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RESEARCH ARTICLE

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A novel circulating miRNA-based signature for the early diagnosis and prognosis prediction of non–small-cell lung cancer

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Abstract

Background: Non–small-cell lung cancer (NSCLC) is a significant public health issue worldwide. The aim of our study was to develop a serum miRNA-based molecular signature for the early detection and prognosis prediction of NSCLC.

Methods: The significantly altered circulating miRNAs were profiled in GSE24709. The top ten upregulated miRNAs were miR-432, miR-942, miR-29c-5p, miR-601, miR-613, miR-520d-3p, miR-1261, miR-132-5p, miR-302b, and miR-154-5p, while the top ten downregulated miRNAs were miR-562, miR-18b, miR-9-3p, miR-154-3p, miR-20b, miR-18a, miR-487a, miR-20a, miR-103, and miR-144. Then, the top four upregulated serum miRNAs (miR-432, miR-942, miR-29c-5p, and miR-601) were validated by real-time quantitative PCR. The clinical significance of two candidate serum miRNAs, miR-942 and miR-601, was further explored.

Results: Our results showed that the expression levels of serum miR-942 and serum miR-601 were significantly upregulated in NSCLC. In addition, serum miR-942 and serum miR-601 showed better performance than CEA, CYFRA21-1, and SCCA for early diagnosis of NSCLC. Combining serum miR-942 and serum miR-601 enhanced the efficacy of detecting early-stage NSCLC. Moreover, high serum miR-942 and serum miR-601 were both associated with adverse clinical variables and poor survival. The NSCLC patients with simultaneously high serum miR-942 and serum miR-601 suffered worst clinical outcome, while those with simultaneously low serum miR-942 and serum miR-601 had most favorable outcome. The multivariate analysis showed that serum miR-942 and serum miR-601 were independent prognostic factors for NSCLC.

Conclusions: Taken together, serum miR-942 and serum miR-601 might serve as a promising molecular signature for the early detection and prognosis prediction of NSCLC.

KEYWORDS

early diagnosis, non–small-cell lung cancer, prognosis prediction, serum miR-601, serum miR-942

1 | INTRODUCTION

Lung cancer is the leading cause of cancer-associated mortality around the world.[1] Approximately 85%-90% of lung cancer are non–small-cell lung cancer (NSCLC).[2] Due to the lack of obvious symptoms and clinical screening strategies, most of the patients with NSCLC are diagnosed at the advanced stage, leading to the dismal prognosis of NSCLC.[3,4] Generally, the 5-year overall survival rate of NSCLC patients at the early stage reaches 70%-90% following surgical treatment.[5] Therefore, it is urgently needed to identify novel biomarkers for the early detection and prognosis prediction of NSCLC.

MicroRNAs (miRNAs) are small, non–protein-coding RNA molecules that regulate gene expression at the posttranscriptional level.[6] Owning to the crucial roles in regulating a variety of biological processes such as proliferation, survival, and apoptosis, abnormal expression of miRNAs has been implicated in tumorigenesis.[7-9] Typically, miRNAs might act as oncomiRs or tumor-suppressive miRNAs in cancer initiation and development. Interestingly, miRNAs are abundant and highly stable in biofluids (plasma, serum, urine, saliva, etc). Therefore, they have become potential biomarkers for the detection and clinical evaluation of human diseases including NSCLC.[10,11] For instance, serum levels of miR-182, miR-205, and miR-200b exhibited good performance for detecting NSCLC at the early stage.[12] The expression level of serum exosomal miR-378 was markedly increased in patients with NSCLC. In addition, upregulation of serum exosomal miR-378 was associated with poor clinical outcome of NSCLC.[13]

In GSE24709, the abnormally expressed circulating miRNAs in NSCLC have been profiled. The top ten upregulated miRNAs were miR-432, miR-942, miR-29c-5p, miR-601, miR-613, miR-520d-3p, miR-1261, miR-132-5p, miR-302b, and miR-154-5p, while the top ten downregulated miRNAs were miR-562, miR-18b, miR-9-3p, miR-154-3p, miR-20b, miR-18a, miR-487a, miR-20a, miR-103, and miR-144. In this study, we first validated the top four upregulated serum miRNAs (miR-432, miR-942, miR-29c-5p, and miR-601) in GSE24709. Our results showed that the expression levels of serum miR-942 and serum miR-601 were significantly increased in patients with NSCLC compared to the healthy controls. Then, the efficacies of serum miR-942 and serum miR-601 as the biomarkers for the early detection and prognosis prediction of NSCLC were investigated. In addition, the combination effects of these two serum miRNAs were also explored.

2 | METHODS

2.1 | Study cohort

This study was approved by the Institutional Review Board of the Affiliated Hospital of Medical School of Ningbo University. All the protocols complied with the Declaration of Helsinki. All enrolled participants provided the written inform consent. In total, 125 patients with NSCLC, 40 patients with benign lung diseases, and 60 healthy volunteers were recruited in this study. The clinicopathological characteristics of this study cohort were listed in Table 1. The NSCLC patients were staged according to the 8th Edition of the American Joint Commission on Cancer tumor-node-metastasis (TNM) staging system. All the patients with NSCLC did not receive any type of treatments such as surgery, chemotherapy, and radiotherapy prior to the collection of serum samples.

2.2 | Serum collection

At least 5 mL peripheral blood specimen was drawn from each participant. The serum was isolated from the whole blood sample with the following two-step centrifugation methodology: 2000 g for 20 minutes at 4°C and then 13 800 g for 15 minutes at 4°C. The extracted serum samples were stored at −80°C until further experiments.

2.3 | Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from 200 μL of serum using the miRNeasy Serum/Plasma Advanced Kit (Qiagen) following the manufacturer's instructions. The purity and concentration of the RNA were evaluated using a NanoDrop system (NanoDrop). The RNA was reverse-transcribed to cDNAs with The SuperScript® IV First-Strand

TABLE 1 Clinicopathological characteristics of the study cohort

Variables	NSCLC	Benign lung diseases	Healthy subjects
Age	61.23 ± 8.15	60.70 ± 9.29	60.28 ± 10.08
Gender			
Female	29 (23.20%)	9 (22.50%)	16 (26.67%)
Male	96 (76.80%)	31 (77.50%)	44 (73.33%)
Smoking history			
No	35 (28.00%)	8 (20.00%)	19 (31.67%)
Yes	90 (72.00%)	32 (80.00%)	41 (68.33%)
Distant metastasis			
No	114 (91.20%)	/	/
Yes	11 (8.80%)		
Lymph node metastasis			
No	67 (53.60%)	/	/
Positive	58 (46.40%)		
TNM stage			
I-II	56 (44.80%)	/	/
III-IV	69 (55.20%)		
Histological grade			
Well/ moderate	80 (64.00%)	/	/
Poor	45 (36.00%)		

Synthesis System (Thermo Fisher Scientific). All RT-qPCR analyses were performed in the Applied Biosystems™ 7500 Real-Time PCR Systems (Applied Biosystems) using the SYBR premix ExTaqII (TaKaRa, Dalian, China). Cel-miR-39 was used as the spike-in control, and the expression levels of circulating miRNAs were calculated with the $2^{-\Delta\Delta Ct}$ method.

2.4 | The enzyme-linked immunosorbent assay (ELISA) assays

Within 1 hour following the collection of serum samples, the expression levels of tumor markers including CEA (MybioSource), CYFRA21-1 (MybioSource), and SCCA (MybioSource) were detected with the corresponding ELISA kits according to the manufacturer's instructions.

2.5 | Statistical analysis

The expression levels of serum miRNAs in different groups were compared with Kruskal-Wallis test or Mann-Whitney U test. Chi-square analysis was performed to evaluate the correlation between serum miR-942/serum miR-601 and clinical variables of NSCLC. The receiver operating characteristic (ROC) curves and the area under the curves (AUC) were used to evaluate the diagnostic performance

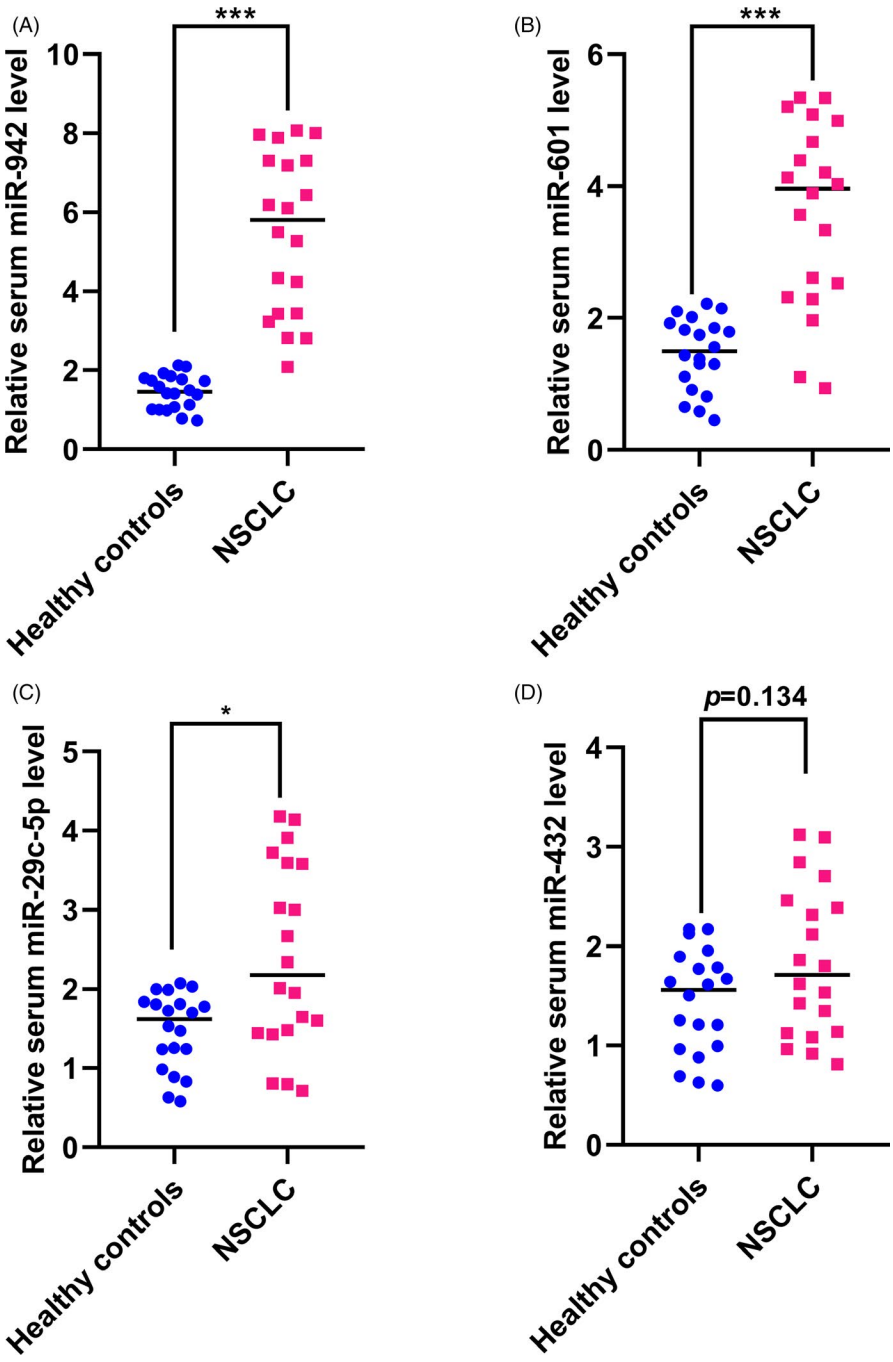

FIGURE 1 Validating the top four upregulated serum miRNAs in GSE24709. A, The expression level of serum miR-942 was significantly increased in NSCLC patients compared to the healthy controls. B, Serum miR-601 was markedly upregulated in NSCLC cases. C, The level of serum miR-29c-5p was slightly increased in patients with NSCLC. D, No significant difference was found for serum miR-432 between NSCLC patients and healthy controls

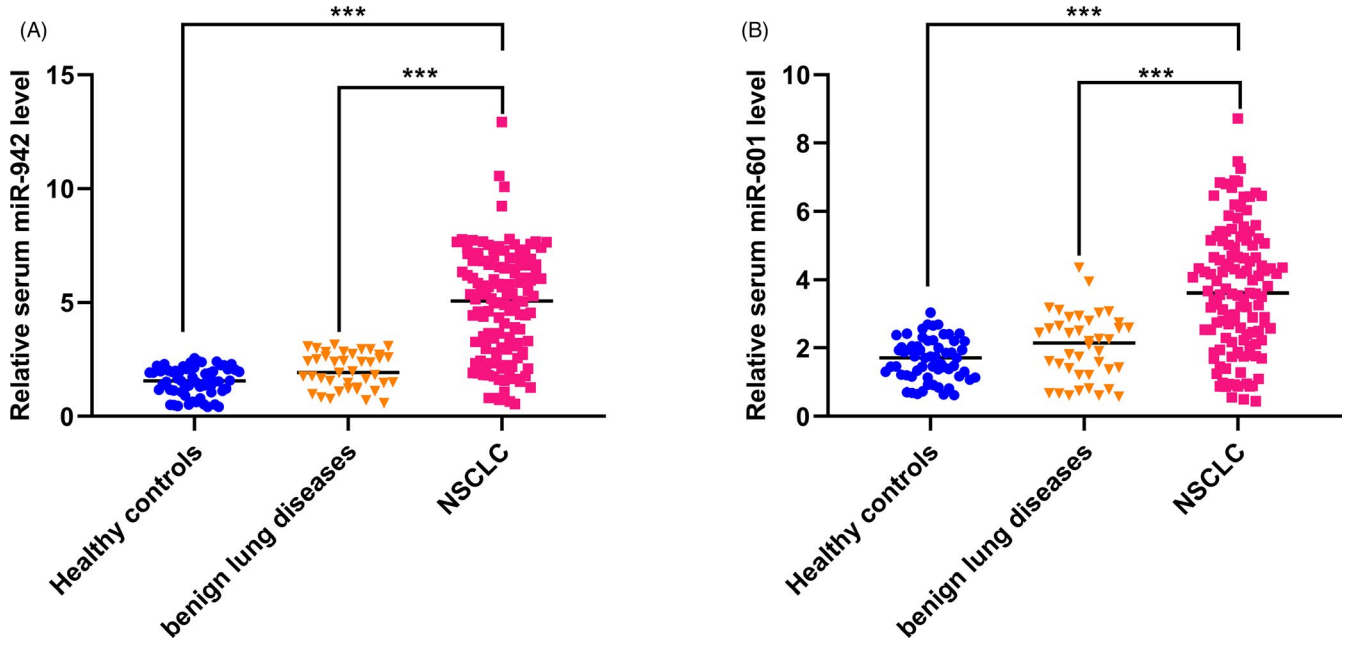

FIGURE 2 Serum miR-942 and serum miR-601 were significantly increased in NSCLC. A, The expression level of serum miR-942 was dramatically upregulated in patients with NSCLC compared to patients with benign lung diseases and healthy controls. B, The serum miR-601 level was higher in patients with NSCLC than in patients with benign lung diseases and healthy controls

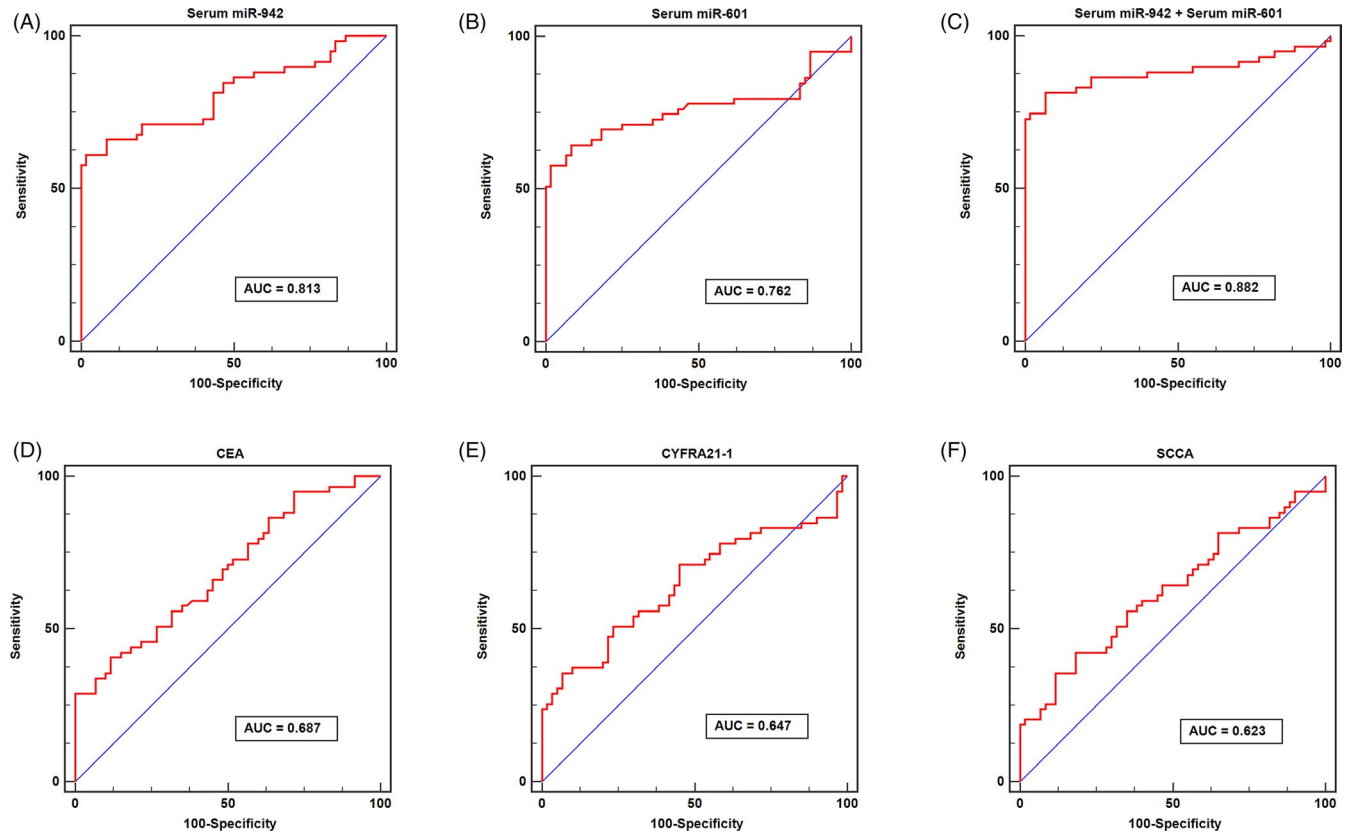

FIGURE 3 The diagnostic value of serum miR-942 and serum miR-601 for early-stage NSCLC. A, The diagnostic performance of serum miR-942 for discriminating early-stage NSCLC from healthy controls. B, The diagnostic performance of serum miR-601 for discriminating early-stage NSCLC from healthy controls. C, The diagnostic performance of combining serum miR-942 and serum miR-601 for discriminating early-stage NSCLC from healthy controls. D-F, The diagnostic performance of CEA, CYFRA21-1, and SCCA for discriminating early-stage NSCLC from healthy controls

TABLE 2 The association between serum miR-942 and clinical variables of NSCLC

Variables	Total (n = 125)	Serum miR-942		p
		Low (n = 63)	High (n = 62)	
Age				
<60	57 (45.60%)	26 (20.80%)	31 (24.80%)	.327
≥60	68 (54.40%)	37 (29.60%)	31 (24.80%)	
Gender				
Female	29 (23.20%)	13 (10.40%)	16 (12.80%)	.493
Male	96 (76.80%)	50 (40.00%)	46 (36.80%)	
Smoking history				
No	35 (28.00%)	17 (13.60%)	18 (14.40%)	.799
Yes	90 (72.00%)	46 (36.80%)	44 (35.20%)	
Distant metastasis				
Negative	114(91.20%)	60 (48.00%)	54 (43.20%)	.108
Positive	11 (8.80%)	3 (2.40%)	8 (6.40%)	
Lymph node metastasis				
Negative	67 (53.60%)	44 (35.20%)	23 (18.40%)	<.001
Positive	58 (46.40%)	19 (15.20%)	39 (31.20%)	
TNM stage				
I-II	56 (44.80%)	37 (29.60%)	19 (15.20%)	.002
III-IV	69 (55.20%)	26 (20.80%)	43 (34.40%)	
Histological grade				
Well/moderate	80 (64.00%)	46 (36.80%)	34 (27.20%)	.034
Poor	45 (36.00%)	17 (13.60%)	28 (22.40%)	

of serum miR-942, serum miR-601, and other tumor biomarkers. Kaplan-Meier plots were used to visualize survival curves, and the differences in survival curves were compared by the log-rank test. Univariate and multivariate analyses were performed to identify the independent risk factors for NSCLC. All statistical tests were conducted with GraphPad Prism 7.0 (GraphPad). A P value < .05 was considered statistically significant.

3 | RESULTS

3.1 | Serum miR-942 and serum miR-601 were upregulated in NSCLC

We profiled the most upregulated serum miRNAs (miR-432, miR-942, miR-29c-5p, and miR-601) in the dataset GSE24709. Then, the data were validated with 20 NSCLC cases and 20 healthy volunteers. Our qRT-PCR results showed that the expression levels of serum miR-942, miR-601, and miR-29c-5p were higher in the patients with NSCLC than in healthy controls (*P < .05, ***P < .001) (Figure 1A-C). However, no significant difference was found for serum miR-432 level between the two compared groups (P = .134, Figure 1D). As serum miR-942 and serum miR-601 were most upregulated in NSCLC, they were selected for further investigation. The expression patterns of serum miR-942 and serum miR-601 were further evaluated with a larger study cohort which included 125 patients with NSCLC, 40 patients with benign lung diseases, and 60 healthy controls. Our results showed that the levels of serum miR-942 and serum miR-601 were both dramatically higher in patients with NSCLC compared to patients with benign lung diseases or healthy controls (***P < .001) (Figure 2A,B).

3.2 | The efficacies of serum miR-942 and serum miR-601 for early diagnosis of NSCLC

There were 56 NSCLC patients at the early TNM stages (stage I-stage II). The diagnostic performance of serum miR-942 and serum miR-601 for early detection of NSCLC was evaluated by the ROC analysis. The AUC values of serum miR-942 and serum miR-601 for discriminating the early-stage NSCLC from healthy controls were 0.813 and 0.762, respectively (Figure 3A,B). Interestingly, combination of serum miR-942 and serum miR-601 increased the discriminative power for early detecting NSCLC, with an AUC value of 0.882 (Figure 3C). For the traditional markers, the AUC values of CEA, CYFRA21-1, and SCCA were 0.687, 0.647, and 0.623, respectively (Figure 3D-F). Therefore, serum miR-942 and serum miR-601 exhibited better performance for the early diagnosis of NSCLC.

TABLE 3 The association between serum miR-601 and clinical variables of NSCLC

Variables	Total (n = 125)	Serum miR-601		p
		Low (n = 63)	High (n = 62)	
Age				
<60	57 (45.60%)	24 (19.20%)	33 (26.40%)	.090
≥60	68 (54.40%)	39 (31.20%)	29 (23.20%)	
Gender				
Female	29 (23.20%)	15 (12.00%)	14 (11.20%)	.871
Male	96 (76.80%)	48 (38.40%)	48 (38.40%)	
Smoking history				
No	35 (28.00%)	14 (11.20%)	21 (16.80%)	.147
Yes	90 (72.00%)	49 (39.20%)	41 (32.80%)	
Distant metastasis				
Negative	114 (91.20%)	58 (46.40%)	56 (44.80%)	.731
Positive	11 (8.80%)	5 (4.00%)	6 (4.80%)	
Lymph node metastasis				
Negative	67 (53.60%)	40 (32.00%)	27 (21.60%)	.025
Positive	58 (46.40%)	23 (18.40%)	35 (28.00%)	
TNM stage				
I-II	56 (44.80%)	40 (32.00%)	16 (12.80%)	<.001
III-IV	69 (55.20%)	23 (18.40%)	46 (36.80%)	
Histological grade				
Well/moderate	80 (64.00%)	43 (34.40%)	37 (29.60%)	.318
Poor	45 (36.00%)	20 (16.00%)	25 (20.00%)	

3.3 | The correlations between serum miR-942/serum miR-601 and clinical variables of NSCLC

The NSCLC patients were split into the high serum miR-942 group and low serum miR-942 group with the median expression of serum miR-942. A total of 62 and 63 patients were in the high and low serum miR-942 group, respectively. The results showed that a higher percentage of patients with positive lymph node metastasis ($P < .001$), or with advanced TNM stages ($P = .001$), or with poor histological grade ($P = .034$) were observed in the high serum miR-942 group. No correlation was found between serum miR-942 and other clinical variables including age, gender, smoking status, and distant metastasis (Table 2). Similarly, for the serum miR-601, it was strongly associated with lymph node metastasis ($P = .025$) and TNM stages ($P < .001$), and it was not correlated with age, gender, smoking status, distant metastasis, and histological grade (Table 3).

3.4 | The prognostic values of serum miR-942/serum miR-601 in NSCLC

The survival analysis showed that the NSCLC patients in the high serum miR-942 group had significantly shorter overall survival (OS) ($P < .001$) and recurrence-free survival (RFS) ($P < .001$) than the patients in the low serum miR-942 group (Figure 4A,B). Similarly, the NSCLC patients in the high serum miR-601 group suffered worse OS ($P < .001$) and RFS ($P < .001$) than the patients in the low serum miR-601 group (Figure 4C,D). Then, we explored the combination effect of serum miR-942 and serum miR-601 on the OS and RFS of NSCLC. Surprisingly, as shown in Figure 5A,B, NSCLC patients in the high serum miR-942+ high serum miR-601 group had worst OS and RFS, while those in the low serum miR-942+ low serum miR-601 group had the most favorable OS and RFS ($P < .001$). The univariate analysis revealed that lymph node metastasis, TNM stage, histological grade, serum miR-942, and serum miR-601 were strongly associated the OS of NSCLC. The multivariate analysis showed that TNM stage (HR = 3.568, 95% CI = 1.804-8.659, $P < .001$), histological grade (HR = 2.206, 95% CI = 1.105-3.768, $P = .018$), serum miR-942 (HR = 2.918, 95% CI = 1.482-5.809, $P < .001$), and serum miR-601 (HR = 2.739, 95% CI = 1.311-5.036, $P = .008$) were independent prognostic factors for NSCLC (Table 4).

4 | DISCUSSION

In the present study, we have shown that the expression levels of serum miR-942 and serum miR-601 were dramatically increased in patients with NSCLC. In addition, these two serum miRNAs had better discriminative power for detecting early-stage NSCLC compared to the traditional molecular biomarkers. Upregulation of serum miR-942 and serum miR-601 was both significantly associated with

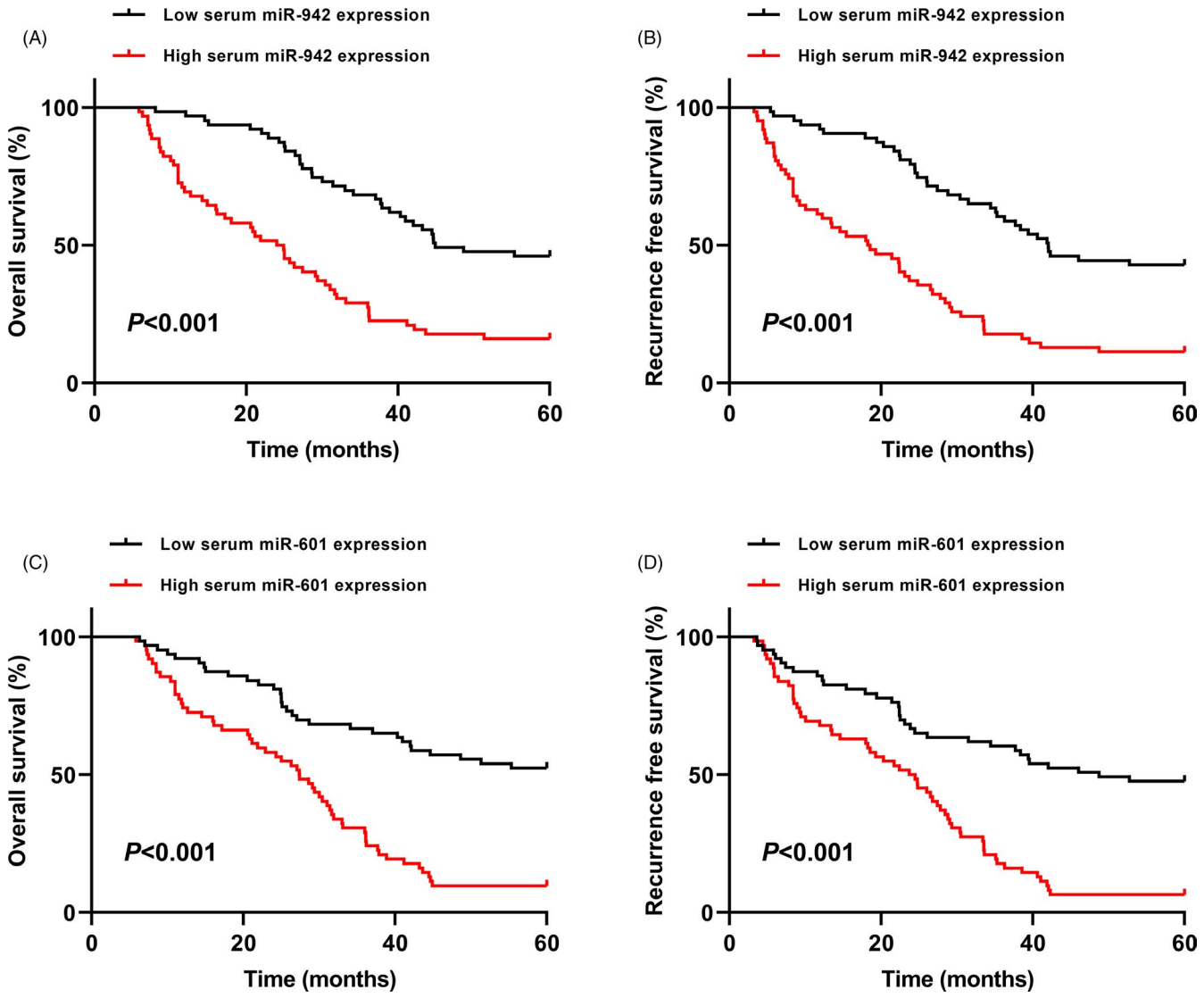

FIGURE 4 The association between serum miR-942/serum miR-601 and OS/RFS in NSCLC. A-B, The NSLCC patients in the high serum miR-942 group had poorer OS and RFS than the patients in the low serum miR-942 group. C-D, The NSLCC patients in the high serum miR-601 group had shorter OS and RFS than the patients in the low serum miR-601 group

unfavorable clinical variables and clinical outcome of NSCLC. Both serum miR-942 and serum miR-601 were identified as independent prognostic factors for NSCLC. Interestingly, combinatory effects of serum miR-942 and serum miR-601 were found for the early detection and prognosis prediction of NSCLC. Therefore, serum miR-942 and serum miR-601 might be a novel molecular signature for detecting NSCLC at the early stage and predicting the clinical outcome of this deadly malignancy.

Consistent with our result, Patnaik et al showed that the expression level of circulating miR-942 was higher in patients with lung adenocarcinoma compared to the controls.[14] The expression level of miR-942 was significantly upregulated in NSCLC tissues and cell lines. Ectopic expression of miR-942 enhanced cell migration, invasion, and angiogenesis in vitro as well as promoted metastasis in vivo, indicating that miR-942 might play a tumor promoting in tumorigenesis of NSCLC.[15] MiR-601 seems to affect many important signaling pathways in lung cancer cells, suggesting it might be a crucial player in NSCLC development.[16] However, the role of both miR-942 and miR-601 in the carcinogenesis of NSCLC needs further investigation.

MiR-942 has also been shown to play an oncogenic role in other types of cancers. For instance, the expression level of miR-942 was upregulated in hepatocellular carcinoma (HCC). Increased expression of miR-942 was strongly correlated with serum alanine transaminase level, tumor size, T stage, lymphatic metastasis, and survival.[17] Similarly, miR-942 was dramatically increased in esophageal squamous cell carcinoma (ESCC), and miR-942 overexpression was associated with unfavorable clinical outcome of ESCC. Enforced expression of miR-942 promoted malignant behaviors of ESCC cells both in vitro and in vivo.[18] MiR-942 was highly expressed in breast

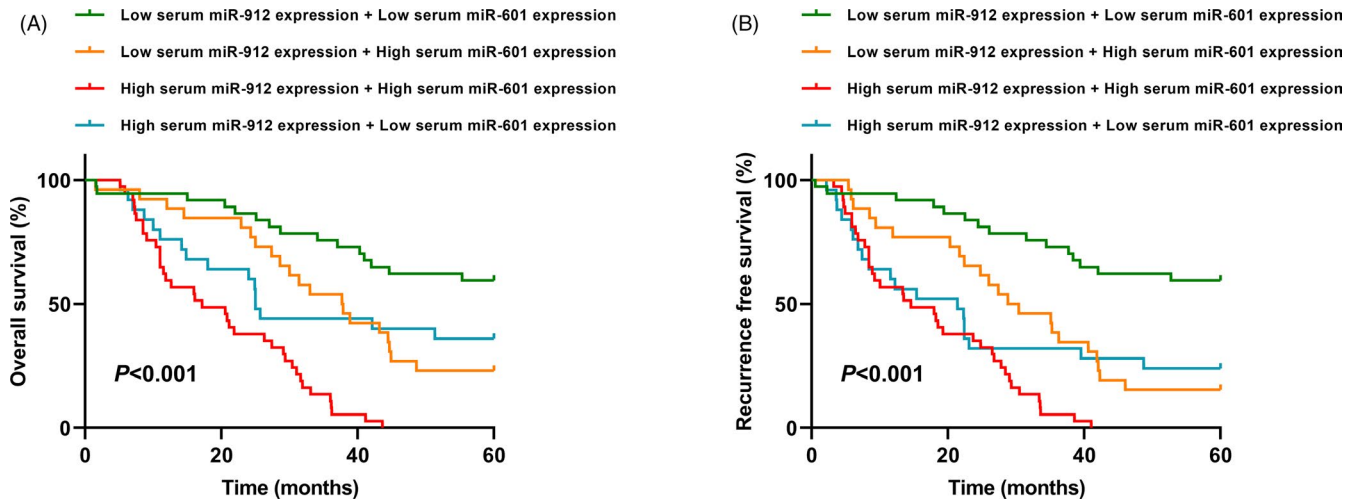

FIGURE 5 The prognostic value of combining serum miR-942 and serum miR-601 in NSCLC. (A-B) Worst OS and RFS were observed in the patients from high serum miR-942+ high serum miR-601 group, while the patients in the low serum miR-942+ low serum miR-601 group had the most favorable OS and RFS

TABLE 4 Univariate and multivariate analyses of the prognostic factors for NSCLC

	Univariate analysis			Multivariate analysis		
Variables	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
Age	1.128	0.725-1.632	.635	1.245	0.681-1.715	.357
Gender	1.005	0.680-1.874	.821	0.973	0.702-1.563	.429
Smoking history	1.325	0.791-2.289	.412	1.468	0.879-2.469	.806
Distant metastasis	0.965	0.518-1.496	.708	0.796	0.428-1.520	.385
Lymph node metastasis	2.510	1.253-4.267	.036	1.852	0.941-3.892	.152
TNM stage	4.539	2.563-12.529	<.001	3.568	1.804-8.659	<.001
Histological grade	2.948	1.408-5.887	.002	2.206	1.105-3.768	.018
Serum miR-942	3.681	1.852-8.974	<.001	2.918	1.482-5.809	<.001
Serum miR-601	3.285	1.608-7.193	<.001	2.739	1.311-5.036	.008

cancer. Downregulation of miR-942 markedly reduced the cell viability, proliferation, migration, and invasion and promoted cell apoptosis. The oncogenic activities of breast cancer cells were increased when the miR-942 was overexpressed.[19]

Similar findings have been reported for miR-601. For instance, the expression level of miR-601 was significantly increased in patients with gastric cancer (GC) compared to patients with adenoma and healthy controls.[20] Similarly, miR-601 was markedly upregulated in GC tissues and cell lines. In addition, miR-601 level was strongly correlated with TNM stage, lymph node metastasis, lymphatic invasion, and distant metastasis. Moreover, GC patients with high tissue miR-601 expression suffered worse OS, and miR-601 was an independent risk factor of NSCLC.[21] It should be noted that miR-601 might play a tumor-suppressive role in tumorigenesis, depending on the cancer types.[22]

In conclusion, our results have demonstrated that the expression levels of serum miR-942 and serum miR-601 are significantly increased in patient with NSCLC. In addition, both serum miR-942 and serum miR-601 effectively detect early-stage NSCLC and are closely associated with the clinical outcome of NSCLC. More importantly, these two serum miRNAs have combinatory effects for the early detection and prognosis prediction of NSLCC. Therefore, serum miR-942 and serum miR-601 might serve as a novel diagnostic and prognostic signature for NSCLC.

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