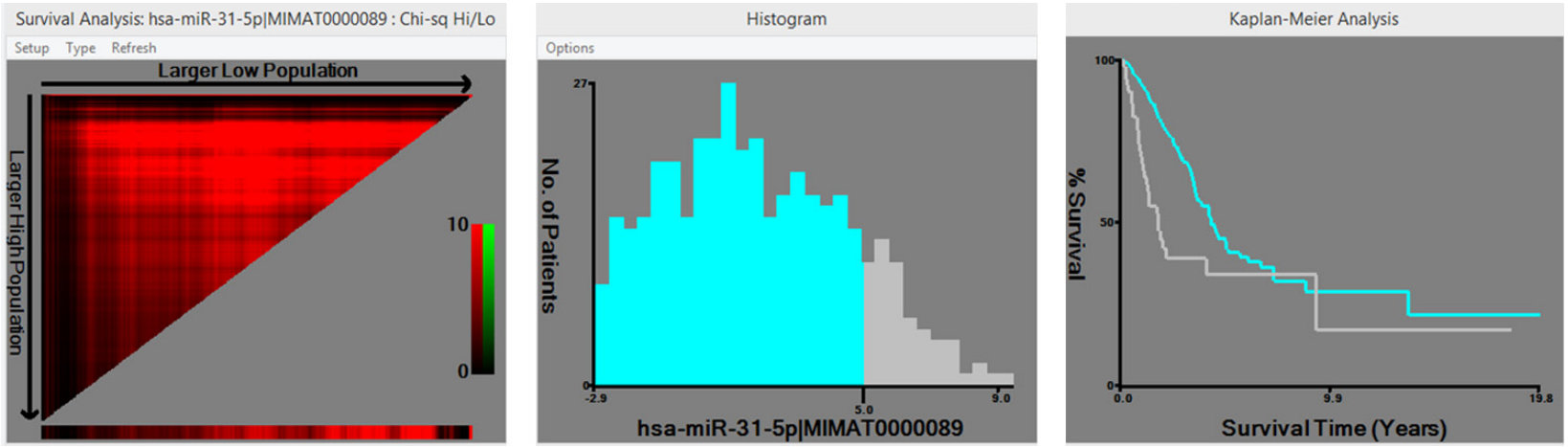

Figure 1. Association between miR-31 expression and long term overall survival in LUAD patients from TCGA data portal.

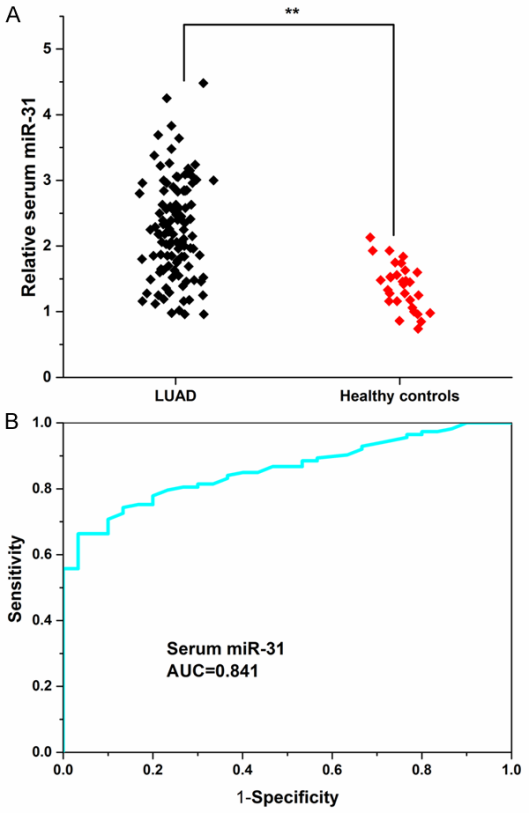

Figure 2. Diagnostic value of serum miR-31 in LUAD.

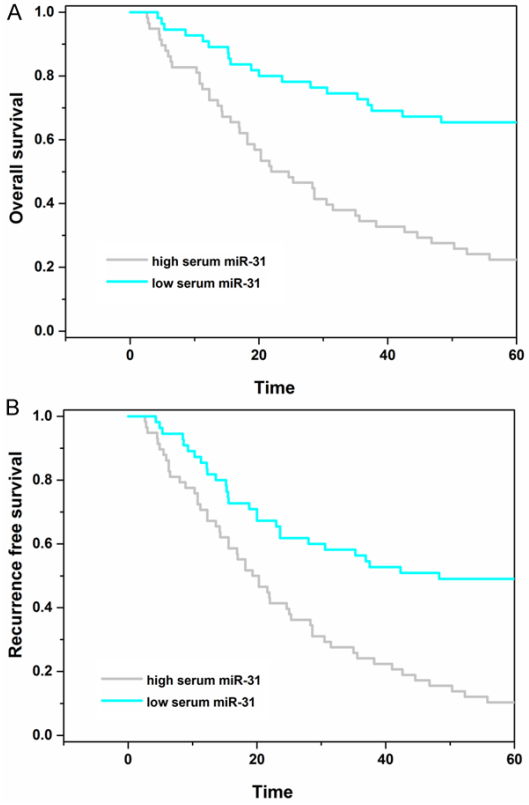

Figure 3. Association between serum miR-31 levels and survival in LUAD patients.

expression levels between the LUAD patients and the healthy controls. Receiver-operating characteristic (ROC) curves and the area under the ROC curve (AUC) were used to assess the diagnostic performance of serum miR-31 levels to discriminate LUAD patients from healthy volunteers. The correlations between the serum miR-31 levels and the clinicopathological parameters were analyzed with Chi-square test. The Kaplan-Meier method was used for the survival analysis and the log-rank test was used to explore the potential statistical difference. Cox's proportional hazard regression test was used to determine the prognostic factors. All *P* values are two-sided and *P* values less than 0.05 were considered to be statistical significant. All of the statistical analyses were performed by OriginPro8.0 (OriginLab Corporation, Northampton, MA, USA).

Results

High tissue miR-31 expression was associated with poor clinical outcome in LUAD

A total of 394 LUAD patients from TCGA cohort were included in this analysis. X tile software was used to find out the most optimal cut-off point to divide the LUAD patient cohort into high miR-31 expression group (gray color, n=53) and low miR-31 expression group (light blue color, n=341) based on the clinical outcome. The survival analysis showed that LUAD patients in the low miR-31 expression group had a better long term overall survival than those in the high miR-31 expression group (**Figure 1**).

Serum miR-31 was upregulated in LUAD and its diagnostic value

We then compared the expression level of serum miR-31 between LUAD patients and healthy volunteers by qRT-PCR. Our results demonstrated serum miR-31 levels were remarkably elevated in LUAD patients when compared to the healthy controls (**P<0.01) (**Figure 2A**). ROC analysis was performed to evaluate the diagnostic performance of serum miR-31 in LUAD. The results showed that serum miR-31 was able to differentiate the LUAD patients from healthy controls, with an AUC value of 0.841 (**Figure 2B**).

Table 2. Univariate and multivariate analyses of prognostic factors in LUAD for 5-year overall survival

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
Serum miR-31						
High vs low	2.384	1.291-3.814	0.01	2.525	1.316-4.283	0.01
Gender						
Male vs female	1.451	0.865-2.132	0.22			
Age						
≥60 vs <60	1.269	0.795-1.833	0.43			
Tumor size (cm)						
≥3 vs <3	1.718	0.961-2.926	0.12			
LN metastasis						
Yes vs no	2.172	1.158-3.549	0.03	2.085	1.092-3.375	0.04
TNM stage						
III-IV vs I-II	2.893	1.502-4.387	<0.01	3.117	1.628-4.928	<0.01
Grade						
III vs I-II	1.850	0.978-3.145	0.07			

The association between serum miR-31 levels and clinicopathological parameters of LUAD

The median value of serum miR-31 levels was used as a cut-off point to divide the LUAD patients into two groups (high serum miR-31 group n=58; low serum miR-31 serum group n=55). Then the association between serum miR-31 levels and clinicopathological parameters of LUAD was analyzed by using. Chi-square test. High serum miR-31 was positively correlated with the presence of lymph node metastasis (LN metastasis, *P*=0.039), TNM stage (*P*=0.010) and pathological grade (*P*=0.014). However, it was not associated with gender, age and tumor size (**Table 1**).

The prognostic significance of serum miR-31 levels in LUAD

Kaplan-Meier survival curves were plotted and log rank analysis was performed to evaluate the prognostic value of serum miR-31 expression for patients with LUAD. The results revealed that LUAD patients in the high serum miR-31 group had both worse 5 year OS and RFS than the patients in the low serum miR-31 group (***P*<0.01) (**Figure 3A**, **3B**).

Univariate analysis showed that 5 year overall survival was strongly associated with serum miR-31 levels (*P*=0.01), LN metastasis (*P*=0.03) and TNM stage (*P*<0.01). Multivariate analysis of these parameters indicated that serum miR-31 was an independent unfavorable prognostic factor for LUAD patients (Hazard ratio =2.525, 95% CI= 1.316-4.283, *P*=0.01) (**Table 2**).

Discussion

In this study, we analyzed the relationship of serum miR-31 expression and the outcome of the patients. Our results found that serum miR-31 levels were increased in LUAD patients and accurately discriminated patients from healthy controls. Furthermore, patients with higher serum miR-31 levels had worse clinicopathological parameters and poorer clinical outcome. Therefore, miR-31 is an oncomiR in LUAD and serum miR-31 might serve as a potential diagnostic and prognostic biomarker for LUAD.

Consistent with prior research, the expression level of miR-31 was significantly upregulated in LUAD patients with lymph node metastasis compared with those without lymph node metastasis. In addition, upregulation of miR-31 predicted poor prognosis in LUAD. Moreover, ectopic expression of miR-31 promoted the proliferation, migration and invasion capacity of lung cancer cells, suggesting miR-31 acted as an oncogene in LUAD [10]. Interestingly, a recent study showed that overexpression of miR-31 specifically in mouse lung could lead to lung hyperplasia, followed by the development of adenoma and adenocarcinoma, suggesting that miR-31 might be a driver of lung tumorigenesis [13]. However, Hou et al reported that miR-31 upregulation inhibited the proliferation of lung adenocarcinoma CSC-like cells both *in vitro* and *in vivo* by targeting MET-PI3K-Akt signaling pathway, and miR-31 downregulation resulted in opposite findings [14]. The contradictory findings of miR-31 in lung cancer indicate that the functional role of miR-31 is extremely complexy and closely correlated with the context. As both tissue and serum miR-31 expression levels are strongly associated with the clinical outcome of LUAD, this molecule definitely plays a critical role in the initiation

and progression of LUAD. Therefore, further studies are necessary in order to elucidate the molecular role of miR-31 in LUAD.

Similarly, miR-31 played an oncogenic role in head and neck squamous cell carcinoma and ARID1A was identified as its downstream target [15]. The expression of miR-31 was significantly upregulated in submucosally invasive colorectal cancer tissues when compared to the paired normal controls, suggesting that deregulation of miR-31 might be important for the early stage of carcinogenesis in colorectal cancer [16]. MiR-31 expression was elevated in cervical cancer tissues and associated with unfavorable clinical variables. In addition, miR-31 overexpression promoted the proliferation, migration and invasion capability of cancer cells [17].

Cyclin-dependent kinase Inhibitor 2A (CDKN2A) is a known tumor suppressor gene. MiR-31, which is located -0.5 Mb telomeric to CDKN2A, is found to commonly deleted various types of cancers [18-20]. Thus miR-31 might play a tumor suppressive role in tumorigenesis. For instance, the expression level of miR-31 was downregulated in liver cancer tissues and associated with poor prognosis. Ectopic expression of miR-31 inhibited cell proliferation by regulating cell cycle proteins and epithelial-mesenchymal transition related proteins [21]. MiR-31 was found to be underexpressed in gastric cancer and downregulation of miR-31 was demonstrated to be a poor prognostic indicator [22].

In conclusion, elevated serum miR-31 level is associated with poor prognosis of lung adenocarcinoma. Our study provides strong evidence that serum miR-31 represents an extremely promising diagnostic and prognostic biomarker for LUAD.

Disclosure of conflict of interest

None.

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