

Expression and Diagnostic Efficacy of miR-126-5p and miR-34c-3p in Serum Extracellular Vesicles of Non-Small Cell Lung Cancer Patients

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Research Article

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Abstract

Background: Non-small cell lung cancer (NSCLC) is a disease with quite grave prognosis. This study explored the diagnostic efficiency of miR-126-5p and miR-34c-3p in serum extracellular vesicles (EVs) in NSCLC patients.

Methods: Serum EVs were extracted from NSCLC patients and healthy people and verified. The expression of miR-126-5p and miR-34c-3p in serum EVs were tested. Correlation of miR-126-5p and miR-34c-3p expression and diagnosis, prognosis and pathological characteristics (age, gender, tumor size, clinical stage, and lymph node metastasis) of NSCLC patients was analyzed. The downstream targets of miR-126-5p and miR-34c-3p were predicted and their roles in diagnosis and prognosis of NSCLC patients were evaluated.

Results: miR-126-5p and miR-34c-3p were poorly expressed in serum EVs of NSCLC patients and their low expressions were associated with clinical stage, lymph node metastasis and prognosis of NSCLC patients and could be used as biomarkers for diagnosis. As the common target genes of miR-126-5p and miR-34c-3p, LYPLA1 and CDK6 were highly expressed in serum EVs and were associated with poor prognosis in NSCLC patients.

Conclusion: Lowly expressed miR-126-5p and miR-34c-3p in serum EVs of NSCLC patients can serve as biomarkers for diagnosis and are linked with prognosis. As common targets of miR-126-5p and miR-34c-3p, LYPLA1 and CDK6 are also associated with poor prognosis in NSCLC patients.

Introduction

Non-small cell lung cancer (NSCLC), one of the most common cancer types, is known to have a gloomy prognosis [1]. Moreover, the cure and survival rates for NSCLC stay low, especially in metastatic disease [2]. Existent methods to diagnose NSCLC are fairly invasive and sometimes unable to detect early-stage NSCLC [3]. In the last decades, although numerous targeted therapies have been advanced with persuasive clinical evidence, the efficacy proved to be ephemeral [4]. Blood biomarkers represent primary tumors and different metastatic lesions, and may be conducive to risk assessment of asymptomatic population, nodule characterization and prognosis, provide information for surgical intervention in diagnosed or resected tumors [5]. Therefore, further research into new diagnostic method is needed to detect early-stage NSCLC and improve the cure and survival rates for NSCLC.

As small membranous vesicles, extracellular vesicles (EVs) are found in blood, urine, and saliva, and can facilitate intercellular communication and affect the homeostasis of blood vessels by transporting molecules like microRNAs (miRNAs) [6, 7]. Additionally, circulating EVs are abundant in serum, one of the most accessible sources [8]. EVs are rich in cancer patients, stable and easy to collect from body fluids, and are good containers for circulating biomarkers, so it is a suitable candidate for lung cancer liquid biopsy [9, 10]. Specific exosomal miRNAs stably expressed in blood have been reported to be reliable biomarkers of several solid malignancies [11, 12]. Several miRNA-panels in EVs isolated from serum have

been proposed for NSCLC diagnosis [9, 13]. For instance, downregulation of miR-126 in NSCLC has been confirmed in various clinical studies. As reported by Wang et al. [14], NSCLC patients at early-stage have lowered miR-126 expression in the blood, consistent with findings of Zhu et al. [15] and Shang et al. [16]. Thereby, miR-126 is regarded a diagnostic marker of early-stage NSCLC. Moreover, a significant association has been identified between decreased miR-126 and NSCLC prognosis [17, 18]. Besides, miR-34c-3p is downregulated both in NSCLC tissues and cell lines [19, 20]. miR-34c-3p is a prognostic factor in KRAS-mutated NSCLC patients and its high expression is linked with longer survival [21]. Exosomes-shuttled low miR-34c-3p is implicated in NSCLC progression [22]. The role and mechanism of miR-126-5p and miR-34c-3p in serum EVs in the prognosis of NSCLC have not been reported at home and abroad. This study attempted to explore the role and mechanism of serum exosomal miR-126-5p and miR-34c-3p in the prognosis of NSCLC, and to provide a new theoretical basis for NSCLC management.

Materials & Methods

Patients

A total of 85 patients with NSCLC (average age: 53.03 ± 4.11 ; 46 males and 39 females) admitted to The Second Affiliated Hospital of Xuzhou Medical University from December 2017 to November 2020 and 33 healthy controls (average age 52.97 ± 3.70 , 17 males and 16 females) selected from physical examination center were included in this study. The inclusion criteria were as follows. All patients with NSCLC were confirmed by histopathological examination and were staged according to the latest TNM stage 8.0 version [23]. All patients with NSCLC were diagnosed by clinical examination and did not receive antibiotics or other relevant treatment before blood sampling. Healthy controls had no tumors in any part of the body. The exclusion criteria were as follows. NSCLC patients with incomplete clinical data, or healthy controls with incomplete physical examination results. No difference was found in gender and age between NSCLC patients and healthy control ($P > 0.05$). All 85 patients were followed up to observe the 5-year overall survival (OS) via telephone or subsequent visit. All included patients had complete clinical data (Table 1).

Table 1
Clinical data

Parameter	Healthy	NSCLC	<i>P</i> value
Age (years)			0.838
≤ 53	18	43	
> 53	15	42	
Sex			0.839
Male	17	46	
Female	16	39	
Tumor size (cm)			-
≤ 5	-	41	
> 5	-	44	
Clinical stage			-
I-II	-	43	
III	-	42	
LNM			-
Negative	-	44	
Positive	-	41	

Extraction of serum EVs

Peripheral blood was collected from all subjects, and serum EVs were separated by ultracentrifugation based on a previous study [24]. Blood samples were coagulated at 4°C for 1 h and centrifuged at 1000 g at 4°C for 15 min to obtain the serum, which was blended with phosphate buffer saline (PBS) at 4:5. Next, the mixture was centrifuged at 4°C at 2000 g for 30 min to remove the cells, and then centrifuged at 4°C at 12000 g for 45 min to remove cell debris. Then, supernatant was centrifuged at 4°C at 110000 g for 2 h to obtain the precipitate. The precipitates were washed with 1 mL PBS, centrifuged at 4°C at 110000 g for 75 min to obtain the EV precipitates, which were resuspended in 150 µL PBS. Bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China) was used for EV quantification. The prepared serum EVs were stored at -80°C for subsequent analysis.

Identification of serum EVs

The EV morphology was observed by transmission electron microscopy (TEM, JEM-1200 EX, JEOL, Ltd. Tokyo, Japan). Western blot analysis was utilized to detect EV marker proteins CD9, CD63, and calnexin,

with the remaining supernatant of EVs as negative control (NC). Nanoparticle tracking analysis (NTA) was employed to measure the size and distribution of EVs.

Western blot analysis

After the EVs were lysed with RIPA lysate and centrifuged to harvest the supernatant and the protein concentration was determined using a BCA kit (Beyotime). The extracted protein was added to the sample buffer and soaked in 95°C water bath for 5 min. After preparing 10% SDS-PAGE, the protein was transferred to PVDF membranes, which were blocked for 2 h and then incubated with primary antibodies CD9 (1:2000, ab223052, Abcam, Cambridge, MA, USA), CD63 (1:1000, ab134045, Abcam) and calnexin (1:2000, ab10286, Abcam) at 4°C overnight. After 3 regimens of TBST washing, the membranes were added with secondary antibody (1:2000, ab205718, Abcam) for 1 h and developed using chemiluminescence reagent and Gel Dol EZ imager (Bio-rad, Hercules, CA, USA). Image J software (NIH, Bethesda, MD, USA) was adopted to analyze the gray value of target bands.

Dual-luciferase assay

Targetscan database (<http://www.targetscan.org>) was employed to predict the binding sites of miR-126-5p/miR-34c-3p and LYPLA1/CDK63. LYPLA1/CDK63 sequences containing miR-126-5p/miR-34c-3p binding sites were synthesized to construct LYPLA1/CDK63 wild type (WT) plasmids (LYPLA1-WT or CDK6-WT) and corresponding mutant (MUT) plasmid LYPLA1-MUT or CDK6-MUT. The constructed plasmids were mixed with mimic NC or mimic and cotransfected into HEK293T cells (ATCC). The luciferase activities were detected 48 h later.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA in serum EVs was acquired using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, UV analysis technology and formaldehyde denaturation electrophoresis were employed to verify high-quality RNA. The cDNA was obtained by TaqMan microRNA reverse transcription kit (Invitrogen) using 1 µg RNA. The qPCR was conducted as per the provided protocol using a qPCR Master Mix of SYBR Green (Bio-Rad) on ABI 7500 thermocycler (Life Technologies, Gaithersburg, MD, USA). PCR primers were designed and synthesized by Sangon Biotech (Shanghai, China) (Table 2), with GAPDH or U6 as internal reference. The relative expression was calculated by $2^{-\Delta\Delta CT}$ method [25].

Table 2
PCR primer sequence

Primer	Sequence (5'-3')
miR-126-5p	F: CATTACTTTTGGTACGCG
	R: CGCGTACCAAAAGTAATAATG
miR-34c-3p	F: AATCACTAACCACACGGCCAGG
	R: CCTGGCCGTGTGGTTAGTGATT
LYPLA1	F: ATGTGCGGCAATAACATGTCAACC
	R: TCAATCAATTGGAGGTAGGAGTTTA
CDK6	F: ATGGAGAAGGACGGCCTGTGCCGC
	R: TCAGGCTGTATTCAGCTCCGAGGT
GAPDH	F: ATGGTTTACATGTTCCAATATG
	R: TTACTCCTTGGAGGCCATGTGG
U6	F: CGCTTCGGCAGCACATATAC
	R: AATATGGAACGCTTCACGA
Note: F: forward; R: reverse.	

Statistical analysis

SPSS 21.0 (IBM Corp. Armonk, NY, USA) and GraphPad Prism 8.01 (GraphPad Software, San Diego, CA, USA) were used for statistical analysis and data plotting. Firstly, a normality and variance homogeneity test was conducted, and the data in compliance with the assumption of normality and homogeneity of variance were compared using the *t*-test (two groups) or two-way analysis of variance (ANOVA). Sidak's multiple comparisons test was utilized for the analysis after ANOVA. A *P* value of < 0.05 was regarded statistically significant.

Results

Identification of serum EVs

EV is a small membrane capsule containing proteins and nucleic acids, such as miRNAs [26, 27]. There are a large number of deregulated miRNAs in patients with NSCLC, and serum EV-shuttled miRNAs can be used as tumor biomarkers of NSCLC [28, 29]. In order to study the relationship between miRNAs in serum EVs of NSCLC patients with and prognosis, we first isolated the serum EVs from NSCLC patients and healthy people by ultracentrifugation, and then characterized the isolated EVs by TEM and NTA. The results showed that the isolated EVs had a bilayer membrane structure (Fig. 1A), and the size distribution was about 100 nm in diameter (Fig. 1B). Western blot analysis showed that specific EV marker proteins

(CD9 and CD63) were present in the part enriched with EVs, but not in the negative control. On the other hand, calnexin was not present in the part enriched with EVs (Fig. 1C). Briefly, EVs were successfully isolated.

Low expression of miR-126-5p and miR-34c-3p in serum EVs of NSCLC patients

The results of database prediction showed that miR-126-5p and miR-34c-3p were lowly expressed in NSCLC patients (Fig. 2A-D). RT-qPCR showed that miR-126-5p and miR-34c-3p in the obtained serum EVs in NSCLC patients was much lower than that in the normal people ($p < 0.01$, Fig. 2E). These results unveiled the low expression of miR-126-5p and miR-34c-3p in serum EVs of NSCLC patients.

Low miR-126-5p and miR-34c-3p in serum EVs are associated with poor prognosis

Next, we analyzed the relationship between miR-126-5p/miR-34c-3p and the prognosis of NSCLC patients. According to the pathological characteristics [age, gender, tumor size, clinical stage and lymph node metastasis (LNM)], 85 patients with NSCLC were allocated into two groups. As shown in Fig. 3A-E, the low expression of miR-126-5p and miR-34c-3p in serum EVs was not related to age, gender and tumor size, but related to clinical stage and positive LNM ($p < 0.05$). The database predicted that NSCLC patients with low miR-126 expression had shorter survival time (Fig. 3F). According to the median expression of miR-126-5p and miR-34c-3p, NSCLC patients were divided into miR-126-5p or miR-34c-3p high expression group and low expression group. By tracking the survival time of these NSCLC patients, we generated the survival curves. Patients with low expression of miR-126-5p or miR-34c-3p in serum EVs had shorter survival time ($p < 0.01$, Fig. 3G). The diagnostic efficacy of miR-126-5p and miR-34c-3p in serum EVs for NSCLC was analyzed by receiver operating characteristic (ROC) curve. The area under the ROC curve was 0.7916 (sensitivity: 0.7412; specificity: 0.7273) and 0.7807 (sensitivity: 0.7412; specificity: 0.8182), respectively (Fig. 3H). In conclusion, miR-126-5p and miR-34c-3p in serum EVs may be diagnostic markers in NSCLC patients and are related to the prognosis.

CDK6 and LYPLA1 are downstream targets of miR-126-5p and miR-34c-3p

In an attempt to explore the downstream of miR-126-5p and miR-34c-3p, we predicted the common target genes through Targetscan and Starbase databases, and finally got 151 target genes (Fig. 4A). The predicted results showed that CDK6 and LYPLA1 were highly expressed in NSCLC (Fig. 4B-C), which was also verified by the literatures [30, 31]. The binding sites of miR-126-5p/miR-34c-3p with CDK6 and LYPLA1 obtained from Targetscan database (Fig. 4D) and dual-luciferase assay ($p < 0.01$, Fig. 4E) confirmed the binding relations. RT-qPCR showed that CDK6 and LYPLA1 were highly expressed in NSCLC patients ($p < 0.01$, Fig. 4F), and negatively correlated with miR-126-5p/miR-34c-3p expression in serum EVs of NSCLC patients ($p < 0.01$, Fig. 4G). In conclusion, CDK6 and LYPLA1 are common targets of miR-126-5p and miR-34c-3p.

Serum levels of CDK6 and LYPLA1 are associated with poor prognosis in NSCLC patients

Next, the relation between CDK6 and LYPLA1 in serum of NSCLC patients and prognosis was analyzed. As shown in Fig. 5A-E, the high expression of CDK6 and LYPLA1 in serum was only associated with clinical stage and LNM ($p < 0.01$). The database predicted that patients with high expression of CDK6 and LYPLA1 had shorter survival time (Fig. 5F). According to the median expression of CDK6 and LYPLA1, NSCLC patients were allocated into two groups, and the survival curves of the two groups were generated. Patients with high expression of CDK6 and LYPLA1 in serum had shorter survival time ($p < 0.01$, Fig. 5G). In addition, ROC curve showed that CDK6 and LYPLA1 had good diagnostic efficacy for NSCLC patients (CDK6: area under the curve: 0.8061, sensitivity: 0.8118, specificity: 0.8788; LYPLA1: area under the curve: 0.7594, sensitivity: 0.6824, specificity: 0.7879) (Fig. 5H). In conclusion, CDK6 and LYPLA1 in serum can be used as diagnostic markers of NSCLC and are associated with poor prognosis.

Discussion

Several blood-based miRNA tests have been developed for lung cancer diagnosis, with high sensitivity and specificity [32–34]. Meanwhile, many functional molecules like miRNAs are stably encapsulated in EVs, which are recognized as promising biomarkers for cancer detection [35]. This study explored the diagnostic efficiency and mechanism of serum exosomal miR-126-5p/miR-34c-3p in NSCLC, and our results supported that miR-126-5p/miR-34c-3p deficiency in serum EVs of NSCLC patients served as biomarkers for diagnosis and prognosis, and their common targets LYPLA1 and CDK6 were also associated with poor prognosis of NSCLC patients.

A lot of miRNAs are abnormally expressed in NSCLC patients, and serum exosomal miRNA editing is potential to non-invasively discriminate normal and NSCLC samples [28, 29]. The database prediction showed that miR-126-5p and miR-34c-3p were poorly expressed in NSCLC patients, consistent with RT-qPCR results that demonstrated miR-126-5p and miR-34c-3p in serum EVs in NSCLC patients were much lowered. Recently studies have highlighted that miR-126-5p is downregulated in NSCLC and influences the prognosis [36, 37]. Exosomes-shuttled low levels of miR-34c-3p may be a diagnostic marker for NSCLC [22]. These results unveiled the low expression of miR-126-5p and miR-34c-3p in serum EVs of NSCLC patients.

miRNAs are potential targets as molecular biomarkers and therapeutic intervention in NSCLC [38, 39]. Therefore, we analyzed the relationship between miR-126-5p/miR-34c-3p and the prognosis of NSCLC patients. In any organ system, TNM staging, LNM, and distant metastasis are tricky problems [40]. Low expression of miR-126-5p and miR-34c-3p in serum EVs was related to clinical stage and positive LNM. The database predicted that NSCLC patients with low miR-126 expression had shorter survival time. High miR-126 expression is significantly associated with a tumor size ≤ 3 cm and favorable prognosis in patients with lung adenocarcinoma (LUAD) [18]. Plasma miR-34a expression was negatively associated with LNM [41]. Low miR-34b expression was correlated with distant metastases in LUAD [42]. miR-34c can be used as an effective biomarker and therapeutic target for lung cancer [43]. Moreover, the survival curves of showed that patients with low expression of miR-126-5p or miR-34c-3p in serum EVs had shorter survival time. The area under the ROC curve of miR-126-5p and miR-34c-3p was 0.7916

(sensitivity: 0.7412; specificity: 0.7273) and 0.7807 (sensitivity: 0.7412; specificity: 0.8182), respectively. A meta-analysis pointed out a positive relation of prolonged OS and elevated miR-126 in NSCLC [44]. miR-126 may be a tumor suppressor in NSCLC and could be a prognostic factor and promising treatment in anticancer therapy [17, 45]. LUAD with higher miR-34a/b/c levels had better OS [46, 47]. miR-34c-3p is a new biomarker for NSCLC patients [21]. In conclusion, miR-126-5p and miR-34c-3p in serum EVs may be applied as diagnostic markers for NSCLC and are related to the prognosis.

Next, we explored the downstream of miR-126-5p and miR-34c-3p. After predicting the common targets through databases, we finally got 151 target genes. Among them, CDK6 and LYPLA1 were highly expressed in NSCLC [30, 31], consistent with our observation. The dual-luciferase assay confirmed the binding relations between miR-126-5p/miR-34c-3p and CDK6/LYPLA1. Besides, CDK6 and LYPLA1 were highly expressed in NSCLC patients and negatively correlated with miR-126-5p/miR-34c-3p expression. In conclusion, CDK6 and LYPLA1 are common targets of miR-126-5p and miR-34c-3p. Furthermore, we analyzed the relation between serum CDK6/LYPLA1 and patient prognosis. High expression of CDK6 and LYPLA1 in serum was only associated with clinical stage and LNM, and patients with high expression of CDK6 and LYPLA1 had shorter survival time. Suppression of LYPLA1 significantly inhibited proliferation, migration and invasion of NSCLC cells [31]. To date, there is no report on the relation between LYPLA1 expression pattern and NSCLC clinical features, which presented the novelty of our study. Higher expression of nuclear CDK6 protein was notably associated with N stage, clinical stage, and differentiation degree, and multivariate analysis demonstrated that high expression of nuclear CDK6 was an independent indicator of OS in LUAD patients [48]. In addition, ROC curve showed that CDK6 and LYPLA1 had good diagnostic efficacy for NSCLC patients. A consistent observation in a recent study revealed that high level of CDK6 indicated a poor prognosis for LUAD patients [49]. Altogether, CDK6 and LYPLA1 in serum can be used as diagnostic and prognostic markers of NSCLCs.

Conclusions

In summary, low expression of miR-126-5p and miR-34c-3p in serum EVs of patients with NSCLC had good diagnostic efficacy and prognosis prediction. LYPLA1 and CDK6, the common target genes of miR-126-5p and miR-34c-3p, are highly expressed in the serum of NSCLC patients, and also have good diagnostic efficacy and are associated with poor prognosis. This study only revealed the relationship between serum exosomal miR-126-5p and miR-34c-3p and the prognosis of NSCLC patients. Whether there are other miRNAs in serum EVs remains to be studied. The efficacy of other target genes downstream of miR-126-5p and miR-34c-3p also needs to be further explored. The roles of miR-126-5p, miR-34c-3p and their downstream targets need in-depth validation in the future.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee of The First Affiliated Hospital of Soochow University. All patients signed the informed consent. This study was in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JZ have given substantial contributions to the conception or the design of the manuscript; WH and QW contributed to acquisition, analysis and interpretation of the data.; All authors have participated to drafting the manuscript, author A revised it critically. All authors read and approved the final version of the manuscript.

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Figures

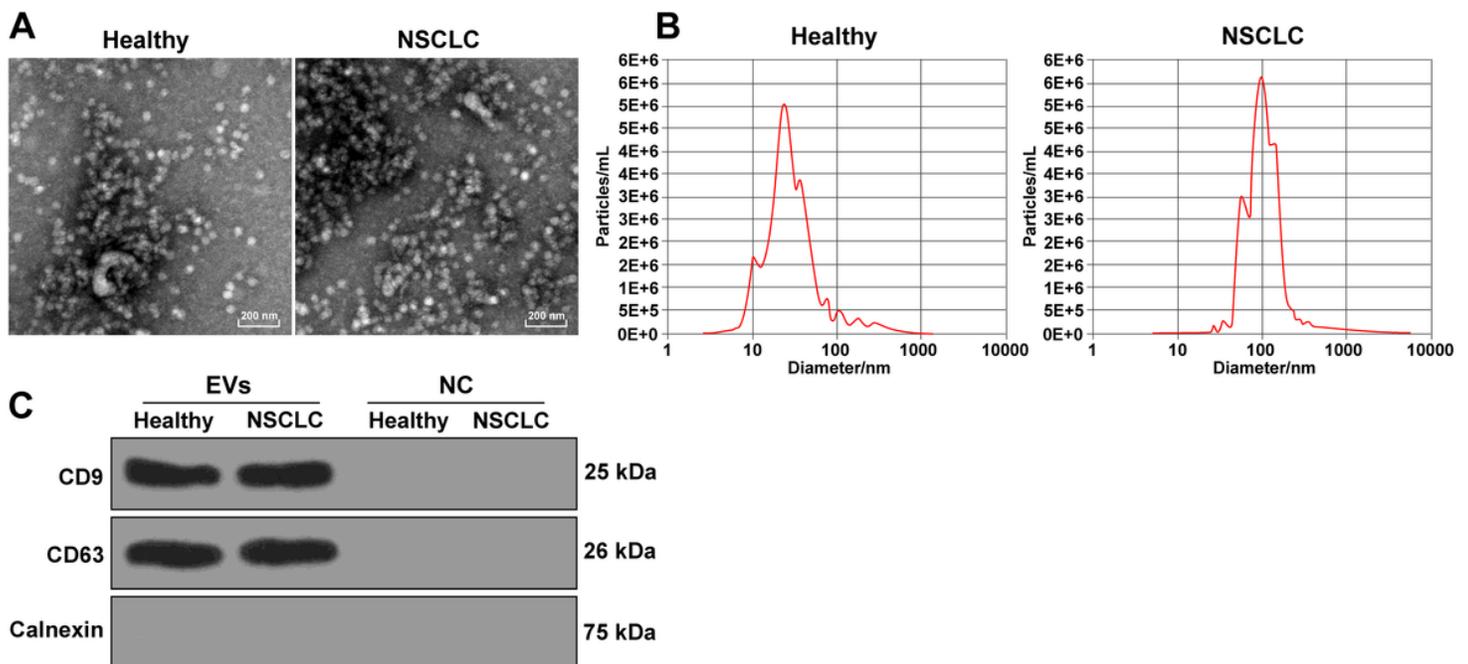


Figure 1

Identification of serum EVs. A: the morphology and size of EVs observed by TEM; B: the size range of serum EVs detected by NTA; C: the expression of CD9, CD63 and calnexin in serum EVs detected by Western blot; NC was the remaining supernatant after EV extraction. The experiment was repeated in triplicate.

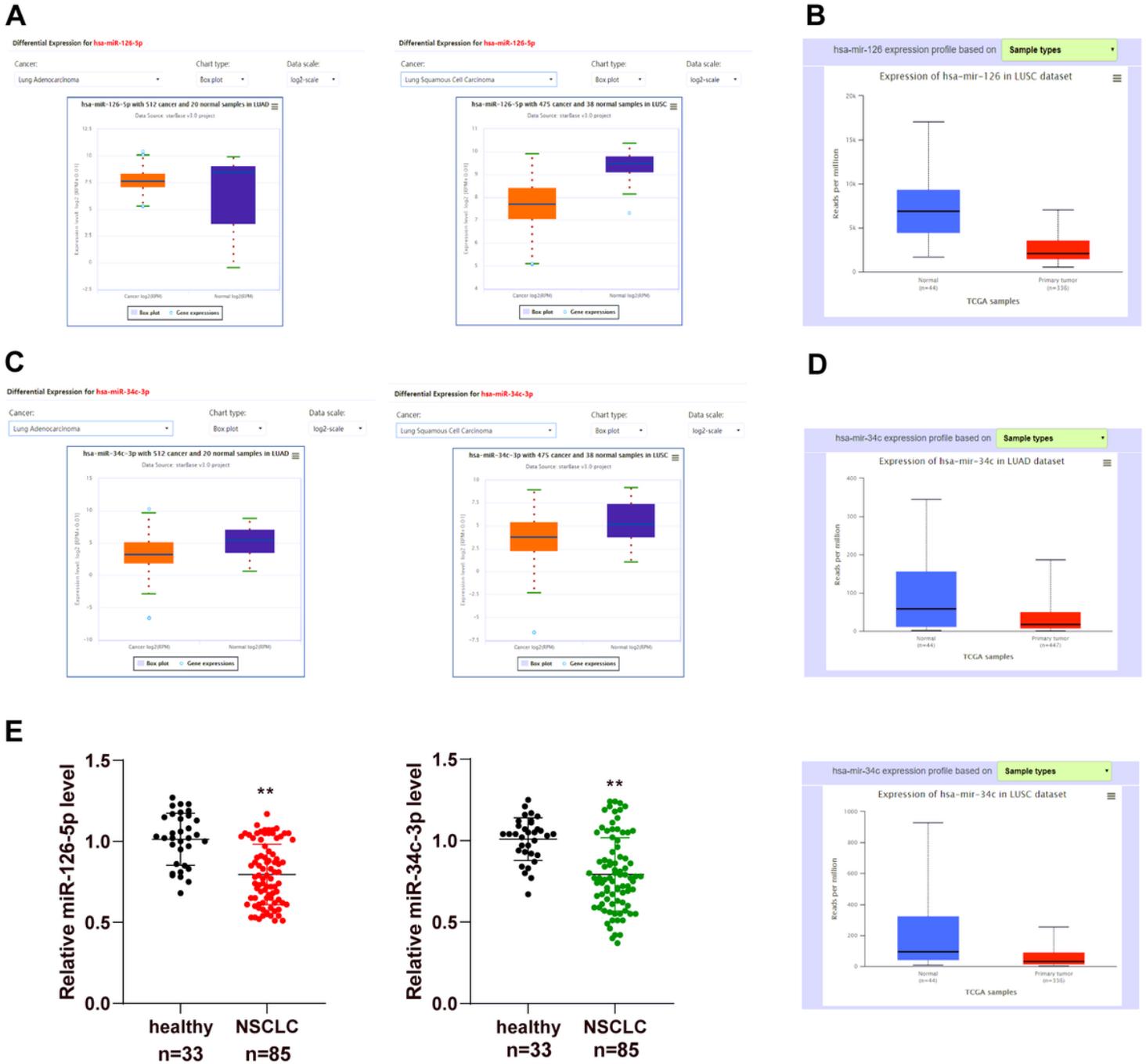


Figure 2

Low expression of miR-126-5p and miR-34c-3p in serum EVs of NSCLC patients. A-D: miR-126-5p and miR-34c-3p expression in NSCLC patients predicted by Starbase and TCGA database; E: miR-126-5p and miR-34c-3p expression in serum EVs of NSCLC patients and healthy people detected by RT-qPCR. All experiments were done in triplicate, and the data in panel E were analyzed by independent sample t test. $**p < 0.01$.

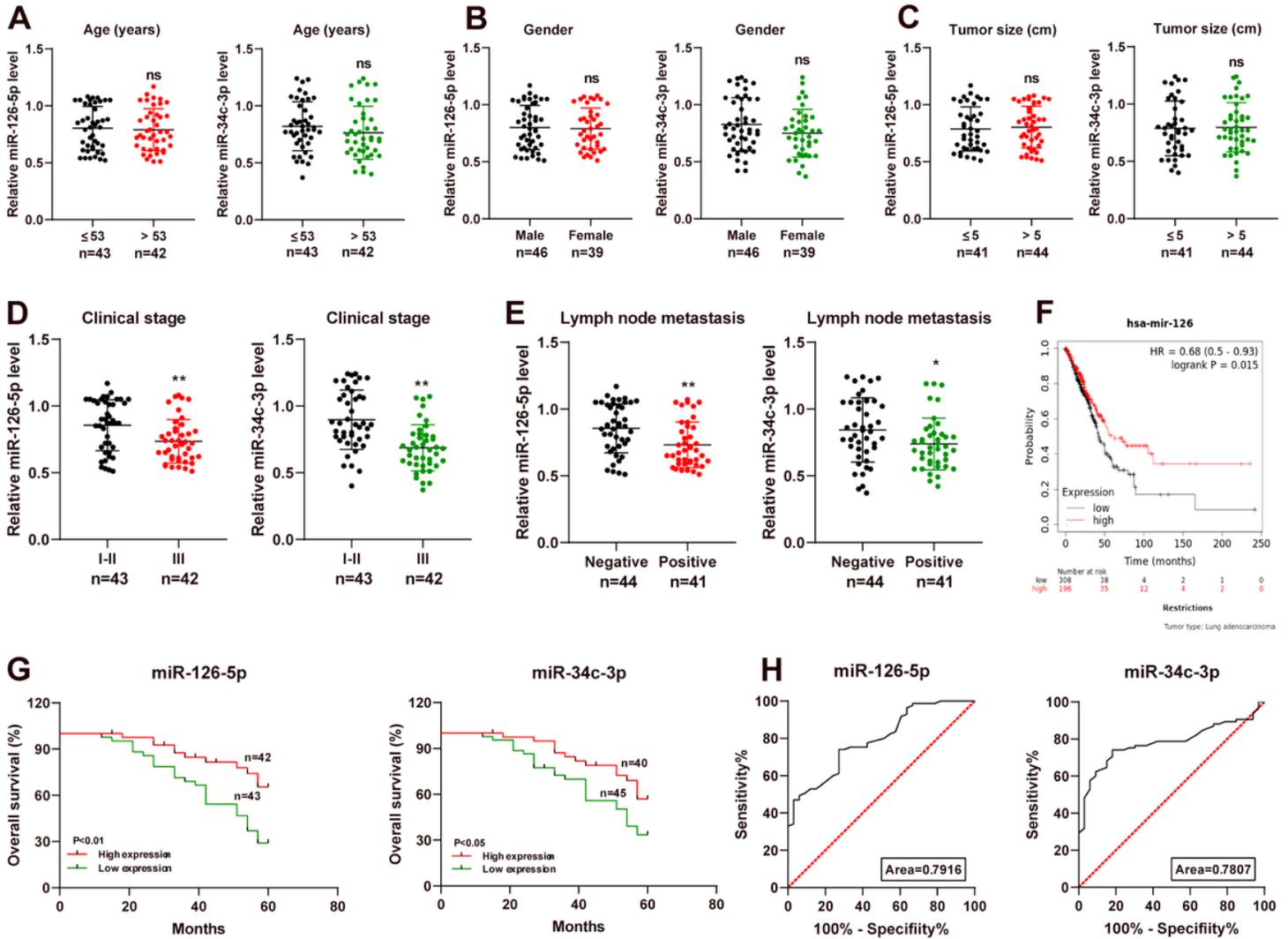


Figure 3

miR-126-5p and miR-34c-3p in serum EVs are associated with poor prognosis. A-E: 85 NSCLC patients were allocated into two groups according to their pathological characteristics, and miR-126-5p/miR-34c-3p expression was tested by RT-qPCR; F: Kaplan-Meier predicted the relation between miR-126 and survival; G: Kaplan-Meier survival analyzed the relation between miR-126-5p or miR-34c-3p in serum EVs and prognosis; H: ROC curve analyzed the diagnostic efficacy of miR-126-5p/miR-34c-3p in serum EVs. All experiments were done in triplicate; data in panels A-E were tested by independent sample t test; data in panel G were compared by log rank test. * $p < 0.05$, ** $p < 0.01$.

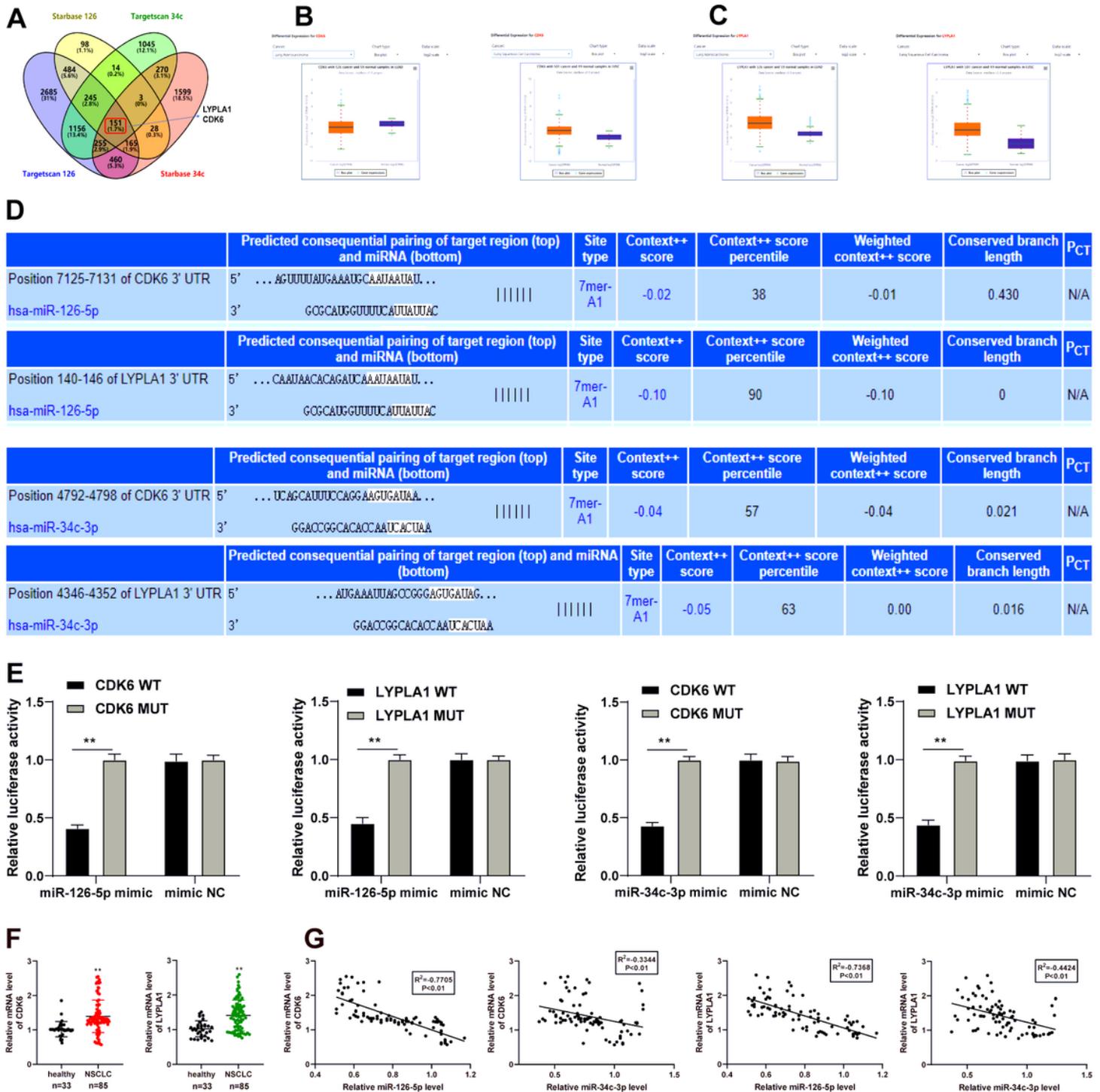


Figure 4

CDK6 and LYPLA1 are downstream targets of miR-126-5p and miR34c-3p. A: Targetscan and Starbase database predicted the downstream targets of miR-126-5p/miR-34c-3p, and the intersection was obtained; B-C: Starbase database predicted the expression of CDK6 and LYPLA1 in NSCLC patients; D: binding sites; E: dual-luciferase assay; F: RT-qPCR tested CDK6 and LYPLA1 expression in serum; G: Pearson correlation analyzed the relation between miR-126-5p/miR-34c-3p and CDK6/LYPLA1. All

experiments were done in triplicate; data in panel E were expressed as mean \pm SD and compared using Two-way ANOVA and Sidak's multiple comparisons test; data in panel F was processed using independent sample t test. ** $p < 0.01$. 126: miR-126-5p; 34c: miR-34c-3p.

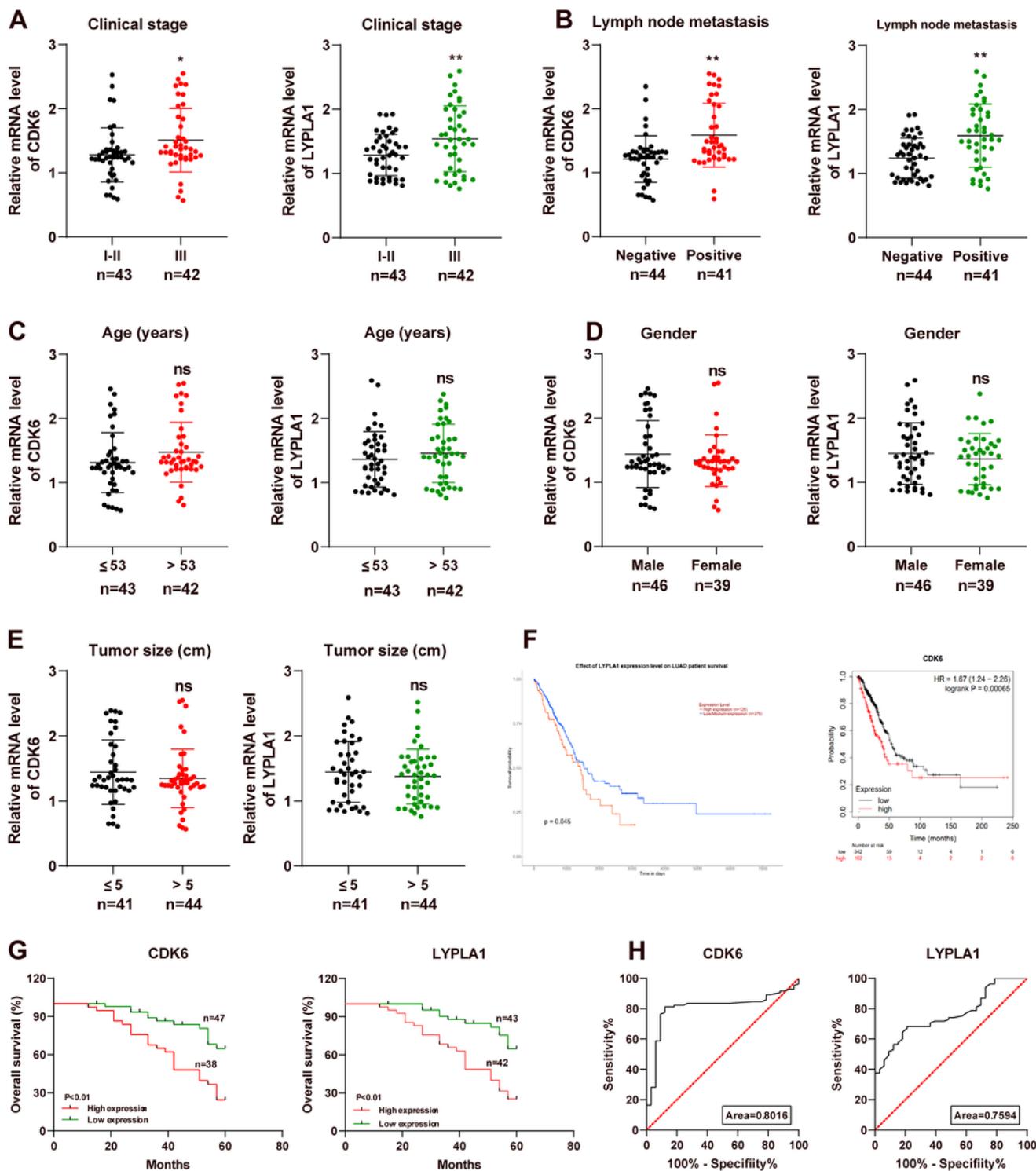


Figure 5

CDK6 and LYPLA1 in serum are associated with poor prognosis of NSCLC patients. A-E: 85 NSCLC patients were allocated into two groups according to their pathological characteristics, and CDK6 and

LYPLA1 expression were tested by RT-qPCR; F: TCGA database and Kaplan-Meier predicted the relation of CDK6/LYPLA1 and NSCLC patient survival; G: Kaplan-Meier analyzed the relation between CDK6/LYPLA1 and the prognosis; H: ROC curve analyzed the diagnostic efficacy of CDK6/LYPLA1 expression in serum of NSCLC patients. All experiments were done in triplicate; data in panels A-E were tested by independent sample t test; data in panel G were compared by log rank test. *p < 0.05, **p < 0.01.