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Original Article

Circulating GRP78 acts as a biomarker in the early diagnosis of lung cancer

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Abstract: Glucose-regulated protein 78 (GRP78) is a major chaperone in endoplasmic reticulum (ER) and is increased in many types of malignant tumors. The role of GRP78 in early lung cancer diagnosis has not been clearly reported. The aim of this study is to detect the circulating level of GRP78 in the plasma of lung cancer patients and to evaluate the role of GRP78 in the early diagnosis of lung cancer. Plasma was collected from 251 lung cancer patients and 105 healthy controls, and the GRP78 expression in each sample was assayed using a commercially available ELISA kit. A receiver operating characteristic curve (ROC curve) was performed to analyze the role of GRP78 in lung cancer diagnosis. The combination of GRP78 and CEA, Cyfra21-1 was then analyzed using SPSS 17.0. The circulating level of GRP78 was increased dramatically in lung cancer patients ($P < 0.0001$) compared with the healthy controls. GRP78 provided a more sensitive and specific diagnosis than CEA in all lung cancer, ADC, and SCC patients, as well as in early (stage I) lung cancer patients. The results also indicated that a combination of GRP78, CEA and Cyfra21-1 could increase the accuracy of lung cancer diagnosis. GRP78 could be used as circulating biomarker in early lung cancer diagnosis.

Keywords: GRP78, lung cancer, biomarker, early diagnosis, combination analysis

Introduction

Lung cancer is the greatest cause of malignant cancer-related deaths worldwide [1]. The mortality was almost 60% for lung cancer patients diagnosed within 1 year and nearly 75% for those diagnosed within 2 years, with a 5-year survival rate less than 16% [2]. The survival rate can reach 70-90% when lung cancer is diagnosed and treated at the earlier stages, which indicates that early detection and treatment of lung cancer is a promising strategy to reduce lung cancer mortality [3]. Low-dose spiral computed tomography (LDCT) is the chief method applied in clinical testing to detect malignant single pulmonary nodules [4]. The largest clinical trial was performed by the National Cancer Institute of the United States which included adult participants by entry criteria: age 55-74, current or former smokers, 30 or more pack-years and still smoking or having done so within the past 15 years. The trial lasted for almost 7 years and the results indicated that LDCT could reduce mortality 20% compared with those who received a chest X ray [5]. However, CT screening generated both interest and controversy, and there is a continuing debate regarding the benefits and risks of lung cancer screening [6]. Over-diagnosis, the high cost, and radiation injuries were the major shortcomings of LDCT in clinical applications [7].

Diagnosis using circulating biomarkers has advantages over CT because it's easily accepted, convenient, noninvasive, and inexpensive. The various types of fluid biomarkers, such as circulating tumor cells (CTC), lipids, secreted proteins, microRNA and circulating tumor DNA, can reflect the existence of primary tumors and metastases [8]. The common biomarkers in clinical diagnosis are carcinoembryonic antigen (CEA) [9], carbohydrate antibody 19-9 (CA199) [10], carbohydrate antibody 12-5 (CA125) [11], chromogranin A [12], pro-gastrin-releasing peptide [13], cytokeratin 19 fragments (Cyfra21-1) [14], and neuron-specific enolase (NSE) [15]. But the limitations of these biomarkers include

Table 1. Clinical characteristics of lung cancer patients and healthy controls

	Lung cancer (251)	Healthy control (106)
Gender		
Male	150	52
Female	101	55
Age		
< 45	28	36
45-65	165	50
> 65	58	20
Subtypes		
ADC	168	
SCC	62	
Others	21	
Stages		
I	96	
II	38	
III	60	
IV	57	
Smoking history		
No	133	
Yes	118	
Differentiation		
Poor	44	
Mediate	52	
High	50	
Unknown	105	
Metastasis		
No	179	
Yes	72	

the lack of sensitivity and specificity, as well as a lack of studies about the role of these biomarkers in the early diagnosis of lung cancer.

Glucose-regulated protein 78 (GRP78) is an endoplasmic reticulum (ER) chaperone widely used as a marker for ER stress [16]. GRP78 secretions are also induced in various tumor cells, including renal cell carcinomas [17], gastric carcinoma [18], colorectal cancer [19], breast cancer [20], and multiple myeloma [21]. As a potential marker in lung cancer, the role of circulating GRP78 in the early diagnosis of lung cancer has not yet been clearly described.

Therefore, in this study, we measured circulating GRP78 levels and investigated its value for the early diagnosis of lung cancer. We also analyzed the diagnostic value of the combination of GRP78 and GEC and Cyfra21-1.

Materials and methods

Sample selection

The study was approved by the medical ethics committee of the Ninth People's Hospital of Chongqing. Lung cancer patients who were diagnosed in the hospital's Thoracic Surgery Department between March 2016 and February 2017 were enrolled in this study. The diagnosis and pathological staging was performed according to the TNM classification of the American Joint Committee for Cancer Staging guidelines, 7th edition [22]. Healthy volunteers were from the physical examination center, and they were not diagnosed with any malignant tumors following a routine physical examination, including CT, routine bloodwork, and assays of tumor markers. The anti-coagulant blood samples were collected from the 251 lung cancer patients and 106 healthy controls. All blood samples were processed within 1 hour according to the following procedure: centrifuge to isolate plasma (1200 g, 20 min, 4°C), second centrifuge to remove remaining blood cells (3000 g, 20 min, 4°C), and the plasma was then transferred to 1.5 ml sterile Eppendorf tubes and frozen at -80°C.

ELISA for GRP78 detection

The enzyme-linked immunosorbent assay (ELISA) kit (ADI-900-214, Enzo Life Sciences) was used to measure the plasma GRP78 levels. All plasma samples were diluted using an assay buffer and added into each well in the detection plate. After 1 hour incubation at room temperature, the liquids were removed from the wells and 50 ul conjugate was added into each well, followed by 1 hour incubating in a shaker. After 3-5 washes with the buffer, a mixed substrate was then added into each well and incubated for 30-min with shaking. Finally, 50 ul of sulfuric acid was conducted to stop the reaction and the optical density (O.D.) was tested at 450 nm on a plate reader (Biotek). The concentrations were calculated according to the standard curves. Standard samples containing recombinant proteins, plasma samples and blank controls were all assayed in duplicate to reduce variation.

Data analysis

The statistical analysis was performed using SPSS 18.0. The unpaired Student's t-test was

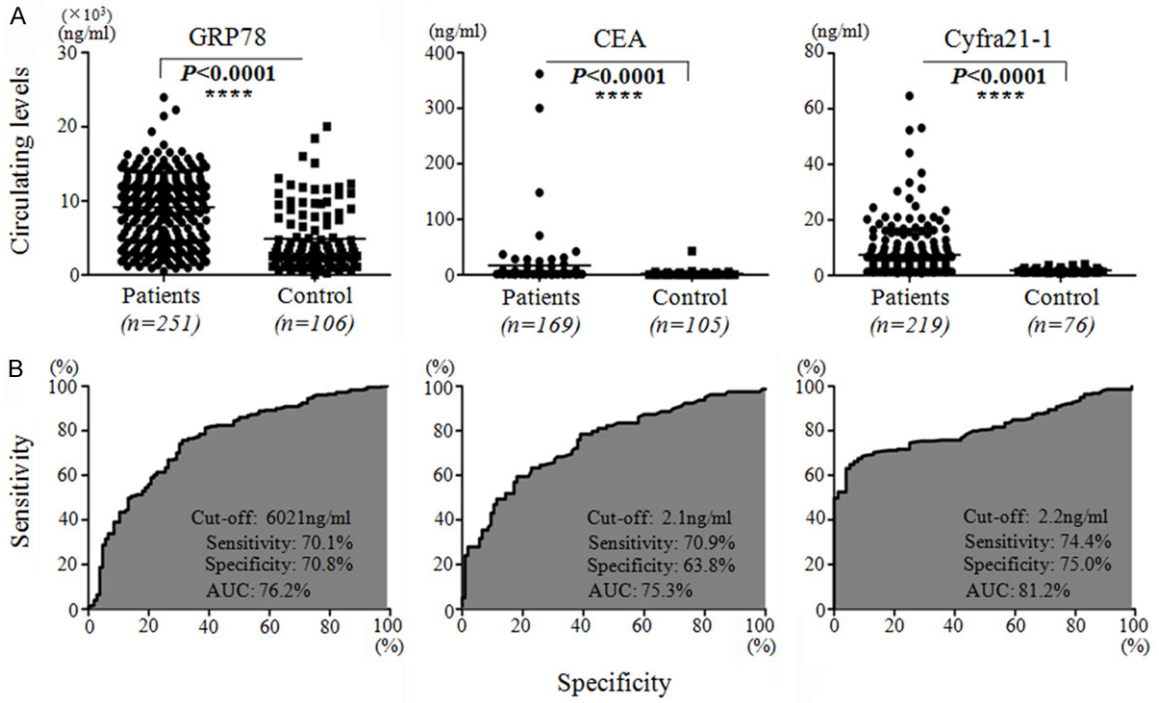

Figure 1. The concentrations and ROC analysis of GRP78, CEA and Cyfra21-1 in all lung cancer patients. A. Concentrations of GRP78, CEA and Cyfra21-1 in all lung cancer patients. The black horizontal lines are median values. *P* values were determined using a Chi-square test, ****P < 0.0001. B. ROC curve analyses of GRP78, CEA and Cyfra21-1 in all lung cancer patients versus the controls.

used for group comparisons between the lung cancer samples and the healthy controls. A two-tailed *p* value < 0.05 was considered to be statistically significant. The sensitivity and specificity of the biomarkers for lung cancer diagnosis were validated using receiver operating characteristic (ROC) curves and areas under the curves (AUC) with a 95% confidence interval (CI). All figures were completed by GraphPad Prism version 5.0 software (GraphPad software, Inc., California, USA).

Results

The characteristics of the participants

There were 251 lung cancer patients and 106 healthy controls in this study. In the healthy controls, the males and females were 52 and 55 cases, respectively. The age breakdown was 36 (< 45 years), 50 (45-65 years), and 20 cases (> 65 years). Among the lung cancer patients, there were 150 males and 101 females. The different subtypes of lung cancer were divided into ADC (168 cases), SCC (62 cases), and others (including small cell lung cancer, large cell cancer and adeno-squamous carcinoma, 21 cases). In terms of staging there were 96 (stage I), 38 (stage II), 60 (stage III) and 57 (stage IV) patients. All the clinical characteristics of the enrolled participants are listed in **Table 1**.

The role of GRP78 in lung cancer diagnosis

ELISA was performed to detect the levels of GRP78 in 251 lung cancer patients and 106 healthy controls. Two classical biomarkers CEA, and Cyfra21-1, were also employed in the analysis. The circulating levels of CEA and Cyfra21-1 were obtained from biomarker detection processes in the Department of Laboratory Medicine in our hospital. The results indicated that all the analyzed biomarkers were dramatically up-regulated in lung cancer patients compared with the healthy controls (all P < 0.0001).

To further evaluate the diagnostic role, an ROC analysis was used to define the sensitivity (SN) and specificity (SP) of GRP78. The results indicated that GRP78 showed 70.1% SN and 70.8% SP, 76.2% AUC, which was higher than CEA (SN: 70.9%, SP: 63.8%, AUC: 75.3%), but less than Cyfra21-1 (SN: 74.4%, SP: 75.0%, AUC: 81.2%)

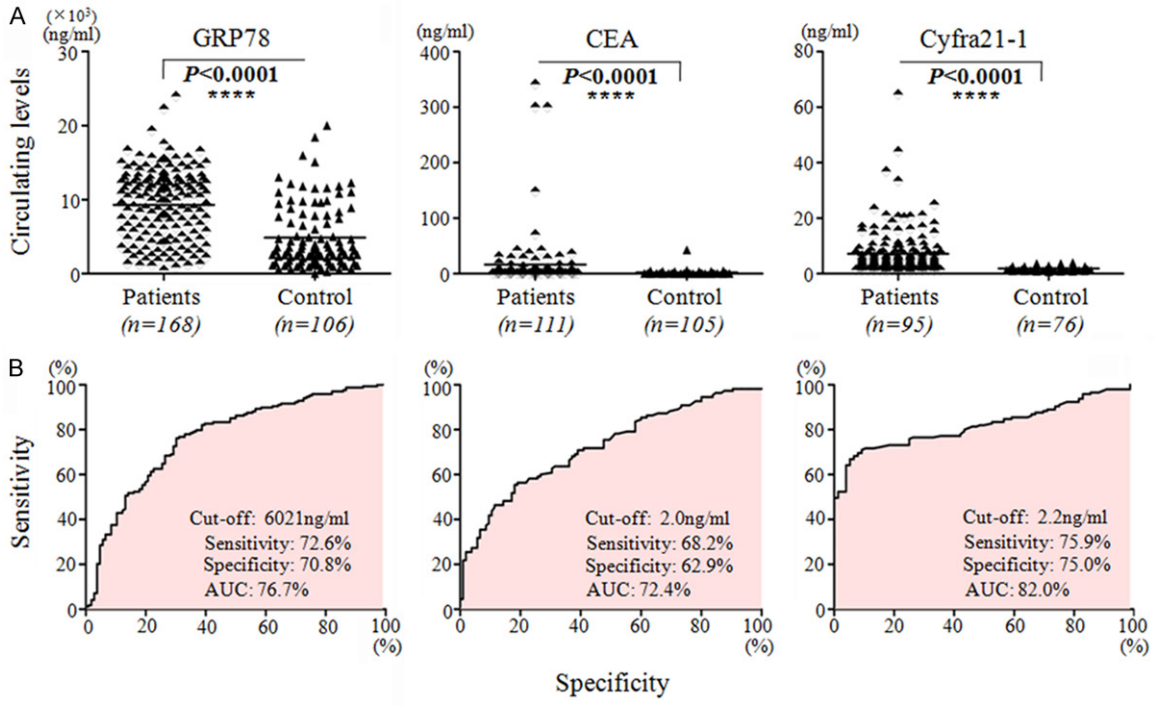

Figure 2. The concentrations and ROC analysis of GRP78, CEA and Cyfra21-1 in ADC patients. A. Concentrations of GRP78, CEA and Cyfra21-1 in ADC patients. The black horizontal lines are median values. *P* values were determined using a Chi-square test, ****P < 0.0001. B. ROC curve analyses of GRP78, CEA and Cyfra21-1 in ADC patients versus the controls.

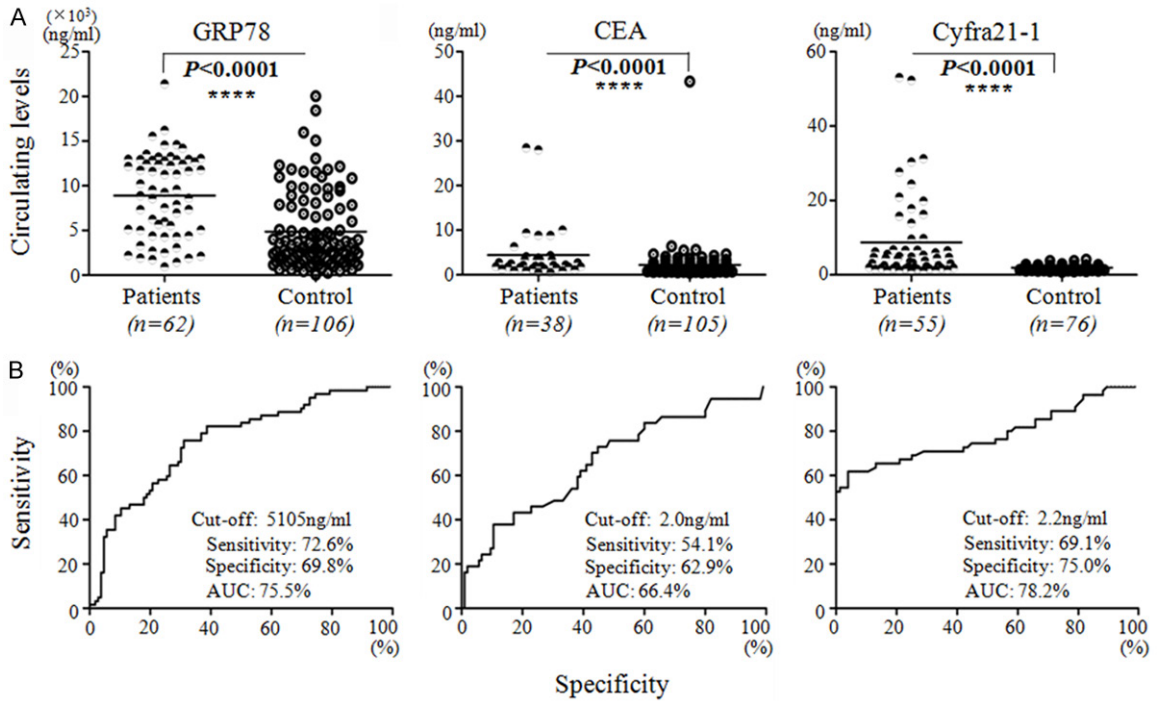

Figure 3. The concentrations and ROC analysis of GRP78, CEA and Cyfra21-1 in SCC patients. A. Concentrations of GRP78, CEA and Cyfra21-1 in SCC patients. The black horizontal lines are median values. *P* values were determined using a Chi-square test, ****P < 0.0001. B: ROC curve analyses of GRP78, CEA and Cyfra21-1 in SCC patients versus the controls.

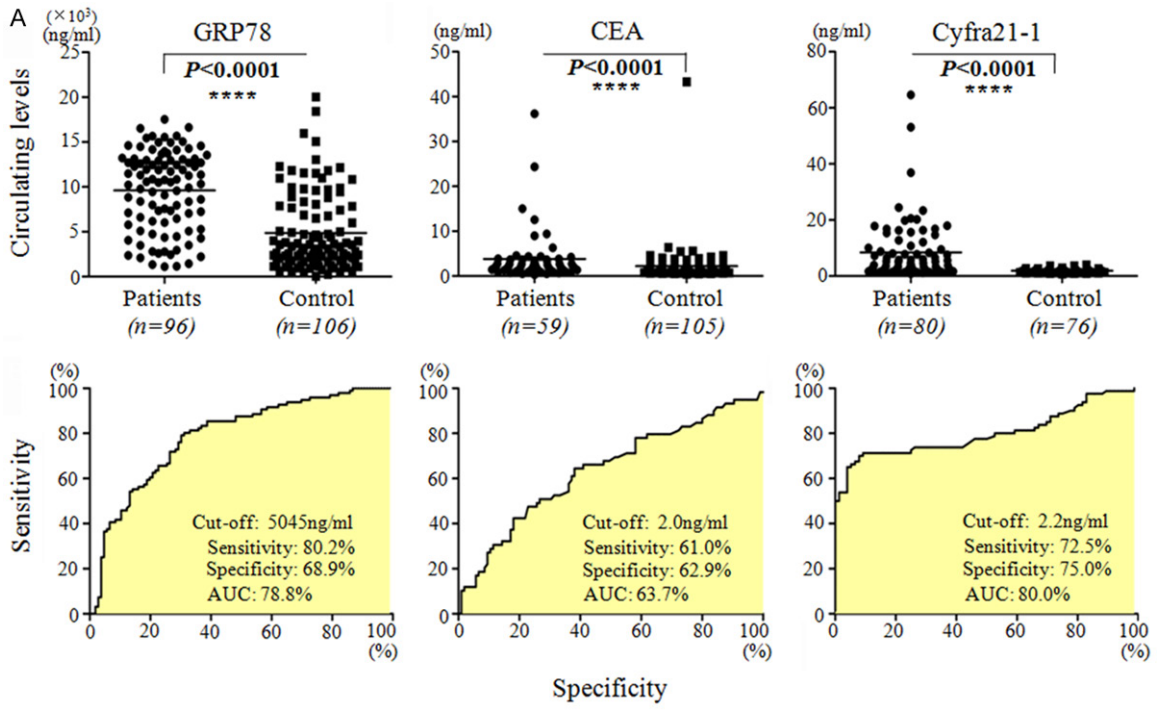

Figure 4. The concentrations and ROC analysis of GRP78, CEA and Cyfra21-1 in early lung cancer patients. A. Concentrations of GRP78, CEA and Cyfra21-1 in early lung cancer patients. The black horizontal lines are median values. *P* values were determined using a Chi-square test, ****P < 0.0001. B. ROC curve analyses of GRP78, CEA and Cyfra21-1 in early lung cancer patients versus the controls.

(**Figure 1B**). Our results demonstrated the value of GRP78 in lung cancer diagnosis.

The diagnostic value of GRP78 in ADC and SCC

Non-small cell lung cancer (NSCLC) makes up about 85% of lung cancer cases. Adenocarcinoma (ADC) and squamous carcinoma (SCC) are two major histological classifications of NSCLC (23). So it was important to evaluate the role of GRP78 in ADC and SCC patients. There were 168 and 62 cases with ADC and SCC, respectively. The results showed that GRP78 levels were also dramatically enhanced in ADC and SCC patients compared with the healthy controls (P < 0.0001), and CEA and Cyfra21-1 levels were also higher in ADC and SCC than in healthy controls (all P < 0.0001) (**Figures 2A** and **3A**).

In the ROC analysis, GRP78 proved more important in ADC (SN: 72.6%, SP: 70.8%, AUC: 76.7%) than in SCC (SN 72.6, SP 69.8%, AUC 75.5), and the same conclusion was also obtained in CEA (ADC: SN 68.2%, SP 62.9%, AUC 72.4%; SCC: SN 54.1%, SP 62.9%, AUC 66.4%) and Cyfra21-1 (ADC: SN 75.9% SP 75.0% AUC 82.0%; SCC: SN 69.1%, SP75.0%, AUC, 78.2%) (**Figures 2B** and **3B**).

The value of GRP78 in the early diagnosis of lung cancer

Early diagnosis is critical in increasing the survival rate of lung cancer, so it was important to evaluate the diagnostic role of GRP78 in early lung cancer. There were total 96 cases of early lung cancer (stage I) enrolled in this study. The ELISA results indicated that GRP78 was clearly increased in early lung cancer patients (P < 0.0001) (**Figure 4A**). The ROC analysis showed GRP78 also played an important role in the early diagnosis of lung cancer (SN 80.2%, SP 68.9%, AUC 78.8%), which was higher than CEA (SN 61.0%, 62.9%, AUC 63.7%) and close to Cyfra21-1 (SN 72.5%, SP 75.0%, AUC 80.0%) (**Figure 4B**).

Construction of diagnostic models for lung cancer combined with GRP78, CEA and Cyfra21-1

It was particularly important to determine whether the combination of biomarkers was more effective than a single biomarker by

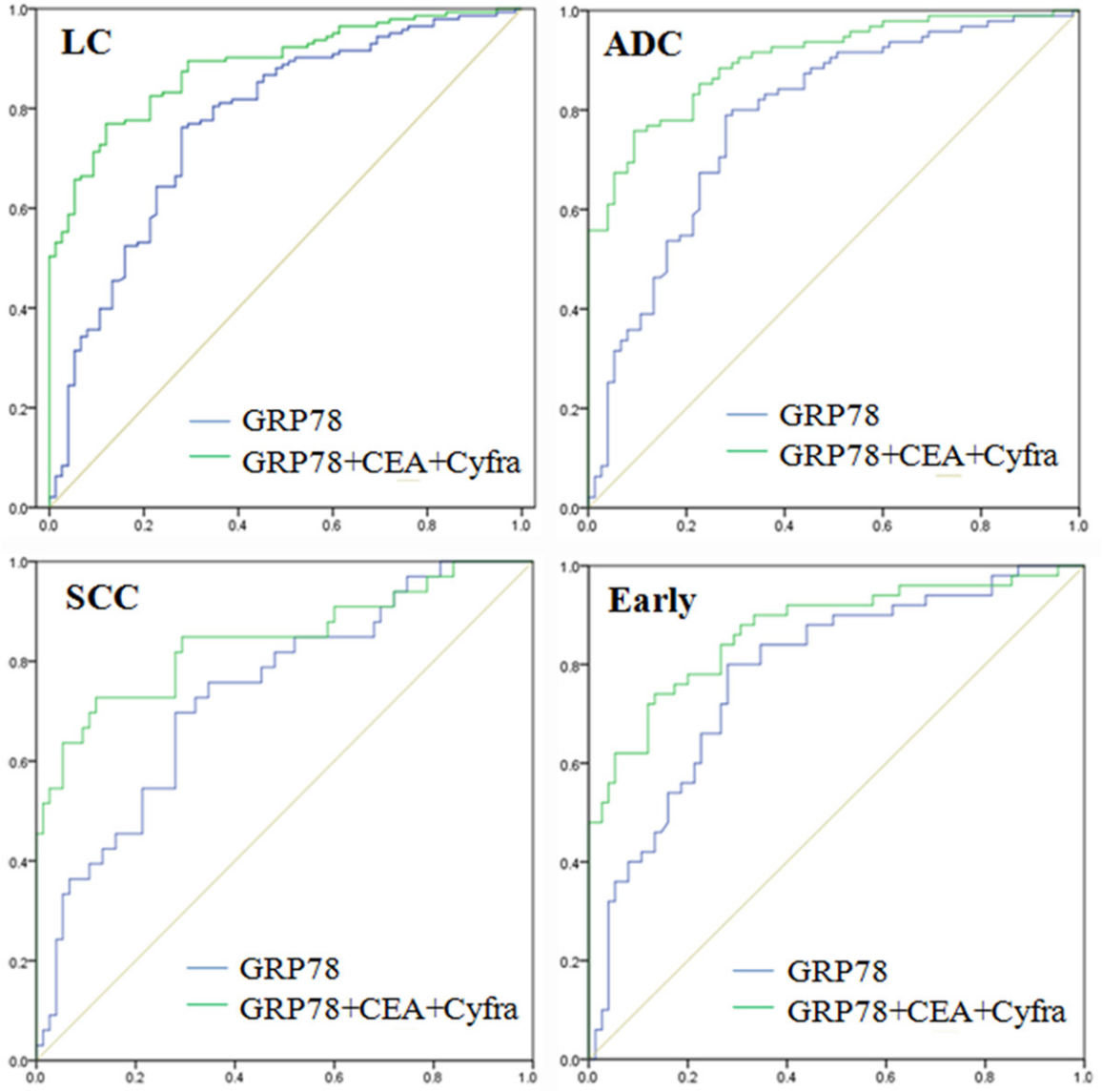

Figure 5. ROC curves of different models of lung cancer patients. LC: all lung cancer, ADC: adenocarcinoma, SCC: squamous carcinoma, Early: stage I patients.

decreasing the false positives and increasing the detection rate. Therefore, a binary logistic regression analysis was performed to analyze the combined accuracy of GRP78, CEA and Cyfra21-1. In all the lung cancer samples, the combination of the three biomarkers increased SN (76.9%), SP (88.0%), and AUC (88.6%) compared with GRP78 alone (**Figure 5**, **Table 2**). And the combination of GRP78, CEA and Cyfra21-1 also increased the diagnostic accuracy in ADC (SN 75.8%, SP 90.7%, AUC 90.1%), SCC (SN 72.7%, SP 88.0%, AUC 84.2%) and early lung cancer (SN 74.0%, SP 86.7%, AUC 86.9%), which indicated the value of these three biomarkers in these specific lung cancer patients (**Figure 5**; **Table 2**).

Discussion

In this study, we found that GRP78 is dramatically increased in lung cancer patients compared with healthy controls ($P < 0.0001$). A ROC analysis also confirmed that GRP78 was valuable in the diagnosis of in lung cancer (SN 70.1%, SP 76.2%, AUC 76.2%), ADC patients (SN: 72.6%, SP: 70.8%, AUC: 76.7%), SCC patients (SN 72.6, SP 69.8%, AUC 75.5) and early lung cancer patients (SN 80.2%, SP

Table 2. The diagnostic efficiency of GRP78 in lung cancer and controls

	AUC (95% CI)	SN (%)	SP (%)	Positive LR	Negative LR
Lung cancer vs. controls					
GRP78	0.771 (0.704-0.838)	76.2	72.0	2.70	0.33
GRP+CEA+CYFRA21-1	0.886 (0.843-0.928)	76.9	88.0	6.35	0.26
ADC vs. controls					
GRP78	0.783 (0.712-0.854)	78.9	72.0	2.82	0.29
GRP+CEA+CYFRA21-1	0.901 (0.856-0.945)	75.8	90.7	8.12	0.27
SCC vs. controls					
GRP78	0.737 (0.636-0.838)	69.7	72.0	2.49	0.42
GRP+CEA+CYFRA21-1	0.842 (0.751-0.934)	72.7	88.0	6.06	0.31
Early vs. controls					
GRP78	0.783 (0.700-0.865)	80.0	72.0	2.86	0.28
GRP+CEA+CYFRA21-1	0.869 (0.802-0.937)	74.0	86.7	5.55	0.30

68.9%, AUC 78.8%). The combination of GRP78, CEA and Cyfra21-1 increased SN, SP and AUC in all lung cancer cases (SN 76.9%, SP 88.0%, AUC 88.6%), ADC (SN 75.8%, SP 90.7%, AUC 90.1%), SCC (SN 72.7%, SP 88.0%, AUC 84.2%), and early lung cancer cases (SN 74.0%, SP 86.7%, AUC 86.9%). These results indicated the valuable role of GRP78 in the early diagnosis of lung cancer.

GRP78 is a major endoplasmic reticulum chaperone and engages in anti-apoptosis activity [24]. Previous studies found that a significantly enhanced expression of GRP78 was observed in various malignant tumors, including prostate cancer, breast cancer, lung cancer, and colon cancer. Wu et al. provided clinical evidence that an elevated expression of GRP78 was found in NSCLC but not in normal lung tissues. And increased GRP78 is closely associated with TNM stages [25]. Zhu et al. found that the enhanced expression of the rs430397 genotype AA of GRP78 in NSCLC protected tumor cells and resulted in a poor prognosis for lung cancer patients, but the precise mechanism of GRP78 action still remains unclear. They also confirmed that elevated GRP78 conferred a tumor survival advantage by blocking apoptotic pathways and contributing to tumor growth [26]. Beside being detected in lung cancer tissues, there were also studies to evaluate the role of GRP78 as a circulating tumor marker. Ma et al. detected GRP78 levels in 163 NSCLC patients and found that although GRP78 had no significant correlations with clinicopathological parameters, higher GRP78 expression related closely to a poor prognosis of NSCLC [27].

Consistent with previous studies, we also found that GRP78 expression is increased in lung cancer patients. And our study had several important advantages. Firstly, we compared the diagnostic efficacy of GRP78 with CEA and Cyfra21-1 which are now applied in clinical diagnosis, and we found that GRP78 had more SN and SP than CEA, but less than Cyfra21-1. The second advantage of our study was that we confirmed the value of GRP78 in early (stage I) lung cancer diagnosis (SN 80.2%, SP 68.9%, AUC 78.8%). Thirdly, we combined GRP78 with CEA and Cyfra21-1 to analyze the role of this panel in lung cancer diagnosis, and the results indicated the panel was more accurate not only in all lung cancer patients, but also in ADC, SCC and early lung cancer patients. To our knowledge, this was the first study of the diagnostic value of the GRP78-related panel in early lung cancer diagnosis. The major limitation of this study was the lack of data to measure the association between GRP78 prognosis. This was because all the enrolled patients were recently diagnosed, so no prognostic/survival information was available.

Conclusion

Our study found that circulating GRP78 can act as a valuable biomarker in early lung cancer diagnosis when combined with CEA and Cyfra21-1.

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Disclosure of conflict of interest

None.

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