

## High *miRNA-221* expression is associated with poor overall survival in patients with non-small cell lung cancer.

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### Abstract

**Objective:** The objective of this study was to investigate *miRNA-221* expression in non-small cell lung cancer (NSCLC) and to determine the correlation between *miRNA-221* expression and overall survival.

**Methods:** Clinical data pertaining to 151 NSCLC patients who underwent complete tumor resection surgery between January 2012 and December 2015 were retrospectively analyzed, and *miRNA-221* expression was detected via quantitative reverse transcription polymerase chain reaction (qRT-PCR). The relationship between *miRNA-221* expression and patient clinical data, including overall survival (OS), was analyzed.

**Results:** The expression level of *miRNA-221* was significantly higher in NSCLC tissues than in adjacent non-tumor tissues ( $P < 0.05$ ). Significantly higher *miRNA-221* expression levels were observed in patients  $\geq 50$  years of age and with advanced disease ( $P < 0.05$ ). Univariate Cox regression analysis showed that clinical stage (OR=1.97, 95% CI: 1.14-2.67), pathological type (OR=2.55, 95% CI: 1.77-5.12), tumor size (OR=1.34, 95% CI: 1.01-2.14) and *miRNA-221* expression (OR=3.08, 95% CI: 1.76-6.31) were risk factors for shorter OS. Multivariate Cox regression analysis showed that clinical stage (OR=3.35, 95% CI: 1.67-5.05), tumor size (OR=2.02, 95% CI: 1.03-3.24) and *miRNA-221* expression (OR=2.58, 95% CI: 1.41-64.85) were independent risk factors for shorter OS. The median OS time in the low *miRNA-221* expression group was 41 months (95% CI: 36.22-45.78), which was significantly higher than that in the high *miRNA-221* expression group (25 months, 95% CI: 5.85-35.15,  $P = 0.011$ ).

**Conclusion:** Our data indicate that higher *miRNA-221* expression levels are associated with shorter OS in patients with NSCLC and that *miRNA-221* may be a molecular marker of patient prognosis in NSCLC.

**Keywords** Non-small cell lung cancer, *miRNA-221*, Gene, Prognosis.

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### Introduction

Lung cancer is the leading cause of cancer-related death worldwide, and almost 85% of lung cancers are classified as non-small cell lung cancer (NSCLC). The majority of affected patients have a very poor prognosis, even during the early stages of the disease, and the 5-year survival rate is only 20-30% [1,2]. Several studies have focused on developing reliable biomarkers predicting therapy responses, which may enable clinicians to predict or improve the prognoses of patients with NSCLC [3-6].

MicroRNAs (miRNAs) are short (19-25 nucleotides in length), non-coding regulatory RNA molecules whose primary function involves silencing gene expression. It has been suggested that the effects of miRNAs are gene and cell specific and that these

molecules may be considered biomarkers of patient prognosis in several human cancers [7-10].

*miRNA-221* is an important miRNA molecule and is considered a type of carcinogenic miRNA, as it has been linked to gastrointestinal stromal tumors [11], glial tumors [2], and prostate cancer [12]. However, studies regarding the association between *miRNA-221* expression and clinical outcomes in NSCLC, especially overall survival (OS), are scarce and have yielded controversial results [13,14]. Further investigations are necessary to explore the prognostic significance of *miRNA-221* in patients with NSCLC. In this study, we evaluated *miRNA-221* expression in tumor tissue samples from patients with NSCLC and analyzed the correlation among *miRNA-221* expression, clinical and pathological factors and prognosis.

## Materials and Methods

### Patients

A total of 151 patients who were diagnosed with NSCLC and underwent complete tumor resection surgery at our hospital from January 2012 to December 2015 were included in this study. The following patients were eligible for inclusion in this study: (1) patients diagnosed via pathology examinations, (2) patients who underwent complete tumor resection surgery, (3) patients who were not treated with radiotherapy, chemotherapy or immunotherapy before surgery and (4) patients with at least 3 months of comprehensive follow-up data. The following patients were excluded from this study: (1) patients with distant metastasis or malignant pleural effusions precluding complete tumor resection and (2) patients with additional tumors. All patients provided informed consent before beginning treatment, and the study was approved by the medical ethics committee of our hospital. All patients received regular examinations and underwent strict follow-up, during which their data were updated, until December 31, 2015.

### Quantitative real-time PCR (qRT-PCR) for miRNA

All tissue samples were put in storage tubes within 30 min of collection for rapid freezing and then stored at -80. miRNA analysis was performed as follows: total RNA tissue samples were isolated using Trizol reagent (Invitrogen), and qRT-PCR was performed using Bulge-Loop miRNA qRT-PCR kits, according to the manufacturer's instructions (RiboBio). Additional information regarding qRT-PCR has been presented in previous studies [15-17]. Briefly, *miRNA-221* was synthesized using U6-specific cDNAs, a specific *miRNA-221* stem loop primer and a U6 reverse primer and then amplified by real-time PCR with an *miRNA-221*-specific forward primer and a universal reverse primer. Each experiment was performed in triplicate. We used U6 small nuclear RNA as an endogenous control. The *miRNA-221* expression level for each sample was calculated based on the  $\Delta$ CT value. The relative *miRNA-221* expression levels in paired tissues obtained from the same patient were determined via the comparative  $2^{-\Delta\Delta CT}$  method [2,15].

### Statistical analysis

T tests and chi-square tests were employed to analyze the correlations between *miRNA-221* expression and patient clinical characteristics. Comparisons between tumor *miRNA-221* expression levels and non-tumorous control *miRNA-221* expression levels were analyzed using the  $2^{-Ct}$  method,  $2^{-Ct} = (Ct_{miRNA221} - Ct_{U6}) - Avg. (Ct_{miRNA221} - Ct_{U6})$ . The  $2^{-Ct}$  median was used as a threshold to divide patients into the following 2 groups: a low *miRNA-221* expression group and a high *miRNA-221* expression group.

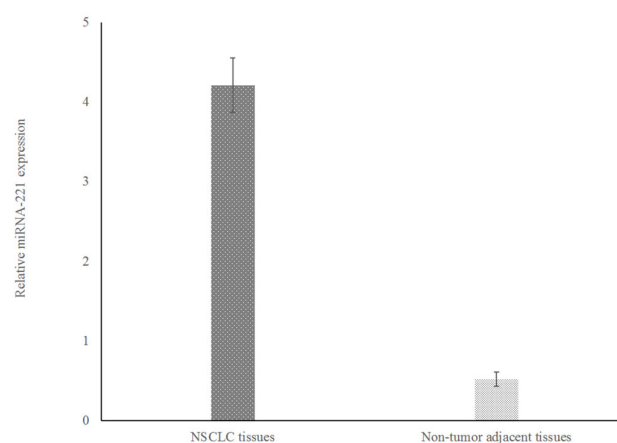
OS curves were plotted via the Kaplan-Meier method, and differences between the groups were analyzed via the log-rank test. Univariate and multivariate analyses of OS were performed using the Cox proportional hazards regression

model. SPSS 13 statistical software was used for all statistical analyses, and  $P < 0.05$  was considered statistically significant.

## Results

A total of 151 NSCLC patients who underwent complete resection were included in the analysis. The median follow-up time was 45 months, the longest follow-up time was 60 months, and the shortest follow-up time was 3 months. Ninety-six patients were male, accounting for 63.6% of the sample, and 55 patients were female, accounting for 36.4% of the sample. The average age was 57.3 years, ranging from 39 to 79 years.

To determine the levels of *miRNA-221* expression in NSCLC tissue samples, we performed qRT-PCR analyses on paired normal and tumor tissue samples from the same patient. As shown in Figure 1, the level of *miRNA-221* expression in NSCLC tissues was significantly higher than that in adjacent non-tumor tissues ( $P < 0.05$ ). Based on the median *miRNA-221* expression level, 79 cases of NSCLC were classified as having high *miRNA-221* expression, and 72 cases were classified as having low expression.



**Figure 1.** *miRNA-221* expression was higher in NSCLC tissues than in matched adjacent non-tumor tissues ( $1.65 \pm 0.34$  vs.  $0.52 \pm 0.09$ ,  $P < 0.05$ ).

The correlations between *miRNA-221* expression and age, gender, clinical stage, pathological type, tumor size, and smoking history are shown in Table 1. Age, clinical stage, and pathological type were correlated with *miRNA-221* gene expression ( $P < 0.05$ ). Moreover, as shown in Table 2, age and TNM stage were correlated with *miRNA-221* expression ( $P < 0.05$ ), indicating that older patients ( $\geq 50$  vs.  $< 50$ ) and patients with more advanced disease (II/III vs. I) were more likely to exhibit high levels of *miRNA-221* expression.

**Table 1.** The correlation between *miRNA-221* expression levels and the clinical characteristics of patients with non-small cell lung cancer.

Characteristics	Samples size	<i>miRNA-221</i> expression	T value	P value
Age (years)			2.59	0.01

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<50	81	4.14 ± 0.48		
≥ 50	70	4.33 ± 0.51		
Gender			0.93	0.357
Male	96	4.32 ± 0.54		
Female	55	4.24 ± 0.46		
Clinical stage			10.09	<0.001
I	54	3.80 ± 0.55		
II,III	97	4.66 ± 0.47		
Pathological type			2.11	0.037
SCC	62	4.31 ± 0.59		
ADC	89	4.12 ± 0.52		
Tumor size(cm)			1.89	0.061
≥ 3	66	4.34 ± 0.45		
<3	85	4.21 ± 0.39		
Smoking history			1.86	0.064
Yes	71	4.01 ± 0.47		
No	80	4.16 ± 0.52		

**Table 2.** The correlation between miRNA-221 expression and the clinical characteristics of patients with non-small cell lung cancer.

Characteristics	Sample size	miRNA-221 expression		$\chi^2$	P value
		high	low		
Age(years)				9.38	0.002
<50	81	33 (40.74)	48 (59.26)		
≥ 50	70	46 (65.71)	24 (34.29)		
Gender				0.01	0.939
Male	96	50 (52.08)	46 (47.92)		

**Table 3.** Univariate analysis of overall survival in patients with non-small cell lung cancer.

Clinical characteristics	$\beta$	Standard error	P value	OR (95% CI)
Sex (1=female, 2=male)	0.46	0.23	0.342	1.59 (0.57-2.46)
Age (1 ≤ 50 years, 2 ≥ 50 years)	0.16	0.12	0.480	1.17 (0.68-1.82)
Clinical stage (I=1, II, III=2)	0.68	0.33	0.036	1.97 (1.14-2.67)
Pathological type (1=SCC, 2=ADC)	0.94	1.45	0.022	2.55 (1.77-5.12)
Tumor size (1 ≤ 3 cm, 2 ≥ 3 cm)	0.29	0.28	0.046	1.34 (1.01-2.14)
Smoking (1=No, 2=Yes)	0.21	0.18	0.858	1.23

Female	55	29 (52.73)	26 (47.27)		
Clinical stage				4.51	0.031
I	54	22 (40.74)	32 (59.26)		
II,III	97	57 (58.76)	40 (41.24)		
Pathological type				0.03	0.993
SCC	62	32 (51.61)	30 (48.39)		
ADC	89	47 (52.81)	42 (47.19)		
Tumor size(cm)				1.23	0.254
≥ 3	66	38 (57.58)	28 (42.42)		
<3	85	41 (48.24)	44 (51.76)		
Smoking history				0.35	0.545
Yes	71	39 (54.93)	32 (45.07)		
No	80	40 (50.00)	40 (50.00)		

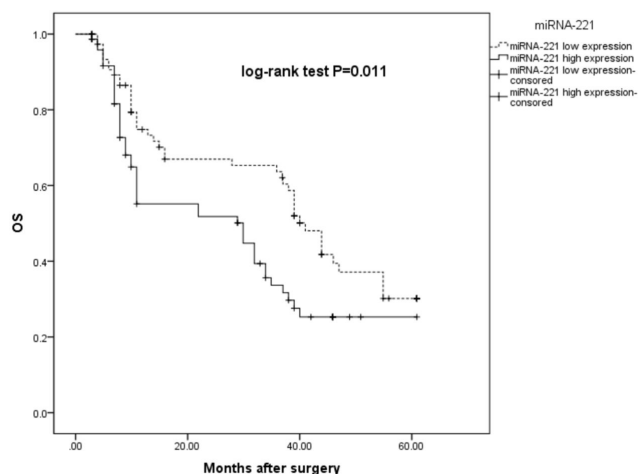
The correlations between clinical characteristics, including miRNA-221 expression levels, and OS are shown in Table 3. Univariate Cox regression analysis showed that advanced clinical stage (II/III), ADC, tumor size ≥ 3 cm and high miRNA-221 expression levels were risk factors for shorter OS. Multivariate Cox regression analysis showed that clinical stage (OR=3.35, 95% CI: 1.67-5.05), tumor size (OR=2.02, 95% CI: 1.03-3.24) and miRNA-221 expression levels (OR=2.58, 95% CI: 1.41-64.85) were independent risk factors for shorter OS, as shown in Table 4.

Patients were stratified into high miRNA-221 expression and low miRNA-221 expression groups to determine the effects of miRNA-221 expression on OS, and we found that the median OS time in the low miRNA-221 expression group was 41 months (95% CI: 36.22-45.78), while the median OS time in the high miRNA-221 expression group was 25 months (95% CI: 5.85-35.15), a difference that was statistically significant (log-rank  $\chi^2=6.520$ , P=0.011), as shown in Figure 2.

				(0.47-1.73)
<i>miRNA-221</i> expression (1=low2=high)	1.12	3.54	0.015	3.08 (1.76-6.31)

**Table 4.** Multivariate analysis of overall survival in patients with non-small cell lung cancer.

Clinical characteristics	$\beta$	standard error	P value	OR (95% CI)
Sex (1=female, 2=male)	0.15	0.44	0.841	1.16 (0.40-2.69)
Age (1 $\leq$ 50 years, 2 $\geq$ 50 years)	0.76	0.41	0.613	2.14 (0.59-3.78)
Clinical stage (I=1, II, III=2)	1.21	3.39	0.013	3.35 (1.67-5.05)
Pathological type (1=SCC, 2=ADC)	0.29	1.46	0.589	2.49 (0.34-4.57)
Tumor size (1 $\leq$ 3 cm, 2 $\geq$ 3 cm)	0.70	0.58	0.039	2.02 (1.03-3.24)
Smoking (1=No, 2=Yes)	0.75	2.61	0.211	2.12 (0.39-5.72)
<i>miRNA-221</i> expression (1=low, 2=high)	0.95	0.42	0.001	2.58 (1.41-4.85)



**Figure 2.** Survival analysis of 151 NSCLC patients using the Kaplan-Meier method. Overall survival was significantly worse in patients with high *miRNA-221* expression levels than in patients with low *miRNA-221* expression levels (log-rank test,  $P=0.011$ ).

## Discussion

In recent years, significant progress has been made regarding NSCLC clinical diagnosis and laboratory testing, but patient prognoses are still poor, and the 5-year survival rate ranges from approximately 20-30% [2]. Exploring the pathogenesis of lung cancer may facilitate development of targeted therapies, resulting in improved patient outcomes [18]. miRNAs can modulate key signaling pathways or genes related to tumorigenesis, and abnormal miRNA expression may induce tumorigenesis [2,3]. Although the processes associated with miRNA-mediated regulation of tumor behavior are very complex and diverse, miRNAs are tissue specific and may be used as tools for the early diagnosis or prediction of lung cancer [19,20].

In this study, we retrospectively collected clinical data pertaining to 151 NSCLC patients who underwent complete tumor resection surgery and detected *miRNA-221* expression in preserved tissue samples using qRT-PCR. We noted higher *miRNA-221* expression in NSCLC tissues than in paired adjacent normal lung tissues, indicating that *miRNA-221* may be used to distinguish cancerous tissues from normal tissues and to determine prognosis in NSCLC patients [10,13].

We then analyzed *miRNA-221* expression levels in lung cancer tissues and divided patients into the following 2 groups based on these levels: a high expression group and a low expression group. All these results showed that *miRNA-221* expression was correlated with age and clinical stage. As shown in Tables 1 and 2, significantly higher *miRNA-221* expression was noted among patients  $\geq$  50 years and with advanced disease (II/III) than among patients  $<$ 50 years and with early disease (I). Yoruker EE showed that *miRNA-221* was overexpressed in papillary thyroid carcinoma and that miRNA expression was correlated with tumor aggressiveness and adverse clinical characteristics. This group also found that *miRNA-221* levels were significantly lower after surgery, indicating that *miRNA-221* expression levels may be correlated with tumor burden [9].

Several studies have shown that high levels of *miRNA-221* expression were correlated with poor prognoses in some tumors, such as hepatocellular carcinoma [21], thyroid cancer [9], and prostate cancer [12], indicating that *miRNA-221* may play a significant role in tumorigenesis or tumor progression. One study showed that the level of *miRNA-221* expression in lung cancer was significantly increased compared to that in benign lung lesions in 40 patients [15]. Overexpression of *miRNA-221* may lead to targeting of some important tumor suppressor genes (for example, *P57*, *FOXO3*, and *DDIT4*) and induce tumor proliferation and invasion. Zhang et al. found that *miRNA-221* targeted the *PUMA* gene and regulated cell apoptosis by directly acting on the 3' UTR of *PUMA* in glioma

[22]. Garofalo et al. found that *miRNA-221* induced TRAIL resistance and enhanced cellular migration by targeting the tumor suppressors PTEN and TIMP3 via activation of the AKT pathway and metalloproteinases in lung and liver cancer [23-25], confirming the role of *miRNA-221* in NSCLC development and prognosis. Moreover, Xu et al. showed that *miRNA-221* overexpression can result in increased H460 cell proliferation and viability. Down-regulation of *miRNA-221* attenuates these effects, which indicates that inhibition of *miRNA-221* may represent a novel treatment approach for human NSCLC [26].

However, studies regarding the correlation between *miRNA-221* expression and NSCLC prognosis in terms of OS are still scarce. In this study, univariate Cox regression analysis showed that advanced clinical stage, adenocarcinoma, tumor size  $\geq 3$  cm and high *miRNA-221* expression are risk factors for shorter OS. The median OS time (25 months) in the high *miRNA-221* expression group was significantly shorter than that (41 months) in the low *miRNA-221* expression group. Multivariate Cox regression analysis showed that clinical stage and *miRNA-221* expression were independent risk factors for shorter OS, suggesting that *miRNA-221* may be a prognostic factor for OS in NSCLC patients. Lv et al. retrospectively analyzed clinical data, including data regarding *miRNA-221* expression, pertaining to 117 NSCLC patients and found that *miRNA-221* expression was an independent risk factor for shorter OS in patients with NSCLC and that the median OS time (35.34 months) in the high *miRNA-221* expression group was significantly shorter than that in the low *miRNA-221* expression group (48.72 months) [13].

Similarly, studies have explored the correlation between *miRNA-221* expression and OS in other tumors. For example, in patients with NK/T-cell lymphoma, higher plasma *miRNA-221* levels were correlated with shorter OS, and plasma *miRNA-221* levels were found to be an independent prognostic factor for OS; therefore, the authors concluded that plasma *miRNA-221* levels may serve as diagnostic and prognostic markers in patients with NK/T-cell lymphoma [27]. Smid et al. noted significantly high *miRNA-221* levels in gastric cancer patients who underwent palliative chemotherapy and that high *miRNA-221* expression levels were correlated with shorter times to disease progression [28]. Moreover, Yuan et al. showed that low *miRNA-221* expression levels were inversely associated with OS and DFS in patients with colorectal cancer (CRC) via combined analyses of patient samples, cell lines, and mouse models [3].

The limitations of this study were its relatively small sample size and retrospective design. Additional studies, especially prospective cohort studies, are needed to evaluate the prognostic value of *miRNA-221* in NSCLC.

In conclusion, we demonstrated that *miRNA-221* may play an important role in NSCLC and that increased *miRNA-221* expression may facilitate tumor progression. Therefore, *miRNA-221* may be a prognostic marker or therapeutic target in patients with NSCLC.

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