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Original Article

Downregulation of serum miR-101 is associated with worse prognosis in non-small cell lung cancer

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Abstract: Non-small cell lung cancer (NSCLC) is the leading cause of cancer-associated deaths, worldwide, and its prognosis is unfavorable. The aim of this study was to detect serum miR-101 levels in NSCLC patients and investigate its potential diagnostic and prognostic value. A total of 93 patients with NSCLC, 40 cases with various benign lung disease, and 55 healthy volunteers, were enrolled. Quantitative RT-PCR was performed to determine relative serum miR-101 levels in our participants. Decreased serum miR-101 expression was observed in patients with NSCLC and was closely associated with aggressive clinical characteristics. In addition, a significant increase in serum miR-101 levels was found in 36 NSCLC cases after tumor resection. Moreover, receiver-operating characteristic (ROC) curve analysis showed that serum miR-101 was an effective indicator for NSCLC diagnosis. Furthermore, Kaplan-Meier survival curve analysis revealed that low serum miR-101 expression predicted poor overall survival and disease-free survival. Finally, multivariate analysis confirmed serum miR-101 expression was an independent prognostic factor for NSCLC patients. In conclusion, serum miR-101 might serve as a potential biomarker for detection and prognosis evaluation of NSCLC.

Keywords: Non-small cell lung cancer, serum miR-101, biomarker, prognosis

Introduction

Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-associated deaths, worldwide, and accounts for approximately 85% of all lung cancer cases [1, 2]. In China, the incidence and mortality of NSCLC have steadily increased over past decades [3, 4]. Although great advancement has been made in lung cancer therapy, 5-year survival rates of patients with NSCLC remain poor. Emerging evidence has revealed that prognosis of patients could be improved with early detection of NSCLC [5]. Thus, identification of effective biomarkers for early diagnosis and prediction of prognosis for NSCLC subjects is urgently needed.

MicroRNAs (miRNAs) are endogenous short non-coding RNAs that function as RNA interference to promote mRNA degradation or inhibit protein translation [6]. Growing evidence has suggested that dysregulation of miRNAs plays essential roles in a wide variety of biological processes including development, differentiation, invasion, proliferation, and apoptosis [7, 8]. As miRNAs are highly stable and readily detectable in plasma/serum, circulating miRNAs have been used for detection and prognosis of NSCLC. For instance, serum miR-195, miR-193b, miR-301, miR-141, and miR-200b have distinguished NSCLC patients from healthy controls, with high accuracy, and have been used as potential biomarkers for NSCLC diagnosis [9, 10].

miR-101, located at chromosome 1p31.3 and chromosome 9p24.1 [11], has been proven to be frequently reduced in various types of cancers including NSCLC [14, 15], glioblastoma [16], papillary thyroid carcinoma [17], gastric cancer [18], breast cancer [19], hepatocellular carcinoma [20, 21], ovarian cancer [22], gallbladder cancer [23], and esophageal cancer [24]. The diagnostic and prognostic roles of serum miR-101 in NSCLC, however, remain poorly known. Thus, the purpose of this study

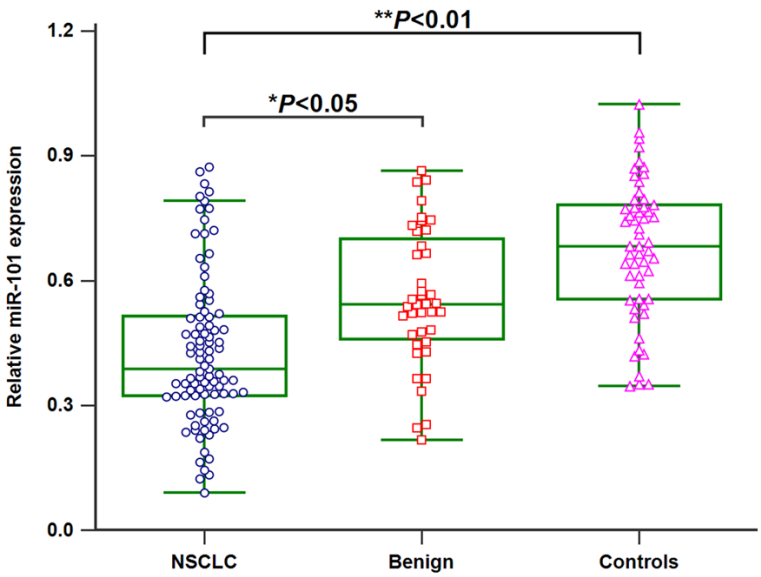

Figure 1. Serum miR-101 levels in NSCLC patients were significantly lower than those in benign lung disease patients and healthy volunteers.

was to investigate the clinical significance of serum miR-101 for patients with NSCLC.

Materials and methods

Ethics statement

This study was conducted with approval of the Ethics Committee of Xinqiao Hospital. All participants provided written informed consent. All serum specimens were handled and made anonymous, according to ethical and legal standards.

Patients and serum preparation

A group of 93 cases, diagnosed with NSCLC, were enrolled in this study. Patients were excluded if they had received chemotherapy or radiotherapy before surgery. This group included 60 men and 33 women. In 93 patients, 45 were adenocarcinomas and 48 were squamous cell carcinomas. Based on the tumor-node-metastasis (TNM) staging system of the Union for International Cancer Control (UICC), 31, 23, 18 and 21 subjects exhibited stage I, II, III, and IV cancer, respectively. Detailed clinical features of these NSCLC subjects are summarized in **Table 1**. Overall survival (OS) was calculated from the date of diagnosis to date of death or last follow up. Disease-free survival (DFS) was calculated from the date of diagnosis to date of recurrence or death or last follow up. Also, 40 cases with various benign lung disease and 55 healthy volunteers were recruited.

Up to 5 mL of peripheral blood was obtained from each participant. Blood samples were centrifuged at 1500 g for 10 minutes, within 30 minutes after collection. Supernatants were divided into aliquots and stored at -80°C until use. Moreover, serum specimens were obtained from 36 NSCLC patients three months after tumor resection.

RNA isolation and quantitative RT-PCR

Total RNA was extracted from serum samples using mirVana PARIS RNA isolation kit (Applied Biosystems, Foster City, USA). Reverse-transcription was carried out with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). PCR reaction was performed, in triplicate, using TaqMan 2× Universal PCR Master Mix and was run on an ABI 7500 Real-Time PCR System (Applied Biosystems, CA, USA). Caenorhabditis elegans miRNA cel-miR-39 was used as a synthetic spike-in control RNA oligonucleotide and relative quantification of miR-101 in serum was calculated with $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Statistical analyses were processed with MedCalc 9.3.9.0 (MedCalc, Mariakerke, Belgium) and GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA) software. *P* value less than 0.05 was considered statistically significant. Significance of serum miR-101 expression between unpaired groups and multiple comparison groups was performed using Mann-Whitney U test and Kruskal-Wallis test, respectively. Categorical variables were compared by Pearson's Chi-square test. Receiver operating characteristic (ROC) curve was generated and area under the curve (AUC) was calculated to evaluate the potential value of serum miR-101 for

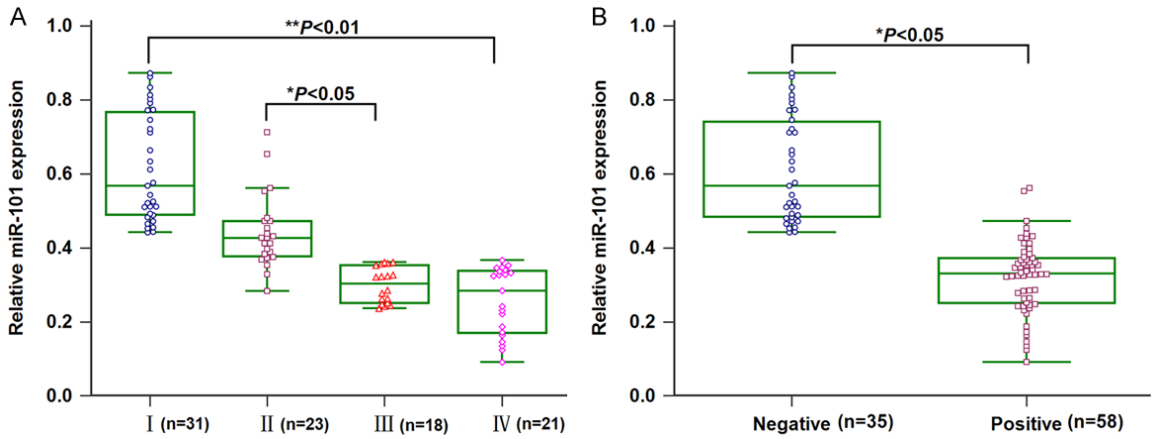

Figure 2. A. Low miR-101 expression was positively correlated with higher TNM stage. B. Patients with lower miR-101 expression experienced more frequent lymph node metastasis.

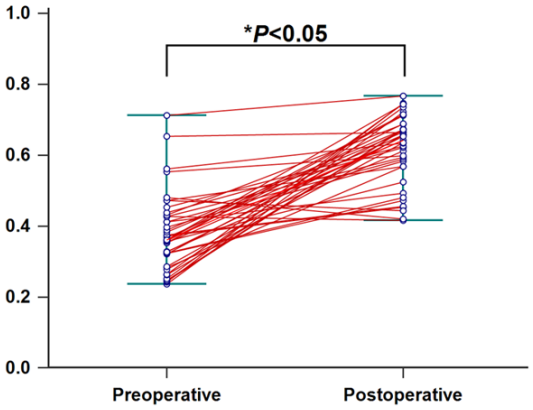

Figure 3. miR-101 levels in post-operative blood samples from 36 NSCLC patients with tumor resection were significantly higher than those in pre-operative samples.

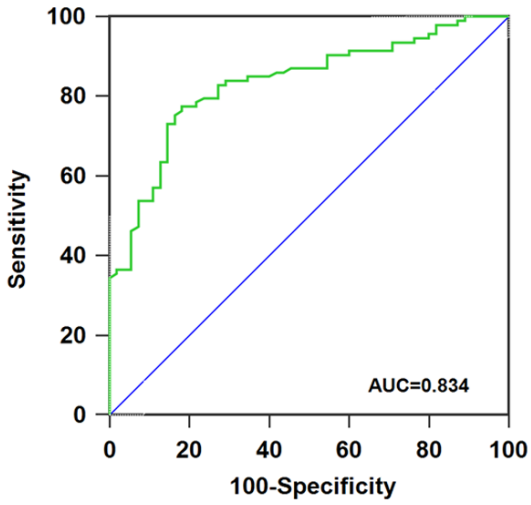

Figure 4. ROC curve to evaluate the value of serum miR-101 levels in 93 NSCLC subjects compared to 55 normal controls.

NSCLC. OS and DFS curves were plotted using Kaplan-Meier survival curve method and log-rank test was used to evaluate differences. Cox proportional-hazards regression analysis was carried out to estimate multivariate hazard ratios for OS and DFS.

Results

Reduced serum miR-101 in NSCLC patients and its diagnostic value

Serum miR-101 levels in 55 healthy volunteers, 40 patients with various benign lung disease, and 93 NSCLC patients were measured by qRT-PCR. We found that serum miR-101 levels in NSCLC patients were greatly lower than those in normal controls (P<0.01), as well as benign lung disease cases (P<0.05, **Figure 1**). As shown in **Figure 2A**, serum miR-101 expression in stage I or II patients was dramatically higher compared to those in IV or III stage cases (P< 0.01 and P<0.05, respectively). Moreover, a significant decrease in serum miR-101 expression was observed in patients with lymph node metastasis compared to those without (P< 0.05, **Figure 2B**).

Subsequently, we assessed serum miR-101 expression levels in 36 paired serum specimens. Serum miR-101 levels in post-operative blood samples were higher than those in pre-operative samples (P<0.05, **Figure 3**). Additionally, ROC curve analysis showed that levels of

Table 1. Association between serum miR-101 expression and different clinical features in NSCLC patients

Clinical parameters	Cases (N=93)	Serum miR-101 expression		*P*
		Low (n=47)	High (n=46)	
Age				0.4638
<60	44	24	20	
≥60	49	23	26	
Gender				0.3140
Male	60	28	32	
Female	33	19	14	
Lymph node metastasis				0.0042
Positive	58	36	22	
Negative	35	11	24	
TNM stage				<0.0001
I/II	54	16	38	
III/IV	39	31	8	
Tumor size				0.1739
<3 cm	36	15	21	
≥3 cm	57	32	25	
Smoking				0.4634
Yes	62	33	29	
No	31	14	17	
Histology				0.2551
Adenocarcinoma	45	20	25	
Squamous cell carcinoma	48	27	21	
Differentiation				0.5976
G1+G2	50	24	26	
G3	43	23	20	

serum miR-101 were significantly distinguishable between NSCLC patients and normal controls, with AUC of 0.834, sensitivity of 77.4%, and specificity of 81.8% (**Figure 4**).

Association between clinical variables and serum miR-101 expression in NSCLC patients

To analyze correlation between serum miR-101 expression and clinicopathological features, we used median miR-101 expression as a cut-off point to divide all NSCLC subjects into a low (n=47) and a high (n=46) expression group. As displayed in **Table 1**, downregulated serum miR-101 expression was strongly associated with advanced TNM stage (*P*<0.0001) and lymph node metastasis (*P*=0.0042). However, there were no obvious changes between serum miR-101 expression and age, gender, tumor size, smoking, histology, and differentiation (all *P*>0.05).

Serum miR-101 correlated with prognosis in NSCLC patients

Kaplan-Meier survival analysis demonstrated that NSCLC patients in low serum miR-101 expression group had shorter OS (*P*=0.024, **Figure 5A**) and DFS (*P*=0.015, **Figure 5B**) than those in the high expression group.

Multivariate Cox regression analysis showed that serum miR-101 expression (HR=2.34, 95% CI=1.08-3.65, *P*=0.031), lymph node metastasis (HR=3.21, 95% CI=1.27-5.39, *P*=0.018), and TN-M stage (HR=4.13, 95% CI=1.46-6.98, *P*=0.006) were closely correlated with OS. In addition, serum miR-101 expression (HR=2.52, 95% CI=1.17-4.06, *P*=0.025), lymph node metastasis (HR=3.53, 95% CI=1.32-5.87, *P*=0.013), and TNM stage (HR=4.42, 95% CI=1.58-7.41, *P*=0.002) were independent prognostic markers for DFS (**Table 2**).

Discussion

miRNAs are stably detectable in blood and circulating miRNAs have been regarded as an ideal class of biomarkers for cancer detection [12, 13]. In this study, we revealed that serum miR-101 expression was significantly underexpressed in NSCLC patients compared with benign lung disease patients and healthy volunteers. In addition, downregulation of serum miR-101 was closely associated with advanced TNM stage and positive lymph node metastasis. Moreover, serum miR-101 levels, in 36 NSCLC cases, were greatly elevated after tumor resection. ROC curve analysis showed that serum miR-101 had a moderate diagnostic value for NSCLC. Furthermore, Kaplan-Meier survival curve analysis showed that NSCLC

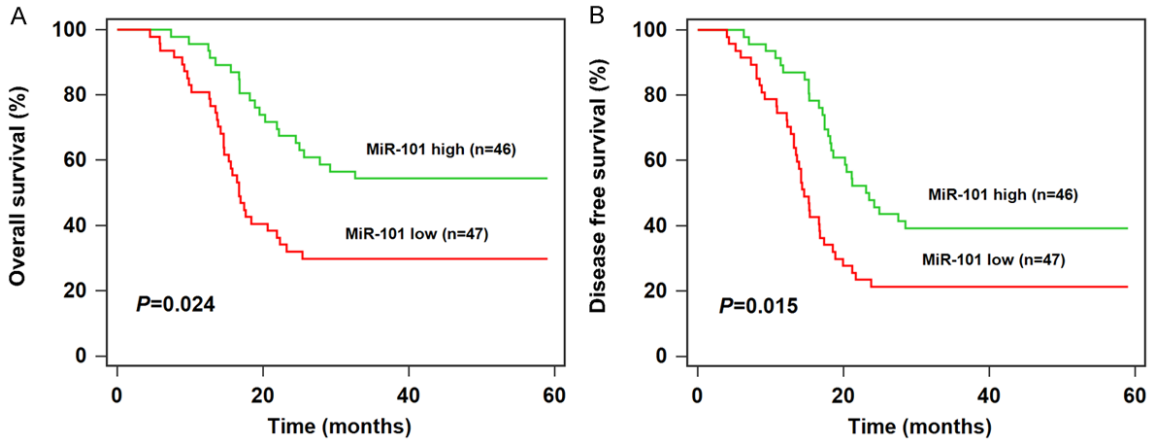

Figure 5. A. Low serum miR-101 expression was significantly associated with worse OS. B. Low serum miR-101 expression was significantly associated with worse DFS.

Table 2. Multivariate analysis of overall survival and disease-free survival in 93 NSCLC cases

Variables		HR (95% CI)	*P* value
Overall survival			
Lymph node metastasis	Positive vs Negative	3.21 (1.27-5.39)	0.018
TNM stage	III/IV vs I/II	4.13 (1.46-6.98)	0.006
Serum miR-101	Low vs High	2.34 (1.08-3.65)	0.031
Disease free survival			
Lymph node metastasis	Positive vs Negative	3.53 (1.32-5.87)	0.013
TNM stage	III/IV vs I/II	4.42 (1.58-7.41)	0.002
Serum miR-101	Low vs High	2.52 (1.17-4.06)	0.025

patients in the low serum miR-101 expression group had a significantly shortened OS and DFS. Finally, multivariate analysis confirmed serum miR-101 expression as an independent prognostic indicator for NSCLC patients. These data suggest that miR-101 might exhibit tumor suppressive properties in NSCLC.

Our findings were consistent with previous results. Yan et al. found that loss of miR-101 expression primarily occurred in lung cancer tissues and cells. Moreover, miR-101 inhibition promoted lung tumorigenesis by targeting DNMT3a-dependent DNA methylation [14]. Zhang and colleagues reported that miR-101 expression was reduced in NSCLC tissues. Enhanced miR-101 expression restrained NSCLC cell proliferation and invasion [15].

miR-101 has also been found to exert tumor-suppressive functions in tumor types other than NSCLC. In glioblastoma, miR-101 was significantly decreased in cancerous tissues and cell lines. Moreover, downregulation of miR-101 promoted cancer cell proliferation, migration, and invasion *in vitro* and *in vivo* by regulating SOX9 [16]. Lin et al. revealed that miR-101 was greatly decreased in papillary thyroid carcinoma tissues and elevated miR-101 expression markedly suppressed cell proliferation in cancer cell lines by directly targeting Rac1 [17]. Loss of miR-101 was observed in gastric cancer tissues and positively correlated with poor clinical outcome. Furthermore, restoration of miR-101 repressed carcinogenesis *in vitro* and *in vivo* through targeting COX-2 [18]. In breast cancer, Wang and colleagues showed that miR-101 expression was under-expressed, both in cancerous samples and cell lines. Forced miR-101 expression remarkably restrained proliferation and stimulated apoptosis *via* degrading Janus kinase 2 [19]. miR-101 expression was dramatically decreased in hepatocellular carcinoma tissues. In addition, ectopic miR-101 expression strongly inhibited cancer cell proliferation, migration, and invasion by inversely targeting Girdin [20], or Mcl-1 [21]. Zheng et al. found that miR-101 expression was downregulated, both in ovarian cancer tissues and cells. Moreover, miR-101 inhibition accelerated cancer cell proliferation and invasion ability through regulating SOCS-2 [22]. In gallbladder cancer, low miR-101 expres-

sion was found in tumor tissues and associated with dismal clinical features. Reduced miR-101 expression promoted carcinogenesis *in vitro* and *in vivo* by negatively targeting ZFX [23]. In esophageal cancer, miR-101 was downregulated in cancer cells and promoted cancer cell viability, while EZH2 was identified as its downstream target [24]. In prostate cancer, *in vitro* and *in vivo* data showed cell proliferation and tumor growth was significantly inhibited by miR-101 upregulation and miR-101 expression was negatively correlated with COX-2 expression [25]. Moreover, Zhang et al. reported that bladder transitional cell carcinoma patients with high miR-101 expression had longer survival times and low miR-101 expression was significantly associated with aggressive clinicopathological factors [26].

In summary, this study demonstrated that decreased miR-101 expression was observed in NSCLC patients and was closely associated with poor prognosis. Therefore, serum miR-101 might serve as a reliable biomarker for early detection and prognosis prediction of NSCLC.

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Disclosure of conflict of interest

None.

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