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Low plasma miR-25 expression is a favorite prognosis factor in non-small cell lung cancer

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Abstract. – OBJECTIVE: Circulating microRNAs (miRNAs) are promising biomarkers for the diagnosis and prognosis prediction of cancer. In the study, we aimed to investigate the potential clinical significance of the plasma miR-25 in non-small cell lung carcinoma (NSCLC).

PATIENTS AND METHODS: We first compared the miRNAs expression pattern between NSCLC tissues and adjacent normal tissues then, bioinformatic analysis of the downstream targets of miR-25 was performed. The diagnostic and prognostic value of the plasma miR-25 in NSCLC was then evaluated.

RESULTS: The expression level of miR-25 was increased in NSCLC tissues compared to the adjacent normal tissues. In addition, bioinformatic analysis of the downstream-targeted genes of miR-25 revealed that many gene ontology functions and pathways were associated with cancer progression. The levels of plasma miR-25 were significantly upregulated in NSCLC patients compared to normal controls. In addition, the plasma miR-25 levels were especially higher in NSCLC patients with positive lymph node metastasis, poorly differentiation or advanced clinical stage. Subsequently, we found that the plasma miR-25 expression levels were dramatically decreased in 45 NSCLC patients after receiving surgical treatment. The receiver operating characteristic (ROC) curve analysis indicated that the plasma miR-25 exhibited high diagnostic sensitivity and specificity to discriminate NSCLC cases from healthy subjects. More interestingly, the combination of the plasma miR-25 and carcinoembryonic antigen (CEA) could effectively enhance the accuracy for distinguishing NSCLC patients from normal controls. Moreover, the plasma miR-25 overexpression was closely correlated with aggressive clinical characteristics and poor survival. Finally, the plasma miR-25 was identified as an independent prognostic marker for the overall survival of NSCLC.

CONCLUSIONS: Collectively, our findings demonstrated that the plasma miR-25 might serve as a novel promising biomarker in the diagnosis and prognosis prediction of NSCLC.

Key Words:
Plasma miR-25, Non-small cell lung cancer, Diagnosis, Prognosis.

Introduction

Lung cancer is one of the most common human cancer types and the leading cause of cancer-related death around the world. In China, more than 700,000 new cases are diagnosed with lung cancer every year[1,2]. Lung cancer includes small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), and NSCLC accounts for about 80-85% of lung cancer diagnoses[3]. Regardless of great advancements in surgery, in radiotherapy and chemotherapy over the past decades, the survival rate of this malignancy remains very unfavorable[4,5]. Therefore, more efforts should be made to identify noninvasive and reliable biomarkers for detecting NSCLC at the early stage and predicting its prognosis. The nucleotides that regulate gene expression by binding to the 3'-untranslated regions of their target mRNAs, lead to the suppression or degradation of the target mRNAs[6-8]. MiRNAs are involved with many cellular processes, such as cell proliferation, growth, invasion, apoptosis, and differentiation[9]. Growing evidence has demonstrated that some miRNAs serve as promising biomarkers for cancer diagnosis, prognosis or predicting treatment responses since they can be detected in plasma or plasma in highly stable form[10-13]. In this regard, the use of plasma miRNAs in NSCLC have been widely reported by previous studies. For instance,

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the high expression levels of miR-21[14], miR-411[15], miR-661[16], and miR-421[17] were found in NSCLC and correlated with worse prognosis, while the levels of miR-15b[18], miR-126[19] were significantly decreased in patients with NSCLC. MiR-25 is one of the commonly up-regulated miRNAs in NSCLC. The overexpression of miR-25 in NSCLC cells stimulated cell migration and invasion via ERK signaling pathway[20]. Moreover, miR-25 expression was markedly increased both in NSCLC tissues and cell lines. MiR-25 inhibition significantly restrained cancer cell proliferation, migration, and invasion by upregulating F-box and WD repeat domain-containing 7 (FBXW7), and *vice versa*[21]. Interestingly, the upregulation of miR-25 was also observed in cells and tissues of small cell lung cancer (SCLC). MiR-25 downregulation significantly suppressed SCLC cell growth, invasion, as well as, resistance to cisplatin by targeting cyclin E2[22]. Whereas, the clinical significance of plasma miR-25 in patients with NSCLC remains poorly known. Hence, in the current study, we first compared the miRNAs expression pattern between NSCLC tissues and adjacent normal tissues; then, the bioinformatic analysis of the downstream targets of miR-25 was performed. The diagnostic and prognostic value of plasma miR-25 in NSCLC was then evaluated.

Patients and Methods

Study Population and Sample Collection

The present study was approved by the Ethics Committee of Guizhou Provincial People's Hospital and Longgang Central Hospital of Shenzhen. The written informed consent was collected from all participants. A total of 114 cases with NSCLC, and 80 age- and sex-matched healthy volunteers were enrolled. NSCLC patients were staged according to the criteria of the Seventh Edition of the American Joint Committee on Cancer tumor-node-metastasis (TNM) staging system. Of all 114 NSCLC subjects, 39 were at TNM stage III/IV, and 75 at TNM stage I/II. The data regarding patient demographics (sex, age) and tumor histopathology (lymph node metastasis, tumor differentiation, TNM stage) were presented in Table I. Approximately 5-ml of venous blood was withdrawn from all NSCLC subjects and healthy controls, and stored into ethylene diamine tetraacetic acid (EDTA) tubes (Becton, Dickinson and Compa-

Table I. Correlation of clinical parameters with plasma miR-25 expression in NSCLC.

Variables	Case	Low expression	High expression	*p*-value
Sex				NS
Male	64	33	31	
Female	50	28	22	
Age				NS
< 55	43	26	17	
≥ 55	71	35	36	
Smoking history				NS
Yes	37	16	21	
No	77	45	32	
Vascular invasion				NS
Yes	11	4	7	
No	103	57	46	
Pathological type				NS
Adenocarcinoma	69	40	29	
Squamous cell carcinoma	45	21	24	
Lymph node metastasis				0.002
Yes	66	27	39	
No	48	34	14	
Tumor differentiation				0.033
Poorly	42	17	25	
Well/Moderately	72	44	28	
TNM stage				<0.0001
I-II	75	52	23	
III-IV	39	9	30	

NS: Not significant.

ny, Melbourne, Australia). Within an hour, the whole blood samples were centrifuged at 1,900 × g for 15 min at 4°C, followed by centrifugation at 12,000 × g for 10 min at 4°C. Then, the plasma samples were stored at -80°C until further analysis. In addition, we collected the plasma samples from 45 patients with NSCLC who receiving tumor resection after 90 days.

Extraction of RNA and Quantitative Reverse Transcription PCR

The total RNA was extracted from 200 μL plasma or tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, the reverse transcription was carried out by the One Step Prime Script miRNA cDNA Synthesis Kit (Takara, Dalian, China) according to the manufacturer's instructions. Quantitative PCR was performed on an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China). Each detection was performed in triplicate. The levels of miRNAs were subsequently calculated using the $2^{-\Delta\Delta Ct}$ method and cel-miR-39 was selected as the normalization control.

Statistical Analysis

Statistical comparisons between the two groups were performed with the Mann-Whitney U test. The association between the plasma miR-25 levels and clinical variables was analyzed with the Chi-square test. The receiver-operating characteristic (ROC) curves and the area under the curve (AUC) were performed to evaluate the feasibility of plasma miR-25 as a diagnostic biomarker. The overall survival (OS) and relapse-free survival (RFS) rates were assessed according to the Kaplan-Meier method, with the log-rank test used for comparison. Univariate and multivariate Cox regression analyses were conducted to estimate the hazard ratios (HR) for OS. OS was defined as the time from diagnosis until the date of death or last follow-up. RFS was defined as the time from diagnosis until the date of disease relapse or progression or death due to any cause. For the investigation on the downstream targeted genes of miR-25, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource (https://david-d.ncifcrf.gov/). All statistical analyses were performed using GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA, USA) and MedCalc 14.8.1 (MedCalc Software, Ostend, Belgium). $p<0.05$ was considered statistically significant.

Results

The Expression Level of MiR-25 Was Upregulated in NSCLC Tissues

We first compared ten miRNA expression patterns between NSCLC tissues and paired adjacent normal tissues. Our results showed that the expression levels of miR-411, miR-21, miR-647, and miR-25 were significantly upregulated in NSCLC tissues compared with the normal tissues. However, the levels of miR-212, miR-296, miR-520b, miR-1258, miR-145, and 409 were remarkably reduced in the tumor samples in comparison with the controlled samples (Figure 1).

Bioinformatic Analysis of the Downstream Targets in MiR-25

The validated downstream-targeted genes of miR-25 were obtained from miRWalk2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/). The GO analysis showed that GO:0006977–DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest, GO:0045727–positive regulation of translation, GO:0000122–negative regulation of transcription from RNA polymerase II promoter, GO:0006366–transcription from RNA polymerase II promoter and GO:0045893–positive regulation of transcription, DNA-templated were top biological processes. GO:0005515–protein binding, GO:0044822–poly(A) RNA binding, GO:0008134–transcription factor binding, GO:0008017–microtubule binding and GO:0043022–ribosome binding were the top enriched molecular functions. GO:0005654–nucleoplasm, GO:0005634–nucleus, GO:0005737–cytoplasm, GO:0005730–nucleolus and GO:0043234–protein complex were the top enriched cellular components (Figure 2A). Our KEGG pathway analysis showed that hsa05219: bladder cancer, hsa05206: microRNAs in cancer, hsa04151: PI3K-Akt signaling pathway, hsa05205: proteoglycans in cancer, and hsa04115: p53 signaling pathway were the top enriched pathways (Figure 2B).

Upregulation of Plasma MiR-25 in NSCLC Patients Compared to Healthy Controls

The expression of plasma miR-25 in all the NSCLC patients and controls was detected using qRT-PCR. As shown in Figure 3A, the plasma miR-25 levels in NSCLC cases were significantly higher than those in healthy volunteers ($p=0.025$). In addition, the expression of plasma

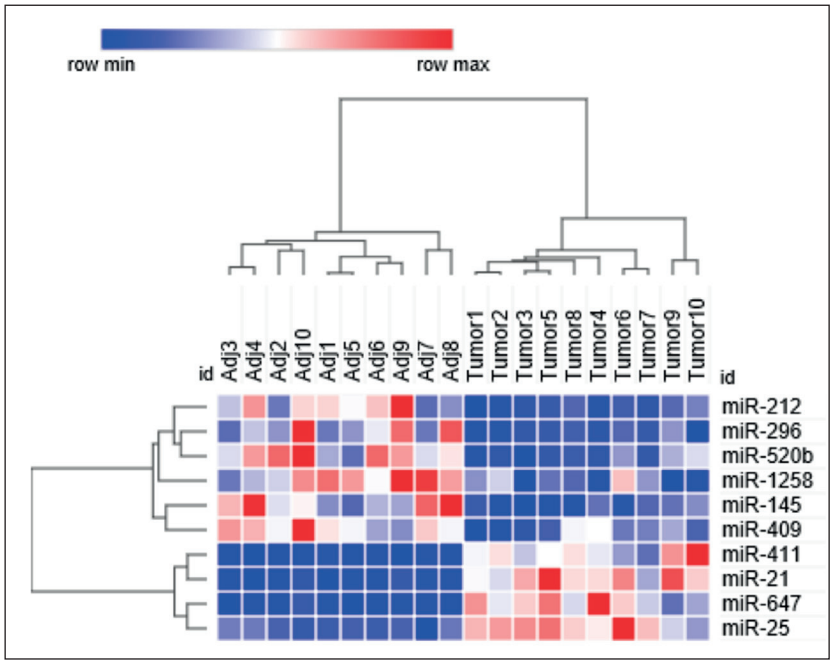

Figure 1. The aberrantly expressed miRNAs between NSCLC tissues and adjacent normal tissues

miR-25 was significantly increased in patients with lymph node metastasis compared with those without lymph node metastasis (p=0.019, Figure 3B). Patients with poorly differentiation have much higher plasma miR-25 expression than that of those with well/moderately differentiation (p=0.037, Figure 3C). Furthermore, the plasma miR-25 expression was dramatically decreased in early-stage NSCLC patients compared with those in advanced-stage cases (p=0.013, Figure 3D).

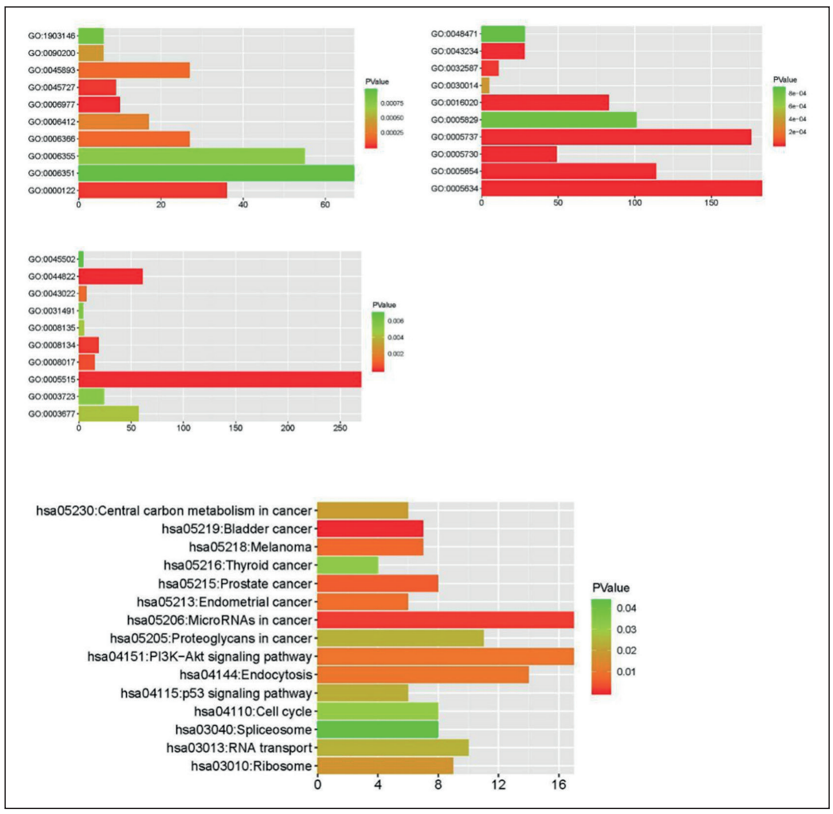

Figure 2. Bioinformatic analysis of the downstream targeted genes of miR-25.

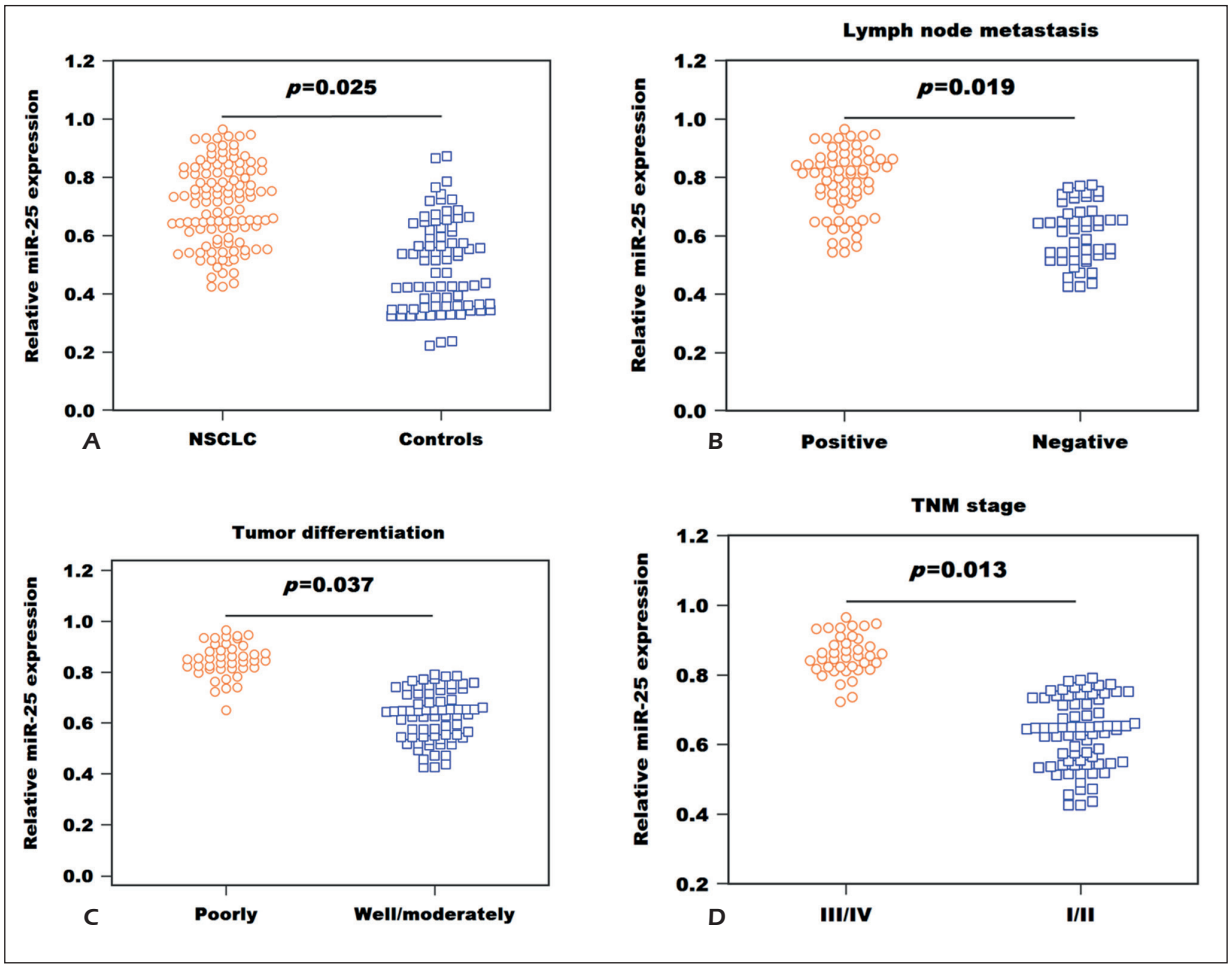

Figure 3. **A**, Plasma miR-25 levels in NSCLC patients and healthy controls. **B**, Plasma miR-25 levels were significantly higher in NSCLC patients with positive lymph node metastasis. **C**, Plasma miR-25 levels were significantly higher in NSCLC patients with poorly differentiation. **D**, Plasma miR-25 levels were significantly higher in NSCLC patients at the advanced clinical stages.

Treatment Response and its Diagnostic Potential

Next, the plasma miR-25 expression levels in 45 paired plasma samples were compared, and we found plasma miR-25 expression levels were greatly reduced in NSCLC subjects after surgical resection, indicating that plasma miR-25 expression was strongly correlated with treatment response (p=0.012, Figure 4). ROC curve analysis was generated to assess the performance of plasma miR-25 and carcinoembryonic antigen (CEA) in discriminating NSCLC patients from normal controls. The plasma miR-25 level had a specificity of 75.0% and a sensitivity of 74.6% to discriminate the NSCLC cases from the controls, with an AUC of 0.832 at the optimal cut-off point (Figure 5A). Since CEA was commonly used for cancer detec-

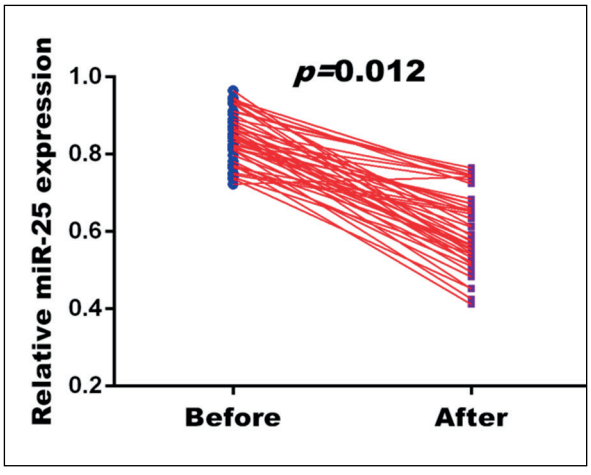

Figure 4. Plasma miR-25 expression levels in 45 NSCLC patients before and after surgical treatment.

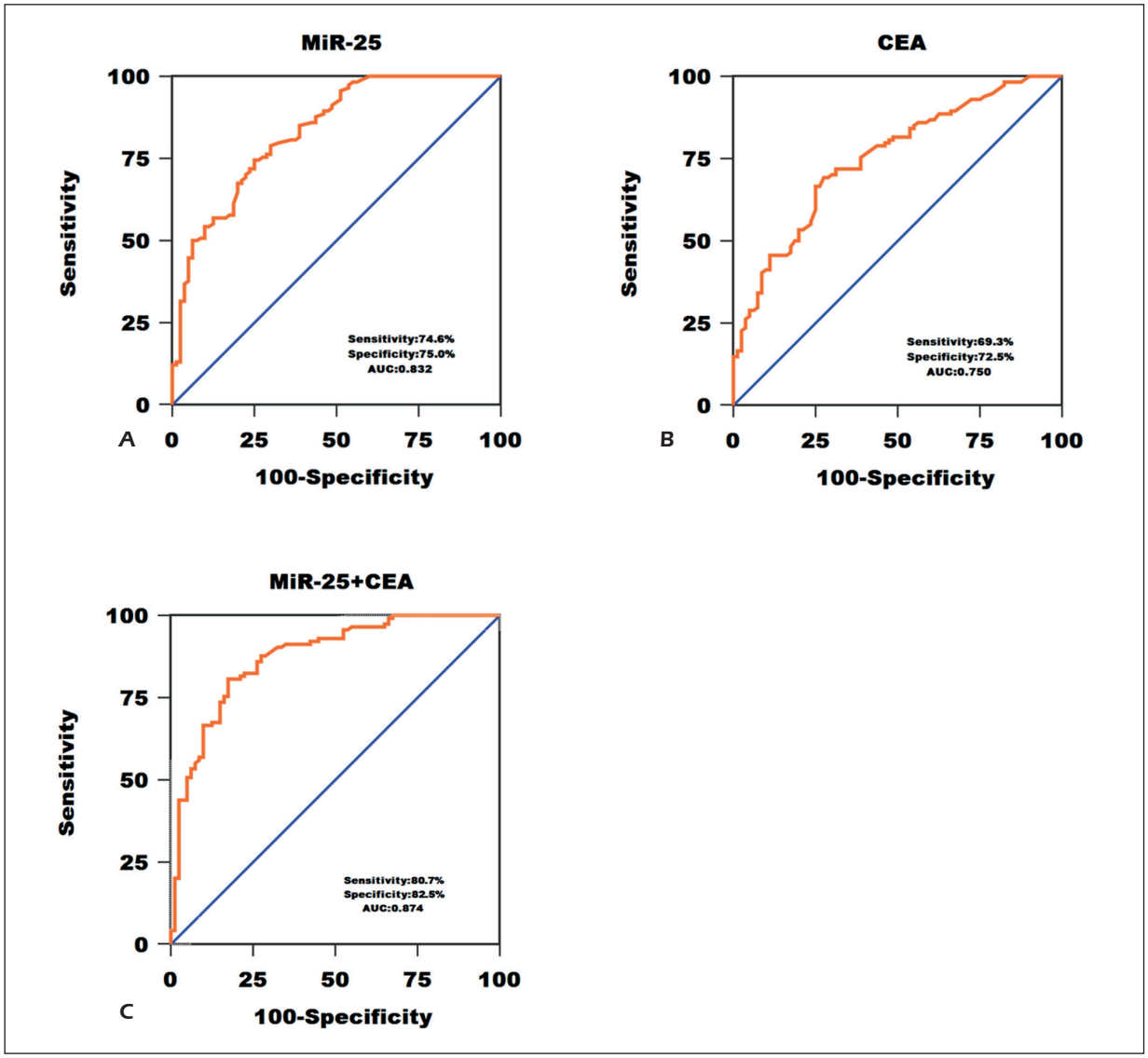

Figure 5. A, ROC curve analysis of miR-25. **B**, ROC curve analysis of CEA. **C**, ROC curve analysis of plasma miR-25 and CEA.

tion, we found the AUC for CEA was 0.750, the specificity and sensitivity were 72.5% and 69.3%, respectively (Figure 5B). Next, the combination of plasma miR-25 and CEA presented further improvement in AUC of 0.874, with a sensitivity of 80.7% and a specificity of 82.5% (Figure 5C).

Correlation Between MiR-25 Expression and Clinical Parameters in NSCLC Patients

The median miR-25 expression level was used as a cut-off value to classify all 114 patients into high plasma miR-25 group (n=53) and low plasma miR-25 group (n=61). As shown in Table I, high plasma miR-25 level was associated with several clinicopathological variables including lymph node metastasis (p=0.002), tumor differentiation (p=0.033) and TNM stage (p<0.001). Yet, there was no significance between miR-25 levels and sex, age, smoking history, vascular invasion or pathological type (all p>0.05).

Correlation between MiR-25 Expression and Prognosis for NSCLC Patients

The Kaplan-Meier curve with log-rank test was performed for NSCLC patients. As displayed in Figure 6A and Figure 6B, the survival time of patients with high plasma miR-25 expression had both shorter OS (p=0.028) and RFS (p=0.012).

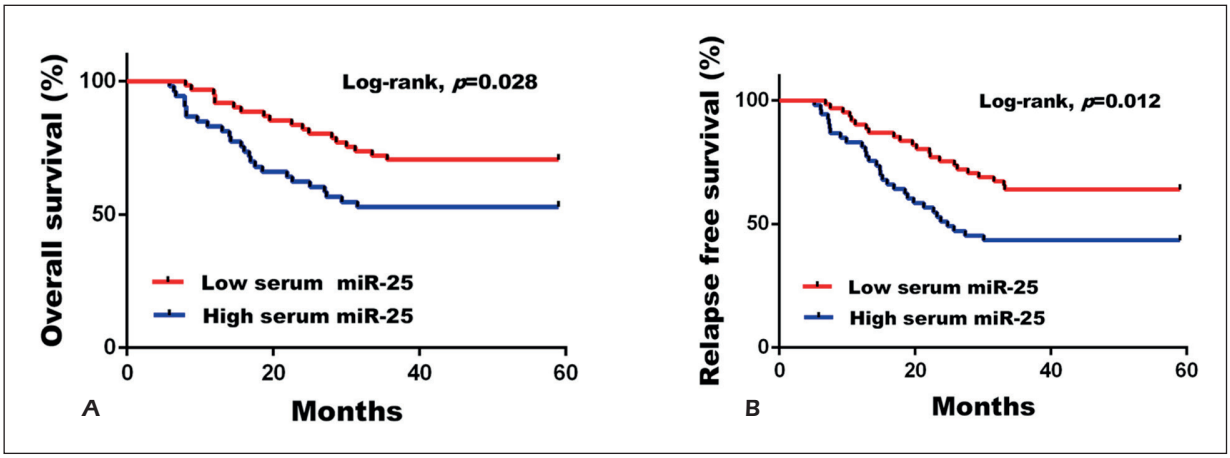

Figure 6. A, OS rate in NSCLC patients with high plasma miR-25 expression was lower than those with low plasma miR-25 expression. **B**, RFS rate in NSCLC patients with high plasma miR-25 expression was lower than those with low plasma miR-25 expression.

The univariate analysis revealed that lymph node metastasis (HR: 5.34, p=0.014), tumor differentiation (HR: 4.52, p=0.028), TNM stage (HR: 6.74, p=0.003), and plasma miR-25 expression (HR: 5.12, p=0.017) were independently associated with the OS. Then, the multivariate analysis confirmed that the lymph node metastasis (HR: 5.82, p=0.009), TNM stage (HR: 7.23, p=0.001), and plasma miR-25 expression (HR: 5.67, p=0.011) were statistically significant predictors for poor OS in NSCLC patients (Table II).

Discussion

Circulating miRNAs have been previously demonstrated to be useful for early detection and prognosis of cancer because of their abundance and stability in circulating blood. In this study, the expression level of miR-25 was increased in NSCLC tissues compared to the adjacent normal tissues. In addition, bioinformatic analysis of the downstream targeted genes of miR-25 showed that many GO functions and KEGG pathways were associated with cancer progression. To the best of our knowledge, this was the first time in which we found that the plasma miR-25 expression was greatly increased in NSCLC patients in comparison with normal controls. Moreover, the plasma miR-25 upregulation was especially higher in NSCLC patients with positive lymph node metastasis, poorly differentiation or advanced clinical stage. The plasma miR-25 levels in 45 NSCLC patients were significantly downregulated after their surgical tumor resection. Next, ROC analysis revealed that the plasma miR-25 could effectively differentiate NSCLC cases and healthy controls. Combined ROC analysis using

Table II. Univariate and multivariate analyses for overall survival in 114 NSCLC cases.

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p	HR (95% CI)	p
Sex	1.28 (0.85-1.79)	0.623	-	-
Age	1.64 (1.02-2.36)	0.542	-	-
Smoking history	3.51 (1.24-6.07)	0.284	-	-
Vascular invasion	3.87 (1.36-6.52)	0.170	-	-
Pathological type	2.72 (1.17-4.49)	0.438	-	-
Lymph node metastasis	5.34 (1.81-9.06)	0.014	5.82 (1.97-9.85)	0.009
Tumor differentiation	4.52 (1.50-7.68)	0.028	-	-
TNM stage	6.74 (2.15-11.53)	0.003	7.23 (2.32-12.38)	0.001
Plasma miR-25	5.12 (1.72-8.71)	0.017	5.67 (1.92-9.44)	0.011

plasma miR-25 and CEA showed an increased AUC value, with higher sensitivity and specificity for improving the accuracy of NSCLC diagnosis. Furthermore, the high plasma miR-25 expression was strongly associated with aggressive clinical parameters. Subsequently, NSCLC patients in high plasma miR-25 expression group had a significantly shorter OS and RFS compared to those in low plasma miR-25 expression group. Finally, both univariate and multivariate analyses showed that the plasma miR-25 was an independent prognostic factor of OS of NSCLC. Taken together, high plasma miR-25 expression was closely correlated with the carcinogenesis of NSCLC, and the results were consistent with the previous studies[20,21]. Besides NSCLC, miR-25 was proposed as an oncogene because its expression was upregulated in various cancer types. In esophageal squamous cell carcinoma (ESCC), the expression of miR-25 was increased in cancerous tissues and cell lines, and the miR-25 overexpression greatly promoted cell migration and invasion[23]. In addition, the miR-25 upregulation was found in the plasma samples, plasma samples, and tissues of ESCC patients. The plasma miR-25 levels in ESCC cases were significantly decreased after surgical resection and could be used to monitor the tumor dynamics[24,25]. In gastric cancer (GC), Zhang et al[26] showed that miR-25 was overexpressed in GC cell lines, the loss of miR-25 could remarkably attenuate cell viability, increase cell apoptosis by upregulating FBXW7 and suppressing oncogenes. He et al[27] found that miR-25 overexpression led to accelerated cell-cycle progression and decreased chemotherapeutic sensitivity of GC cells to cisplatin, while miR-25 inhibition showed opposite effects. Zhang et al[28] revealed that miR-25 expression was significantly elevated both in clinical samples and cell lines of ovarian cancer. The ectopic expression of miR-25 expression induced cell proliferation and repressed cell apoptosis by degrading Bim. In hepatocellular carcinoma (HCC), miR-25 was highly amplified in cancerous tissues when compared with normal samples. The overexpression of miR-25 dramatically promoted the tumorigenesis and stimulated the epithelial-mesenchymal transition (EMT) by directly targeting RhoGDI1, and the adverse association of miR-25 upregulation with OS of HCC patients was demonstrated[29,30]. In melanoma, the miR-25 upregulation occurred more frequently in cancer tissues and cell lines. Reduced miR-25 expression dramatically inhibited carcinogenesis via negatively regulating DKK3 through the WNT/β-Catenin pathway[31]. Of note, miR-25 might also function as a tumor suppressor. Hua et al[32] characterized the tumor-suppressive activity of miR-25 in nasopharyngeal carcinoma, and it could mediate cell growth suppression and motility inhibition by regulating MALAT1 expression. Likewise, *in vitro* and *in vivo* analysis revealed that miR-25 acted as a tumor suppressor in prostate cancer by silencing αv- and α6-Integrin Expression[33]. These findings suggested that the functional role of miR-25 in tumorigenesis might be depended on the tumor types. Therefore, further studies are warranted to elucidate the detailed role of miR-25 in the initiation and development of cancer.

Conclusions

Collectively, we have demonstrated that the plasma miR-25 levels were significantly increased in patients with NSCLC. In addition, the high plasma miR-25 expression was strongly associated with worse clinical variables and shorter survival, indicating that the plasma miR-25 could be used as a promising biomarker in the diagnosis and prognosis of NSCLC.

Acknowledgment

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Conflict of Interests

The Authors declared no conflict of interests.

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