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A combined biomarker pattern improves the discrimination of lung cancer

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Abstract

A total of 227 sera were analysed using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) to find novel serum biomarkers for lung cancer. The results showed that the 11.53, 11.70, 13.78, 13.90 and 14.07 k *m/z* peaks identified as native serum amyloid A (SAA), SAA with N-terminal Arg cleaved, native transthyretin (TTR) and its two variants significantly differentiated lung cancer sera from normal control sera (p <0.01). A 'biomarker pattern' combining SAA and TTR was tested to distinguish lung cancer patients from normal control individuals, and the diagnostic positive rate of lung cancer was improved to 91.6%.

Keywords: *SELDI; lung cancer; biomarker; TTR; SAA*

Introduction

Currently, as one of the life-threatening diseases, cancer is the second leading cause of death worldwide. Moreover, lung cancer morbidity and mortality rank first among the various forms of cancer. It is very difficult to diagnose lung cancer in the early stages, and very few biomarkers have been accepted for clinical diagnosis. Despite improvement in diagnostic imaging and therapeutic methods over the past decades, the 5-year patient survival rate remains at 14% (Chen et al. 2003, Granville & Dennis 2005, Xiao et al. 2005). Consequently, it is of critical importance to discover specific and useful biomarkers for lung cancer.

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS), a proteomic technology, has the advantage of high throughput, fast detection and high sensitivity. It has been used extensively for screening serum biomarkers of different cancers. An increasing number of cancer-related biomarkers for diagnosis, progression and prognosis have been successfully identified by SELDI-TOF-MS (Yu et al. 2005, Ward et al. 2006a and b, Adam et al. 2002, Petricoin et al. 2002, Chen et al. 2004). In a previous study, we used SELDI-TOF MS to carry out protein expression profiling of lung cancer sera and to set up a biomarker pattern related to lung cancer using an automated decision tree algorithm (Xiao et al. 2004). However, no biomarker was identified due to the limitations of SELDI-TOF MS (Poon 2007). 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. Therefore, the identification of candidates and the evaluation of their uses as potential biomarkers for cancer have attracted more attention.

In the present study, we used SELDI-TOF MS to carry out protein expression profiling of lung cancer sera and to establish a biomarker pattern of serum amyloid A (SAA) and transthyretin (TTR). This biomarker pattern was demonstrated to be useful for discriminating lung cancer from both benign lung diseases and from a normal

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control group, suggesting a potential value in lung cancer diagnosis.

Materials and methods

Serum samples

A total of 146 sera of lung cancer, including 49 adenocarcinoma, 73 squamous cell carcinoma and 24 small-cell lung carcinoma (Table 1), and 41 sera of benign lung diseases were collected from the Department of Respiratory Medicine, Second Hospital of Xi'an Jiaotong University. Forty sera from a general survey of healthy individuals were used as normal controls and were provided by the State Sport and Physical Culture Administrator. All the sera were collected during October 2003 to July 2005. Sera were collected before any treatment, and were collected into a 10-ml vacutainer and kept at 4°C for 1 h for clotting, then centrifuged at 4000 rpm for 30 min, immediately aliquoted and stored at –80°C.

Protein chip profiling analysis

Serum protein profiling analysis was performed on CM10 (weak cation exchange) protein chips (Ciphergen, Fremont, CA, USA) according to the method described previously (Liu et al. 2008). The chips were assayed on a PBS-II ProteinChip Reader (Ciphergen). An automated analytical protocol was used to control the data acquisition process in most of the sample analysis. Data were collected by averaging 128 laser 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 Biosystem Inc.). We have achieved a mass accuracy of 1000 ppm for protein and polypeptide of 3000–30 000 mass/charge (*m/z*) in this system. Serum samples from patients and normal controls were run concurrently to minimize experimental variation.

Table 1. Characteristics of lung cancer and benign lung disease patients.

Characteristics	*n*
Lung cancer group	
Total patients	146
Male	105
Female	41
Age (years), mean (range)	65 (41–76)
Disease stage	
I	18
II	37
III	59
IV	32
Tumour histology	
Squamous cell carcinoma	73
Adenocarcinoma	49
Small cell lung cancer	24
Benign lung disease group	
Total patients	41
Pulmonary tuberculosis	7
Tuberculous pleurisy	20
Pneumonia	14

Tricine gel electrophoresis and ESI-MS/MS identification

Two serum samples from lung cancer and normal groups were run on Tricine-SDS-PAGE gels (49.5% bis acrylamide) and then the gels were stained with CBB R250. One sample from each group (lung cancer and normal group) was selected and analysed, differentially expressed bands were manually excised from gels, washed with Milli-Q water three times, destained in 25 mM NH_4HCO_3/50% acetonitrile, then dried in 100% ACN. The dried gel pieces were incubated in 10 µl trypsin solution (12.5 ng ml^{-1} trypsin dissolved in 25 mM NH_4HCO_3) for 16–20 h at 37°C. The tryptic peptide mixture was extracted and purified with Millipore ZIPTIP C18 column ((Millipore, Bedford, MA, USA). The resultant peptides were analysed with Qstar Pulser I Q-TOF-MS (Applied Biosystems/MDS Sciex, Toronto, Canada) mounted with NanoESI source. The protein identification was achieved either by PMF or by MS/MS fragment ion using MASCOT software by searching the Swiss-Prot database. The search was performed allowing one missed cleavage and possible modifications.

Immunoprecipitation

Twenty-five microlitres of Protein G sepharose beads (Sigma, St Louis, MO, USA) were washed three times with PBS. After the careful removal of supernatant, 10 µl TTR or SAA antibody was diluted (1:20) in immunoprecipitation buffer (1× PBS, 0.1% Triton X-100, pH 7.4), then blended with 25 µl of pre-cleared Protein G sepharose beads for 3 h at room temperature with tumbling. After washing three times with PBS, 10 µl serum sample was used at 1:20 dilution and then added to beads with tumbling overnight at 4°C. After three washes with PBS, the beads were eluted with organic elution buffer (33.3% isopropanol/16.7% acetonitrile/0.1% trifluoroacetic acid) (Fung et al.2005). The eluate was analysed using CM10 chips. Pure TTR protein from human plasma (P1742; Sigma) was used as the positive control. The negative control was obtained by adding an equal volume of PBS buffer instead of the specific antibody or adding an equal volume of PBS buffer instead of serum sample.

ELISA assay

Total TTR level was quantified directly by ELISA with purified commercial polyclonal antibody against TTR (polyclonal rabbit antihuman, cat. no. A0002; Dako, Glostrup, Denmark) and pure TTR protein was used as an antigen for standard calibration in each assay. SAA was measured by OD value (no pure SAA protein). 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 of a 96-well plate. After incubating 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% BSA. Then, 100 µl TTR antibody (1:2000 dilution) and SAA antibody (polyclonal rabbit antihuman, cat. no. sc-20651, 1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA,) were added and incubated for 1.5 h at 37°C. After three washes, 100 µl secondary antibody was added to each well (1:6000 diluted goat antirabbit IgG-HRP; Golden Bridge Biotechnology Co., Beijing, China) and incubated for 1.5 h at 37°C. After washing, the reaction was developed with OPD and terminated by 3 M H_2SO_4. Absorbencies were measured on a microplate reader (Bio-Rad model 550; Bio-Rad, Hercules, CA, USA) at 490 nm. The same normal sera or lung cancer sera were used as positive controls while a primary antibody-depleted serum sample was used as a negative control. All analyses were assayed in duplicates.

Statistical analysis

SELDI-TOF-MS data were analysed by Biomarker Wizard Software version 3.1 (Ciphergen Biosystems Inc.). SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA) was used to conduct all statistical comparisons. A non-parametric test (Mann–Whitney *U*-test) was employed to compare the different groups in ELISA. Two-tailed *p*-value of 0.05 or less was considered to be statistically significant. ROC (receiver-operating characteristic) analysis was used to detect the optimal cut-off points (i.e. those with the highest total accuracy) for separating lung cancer from other tested groups.

Results

The discovery of lung cancer biomarkers by SELDI-TOF-MS

Two hundred and twenty-seven serum samples were screened on CM10 chips. After normalization with the intensity of total ions, five peaks were found to have strong discriminatory values in the tested groups, based on their peak intensities both by Biomarker Wizard Software analysis and visually comparing mass spectrum profiles. The intensities of 11.53 and 11.70 k *m/z* protein peaks were higher in lung cancer than in normal controls (p <0.01), whereas they were lower than in the benign lung diseases group (p >0.05, no statistical significances); in contrast, the intensities of 13.78, 13.90 and 14.07 k *m/z* protein peaks were lower in lung cancer than in normal controls (p <0.01), whereas they were higher than in the benign lung diseases group (p <0.01). Although there was overlap in the tested groups, visible discrimination between sera of the lung cancer and control groups could be achieved when a non-parametric Mann–Whitney *U*-test and ROC curve were used (Table 2; Figure 1). This suggested that these five peaks were potential biomarkers for lung cancer diagnosis.

When the clinicopathological stage of samples was determined after surgery using the standard criteria based on the revised version of the International System for Staging Lung Cancer, which is adopted by the American Joint Committee on Cancer and the International Union Against Cancer (Mountain 1997), changes of 11.53, 11.70, 13.78, 13.90 and 14.07 k *m/z* protein peaks were found to be correlated with the clinical stage of lung cancer. The intensities of 11.53 and 11.70 k *m/z* protein peaks were higher in stages III and IV than in stages I and II (p >0.05 and p <0.05, respectively) using multiple comparisons of dependent variables (one-way ANOVA). In contrast, the intensities of the 13.78, 13.90 and 14.07 k *m/z* protein peaks were lower in stages III and IV than in stages I and II (p >0.05, no statistical significances).

Similarly, the intensities of the 11.53 and 11.70 k *m/z* protein peaks were much higher in patients with squamous

Table 2. Comparison of sensitivities and specificities of five candidate protein peaks[a].

Candidate biomarkers (k *m/z*)	Normal vs lung caner			Benign vs lung cancer		
	Cut-off values (peak intensity)	Sensitivity (%)	Specificity (%)	Cut-off values (peak intensity)	Sensitivity (%)	Specificity (%)
11.53	2.07	80.8 (118/146)	85 (34/40)	–	–	–
11.70	4.79	76.7 (112/146)	80 (32/40)	–	–	–
13.78	18.35	65.8 (96/146)	72.5 (29/40)	10.76	56.1 (23/41)	66.4 (97/146)
13.90	15.8	65.8 (96/146)	67.5 (27/40)	10.21	63.4 (26/41)	67.8 (99/146)
14.07	17.4	60.3 (88/146)	62.5 (25/40)	11.37	63.4 (26/41)	74.7 (109/146)

[a]Values of five candidate protein peak 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. '–' p >0.05, no statistically significant difference.

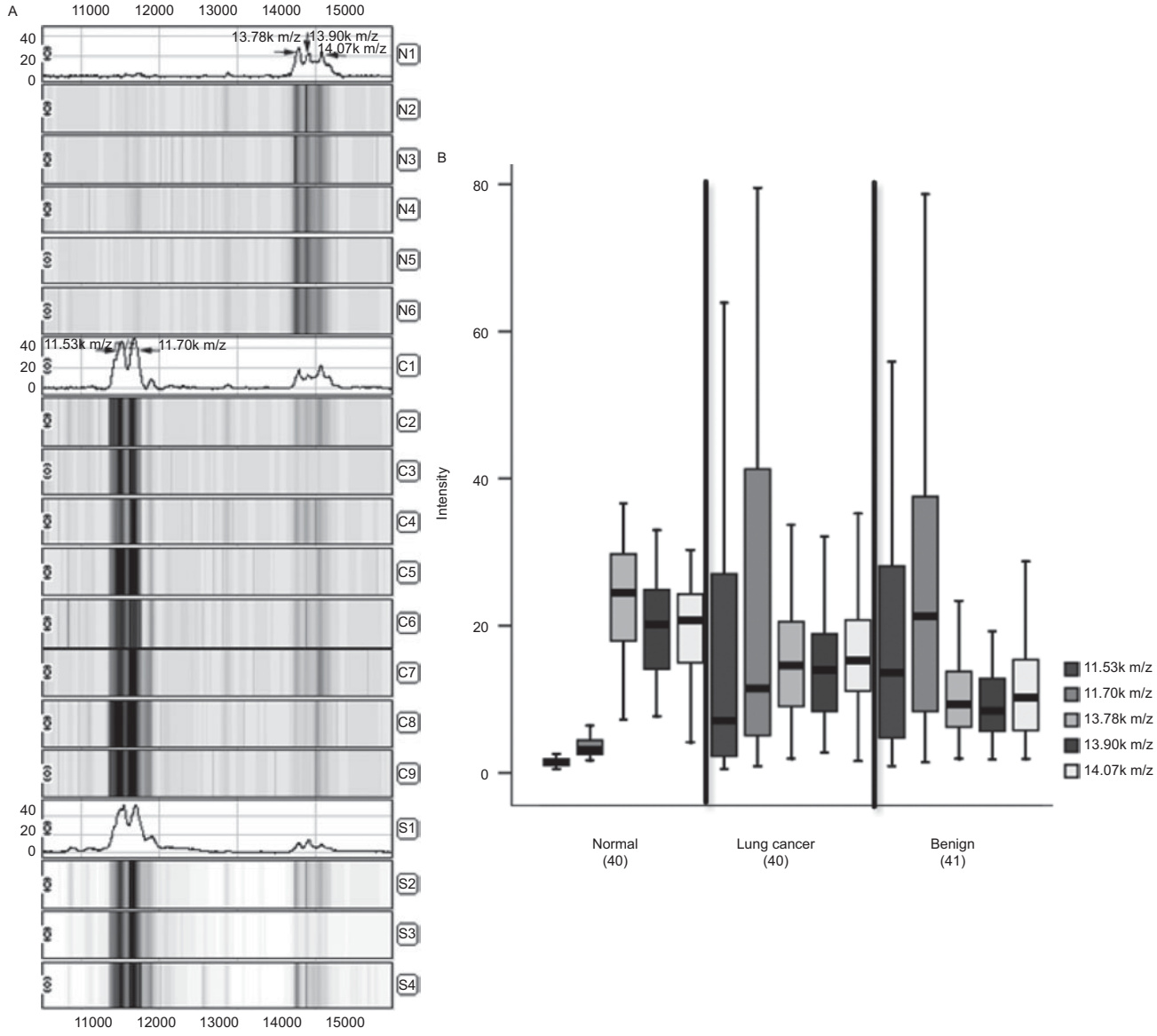

Figure 1. (A) SELDI spectra showing five protein peaks at 11.53, 11.70, 13.78, 13.90 and 14.07 k *m/z* on CM10 chips. Normal controls, lung cancer and benign lung diseases are shown from top to bottom. The *x*-axis and *y*-axes show *m/z* and the intensity of the peak, respectively. N1–6, normal 1–6; C1–9, lung cancer 1–9; B1–4, benign lung diseases 1–4. (B) Boxplot of intensity values for these five protein peaks from lung cancers (Lung cancer), benign lung diseases (Benign) and age-matched healthy controls (Normal).

cell carcinoma than in patients with adenocarcinoma and small-cell lung carcinoma (p >0.05, no statistical significances); in contrast, the intensities of the 13.78, 13.90 and 14.07 k *m/z* protein peaks were lower in patients with squamous cell carcinoma than in patients with adenocarcinoma and small-cell lung carcinoma (p >0.05, no statistical significances, Figure 2).

The biomarkers were identified as SAA and TTR

Equal quantities of lung cancer and normal individual sera were separated using Tricine-SDS-PAGE and the differential protein bands were detected on CBB R250 stained gels according to the image analysis software. Comparing lung cancer sera with normal sera, a protein band at about 11 kDa was significantly upregulated and a 16-kDa protein band was downregulated (Figure 3A). The differentially expressed bands were 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 11 kDa was identified as SAA (Figure 3B) (molecular weight 11682.70 Da, Swiss-Prot accession no. P02735); at about 16 kDa it was identified as native TTR (Figure 3C) (molecular weight 13761.41 Da, Swiss-Prot accession no. P02766). Because the migration

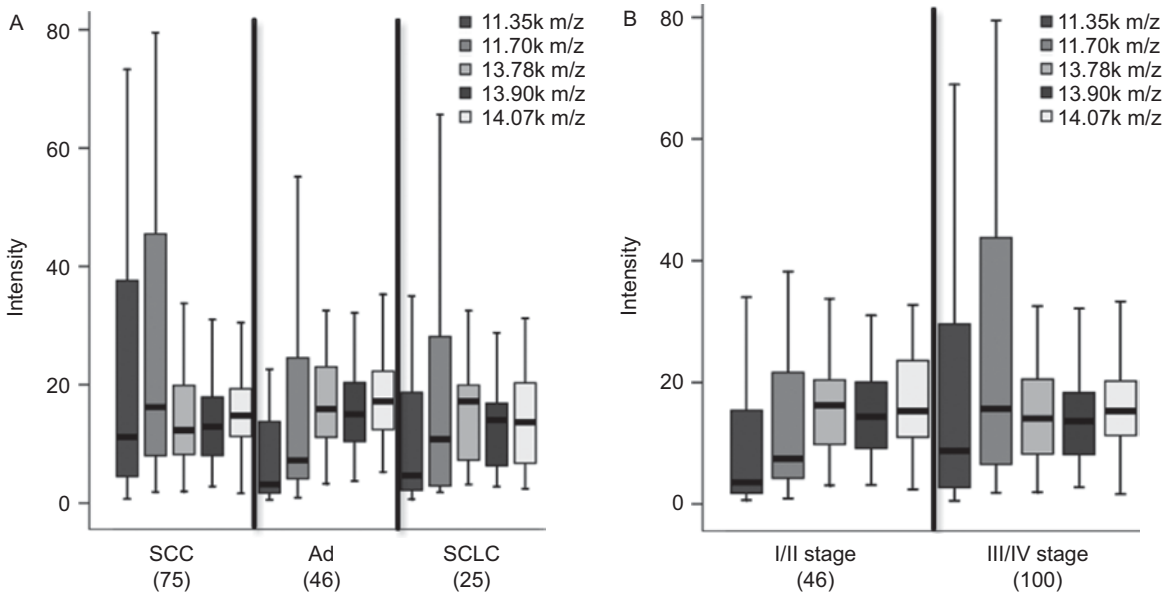

Figure 2. (A) Boxplot of intensity values for 11.53, 11.70, 13.78, 13.90 and 14.07 k *m/z* protein peaks from SCC, Ad and SCLC. (B) Boxplot of intensity values for 11.53, 11.70, 13.78, 13.90 and 14.07 k *m/z* protein peaks from I/II stages and III/IV stages. SCC, squamous cell carcinoma; Ad, adenocarcinoma; SCLC, small cell carcinoma.

rate for proteins with molecular masses <20 kDa may lie more on structural features rather than mass, the differentially expressed band at 16 kDa was identified as TTR (Altland & Winter 2003). It has been reported that the mass spectra are dominated by the presence of intact SAA (molecular weight 11 682.7 Da) and a naturally occurring post-translational modification in which the N-terminal Arg cleaved SAA (R-, N-terminal; molecular weight 11 526.5 Da) (Kiernan et al. 2003). The peaks at 13 880 Da and 14 066 Da contained wild-type TTR conjugated to cysteine (Mcal_13 881 Da) and glutathione (Mcal_ 14 067 Da), respectively (Lim et al. 2003a, b, Gericke et al. 2005, Schweigert et al. 2004, Bergen et al. 2004).

The identification of SAA and TTR was confirmed by immunoprecipitation

In order to confirm the proteins identified by ESI-MS/MS, immunoprecipitation of sera from lung cancer patients and normal individuals was carried out with commercial SAA and TTR antibodies, respectively, and the eluate of the precipitate was analysed by SELDI-TOF-MS on a CM10 protein chip. The 11.53 and 11.70 k *m/z* protein peaks were successfully captured by the eluate of the precipitate of the serum with SAA antibody; however, eluate of the precipitate of the serum without SAA antibody captured weakly non-specific peaks (Figure 4A); in the same way, the 13.78, 13.90 and 14.07 k *m/z* peaks were specifically captured by the eluate of the precipitate of the serum with TTR antibody, whereas they were not captured by the eluate of the precipitate of the serum without TTR antibody; moreover, 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 (Figure 4B). Therefore, the 13.90 and 14.07 k *m/z* peaks may represent different variants of native TTR (Lim et al. 2003a and b).

To confirm this theory, some serum samples were treated with DL-dithiothreitol (DTT) to destroy disulfide bonds and then analysed on CM10 chips. We found that the intensities of the 13.90 and 14.07 k *m/z* peaks were significantly lower, or even disappeared, whereas the intensity of the 13.78 k *m/z* peak was almost unchanged (Figure 5). Thus, the combined analysis using immunoprecipitation, Tricine-SDS-PAGE and ESI-MS/MS strongly suggested that the 11.53 and 11.70 k *m/z* protein peaks observed with SELDI-TOF-MS on a CM10 chip were native SAA and a variant of SAA, while the 13.78, 13.90 and 14.07 k *m/z* peaks were native TTR and two variants of TTR.

The validation by ELISA assay

Based on the identified SAA and TTR, we randomly selected the sera of 79 lung cancer patients, 40 normal individuals and 36 benign lung disease patients from the same set of sera with which CM10 chips were run and performed an ELISA assay. It was found that the levels of SAA (OD value) in lung cancer sera were significantly higher than those in the normal control group (p <0.01), whereas they were lower than in the benign lung diseases group (p >0.05, no statistical significance); the levels of TTR (μg ml^{-1}) in lung cancer sera were significantly lower than those in the normal control group (p <0.01), whereas they were higher than in the benign lung

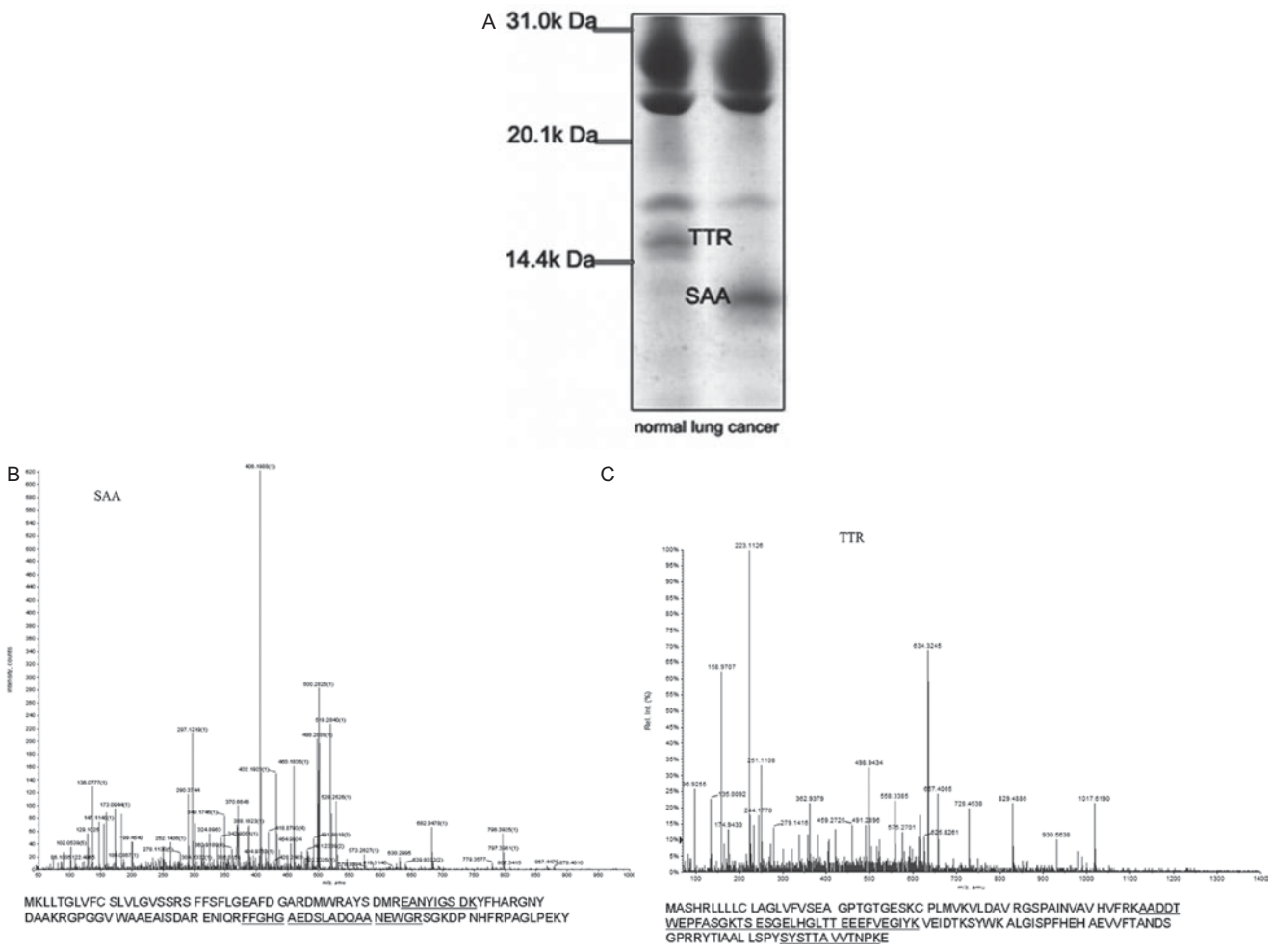

Figure 3. (A) Tricine-SDS-PAGE gel electrophoresis with Coomassie Brilliant Blue staining for normal and lung cancer sera. The differentially expressed bands between the two groups were at 11 kDa and 16 kDa, and were identified as serum amyloid A (SAA) and transthyretin (TTR). (B) Tandem mass spectrometry spectra of the peptide at 498.73 *m/z,* and amino acid sequences of SAA and the two matched peptide sequences are underlined. (C) Tandem mass spectrometry spectra of the peptide at 634.3 *m/z,* and amino acid sequences of TTR and the three matched peptide sequences are underlined.

diseases group (p <0.01). Results from ELISA consisted of data from chip arrays. When ROC curve analysis was used to detect the optimal cut-off points of SAA and TTR for better discrimination of lung cancer patients from other groups, at an optimal cut-off point of 0.051 OD of SAA for differentiating lung cancer sera from normal sera, the sensitivity was 60.8% and the specificity was 60.0%; at an optimal cut-off point of 115 μg ml^{-1} of TTR for differentiating lung cancer sera from normal sera, the sensitivity was 78.5%, the specificity was 77.55%. When the optimal cut-off point was 88.5 μg ml^{-1} of TTR for differentiating lung cancer sera from benign lung disease sera, the sensitivity was 66.7%, the specificity was 64.4%. Although the level of SAA was decreased in the lung cancer group compared with the benign lung diseases group, no statistically significant differences were obtained (p >0.05), so we did not measure the sensitivity and specificity (Figure 6, Table 3).

The sensitivity and specificity were improved with the 'biomarker pattern'

Bu using SELDI and ELISA, we found that SAA and TTR constituted a 'biomarker pattern' of lung cancer. Using the 'biomarker pattern' to diagnose lung cancer, the sensitivity was improved to 93.7% and the specificity was increased to 87.5%; the rate of accuracy was increased to 91.6% (Table 4).

Discussion

Recently, the focus of cancer research has expanded from genetic information in the human genome to protein expression analyses. SELDI-TOF-MS has frequently been used and has demonstrated considerable promise for the discovery of biomarkers in cancer (Gericke et al. 2005). In our study, five peaks were first screened

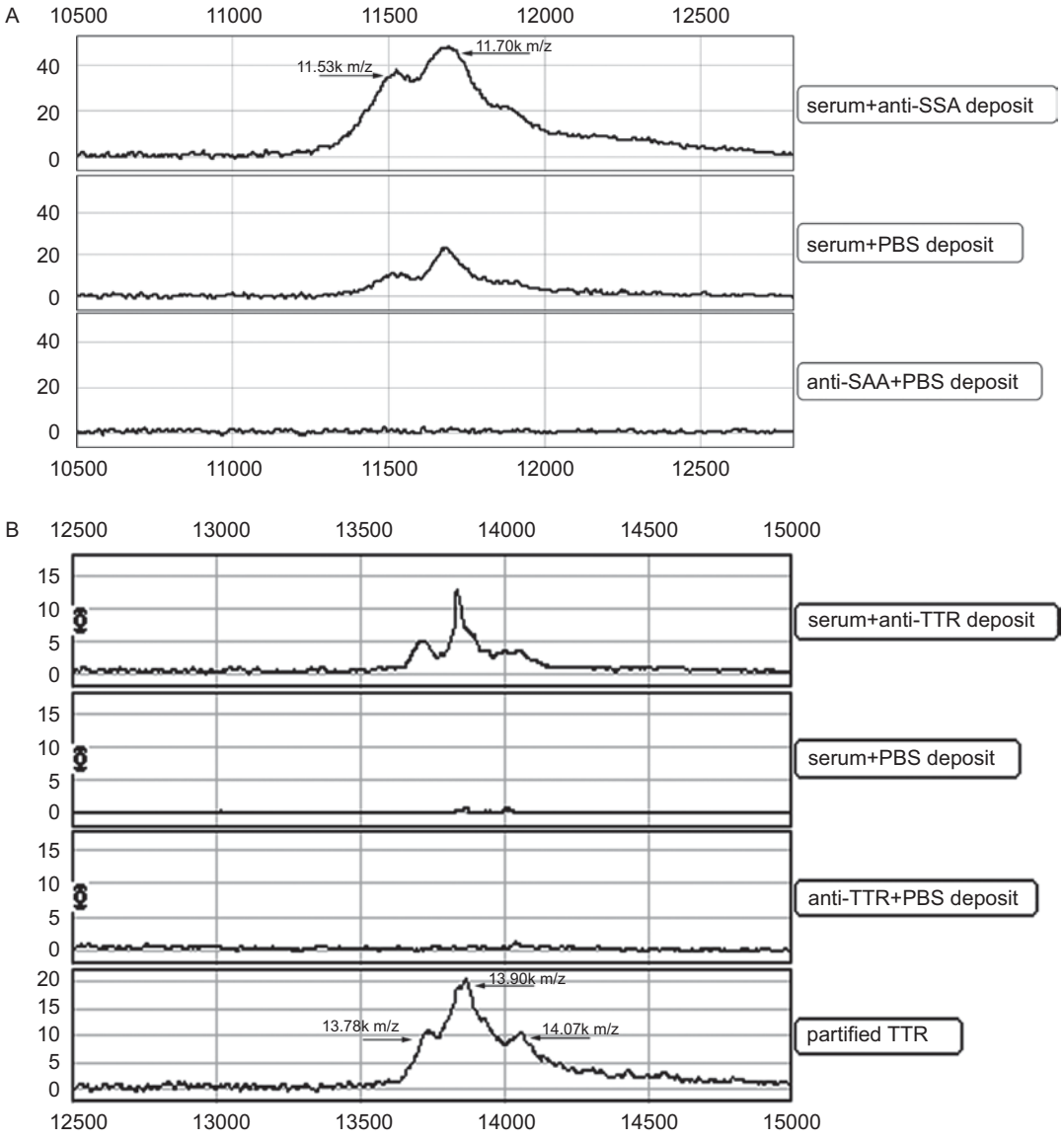

Figure 4. (A) CM10 protein profiling shows the successful depletion of serum amyloid A (SAA) peaks in one of the lung cancer serum samples immunoprecipitated with SAA antibody. From top to bottom: eluate of precipitate of the serum with SAA antibody; eluate of precipitate of the serum without SAA antibody; eluate of precipitate of PBS with SAA antibody. (B) CM10 protein profiling shows the successful depletion of transthyretin (TTR) peaks in one of the normal serum samples immunoprecipitated with TTR antibody. From top to bottom: eluate of precipitate of the serum with TTR antibody; eluate of precipitate of the serum without TTR antibody; eluate of precipitate of PBS with TTR antibody.

as candidate biomarkers for lung cancer using SELDI protein chips. Because SELDI technology is limited in supporting direct protein identification on chips, the combined analysis using immunoprecipitation, Tricine-SDS-PAGE and ESI-MS/MS strongly suggested that the 11.53 and 11.70 k *m/z* protein peaks were native SAA and a variant of SAA, the 13.78, 13.90 and 14.07 k *m/z* peaks were native TTR and two variants of TTR.

In the present study, we identified native TTR and its two variants (they are most likely cysteinylated TTR and glutathionylated TTR according to their molecular weight and related reports) (Bergen et al. 2004, Schweigert et al. 2004, Basu & Sasmul 1988) and found them to be decreased in lung cancer sera (Liu et al. 2007a). Furthermore, native TTR and the 13.90 k *m/z* variant decreased more obviously than the 14.07 k *m/z* variant in lung cancer. It has been shown that the TTR level in lung cancer sera was correlated with clinical stage; it was higher in stages III and IV than in stages I and II. The level of TTR in sera is mainly impacted by liver function, malnutrition and acute inflammation (Imanishi 1981, Marten et al. 1996, Ritchie et al. 1999). Because lung cancer at an early stage is usually followed by inflammation, severe liver disease and cachexia are generally found in the terminal stage of lung cancer; therefore TTR decreases in lung cancer

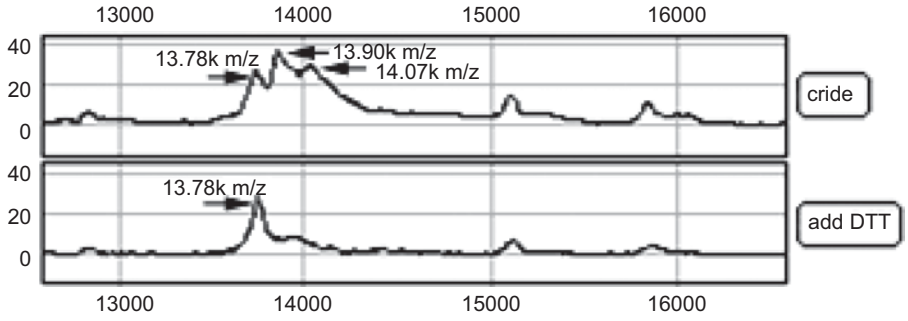

Figure 5. SELDI spectra show protein peaks of transthyretin (TTR) on CM10 chips. The *x*- and *y*- axes express *m/z* and the intensity of the peak, respectively. Crude, serum without treatment; add DTT, serum with 0.1 M dithiothreitol. TTRs are indicated in bold with black arrows.

sera. The content of TTR in sera will increase after tumour excision or on improving the nutritional status (Ellegård & Bosaeus 2008, Ihara et al. 2003). The decrease of TTR expression in sera of benign lung diseases (including pneumonia and tuberculosis) is related to inflammation. When inflammation disappeared, TTR expression was increased (Liu et al. 2007b). The mechanisms for this phenomenon need further study for verification.

SAA is a family of proteins encoded in a multigene complex. Acute-phase isotypes SAA1 and SAA2 are synthesized in response to inflammatory cytokines (Yamada 1999, Malle & de Beer 1996). Under normal physiological conditions, SAA is produced by the liver parenchymal cells and secreted into the blood (Raynes et al. 1991), and it quickly combines with high-density lipoproteins. Usually, 90% of the SAA protein is a combination of high-density lipoproteins (Bausserman et al. 1984). Despite the widespread existence of SAA, it is in low levels in normal sera (d'Eril et al. 2001). When cancer occurs, the SAA level in the blood rises sharply. The elevation of SAA was detected in sera of renal cell carcinoma and colon cancer patients (Kimura et al. 2001, Glojnaric et al. 2001). In the serum of prostate cancer, the content of SAA increased 500-fold more than in the removed tumour. As SAA expression is higher in sera of metastatic diseases than in restrictive diseases, it could be used as one of the protein indicators to assess the certain types of cancer recurrence, metastasis and prognosis (Kaneti et al.1984). In addition, SAA level is also increased in various injuries or other diseases, such as trauma, infection, inflammation, rheumatoid arthritis and amyloidosis disease (Benson & Cohen 1979). However, Cho et al. (2004) found that in the 31 relapsed NPC patients, infection resulting in neutropenic fever was associated with SAA elevation on only two occasions. Therefore, the majority of the SAA elevation in the relapsed patients appeared to be unrelated to the benign cause of infection and inflammation, but to be associated with the relapse. The reasons for the association of an acute-phase protein with cancer relapse remains largely unknown (Cho et al. 2004). The relationship between SAA and lung cancer has been reported (Howard et al. 2003). In our study, we found that SAA expression was increased in the sera of lung cancer patients, and it had the highest expression in squamous cell carcinoma. SAA expression in sera was correlated with the clinical stage of lung cancer; its expression level was higher when the condition was worse. Obstructive pneumonia most likely occurs in lung cancer patients, especially in advanced stages, therefore, it may be one of the reasons why elevation of SAA was higher in III/IV stages than in I/II stages. On the other hand, SAA elevation was also detected in some patients with pneumonia and pulmonary tuberculosis. However, SAA in pulmonary inflammation was temporarily elevated and recovered soon after the elimination of infection, which may represent a primary difference between benign and malignant lung diseases.

Because the expression of SAA or TTR was increased or decreased in sera of different tumours, the specificity was not high for SAA or TTR to diagnose lung cancer separately. In our study, we found that SAA and TTR formed a specific 'biomarker pattern' of lung cancer; using this 'biomarker pattern' to diagnose lung cancer, the sensitivity and specificity were greatly improved. The diagnostic accuracy could be increased to 91.6%. We showed SAA plus TTR could discriminate lung cancer from benign diseases. We were not be able to explain the reasons or mechanisms why the biomarkers went up or down in the benign group, in fact, even in the lung cancer group. Inflammation is just reported to be a possible factor in influencing the TTR and SAA expression.

In summary, five serum biomarkers were screened and identified by SELDI-TOF-MS and ESI-MS/MS; these could help in the diagnosis and prognosis of lung cancer. At the same time, we found that the 'combination of SAA and TTR' improved the sensitivity and specificity for cancer diagnosis compared with SAA or TTR alone. This suggests a more accurate diagnosis for cancer.

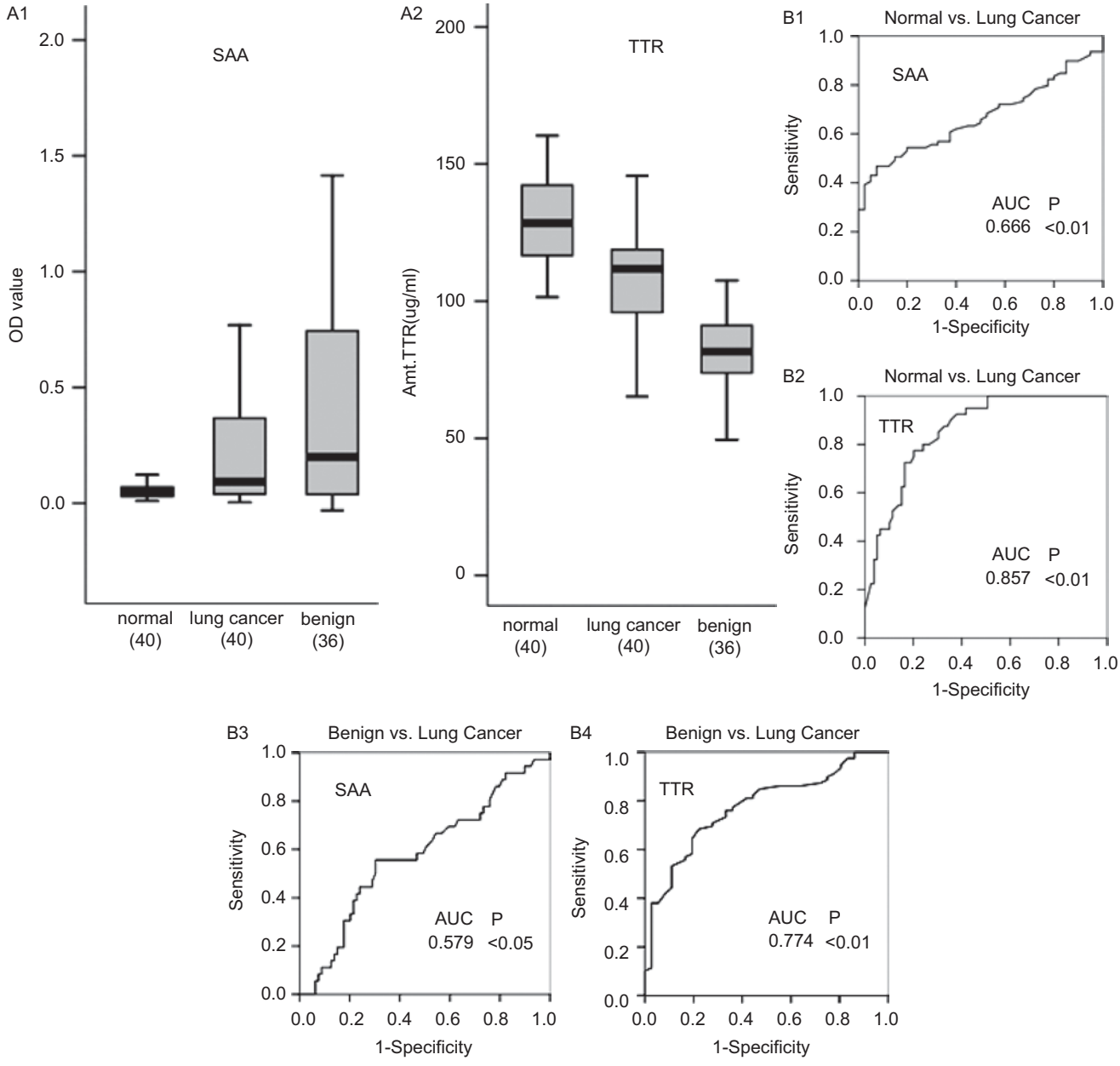

Figure 6. (A1 and A2) Boxplot of intensity values obtained using enzyme-linked immunosorbent assay for serum transthyretin (TTR) concentrations and serum amyloid A (SAA) concentrations from lung cancers (lung), age-matched healthy controls (normal) and benign lung diseases (benign). (B1 and B2) Receiver-operating characteristic (ROC) curves of SAA and TTR between lung cancer and age-matched healthy controls. (B3 and B4) ROC curves of SAA and TTR between lung cancer and benign lung disease groups. AUC, area under the curve; *p*-values were estimated by Mann–Whitney *U*-test for two-group comparison.

Table 3. Comparison of serum amyloid A (SAA) and transthyretin (TTR) in sera of three groups[a].

Candidate biomarkers	Normal vs lung caner			Benign vs lung cancer		
	Cut-off values	Sensitivity (%)	Specificity (%)	Cut-off values	Sensitivity (%)	Specificity (%)
SAA	0.051 (OD)	60.8 (48/79)	60.0 (24/40)	–	–	–
TTR	115 (μg ml^{-1})	78.5 (62/79)	77.5 (31/40)	88.5 (μg ml^{-1})	66.7 (24/36)	64.4 (58/79)

[a]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. Comparison of serum transthyretin (TTR) and serum amyloid A (SAA) between lung cancer and normal groups.

	Normal vs lung cancer		
	TTR	SAA	TTR and SAA
Sensitivity(true positive)	78.5% (62/79)	60.8% (48/79)	93.7% (74/79)
Specificity(true negative)	77.5% (31/40)	60.0% (24/40)	87.5% (35/40)
Accuracy	78.2% (93/119)	60.5% (72/119)	91.6% (109/119)
False-positive rate	22.5% (9/40)	40.0% (16/40)	12.5% (5/40)

Declaration of interest

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