

Transthyretin as a potential biomarker for the differential diagnosis between lung cancer and lung infection

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Abstract. Satisfactory biomarkers for screening and early diagnosis of lung cancer remain scarce and require further investigation. The aim of the present study was to examine the changes of the biochemical and protein composition in the serum and pleural effusion from lung cancer and lung infection (bacterial pneumonia) patients. A total of 92 patients with lung cancer, 38 with bacterial pneumonia and 42 healthy controls were enrolled in the study. The serum levels of cholesterol, apolipoprotein A and transthyretin (TTR) in the lung cancer patients were higher than that of the lung infection patients ($P<0.05$). The levels of TTR were higher, whereas the activity of adenosine deaminase (ADA) was lower in the pleural effusion from the lung cancer patients compared to the lung infection patients ($P<0.05$). Furthermore, the pleural effusion/serum TTR ratios in the lung cancer patients were higher, whereas the ratios of ADA were lower ($P<0.05$). By matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis, four major peaks corresponding to native TTR, Sul-TTR, Cys-TTR and Cysgly-TTR were observed in the serum of the lung cancer and lung infection patients. A significant increase was found in the proportion of Cysgly-TTR in the pleural effusion from the patients with lung cancer. The data indicated that a combination of pleural effusion/serum TTR ratios and modified TTR may be beneficial for the differential diagnosis between lung cancer and lung infection.

Introduction

Lung cancer is one of the most common malignant tumors worldwide, with a 5-year survival rate of 14% (1). Thus far, the statistics for cancer occurrence and outcome show that lung cancer remains a primary cause of mortality from cancer (2,3). Since the incidence and mortality of lung cancer increases significantly every year, it represents a major economic burden to society. Currently, the screening and early diagnosis of lung cancer in clinics relies mainly on magnetic resonance imaging (MRI) and computed tomography (CT) imaging, whereas the final diagnosis is established on the basis of histopathological examination results. The early clinical manifestations of lung cancer patients are relatively mild and not typical, easily overlooked or confused with benign inflammatory disease, such as lung infection. Therefore, early identification and diagnosis of lung cancer is significant since lung cancer may be curable in its early stages (1).

Thus far, increasing attention has focused on searching for improved biomarkers that function not only to detect lung cancer at an early stage but also to explore the molecular mechanisms that underlie cancer development. Tumor markers, including carcinoembryonic antigen (CEA), carbohydrate antigen 125 (CA125), neuron-specific enolase (NSE), squamous cell carcinoma antigen (SCCAg) and cytokeratin-19 fragments (Cyfra21-1), are clinically applied for the early detection of lung cancer (4,5). However, the sensitivity and specificity of these biomarkers are not adequate (6-8). Combined detection using a panel of biomarkers can improve the sensitivity and accuracy in lung cancer diagnosis, but consequently results in a lower specificity and increased financial burden to patients. Determining the diagnosis and establishing a more appropriate treatment in early stage patients with lung cancer remains a challenge. Additional biomarkers to benefit early diagnosis of lung cancer are required. Since malignant pleural effusion is a common complication of lung cancer patients, biochemical and pathogenic microorganisms analysis in pleural fluid contribute to differential diagnosis between lung cancer and benign inflammatory diseases.

Currently, several proteomic approaches have been used for the identification of cancer biomarkers, including two-dimensional electrophoresis (2-DE) and mass spectrometry (MS). Monitoring the protein expression pattern by proteomic technologies contributes to the early detection of potentially novel

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cancer biomarkers. Proteomics has proved to be a powerful tool in clinical diagnosis and biomarker discovery, particularly in the identification of specific post-translational modifications (9). Previously, those proteomic approaches have been applied to screen biomarkers for early diagnosis of lung cancer (10-13). The transthyretin (TTR) monomer has been shown to be upregulated in the sera of adenocarcinoma lung cancer patients using 2-DE coupled to matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF-MS) peptide mass fingerprinting (14). Previous studies have shown that TTR may be a novel serum biomarker for distinguishing lung cancer patients from normal control individuals using surface-enhanced LDI-TOF-MS (15,16).

TTR is a normal serum protein synthesized primarily in the liver, the choroid plexus and the retina (17). As a homotetramer in plasma, TTR binds and transports the thyroid hormones and the retinol-binding protein-retinal complex (18). The decreased serum concentration of TTR has been used as a marker to evaluate malnutritional/inflammatory status under a variety of conditions (19-21). The implication of TTR in the formation of amyloid deposits in familial amyloidosis and senile systemic amyloidosis has been shown previously (22,23). In addition, a number of studies have shown the potential value of serum TTR in cancer diagnosis, including ovarian (21,24), endometrial (25) and lung cancer (14-16).

The aim of the present study was to investigate the changes of the biochemical and protein composition between lung cancer and lung infection patients in order to screen biomarkers for the differential diagnosis of malignant pleural effusions. An MS-based proteomic approach, MALDI-TOF-MS, was applied to characterize TTR variants in serum and pleural effusion of patients with lung cancer and lung infection.

Patients and methods

Patients and samples. The serum and pleural effusion samples were obtained from Tianjin Chest Hospital including 92 patients with lung cancer and 38 patients with lung infection (bacterial pneumonia). The serum and pleural effusion samples were collected prior to any clinical treatment. Control serum samples were also obtained from 42 healthy adult volunteers. The diagnosis of lung cancer and lung infection was based on the clinical outcome, MRI/CT imaging and laboratory findings. Stage and histological classification were performed according to the World Health Organization 1999 criteria for lung cancer classification (26). The detailed clinical characteristics of the participants are shown in Table I.

The Medical Ethics and Human Clinical Trial Committee of Tianjin Medical University (Tianjin, China) approved the study and informed consent was obtained from all the study subjects.

Sample processing. The serum samples were collected and maintained at 4°C for 1 h for clotting, subsequently centrifuged at 1,700 × g for 15 min and immediately aliquoted and stored at -80°C. All the serum samples were only allowed to thaw once. The pleural effusion samples were collected from the patients with lung cancer-induced malignant pleural effusion and pleural effusion induced by lung infection. The effusions were collected in sterile tubes and centrifuged immediately at

Table I. Clinical characteristics of the subjects in each group.

Characteristics	Lung cancer (n=92)	Lung infection (n=38)	Healthy controls (n=42)
Gender, n			
Male	60	23	24
Female	32	15	18
Mean age, years (range)	65.8 (48-83)	54.9 (18-78)	58.6 (45-65)
Lung cancer histology, n			
Adenocarcinoma	45		
Squamous cell lung cancer	30		
Small cell lung cancer	17		
Disease stages, n			
I	10		
II	36		
III	28		
IV	18		

4°C. The cell-free supernatants were collected and the aliquots were stored at -80°C until use.

Clinical and laboratory measurements. Simultaneous to the collection of the serum and pleural effusion samples, the following clinical and laboratory data were obtained: Age, gender, triglycerides (TG), cholesterol (CHO), apolipoprotein A (ApoA), ApoB, glucose (GLU), TTR, total protein (TP), albumin (ALB), adenosine deaminase (ADA) and lactate dehydrogenase (LDH). Regarding the laboratory features, TG, CHO, GLU, TP, ALB, ADA and LDH were measured using a Toshiba TBA-120 auto-analyzer (Toshiba Medical Systems Co., Ltd., Tokyo, Japan). The concentration of TTR, ApoA and ApoB were measured by the immunonephelometric method using an automatic clinical analyzer (TBA-40, Toshiba Medical Systems Co., Ltd.).

MALDI-TOF-MS analysis of TTR. All the experiments were performed with a MALDI-TOF-MS (Shimadzu/Kratos, Manchester, UK) operated at a wavelength of 337 nm. The optimal spectra of TTR were obtained at an ion-accelerating voltage of 27.5 kV and a reflectron voltage of 30 kV. The spectra were calculated by using external calibration with [M+H]⁺ ions produced from horse cytochrome *c* (12,361.96 m/z) and horse myoglobin (16,952.27 m/z). The matrix was a saturated solution of sinapinic acid in acetonitrile plus water (1:2, v/v) containing 0.1% trifluoroacetic acid. The samples were deposited onto the sample probe assembly. MALDI-TOF-MS data were analyzed using Launchpad software version 2.4 (Kratos Analytical, Manchester, UK) (27).

Statistical analysis. Data were expressed as mean ± standard deviation. Data were processed with SPSS software 13.0. (SPSS, Inc., Chicago, IL, USA). Statistical analysis was performed using the independent samples t-test between the groups. P<0.05 was considered to indicate a statistically significant difference.

Table II. Concentrations of the biochemical indicators in the serum and pleural effusion.

Indicators	Serum		Pleural effusion	
	Lung cancer (n=92)	Lung infection (n=38)	Lung cancer (n=92)	Lung infection (n=38)
TG, mmol/l	1.21±0.55	1.02±0.33	0.33±0.35	0.26±0.13
CHO, mmol/l	4.27±0.90 ^a	3.67±0.86	1.46±0.61	1.47±0.72
ApoA, g/l	1.05±0.17 ^a	0.94±0.18	0.43±0.12	0.47±0.28
ApoB, g/l	1.03±0.17	0.96±0.14	0.53±0.11	0.53±0.14
GLU, mmol/l	5.62±1.36	5.91±2.25	4.82±2.29	4.24±2.17
TTR, mg/l	180.12±50.16 ^a	150.08±50.18	100.45±40.22 ^a	68.36±35.29
TP, g/l	60.64±7.18	62.31±7.10	35.91±7.26	35.36±11.53
ALB, g/l	37.58±3.55	36.46±4.72	23.16±5.36	21.44±7.95
ADA, U/l	8.21±10.59	7.90±2.35	5.85±3.16 ^a	15.66±8.89
LDH, U/l	224.72±196	190.73±63.60	319.87±346.25	212.60±131.76

Values are expressed as the mean ± SD. ^aP<0.05, as calculated by the Student's t-test. TG, triglycerides; CHO, cholesterol; Apo, apolipoprotein; GLU, glucose; TTR, transthyretin; TP, total protein; ALB, albumin; ADA, adenosine deaminase; LDH, lactate dehydrogenase.

Results

Concentrations of the biochemical indicators in lung cancer and lung infection patients. The biochemical indicators in the serum and pleural effusion of the two groups of patients were detected. The results showed that the serum levels of CHO, ApoA and TTR in lung cancer patients were higher than that of the lung infection patients (P<0.05). The levels of TTR were higher, whereas the activity of ADA was lower (P<0.05) in the pleural effusion of lung cancer patients compared to lung infection patients (Table II).

Pleural effusion/serum ratios of biochemical indicators in lung cancer and lung infection patients. To further compare the changes of the biochemical indicators between the two groups of patients, the pleural effusion/serum ratios were calculated and analyzed. The results showed that the pleural effusion/serum TTR ratios were higher (P<0.05) in patients with lung cancer compared to lung infection patients, whereas the ratios of ADA were lower (P<0.05) in lung cancer patients. There were no significant differences with regards to the other biochemical indicators (Table III).

Four major TTR peaks were detected by MALDI-TOF-MS. The modified TTR isoforms were detected by MALDI-TOF-MS. The proportion of TTR isoforms in serum and pleural effusion was further analyzed. As shown in Fig. 1, four major peaks, which were native TTR (13,749.86±1.48 m/z), Sul-TTR (13,829.63±2.76 m/z), Cys-TTR (13,870.70±2.70 m/z) and Cysgly-TTR (13,927±5.77 m/z), were observed in the mass spectrum of the serum samples from the patients with lung cancer, lung infection and the healthy volunteers. In addition, the proportion of modified TTR isoforms showed no significant differences among the three groups. The proportion of Cysgly-TTR in the pleural effusion of the patients with lung cancer significantly increased compared to the lung infection patients. The results indicated that the proportion of

Table III. Pleural effusion to serum concentration ratios of the biochemical indicators.

Indicators	Lung cancer (n=92)	Lung infection (n=38)
TG, mmol/l	0.29±0.25	0.28±0.14
CHO, mmol/l	0.35±0.17	0.42±0.23
ApoA, g/l	0.41±0.12	0.53±0.32
ApoB, g/l	0.54±0.18	0.55±0.14
GLU, mmol/l	0.83±0.35	0.74±0.27
TTR, mg/l	0.61±0.19 ^a	0.48±0.23
TP, g/l	0.59±0.12	0.56±0.17
ALB, g/l	0.65±0.14	0.57±0.19
ADA, U/l	0.96±0.49 ^a	1.99±1.08
LDH, U/l	1.56±1.84	1.30±0.81

Values are expressed as the mean ± SD. ^aP<0.05. TG, triglycerides; CHO, cholesterol; Apo, apolipoprotein; GLU, glucose; TTR, transthyretin; TP, total protein; ALB, albumin; ADA, adenosine deaminase; LDH, lactate dehydrogenase.

Cysgly-TTR varied in the pleural effusion between the lung cancer and lung infection patients.

Discussion

Satisfactory biomarkers for screening and early diagnosis of lung cancer remain limited and require further investigation. The identification of novel biomarkers with potential diagnostic value is essential for the development of novel therapeutic strategies in lung cancer. During previous years, a few candidate cancer biomarkers, including CEA, CA125, NSE, SCCAg and Cyfra21-1, have been widely used for the early detection of lung cancer. However, the sensitivity and specificity of these biomarkers are not adequate in establishing pathological diagnosis. Therefore, identification of novel

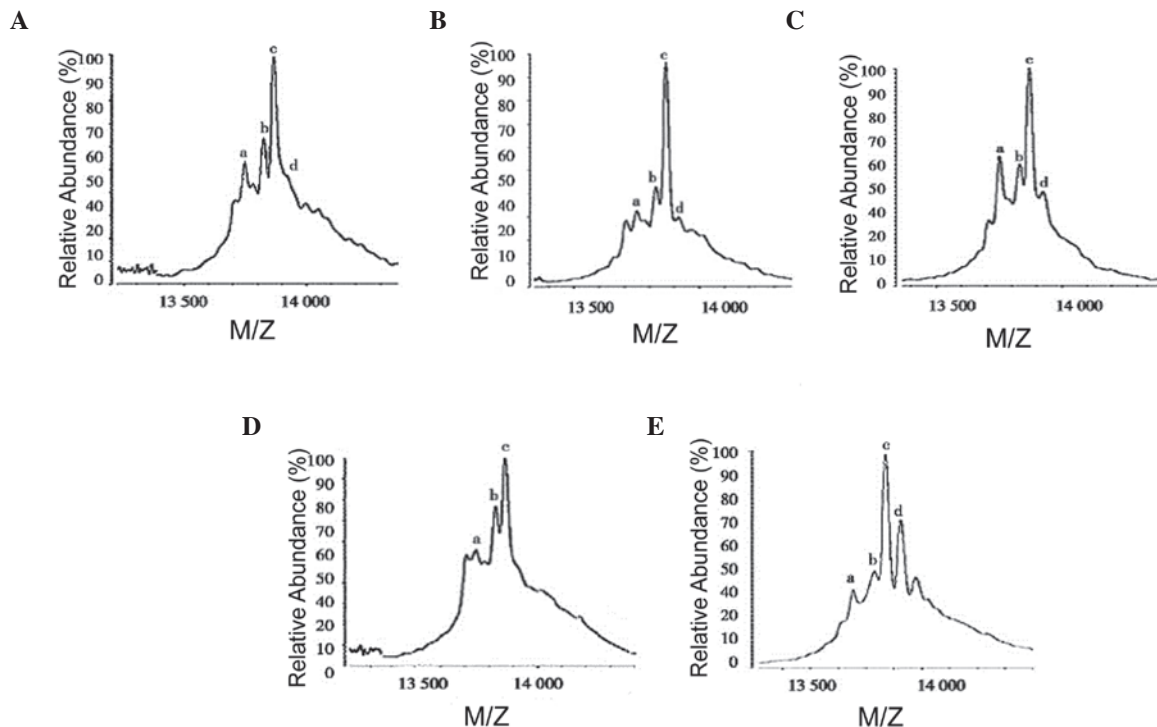


Figure 1. Transthyretin (TTR) modifications in serum and pleural effusion identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Four major TTR peaks, (a) native TTR ($13,749.86 \pm 1.48$ m/z); (b), Sul-TTR ($13,829.63 \pm 2.76$ m/z); (c), Cys-TTR ($13,870.70 \pm 2.70$ m/z); and (d), Cysgly-TTR ($13,927 \pm 5.77$ m/z); were identified in the serum of (A) patients with lung infection; (B) patients with lung cancer; and (C) healthy volunteers. A significant increase was shown in the proportion of Cysgly-TTR in the pleural effusion of (E) patients with lung cancer compared to (D) lung infection patients.

biomarkers that are specific for lung cancer appears to be an important challenge for clinical pathologists.

In the present study, ten biochemical indicators in the serum and pleural effusion from patients with lung cancer and lung infection were detected. The results showed that higher levels of CHO, ApoA and TTR were found in the serum of patients with lung cancer compared to lung infection patients. The levels of CHO and ApoA are closely associated with dietary factors and are nonspecific for the differential diagnosis of lung disease. The changes in pleural effusion may contain information that directly reflects the pathological status for pulmonary diseases. Therefore, the levels of biochemical indicators were measured in the pleural effusion. Higher levels of TTR were found in the pleural effusion of lung cancer patients compared to lung infection patients, whereas the activity of ADA was lower in lung cancer patients. The pleural effusion/serum ratios of the biochemical indicators were further analyzed, and the results showed higher pleural effusion/serum TTR ratios in the lung cancer patients compared to the lung infection patients, whereas the ratios of ADA in lung cancer patients were lower. The results indicated that TTR and ADA may directly reflect the pathological state of pulmonary diseases.

TTR is a homotetrameric protein composed of four 127-amino acid residues subunits synthesized mainly in the liver. The normal concentration of TTR in the blood ranges 20-40 mg/dl. As a well-known negative acute-phase protein, a decreased serum concentration of TTR has been reported in cases of severe liver disease, malnutrition and acute inflammation. In addition, TTR was found to decrease in the sera of patients with ovarian and endometrial cancers (24,25), and the

mechanisms it is involved in remain unknown. Elevated levels of TTR were detected in the aqueous humor of patients with primary open-angle glaucoma and were considered to play a role in the onset of glaucoma (28). Previously, certain studies have shown the potential value of serum TTR in lung cancer diagnosis (14-16).

The results of the present study showed that the TTR levels in the serum and pleural effusion of patients with lung cancer were significantly higher compared to the TTR in lung infection patients. The results were consistent with the study by Liu *et al* (15), which showed decreased levels of TTR in the sera of lung cancer and benign lung disease patients compared to normal sera, and in addition, the decreased level of TTR in benign lung diseases was more evident compared to the patients with lung cancer. Recently, Wang *et al* (29) found a relatively increased level of TTR in the effusions and sera of lung cancer patients compared to the benign inflammatory disease samples. Collectively, the accumulated data indicated a potential value of TTR in lung cancer diagnosis. There are several potential mechanisms for the relatively higher level of TTR in lung cancer patients compared to benign inflammatory disease, such as lung infection. First, TTR is synthesized mainly in the liver and the dysfunction of the liver may contribute to the reduced synthesis of TTR. Second, as a negative acute-phase protein, TTR is downregulated during inflammation, which may be the reason for the relatively lower level of TTR in benign inflammatory disease. Third, overexpression of TTR was detected in lung cancer tissue cells and may be secreted into serum and pleural effusion to supplement the decreasing TTR in the sera and pleural effusion of lung cancer patients (15).

Currently, pathologists have sought to utilize proteomic technologies, such as MS, for the identification of useful biomarkers and therapeutic targets in lung cancer. MS could represent a powerful and sensitive tool for screening protein profiling, as well as providing high-dimensional information regarding proteins (including post-translational proteins). These MS-based proteomics technologies offer novel approaches in identifying the potential biomarkers for lung cancer diagnosis and clinical management of this disease. The detection of TTR isoforms by MS would aid in the analysis of lung cancer pathogenesis and the investigation of biomarker panels for clinical practice.

In order to identify TTR isoforms and further explore their role in lung cancer diagnosis, MALDI-TOF-MS was used in the present study to identify the relative abundance, types and proportion of TTR modification in serum and pleural effusion of patients with lung cancer and lung infection. The present results showed that four major peaks were observed in the mass spectrum of serum samples from patients with lung cancer, lung infection and healthy volunteers, including native TTR, Sul-TTR, Cys-TTR and Cysgly-TTR. In addition, the proportion of modified TTR isoforms showed no significant differences among the three groups. Notably, the proportion of Cysgly-TTR in the pleural effusion of patients with lung cancer significantly increased compared to the lung infection patients. The pleural effusion samples obtained from local lesions reflect the pathological state more accurately. Therefore, an increased proportion of Cysgly-TTR in the pleural effusion of patients with lung cancer may have a potential diagnostic value. The role for TTR post-translational modification involved in the pathogenesis of lung cancer requires further investigation.

In conclusion, higher pleural effusion/serum TTR ratios were demonstrated in lung cancer patients compared to lung infection patients. Furthermore, four modified TTRs were identified in lung cancer by MALDI-TOF-MS and the proportion of Cysgly-TTR was significantly increased in the pleural effusion of patients with lung cancer. The results indicated that a combination of pleural effusion/serum TTR ratios and modified TTR may contribute to the differential diagnosis between lung cancer and lung infection.

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