

## Original Article

# Reduction of Plasma MicroRNA-21 is Associated with Chemotherapeutic Response in Patients with Non-small Cell Lung Cancer

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## ABSTRACT

**Objective:** To examine plasma microRNA-21 (miR-21) level in patients with non-small cell lung cancer (NSCLC) and its potential correlation with chemotherapeutic response.

**Methods:** 77 NSCLC patients and 36 age and sex-matched healthy controls were included. Plasma miR-21 concentration was examined using a quantitative real-time reverse transcription polymerase chain reaction assay (qRT-PCR). Potential correlation between plasma miR-21 concentrations with chemotherapeutic responses was analyzed in 35 patients with advanced NSCLC (stages IIIB and IV).

**Results:** Plasma miR-21 was significantly higher in NSCLC patients relative to the healthy controls ( $P < 0.0001$ ). As a biomarker, plasma miR-21 had a receiver operating characteristic (ROC) curve area of 0.729 with 61.04% sensitivity and 83.33% specificity. Chemotherapeutic response in the 35 patients with advanced NSCLC (stages IIIB and IV) included partial response (PR) (n=11), stable disease and progression disease (SD+PD) (n=24). The overall response rate (CR+PR) was 31.4%. Plasma miR-21 in patients who achieved PR was significantly lower than those who did not respond (SD+PD) ( $P = 0.0487$ ), and comparable to that of the healthy controls ( $P = 0.2744$ ).

**Conclusion:** Plasma miR-21 is a good biomarker for NSCLC, and could be used to predict responses to chemotherapy.

**Key words:** NSCLC; miR-21; Biomarker; Plasma; Chemotherapeutic response

## INTRODUCTION

Lung cancer is one of the most common malignant tumors and is also the leading cause of cancer-related death in the world, 80% of which are from non-small cell lung cancer (NSCLC). Five-year survival rate of NSCLC patients is only 15%-20%. The two main reasons for such poor prognosis are paucity in reliable plasma markers for NSCLC and common chemo-resistance in therapy.

MicroRNAs (miRNAs) are 18- to 25-nucleotide, noncoding RNA molecules that regulate the translation of many genes. MiRNAs expression mainly by base-pairing to the 3'-UTR of the target mRNA and play a role in regulation of gene expression<sup>[1]</sup>. At cellular level, miRNAs play critical roles in differentiation, proliferation, apoptosis, and metabolism and have ultimately been linked to cancer development<sup>[2]</sup>. It was demonstrated that miRNA is important for development of human cancers including leukemia<sup>[3]</sup>, neuroblastoma<sup>[4]</sup>, pituitary adenoma<sup>[5]</sup>, breast cancer<sup>[6]</sup>, thyroid cancer<sup>[7]</sup>, and many

other cancers<sup>[8-16]</sup>. Over 50% of annotated human miRNA genes are located within regions of cancer-associated genomic regions or fragile sites, and they may have important functions similar to oncogenes or tumor suppressors<sup>[3]</sup>. Various studies have shown that miRNA may become useful biomarker for cancer diagnosis, therapy and prognosis. Since 1999, tumor-associated RNAs have been detected in serum or plasma from patients suffering from nasopharyngeal, breast, and colon cancers<sup>[17-21]</sup>. More recently, several studies also suggested that circulating miRNAs existed in serum and plasma<sup>[22-29]</sup>, raising the possibility of using miRNAs as novel non-invasive molecular markers for cancer detection. The miR-21 gene is located at the 10th intron of coding region for TMEM49 at chromosome 17. Multiple studies showed that miR-21 might be one of the most relevant oncogene-like factors among the class of miRNAs<sup>[30]</sup>. It has been implicated in not only the promotion of tumor growth, proliferation, anti-apoptosis, but also tumor invasion, migration. In addition, it is associated to the response to chemotherapy and prognosis. MiR-21's targets include PTEN, PDCD4, MTAP, and TGF- $\beta$ , all of which regulate cell cycle. Their aberrant expression in various cancers suggests them to be a novel class of oncogenes or tumor suppressor gene. Zhang et al<sup>[31]</sup> demonstrated that miR-21 post-transcriptionally down-regulated the expression of tumor suppressor PTEN and stimulated growth and invasion of

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NSCLC. Their data showed that miR-21 was possible to become a potential therapeutic target for NSCLC. In the present study, we assessed the feasibility of using plasma miR-21 as a non-invasive tool to diagnose NSCLC and predict treatment outcome.

## MATERIAL AND METHODS

### Patients and Plasma Samples

A total of 113 subjects were enrolled in this study, including 77 NSCLC patients ((September 2009-July 2010), 39 patients with tumor resection (stages I, II and IIIA) and 38 without (stages IIIB and IV)), and 36 age- and sex-matched healthy volunteers as controls. NSCLC patients were recruited at Department of Oncology, First Affiliated Hospital of Nanjing Medical University, China. Blood samples were taken before chemotherapy in both operable and non-operable patients. All patients underwent stage classification according to AJCC/UICC guidelines. None of these patients had received adjuvant chemotherapy or radiotherapy before admission. Informed consents were obtained from all enrolled subjects and the local Ethics Committee approved the protocol.

### Chemotherapy and Clinical Response Evaluation

Seventy-seven NSCLC patients were routinely treated by cisplatin- or carboplatin-based chemotherapy, and the clinical response was evaluated after 2-3 cycles of chemotherapy. Response to chemotherapy was graded by standard WHO criteria. Patients received chemotherapy every 3-4 weeks.

### Selection and Validation of Internal Control of Plasma miRNA

At first, miR-16 was selected as a control based on previous reports<sup>[32-34]</sup>, and was measured using qRT-PCR on a small set of plasma samples (20 NSCLCs and 20 controls). These samples were processed under the exactly same conditions.

### Isolation of Human Plasma

For miRNA detection, whole blood samples (5 ml per patient) were collected from subjects via a direct venous puncture into tubes containing EDTA, which were then centrifuged at  $2000 \times g$  for 10 min, and the supernatant (plasma) was carefully transferred into an RNase-free tube for RNA extraction. The resultant plasma was aliquoted into Eppendorf tubes and stored at  $-80^{\circ}\text{C}$ .

### Plasma RNA Extraction

Total RNA was extracted from 400 $\mu\text{l}$  plasma using mirVana PARIS Kit (Ambion), and finally eluted into 100 $\mu\text{l}$  pre-heated ( $95^{\circ}\text{C}$ ) Elution Solution according to the manufacturer's protocol. To allow normalization of sample-to-sample variation in the RNA isolation step, synthetic cel-miR-39 was added to each sample as described by Mitchell et al<sup>[22]</sup>.

### MiR-21 Quantification by qRT-PCR

The extracted total RNA was polyadenylated by

poly(A) polymerase and then reversely transcribed to cDNA in a final volume of 20 $\mu\text{l}$  using miScript Reverse Transcription kit (Qiagen, Germany). Real-time PCR was performed in duplicate measurements using miScript SYBR Green PCR kit (Qiagen, Germany) with the manufacture-provided miScript Universal primer and the miRNA-specific forward primers in the ABI PRISM 7300 Real-time PCR system (Applied Biosystems). The miRNA-specific primer sequences were 5' UAGCAGCACGUAAAUAUUGGCG3' for miR-16; 5' UAGCUUAUCAGACUGAUGUUGA3' for miR-21; 5'UCACCGGGUGUAAAUCAGCUUG3' for cel-miR-39. The miRNA-specific primer sequences were designed based on the miRNA sequences obtained from the miRBase database (<http://microrna.sanger.ac.uk>). Each amplification reaction was performed in a final volume of 25 $\mu\text{l}$  containing 2 $\mu\text{l}$  of cDNA, 2.5 $\mu\text{l}$  of each primer and 12.5 $\mu\text{l}$  of 1 $\times$ SYBR Green PCR Master mix. The amplification profile was: denaturation at  $95^{\circ}\text{C}$  for 15min, followed by 40 cycles of  $94^{\circ}\text{C}$  for 15s,  $57^{\circ}\text{C}$  for 30s and  $70^{\circ}\text{C}$  for 34s, in which fluorescence was acquired. At the end of the PCR cycles, melting curve analyses were performed as well as electrophoresis of the products on 2% agarose gels in order to validate the specific generation of the expected PCR products. Each sample was tested in duplicates. The expression levels of miR-21 were normalized to miR-16, and were calculated utilizing the  $2^{-\Delta\Delta\text{Ct}}$  method, where  $\Delta\Delta\text{Ct} = (\text{Ct}_{\text{miR-21}} - \text{Ct}_{\text{miR-16}})_{\text{NSCLC}} - (\text{Ct}_{\text{miR-21}} - \text{Ct}_{\text{miR-16}})_{\text{Meannormal}}$  and  $\text{Ct}' = (\text{Ct}_{\text{miR-21}} - \text{Ct}_{\text{miR-16}})_{\text{pro-chemotherapy}} - (\text{Ct}_{\text{miR-21}} - \text{Ct}_{\text{miR-16}})_{\text{post-chemotherapy}}$ , and Ct is the threshold cycle to detect fluorescence<sup>[35]</sup>.

### Statistical Analysis

The significance of plasma miR-21 levels was determined by the Mann-Whitney test, Wilcoxon test, Independent-Samples t test, the  $\chi^2$  test or Kruskal-Wallis test where appropriate. The probability of progression-free survival (PFS) as a function of time was determined by the Kaplan-Meier method. Receiver operating characteristic (ROC) curves were established for discriminating NSCLC and controls. All *P* values are two-sided and less than 0.05 was considered statistically significant. All statistical calculations were performed by GraphPad Prism V4.03 or the SPSS 16.0 software.

## RESULTS

### Patient Characteristics

A total of 113 participants including 77 NSCLC patients and 36 healthy controls were recruited. Patient characteristics are summarized in Table 1. There were no significant differences of age or gender between NSCLC patients and healthy controls ( $P=0.0890$ , Independent-Samples t test,  $P=0.8420$ ,  $\chi^2$  test).

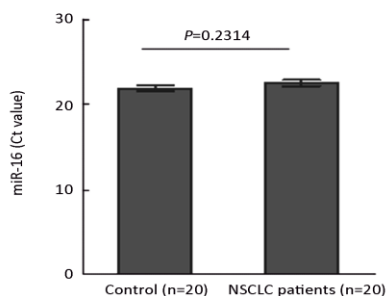
### The Validation of Internal Control on a Small Set of Plasma Samples

RNU6B and miR-16 have been proposed as the internal normalization control for miRNA quantification. In our experiment, miR-16 was selected as an internal

control. We examined the levels of miR-16 by using qRT-PCR in the plasma of 20 controls and of 20 NSCLC patients. Our data showed that no significant difference was observed in terms of Ct values of miR-16 ( $P=0.2314$ , Mann-Whitney U test, Figure 1) between controls and NSCLC samples. Therefore, miR-16 was served as an internal control.

**Table 1.** Characteristics of enrolled subjects

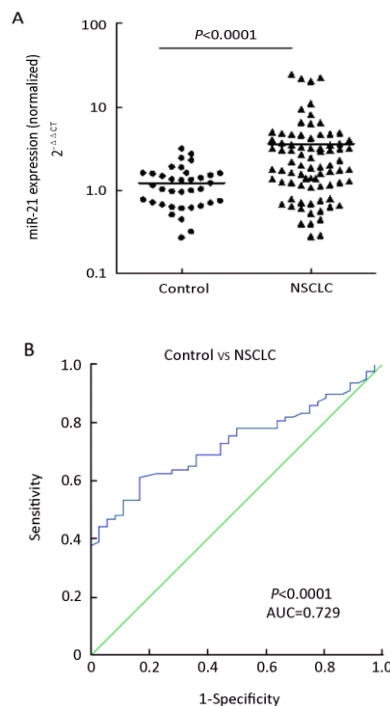
Characteristics	NSCLC	Healthy controls	P value
Age			
$\bar{x} \pm s$	59.6±9.1	56.4±9.2	0.0890
<59	37		
>59	42		0.7495
Gender			
Male	55	27	
Female	22	9	0.8420
Smoking status			
Smoker	40		
Never smoker	37		0.3290
Histological			
Adenocarcinoma	44		
Squamous-cell	21		
Large cell	2		
Unclassified	10		0.1855
TNM stage			
I-II	24		
III-IV	53		0.2812
T1-2	31		
T3-4	46		0.2621
Lymph node			
N0	47		
N+	30		0.7140
Metastasis			
M0	46		
M1	31		0.7633
Operation status			
Yes	39		
No	38		0.5276



**Figure 1.** MiR-16 levels were tested as a baseline internal control. Levels of miR-16 in the plasma of 20 controls and 20 NSCLC patients were measured by qRT-PCR.

**The Diagnostic Value of miR-21 in Plasma for NSCLC**  
qRT-PCR analysis indicated that the NSCLC patients

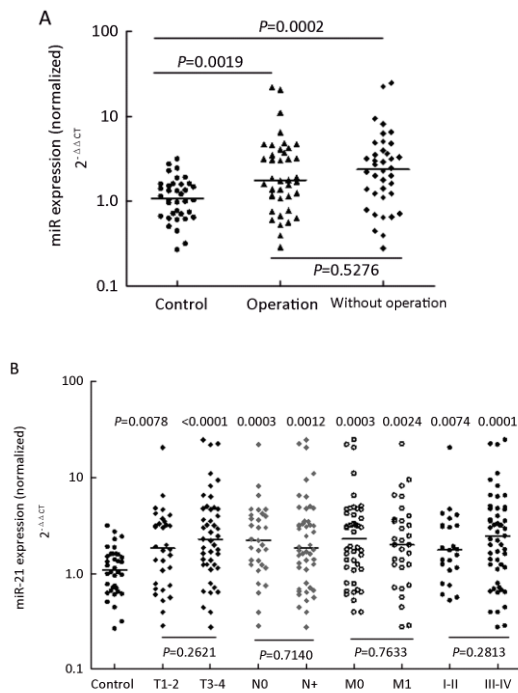
had significant higher levels of miR-21 in their plasma than in the controls ( $P<0.0001$ , Figure 2A). ROC curve analyses also showed that miR-21 yielded an area of 0.729 under ROC. At the cutoff value of 1.630 for this marker, the sensitivity and the specificity were 61.04% and 83.33%, respectively (95%CI: 0.639-0.819, Figure 2B). The odds ratio (OR) for cases with miR-21 >1.630 being associated with NSCLC was 7.833 (95%CI: 2.914-21.060). These results demonstrated that miR-21 levels possessed good diagnostic value for NSCLC patients.



**Figure 2.** MiR-21 levels in NSCLC patients and healthy controls. A: Scatter plots of plasma levels of miR-21 in healthy subjects (n=36) and NSCLC patients (n=77). Expression levels of the miR-21(Log10 scale at Y-axis) are normalized to miR-16. The line represents the median value. Mann-Whitney U test was used to determine statistical significance. B: Receiver operating characteristics (ROC) curve analysis using plasma miR-21 for discriminating NSCLC.

**Relationship between Plasma Level of miR-21 and Clinical Characteristics**

We examined the correlation between the expression of miR-21 and clinical parameters. Statistical analysis studies revealed that operable patients of NSCLC still had significantly higher levels of miR-21 in their plasma than the controls ( $P=0.0019$ , Figure 3A). No significant association was found between the miR-21 and gender, age, smoking status or history classification ( $P>0.05$ ). There is no difference between the plasma miR-21 levels of T1-2 patients and those of T3-4 patients, but both of them were significantly higher than controls. The plasma levels of miR-21 in N0 and N+, M0 and M1, or stage I-II and III-IV patients showed the same negative results as above (Figure 3B). So we suggested that there was no clear correlation between miR-21 and TNM staging.

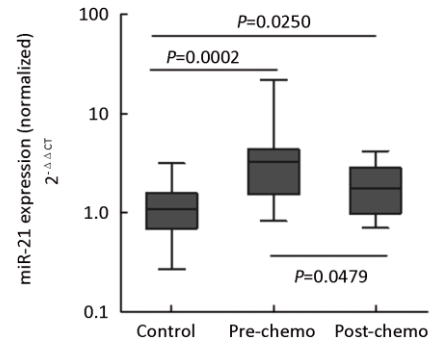


**Figure 3.** The correlation between the expression of miR-21 and clinical parameters. A: MiR-21 levels were measured in NSCLC patients with or without operation. The line represents the median value. B: MiR-21 levels in patients at different TNM stages were measured. The line represents the median value.

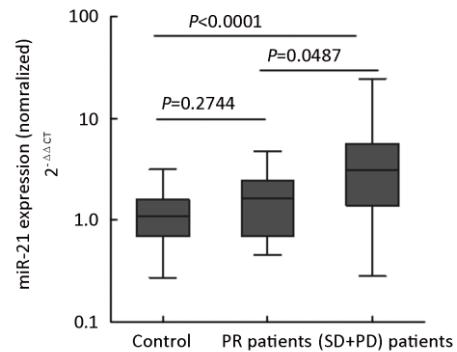
#### The Relationship between miR-21 Level in Plasma of NSCLC Patients and Effects of Chemotherapy

The concentration of miR-21 was analyzed in paired pre- and post-chemotherapy plasma samples from 2 TNM stage I, 3 with stage II, and 8 stage III patients. The plasma miR-21 levels of these 13 post-operative patients were still higher than those of normal controls ( $P=0.0002$ ). They were then received 2-3 cycles of platinum-based combination chemotherapy. MiR-21 levels were significantly reduced in post-chemotherapy samples compared with the levels in pre-chemotherapy samples ( $P=0.0479$ , Wilcoxon tests). And if compared to controls, chemotherapy seemingly resulted in a dramatically decreased plasma miR-21 level ( $P=0.0002$  for pre-,  $P=0.0250$  for post-chemotherapy, Mann-Whitney test; Figure 4). These findings showed that surgery can not completely reduce the level of miR-21 to baseline and there could be a possible correlation between therapeutic effect and the plasma miR-21 level. Another 35 patients (stages IIIB and IV) were evaluated for responses to chemotherapy. Response was graded by standard WHO criteria. After 2-3 cycles of platinum-based chemotherapy, 11 patients acquired partial response (PR), 24 patients acquired stable disease or progression disease (SD+PD) and no patients acquired complete response (CR). The total effective power (CR+PR) is 31.4%. Importantly, the levels of plasma miR-21 in PR samples were lower than that of (SD+PD) samples ( $P=0.0487$ , Mann-Whitney test). Furthermore, the plasma miR-21 levels of 11 PR samples were close to those of healthy controls ( $P=0.2744$ ,

Mann-Whitney test; Figure 5). These results indicated that the quantity of miR-21 in plasma could be used as a potential biomarker for predicting platinum-based chemo-sensitivity of NSCLC.



**Figure 4.** Shows the measurement of plasma miR-21 level in healthy controls and 13 post-operative patients before and after chemotherapy. Expression levels of the miR-21 (Log10 scale at Y-axis) are normalized to miR-16. Statistically significant differences were determined using Mann-Whitney U test. pre-chemo: pre-chemotherapy; post-chemo: post-chemotherapy.



**Figure 5.** Analysis of the miR-21 plasma levels in PR and (SD+PD) samples. Expression levels of the miR-21 (Log10 scale at Y-axis) are normalized to miR-16. Statistically significant differences were determined using Mann-Whitney U test. PR: partial response; SD: stable disease; PD: progression disease

#### Relationship between MiR-21 Plasma Level and Treatment Outcome in NSCLC Patients

To determine whether the miR-21 level could serve as a prognostic marker of chemotherapy, we reviewed follow-up care for the 77 NSCLC patients. During the follow-up, 4 operable patients and 13 non-operable patients occurred to progress, and 9 of all patients, operable and non-operable, died. At 12 month, analysis indicated that the median time to progression of 17 PD patients was 4.3 months (95% CI, 1.476-7.124).

#### DISCUSSION

In this study, we found that the plasma levels of miR-21 in NSCLC patients were markedly higher than those in healthy controls. MiR-21 had important diagnostic value for NSCLC and yielded AUC of 0.729

with 61.04% sensitivity and 83.33% specificity (95% CI 0.639-0.819). The result demonstrated that plasma miR-21 could be a potential noninvasive biomarker for NSCLC. There was no significant difference in the expression levels of miR-21 when stage I-II patients were compared with stage III-IV patients, indicating that miR-21 remained relatively high level after surgery and miR-21 over-expression is an early event in lung carcinogenesis.

Higher levels of miR-21 expression have been reported in non-smoker NSCLC than smoker ones<sup>[36]</sup>. Nevertheless, there was no difference between the plasma miR21 levels of smokers and non-smokers in our study. Either way, it has not been confirmed whether this was associated with sample size or ethnics. Based on this we suggested that miR-21 might not be involved in the carcinogenesis of NSCLC in non-smokers which should be further investigated. Among all patients, early stage patients received operation. After surgery, however, plasma miR-21 levels remained higher than controls. This result indicated that surgery can decrease miRNA expression, but not to normal level. Several more recent studies have documented that tumor cells released functional miRNA-enriched microparticles into the blood<sup>[37-40]</sup>. Nevertheless, the mechanism of plasma miRNAs generation and physiological function are unclear.

The kinetics and metabolism of the plasma miRNAs have not been clearly elucidated, but the plasma miRNAs showed potentials to be new therapeutic targets in treating NSCLC. Our study explored the possible association between miR-21 and clinical response of chemotherapy in NSCLC. We hypothesize that the plasma miR-21 expression level may help in predicting a patient's response to platinum-based combination chemotherapy, commonly used for the treatment of NSCLC in the clinic and lower levels of miR-21 expression in plasma correlates with better response to platinum-based chemotherapy in NSCLC.

Platinum-based chemotherapy is the mainstream method of treatment against advanced NSCLC and for early stage patients who had high risk factors. However, tumor resistance to chemotherapy is the leading cause of poor treatment outcome. Thus, predictive markers of sensitivity to adjuvant therapy and new therapeutic targets are urgently needed in NSCLC. Recent studies suggest that miR-21 might be potential biomarker and drug targets to predict chemotherapy's efficiency. Meng et al<sup>[41]</sup> found that inhibition of miR-21 expression in cholangiocarcinoma cells can increase cell sensitivity to gemcitabine. Si et al<sup>[42]</sup> reported that suppression of over-expressed miR-21 in MCF-7 cells was associated with increased cell apoptosis and decreased proliferation. Then, Schetter et al<sup>[43]</sup> first analyzed the correlation between miR-21 expression, 5-FU activity and therapeutic outcomes in colon cancer patients, showing that high miR-21 expression was associated with a poor response to 5-FU-based chemotherapy and poor prognosis. Yan et al<sup>[44]</sup> showed that overexpression of miR-21 was correlated with disease progression and poor prognosis

in breast cancer. Recently, Hwang et al<sup>[45]</sup> investigated whether miR-21 can predict clinical outcome in pancreatic ductal adenocarcinoma (PDAC) patients treated with adjuvant therapy. The results suggested that low miR-21 expression was seen by adjuvant treatment in PDAC patients and anti-miR-21 increased anticancer drug activity in vitro. Gao et al<sup>[46]</sup> indicated that high-level miR-21 in squamous cell lung carcinoma tissue was associated with shortened survival time. On the contrary, Voortman and his colleagues<sup>[47]</sup> suggested that there was no significant association between the tested microRNAs (miR-21, miR-29b, miR-34a/b/c, miR-155 and let-7a) expression and survival as well as predicted chemotherapy response in resected NSCLC patients. Our results demonstrated that high plasma miR-21 level is associated with poor treatment response in NSCLC patients. Furthermore, the results suggested that tumor resection can not completely return miRNAs level to baseline level and chemotherapy may be a powerful and useful elimination tool. Because patients with advanced lung cancer are difficult to obtain tissue samples, plasma miR-21 may serve as a potential biomarker for diagnosis, therapy assessment and prognosis for NSCLC.

However, some issues should be considered when interpreting the results of this study. First, as the sample size is small, further validations of this marker in large cohorts and independent data set are necessary. Second, it is unable to eliminate other chemotherapy drugs' effect on miR-21 levels. Third, because the follow-up time is short, the correlation between levels of miR-21 in plasma and prognosis cannot be analyzed. Another limitation is that the molecular mechanism of miR-21 elevation and its role in NSCLC development and progression is currently unclear. Whether plasma miR-21 could be released from the primary tumor tissues remains controversial, thus further studies are warranted.

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