

Original Article

Identification of plasma microRNA-21 as a biomarker for early detection and chemosensitivity of non-small cell lung cancer

Juan Wei, Wen Gao, Cheng-Jun Zhu, Yi-Qian Liu, Zhu Mei, Ting Cheng and Yong-Qian Shu

Abstract

Studies have shown cell-free microRNA (miRNA) circulating in the serum and plasma with specific expression in cancer, indicating the potential of using miRNAs as biomarkers for cancer diagnosis and therapy. This study was to investigate whether plasma miRNA-21 (miR-21) can be used as a biomarker for the early detection of non-small cell lung cancer (NSCLC) and to explore its association with clinicopathologic features and sensitivity to platinum-based chemotherapy. We used real-time RT-PCR to investigate the expression of miR-21 in the plasma of 63 NSCLC patients and 30 healthy controls and correlated the findings with early diagnosis, pathologic parameters, and treatment. Thirty-five patients (stages IIIB and IV) were evaluable for chemotherapeutic responses: 11 had partial response (PR); 24 had stable and progressive disease (SD+PD). Plasma miR-21 was significantly higher in NSCLC patients than in age- and sex-matched controls ($P < 0.001$). miR-21 was related to TNM stage ($P < 0.001$), but not related to age, sex, smoking status, histological classification, lymph node status, and metastasis (all $P > 0.05$). This marker yielded a receiver operating characteristic (ROC) curve area of 0.775 (95% CI: 0.681 – 0.868) with 76.2% sensitivity and 70.0% specificity. Importantly, miR-21 plasma levels in PR samples were several folds lower than that in SD plus PD samples ($P = 0.049$), and were close to that in healthy controls ($P = 0.130$). Plasma miR-21 can serve as a circulating tumor biomarker for the early diagnosis of NSCLC and is related to the sensitivity to platinum-base chemotherapy.

Key words Lung neoplasm, non-small cell, miRNA-21, biomarker, plasma, early detection, chemosensitivity

Lung cancer, one of the most common malignant tumors, is a serious disease threatening human life and health. Of the pathologic types of lung cancer, non-small cell lung cancer (NSCLC) is the most prevalent (80%). The lack of effective early diagnostic and treatment methods are the main causes of high mortality in patients with lung cancer, with the 5-year survival rate at 15% to 20%. MicroRNA (miRNA) is a class of 20 to 25 nt long non-coding RNA found *in vivo*. miRNA genes are transcribed in the nucleus to primary transcripts

(pri-miRNA)^[1]. After digestion by Drosha, precursor miRNA (pre-miRNA) is then formed and is transported to the cytoplasm and digested by Dicer to produce mature miRNA^[2,3], which regulates gene expression mainly through its partially complementary to 3'-untranslated region (3' UTR) of target mRNA^[4].

Among their functional roles, miRNAs regulate development, organogenesis, hematopoiesis, cell proliferation and apoptosis, and tumorigenesis and progression. It has been confirmed that miRNAs are involved in the development of a variety of tumors, such as leukemia^[5], neuroblastoma^[6], pituitary adenoma^[7], breast cancer^[8], thyroid cancer^[9], hepatocarcinoma^[10], colorectal cancer^[11], and lung cancer^[12]. The up- or down-regulation of different miRNAs in these tumor tissues were found with most of the miRNA targets located in regions of tumor-related genome, fragile sites, loss of heterozygosity, and amplified regions^[5], thereby exhibiting the same effect as oncogenes or tumor

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suppressor genes.

An increasing number of studies have shown that analysis of miRNA expression has a potential application value in tumor diagnosis, treatment, and prognosis prediction. It has been observed that miRNAs are closely related to tumor development and stably exist in serum/plasma^[13-15]. The origin of miRNAs is unclear and lacks experimental evidence, but current assumptions of the origin include cancer cells in peripheral blood^[16] or tumor-associated bodies^[17,18]. The first miRNA marker found in serum is miRNA-21 (miR-21). Lawrie *et al.*^[19] proved that high expression of miR-15, miR-21, and miR-210 existed in the serum of patients with diffuse large B-cell lymphoma (DLBCL) using real-time fluorescence quantitative PCR, and miR-21 with largest differential expression is related to lymphoma recurrence and patient survival. Subsequently, Mitchell *et al.*^[16] found that miR-141 was highly expressed in patients with metastatic prostate cancer. The level of miR-141 expression is highly specific and sensitive for the diagnosis of prostate cancer and thus, can serve as a marker. In addition, the level of miR-184 expression in the plasma of patients with tongue squamous cell carcinoma was significantly higher than that in healthy people and was decreased significantly after tumor removal and corresponding treatment^[20]. Ng *et al.*^[21] found that the expression levels of miR-17-3p and miR-92 in the plasma and tissue of patients with rectal cancer were significantly increased and that the expression of miR-92 was significantly reduced after tumor resection, suggesting that miR-92 can function as a serum molecular marker for the diagnosis of colorectal cancer at stage I-IV, whereas early diagnosis can improve survival and prognosis. The expression of miR-155 in patients with progesterone receptor-positive breast cancer was higher than that in progesterone receptor-negative patients, suggesting that miR-155 can serve as a molecular marker differentiating hormone-sensitive breast cancer from non-sensitive ones^[22]. These studies indicate that plasma/serum miRNAs may function as potential biomarkers for the non-invasive diagnosis, treatment, and prognosis of cancers.

In the present study, we measured the plasma level of miR-21 and determined its relationship to the early diagnosis of NSCLC, efficacy of chemotherapy, and clinicopathologic characteristics of patients with NSCLC.

Materials and Methods

Study subjects

Peripheral blood specimens from 63 patients with pathologically confirmed NSCLC were collected between October 2009 and August 2010 from the Departments of

Thoracic Surgery and Oncology in the First Affiliated Hospital of Nanjing Medical University. Of the 63 patients, 45 were men and 18 were women, aged of 36 to 83 years with a median of 61 years. The patients did not undergo chemotherapy or radiotherapy prior to admission. Venous blood was drawn before surgery for patients with early stage NSCLC. For those with advanced NSCLC, CT was used to confirm measurable tumor lesions. In addition, functional scores before chemotherapy were greater than 60, with blood and kidney function in the normal range and a normal electrocardiogram. Venous blood was drawn before chemotherapy for all patients. According to the 7th edition of TNM staging of the Union for International Cancer Control (UICC) and American Joint Committee on Cancer (AJCC) in 2009, 18 cases were classified as stage I, 5 as stage II, 5 as stage III, and 35 as stage IV. Thirty-eight patients had adenocarcinoma, 15 had squamous cell carcinoma, 1 had large cell carcinoma, and 9 had unclassified tumors. In addition, 30 had lymph node metastasis and 33 had no lymph node metastasis. The control group was composed of 30 healthy people: 20 men and 10 women aged 41 to 75 years with a median of 57 years. There was no statistical difference in age ($P = 0.086$, Mann-Whitney test) and sex ($P = 0.803$, χ^2 test) between NSCLC patients and the control group.

Chemotherapy regimen and the evaluation of therapeutic efficacy

Of the 63 patients, 40 underwent cisplatin- or carboplatin-based chemotherapy; 35 had measurable lesions. The chemotherapy regimen was repeated every 3 to 4 weeks. After 2 to 3 cycles of treatment, an objective evaluation was made according to the standards of Evaluation Criteria in Solid Tumors recommended by the WHO, defined as complete remission (CR), partial remission (PR), stable disease (SD), and progressive disease (PD).

Real-time quantitative RT-PCR detection of miR-21 expression in plasma

Peripheral blood specimen (5 mL) was conventionally drawn from each patient before surgery or chemotherapy. EDTA was used as an anticoagulant. Plasma and blood cells were separated by centrifugation at $1200 \times g$ for 10 min at 4°C , and stored in 1.5 mL RNase-free tubes at -80°C . Peripheral blood was processed within 4 h.

Plasma (400 μL) from each sample was centrifuged at $1200 \times g$ for 10 min at 4°C before RNA extraction. Total RNA was extracted using mirVana PARIS kit according to manufacturer's instruction (Ambion, USA). To eliminate differences among samples, synthetic

Caenorhabditis elegans cel-miR-39, which has no homologous gene in human, was added into each sample. RNA was dissolved in 100 μ L of RNase-free water. Absorbance at 260 and 280 nm was measured using a spectrophotometer to determine the concentration and purity of RNA.

The expression of miR-21 in NSCLC patients and healthy controls was detected by using miScript SYBR Green PCR kit (Qiagen, Germany). The reaction was performed in a real-time quantitative PCR machine ABI7300HT. Reverse transcription of miRNA was performed according to manufacturer's instruction using the miScript Reverse Transcription kit (Qiagen). The first chain of cDNA was reverse transcribed from 2 μ L of total RNA using specific stem-loop structure RT primers of miR-21. The amount of RNA was determined by the volume of plasma: $(2.0/80) \times 400 = 10$ mL, 2 μ L of RNA corresponding to 10 μ L plasma^[16]. The reverse transcription reaction was carried out at 37°C for 60 min and at 95°C for 5 min. Subsequently, PCR was performed using the first chain of cDNA as the template. The reaction system contained 2 μ L of cDNA, 12.5 μ L of 1 \times SYBR Green I Master mix, 2.5 μ L of miRNA specific primers, and 2.5 μ L of upstream universal primer. The reaction conditions were as follows: denatured at 95°C for 15 min, 40 cycles of 95°C for 15 s, 57°C for 30 s, and 70°C for 34 s. miR-16 was used as an internal reference for normalization. An average of triplicate findings was considered as the result for each sample. The sequence of cel-miR-39 was 5'-UCACCGGGUGUAAAUCAGCUUG-3'; the sequence of miR-16 primer was 5'-UAGCAG-CACGUAAAUAUUGGCG-3'; the sequence of miR-21 primer was 5'-UAGCUUAUCAGACUGAUGUUGA-3' (synthesized by Qiagen). Finally, Ct values were measured using melting curve analysis to represent the expression of target gene mRNA. Relative changes of gene expression were represented by $2^{-\Delta\Delta C_t}$, the difference between the original copy number of miR-21 in the NSCLC group and that in the normal group. $\Delta\Delta C_t = (C_{t_{miR-21}} - C_{t_{miR-16}})$ of the NSCLC group - $(C_{t_{miR-21}} - C_{t_{miR-16}})_{mean}$ of the control group.

Statistical analysis

GraphPad Prism V4.03 and SPSS16.0 statistical softwares were used for statistical analysis. Log10 normality conversion was used to analyze the relative expression of miR-21. The Mann-Whitney *U* test was used for data comparison between two groups. The Kruskal-Wallis *H* test was used for comparison among multiple groups. The receiver operating characteristic (ROC) curve was drawn to evaluate the diagnosis value of plasma miR-21 for NSCLC. $P < 0.05$ indicates a statistical significance.

Results

The expression levels of plasma miR-21 in NSCLC group and healthy control group detected by real-time quantitative RT-PCR

Using miR-16 as an internal reference, the expression level of plasma miR-21 was detected by real-time fluorescence quantitative PCR. The quantitative PCR amplification and melting curves reveal the expression levels of miR-21 and miR-16 (Figure 1).

Relationship between plasma miR-21 expression and clinicopathologic features of NSCLC

PCR results showed that miR-21 expression was significantly higher in the NSCLC group than in the control group ($P < 0.001$) (Figure 2). miR-21 expression was not related to age, sex, smoking status, pathologic type, lymph node metastasis, and distant metastasis (all $P > 0.05$), but related to TNM stage ($P < 0.001$). Plasma miR-21 expression was higher in stage T3–4 patients than in stage T1–2 patients ($P = 0.036$) and was higher in stage III–IV patients than in stage I–II patients ($P = 0.043$) (Figure 2), indicating that plasma miR-21 is important for the diagnosis of stage I–IV NSCLC. The area under the ROC curve (AUC) for plasma miR-21 was 0.775 (95% CI: 0.681–0.868), the sensitivity and specificity were 76.2% and 70.0%, respectively, and the value of the cut-off point was 1.308 (Figure 3).

Relationship between plasma miR-21 expression and chemotherapeutic efficacy

Thirty-five patients with advanced NSCLC who underwent 2 to 3 cycles of platinum-base chemotherapy were followed up: 11 had PR, 13 had PD, 11 had SD, and none achieved CR after chemotherapy. The expression of plasma miR-21 was significantly higher in the PD plus SD group than in the PR group ($P = 0.049$), whereas no statistical significance was found between the PR group and the control group ($P = 0.130$) (Figure 4), suggesting that plasma miR-21 expression may correlate with sensitivity to chemotherapy in NSCLC.

Discussion

miR-21 is overexpressed in lung cancer, breast cancer, glioblastoma, gastric cancer, pancreatic cancer, pancreatic neuroendocrine tumor, liver cancer, bile duct cancer, colon cancer, and prostate cancer^[23]. miR-21 is encoded by a single gene, which locates at the fragile site FRA17B on 17p23.2 and is overlapped with the

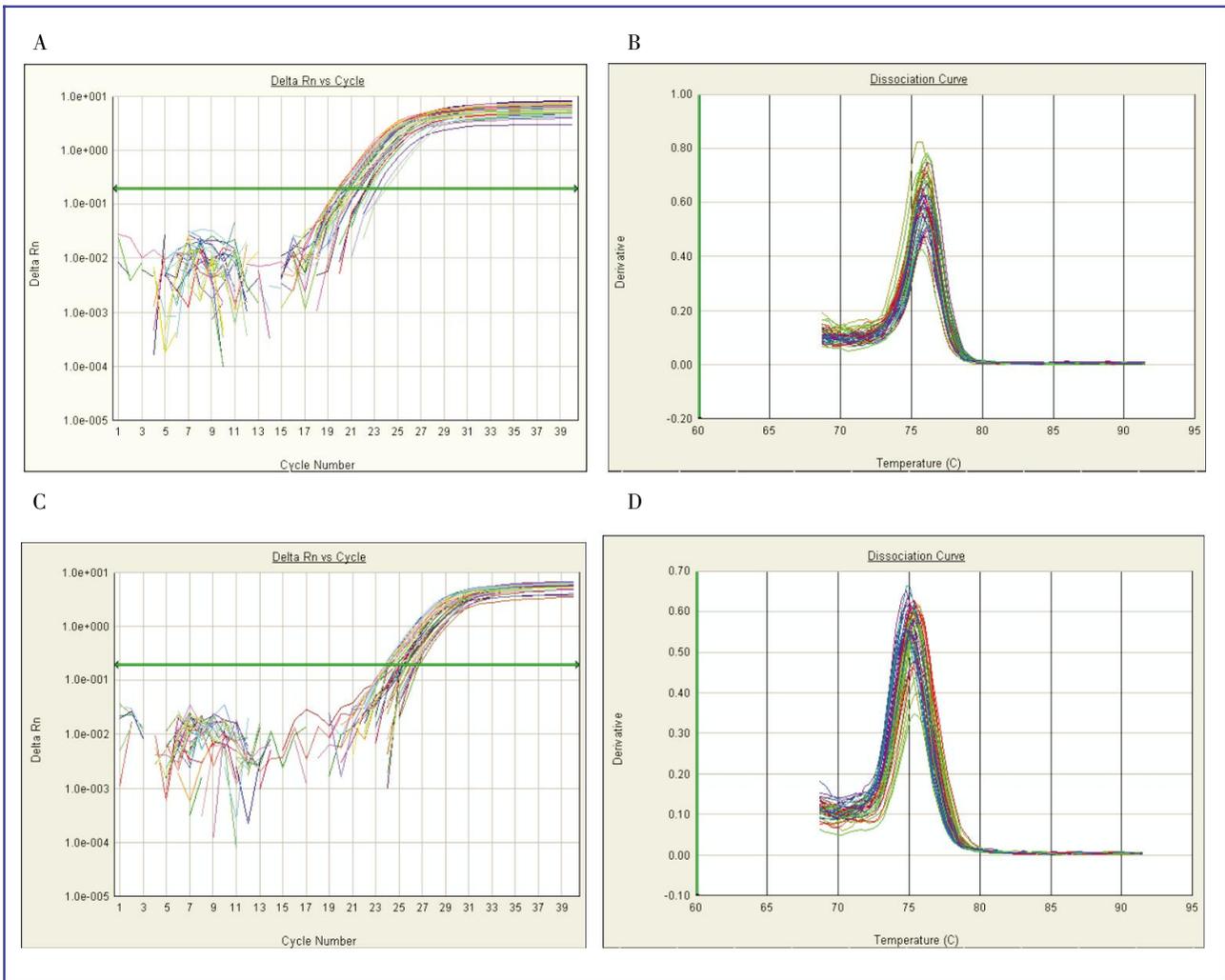


Figure 1. The amplification and melting curves of miR-16 and miR-21. A, amplification curve of miR-16; B, melting curve of miR-16; C, amplification curve of miR-21; D, melting curve of miR-21. Quantitative fluorescence amplification curves show the relationship between fluorescence and the number of cycles. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold. Ct levels are inversely proportionate to the amount of target nucleic acid in the sample: the lower the Ct level, the greater the amount of target nucleic acid in the sample. Ct \leq 29 indicates strong positive reactions indicative of abundant target nucleic acid in the sample. Melting curve is the quality control of amplification. It is used to analyze the homogeneity of PCR products. The results show that the Ct value of miR-16 is about 21, the Ct value of miR-21 is about 25, and the Tm is about 75°C. Impure or abnormal broaden peaks do not appear in the figure, indicating that there is no contamination, primer dimmers, or nonspecific amplification.

gene encoding transmembrane protein 49 (TMEM49, also known as VMP-1)^[24]. miR-21 contains 22 nucleotides and is separated from a 3400 nt pre-transcript^[25]. Abnormal expression of miR-21 may be related to transcription and post-transcriptional regulation^[24]. miR-21 regulates cell differentiation, proliferation, and apoptosis and is involved in tumor cell invasion, vascular infiltration, and metastasis by regulating target genes, including tropomyosin 1 (*TPM1*), programmed cell death gene 4 (*PDCD4*), methyl-adenosine phosphatase (*MTAP*), *SOX5*, genes in the p53-mediated pathway and transforming growth factor (TGF- β) pathway, *Bcl-2*,

phosphatase and tensin homolog (*PTEN*). In addition, the high expression of miR-21 in patients with colorectal cancer is correlated to clinical stage, lymph node metastasis, and distant metastasis^[26]; stages of breast cancer and liver metastases accompany with the increasing expression level of miR-21^[8]. Several studies have also confirmed that miR-21 plays an important clinical role in tumor diagnosis and evaluation. Schetter *et al.*^[27] found that high expression of miR-21 in colon cancer is related to poor efficacy of treatment and prognosis. Yan *et al.*^[28] found that overexpression of miR-21 in breast cancer is associated to lymph node

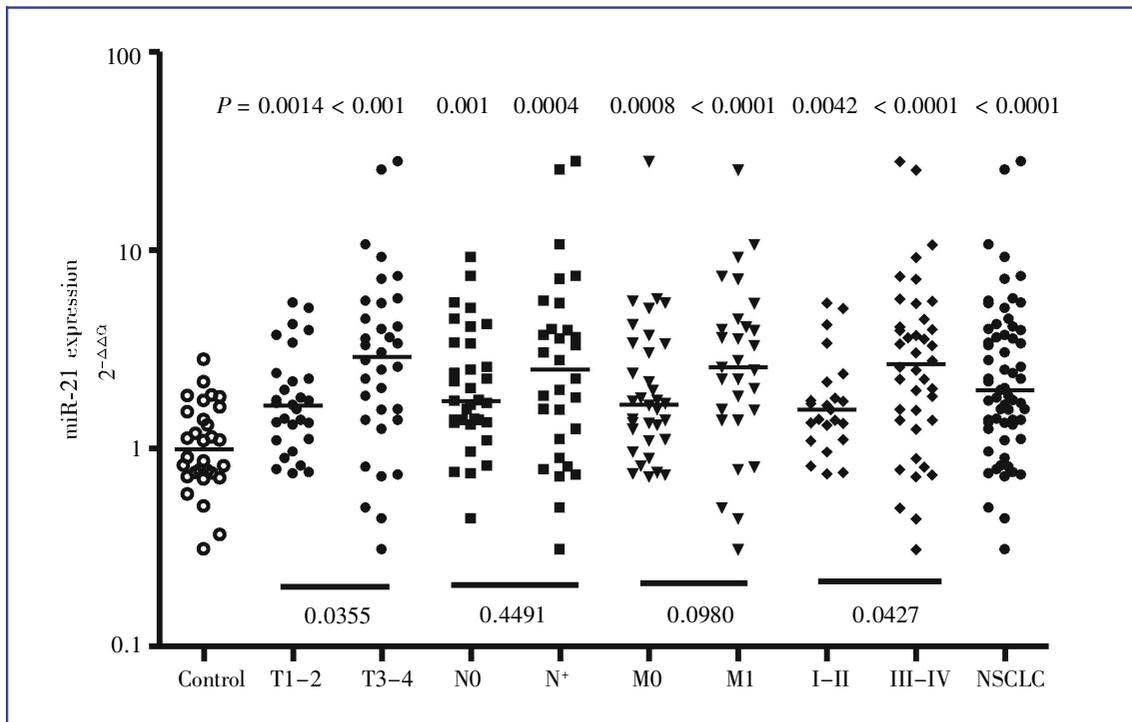


Figure 2. The correlation between the expression of miR-21 and clinical parameters of non-small cell lung cancer (NSCLC) analyzed by Mann-Whitney *U* test. The expression of miR-21 is significantly higher in NSCLC than in normal control ($P = 0.001$), higher in stage T3-4 NSCLC than in stage T1-2 NSCLC ($P = 0.036$), and higher in stage III-IV NSCLC than in stage I-II NSCLC ($P = 0.043$).

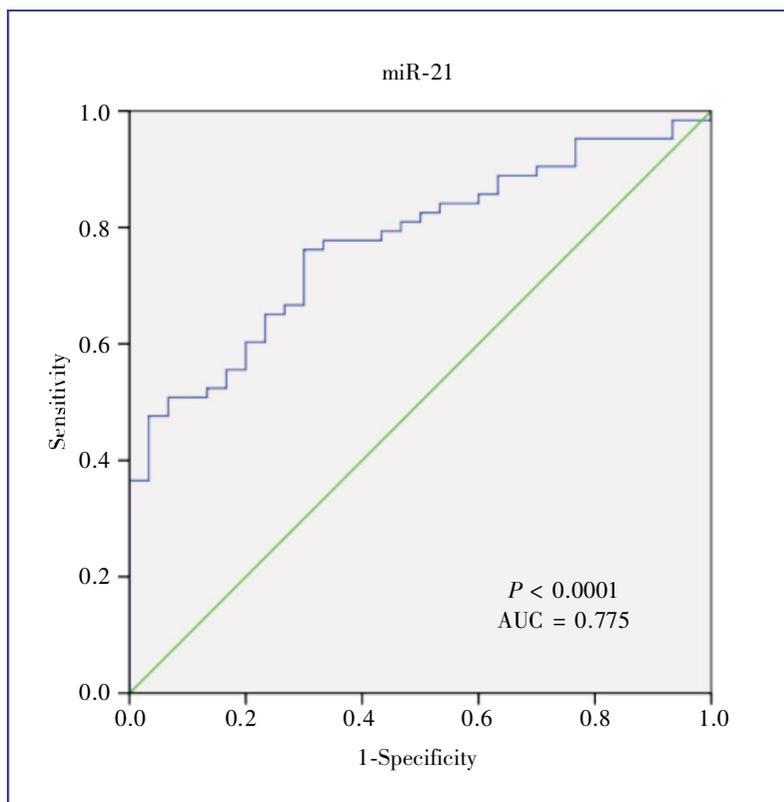


Figure 3. Diagnostic value of plasma miR-21 for NSCLC analyzed with receiver operating characteristics (ROC) curve. Plasma miR-21 yielded an (area under the ROC curve (AUC) of 0.775 (95% CI: 0.681 – 0.868) with 76.2% sensitivity and 70.0% specificity. The cut-off value was 1.308.

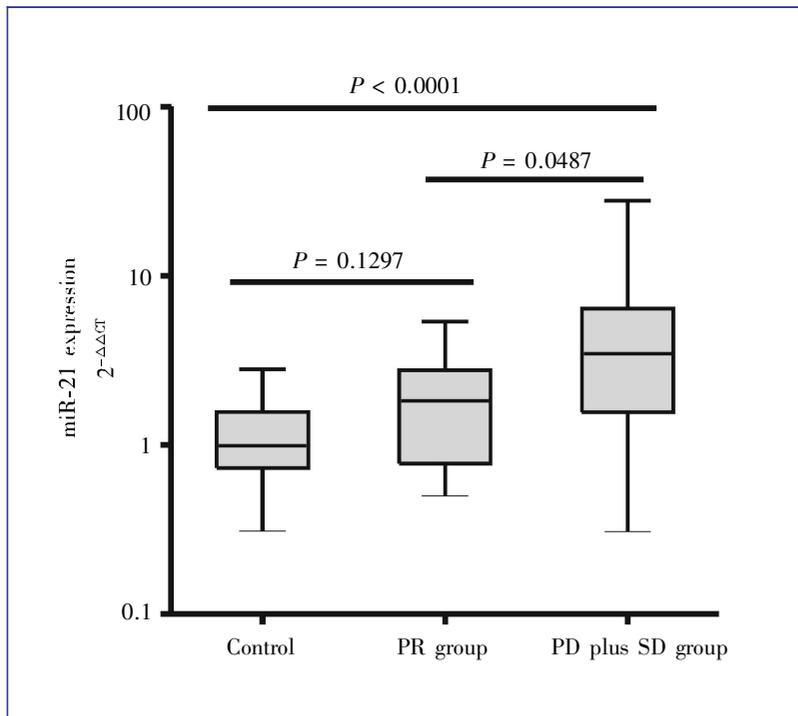


Figure 4. Plasma level of miR-21 in patients with partial remission (PR) or progressive disease (PD) and stable disease (SD). Expression levels of the miR-21 (Log10 scale at Y-axis) are normalized to miR-16. Statistically significant differences were determined using the Mann-Whitney *U* test. Plasma level of miR-21 is significantly higher in PD plus SD samples than in PR samples ($P < 0.05$) and healthy control samples ($P < 0.001$), and is similar between PR samples and control samples. The quantity of miR-21 in plasma may be a potential biomarker for predicting platinum-based chemo-sensitivity of NSCLC.

metastasis and poor prognosis. Gao *et al.*^[29] revealed that high expression of miR-21 in lung squamous cell carcinoma indicates poor prognosis. However, it has been reported that miR-21 only serves as a diagnostic marker in gastric cancer with no correlation to prognosis^[23].

In this study, we used real-time quantitative PCR to determine the expression of plasma miR-21 in patients with NSCLC. Plasma miR-21 expression was significantly higher in NSCLC patients than in the control group ($P < 0.0001$). ROC curve analysis showed the AUC was 0.775; the sensitivity and specificity were 76.19% and 70.0%. miR-21 expression was significantly related to TNM stage. The expression of plasma miR-21 was higher in patients with advanced NSCLC than in those with early stage NSCLC, suggesting that miR-21 expression may be related to factors associated with tumor progression. In particular, the expression of plasma miR-21 was significantly higher in patients with stage T1–2, N0 or I–II NSCLC than in the control group, suggesting that plasma miR-21 can be used as an effective, non-invasive marker for early NSCLC diagnosis.

Recently, studies have shown that miRNA-based treatment has a potential effect. Meng *et al.*^[30] found that the application of anti-miR-21 AMOs (anti-miRNA oligonucleotides) increase sensitivity of cholangiocarcinoma cells to gemcitabine. Si *et al.*^[31] treated miR-21/AMOs-transfected cancer cells with topotecan and found that antisense inhibition of

miR-21-mediated cell growth was significantly inhibited. Li *et al.*^[32] found that overexpression of miR-21 in malignant glioma cells leads to tumor cell resistance to chemotherapeutic drugs and that inhibiting the expression of miR-21 in malignant glioma U373MG cells by specific AMOs increases the sensitivity of U373MG cells Vm-26, indicating that miR-21 can be used to increase efficacy of drugs on malignant glioma. Hwang *et al.*^[33] retrospectively studied the effect of miR-21 on the chemotherapy efficacy and prognosis of pancreatic cancer: patients with low expression of miR-21 have significantly prolonged overall and disease-free survival after chemotherapy; antisense inhibition of miR-21 can enhance chemotherapeutic sensitivity. We followed up 35 patients with advanced NSCLC treated with platinum chemotherapy and found that the expression of plasma miR-21 in SD and PD patients was higher than that in PR patients. The expression of plasma miR-21 was low in 11 PR patients, which showed no significant difference when compared to that in the control group. Thus, miR-21 may be related to platinum sensitivity. The high expression of plasma miR-21 may also be involved in cell resistance to platinum chemotherapy. Inhibiting miR-21 expression is likely to restore cell sensitivity to platinum chemotherapy. Therefore, our study provides a favorable basis to use miR-21 as a predictor of sensitivity to platinum chemotherapy. Due to a short follow-up duration, we cannot calculate the overall and disease-free survival, but we will continue to explore the relationship between plasma miR-21 expression and

NSCLC prognosis.

In summary, our study demonstrated that the expression of plasma miR-21 is sensitive and specific for the early diagnosis of NSCLC and thus can be used as a potential biomarker for NSCLC diagnosis. Plasma miR-21 expression reflects sensitivity to platinum chemotherapy for NSCLC, suggesting that plasma miR-21 is a predictive indicator for sensitivity to platinum chemotherapy and may be a therapeutic target that reverse resistance to platinum chemotherapy.

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