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ORIGINAL ARTICLE

Prognostic Significance of Serum miR-22, miR-125b, and miR-15b in Non-Small Cell Lung Cancer Patients

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SUMMARY

Background: **Studies have shown that miRNA (miR) can be stably detected in serum, and aberrant expression of various miRNAs has shown diagnostic value in non-small cell lung cancer (NSCLC) patients. However, the role of miRNA in the context of prognosis has not been extensively investigated. Our previous study reported that miR-22, miR-125b, and miR-15b in serum had potential for use as tumor markers for auxiliary diagnosing of NSCLC. Therefore, the objective of this study was to detect the levels of miR-22, miR-125b, and miR-15b in serum from NSCLC patients and explore the potential prognostic significance of the three selected miRNAs.**
Methods: **The relative expression of miR-22, miR-125b, and miR-15b in 74 patients with advanced NSCLC in pre- and post-chemotherapy were detected by real-time quantitative polymerase chain reaction.**
Results: **Serum level of miR-125b significantly decreased after chemotherapy ($p < 0.05$) and the levels of miR-15b significantly increased ($p < 0.01$), while there was no change in the level of serum miR-22 ($Z = 0.716$, $p > 0.05$). Compared with pre-chemotherapy, serum miR-125b expression in advanced NSCLC patients of responders (CR + PR) were significantly decreased post-chemotherapy ($p < 0.05$); serum miR-15b expression in advanced NSCLC patients of responders (CR + PR) were increased ($p < 0.01$). The chemotherapy sensitivity of advanced NSCLC patients with high expression of miR-125b was lower than that of NSCLC patients with low expression ($p < 0.05$). The chemotherapy sensitivity of advanced NSCLC patients with high expression of miR-15b was higher than that of NSCLC patients with low expression ($p < 0.05$). High levels of serum miR-125b and low levels of serum miR-15b were related to poor overall survival ($p < 0.05$).**
Conclusions: **The serum levels of miR-125b and miR-15b in advanced NSCLC patients were changed pre- and post-chemotherapy and these changes were associated with chemotherapeutic response. Serum miR-125b and miR-15b have certain potential clinical value for chemotherapeutic response in advanced NSCLC. The serum levels of miR-125b and miR-15b in patients with advanced NSCLC before treatment may be used to estimate the overall survival.**
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KEY WORDS

non-small cell lung cancer, miRNA, chemotherapeutic response

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INTRODUCTION

Lung cancer is one of the major cancer diseases in the world. An estimated 1.3 million people will die from lung cancer each year [1]. Approximately 80% of lung cancer patients are non-small-cell lung cancer (NSCLC). The main types of NSCLC are adenocarcinomas and squamous cell carcinoma [2]. More than 75% of patients of NSCLC are diagnosed at an advanced stage, making them miss the optimal timing of surgery [3]. The main treatment method for advanced NSCLC is chemotherapy. The practice proved that combined chemotherapy can improve the survival rate of advanced NSCLC [4]. However, the effective rate of first-line chemotherapy is only 30%. Clinical resistance to chemotherapeutic drugs may result in not only treatment failure, but also in severe damage to the immune system and loss of optimal timing of other therapy [5,6]. Therefore, clinicians need to monitor tumor progression and optimize therapeutic strategies in time.

Circulating tumor markers are already used for cancer detection since there are blood tests available and cost is relatively low. Useful tumor markers are valuable for clinical diagnosis, prognostic evaluation, and therapy monitoring [7,8]. Serum miRNAs are known to be candidates for tumor biomarkers. miRNAs are single stranded, small (18 - 25 nucleotides), noncoding RNA molecules, which can regulate various cell functions by affecting target protein expression [9,10]. Analysis of miRNA expressions has a potential application value in cancer diagnosis [11,12]. Our previous study reported that miR-22, miR-125b, and miR-15b in serum had potential for use as a tumor marker for auxiliary diagnosing of NSCLC [13]. However, the clinical values of serum miRNAs in the context of prognosis prediction have not been extensively investigated. Therefore, in this study, we measured the relative serum expressions of miR-22, miR-125b, and miR-15b in NSCLC patients of pre- and post-chemotherapy and explore the potential prognostic significance of the three selected miRNAs.

MATERIALS AND METHODS

Study subjects

Seventy-four patients with advanced NSCLC referred to the Beijing Chest Hospital between August 2012 and October 2013, were enrolled in the study. Among the study subjects, 39 were male and 35 were female. The median age is 50.1 years (range, 41 - 73 years). The subtypes include 33 squamous-cell lung carcinoma and 41 adenocarcinoma. TNM classification was performed according to the Union for International Cancer Control (UICC) in 2009, there are 19 stage III and 55 stage IV patients. All cases were pathologically diagnosed with advanced NSCLC and had never received previous treatment. Cases with a family history of malignant tumors or with severe complications were excluded. This study was approved by the ethics committees of Beijing Tuberculosis Thoracic Tumor Institute.

Chemotherapy regimens and the evaluation of chemotherapeutic response

All advanced NSCLC patients were treated with cisplatin-based combination chemotherapy at a dose of 75 mg/m^2 of body surface area. The chemotherapy cycles were given every three weeks. The chemotherapeutic response after 2 cycles was evaluated on the advanced NSCLC patients whose routine blood parameters, liver, and kidney functions and physical conditions were appropriate for chemotherapy. The evaluation of the response rate was based on the standards of WHO Response Evaluation Criteria in Solid Tumors (RECIST). Chemotherapeutic response was categorized as a group of sensitive to chemotherapy including complete remission (CR) or partial remission (PR) and group of non-response including stable disease (SD) or progressive disease (PD).

Extraction of miRNA and real-time quantitative PCR (qRT-PCR)

Venous blood samples (1 mL) were obtained by the nurses from 74 patients of advanced NSCLC prior to any treatments and after completion of the second chemotherapy cycle. The blood samples were centrifuged at 4,000 rpm for 15 minutes, then the supernatant sera were separated and stored at -80°C until they were used for analyses. The miRNA was extracted from sera using the miRNA extraction kit (Tiangen Biology Co., Ltd Beijing, China) according to the manufacturer's instructions. The concentration and purity of miRNA were determined by using an ultraviolet spectrophotometer to measure the absorbance at 260 nm and 280 nm.

To synthesize cDNA, a reverse transcription reaction was performed using a kit (Tiangen Biology Co., Ltd Beijing, China). The reaction conditions were carried out at 37°C for 60 minutes and 95°C for 5 minutes. Subsequently, qRT-PCR was performed using SYBR qRT-PCR Kit (Tiangen Biology Co., Ltd Beijing, China). Amplification was carried out in a real-time PCR system of Light Cycler-480 (Roche Diagnostics, Basel, Sweden) under the following conditions: 94°C for 2 minutes, 45 cycles of 94°C for 20 seconds and 60°C for 34 seconds. miR-103 was used as an internal control for the serum samples and normalization of the relative expression of the three selected miRNAs [14]. All experiments were performed in triplicate. The mean cycle threshold (Ct) values of serum miR-22, miR-15b, and miR-125b were calculated. The relative expression levels of the three miRNAs were normalized by the $2^{-\Delta\Delta Ct}$ method, ΔCt = Ct (miR-22, miR-15b or miR-125b) - Ct (reference miR-103).

Statistical analysis

The statistical analyses were performed using SPSS 17.0 software and the data of serum miRNA levels were expressed as median (interquartile space). The Mann-Whitney *U* test was used for data comparison between

Table 1. Relative expressions of miRNAs in NSCLC patients.

Group	miR-22	miR-125b	miR-15b
pre-chemotherapy	4.56 (2.30 - 9.66)	1.26 (0.29 - 2.91)	0.78 (0.44 - 2.58)
post-chemotherapy	4.51 (2.42 - 8.43)	0.82 (0.12 - 2.14)	1.39 (0.67 - 9.79)
Z value	0.364	2.196	2.650
p-value	0.716	0.028	0.008

Note: NSCLC - non-small cell lung cancer.

Table 2. Relative expressions of miRNA-125b in advanced NSCLC patients.

Group	n	pre-chemotherapy	post-chemotherapy	Z value	p-value
CR + PR	34	1.22 (0.26 - 2.83)	0.93 (0.14 - 2.18)	2.128	0.033
PD + SD	40	1.32 (0.31 - 2.97)	1.29 (0.27 - 2.88)	0.245	0.806

Note: NSCLC - non-small cell lung cancer, CR - complete response, PR - partial response, SD - stable disease, PD - progression disease.

Table 3. Relative expressions of miRNA-15b in advanced NSCLC patients.

Group	n	pre-chemotherapy	post-chemotherapy	Z value	p-value
CR + PR	34	0.79 (0.41 - 2.48)	1.65 (0.86 - 4.96)	2.794	0.005 [a]
PD + SD	40	0.65 (0.32 - 2.31)	0.73 (0.35 - 2.42)	0.386	0.699 [b]

Note: NSCLC - non-small cell lung cancer, CR - complete response, PR - partial response, SD - stable disease, PD - progression disease.

Table 4. Relative expressions of miRNAs and chemotherapeutic effects.

Group	advanced NSCLC	CR + PR	χ^2 value	p-value
miR-125b (high)	43/74 (58.1%)	15/43 (34.9%)		
miR-125b (low)	31/74 (41.9%)	16/31 (51.6%)	5.879	0.015
miR-15b (high)	34/74 (45.9%)	18/34 (52.9%)	4.795	0.029
miR-15b (low)	40/74 (54.1%)	15/40 (37.5%)		

Note: NSCLC - non-small cell lung cancer, CR - complete response, PR - partial response.

the two groups. The χ^2 test was used to assess the relationships between serum miRNA levels and chemotherapeutic response. The correlation between relative levels of the serum miRNAs and the survival time was analyzed by the Kaplan-Meier method. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Relative expressions of serum miRNAs in the advanced NSCLC patients

To evaluate the relationships between the three selected miRNAs and chemotherapeutic response, the expression levels of serum miRNAs were detected in 74 patients with advanced NSCLC before treatment and after 2 cycles of chemotherapy. As shown in Table 1 and Figure

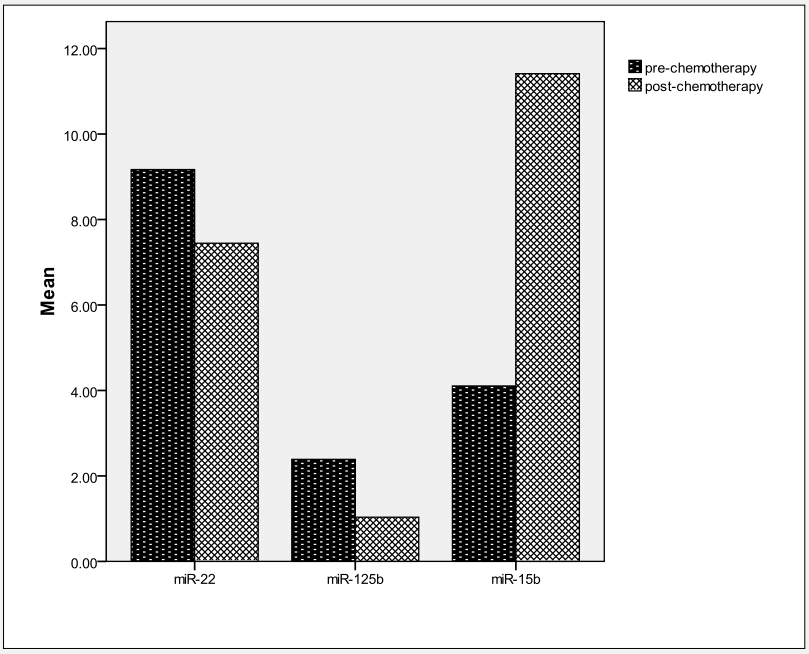

Figure 1. Relative expressions of serum miRNAs pre- and post-chemotherapy in advanced NSCLC patients.

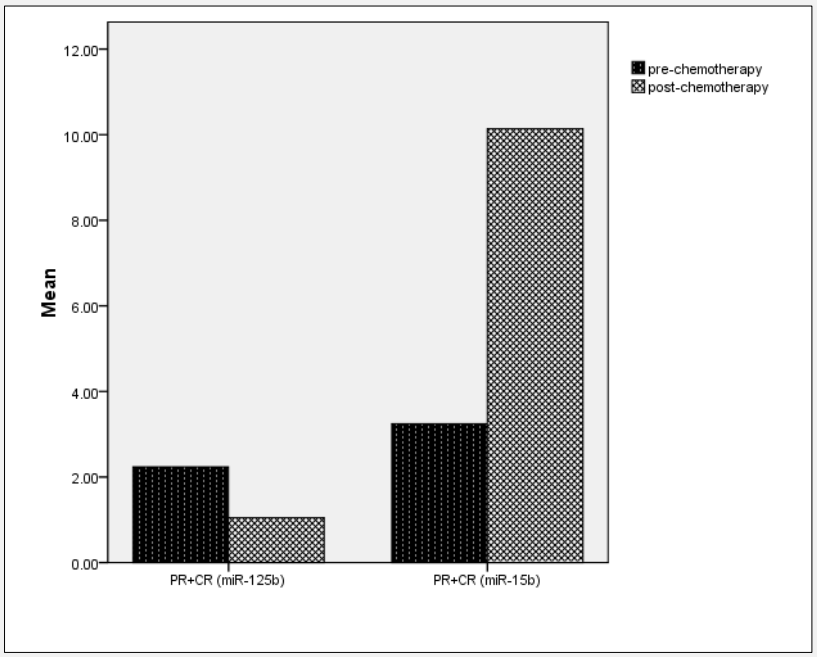

Figure 2. Relative expressions of serum miRNAs pre- and post-chemotherapy in advanced NSCLC patients of responders (PR + CR).

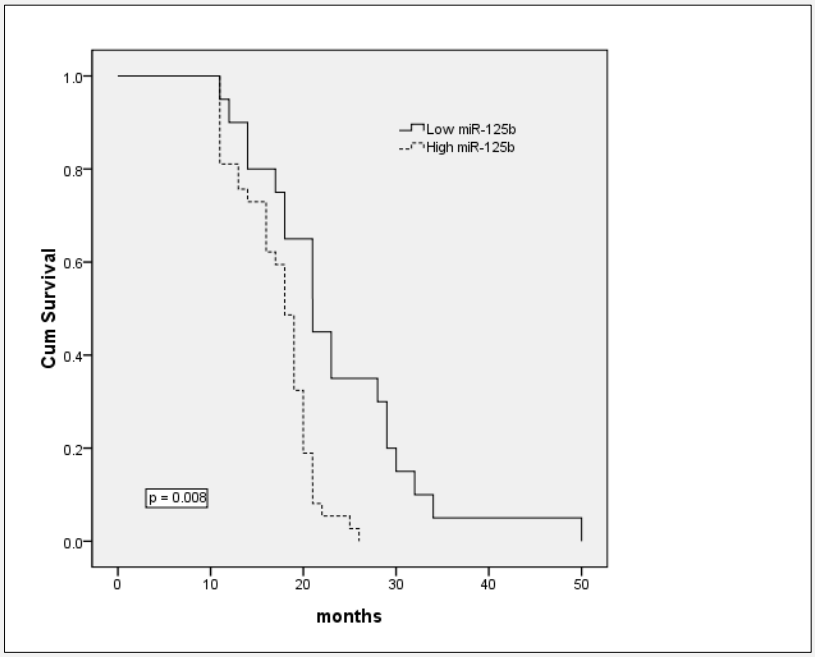

Figure 3. Relationship between the expression of serum miR-125b pre-chemotherapy and the OS in advanced NSCLC patients.

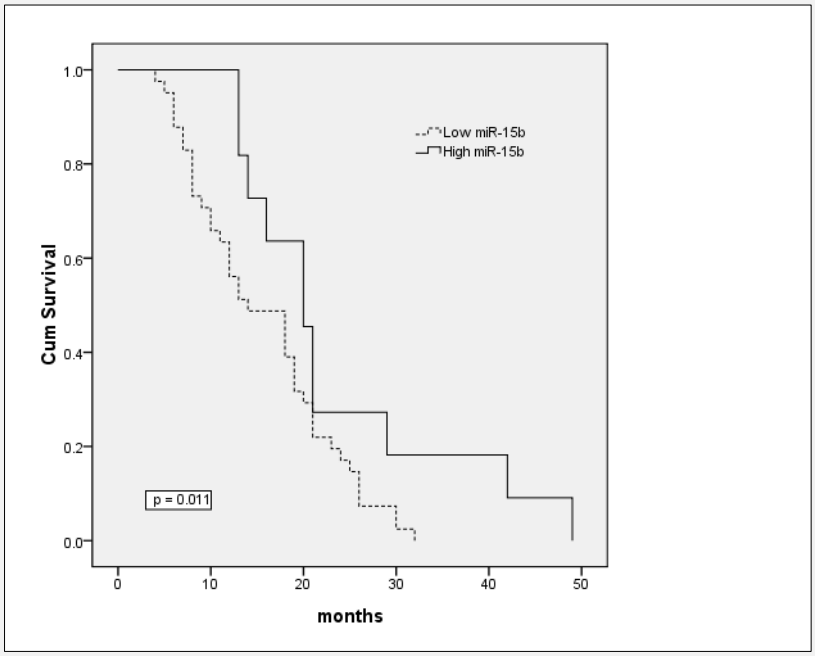

Figure 4. Relationship between the expression of serum miR-15b pre-chemotherapy and the OS in advanced NSCLC patients.

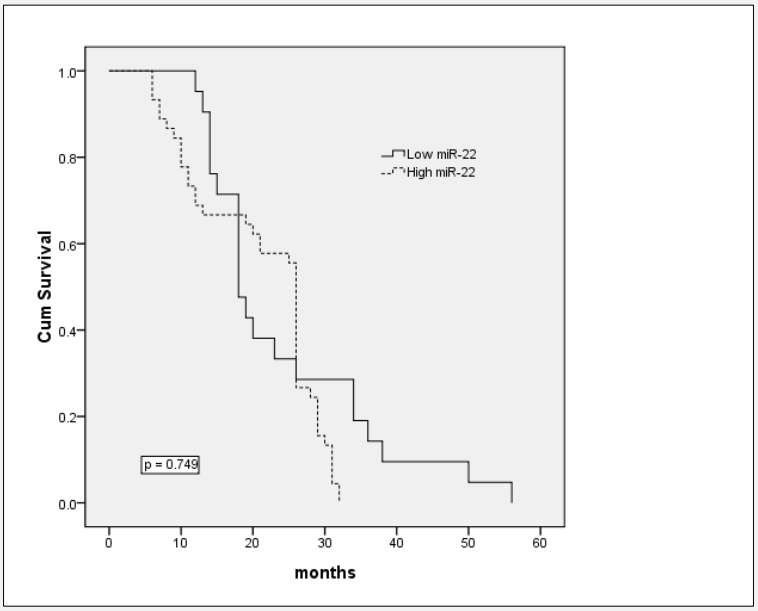

Figure 5. Relationship between the expression of serum miR-22 pre-chemotherapy and the OS in advanced NSCLC patients.

1, serum level of miR-125b significantly decreased after chemotherapy, the difference was statistically significant ($Z = 2.196$, $p < 0.05$). The levels of miR-15b significantly increased after 2 cycles of chemotherapy ($Z = 2.650$, $p < 0.01$). While there was no change in the level of serum miR-22 ($Z = 0.716$, $p > 0.05$).

Chemotherapeutic effects of the advanced NSCLC patients

Chemotherapeutic effects after 2 cycles were evaluated on the 74 advanced NSCLC patients. According to the evaluation criteria of Solid Tumor Chemotherapeutic Effects, all the patients were partitioned into 4 groups as follows: 0 complete response (CR), 34 (45.9%) partial response (PR), 33 (44.6%) stable disease (SD), and 7 (9.5%) progression disease (PD).

The relationship between chemotherapeutic effects and the expressions of serum miRNAs pre- and post-chemotherapy in advanced NSCLC patients

As shown in Table 2 and Figure 2, compared with pre-chemotherapy, levels of serum miR-125b in advanced NSCLC patients of responders (CR + PR) were significantly decreased post-chemotherapy ($Z = 2.128$, $p < 0.05$). Table 3 and Figure 2 showed that serum miR-15b levels in advanced NSCLC patients of responders (CR + PR) were significantly increased ($Z = 2.794$, $p < 0.01$). While the serum relative expressions of miR-125b and miR-15b showed no changes in the patients with advanced NSCLC of non-responders (SD + PD) ($p > 0.05$).

The correlation between chemotherapeutic effects and the expressions of miRNA-125b and miRNA-15b pre-chemotherapy in patients with advanced NSCLC

According to the mean miRNA expressions of pre-chemotherapy, all advanced NSCLC patients could be divided into either low-level or high-level groups. The chemotherapeutic effect was significantly related to the relative expressions of miRNA-125b and miRNA-15b before treatment (Table 4). Among the advanced NSCLC patients with high expression of miR-125b, 34.9% responded to chemotherapy with either CR or PR. Of the advanced NSCLC patients with low expression of miR-125b, 51.6% responded to chemotherapy, the difference was statistically significant ($\chi^2 = 5.879$, $p < 0.05$). Among the advanced NSCLC patients with low expression of miR-15b, 37.5% responded to chemotherapy with either CR or PR. Among the NSCLC patients with high expression of miR-15b, 52.9% responded to chemotherapy ($\chi^2 = 4.795$, $p < 0.05$).

The relationship between the levels of serum of the selected miRNAs and the OS in patients with advanced NSCLC

The overall survival (OS) time of patients was calculated by telephone follow-ups. All 74 patients with advanced NSCLC completed a 5-year follow-up. The relationship between the three miRNA levels and the OS was evaluated by the Kaplan-Meier method. As shown

in Figure 3 and Figure 4, serum miR-125b high expression and serum miR-15b low expression were significantly correlated with poor OS (all $p < 0.05$). There was no significant relationship between the serum miR-22 level and OS ($p > 0.05$, Figure 5).

DISCUSSION

The main treatment against advanced lung cancer is chemotherapy. The efficacy of the chemotherapy applied is mostly determined by evaluations of solid tumor method. However, this method not only needs a long time (42 days at least) evaluating the changes of the tumor, but also expensive imaging data. Evaluations of solid tumor method is sometimes unsuitable for chemotherapeutic response, such as in lung cancer patients with diffuse nodules or pleural effusions [15,16]. Therefore, the novel reference indexes for monitoring treatment are urgently needed. It would be very helpful to know the chemotherapeutic response earlier, so as to adjust the therapy to each NSCLC individual and change the regimen in time. Tumor markers have been extensively used for prognosis prediction and monitoring treatment in patients with NSCLC [17].

Serum miRNAs as the potential non-invasive tumor markers, the study of serum miRNAs regarding prognosis prediction, and monitoring treatment in patients with NSCLC are being investigated. An increasing number of studies have reported that miRNAs have important roles in the development of various malignant tumors. Analysis of miRNA expressions has a potential application value in lung cancer treatment and prognosis prediction [18-20]. Skrzypski et al. [21] found that miR-662 and miR-192 might be the independent prognostic factors in the patients of lung squamous cell carcinoma. Ge et al. [22] showed that low miR-148b level was significantly related with lymph node or distant metastasis, miR-148b level was independently related with OS of patients with NSCLC. Tian et al. [23] found that serum miR-106a was associated with the prognosis of lung cancer patients and can be used to assess the effectiveness of chemotherapy. Dejima et al. [24] reported that the relative expressions of miR-21 and mir-4257 in plasma exosomes have predictive value for recurrence in patients of lung cancer. These previous findings suggest that miRNAs may participate in the physiological process of lung cancer.

Our research showed that the expressions of serum miR-125b in patients with advanced NSCLC of responders (CR + PR) were significantly decreased after chemotherapy ($p < 0.05$), while the levels of serum miR-15b were significantly increased ($p < 0.01$). We also found that high expressions of serum miR-125b and low expressions of serum miR-15b were significantly correlated with poor OS ($p < 0.05$). Up to now, our research is the first to report that serum miR-15b expressions are significantly related to chemotherapeutic response and OS of advanced NSCLC patients. These results indicated that the relative expressions of serum miR-125b and miR-15b in advanced NSCLC patients were changed pre- and post-chemotherapy, and the changes were associated with chemotherapeutic response. The serum miR-125b and miR-15b have potential clinical value for predicting chemotherapeutic response in advanced NSCLC. The relative expressions of serum miR-125b and miR-15b in patients of advanced NSCLC before treatment may be used to estimate the OS.

A previous study reported that miR-125b may be a potential independent prognostic factor for survival and recurrence of clear-cell renal cell carcinoma patients after nephrectomy [25]. Another study showed that miR-125b was associated with breast cancer chemoresistance and could be a significant prognostic response marker for breast cancer therapy [26]. Cui et al. [27] reported that miR-125b was significantly correlated with the evaluation of chemotherapy, and high expression of miR-125b was significantly associated with poor OS, which is consistent with the findings of our research. Zhao et al. [28] showed that upregulation of miR-15b could suppress phosphatidylethanolamine-binding protein 4 expression and increased chemoresistance of lung cancer cells to cisplatin. Ji et al. [29] reported that miR-15b could be used as a valuable biomarker of colorectal cancer for chemotherapeutic response and prognosis prediction. There was no prior study related to serum miR-15b expression level for chemotherapeutic response in NSCLC patients. Therefore, the relationship between serum miR-15b expression and chemotherapeutic response in patients with NSCLC still needs further investigation.

CONCLUSION

Taken together, our research showed that the expression levels of serum miR-125b and miR-15b in advanced NSCLC patients were significantly changed pre- and post-chemotherapy, and the changes were associated with chemotherapeutic response. These results indicated that detecting the serum expression levels of miRNAs have certain potential clinical value for chemotherapeutic response in advanced NSCLC. Our findings also showed that the relative expressions of serum miRNAs pre-chemotherapy in advanced NSCLC patients were associated with chemotherapeutic response and may be used to estimate the OS. The prognosis for NSCLC patients who received cisplatin-based combination chemotherapy can be estimated by detecting the relative expressions of serum miR-125b and miR-15b before treatment.

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Declaration of Interest:
The authors declare no conflict of interest.

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