Reduced transthyretin expression in sera of lung cancer

Liyun Liu,[1,2] Jifu Liu,[3] Songwei Dai,[1,2] Xiaomin Wang,[1,2] Shanshan Wu,[3] Jianing Wang,[1,2] Lingyun Huang,[1,2] Xueyuan Xiao[1,2,4] and Dacheng He[1,2,4]

[1]Key laboratory for Cell Proliferation and Regulation Biology, Ministry of Education, Beijing Normal University; [2]Universities' Confederated Institute of Proteomics, Beijing 100875; [3]Department of Thoracic Surgery, General Hospital of Beijing Unit, PLA, Beijing 100700, China

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Lung cancer is a leading cause of cancer death worldwide, and very few specific biomarkers can be used in its clinical diagnosis. Using surface-enhanced laser desorption–ionization time-of-flight mass spectrometry (MS) to find novel serum biomarkers for lung cancer, we analyzed 227 serum samples, including 146 lung cancers, 41 benign lung diseases and 40 normal individuals. Three peaks, at 13.78, 13.90 and 14.07 k m/z, were significantly lower in lung cancer sera compared with sera from normal individuals ($P < 0.001$), whereas these peaks were higher than those in the sera of benign lung diseases ($P < 0.001$). The peaks were identified as native transthyretin (TTR) and its two variants by one-dimensional polyacrylamide gel electrophoresis, ESI-MS/MS, immunoprecipitation and western blot analysis. An enzyme-linked immunosorbent assay indicated that TTR levels were consistent with surface-enhanced laser desorption–ionization analysis in all groups tested. It gave 78.5% sensitivity and 77.5% specificity for lung cancer versus normal at the cut-off point 115 μg/mL, and 66.7% sensitivity and 64.4% specificity for lung cancer versus benign lung diseases at the cut-off point 88.5 μg/mL. Therefore, TTR may be useful as a biomarker to improve the diagnosis of lung cancer. (*Cancer Sci* 2007; 98: 1617–1624)

Lung cancer is a leading cause of malignancy-related death in China. The 5-year patient survival rate remains at 14% despite diagnostic imaging and therapeutic improvements over the past decades.[1–3] Some tumor markers, including p53, neuro-specific enolase (NSE), carcinoembryonic antigen (CEA) and Cyfra21-1, have been investigated and are used commonly as lung cancer biomarkers;[4,5] however, very few have been accepted for clinical diagnosis, progression and prognosis, either because they lack specificity or because of conflicting reports. Therefore, to discover specific and facilitative biomarkers for lung cancer is critically needed.

Currently, screening of cancer biomarkers has become a hot field in serum proteomics. Serum proteins may serve as indicators of diseases and are also valuable resources for biomarker discovery. An increasing number of cancer-related biomarkers for diagnosis, progression and prognosis have been identified successfully using SELDI technology.[6–12] In a previous study, we used SELDI technology to carry out protein expression profiling of sera from lung cancer and to set up a biomarker pattern related to lung cancer using an automated decision tree algorithm. However, no biomarkers were identified due to the limitations of SELDI technology.[13] Identification of these candidates will not only assist in exploring the mechanisms of carcinogenesis, but will also facilitate the development of traditional multiprotein antibody arrays for early detection of cancer. Recently, as a parallel effort in the discovery of biomarkers, the identification of candidates and evaluation of their use as potential biomarkers for cancer has been given more attention.

Transthyretin is a 55-kDa homotetrameric protein involved in the transport of thyroid hormones in blood,[14,15] and plays a major role in retinol metabolism. It is synthesized partly from liver, partly from extrahepatic tissues such as retinal pigment epithelium, choroids plexus and islet A and B cells.[16–18] As a highly abundant protein, TTR binds to circulating RBP to prevent glomerular filtration of low molecular mass RBP.[19–23] The levels of TTR can be decreased in cases of severe liver disease, malnutrition and acute inflammation.[24–26] In addition, the level of TTR was found to be decreased in the sera of patients with ovarian cancer and advanced cervical and endometrial carcinomas.[27] In the present study, TTR was demonstrated to be a useful biomarker for discriminating lung cancer from both benign lung diseases and a normal control group, suggesting its potential value in lung cancer diagnosis.

Materials and Methods

Serum samples. A total of 227 sera were obtained from the Department of Thoracic Surgery, General Hospital of Beijing Unit, PLA, between December 2003 and July 2005 (Table 1). Sera were collected before any treatment. Sera were collected in 10-mL vacutainers and kept at 4°C for 1 h for clotting, then centrifuged at 1700*g* for 30 min, immediately aliquoted and stored at –80°C. All sera were allowed to thaw once only. Lung cancer staging was based on the tumor–node–metastasis and staging classification of 1989 and remained comparable with the new World Health Organization criteria classification of 1997. The study was approved by the Medical Ethics and Human Clinical Trial Committee at the General Hospital of Beijing Unit, PLA and the First Affiliated Hospital of Jilin University. Informed consent was obtained from all patients and members of the control group.

Protein chip profiling analysis. Serum protein profiling analysis was carried out using CM10 protein chips (Ciphergen, Fremont, CA, USA).[28] Briefly, 20 μL of 9 mol/L urea, 1% CHAPS in PBS (pH 7.4) was added to 10 μL of each serum sample. The mixture was vortexed at 4°C for 30 min and diluted 1:9 in binding buffer (100 mmol/L NaAc [pH 4.0]), and 100 μL of diluted sample was then applied to each spot. After binding at room temperature on a platform shaker at 1400*g*/min for 35 min, the chips were washed three times for 5 min each with 200 μL washing buffer. After air drying, 0.5 μL saturated sinapinic acid was applied twice to each spot. The chips were read using a PBS-IIc ProteinChip Reader (Ciphergen). An automated analytical protocol was used to control the data acquisition process in most of the sample analyses. Data were collected by averaging 128 laser

[4]To whom correspondence should be addressed.
E-mail: xyxiao@bnu.edu.cn, dhe@bnu.edu.cn

Abbreviations: 1-D PAGE, one-dimensional polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; PBS, phosphate-buffered saline; ROC, receiver operating characteristic; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; SELDI, surface-enhanced laser desorption–ionization; TOF-MS, time of flight–mass spectrometry; TTR, transthyretin.

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Table 1. Characteristics of lung cancer patients

Characteristic	No. patients
Total patients	146
Male	105
Female	41
Mean age in years (range)	65 (41–76)
Disease stage	
I	18
II	37
III	59
IV	32
Tumor histology	
Squamous cell	73
Adenocarcinoma	49
Small cell lung cancer	24

shots at an intensity of 220 in the positive mode and a detector sensitivity of 9, and in the optimization range from 3000 to 50 000 Da. Mass accuracy was calibrated externally using all-in-one peptide or all-in-one protein (Ciphergen). We achieved a mass accuracy of 0.1% for protein and polypeptides of 3000–30 000 mass/charge (m/z) with this system. Serum samples from patients and normal controls were run concurrently to minimize experimental variation.

One-dimensional gel and in-gel digestion followed by ESI-MS/MS identification. Equal quantitative serum samples from lung cancer patients and normal individuals were run on 15% SDS-PAGE gels, which were then silver stained. Differentially expressed bands below 16 kDa were excised manually from gels and transferred to 0.5 mL siliconized microfuge tubes. The gel pieces were washed with deionized water three times before the addition of 0.1 mL destaining solution (100 mM sodium thiosulfate and 30 mM potassium ferricyanide solutions, mixed in a 1:1 [v/v] ratio) and incubation at room temperature for 15 min. Then, 25 mM NH_4HCO_3/50% acetonitrile was added to samples until the gel pieces were destained completely. Finally, the gel pieces were washed with 100% acetonitrile for 5 min. The dried gel pieces were then incubated in 10 μL trypsin solution (12.5 ng/mL trypsin dissolved in 25 mM NH_4HCO_3) overnight at 37°C. The tryptic peptide mixture was extracted and purified using a ZIPTIP C18 column (Millipore, Bedford, MA, USA). The resultant peptides were analyzed using Qstar Pulser I Q-TOF-MS (Applied Biosystems/MDS Sciex, Toronto, Canada) mounted with a NanoESI source. The protein identification was determined by MS/MS fragment ions using peptide fragment search and sequence tags for mass matching and protein identity. The search allowed one missed cleavage and possible modifications.

Western blot analysis. The total amount of serum proteins was determined by Bradford assay using bovine serum albumin as a standard (Sigma-Aldrich, St Louis, MO, USA). Aliquots of sera samples were separated by 15% SDS-PAGE, and then transferred to nitrocellulose membranes (Amershan Biosciences, Buckinghamshire, UK). After blocking with 5% skim milk/Tween-20/Tris-buffered saline for 1 h at 37°C, the membranes were incubated with TTR antibody (polyclonal rabbit antihuman, catalog number A0002; Dako, Glostrup, Denmark) at a 1:2000 dilution for 1 h at 37°C, and then with alkaline phosphatase-conjugated goat antirabbit IgG (1:3000; Golden Bridge, Beijing, China) as the secondary antibody for 1 h at 37°C. Color substrate NBT/BCIP (Promega, Madison, WI, USA) was added and incubated for 5 min for color development.[29]

Immunoprecipitation. Protein G Sepharose beads (25 μL; Sigma-Aldrich) were washed three times with PBS. After the careful removal of supernatant, 10 μL TTR antibody was diluted 1:20 in immunoprecipitation buffer (1× PBS, 0.1% Triton X-100 [pH 7.4]), then blended with 25 μL precleared Protein G Sepharose beads for 3 h at room temperature with tumbling. After three washes in PBS, 10-μL serum samples were added to the beads at a dilution of 1:20 and incubated overnight at 4°C with tumbling. After a further three washes with PBS, half of the beads were eluted with organic elution buffer (33.3% isopropanol/16.7% acetonitrile/0.1% trifluoracetic acid).[15] Supernatant and eluate were analyzed using CM10 chips and SDS-PAGE gels. Pure TTR protein from human plasma (P1742; SIgma-Aldrich) was used as the positive control. The negative controls were set up by adding an equal volume of PBS buffer instead of the specific antibody or adding an equal volume of PBS buffer instead of serum samples.

Quantitative validation by ELISA. Total TTR level was quantified using direct ELISA with purified commercial polyclonal antibody against TTR. Pure TTR protein was used as an antigen for standard calibration in each assay. Individual serum samples were diluted 1:10 with coating buffer (0.05 M carbonate buffer [pH 9.6]), and then 100 μL diluted serum was added to each well in a 96-well plate. After incubation overnight at 4°C, the wells were washed three times with washing buffer (1× PBS, 0.05% Tween-20 [pH 7.4]) and blocked with 3% bovine serum albumin. Then, 100 μL TTR antibody (diluted 1:2000) was added and incubated for 1.5 h at 37°C. After three washes, 100 μL secondary antibody (1:6000 dilution, goat antirabbit IgG–horseradish peroxidase (HRP; Golden Bridge) was added to each well and incubated for 1.5 h at 37°C. After washing, the reaction was developed with o-phenylene diamine dihydrochloride (OPD) and terminated by the addition of 3 M H_2SO_4. Absorbance was measured using a microplate reader (model 550; Bio-Rad, Hercules, CA, USA) at 490 nm. The same normal sera were used as a positive control whereas a primary antibody-depleted serum sample was used as a negative control. All analyses were carried out in duplicate.

Statistical analysis. SELDI-TOF-MS data were analyzed using Biomarker Wizard software version 3.1 (Ciphergen). All spectra were baseline subtracted using a fitting window of eight times expected peak width and qualified mass peaks (S/N > 5); m/z between 3000 and 50 000 Da were autodetected. Peak clusters were completed using second-pass peak selection (S/N > 2, within 0.3% mass window) and estimated peaks were added. The peak intensities were normalized to the total ion current of m/z between 1500 and 50 000 Da. A non-parametric test (Mann–Whitney *U*-test) was used here to compare the marker peak intensities from the different groups. When $P < 0.05$, the proteins were defined as positive proteins.

SPSS software version 13.0 (SPSS, Chicago, IL, USA) was used to conduct all statistical comparisons. A non-parametric test (Mann–Whitney *U*-test) was used to compare ELISA results from the different groups. Two-tailed *P*-values of 0.05 or less were considered statistically significant. ROC curve analysis was used to detect the optimal cut-off points (i.e. those with the highest total accuracy) for separating lung cancer from the other groups tested.

Results

Three peaks at 13.78, 13.90 and 14.07 k m/z were significantly decreased in lung cancer patients. Two hundred and twenty-seven serum samples were screened on CM10 chips. After normalization with the intensity of total ions, 15 peaks were found to have discriminatory values in the groups tested, based on their mass intensity both by Biomarker Wizard Software analysis and visually comparing mass spectrum profiles. Among the 15 peaks, the peak at 11 697 m/z had the highest discriminatory power and was identified as serum amyloid A (SAA) (data not shown). The peaks at 13.78 (13777.69 ± 3.29), 13.90 (13899.78 ± 8.91) and 14.07 (14065.02 ± 10.34) k m/z were used to distinguish lung

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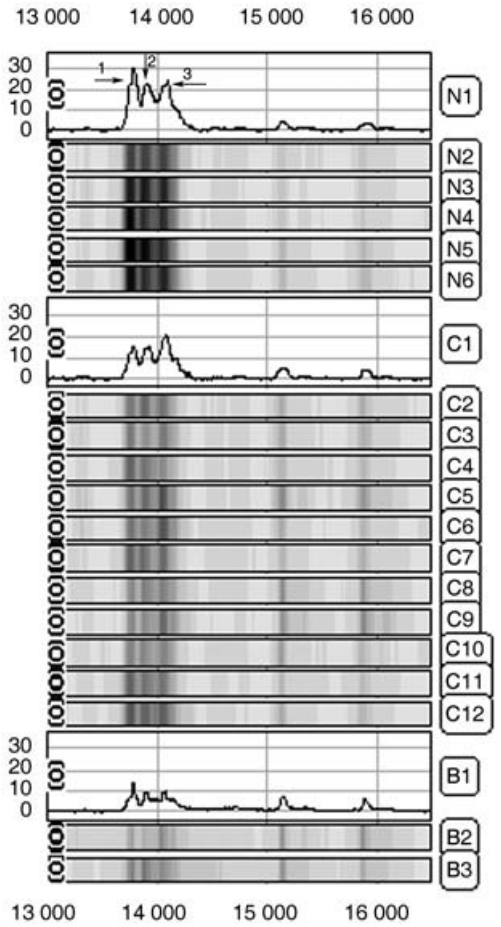

Fig. 1. Surface-enhanced laser desorption–ionization spectra showed three protein peaks at 13.78, 13.90 and 14.07 k m/z on CM10 chips. Normal control, lung cancer and benign lung diseases are shown from top to bottom. The *x*-axis and *y*-axis express m/z and the intensity of the peaks, respectively. Three biomarkers are indicated in bold with black arrows. Nos 1, 2 and 3 represent the 13.78, 13.90 and 14.07 k m/z protein peaks, respectively. N1–6, normal 1–6; C1–12, lung cancer 1–12; B1–3, benign lung diseases 1–3.

cancer from other control groups. These were found to decrease in the lung cancer and benign lung diseases groups (Fig. 1). The average intensities of the 13.78, 13.90 and 14.07 k m/z peaks were 15.45, 14.21 and 16.13 for lung cancer sera, 10.43, 9.64 and 10.77 for benign lung diseases sera, and 23.99, 20.05 and 19.65 for normal sera, respectively. Although there was overlap in the tested groups, visible discrimination between sera of lung cancer and control groups could be achieved when a non-parametric Mann–Whitney *U*-test and ROC curve were used (Table 2; Fig. 2). It was suggested that these three peaks were potential biomarkers for lung cancer diagnosis.

Isolation and identification of the peaks as TTR. To identify proteins that corresponded to the observed 13.78, 13.90 and 14.07 k m/z peaks on the CM10 chips, lung cancer and normal control sera were analyzed by 1-D SDS-PAGE gels with silver staining. In each case, a protein band with an apparent molecular weight of ~16 kDa by SDS-PAGE was found to be significantly downregulated in sera of lung cancer patients compared with normal sera (Fig. 3a). The differentially expressed band at ~16 kDa could very possibly be the protein corresponding to the peaks observed on chips, as migration rates for proteins with molecular masses less than 20 kDa may become more dependent on differences in structural features rather than mass.[30] To confirm this, the differentially expressed band at approximately 16 kDa was excised from the gels for trypsin digestion and identification by ESI-MS/MS. The MS/MS fragment ions were searched using peptide fragment search and sequence tag methods. The candidate biomarker at approximately 16 kDa was identified as native TTR (molecular weight 13761.41 Da, Swiss-Prot accession no. P02766). Three tryptic fragments, 819.4, 697.8 and 634.3 m/z, from the ~16-kDa band were all identified as native TTR and the scores of the tandem MS spectra were 661, 681 and 586 by sequence tag methods (Fig. 3b,c). The same protein identification procedures were repeated on multiple normal and cancer serum samples, and each time the excised protein bands were identified as native TTR.

To further confirm the identities, western blotting with TTR antibody was carried out on a small set of the sera from lung cancer and normal individuals, and only a single specific band at ~16 kDa was observed clearly on both membranes probed with TTR antibody. Furthermore, in the lung cancer group this band was overexpressed more than in the normal individuals (Fig. 3d). Importantly, when pure TTR protein was used as a positive control, the specific band at ~16 kDa was also observed by western blotting with TTR antibody (data not shown). Thus, it is strongly suggested that the differentially expressed band at ~16 kDa was TTR.

Validation of the 13.78, 13.90 and 14.07 k m/z peaks as TTR on CM10 chips. It was possible that the band at ~16 kDa may have represented another protein or may have not been a single protein. As such, immunoprecipitation with commercial TTR antibody was carried out in order to ensure that the 13.78, 13.90 and 14.07 k m/z peaks on the chips actually corresponded to the TTR protein identified by ESI-MS/MS. The supernatant and eluate resulting from the immunoprecipitation of lung cancer and normal individual sera with TTR antibody were analyzed by SELDI-TOF-MS and 1-D PAGE, respectively. It was not surprising to find that selective removal of the three peaks at 13.78, 13.90 and 14.07 k m/z was observed in the sera of lung cancer patients and normal individuals treated with TTR antibody. It was noteworthy that the intensities of the three peaks were spontaneously decreased or depleted, whereas the intensities of other peaks in the same samples did not change (Fig. 4a). An example of 1-D PAGE showed that only one band at ~16 kDa could be observed clearly in the serum sample immunoprecipitated with TTR antibody (Fig. 4b). Moreover, it was exciting to notice

Table 2. Comparison of sensitivities and specificities of the 13.78, 13.90 and 14.07 k m/z protein peaks[†]

Biomarker (k m/z)	Normal versus lung caner			Benign versus lung cancer		
	Cut-off point (peak intensity)	Specificity (%)	Sensitivity (%)	Cut-off point (peak intensity)	Specificity (%)	Sensitivity (%)
13.78	18.35	65.8 (96/146)	72.5 (29/40)	10.76	56.1 (23/41)	66.4 (97/146)
13.90	15.80	65.8 (96/146)	67.5 (27/40)	10.21	63.4 (26/41)	67.8 (99/146)
14.07	17.40	60.3 (88/146)	62.5 (25/40)	11.37	63.4 (26/41)	74.7 (109/146)

[†]Values of the 13.78, 13.90 and 14.07 k m/z intensities greater than or equal to the cut-off value were considered the 'positive group' to compute the true positive ratio (sensitivity) and false positive ratio (1-specificity) for each cut-off value of the peaks.

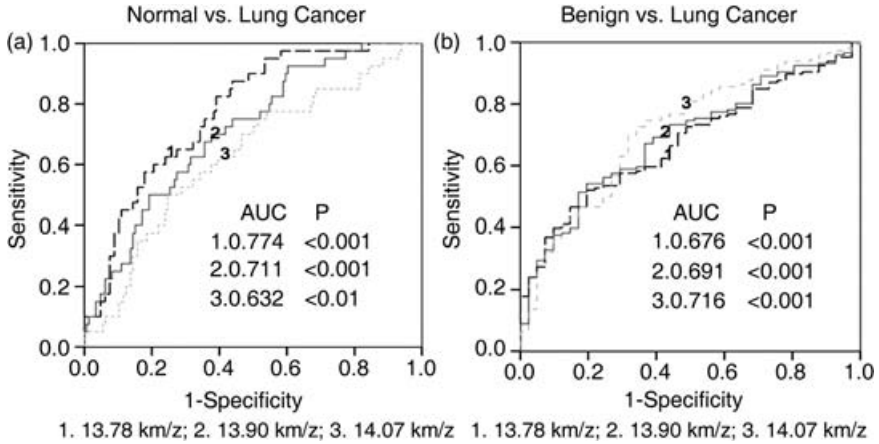

Fig. 2. Comparison of receiver operating characteristic (ROC) curves among 13.78, 13.90 and 14.07 k m/z peaks. (a) ROC curves between lung cancer and the normal control. (b) ROC curves between lung cancer and benign lung diseases. AUC, area under the curve. *P*-values were estimated using the Mann–Whitney *U*-test for two-group comparison.

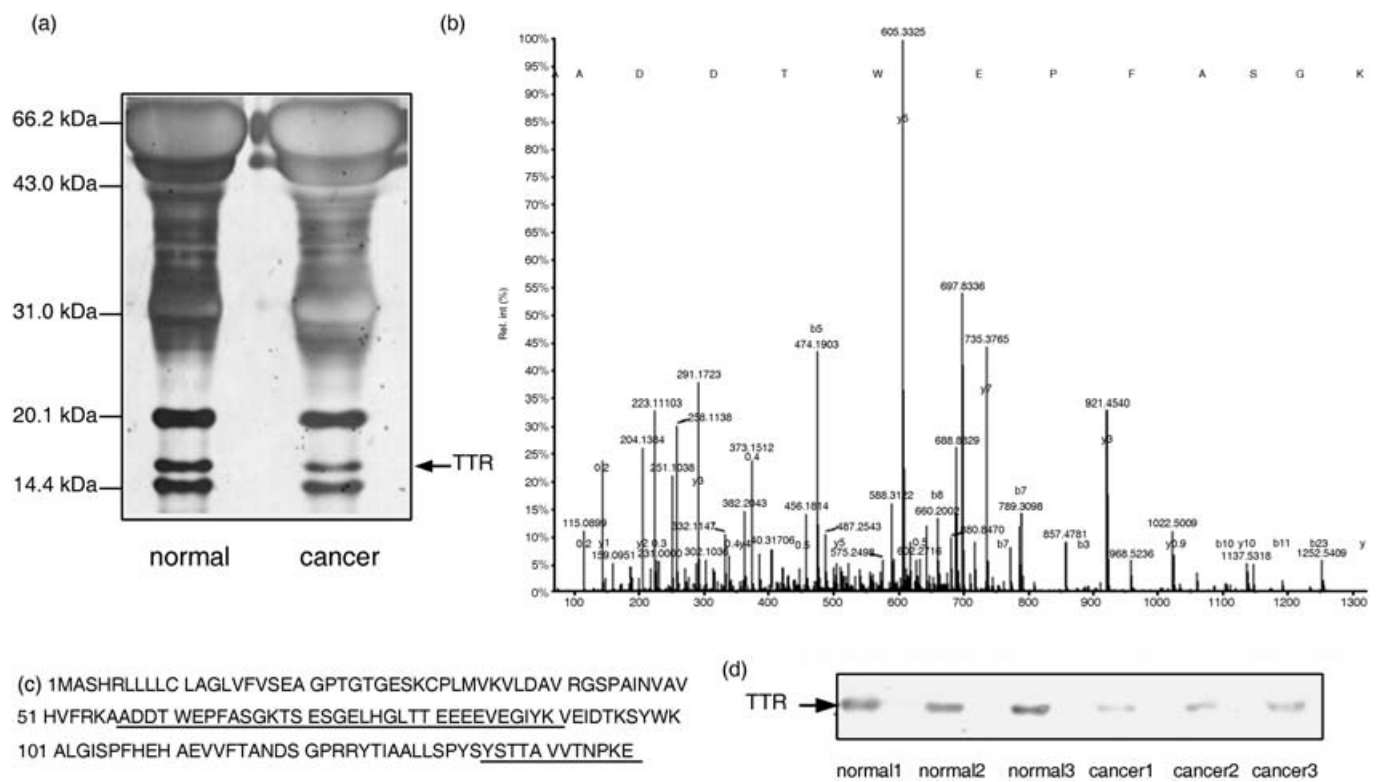

Fig. 3. (a) Sodium dodecylsulfate–polyacrylamide gel electrophoresis (15% gel) with silver staining for normal and lung cancer sera. The differentially expressed band between the two groups was at ~16 kDa, and is indicated by the black arrow. (b) Tandem mass spectrometry spectra of the peptide at 697.82 m/z, for which the sequence was confirmed from the labeled b- and y-ions. (c) Amino acid sequences of transthyretin (TTR). The three matched peptide sequences are underlined. (d) The level of TTR was detected in sera of three normal individuals and lung cancer patients by western blotting. Normal 1–3 represents sera of normal individuals, and cancer 1–3 represents sera of lung cancer patients. The TTR band is indicated by the bold black arrow.

that three peaks at 13.78, 13.90 and 14.07 k m/z were observed when pure TTR protein was examined by SELDI-TOF-MS on a CM10 chip. Empirical validation indicated that all three peaks were TTR, and the 13.78, 13.90 and 14.07 k m/z peaks were variants because native TTR in the serum is more likely to be modified post-translationally in the form of S-cysteinylation and S-glutathionylation.[31] To confirm this guess, some serum samples were treated with DL-dithiothreitol (DTT) to destroy disulfide bonds and then analyzed on CM10 chips. It was inspiring to find that the intensities of the 13.90 and 14.07 k m/z peaks were significantly lowered, or even disappeared, whereas the intensity of the 13.78 k m/z peak was almost unchanged (data not shown). Thus, the combined analysis using immunodepletion, SDS-PAGE and western blotting strongly suggested that the 13.78, 13.90 and 14.07 k m/z peaks observed with SELDI-TOF-MS were native TTR and two variants of TTR.

Serous levels of TTR were significantly decreased in lung cancer patients. Although the discovery of biomarkers by SELDI-TOF-MS depends mainly on peak intensity, the value may be variable due to laser energy and detector sensitivity. In addition, easy operation and reproduction are important factors for clinical usage. Therefore, we used an ELISA to further validate our quantitative analysis of TTR in the sera, including 79 lung cancer patients, 36 benign lung disease patients and 40 normal individuals, as

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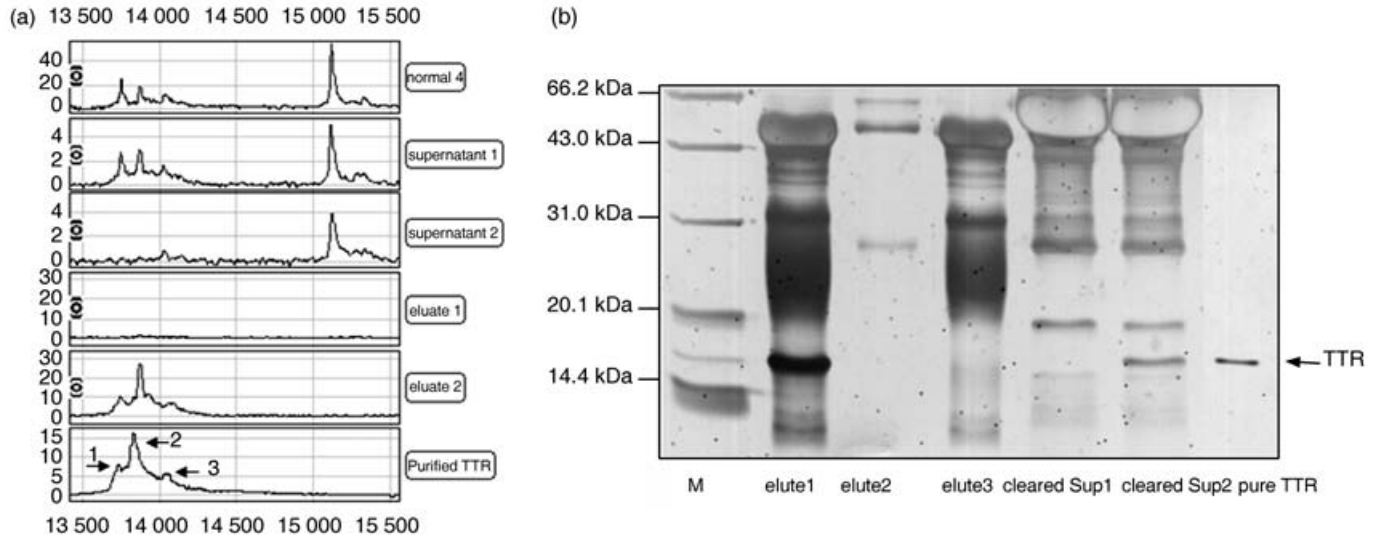

Fig. 4. (a) CM10 protein profiling showed the successful depletion of transthyretin (TTR) peaks in one of the normal serum samples immunoprecipitated with TTR antibody. From top to bottom: untreated normal serum; supernatant of the serum without TTR antibody; supernatant of the serum with TTR antibody; eluate of precipitate of the serum without TTR antibody; eluate of precipitate of the serum with TTR antibody; purified TTR. Black arrows identify depleted normal serum markers. Nos 1, 2 and 3 represent native TTR and its two variants. (b) Immunoprecipitation results of one of the normal serum samples shown by one-dimensional polyacrylamide gel electrophoresis. M, protein marker; elute 1, eluate of precipitate of the normal serum with TTR antibody; elute 2, eluate of precipitate of the normal serum without TTR antibody; elute 3, eluate of precipitate of the TTR antibody without serum; cleared Sup1, supernatant of the normal serum with TTR antibody; cleared Sup2, supernatant of the normal serum without TTR antibody; pure TTR, purified TTR protein. The TTR band is indicated by the bold black arrow.

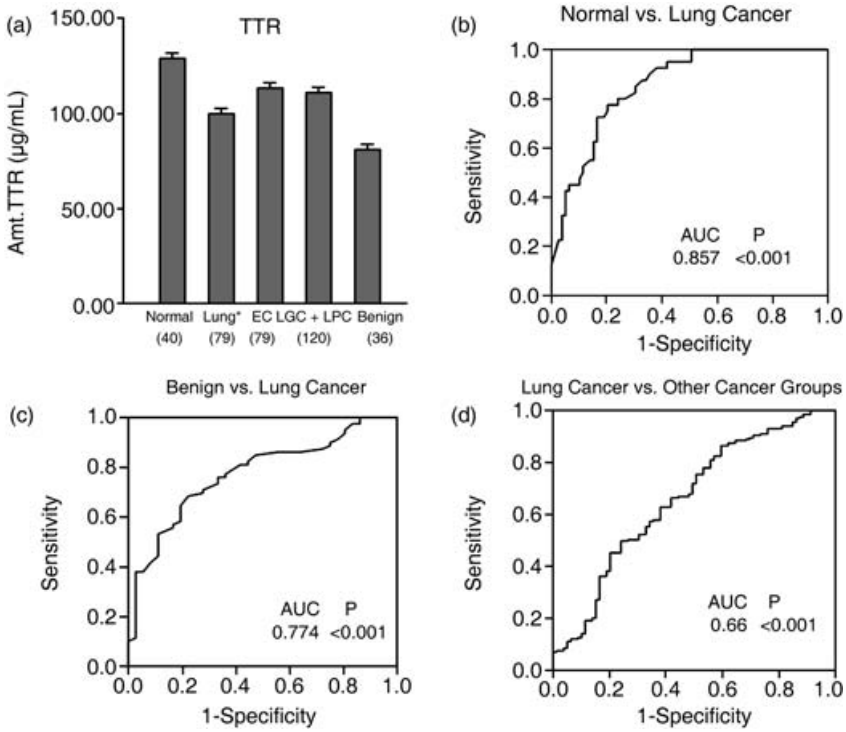

Fig. 5. (a) Histogram of intensity values obtained using enzyme-linked immunosorbant assay for serum transthyretin (TTR) concentrations from lung cancers (Lung), laryngeal carcinomas (LGC), laryngopharyngeal carcinomas (LPC), esophageal cancers (EC), age-matched healthy controls (Normal) and benign lung diseases (Benign). *$P < 0.001$, using a two-group *t*-test (Mann–Whitney test) comparing serum TTR concentrations for lung cancer versus the other tested cancer groups, benign lung disease groups and healthy control group. (b) Receiver operating characteristic (ROC) curves between lung cancer and age-matched healthy controls. (c) ROC curves between the lung cancer and benign lung disease groups. (d) ROC curves between the lung cancer and other tested cancer groups. AUC, area under the curve. *P*-values were estimated by Mann–Whitney *U*-test for two-group comparison.

Table 3. Comparison of the transthyretin (TTR) content of lung cancer versus benign lung diseases and normal groups†

Biomarker	Normal versus lung caner			Benign versus lung cancer		
	Cut-off point (μg/mL)	Sensitivity (%)	Specificity (%)	Cut-off point (μg/mL)	Sensitivity (%)	Specificity (%)
TTR	115	78.5 (62/79)	77.5 (31/40)	88.5	66.7 (24/36)	64.4 (58/79)

†Values of the TTR content greater than or equal to the cut-off value were considered the 'positive group' to compute the true positive ratio (sensitivity) and the false positive ratio (1-specificity) for each cut-off value of the TTR content.

Table 4. Relationship between serum transthyretin (TTR) levels and important parameters in 79 patients with lung cancer (mean ± SD)

Parameter	Sex		Age (years)			Histology†			Stage of cancer†	
	Male	Female	≤50	50–60	>60	Ad	Scc	Sclc	I/II	III/IV
No. patients	46	33	20	22	33	30	35	10	16	59
TTR (μg/mL)	87.30 ± 13.06	120.74 ± 11.97	104.88 ± 18.18	101.23 ± 19.79	100.06 ± 23.48	103.67 ± 22.02	99.28 ± 19.88	106.03 ± 23.09	90.31 ± 18.67	104.78 ± 20.58

†Four serum samples had intangible clinicopathological features. Ad, adenomcarcinoma; Scc, squamous cell carcinoma; Sclc, small cell lung carcinoma.

well as other cancer groups, including 120 laryngeal carcinomas and laryngopharyngeal carcinomas and 79 esophageal cancers. The average total TTR level for lung cancer serous samples (101.3 ± 20.8 μg/mL) was significantly lower than that in the normal control group (129.3 ± 15.6 μg/mL, $P < 0.001$), esophageal cancer group (114.2 ± 22.5 μg/mL, $P < 0.001$) and the laryngeal carcinoma + laryngopharyngeal carcinoma group (112.7 ± 19.0, $P < 0.001$), whereas it was higher than in the benign lung diseases samples (81.5 ± 17.8 μg/mL, $P < 0.001$) (Fig. 5a). When ROC curve analysis was used to detect the optimal cut-off points of TTR for better discrimination of lung cancer patients from other groups, 78.5% lung cancer serous samples were below this cut-off, whereas 77.5% normal serous samples were above it, at an optimal cut-off point of 115 μg/mL. When the optimal cut-off point was 88.5 μg/mL, 64.4% lung cancer serous samples were above this cut-off and 66.7% benign lung disease serous samples were below this cut-off (Table 3; Fig. 5b–d). This demonstrated that using ELISA to measure the sum of all forms of TTR could also discriminate lung cancer patients from benign lung disease patients and normal individuals.

The TTR levels detected were lower in men than in women ($P < 0.001$) and higher in stage III/IV than stage I/II ($P < 0.05$). Although TTR levels decreased with age and were lower in squamous cell carcinomas than in adenomcarcinomas and small cell lung carcinomas, no statistical significances were obtained ($P > 0.05$) (Table 4).

Discussion

As a powerful and sensitive tool to screen protein profiling, SELDI-TOF-MS not only offers discriminatory power for separating any given set of cases and control samples based on their peak intensities, but also provides high-dimensional information about proteins (e.g. post-translational proteins). Therefore, it is becoming more and more useful in the discovery of cancer-related biomarkers. Recently, pathologists have sought to discover the identities of biomarkers in the pattern of disease-related biomarkers in order to understand their significance in disease pathogenesis. Identification of individual differentially expressed proteins with potential diagnostic value is essential for the development of routine clinical analysis, as well as being helpful for exploring the mechanisms of carcinogenesis. In the present study, three peaks at 13.78, 13.90 and 14.07 k m/z were first selected as candidate biomarkers for lung cancer using CM10 chips. Because SELDI technology was limited in supporting direct identification on chips, 1-D PAGE and ESI-MS/MS technologies were used together to separate the candidates. TTR was finally identified as a potential biomarker for lung cancer. It was also found that the 13.90 and 14.07 k m/z peaks were two variants of TTR and the location of the TTR band in SDS-PAGE occurred due to its post-translational modification.

Native TTR is a homotetramer in blood that is synthesized mainly by liver, choroids plexus of brain and pancreatic islet A and B cells. Under physiological conditions, TTR functions as a carrier for both thyroxine and retinol (vitamin A).[14–23] Genetic differences, such as L55P point mutations in TTR, produce an altered primary sequence in the protein. These variant proteins cause the eventual formation of insoluble protein fibrils (amyloid) in various tissues and organs. The deposited amyloid eventually leads to organ dysfunction and ultimately death when major organs such as the heart are involved. The mechanisms by which soluble proteins selfassemble into a fibrillary structure are unknown. However, some variants of TTR exist in sera due to modifications on one readily accessible cysteine that forms adducts with other molecules, including cysteine, cysteinylglycine and glutathione; these can be detected based on their mass shift.[14,32] Typically, native TTR accounts for only 5–15% of total TTR circulating in plasma. The other 85–95% is modified

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post-translationally in the form of S-sulfonation and S-thiolation.[31] Importantly, it has been reported that the levels of TTR could decrease in cases of severe liver disease, malnutrition and acute inflammation.[24–26] In addition, TTR was found to decline in the sera of patients with ovarian cancer, advanced cervical and endometrial carcinomas,[27] and the mechanisms for this are unknown. In the present study, we successfully identified native TTR and its two variants (they are most likely cysteinylated TTR and glutathionylated TTR according to their molecular weight and related reports)[33–35] and found them to be decreased in lung cancer sera using western blotting, immunopreciptation and SELDI-TOF-MS. The identities were further confirmed by comparison of their m/z with pure TTR using SELDI analysis. Interestingly, it was also found that native TTR and the 13.90 k m/z variant were more obviously decreased than the 14.07 k m/z variant in lung cancer. Combined with other reports, our results inferred that a combination of variants of TTR may be associated with certain diseases and this specific biomarker pattern may be very useful for cancer diagnosis. The advantage of SELDI technology in the discovery of novel cancer-related biomarkers should be paid more attention.

Although TTR was not the accepted biomarker for lung cancer, TTR as a new serum biomarker would be useful for lung cancer diagnosis, especially when was used together with another biomarker, SAA,[36,37] which improved the positive diagnostic rate of lung cancer from 78.5 to 93.7%. Importantly, this combined pattern does not exist in other cancers examined in the present paper (data not shown).

Changes in the levels of TTR in serum samples is mainly due to liver activity, which could be converted to the synthesis of acute-phase response proteins and result in a dramatic drop in TTR.[29,38,39] In the present study, the level of TTR was significantly decreased in the sera of lung cancer and benign lung disease patients compared with normal sera. First, we speculated that the lowest level of TTR may occur in lung cancer patients due to liver dysfunction and inflammation. Unexpectedly, the decreased level of TTR in the sera of benign lung diseases was more obvious than that in lung cancer sera. To investigate this obscure phenomenon, expression of TTR was examined in primary lung cancer tissues and non-cancerous lung tissues by immunohistochemistry. It was surprising to note that TTR could be detected in primary lung cancer cells and both the nuclei and cytoplasm of cancer cells showed positive staining. Importantly, some secretary bubbles with stronger TTR staining were observed in the luminal aspect or in the cytoplasm of some lung cancer cells. However, immunoreactivity was not found in either non-cancerous lung tissues or stroma of lung tissue (data not shown). Therefore, we postulate that the contrary results of TTR expression levels in sera and tissues from lung cancer patients bear two explanations. First, the reduction of TTR synthesis in liver should be considered. Second, synthesized TTR in lung cancer tissue cells may be secreted into serum and partially supplement the decreasing TTR in sera of lung cancer patients. However, based only on the immunohistochemical results and fragmentary nature of our current knowledge, it is not possible to draw conclusions about why and how TTR could be involved in tumorgenesis; it may be involved in functions of cancer cell proliferation or apoptosis according to its nuclear staining.

In addition, proteases and protease inhibitors play integral roles in the host response.[40,41] It has become clear that different subsets of proteases could actually be expressed by tumor cells themselves in different types of cancers.[42,43] The former are thought to function in the development of the diseases and may be valuable for cancer diagnosis. Host response proteins exposed to the cancer microenvironment are processed by different proteases; thus, different byproducts or modified forms of host response proteins, even the same parent protein, could be produced and re-enter the circulatory system. For example, inter-α trypsin inhibitor heavy chain 4 was found to exhibit differential modifications in ovarian, breast, colon and prostate cancers.[15,44] Therefore, finding which modified forms of host response protein serve which cancer types will facilitate their inclusion in future diagnostic multimarker panels.

In conclusion, three biomarkers (native TTR and its two variants) were found to be decreased in sera of lung cancer and benign lung disease compared with normal sera. Furthermore, TTR could discriminate lung cancer patients from benign lung disease patients. Therefore, the results suggest that TTR are potential biomarkers for lung cancer. Of course, further validation in a large number of serum samples is needed. It is noteworthy that to our knowledge this is the first study to show that TTR can be synthesized in lung cancer cells. However, further studies exploring why TTR is synthesized in lung cancer cells and the biological functions of TTR in lung cancer are required in order to elucidate the role of TTR in carcinogenesis.

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