



Measuring serum human epididymis secretory protein autoantibody as an early biomarker of lung cancer

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Background: Lung cancer (LC) is one of the most common types of malignant tumors and is the most prominent cause of tumor-related death worldwide. LC is a heterogeneous disease caused by somatic cell mutations and dysregulation in several signaling pathways. Understanding these pathways provides the basis for detecting LC. LC screening and diagnosis in current clinic still rely on computed tomography (CT), but its high false positive rates and cost may prevent it from being a routine screening method. Therefore, the discovery of new non-invasive and more valuable biomarkers may present an improved diagnostic approach for LC, and potentially provide more useful information for the prognosis and treatment of LC in patients. This study investigated the potential of detecting serum autoantibodies produced against human epididymis secretory protein 4 (HE4) for LC diagnosis in high-risk groups.

Methods: Serum samples from 61 patients with LC were included in this study, and another 53 serum samples from healthy donors or benign lung diseases (BLD) patients were collected as the control group. The samples were analyzed with enzyme-linked immunosorbent assays (ELISA).

Results: ELISA results showed significantly higher levels of serum autoantibodies against HE4 in samples from LC patients compared to the control group ($P < 0.001$). Analysis of HE4 autoantibodies showed a receiver operating characteristic (ROC) curve indicating 67.21% sensitivity, 96.23% specificity, and an area under the curve (AUC) of 0.848. Levels of HE4 autoantibodies can discriminate early-stage LC patients from the control group with a 54.76% sensitivity.

Conclusions: Detecting serum HE4 autoantibody levels may be a potential biomarker in high-risk groups of LC. We present a new method for the diagnosis of LC in the clinic.

Keywords: Tumor-associated antibodies (TAAbs); biomarker; lung cancer (LC); diagnosis

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Introduction

Lung cancer (LC) is one of the most common types of malignant tumors and is the most prominent cause of tumor-related death worldwide. According to a report from the International Agency for Research on Cancer's report in 2018 (GLOBOCAN 2018), there are approximately 2.1 million cases of LC globally, accounting for 11.6% of all malignant tumors. Additionally, 1.8 million LC deaths were

reported, accounting for 18.4% of all malignant tumors (1). There are two main types of LC: small cell LC (SCLC) and non-small cell LC (NSCLC). NSCLC accounts for about 80–90% of LC, consisting of adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma (LCC) (2,3). In most cases, LC is not caused by point mutations, but is caused by a combination of multiple molecular changes leads to its formation. LC is a

heterogeneous diseases caused by somatic cell mutations and dysregulation of multiple signaling pathways. Mutations in genes such as p53, p16, and Rb results in aberrant function of receptor tyrosine kinases (RTK), such as epidermal growth factor receptor (EGFR), and insulin-like growth factor 1 (IGF-1R) (4). Understanding these pathways provides a molecular basis for detecting LC. Current methods for screening and diagnosis in the clinic still rely on computed tomography (CT), but its high false positive rates and cost may prevent it from being a routine screening method (5-7). Therefore, the discovery of new non-invasive and more valuable biomarkers can present an improved diagnostic approach for LC, and potentially provide more useful information for the prognosis and treatment of LC patients in clinic.

Human epididymis secretory protein 4 (HE4), an oncogene, was firstly found in distal human epididymis epithelium cells in 1991 by Kirechhoff *et al.* (8), located on chromosome 20q 12-13.1, this gene which encodes a small molecular protein associated with sperm maturation belonging to the WAP four-disulfide core domain (WFDC) family (9). HE4 is mainly expressed in reproductive system cells, such as in ovaries, mammary gland epithelium, epididymis and vas deferens epithelium. However, it is also found in human respiratory tract and lungs (10,11). HE4 is involved in the natural immune process in the respiratory tract, and in the mouth and nasal cavity, and may be related to the lung ADC (12). In addition, several studies have shown that elevated serum HE4 levels may be associated with ovarian cancer, and that detection of HE4 can even be used as a marker for early ovarian cancer (13,14). Moore *et al.* found that detection of serum HE4 is more specific in early ovarian cancer screening compared with conventional carbohydrate antigen-125 (CA-125) (15). HE4 is overexpressed in most lung ADCs and in many lung SCCs, therefore, HE4 is an attractive target for LC diagnosis and prognosis of LC (12). Other relevant studies have also showed that serum HE4 can be used as a potential diagnostic marker for NSCLC (16-18). HE4 is a tumor-associated antigens (TAAs), which originates from tumor cells and are typically over-expressed, misfolded or abnormally degraded in patients with malignant tumors (19). Tumor-associated antibodies (TAAbs) are specifically produced in an immune response against TAAs, where detection of TAAbs can improve early diagnosis of malignant tumors with higher sensitivity and specificity than TAAs (20-22). In addition, serum TAAbs can still be detected by relevant technologies even if the concentration of TAAs in peripheral blood is very

low and cannot be detected (23).

However, the feasibility of using TAAbs as biomarkers in LC remains unclear. The present study aims to evaluate the clinical significance of HE4 autoantibody expression in serums samples from LC patients.

Methods

Patients

A total of 61 serum samples from patients with LC referred to the China-Japan Union Hospital of Jilin University between May 2019 and July 2019 were included in this study. Serum samples were collected before the patients underwent LC surgery. Clinical characteristics from the patients are summarized in *Table 1*. All LC patients were confirmed by pathological examination.

Controls

A total of 24 serum samples were collected from patients with benign lung diseases (BLD) (infectious diseases, chronic obstructive pulmonary diseases and tuberculosis). Serum sample from 29 healthy donors were also collected. In contrast with patients from the LC group, control group donors have similar age and sex ration than patients in the LC group. The study was approved by the ethics committee of the China-Japan Union Hospital of Jilin University (approval number 2019071610). Informed consent was obtained from the participants in the study.

Laboratory methods

A blood sample was collected and serum was separated and stored at -80°C until tested. One hundred μL of 1 $\mu\text{g}/\text{L}$ diluted human recombinant protein of HE4 (12609-H08H, Sino Biological Inc., Beijing, China) was adsorbed to 96-well plates, covered and incubated overnight at 4°C . After the recombinant protein coated plate was washed three times with PBS, subsequent to block with PBS containing 5% bovine serum albumin (BSA). After washing, 100 μL serum sample diluted 1:200 in PBS containing 0.5% BSA was added to each well, meanwhile, 100 μL blocking buffer and rabbit anti-human HE4 monoclonal antibody (12609-R109, Sino Biological Inc., Beijing, China) diluted 1:10000 was also added to the negative and positive control wells, each sample was tested in duplicate. Following 2h incubation at 37°C , the plate was washed three times and 100 μL goat

Table 1 Characteristics of samples

Characteristics	Patients (N=61)	Controls (N=53)	P value
Male rate, n (%)	20 (32.79%)	19 (35.85%)	0.731 ^a
Median age, years [range]	54.84±7.67 [44–70]	55.17±6.00 [44–60]	0.075 ^b
Smoking history, n (%)			
≤20 years	44 (72.13)	38 (71.70)	0.959 ^a
>20 years	17 (22.87)	15 (28.30)	
Weight loss (WL), n (%)			
≤5% (body weight)	41 (67.21)	–	
>5% (body weight)	20 (32.79)	–	
Performance status (PS), n (%)			
0–1	40 (65.57)	–	
2–4	21 (34.43)	–	
Tumor type, n (%)			
ADC	44 (72.13)	–	
SCC	15 (24.59)	–	
SCLC	2 (3.28)	–	
Stage, n (%)			
I	28 (45.90)	–	
II	14 (22.95)	–	
III	16 (26.23)	–	
IV	3 (4.92)	–	

^a, Pearson Chi-square test; ^b, independent sample *t*-test.

anti-rabbit and goat anti-human secondary antibody labeled with horseradish peroxidase (HRP) (SSA018, SSA016, Sino Biological Inc, Beijing, China) diluted 1:20,000 in 0.5% BSA was added to the each well and incubated at 37 °C for 2 h. After washed five times, 100 μL of tetramethylbenzidine (TMB) substrate solution (P0209, Beyotime Biotechnology Inc., Shanghai, China) was added. After incubating at room temperature in the dark for 15 min, 100 μL stop solution was added, and the optical density (OD) was measured at 450 nm with a reference wavelength of 620 nm in an ELISA plate reader.

Statistical analysis

SPSS 25.0 software was used for statistical analysis.

T-test was used for the age difference between groups, gender and smoking history was represented by rate or constituent ratio, Chi-square test was used to compare the difference between groups. The measured OD values of the two groups of serum samples were taken as the mean value of the duplicate wells. The statistical OD values were expressed as mean ± standard deviation ($\bar{x} \pm s$). Because the sample data did not have a normal distribution, the comparison of data between groups was performed using medians, and the nonparametric Mann-Whitney U test was used to compare the difference between groups. The mean of the OD values of control group plus two standard deviations ($\bar{x} \pm s$) was used as the cut-off value. A receiver operating characteristic (ROC) curve was used to compare the diagnostic value of the anti-HE4 autoantibody, and $P < 0.05$ was considered to indicate a statistically significant result.

Results

HE4 autoantibody levels detection in the serum from LC patients

Serum levels of autoantibody to HE4 were significantly higher in patients with LC (0.487 ± 0.248) than control subjects (0.208 ± 0.098) ($Z = 6.389$, $P < 0.001$). A significant change was observed in the BLD group (0.247 ± 0.124) compared to healthy donors group (0.175 ± 0.056) ($Z = -2.314$, $P = 0.021$). Patients with either SCC or ADC had increased levels of HE4 autoantibodies. OD values in samples from ADC patients (0.535 ± 0.260) were significantly higher than in SCC patients (0.336 ± 0.154) ($Z = -2.768$, $P = 0.006$) (*Figure 1*). However, performance status (PS) ($P = 0.390$) and weight loss (WL) ($P = 0.261$), showed that the levels of HE4 autoantibody levels were not significantly changed in the patient group, but were significantly different between clinical stages ($P = 0.022$) (*Figure 2*).

Diagnostic value of serum HE4 autoantibody in LC

According to the cut-off value of 0.403, serum HE4 autoantibody sensitivity was 67.21%, the specificity was 96.23% and the Youden index was 0.648 (*Table 2*). Sensitivity of detecting serum HE4 autoantibody in an early stages of LC was 54.76% with 23 positive samples in early-stage LC groups. Evaluation of HE4 autoantibodies in LC by ROC curve analysis showed an AUC value of 0.848 (95% CI: 0.776–0.920) (*Figure 3*).

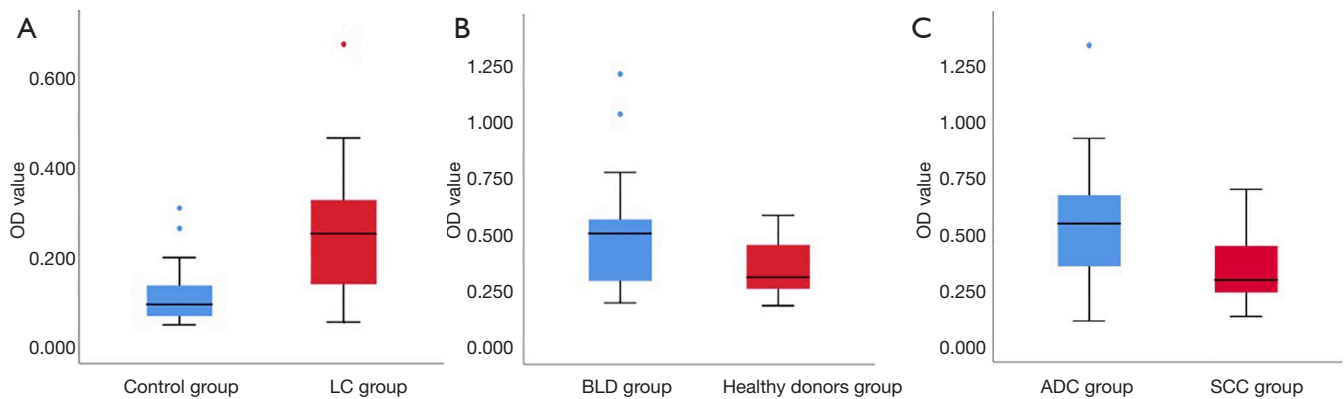


Figure 1 Serum levels of autoantibody to HE4 in different groups. (A) The serum levels of autoantibody to HE4 were significantly higher in patients with LC than control subjects ($P < 0.001$). (B) The serum levels of autoantibody to HE4 were significantly higher in BLD patients than healthy donors ($P = 0.021$). (C) The serum levels of autoantibody to HE4 were significantly higher in ADC patients than SCC patients ($P = 0.006$). LC, lung cancer; BLD, benign lung disease; ADC, adenocarcinoma; SCC, squamous cell carcinoma.

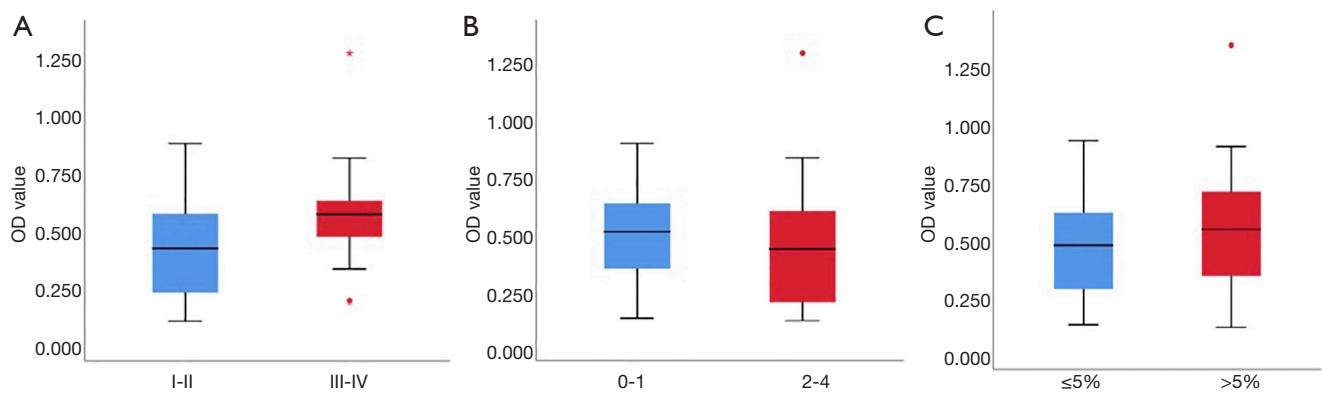


Figure 2 Serum levels of autoantibody to HE4 in different subgroups of LC patients. (A) The serum levels of autoantibody to HE4 were significantly higher in patients at III-IV stage than I-II stage ($P = 0.022$). (B) The serum levels of autoantibody to HE4 were not significantly changed in patients with PS 0-1 and 2-4 ($P = 0.390$). (C) The serum levels of autoantibody to HE4 were not significantly changed in patients with WL $\leq 5\%$ and WL $> 5\%$ ($P = 0.261$). WL, weight loss.

Table 2 The positive rate of serum HE4 autoantibody in two groups

Groups	Positive rate (%)	Sensitivity (%)	Specificity (%)	P value
LC (N=61)	41 (67.21)	67.21	96.23	<0.001
Control (N=53)	2 (3.77)			

Discussion

Diagnostic methods for early LC detection by peripheral blood tests are preferred as they are more convenient and

quicker. Therefore, a series of tumor biomarkers, such as neuron-specific enolase (NSE) and carcino-embryonic antigen (CEA), are often used in the clinic, but detection sensitivities and specificities are not satisfactory. Tumor-associated autoantibodies (TAAbs) have been studied for many years, where certain TAAbs have potential diagnostic value (24-29). However, due to limitations such as sample quality and detection methods, further validation is required. The diagnostic value of the same TAAb varies between different studies. For example, sensitivity of detecting p53 autoantibodies was 12.6% in a German study (30), but was a reported 40.3% in a Japanese study (31). This may be related to the pathological subtypes of the

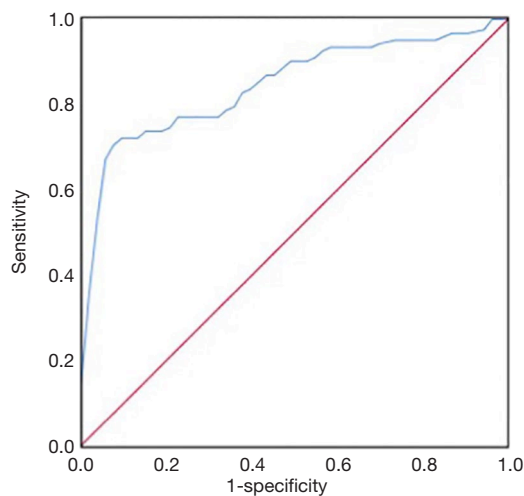


Figure 3 The sensitivity of serum HE4 autoantibody was 67.21%, the specificity was 96.23%. ROC curve analysis showed an AUC of 0.848 (95% CI: 0.776–0.920) for HE4 autoantibody for LC. ROC, receiver operating characteristic; AUC, area under the curve; LC, lung cancer.

samples, since the former study mainly included the ADC and SCLC patients, while the latter study included a certain number of SCC patients. This suggested that p53 autoantibody levels may be higher in peripheral blood in SCC patients. Park *et al.* confirmed that the sensitivity of p53 autoantibody detection was 34.1% in patients with SCC (32). Therefore, in order to avoid such bias, ADC and SCC patients were both included in this study.

Although many TAAbs have been used for early diagnosis and prognosis in LC, they still can not be used in clinic due to their low sensitivities and specificities. Therefore, discovery of new TAAbs with high sensitivity and specificity is necessary. In this study, HE4 autoantibodies were studied to find its diagnostic potential for LC. Although detection of HE4 autoantibodies have been proved to have satisfactory diagnostic value in ovarian and breast cancers (15), there are no studies evaluating its use for LC. Therefore, the results of this study may provide supporting information for other TAAbs that are being researched for LC.

In this study serum samples from 61 LC patients were detected by ELISA, including 44 cases of ADC, 15 cases of SCC and 2 cases of SCLC, serum levels of HE4 autoantibodies in 24 cases of BLD patients and 29 healthy donors were also detected. HE4 autoantibody levels in patients with LC group was significantly higher than in the BLD group and healthy donors group ($P < 0.001$),

In addition, HE4 autoantibody levels were significantly different ($P = 0.021$) in the BLD group compared with the healthy donors group, this may be related to the increase in HE4 levels in inflammatory tissues (33). Therefore, detection of HE4 autoantibody levels can be used to detect LC in high risk populations, and to distinguish between BLD patients and healthy people. Analysis of different LC pathology subtypes showed that serum levels of HE4 autoantibody in the ADC group was significantly higher than in SCC group ($P = 0.006$), which was consistent with results reported by Bingle *et al.* (12). Analysis of different clinical stages showed that serum HE4 autoantibody levels were significantly different ($P = 0.022$), patients at more advanced stages had higher serum HE4 autoantibody levels than ones at early stages, which may because patients with advanced cancer have enhance the immune responses, where immune cells are constantly releasing autoantibodies. According to ROC curve analysis of serum HE4 autoantibody detection in LC diagnosis, sensitivity, specificity and AUC were 67.21%, 96.23%, and 0.848 respectively. Hence, our results indicated that serum HE4 autoantibody has potential diagnostic value for LC. In addition, the detection sensitivity of serum HE4 autoantibody in early LC was 54.76%, suggesting a potential test for early LC in clinic.

However, there are some limitations in this study. First, the sample size included was small, so there may be errors in representation. Second, healthy donors were not examined by bronchoscopy which may have generated errors in diagnosis. Third, ADC accounts for the majority of pathological subtypes in the LC group, so there may be some bias in comparing the ADC group to the SCC group. Fourth, ELISAs were the only detection method used in this study. Therefore, the value of serum HE4 autoantibodies for LC diagnosis still requires further validation, including a larger sample size and with samples from other centers.

Conclusions

In this study, we found that serum HE4 autoantibody is a potential biomarker for LC diagnosis. Additionally, we present a potential method for early LC screening in high-risk populations.

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Footnote

Conflicts of Interest: The authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.11.50>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of the China-Japan Union Hospital of Jilin University (approval number 2019071610). Informed consent was obtained from the participants in the study.

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