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Original Article

Apelin is a novel circulating biomarker for the diagnosis of lung cancer

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Abstract: Apelin was an important factor tumor development but its role in diagnosis of lung cancer remained unknown. In this study, ELISA assay was performed to measure the circulating level of Apelin in plasma samples from 129 lung cancer patients and 57 healthy controls. The aim of this study was to evaluate whether the circulating Apelin could act as biomarker in lung cancer diagnosis. The results indicated that plasma Apelin levels were significantly lower in lung cancer patients than in healthy controls (P<0.0001). Receiver operating characteristic (ROC) analysis showed that Apelin displayed higher SN/SP and AUC (SN: 84.1%, SP: 71.5%, AUC: 77.3%) than two clinical existing biomarkers: CEA (SN: 74.1%, SP: 73.5%, AUC: 81.01%) and Cyfra21-1 (SN: 81.5%, SP: 70.2%, AUC: 78.9%). In addition, the plasma levels of Apelin could contribute to diagnosis of adenocarcinoma patients with high efficiency (SN: 84.2%, SP: 71.4%, AUC: 77.7%). Besides, the expression of Apelin was related to metastasis (P<0.01) in lung cancer patients. Combined analysis of Apelin with CEA and Cyfra21-1 was more effective for lung cancer diagnosis (AUC: 0.980, 95% CI, 0.952-1.000) and lung adenocarcinoma diagnosis (AUC: 0.970, 95% CI: 0.925-1.000) than Apelin alone. All these results showed that Apelin can be used as a novel biomarker for the diagnosis of lung cancer with high sensitivity and specificity.

Keywords: Apelin, lung cancer, diagnosis, ADC, combination analysis

Introduction

Apelin was a secreted peptide, which was identified as the endogenous ligand of the G-protein-coupled cell surface receptor APJ [1]. Apelin was widely expressed in many organs including heart, lung, kidney, brain, liver and breast [2]. The interaction of Apelin/APJ system was involved in many biological functions such as regulation of metabolism process by inhibiting insulin secretion stimulated by glucose [3], regulation of cell apoptosis and migration [4], and reduction of pulmonary inflammation [5]. The most important role of Apelin was to stimulate the tube formation of endothelial cells (EC) and played an important role in formation of blood vessels [6]. Apelin-deficient mice developed narrow blood vessels compared with enlarged blood vessels which were observed in Apelin-overexpression mice [7].

The development of vascular system plays an important role in tumor initiation and distant metastasis, which requires the participation of many signaling pathways. Many membrane receptors act at different stages from the formation of primitive network by vasculogenesis to remodeling into vascular tree by angiogenesis [8]. Among all angiogenesis factors involved in tumoregenesis, Apelin was one of the important factors in formation of vascular system in both primary and metastasis sites of tumors. Recent studies indicated that high level of Aplein was expressed in human breast cancer cell line Hs578T and overexpression of Apelin dramatically increased tumor growth in malignant gliomas, suggesting that high level Apelin associated closely with tumor development [9]. In an assay of non-small cell lung cancer (NSCLC) patients and cell lines, the results indicated that Apelin was highly expressed at both

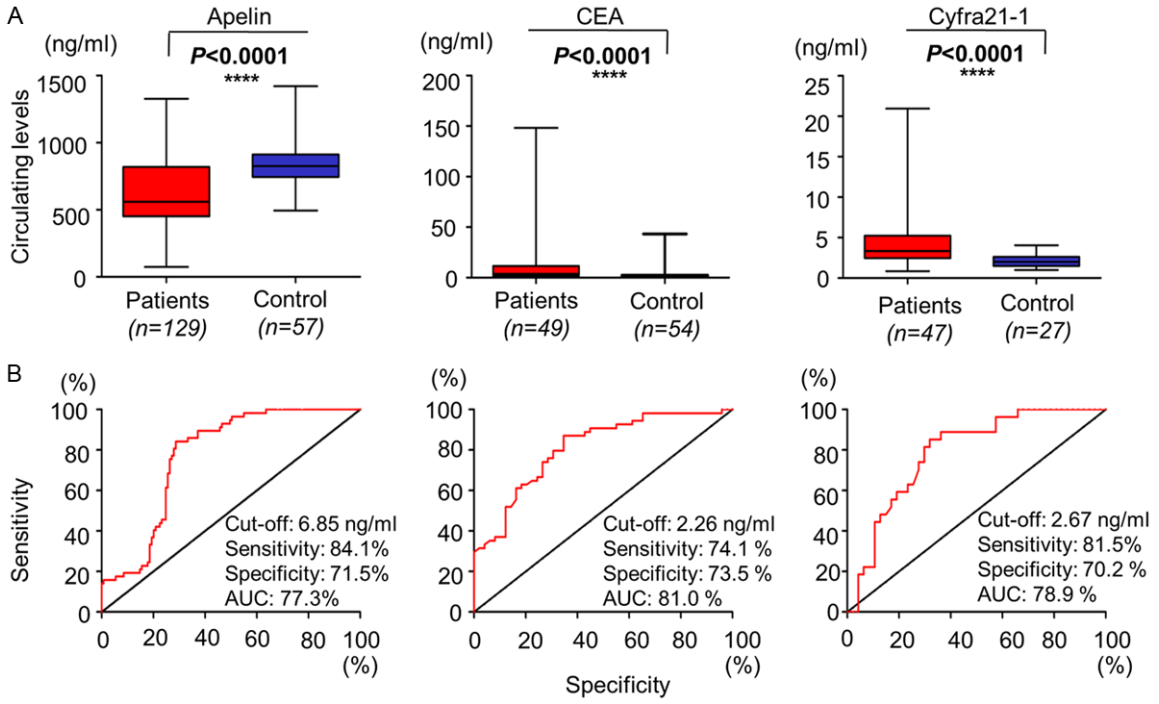

Figure 1. The concentrations and ROC curve analyses of Apelin, CEA and Cyfra21-1 in all lung cancer patients. A. Concentrations; B. ROC curves. The black horizontal lines are median values. *P* values were determined by the Chi-square test (****P<0.0001).

mRNA and protein levels and overexpression of Apelin significantly stimulated formation of tumor microvessels without influencing proliferation of NSCLC cells, and high Apelin protein levels related dramatically to poor survival of NSCLC [10].

However, the role of circulating Apelin in lung cancer diagnosis has not been reported yet. In this study, ELISA detection was performed to measure the circulating plasma Apelin levels in 129 lung cancer patients and 57 healthy controls. The aim of this study was to evaluate the role of circulating Apelin in lung cancer diagnosis.

Materials and methods

Study participants

Blood samples of lung cancer patients were collected from the Department of Respiration Thoracic Surgery and Center of Lung Cancer in West China Hospital between March, 2014 and February, 2016. Blood samples were collected within two weeks after the first biopsy-proven lung cancer diagnosis and prior to any treatment methods including surgical procedure, radiotherapy and chemotherapy. Healthy volunteers were all from the Center of Physical Examination in West China Hospital, and blood samples were collected during the routine physical examination. The definition of healthy controls was not diagnosis of malignancy or benign tumors and suspicious nodules in CT scanning and blood routine examination and tumor markers tests. All plasmas were prepared according to the standard protocols by centrifuge of the anticoagulant whole blood (5 ml) at 800 g and 4°C for 20 min. The isolated plasma was then centrifuged (800 g, 20 min, 4°C) to remove the remaining red blood cells and leukocytes. The plasma was then transferred to 1.5 ml sterile Eppendorf tubes and stored in -80°C refrigerator.

Validation with large samples by ELISA

The ELISA kits for circulating Apelin assay were purchased from Abnova (KA1681). All plasma samples and kit components were equilibrated to room temperature before assay. The detection procedures were performed in accordance with the manufacturer's manual. Firstly, 100 µl anti-Apelin antibody was added to detection wells and incubated 1.5 hours at room temper-

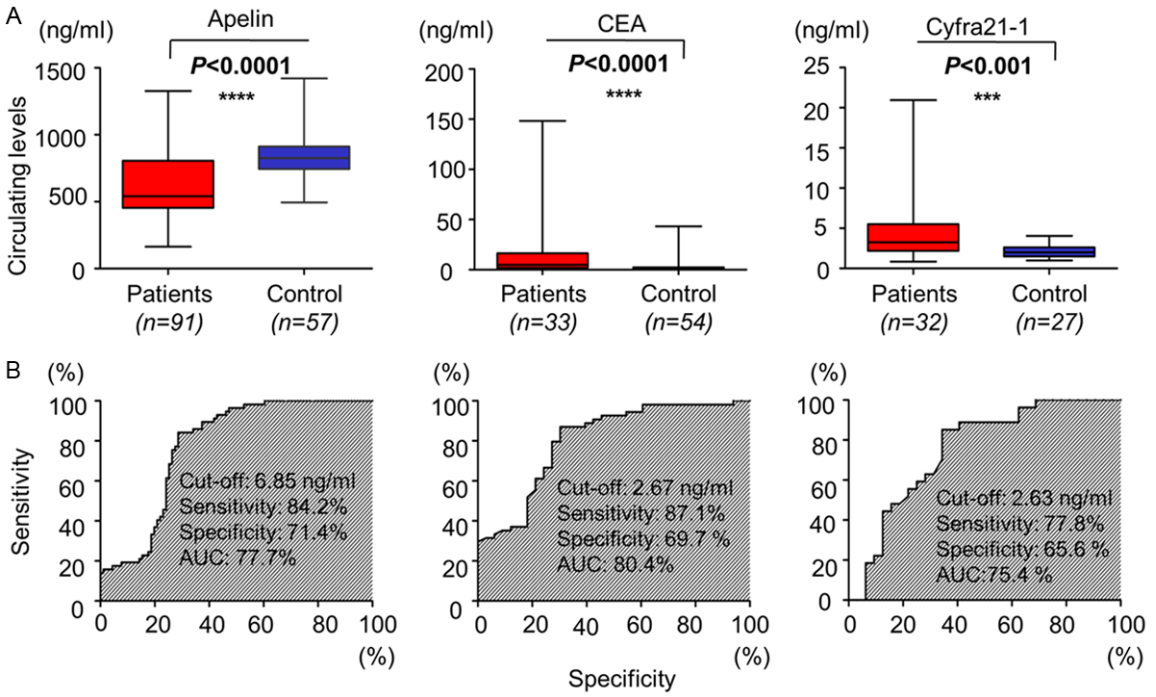

Figure 2. The concentrations and ROC curve analyses of Apelin, CEA and Cyfra21-1 in ADC patients. A. Concentrations; B. ROC curves. The black horizontal lines are median values. *P* values were determined by the Chi-square test (***P<0.001, ****P<0.0001).

ature. Then the Apelin standard and plasma samples were diluted by sample reagents and then added into each well and incubated 2.5 hours with gentle shaking. After 3-5 washes with buffer, HRP-Streptavidin solution was added to each well and incubated 45-min at room temperature. Color development was achieved by adding of the TMB one-step substrate reagent and incubated for 30-min at room temperature in the dark with gentle shaking. Finally, 50 μl sulfuric acid was conducted to stop 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.

Statistical analysis

The standard curve of Apelin was created according to the instruction manual of ELISA kits. Concentrations of all tested samples were calculated according the formula from standard curves. Chi-square test in SPSS17.0 was used to analyze the protein levels between lung cancer patients and healthy volunteers. The sensitivity and specificity of all biomarkers for lung cancer diagnosis were evaluated by receiver operating characteristic (ROC) curves and areas under the curves (AUC) with 95% confidence interval (CI). The best cut-off value for diagnosis was determined by maximizing the specificity and sensitivity at 95% CI. A two-tailed *P* value less than 0.05 was considered significant. All figures were completed by GraphPad Prism version 5 for windows.

Results

The role of Apelin as novel biomarkers in lung cancer diagnosis

ELISA was then used to analyze the diagnosis role of Apelin in lung cancer patients (129 cases) and healthy controls (59 cases) with clear and sufficient clinical records. In the lung cancer patients the plasma Apelin levels were dramatically lower than that of the healthy controls (P<0.0001) (**Figure 1A**). To further explore the diagnosis accuracy of Apelin, Receive operating characteristic (ROC) analysis was used to define the sensitivity (SN) and specificity (SP) of Apelin. The results indicated that Apelin displayed a relatively high SN/SP and AUC (SN:

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

	Lung cancer (129)	Healthy control (57)
Gender		
Male	71	36
Female	58	21
Age		
<45	17	17
45-65	81	30
>65	31	10
Subtypes		
ADC	91	
SCC	34	
Others	4	
Stages		
Early	56	
Advanced	73	
Smoke history		
Yes	44	
No	51	
Unknown	34	
Metastasis		
Yes	56	
No	73	

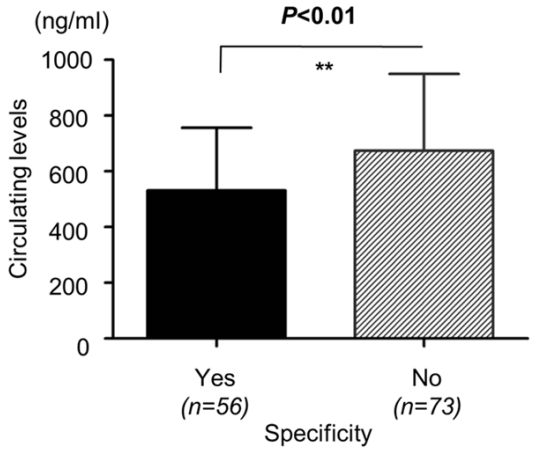

Figure 3. The association analysis between Apelin levels and metastasis. Yes: patients with metastasis, No: patients without metastasis. **P<0.01.

84.2%, SP: 71.5%, AUC: 77.3%) (**Figure 1A**). And we also analyzed 2 clinical existing biomarkers: CEA and Cyfra21-1 in lung cancer and healthy samples and compared their diagnostic efficacy with Apelin (**Figure 1A**). The results indicated that both SN and SP of CEA and Cyfra21-1 were less than Aplein (CEA: SN 74.1%, SP 73.5%, AUC 81%; Cyfra21-1: SN 81.5%, SP 70.2%, AUC 78.9%) (**Figure 1B**). Our results demonstrated that Apelin was more valuable than CEA and Cyfra 21-1 in lung cancer diagnosis.

Diagnostic value of Apelin in ADC patients

Adenocarcinoma (ADC) was the major subtype of lung cancer; therefore, it is important to identify the role of Apelin in ADC diagnosis. The results showed the plasma Apelin levels of ADC patients were also significant lower than t healthy controls (P<0.0001), while CEA (P< 0.0001) and Cyfra21-1 (P<0.0001) were significantly increased in ADC patients (**Figure 2A**). Receive operating characteristic (ROC) analysis indicated that Apelin had higher diagnostic efficiency in ADC patients (SN: 84.2%, SP: 71.4%, AUC: 77.7%) than Cyfra21-1 (SN: 77.8%, SP: 65.6%, AUC: 75.4%), but less than CEA (SN: 87.1%, SP: 69.7%, AUC: 80.4%) (**Figure 2B**). The results indicated that Apelin was also important in ADC diagnosis.

Relationship between Apelin levels and lung cancer clinical characteristics

We further analyzed whether Apelin levels were related to the important clinical characteristics (**Table 1**). And we found that expression of Apelin was dramatically lower in patients with metastasis than those without metastasis (P<0.01) (**Figure 3**). And our results suggested that no significantly association was found between Apelin levels and histological classification, stages, age, smoke history and gender (all P>0.05, data not showed).

ROC analyses of Apelin and the construction of diagnostic models for lung cancer

ROC curves based on the results of ELISA were used to confirm the diagnostic efficiency of Apelin plasma levels for lung cancer. The efficiencies of the two classic markers, CEA and Cyfra21-1 were also included (**Table 2A**). Binary logistic regression analysis showed combination of Apelin, CEA and Cyfra21-1 improved diagnostic accuracy of NSCLCs (AUC: 0.980, 95% CI, 0.952-1.000) (**Figure 4** left, green line) and combination of these three biomarkers also showed enhanced sensitivity and specificity (SN: 95.7%, SP: 96.2%).

Table 2. The diagnostic efficiency of model in all lung cancer (A) and ADC (B) patients

A.	AUC (95% CI)	SN (%)	SP (%)	Positive LR	Negative LR
Lung cancer vs. Controls					
Apelin+CEA+CYFRA21-1	0.980 (0.952-1.000)	95.7	96.2	24.9	0.04
Apelin	0.933 (0.878-0.987)	85.1	88.5	7.38	0.17
B.	**AUC (95% CI)**	**SN (%)**	**SP (%)**	**Positive LR**	**Negative LR**
ADC vs. Control					
Apelin+CEA+CYFRA21-1	0.970 (0.925-1.000)	93.8	100		0.06
Apelin	0.935 (0.877-0.993)	84.4	88.5	7.31	0.18

We further analyzed the diagnostic efficiency of these three biomarkers in ADC patients. The results showed combination of these three biomarkers also improved diagnostic accuracy in ADC patients (AUC: 0.970, 95% CI: 0.925-1.000) (**Figure 4** right, green line), and also showed enhanced sensitivity and specificity (SN: 93.8%, SP: 100%), which indicated that these biomarkers were also important in ADC diagnosis (**Table 2B**).

Discussion

Lung cancer is the leading cause of cancer-related death worldwide [11] with approximately 86,380 of male and 71,660 of female death in 2015 in United States [12]. In China, the morbidity and mortality of this disease were 73.3 and 61.0 per 100,000 respectively in 2015 [13]. The circulating markers were widely used in diagnosis of lung cancer due to its non-invasive and low cost, including carcinoembryonic antigen (CEA) [14], squamous cell carcinoma antigen (SCC) [15], carbohydrate antigen (CA125) [16], cytokeratin 19 fragment (CYFRA 21-1) [17] and neuron specific enolase (NSE) [18], but low specificity and sensitivity limited their clinical value in lung cancer diagnosis. Therefore, the search for new circulating biomarkers was very important in lung cancer diagnosis and treatment surveillance.

In this study, results showed that the plasma level of Apelin was significantly decreased in 129 lung cancer patients compared with 57 healthy volunteers. We also tested 2 existing clinical biomarkers, CEA and Cyfra21-1 and compared their diagnostic efficacy to that of Apelin. It is showed that Apelin displayed a higher SN/SP and AUC in both NSCLCs and ADC patients' diagnosis. In association analysis between Apelin and clinical characteristics, the results indicated that Apelin was significantly lower in patients with metastasis than those without metastasis. Then, we identified that the efficiency of Apelin in combination with CEA and Cyfra21-1 was superior to that of single biomarkers for the discrimination of NSCLCs from healthy controls, with a sensitivity of 95.7% and a specificity of 96.2%. In addition, the combination of Apelin with CEA and Cyfra21-1 improved the diagnosis sensitivity to 93.8% in ADCs, with a specificity of 100%. Therefore integrated analyses of plasma Apelin, CEA and Cyfra21-1 may provide a better panel of diagnostic tools for the rapid and reliable diagnosis of NSCLCs and ADCs.

Apelin was a well-known factor and acted as promoter in tumor development by increasing formation of microvessels but the role of Apelin in cancer diagnosis was less reported. Recently, some researches pay attention on Apelin as a biomarker in different diseases. Maa et al. showed that plasma Apelin was a novel biomarker for predicting type 2 diabetes diseases, while the sensitivity and specificity of plasma Apelin for this prediction was 63.2% and 58.9%, respectively [19]. Goetzea et al. showed that plasma concentrations of Apelin were decreased in patients with chronic parenchymal lung disease and preserved cardiac function, and combination of Apelin-36 and proBNP may be a new diagnostic approach in distinguishing pulmonary from cardiovascular causes of dyspnea [20]. The diagnostic value of serum Apelin was also evaluated in early-onset neonatal sepsis in full term neonates [21] and gastroesophageal cancer (GEC) [22].

To our knowledge, our study was the report in that plasma Apelin acted as valuable biomarker in lung cancer diagnosis, either in single or combination with CEA and Cyfra21-1. However, there were still some limitations of this study. The first was the small sample size of lung cancer patients (129 cases) and controls (57 cases) and we could not make further analysis

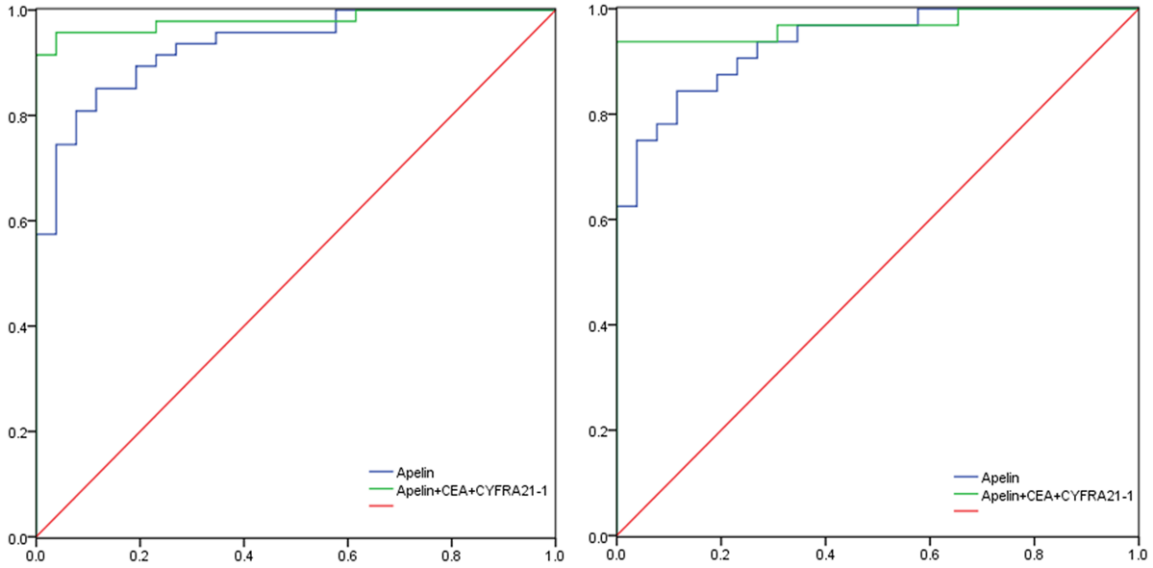

Figure 4. The combination analysis of Apelin, CEA and Cyfra21-1. Left: all lung cancer patients; Right: ADC patients.

such as the role of Apelin in different subtypes and early stages of lung cancer. The second limitation of our study was the lack of analysis between Apelin levels and poor prognosis, the reason was that the enrolled patients were diagnosed within past two years and no prognosis information were available. Further studies should focus on the role of Apelin in prediction of poor prognosis and the mechanisms of Apelin in tumor initiation and metastasis.

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Disclosure of conflict of interest

None.

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