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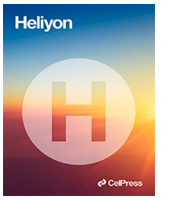

Combinations of plasma cfDNA concentration, integrity and tumor markers are promising biomarkers for early diagnosis of non-small cell lung cancer

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ABSTRACT

Background: Circulating cell-free DNA (cfDNA) concentration and integrity as noninvasive biomarkers play an important role in cancer diagnosis, prognosis and therapy monitoring. However, few studies have been conducted on the combination of plasma cfDNA concentration, integrity and tumor markers (CEA, CA125, NSE and CYFRA21-1) for cancer detection. Thus, the purpose of this study was to investigate the diagnostic value of combining plasma cfDNA concentration, integrity and tumor markers in early detection of non-small cell lung cancer (NSCLC).

Methods: Plasma cfDNA concentration from 50 healthy controls and 84 NSCLC patients were assessed by quantitative real-time PCR of ALU repeated sequence. Plasma cfDNA integrity was calculated as the ratio of long to short fragments (ALU115/60).

Results: Plasma cfDNA concentration (ALU60 and ALU115) and integrity ALU115/60 were significantly higher in NSCLC patients with stage III/IV than in healthy controls ($p = 0.0002$, $p < 0.0001$, and $p = 0.0093$, respectively). The receiver operating characteristic (ROC) curve for discriminating NSCLC patients from healthy controls had an area under the curve (AUC) of 0.936 (95 % CI, 0.939–0.996). Moreover, the combination of plasma cfDNA concentration, integrity and tumor markers (CEA, CA125, NSE and CYFRA21-1) had higher diagnostic performance than either plasma cfDNA concentration alone, integrity alone or tumor markers alone, with sensitivity, specificity and AUC value of 94.05%, 90.00% and 0.968, respectively. These results demonstrated that the combination of plasma cfDNA concentration, integrity and tumor markers could significantly improve the diagnostic accuracy of NSCLC.

Conclusion: Combination of plasma cfDNA concentration, integrity and tumor markers is a promising biomarker for early diagnosis of NSCLC.

1. Introduction

Cancer remains the leading cause of death in countries around the world and a significant barrier to increasing life expectancy [1].

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According to the latest report of Global Cancer Statistics 2020, it is estimated that 19.3 million new cases and 10.0 million cancer deaths were occurred in 2020 year [2]. Most conspicuously, lung cancer incidence and mortality rates second (11.4 %) and first (18 %) among all cancers, representing approximately 2.2 million new cases and 1.8 million deaths [2]. Lung cancer is made up of two main histologically heterogeneous subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which is the most prevalent pathological type of lung cancer, accounting for approximately 85 % of all lung cancers cases [3]. The majority of NSCLC patients are at an incurable stage (stage III/IV) at the time of diagnosis and 5-year overall survival rate was less than 5% [4]. The key to successful treatment of NSCLC is early diagnosis; however, the existing methods of chest radiographs, computed tomography (CT) scans and bronchoscopy were inefficient in diagnosing early patients with NSCLC [5–8]. In addition, conventional tumor markers, such as carcinoembryonic antigen (CEA) [9], carbohydrate antigen 125 (CA125) [10], cytokeratin 19 fragments (CYFRA21-1) [11] and neuron specific enolase (NSE) [12] had low sensitivity or specificity in diagnosing early NSCLC. Therefore, a new screening tool with high sensitivity and specificity is urgently needed.

Recently, circulating cell-free DNA (cfDNA) in blood is a proposed diagnostic biomarker for various malignancies [13–15]. Plasma/serum cfDNA is a double-stranded DNA fragment released into the bloodstream from tumor cells and/or normal cells, which provide a noninvasive diagnostic avenue for cancer patients [16]. The available evidence suggested that cfDNA released from necrotic tumor cells varies in size, whereas cfDNA released from apoptotic cells is uniformly truncated into 185 - 200bp fragments. Because the main source of cfDNA in healthy individuals is apoptotic cells, whereas the main source of cfDNA in cancer patients is necrotic tumors cells, therefore, quantification of cfDNA fragments could be a promising biomarker for malignancies detection [17,18]. Numerous studies have demonstrated that the concentration of cfDNA was higher in various types of cancers such as breast cancer [19], colorectal cancer [20], ovarian cancer [21], gastric cancer [22], testicular germ cell cancer [23], and leukemia [24] than in healthy individuals. In addition, cfDNA integrity, calculated as the ratio of long to short cfDNA fragments, was also prominently higher in patients with various cancers than in healthy individuals and was closely associated with the progression and prognosis in several types of cancers [25,26]. These results demonstrated that plasma/serum cfDNA concentration and integrity is a promising noninvasive biomarker for early cancer diagnosis.

Currently, the application of cfDNA in the detection and monitoring of NSCLC has been widely reported, mainly focusing on gene mutation detection [27,28], DNA methylation detection [29,30] and cfDNA concentration and integrity detection [31,32]. Although plasma/serum cfDNA concentration and integrity play an important role in the diagnosis and prognosis of NSCLC, however, little is known about the diagnostic values of combined plasma cfDNA concentration, integrity and tumor markers (CEA, CA125, NSE and CYFRA21-1) for NSCLC detection. To investigate the diagnostic values of combined plasma cfDNA concentration, integrity and tumor markers for NSCLC, the sensitivity, specificity and AUC values were evaluated. The concentration and integrity of plasma cfDNA in 84 NSCLC patients and 50 healthy individuals were measured by quantitative real-time PCR of ALU repeated sequence (ALU-qPCR) with two sets of primers (60 and 115bp) amplifying different lengths of cfDNA fragments. Because ALU repeated sequence is short interspersed element, typically 300bp, which is the most abundant sequence in the human genome, accounting for more than 10 % of the human genome [33–36]. In addition, the cfDNA fragments normally at a low concentration, thus qPCR of ALU repeated sequence can significantly improve the sensitivity and accuracy of cfDNA detection.

Our findings revealed that plasma cfDNA concentration and integrity were significantly higher in NSCLC patients than in healthy controls, indicating that plasma cfDNA concentration and integrity is a promising biomarker for differentiating NSCLC patients from healthy controls. Furthermore, combined plasma cfDNA concentration, integrity and tumor markers had higher diagnostic value than either plasma cfDNA concentration alone, integrity alone, or tumor markers alone. These preliminary findings highlight the important clinical value of combined determination of plasma cfDNA concentration, integrity and tumor markers and provide a new strategy for early diagnosis of NSCLC.

2. Materials and methods

2.1. Clinical and pathologic characteristics of NSCLC patients

Fifty healthy individuals and 84 NSCLC patients, including 49 males and 35 females were recruited from the People's Hospital of Chongqing Liang Jiang New Area. NSCLC patients were selected by the database coordinator based on those patients treated between 2020 and 2022 year. Patient inclusion criteria: none had received anti-tumor treatment such as surgery, radiotherapy or chemotherapy before blood collection, and all patients were confirmed by histopathology. Exclusion criteria: all patients were excluded from 4 infectious diseases (including hepatitis B, hepatitis C, HIV, syphilis) and serious organ damage diseases. Healthy individuals' inclusion criteria: the physical examination results (including blood routine, urine routine, liver function, kidney function and other routine items) are normal. Among 84 NSCLC patients, stage I ($n = 27$), stage II ($n = 13$), stage III ($n = 18$), and stage IV ($n = 26$) according to Union for International Cancer Control (UICC) criteria. This study was approved by the Medical Ethics Committee of the People's Hospital of Chongqing Liang Jiang New Area and was conducted in line with the relevant guidelines and regulations. All subjects provided written informed consent before enrollment.

2.2. Plasma samples collection and cfDNA extraction

Plasma samples from 50 healthy individuals and 84 NSCLC patients were assessed. Two mL of antecubital peripheral venous blood was collected into EDTA-containing tubes, stored at 4 °C, and processed within 6 h. All blood samples were centrifugation at 1000*g* for 10 min and then supercentrifugation at 15000g for 10 min to remove potentially contaminating cells. One mL of plasma was

cryopreserved at −80 °C until use. Then, cfDNA was extracted from 1 mL of plasma by using a QIAamp circulating nucleic acid kit (Qiagen, Germany) according to the manufacturer's instructions. The extracted plasma cfDNA was stored at −80 °C until further use.

2.3. Measurement of plasma cfDNA concentration and integrity

The concentration of plasma cfDNA fragments was quantified by quantitative real-time PCR. The target amplicon in this study was a consensus human *ALU* repeated sequence. We designed two sets of primers for amplifying different lengths of *ALU* fragments, namely, 60bp amplicon (ALU60) and 115bp amplicon (ALU115). Probe and primer sequences were obtained from our previous study [37]. The primer sets for the 60bp amplicon (ALU60) amplifies both shorter and longer DNA fragments, whereas the primer sets for the 115bp amplicon (ALU115) amplifies only longer DNA fragments. Therefore, ALU60-qPCR results represent the total amount of plasma cfDNA, ALU115-qPCR results represent the amounts of cfDNA released from tumor cells. Plasma cfDNA integrity was calculated as the ratio of qPCR results (ALU115-qPCR/ALU60-qPCR).

The reaction mix included 2 μL cfDNA template, 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), 1 μL probe (8 μM), 10 μL qPCR SuperMix-UDG (Invitrogen, USA) and 5 μL RNase-free H_2O in a total volume of 20 μL. The following conditions were used for the qPCR reaction in a CFX96 Real-Time fluorescence quantitative PCR instrument (Bio-Rad, USA): DNA polymerase was activated at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 60 s. A standard curve was established using genomic DNA (100 ng-10 pg) from healthy controls to quantify the concentration of ALU fragments (ALU60, ALU115) in plasma cfDNA according to our previous study [37]. In addition, a negative control has been included in each of the reaction plates. The mean values were calculated on the basis of duplicate reactions.

2.4. Measurement of plasma tumor markers (CEA, CA125, NSE and CYFRA21-1) levels

The levels of CEA, CA125, NSE and CYFRA21-1 were measured by chemiluminescent immunoassay. The reference range of CEA, CA125, NSE and CYFRA21-1 were 0–5 ng/mL, 0–35 U/mL, 0–15.2 ng/mL, and 0–3.3 ng/mL, respectively.

2.5. Combined diagnostic values of plasma cfDNA concentration, integrity and tumor markers

To assess the diagnostic values of combining plasma cfDNA concentration, integrity and tumor markers for NSCLC, the sensitivity, specificity and AUC values were evaluated. A schematic presentation of the technical approach in this study was provided in Supplementary Materials Fig. S1.

2.6. Statistical analysis

The Mann Whitney test (non-parametric test) was used to compare plasma cfDNA concentration and integrity between NSCLC patients group and healthy controls group. The One-way ANOVA test (Kruskal-Wallis test) was used to compare NSCLC patients within

Table 1
Clinicopathologic characteristics in patients with NSCLC and healthy controls.

Clinical characteristics	NSCLC patients	Healthy controls
No. pts	84	50
Age		
Mean	60.38 ± 9.61	48.18 ± 8.89
Sex		
Male	49 (58.33 %)	21 (42.00 %)
Female	35 (41.67 %)	29 (58.00 %)
UICC primary tumor		
T1	27 (32.14 %)	
T2	25 (29.76 %)	
T3	13 (15.48 %)	
T4	19 (22.62 %)	
UICC regional lymph nodes		
N0	41 (48.81 %)	
N1	10 (11.90 %)	
N2	17 (20.24 %)	
N3	16 (19.05 %)	
UICC distant metastasis		
M0	58 (69.05 %)	
M1	26 (30.95 %)	
UICC stage		
I	27 (32.14 %)	
II	13 (15.48 %)	
III	18 (21.43 %)	
IV	26 (30.95 %)	

Abbreviations: NSCLC, non-small cell lung cancer; UICC, Union for International Cancer Control.

each stage (stage I/II, stage III/IV) and healthy controls. The receiver operating characteristic (ROC) curve analysis was used to assess the clinical diagnosis value of plasma cfDNA in discriminating NSCLC patients from healthy controls. All statistical analysis was carried out using the SPSS software (version 22.0) and the figures were generated using the GraphPad Prism 7.0 software. Results were considered statistically significance if $p < 0.05$ (two-tailed).

3. Results

3.1. Clinical and pathologic characteristics of NSCLC patients

There were 84 patients with NSCLC and 50 healthy controls in this research. The mean age was 48.18 ± 8.89 (standard deviation, SD) years for healthy controls and 60.38 ± 9.61 years for patients with NSCLC. Among 84 patients with NSCLC, 27, 13, 18 and 26 were belong to stage I, stage II, stage III and stage IV, respectively, according to Tumor Node Metastasis (TNM) classification. In addition, 43 patients (51.19 %) had regional lymph node metastases (LNM) and 26 patients (30.95 %) had distant metastasis. Table 1 presents a detailed list of histopathological characteristics of NSCLC patients. Furthermore, age and gender parameters have no effect on plasma cfDNA concentration and integrity in NSCLC patients group and healthy controls group (Table 2). What's more, the mean values of tumor markers (CEA, CA125, NSE and CYFRA21-1) were significantly higher in NSCLC patients than in healthy controls (Supplementary Table S1).

3.2. Comparison of plasma cfDNA concentration in NSCLC patients and healthy controls

To assess the concentration of plasma cfDNA in NSCLC patients and healthy controls, plasma samples from these two groups were collected preoperatively and amplified two different lengths of cfDNA fragment by quantitative real-time PCR of ALU repeated sequence (ALU - qPCR). The mean concentration of ALU60 fragment in healthy controls and NSCLC patients with stage I/II, and stage III/IV were 43.03, 53.75, and 75.74 ng/mL, respectively (Table 3). The mean value of ALU60 fragment was significantly higher in patients with stage III/IV than in healthy controls ($p = 0.0002$, Fig. 1a). However, there was no significantly difference between patients with stage I/II and stage III/IV ($p = 0.1137$). Similarly, the mean concentration of ALU115 fragment in patients with stage I/II and stage III/IV were 87.16 and 122.80 ng/mL, which were significantly higher than in healthy controls ($p = 0.0015$ and $p < 0.0001$, Table 3; Fig. 1b). In addition, the mean value of ALU115 fragment was significantly higher in patients with stage III/IV than in stage I/II NSCLC ($p = 0.0006$, Fig. 1b).

The diagnostic value of ALU fragments for differentiating NSCLC patients from healthy controls was assessed by ROC curves. The area under curves (AUCs) for differentiating NSCLC patients with stage I/II, stage III/IV from healthy controls by two ALU fragments concentration were 0.608 (95 % CI: 0.487–0.730), 0.732 (95 % CI: 0.630–0.835), and 0.740 (95 % CI: 0.638–0.841), 0.927(95 % CI: 0.871–0.983), respectively (Fig. 1c and d; Table 4). These results suggested that quantification of plasma cfDNA fragments ALU60 and ALU115 could be used as potential biomarkers for discriminating NSCLC patients from healthy controls.

3.3. Comparison of plasma cfDNA integrity in NSCLC patients and healthy controls

The plasma cfDNA integrity, which represents the ratio of longer cfDNA fragments to total plasma cfDNA fragments, was calculated as the ratio of ALU115/60 of each sample. The mean plasma cfDNA integrity in healthy controls and in NSCLC patients with stage I/II, and stage III/IV were 1.43, 1.75 and 1.91, respectively (Table 3). The mean value of plasma cfDNA integrity was significantly higher in patients with stage III/IV than in healthy controls ($p = 0.0093$, Fig. 2a). A trend of elevation in patients with stage III/IV was observed although there was no difference between patients with stage I/II and stage III/IV (Fig. 2a, $p > 0.9999$).

Furthermore, the AUCs of the ROC curve for differentiating NSCLC patients with stage I/II, stage III/IV from healthy controls by plasma cfDNA integrity were 0.638 (95 % CI: 0.523–0.753) and 0.678 (95 % CI: 0.569–0.787), respectively (Fig. 2b; Table 4). These

Table 2
Correlation between demographic characteristics and plasma cfDNA concentration or integrity in NSCLC patients and healthy control.

	N	*ALU*60 (ng/mL)	*p*	*ALU*115 (ng/mL)	*p*	*ALU*115/60	*p*
Healthy controls							
Age (years)							
35-60	45	43.14	0.8615	59.06	0.4777	1.42	0.7320
61-85	5	41.97		52.40		1.494	
Sex							
Male	21	47.24	0.0695	60.92	0.4453	1.339	0.2406
Female	29	39.98		56.57		1.492	
NSCLC patients							
Age (years)							
35-60	44	59.08	0.3906	100.10	0.6835	1.889	0.3454
61-85	40	72.08		112.10		1.776	
Sex							
Male	49	70.88	0.5026	113.20	0.1355	1.897	0.7538
Female	45	57.41		95.52		1.749	

Table 3
Concentration and integrity of plasma cfDNA in NSCLC patients and healthy controls.

	Controls	Stage I/II	Stage III/IV	Stage I/IV
No. pts	50	40	44	84
*ALU*60 (ng/mL)				
Mean	43.03	53.75	75.74	65.27
95 % CI	39.05–47.00	45.47–62.03	60.15–91.33	56.07–74.48
Range	19.63–76.04	21.23–120.20	26.10–274.40	21.23–274.40
Median	44.43	46.92	57.36	53.93
P value		0.2684	0.0002	0.0007
*ALU*115 (ng/mL)				
Mean	58.40	87.16	122.80	105.80
95 % CI	52.81–63.98	74.44–99.88	107.30–138.30	95.17–116.50
Range	21.53–102.30	43.33–197.70	40.47–305.50	40.47–305.50
Median	60.42	74.64	111.00	94.61
P value		0.0015	<0.0001	<0.0001
*ALU*115/60				
Mean	1.43	1.75	1.91	1.84
95 % CI	1.30–1.56	1.56–1.94	1.68–2.14	1.69–1.98
Range	0.54–2.42	1.00–3.15	0.83–3.58	0.83–3.58
Median	1.40	1.51	1.77	1.53
P value		0.0712	0.0093	0.0020

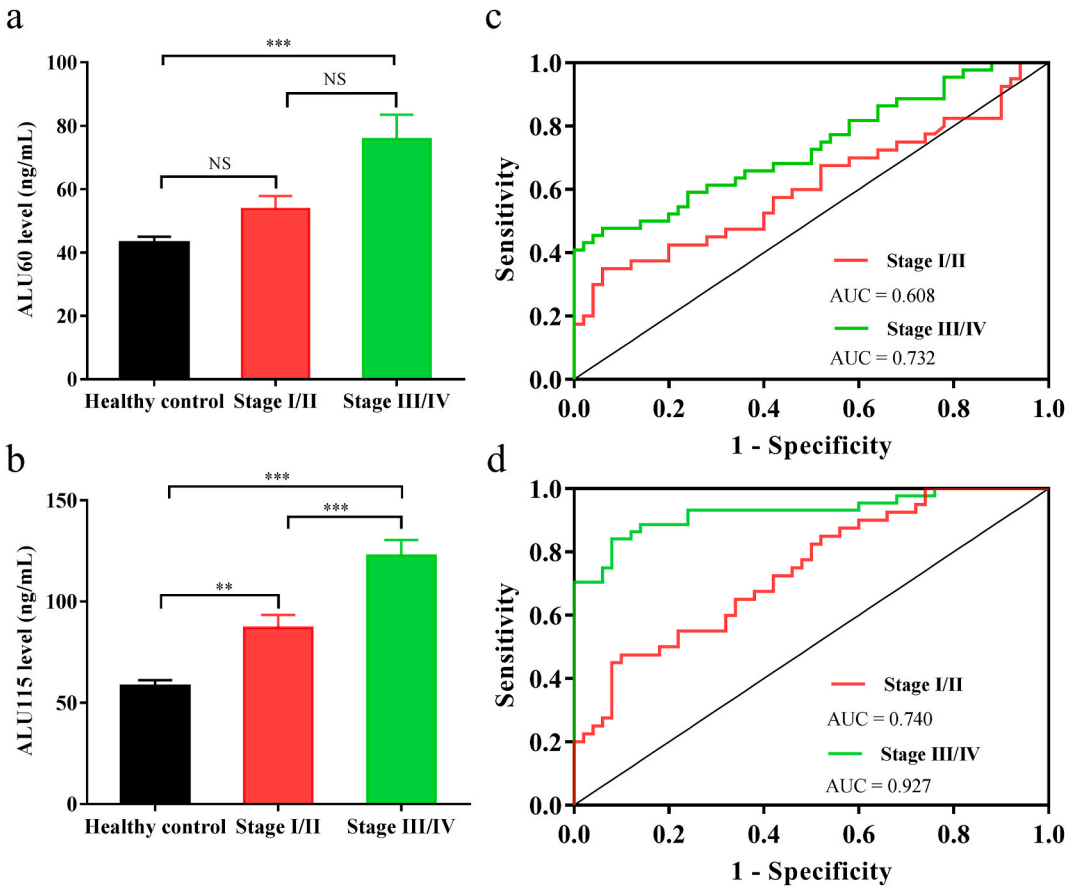

Fig. 1. Comparison of plasma cfDNA concentrations in NSCLC patients and healthy controls. (**a**) The mean concentration of ALU60 fragment was significantly higher in NSCLC patients with stage III/IV than in healthy controls. (**b**) The mean concentration of ALU115 fragment was significantly higher in NSCLC patients with stage I/II and stage III/IV than in healthy controls. Moreover, the mean concentration of ALU115 fragment was also significantly higher in patients with stage III/IV than stage I/II. (**c - d**) Receiver operating characteristic curves for distinguishing NSCLC patients from healthy controls. **P < 0.01, ***P < 0.001, NS: no significant difference.

Table 4
Diagnostic values of plasma cfDNA concentration and integrity in NSCLC patients.

	Cut off (ng/mL)	Sensitivity (%)	Specificity (%)	AUC	95 % CI
*ALU*60					
Stage I/II	61.05	35.00	94.00	0.608	0.487–0.730
Stage III/IV	62.24	47.73	94.00	0.732	0.630–0.835
Stage I/IV	61.05	41.67	94.00	0.673	0.584–0.762
*ALU*115					
Stage I/II	78.88	47.50	90.00	0.740	0.638–0.841
Stage III/IV	84.75	84.09	92.00	0.927	0.871–0.983
Stage I/IV	81.39	65.48	92.00	0.838	0.773–0.903
*ALU*115/60					
Stage I/II	1.26	87.50	40.00	0.638	0.523–0.753
Stage III/IV	1.74	52.27	80.00	0.678	0.569–0.787
Stage I/IV	1.92	38.10	88.00	0.659	0.566–0.752

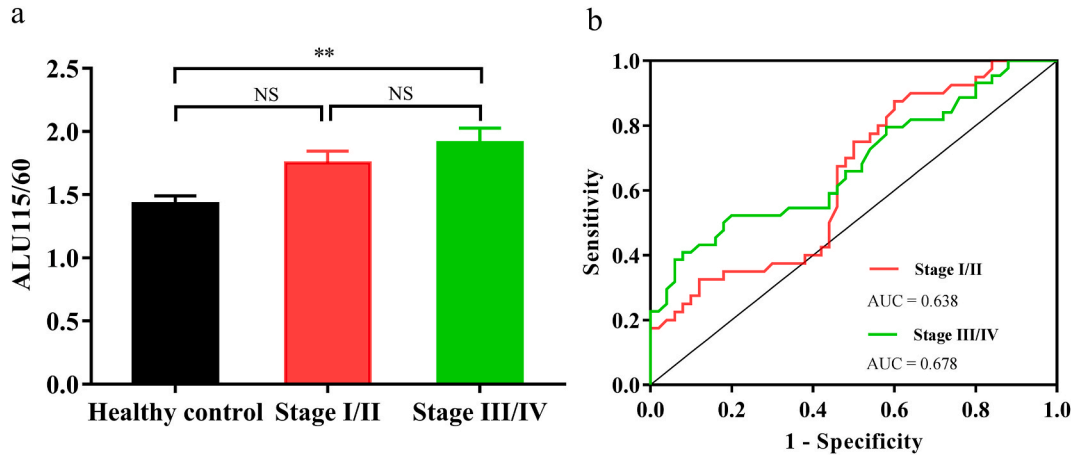

Fig. 2. Comparison of plasma cfDNA integrity in NSCLC patients and healthy controls. (**a**) The mean plasma cfDNA integrity ALU115/60 was significantly higher in patients with stage III/IV than in healthy controls. (**b**) Receiver operating characteristic curves for distinguishing NSCLC patients from healthy controls. **P < 0.01, NS: no significant difference.

results indicated that plasma cfDNA integrity ALU115/60 might be a candidate biomarker for distinguishing NSCLC patients from healthy controls.

3.4. Correlation analysis between plasma cfDNA and lymph node metastasis

The degree of lymph node metastasis (LNM) is one of the most important factors for the staging and prognosis of patients with NSCLC. To explore the correlation between plasma cfDNA and lymph node metastasis in patients with NSCLC, we compared the concentration and integrity of plasma cfDNA in LNM-positive and LNM-negative patients. The mean concentrations of ALU60 fragment in 47 LNM-positive and 37 LNM-negative patients were 76.18 ng/mL and 51.41 ng/mL. In addition, the mean concentrations of ALU115 fragment in 47 LNM-positive and 37 LNM-negative patients were 127.00 ng/mL and 78.95 ng/mL. These mean values were significantly higher in LNM-positive patients than in LNM-negative patients ($P = 0.0133$ and $P < 0.0001$, respectively; Fig. 3a and b). The AUCs of ROC curve for differentiating LNM-positive patients from LNM-negative patients by ALU60, ALU115 fragment concentration were 0.657 (95 % CI: 0.541–0.773, Figs. 3d) and 0.816 (95 % CI: 0.725–0.906, Fig. 3e).

What's more, the mean values of plasma cfDNA integrity in 47 LNM-positive patients and 37 LNM-negative patients were 1.95 and 1.69. However, there was no significantly difference between LNM-positive patients and LNM-negative patients ($p = 0.2225$, Fig. 3c). The AUC of ROC curve for distinguishing LNM-positive patients from LNM-negative patients by plasma cfDNA integrity was 0.578 (95 % CI: 0.456–0.700, Fig. 3f). These results showed that quantification of plasma cfDNA concentration is more useful than plasma cfDNA integrity in predicting lymph node metastasis in NSCLC patients.

3.5. Correlation analysis between plasma cfDNA and EGFR mutation

Epidermal growth factor receptor (*EGFR*) gene mutation was common in NSCLC patients, to investigate the correlation between *EGFR* mutation and plasma cfDNA in NSCLC patients, the concentration and integrity of plasma cfDNA in patients with *EGFR* mutation and *EGFR* wild-type were assessed. The mean concentrations of plasma cfDNA ALU60 fragment in 20 patients with *EGFR* wild-type and

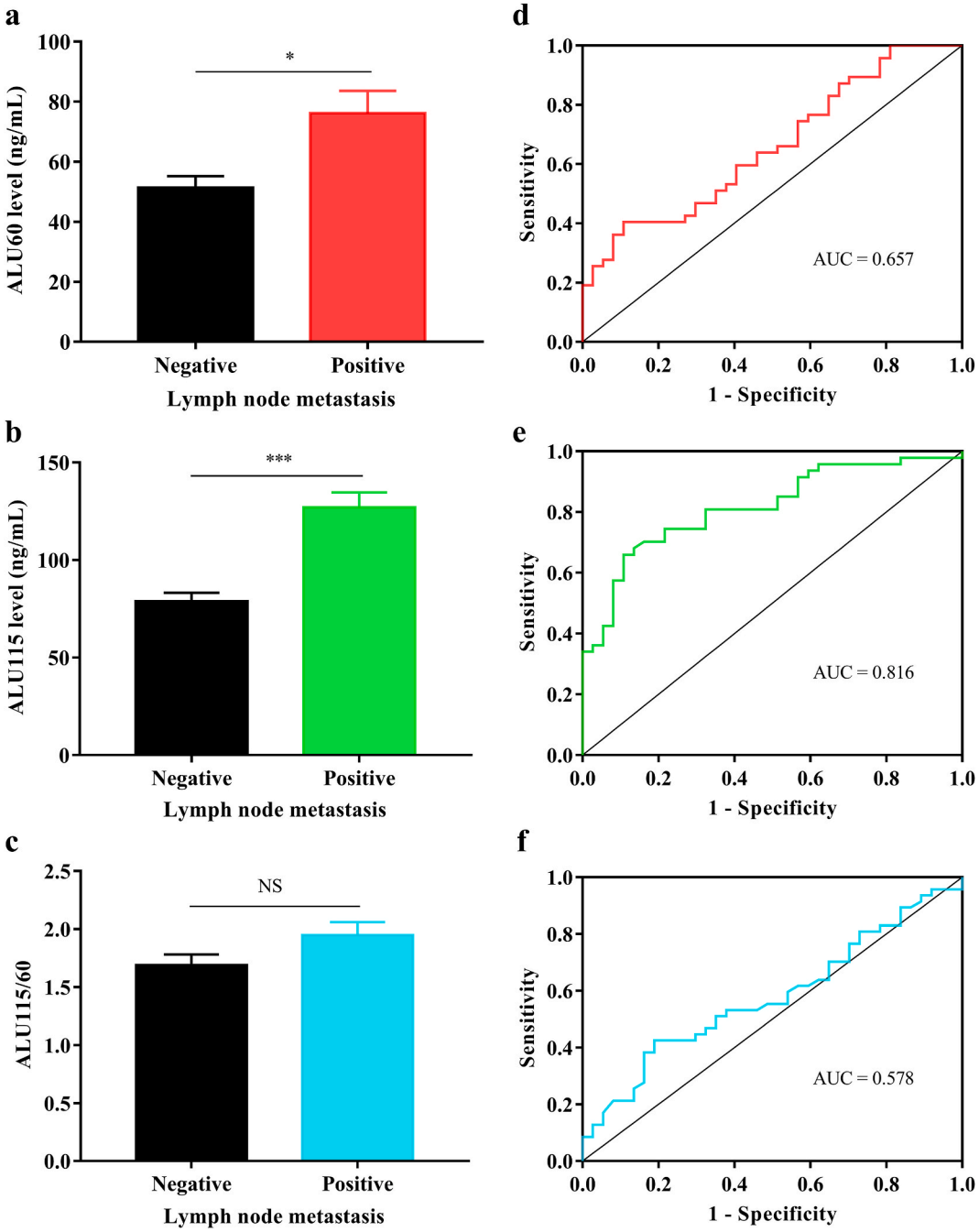

Fig. 3. Correlation analyses between plasma cfDNA and lymph node metastasis. (**a**) The mean concentration of ALU60 fragment was significant higher in LNM-positive patients than LNM-negative patients. (**b**) The mean concentration of ALU115 fragment was significant higher in LNM-positive patients than LNM-negative patients. (**c**) There was no difference in plasma cfDNA integrity between LNM-positive patients and LNM-negative patients. (**d - f**) Receiver operating characteristic curves for differentiating LNM-positive from LNM-negative patients. *P < 0.05, ***P < 0.001, NS: no significant difference.

9 patients with *EGFR* mutation were 67.85 ng/mL and 52.53 ng/mL, and the corresponding values of ALU115 fragment were 105.5 ng/mL and 81.68 ng/mL. However, there was no significant difference between *EGFR* mutation and *EGFR* wild-type patients ($p = 0.6601$ and $p = 0.1300$, Supplementary Materials Fig. S2). Moreover, the mean values of plasma cfDNA integrity in *EGFR* mutation and *EGFR* wild-type patients were 1.65 and 1.76. Nevertheless, there was also no significantly difference between *EGFR* mutation and *EGFR* wild-type patients ($p = 0.9172$, Supplementary Materials Fig. S2).

The AUCs of ROC curve for discriminating *EGFR* mutation and *EGFR* wild-type patients by ALU60, ALU115 fragment concentration

and plasma cfDNA integrity were 0.556 (95 % CI: 0.342–0.769), 0.606 (95 % CI: 0.398–0.813) and 0.514 (95 % CI: 0.287–0.741), respectively. These results indicated that *EGFR* mutation may not correlate with plasma cfDNA concentration and integrity in NSCLC patients.

3.6. Comparison of diagnostic performance of plasma cfDNA and tumor-related markers

To compare the diagnostic performance of plasma cfDNA and tumor-related markers (CEA, CA125, NSE and CYFRA21-1) in differentiating NSCLC patients from healthy individuals, the sensitivity, specificity and AUC values were evaluated. The sensitivity, specificity and AUC values of the combination of ALU60 + ALU115, ALU115 + ALU115/60 for NSCLC patients were 85.71%, 90.00% and 0.914; 70.24%, 98.00% and 0.864, respectively (Table 5). Particularly, the combination of ALU60 + ALU115 + ALU115/60 had optimum sensitivity, specificity and AUC values, with 90.48%, 92.00% and 0.936, respectively (Fig. 4a, Table 5). These results demonstrated that the combination of plasma cfDNA concentration and integrity had higher diagnostic performance than either plasma cfDNA concentration alone or integrity alone.

Similarly, the sensitivity, specificity and AUC values of CEA, CA125, NSE and CYFRA21-1 for NSCLC patients were 83.33%, 58.00% and 0.785; 52.38%, 88.00% and 0.752; 69.05%, 84.00% and 0.852; 77.38%, 88.00% and 0.888, respectively (Table 5). Notably, the combination of CA125 + CYFRA21-1+NSE had the optimum sensitivity, specificity and AUC values, with 78.57%, 94.00% and 0.918, respectively (Fig. 4b, Table 5). Furthermore, we also observed that the combination of ALU60 + ALU115 + ALU115/60 had a higher AUC value than these four tumor markers and their combinations (Table 5). These results indicated that the combination of plasma cfDNA concentration and integrity (ALU60 + ALU115 + ALU115/60) has better diagnostic performance than these tumor-related markers (CEA, CA125, NSE and CYFRA21-1) in the detection of NSCLC.

3.7. Diagnostic value of combined plasma cfDNA and tumor markers in NSCLC patients

To investigate the diagnostic value of combined plasma cfDNA concentration, integrity and tumor markers for early detection of NSCLC patients, the sensitivity, specificity and AUC values were analyzed. Except for the combination of ALU60 + ALU115 + ALU115/60 + CA125, the AUC values of other combinations were higher than the combination of ALU60 + ALU115 + ALU115/60 (Table 6). Moreover, the combination of ALU60 + ALU115 + ALU115/60 + CA125+CYFRA21-1+NSE had the maximum AUC value compared to other combinations (Fig. 5b, Table 6).

What's more, we found that the combinations of plasma cfDNA concentration, integrity and tumor markers (CEA, CA125, NSE and CYFRA21-1) had higher AUC values than either plasma cfDNA concentration alone, integrity alone or tumor markers alone (Fig. 5a). These results demonstrated that the combination of plasma cfDNA concentration, integrity and tumor markers could significantly improve the diagnostic accuracy for NSCLC.

Table 5
Diagnostic performances of plasma cfDNA and tumor markers in NSCLC patients.

Markers	Sensitivity (%)	Specificity (%)	AUC	*P*
*ALU*60	41.67	94.00	0.673	0.0007
*ALU*115	65.48	92.00	0.838	<0.0001
*ALU*115/60	38.10	88.00	0.659	0.0020
*ALU*60 + *ALU*115	85.71	90.00	0.914	<0.0001
*ALU*60 + *ALU*115/60	35.71	100.00	0.670	0.0011
*ALU*115 + *ALU*115/60	70.24	98.00	0.864	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60	90.48	92.00	0.936	<0.0001
CEA	83.33	58.00	0.785	<0.0001
CA125	52.38	88.00	0.752	<0.0001
CYFRA21-1	77.38	88.00	0.888	<0.0001
NSE	69.05	84.00	0.852	<0.0001
CEA + CA125	66.67	88.00	0.824	<0.0001
CEA + CYFRA21-1	83.33	92.00	0.906	<0.0001
CEA + NSE	82.14	82.00	0.892	<0.0001
CA125+CYFRA21-1	76.19	88.00	0.886	<0.0001
CA125 + NSE	76.19	82.00	0.853	<0.0001
CYFRA21-1+NSE	78.57	94.00	0.917	<0.0001
CEA + CA125+CYFRA21-1	82.14	92.00	0.903	<0.0001
CEA + CA125 + NSE	76.19	90.00	0.893	<0.0001
CEA + CYFRA21-1+NSE	78.57	94.00	0.915	<0.0001
CA125+CYFRA21-1+NSE	78.57	94.00	0.918	<0.0001
CEA + CA125+CYFRA21-1+NSE	78.57	94.00	0.917	<0.0001

Abbreviations: CEA: carcinoembryonic antigen; CA125: carbohydrate antigen 125; NSE: neuron specific enolase; CYFRA21-1: cytokeratin 19 fragments.

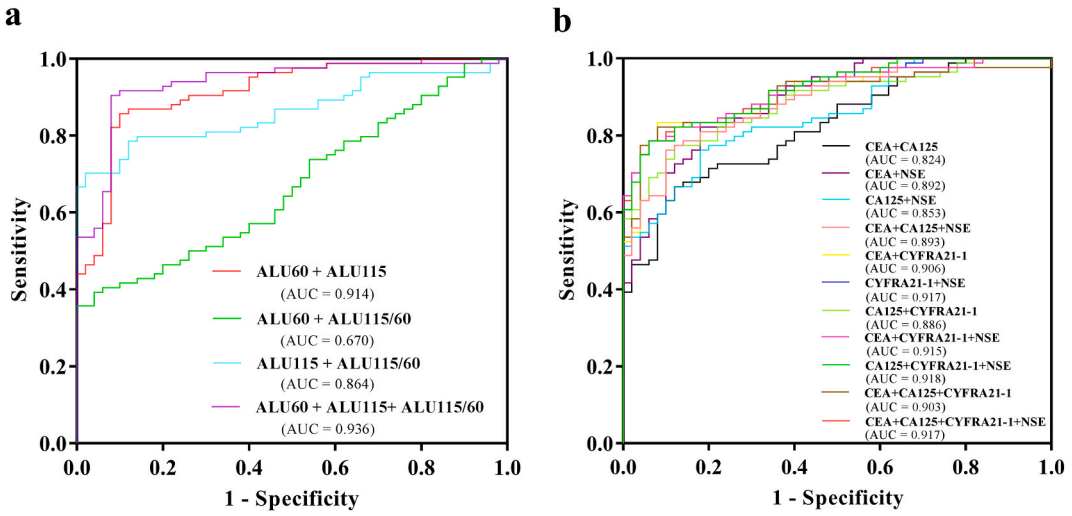

Fig. 4. Comparison of diagnostic performance of plasma cfDNA and tumor markers in NSCLC patients. (**a**) ROC curves of the combinations of ALU60, ALU115 and ALU115/60 for differentiating NSCLC patients from healthy controls. (**b**) ROC curves of the combinations of CEA, CA125, CYFRA21-1 and NSE for differentiating NSCLC patients from healthy controls.

Table 6
Diagnostic values of combined plasma cfDNA and tumor biomarkers for NSCLC patients.

Markers	Sensitivity	Specificity	AUC	*P*
*ALU*60 + *ALU*115+ *ALU*115/60+ CEA	90.48	92.00	0.943	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60+ CA125	94.05	86.00	0.936	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60+ CYFRA21-1	97.62	88.00	0.964	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60 + NSE	95.24	88.00	0.959	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60+ CEA + CA125	95.24	86.00	0.945	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60+ CEA + CYFRA21-1	92.86	92.00	0.966	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60+ CEA + NSE	90.48	90.00	0.959	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60+ CA125+ CYFRA21-1	95.24	90.00	0.965	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60+ CA125 + NSE	96.43	88.00	0.959	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60+ CYFRA21-1+NSE	92.86	90.00	0.967	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60+ CEA + CA125+ CYFRA21-1	92.86	92.00	0.966	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60+ CEA + CA125 + NSE	88.10	92.00	0.960	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60+ CEA + CYFRA21-1+NSE	92.86	90.00	0.967	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60+ CA125+ CYFRA21-1+NSE	94.05	90.00	0.968	<0.0001
*ALU*60 + *ALU*115+ *ALU*115/60+ CEA + CA125+ CYFRA21-1+ NSE	92.86	90.00	0.967	<0.0001

4. Discussion

Circulating cell-free DNA (cfDNA) is a promising novel biomarker for malignancies because it contains DNA fragments released from tumor cells [38]. Extensive studies have demonstrated that plasma/serum cfDNA concentration or integrity was higher in patients with various types of cancers such as colorectal cancer [20,26], breast cancer [19,25], hepatocellular carcinoma [39,40] and prostate cancer [41]. These results showed that plasma/serum cfDNA concentration and integrity could be a noninvasive biomarker, which provide important complementary information for diagnosis, prognosis and treatment monitoring in cancer patients.

In this study, ALU repeated sequence was selected as the target because it is the most abundant elements in the human genome, accounting for more than 10% of the human genome [34], with a copy number of 1.4×10^6 per genome [33], thus qPCR of ALU elements can significantly improve the sensitivity and accuracy of the cfDNA detection. Our results revealed that the concentration of plasma cfDNA (ALU60, ALU115) was significantly higher in NSCLC patients than in healthy controls, which was consistent with the previous studies [37]. ROC analysis suggested that ALU115 fragment had higher AUC value (AUC = 0.838) compared to ALU60 fragment for differentiating NSCLC patients from healthy controls. Besides, ALU115 fragment had higher sensitivity (65.48% vs. 41.67%) although ALU60 fragment had slightly higher specificity (94% vs. 92%). These results demonstrated that ALU115 fragment could be a better indicator for NSCLC diagnosis. Furthermore, plasma cfDNA integrity ALU115/60 was significantly higher in NSCLC patients than in healthy controls, which was also consistent with the previous studies [37]. However, the AUC value of plasma cfDNA integrity ALU115/60 was less than plasma cfDNA concentration in distinguishing NSCLC patients from healthy controls, suggesting that plasma cfDNA concentration (especially ALU115 fragment) is a better biomarker for early diagnosis of NSCLC.

In addition, our findings revealed that the concentration and integrity of plasma cfDNA were not associated with demographic

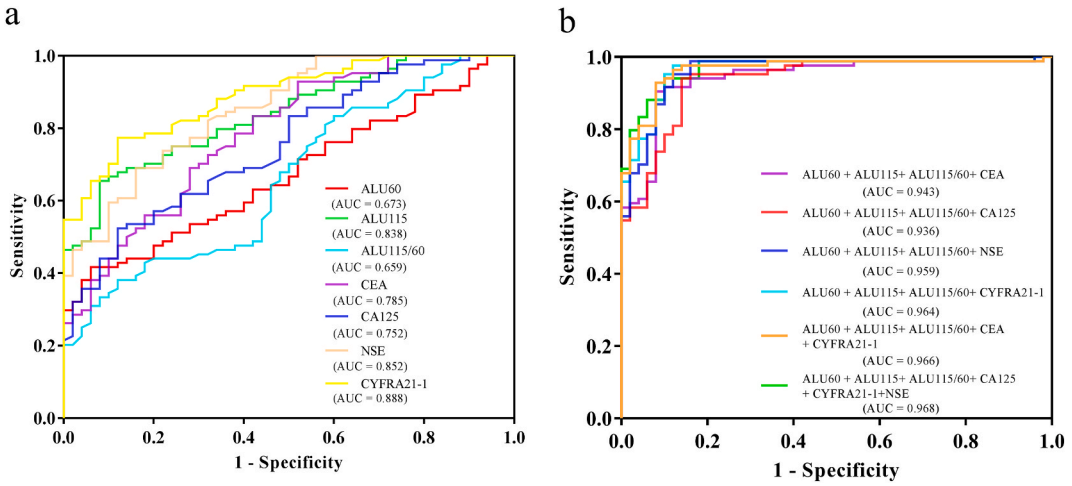

Fig. 5. Diagnostic values of combined plasma cfDNA concentration, integrity and tumor biomarkers for NSCLC patients. (**a**) ROC curves of ALU60, ALU115, ALU115/60, CEA, CA125, NSE and CYFRA21-1 for differentiating NSCLC patients from healthy controls. (**b**) ROC curves of the combinations of plasma cfDNA concentration, integrity and tumor markers for distinguishing NSCLC patients from healthy controls.

characteristics (age and gender) in healthy control group and NSCLC patients group. However, there was a significantly difference in age between NSCLC patients group and healthy control group ($p < 0.0001$). Although there was a significant difference in age between NSCLC patients and healthy controls, we hypothesized that this difference could be attributed to the presence of cancer rather than aging. This viewpoint is supported by numerous published articles.

What's more, our results also revealed that the combination of plasma cfDNA concentration and integrity had higher sensitivity and AUC values than either plasma cfDNA concentration alone or plasma cfDNA integrity alone. Particularly, the combination of ALU60 + ALU115 + ALU115/60 had the optimum sensitivity, specificity and AUC value. In addition, Zhou's study revealed that the combination of plasma cfDNA concentration and integrity had higher AUC value than plasma cfDNA concentration (0.90 vs. 0.86) or integrity (0.90 vs. 0.72) for differentiating ovarian cancer patients from healthy controls [42]. Srdjan's study revealed that the combination of plasma cfDNA concentration and cfDNA integrity had higher AUC value than plasma cfDNA concentration (0.84 vs. 0.81) or integrity (0.84 vs. 0.60) for differentiating ovarian cancer patients from healthy controls [43]. Our result was consistent with these previous studies. These results demonstrated that the combination of plasma cfDNA concentration and integrity could significantly improve the diagnostic accuracy of cancers. Meanwhile, the combination of ALU60 + ALU115 + ALU115/60 had higher sensitivity and AUC value than these tumor markers and combinations of these tumor markers, implying that the combination of plasma cfDNA concentration and integrity is superior to tumor-related markers in the diagnosis of NSCLC. What's more important, we are the first to investigate the diagnostic value of combining plasma cfDNA concentration, integrity and tumor markers (CEA, CA125, NSE and CYFRA21-1) in the detection of NSCLC. Our findings demonstrated that the combinations of plasma cfDNA concentration, integrity and tumor markers had higher AUC values than either plasma cfDNA concentration alone, integrity alone, or tumor markers alone, which was consistent with the Wei's findings [44]. Notably, the combination of plasma cfDNA concentration, integrity and tumor markers in our study had higher sensitivity, specificity and AUC values compared to Wei's study (94.05 % vs. 83.33 %, 90.00 % vs. 85.29 %, 0.968 vs. 0.915). These findings suggested that the combination of plasma cfDNA concentration, integrity and tumor markers could significantly improve the diagnostic accuracy of NSCLC.

Furthermore, we also analyzed the correlation between plasma cfDNA concentration, integrity and some clinicopathological characteristics such as lymph node metastasis (LNM) and *EGFR* mutation. Our findings revealed that plasma cfDNA concentration was significantly higher in LNM-positive patients than in LNM-negative patients. Particularly, the ALU115 fragment had higher sensitivity and AUC value compared to ALU60 fragment (65.96% vs. 40.43%, 0.816 vs. 0.657), implying that ALU115 fragment is superior in predicting lymph node metastasis in NSCLC patients. Noteworthy, plasma cfDNA concentration exhibited higher sensitivity, specificity and AUC value than plasma cfDNA integrity in predicting lymph node metastases. These findings indicated that plasma cfDNA concentration (ALU115 fragment) was more competitive than plasma cfDNA integrity in predicting lymph node metastasis in NSCLC patients. The result of this study was consistent with our previous findings [45]. However, there was no significantly difference in plasma cfDNA concentration and integrity between NSCLC patients with *EGFR* wild-type and *EGFR* mutation. We considered that the reasons for this result are as follows, one possibility is that the number of NSCLC patients with *EGFR* wild-type or *EGFR* mutation in this study is too small to accurately reflect the real result; another possibility is that the presence of individual differences. In addition, we also evaluated the performance of tumor markers (CEA, CA125, NSE, and CYFRA21-1) in distinguishing LNM-positive and LNM-negative patients, as well as between EGFR wild-type and EGFR mutant patients. We found no significant difference in distinguishing LNM-positive and LNM-negative patients by tumor markers (CEA, CA125, NSE and CYFRA21-1). Likewise, there was also no significant difference in distinguishing EGFR wild-type and EGFR mutant patients by tumor markers (CEA, CA125, NSE, and CYFRA21-1).

Although our results have important implications, there are several limitations to consider. Firstly, the sample size of NSCLC

patients and healthy individuals in this study is relatively small and may not accurately reflect the true results, thus larger cohort studies with NSCLC patients and healthy individuals are required in the future studies. Secondly, there was no patient with benign lung disease group in this study, which may not distinguish NSCLC patients from patients with benign lung disease; therefore, patients with benign lung disease group needs to be considered in the future studies. Thirdly, we only considered lymph nodes metastasis and *EGFR* mutation, and ignored the effects of smoking, obesity, hypertension, hyperlipidemia, diabetes and other variables on the results, thus, these factors need to be taken into account in future studies. In summary, our findings are expected to provide new perspectives for the early diagnosis of NSCLC.

5. Conclusions

Plasma cfDNA concentration and integrity were significantly higher in NSCLC patients than in healthy controls. Furthermore, the combination of plasma cfDNA concentration, integrity and tumor markers (CEA, CA125, NSE, and CYFRA21-1) could significantly improve the diagnostic accuracy of NSCLC.

Ethics statement

The research project was approved by the ethics committee of the People's Hospital of Chongqing Liang Jiang New Area (No. 20210428). We have reached an agreement with all participants. All procedures were carried out in accordance with the Helsinki Declaration and Chinese policies. We confirmed that informed consent was obtained from all patients for the publication of all their data and/or images.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Sai Ren: Conceptualization, Formal analysis, Funding acquisition, Methodology, Writing – original draft, Writing – review & editing. **Guichuan Zeng:** Conceptualization, Investigation, Software, Writing – original draft, Writing – review & editing. **Yuling Yi:** Formal analysis, Investigation, Methodology, Resources, Software, Validation. **Ling Liu:** Formal analysis, Investigation, Methodology, Resources, Software, Validation. **Hongmei Tu:** Investigation, Methodology, Resources, Software, Visualization. **Tingjia Chai:** Investigation, Methodology, Resources, Software, Validation, Visualization. **Liyi Hu:** Conceptualization, Data curation, Funding acquisition, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20851.

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