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ORIGINAL ARTICLE

Up-Regulation of miR-21 Expression Predicate Advanced Clinicopathological Features and Poor Prognosis in Patients with Non-Small Cell Lung Cancer

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Abstract MicroRNAs (miRNAs) are endogenous small (19–24 nt long) noncoding RNAs that regulate gene expression in a sequence specific manner. An increasing association between miRNA and cancer has been recently reported. Lung cancer is globally responsible for 1.4 million deaths annually and is the leading cause of cancer-related deaths in both women and men. In this study, we investigated the miR-21 expression in non-small cell lung cancer (NSCLC) to evaluate their value in prognosis of this tumor. Here, we assess miR-21 expression in NSCLC and its clinical significance including survival analysis. The expression of miR-21 in matched normal and tumor tissues of NSCLC was evaluated using a quantitative real-time RT-PCR. A Kaplan–Meier survival curve was generated following a logrank test. It was observed that miR-21 expression was up-regulated in NSCLC tissues compared with noncancerous lung tissues (mean ± SD: 6.7 ± 2.3 vs. 3.7 ± 1.5, $P < 0.001$). The up-regulation of miR-21 in NSCLC cancer tissues was also significantly correlated with aggressive clinicopathological features. We found that the patients with high miR-21 expression have a higher tumor grade ($P = 0.027$) and are in higher risk of lymph node metastasis ($P = 0.021$). Moreover, the results of Kaplan–Meier analyses showed that NSCLC patients with the high miR-21 expression tend to have shorter overall survival and progression free survival ($P < 0.001$). The multivariate analysis clearly indicated that the high miR-21 expression in biopsy samples may be considered as an independent prognostic factor in NSCLC for decreased survival (RR 3.88; 95%CI, 2.47–6.11). Our data indicate the potential of miR-21 as a novel prognostic biomarker for NSCLC. Large well-designed studies with diverse populations and functional evaluations are warranted to confirm and extend our findings.

Keywords Lung cancer · miR-21 · Prognosis

Introduction

Lung cancer is globally responsible for 1.4 million deaths annually and is the leading cause of cancer-related deaths in both women and men [1]. Approximately 80 % of the lung cancer patients have non-small cell lung cancer (NSCLC). Once diagnosed, survival rates are low. Although the 5-year survival rate has slightly improved over the last 3 decades, that of early-stage patients is still worse than colon, gastric and breast cancer patients. Even diagnosed early, graed I lung cancer patients have only a 60–70 % five-year survival rate [2–4]. Successful surgical resection remains the only curative treatment for lung cancer, highlighting the need for novel diagnostic and therapeutic strategies. Identifying factors that are associated with aggressive disease may lead to the development of novel biomarkers and identification of therapeutic targets that can help reduce the burden of this disease.

MicroRNAs (miRNAs) are endogenous small (19–24 nt long) noncoding RNAs that regulate gene expression in a sequence specific manner. This is primarily accomplished through binding to 3′UTR of target mRNAs, either targeting the transcripts for degradation or blocking their translation [5]. As a post-transcriptional regulator, miRNAs bind to complementary sequences on target messenger RNAs (mRNAs), resulting in mRNA degradation or translational repression [6]. Through the post-transcriptional path, miRNAs have been shown to play fundamental roles in diverse biological and

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pathological processes, including cell proliferation, differentiation, apoptosis, and carcinogenesis [7–9].

Human miR-21 (hsa-miR-21) was cloned from HeLa cell total RNA and is highly conserved among species including human, rat, mouse, fish and frog [10]. It is located on chromosome 17q23-1 overlapping with the TMEM49 gene, a human homologue of rat vacuole membrane protein-1. MiR-21 encodes a single hairpin and is regulated by its own promoter containing binding sites for AP-1 and PU.1 transcription factors [11]. Experimental data has shown that miR-21 functions in many cell types as an anti-apoptotic and pro-survival factor and plays a significant role in cancer biology and prognosis [12]. Asangani et al. [13] transfected Colo206f cells with miR-21 and found significant suppression of PDCD4 proteins in vitro. A role as oncogene has been suggested for miR-21 that is deregulated in lung cancer and other cancers [14–16]. MiR-21 was reported to be a prognostic factor for colorectal cancer [17], prognostic cancer [16], gastric cancer [16] and et al. [18].

In this study, we investigated the miR-21 expression in NSCLC to evaluate their value in prognosis of this tumor. To this end, we examined the up-regulation of miR-21 in 204 NSCLC cases by means of TaqMan real-time reverse-transcription PCR (RT-PCR). We report here that miR-21 is frequently overexpressed in NSCLC. More importantly, those patients with elevated miR- 21 expressions were also found to have significantly worse prognosis; and this prognostic impact appears to be independent of other factors in multivariate Cox regression analysis Table 2.

Materials and Methods

Ethics Statement

The study was approved by No.88 Hospital of People's republic of China. Written informed consent was obtained for the acquisition and use of patient tissue samples and anonymized clinical data.

Samples and Cases

For comparison of the reliability of real-time PCR for detection of miRNA in formalin-fixed paraffin-embedded tissues (FFPETs), 204 pairs of samples (including 204 NSCLC samples and normal adjacent tissues) of FFPETs were collected from March 2001 to December 2007 at No.88 Hospital of People's republic of China. For further miR-21 quantitative analysis, FFPETs of NSCLC samples and paired noncancerous tissues were prepared.

The diagnosis and histological grade of each case were independently confirmed by two pathologists based on WHO classification. The clinical graed was classified according to the American Joint Committee on Cancer (AJCC, Seventh Edition, 2010) tumor-lymph node-metastasis (TNM) classification system.

RNA Extraction

For real-time PCR analysis of miRNA, total RNA from FFPETs was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Briefly, plugs were punched out (1.5 mm × 1.5 mm) of a paraffin block. Samples were deparaffinized three times in 1 mL ACS grade xylene with incubation at 60 °C for 10 min, followed by a wash with 100 % ACS grade ethanol and air drying at room temperature. Samples were then incubated with proteinase K (Merck) at 55 °C overnight, shaking every 2 h. RNA samples were resuspended in RNase-free water after the final precipitation step. RNA quality and quantity were assessed using a biophotometer (Eppendorf). The paraffin plugs were enriched for tumor tissue under microscope control using H&E-stained sections of the same sample for guidance.

The RNA concentration and purity were assessed by UV spectrophotometry (A260/A280 ratio of 1.8–2.0). Total RNA samples were reverse transcribed to cDNA using a TaqMan® microRNA assay miRNA-specific stem-loop primer and the TaqMan® microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The PCR was performed using the TaqMan® Universal PCR Master Mix and a 7500 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions, and previously published primer sequences [19]. The cycling programme involved preliminary denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s and elongation at 60 °C for 60 s. The U6 small nuclear (sn)RNA was amplified as an internal control using previously published primer sequences. Each sample was analysed in triplicate. Levels of miR-21 were analysed quantitatively relative to U6 snRNA by the $2^{-\Delta\Delta CT}$ method using the equation, relative quantity $=2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = (CT^{\text{miR-21}} - CT^{U6})_{\text{cancer}} - (CT^{\text{miR-21}} - CT^{U6})_{\text{normal adjacent tissues}}$, and CT is the cycle threshold for each specimen.

Statistical Analyses

To analyze baseline characteristics, we used Chi-square tests for the categorical data and Mann–Whitney tests for continuous data when comparing patient and control baseline data. Associations between miR-21 expression and over survival of the patients with thyroid cancer were estimated using adjusted relative risks and 95 % confidence intervals (95 % CIs) from multivariate logistic regression.

Survival time was calculated from the date of NSCLC diagnosis to the date of death or last follow-up. Survival analysis

was estimated using the Kaplan–Meier method, log-rank test, and Cox-proportional hazards regression model. The significance level of all tests was set at $P < 0.05$. The software of SPSS version13.0 for Windows (SPSS, Inc., Chicago, IL) and SAS 9.1 (SAS Institute, Cary, NC) were used for statistical analysis.

Results

Clinicopathologic Characteristics of the Patients

The clinicopathologic characteristics of the patients and follow-up data are summarized in Table 1. The median age of the NSCLC patients at diagnosis was 47.9 year (range, 25–84 year). Among the 204 NSCLC cases, 126 (61.8 %) were diagnosed as adenocarcinoma (ADC), 1 (1 %), 56 (27.5 %) squamous cell carcinoma (SCC) and 12 (5.9 %) large cell carcinoma (LCC). The tumor graed consisted of grade Ia (n = 43, 21.1 %), grade Ib (n = 71, 34.8 %), grade IIa (n = 26, 12.7 %), grade IIb (n = 36, 17.6 %) and grade IIIa (n = 28, 13.7 %). Lymph node involvement was found in 107 (52.5 %) patients. The median follow-up time was 46.7 months (range, 5.0–71.0 months) after the primary surgical treatment (Table 2).

Table 1 Correlation of miR-21 expression with clinicopathological features of non-small cell lung cancer (n = 204)

Clinicopathological features	No. of cases	miR-21 expression		P
		High	Low	
Mean age (year)				
< 65	100	45	55	0.261
≥ 65	104	55	449	
Gender				
Male	98	52	46	0.209
Female	106	48	58	
Tumor garde				
Ia	43	12	31	**0.027**
Ib	71	29	42	
IIa	26	14	12	
IIb	36	24	12	
IIIc	28	21	7	
Histology				
ADC	126	61	65	0.301
SCC	56	27	29	
LCC	12	5	7	
others	10	5	5	
Lymph node metastasis				
Negative	97	29	68	**0.021**
Positive	107	71	36	
Tumor size (cm)				
≥ 6	105	55	50	0.354
< 6	99	45	54	
Smoking				
Never	45	23	22	0.412
Former	129	62	67	
Smoking	30	15	15	
Chemotherapy				
Yes	121	61	60	0.670
No	83	39	44	

The p-values were assessed by $\chi 2$ test and significant p-values are in bold. ADC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma

MiR-21 Up-Regulation in NSCLC Tissues

MiR-21 expression was detected in 204 pairs of NSCLC tissues and adjacent non-neoplastic lung tissues normalized to RNU6B. It was found that the expression of miR-21 was distinctly increased in NSCLC tissues compared with non-neoplastic lung tissues (mean ± SD: 6.7 ± 2.3 vs. 3.7 ± 1.5, $P < 0.001$, Fig. 1). In addition, miR-21 expression in high-grade (IIb-IIIa; 7.9 ± 2.1) and low-grade (Ia-IIa; 5.1 ± 1.6) NSCLC tissues were both significantly higher than that in non-neoplastic brain tissues There was also a significant difference in miR-21 expression between high-grade (IIb-IIIa) and low-grade (Ia-IIa) NSCLC tissue specimens ($P < 0.001$, Fig. 2).

Expression of miR-21 and Clinicopathological Features

We then analyzed the association between miR-21 expression and clinicopathological parameters in NSCLC. NSCLC tissues expressing miR-21 at levels less than the median expression level (5.0) were assigned to the low expression group (mean expression value 4.6, n = 104), and those samples with expression above the median value were assigned to the high

Table 2 Multivariate analyses of different prognostic parameters in patients with non-small cell lung cancer by Cox regression analysis

Parameter	Risk ratio [1]	95 % CI [2]	P [3]
Age	1.81	0.43–2.74	0.552
Gender	1.45	0.56–2.45	0.453
Tumor graed	2.47	1.32–4.38	**0.012**
Histology	1.87	0.86–5.01	0.08
Lymph node metastasis	2.97	1.26–4.32	**0.020**
Tumor size (cm)	2.12	0.97–3.67	0.064
Smoking status	2.08	0.89–5.98	0.092
Chemotherapy	1.67	0.65–3.34	0.327
miR-21 expression	3.88	2.47–6.11	**< 0.001**

[1] RR; relative risk

[2] CI; confidence interval

[3] P-value,0.05 statistically significant and significant p-values are in bold

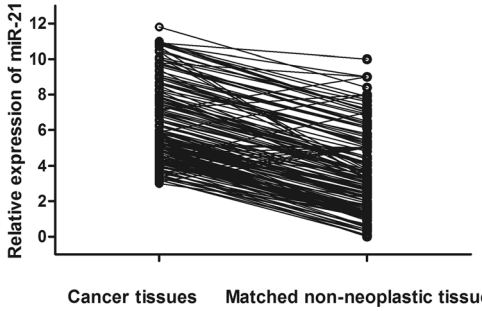

Fig. 1 miR-21 expression in 204 pairs of NSCLC and adjacent non-neoplatic lung tissues detected by quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The expression of miR-21 was distinctly increased in NSCLC tissues compared with non-neoplastic lung tissues (mean ± SD: 6.7 ± 2.3 vs. 3.7 ± 1.5, $P < 0.001$)

expression group (mean expression value 8.3, $n = 100$). The high level of miR-21 expression was significantly more common in NSCLC tissues with advanced pathologic grade than those with low pathologic grade ($P < 0.001$, Table 1). Besides, miR-21 up-regulation group has a higher rate of lymph node metastasis ($P = 0.021$). In this study, no significant association between miR-21 expression and tumor size was detected ($P = 0.354$). When the smoking status was considered, the expression of miR-21 was not different among the current smoker, former smoker or never smoker ($P = 0.412$).

MiR-21 Expression and Survival in Patients with NSCLC

In the 5 years' follow-up, the association between miR-21 expression and prognosis was detected. The overall survival of pediatric patients with NSCLC with high miR-21 expression were significantly shorter than those with low miR-21 expression ($P < 0.001$, Fig. 3a). However, when the pathological graeds were considered, the higher miR-17 expression was a risk of poor prognosis in both the high-grade NSCLC (IIb-IIIa, $P < 0.001$, Fig. 3b) and the low-grade NSCLC (Ia-IIa, $P = 0.002$, Fig. 3c).

When the progression-free survival was considered, the progression-free survival of the patients with high miR-21 expression was shorter ($P < 0.001$, Fig. 4a). The higher miR-17 expression was a risk of shorter progression-free survival in both the high-grade NSCLC (IIb-IIIa, $P < 0.001$, Fig. 4b) and the low-grade NSCLC (Ia-IIa, $P = 0.002$, Fig. 4c) stratifying by the pathological graeds.

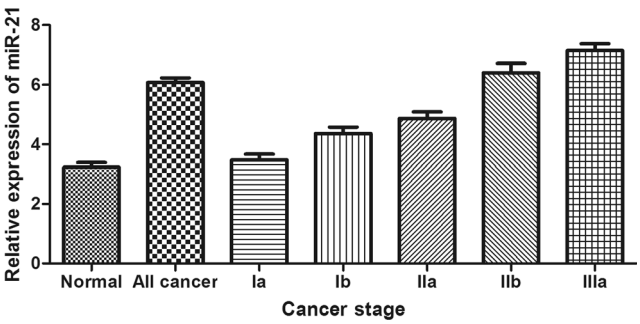

Fig. 2 miR-21 expression in 204 pairs of NSCLC and adjacent non-neoplatic lung tissues detected by quantitative real-time polymerase chain reaction (qRT-PCR) analysis according to different tumor graeds (normal, all cancer and graed Ia-IIIa)

Multivariate cox Proportional Hazard Analysis

In the Cox proportional hazard model, it was confirmed that miR-21 expression in the biopsy samples (RR 3.88; 95%CI, 2.47–6.11), tumor graed (RR 2.47; 95 % CI, 1.32–4.38) and lymph node metastasis (RR, 2.97; 95 % CI, 1.26–4.32) were predictor of poor prognosis of the patients with thyroid cancer. Our results showed that age (RR, 1.81;RR, 0.43–2.74), gender (RR, 1.45; 95 % CI, 0.56–2.45), tumor size (RR, 2.12; 95 % CI, 0.97–3.67), histology(RR, 1.87; 95 % CI, 0.86–5.01), smoking status (RR, 2.08; 95 % CI, 0.89–5.98) and chemotherapy(RR, 1.67; 95 % CI, 0.65–3.34) were not independent predictor of the survival of patients with NSCLC.

Discussion

Although with the development of early diagnosis and surgical techniques, the patients with NSCLC still often demonstrated a relatively poor prognosis. The molecular biomarkers for early diagnosis and predictor of prognosis are desperately required now. A lot of work has been conducted in an attempt to identify biomarkers with diagnostic and prognostic implications for NSCLC. MiRNAs have been demonstrated to be critical regulators of carcinogenesis and tumor progression in this malignancy. In this study, we observed that miR-21 expression was up-regulated in NSCLC tissues compared with no cancerous lung tissues. The up-regulation of miR-21 in NSCLC cancer tissues was also significantly correlated with aggressive clinic pathological features. Besides, patients with high miR-21 expression have higher tumor staging and are in higher risk of lymph node metastasis. Moreover, the results of Kaplan–Meier analyses showed that NSCLC patients with the high miR-21 expression tend to have shorter overall survival and progression free survival. The multivariate analysis clearly indicated that the high miR-21 expression in biopsy samples may be considered as an independent prognostic factor in NSCLC for decreased survival.

At present, the lack of knowledge regarding miRNA gene targets hampers a full understanding of the biological functions that may be disregulated by aberrant miRNA expression. Various cancer-associated genes, such as miR-126, mR-195 and let-7c were reported to be associated with kinds of cancers [20–22]. For the up-regulated miR-21, its potential target genes include the tumor suppressors TIMP3 and PDCD4. It is noteworthy that TPM1 has recently been shown to be a target of miR-21 [23]. Interestingly, miR-21, miR-31,

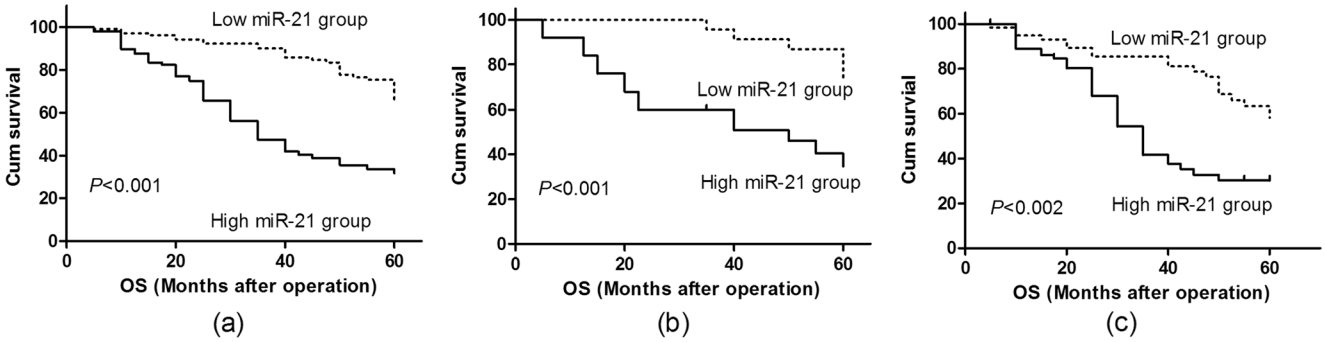

Fig. 3 Kaplan-Meier survival curves for NSCLC patients with high or low expression of miR-21. **a** The 5-year overall survival rate of all 204 NSCLC patients with high or low miR-21 expression; **b** The 5-year overall survival rate of 140 NSCLC patients with low pathological grades (tumor graed Ia ~ IIa) in high or low miR-21 expression group; **c** The 5-year overall survival rate of 64 NSCLC patients with advanced pathological grades (tumor graed IIb ~ IIIa) in high or low miR-21 expression group

miR-335, and miR-320 may target the same gene, RAS p21 protein activator (RASA1). In NSCLC, the anticipated targets of down-regulated miRNAs are expected to be oncogenes or genes encoding proteins with potential oncogenic functions. For example, miR-31 and miR-320 may, respectively, target RAB1B, RAB6B, RAB14, and RAB18 (members of the RAS oncogene family). However, with few exceptions, the physiologic targets of miRNAs remain to be identified. Further investigative focus on these miRNAs and their functions in the pathogenesis of NSCLC is warranted.

MiR-21 is an oncogenic microRNA with a suggested role in multiple cancer types. Overexpression of miR-21 has been found in at least 18 malignancies indicating that altered expression of miR-21 may be a common mechanism in carcinogenesis [24, 25]. The cause of miR-21 overexpression in cancer is still being elucidated. The altered activity of at least four cancer related regulatory factors is thought to contribute to the altered expression of miR-21, including signal transducer and activator of transcription 3 (STAT3), activator protein 1 (AP-1), transforming growth factor β (TGFβ), and epidermal growth factor receptor (EGFR). STAT3 is a transcription factor that is downstream of IL-6 (interleukin 6) and can influence cell transformation. The miR-21 promoter has STAT3 binding sites and IL-6 can induce the expression of miR-21 in a STAT3 dependent manner indicating that altered inflammatory states may be affecting miR-21 expression in the context of cancer [26].

MiRNA is now more and more popular used in the early diagnosis and long prognosis for kinds of disease, especially cancers. MiR-21 was found to be significantly up-regulated in NSCLC in both our and previous studies [27]. In addition, miR-21 and its precursor have been reported to be up-regulated in other kinds of tumors, including malignant cholangiocytes, glioblastomas, and malignancies of the colon, breast, pancreas, prostate and stomach [12, 28–30]. Komatsu S et al. retrospectively examined the association between plasma miRNA concentrations and prognosis of gastric carcinoma [31]. The results showed that the postoperative cause-specific survival rate of patients with high plasma miR-21 concentration was significantly poorer than those with a low concentration ($p = 0.0451$) and they concluded that the level of circulating miR-21 could be a reliable prognostic marker in the plasma of patients with gastric carcinoma. A previous study focused on the correction the expression of miR-21 and prognosis of glioblastomas [32]. Motonobu et al. tested if the expression of miR-21 was associated with prognosis and disease

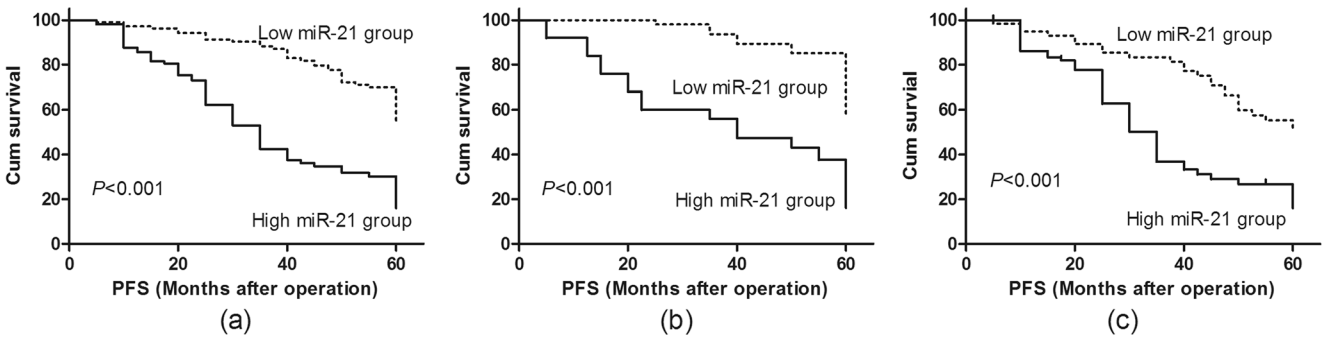

Fig. 4 Kaplan-Meier progression free survival curves for NSCLC patients with high or low expression of miR-21. **a** Ther overall progression free survival rate of all 204 NSCLC patients with high or low miR-21 expression; **b** The progression free survival rate of 140 NSCLC patients with low pathological grades (tumor graed Ia ~ IIa) in high or low miR-21 expression group; **c** The progression free survival survival rate of 64 NSCLC patients with advanced pathological grades (tumor graed IIb ~ IIIa) in high or low miR-21 expression group

progression in early graed lung adenocarcinoma in a retrospective analysis of three cohorts [27]. The expression of miR-21 was measured by quantitative RT-PCR in tissues from 317 non small cell lung cancer (NSCLC) patients that originated from Maryland, Norway and Japan. The results showed that elevated miR-21, miR-17, miR-155 were associated with worse cancer-specific mortality in the Maryland cohort. These were evaluated in two additional cohorts and only miR-21 was associated with worse cancer-specific mortality in the Norwegian cohort (HR 2.78, 1.22–6.31) and worse relapse free survival in the Japanese cohort (HR 2.82, 1.57–5.07). The results in this study, which is from a Chinese cohort, demonstrated a similar conclusion as that study and this suggests that expression of miR-21 may contribute to lung carcinogenesis and serve as a therapeutic target or early graed prognostic biomarker for lung cancer.

In conclusion, our results have demonstrated that the levels of miR-21 are higher in NSCLC tissues than those in matched normal mucosas and correlated with disease stage and the presence of lymph node metastasis. These findings enhance our understanding of the role of miR-21 in NSCLC progression and suggest that miR-21 may function as microtumor promoter genes in NSCLC. These findings suggest the potential clinical use of microRNA measurements, particularly in estimating prognosis for patients with NSCLC. Large well-designed studies with diverse populations and functional evaluations are warranted to confirm and extend our findings. Examining new targets and other biological experiments will clarify the functions and roles of microRNAs in NSCLC.

Competing Interests The authors declare that they have no competing interests.

Authors' Contribution LT, XJL, XHL and CYW provided the conduction of the whole project, LT, WYS, YFZ, XJL, XHL and CYW performed the research, LT, WYS, YFZ, XJL, XHL and CYW drafted the manuscript; LT and CYW contributed to revise the manuscript. All authors read and approved the final manuscript.

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