# Serum Amyloid A Protein: A Potential Biomarker Correlated With Clinical Stage of Lung Cancer[1]

DAN-HUI LIU[a,#], XIAO-MIN WANG[a,#], LI-JUAN ZHANG[a], SONG-WEI DAI[a], LI-YUN LIU[a], JI-FU LIU[b], SHAN-SHAN WU[b], SHUAN-YING YANG[c], SAM FU[d], XUE-YUAN XIAO[a,*], AND DA-CHENG HE[a,2]

[a]*Key Laboratory of Cell Proliferation and Regulation of Ministry of Education, Beijing Normal University, Beijing 100875, China;* [b]*Department of Thoracic Surgery, General Hospital of Beijing Command, PLA, Beijing 100700, China;* [c]*Department of Respiratory Medicine, Second Hospital of Xi'an Jiaotong University, Xi'an 710004, Shaanxi, China;* [d]*Ciphergen Biosystems, Inc., Fremont, CA 94555, USA*

**Objective** To identify serum diagnosis or progression biomarkers in patients with lung cancer using protein chip profiling analysis. **Method** Profiling analysis was performed on 450 sera collected from 213 patients with lung cancer, 19 with pneumonia, 16 with pulmonary tuberculosis, 65 with laryngeal carcinoma, 55 with laryngopharyngeal carcinoma patients, and 82 normal individuals. A new strategy was developed to identify the biomarkers on chip by trypsin pre-digestion. **Results** Profiling analysis demonstrated that an 11.6kDa protein was significantly elevated in lung cancer patients, compared with the control groups ($P$<0.001). The level and percentage of 11.6kDa protein progressively increased with the clinical stages I-IV and were also higher in patients with squamous cell carcinoma than in other subtypes. This biomarker could be decreased after operation or chemotherapy. On the other hand, 11.6kDa protein was also increased in 50% benign diseases of lung and 13% of other cancer controls. After trypsin pre-digestion, a set of new peptide biomarkers was noticed to appear only in the samples containing a 11.6kDa peak. Further identification showed that 2177Da was a fragment of serum amyloid A (SAA, MW 11.6kDa). Two of the new peaks, 1550Da and 1611Da, were defined from the same protein by database searching. This result was further confirmed by partial purification of 11.6kDa protein and MS analysis. **Conclusion** SAA is a useful biomarker to monitor the progression of lung cancer and can directly identify some biomarkers on chip.

**Key words**: Lung cancer; Serum amyloid A; On chip identification; Surface enhanced laser desorption/ionization; Biomarker

## INTRODUCTION

Currently screening cancer biomarkers is a hot field in serum proteomics because serum proteins may often serve as indicators of disease and are a rich source of biomarkers. Some biomarkers for diagnosis, progression, and prognosis of cancer have been identified by either surface-enhanced laser desorption/ionization (SELDI)[1-9] or 2D gel[10-13]. SELDI is a useful and powerful platform to analyze the raw mixture samples directly and discover biomarkers in a large number of samples with its fast screening ability and the superior visibility of results, which avoids the complicacy of personal difference.

Up to now, lung cancer is the leading cause of malignancy-related deaths in China, and the five-year survival rate of patients is about 14%, despite diagnostic imaging and therapeutic improvements over the past decade[14]. Some tumor markers, including p53, NES, CEA, Cyfra21-1, and CA19-9, have been investigated and are commonly used as lung cancer biomarkers[15-18]. However, few biomarkers have been accepted as indicators of clinical diagnosis, progression and/or prognosis, either because of lacking specificity or because of conflicting reports. Therefore, to discover specific or novel biomarkers of lung cancer is urgently needed.

Recently, some new potential biomarkers for lung cancer diagnosis have been reported, one of which is serum amyloid A (SAA) which is elevated in

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[2]Correspondence should be addressed to Dr. Da-Cheng HE and Xue-Yuan XIAO, Beijing Normal University, Key Laboratory of Cell Proliferation and Regulation of Ministry of Education, 19 Xinjiekouwai St., Beijing 100875, China. Fax: 86-10-58805042. E-mail: dhe@bnu.edu.cn, xyxiao@bnu.edu.cn.

[#] These authors contribute equally to this article.

Biographical note of the first author: Dan-Hui LIU, female, born in 1980, Ph. D. of Key Laboratory of Cell Proliferation and Regulation of Ministry of Education, Beijing Normal University, majoring in cell biology and proteomics; Xiao-Min WANG, male, born in 1982, graduate student of Key Laboratory of Cell Proliferation and Regulation of Ministry of Education, Beijing Normal University, majoring in cell biology and proteomics.

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various cancers[19-20]. The level of SAA is increased in lung cancer patients[21-24], whereas the relatively small number of samples and lack of clinicopathological staging in such patients are the shortcomings. On the other hand, in our previous study, sera from hundreds of lung cancer patients and normal individuals were screened by ProteinChip Array and the classification tree with 5 biomarkers has been set up[25-26]. Although its sensitivity and specificity in blind test are better than any of the clinical serological tests for lung cancer, none of the biomarkers has been identified due to the technique limitation. In fact, among the biomarkers discovered by SELDI in various cancers, only a few have been identified up to date. In the present study, a new strategy was developed to directly identify the new peptide biomarkers on chip instead of purified biomarkers by SDS-PAGE or some other techniques.

## MATERIALS AND METHODS

### *Serum Samples*

Sera from patients with initial diagnosis of lung cancer were procured from the Department of Thoracic Surgery, General Hospital of Beijing Command and the Department of Respiratory Medicine, Second Hospital of Xi'an Jiaotong University. The study population of lung cancer patients is described in Table 1. In addition, serum samples were collected from lung cancer patients one week before treatment and after two courses of chemotherapy or one week after operation for further protein chip profiling study. These serum samples were obtained from patients at the Department of Respiratory Medicine, Second Hospital of Xi'an Jiaotong University. Of the patients, 13 were treated with vinorelbine and cisplatin combination chemotherapy and 7 with operation. Vinorelbine was administered at a dose of 25 mg/kg of body weight and cisplatin was given 80 mg/kg of body weight. One hundred and fifty-five sera from 55 patients with laryngopharyngeal carcinomas (LPG), 65 with laryngeal carcinomas (LGC), 19 with penumonia, and 16 with pulmonary tuberculosis (TB) as non-lung cancer controls were collected from the Department of Otolaryngology-Head and Neck Surgery, Third Hospital of Jilin University and the Department of Respiratory Medicine, Second Hospital of Xi'an Jiaotong University. Sera under the study were collected between December 2002 and July 2004. After informed consent was obtained from the patients, five milliliters of blood was collected into a 10 mL vacutainer and kept at 4℃ for 1 h. Each blood sample was allowed to clot and centrifuged at 4000 rpm for 20 min. Sera were collected, aliquoted, and kept at -80℃ for analysis. Eighty-two normal serum samples from 50 males and 32 females aged 40-70 years were obtained from staff of the State Sport Administration in a general health examination. The processing, collection, and storage protocols of serum samples for normal individuals were exactly the same as aforementioned for the patients.

TABLE 1

Characteristics of Lung Cancer Patients

| Characteristics | No. of Patients |
|---|---|
| Lung Cancer Group | |
| Total Patients | 193 |
| Male | 135 |
| Female | 58 |
| Mean Age in Years ( Range) | 65(41-76) |
| Disease Stage | |
| I | 20 |
| II | 43 |
| III | 67 |
| IV | 63 |
| Tumor Histology | |
| Squamous Cell | 97 |
| Adenomcarcinoma | 65 |
| Samll Cell Lung Cancer | 32 |

### *Protein Chip Profiling Analysis*

Serum protein profiling was performed on WCX2 protein chips[27]. Briefly, sera were prepared by vortexing 10 μL of serum with 20 μL 8 mol/L urea at 4℃ for 30 min. WCX2 chips were pretreated with 5 μL 10 mmol/L HCl each array and rinsed 3 times with M-Q-water and then put into a bioprocessor (Ciphergen Biosystems Inc., USA). Serum/urea mixture samples of 100 μL each diluted with binding buffer (1:9) were applied to each well and the bioprocessors were shaken at 250 rpm for 35 min. After washed 3 times with the buffer (100 mmol/L NaAc, pH 4.0), 0.5 μL of a saturated solution of SPA was applied twice onto each chip array. The chips were placed in a ProteinChip Reader (PBSII-C) and time-of-flight spectra were generated by averaging 128 laser shots collected in the positive mode at a laser intensity of 210, detector sensitivity of 9, and in the optimization range from 3000 to 50 000 Da with high mass of 200 000 Da. Mass accuracy was calibrated externally using the all-in-one peptide and all-in-one protein molecular mass standard. Serum samples from patients and normal controls were run

concurrently to minimize experimental variations. The reproducibility of mass location and intensity between chips was determined using the pooled normal serum quality control sample. Twenty-two peaks were selected manually throughout the range 3000-30 000Da.

For peptide profiling analysis, each serum sample was diluted ten-fold with 25 mmol/L $NH_4HCO_3$ (pH 8.0), then the mixture was digested with 0.01 μg/μL trypsin in a well sealed humidity chamber for 4 h at 37℃. The tryptic peptide mixture was loaded on the spots of NP20 chips and air-dried, then 1 μL of 20% saturated CHCA was added. Peptide profilings were analyzed on Ciphergen PCS 4000 ProteinChip Reader with the high laser intensity 2800, low laser intensity 2800. The shots were kept and the optimization mass ranged 1000-10 000 Da. A protein or peptide retentate map was generated in which the individual proteins were displayed as unique peaks based on their mass and charge (m/z). The spectra were calibrated with external calibrant peptide-all-in-one (Ciphergen Biosystems, Inc., USA).

*Protein Identification*

Tryptic-digested peptides were measured with ProteinChip Reader PCS 4000 and selected peptides were identified by direct sequencing using a ABI Q-Star quadrupole tandem mass spectrometer equipped with Ciphergen ProteinChip Interface PCI 1000[28]. After the spectra were reviewed, the interested ion was selected and introduced into the collision cell for CID fragmentation. The CID spectral data were submitted to the database-mining tools (Mascot, Matrix Sciences) or Protein Prospector MS-Tag (UCSF) for identification. On the other hand, 2D electrophoresis and MS analysis were used to validate the identifications of the proteins.

*Bioinformatics and Statistical Methods*

The protein chip profiling spectra from the digested serum samples were collected and analyzed by Ciphergen Biomarker Software 3.0.2. Using the Biomarker Wizard Mode, consistent biomarker peaks across multiple serum samples after normalization were compared with searches for biomarkers that were differentially elevated in one subtype of lung cancer group relative to the other two subtypes according to the instruction manual of the software. A nonparametric test (Mann-Whitney U test) was employed to compare the marker peak intensities between lung cancer with normal groups, between lung cancer and other carcinoma (laryngeal carcinoma and laryngopharyngeal carcinoma) groups, and between lung cancer and TB groups. Difference was defined as significant at $P<0.05$. Then we employed one-way ANOVA to analyze the intensity distribution of the 11.6KD biomarker in the subtypes of lung cancer.

## RESULTS

*11.6 kDa Biomarker Correlated With the Clinicopathological Stage of Lung Cancer*

Four hundreds and thirty serum samples were screened by WCX2 ProteinChip Array. A biomarker 11.6kDa was found abundantly present in most lung cancer patients. The average peak intensities ( $\bar{x} \pm s$ ) of the 11.6kDa biomarker in 193 patients with lung cancer (19.3±20.6, $P<0.000$) were significantly higher than those in 120 patients with laryngeal carcinoma and laryngopharyngeal carcinoma (3.9±3.8, $P<0.001$), 35 patients with pneumonia and pulmonary tuberculosis (8.9±8.2, $P<0.19$), and 82 normal individuals (2.1±0.7, $P<0.000$) when a nonparametric test of Mann-Whitney U test was employed (Fig. 1A). Particularly, when the clinicopatholgocial

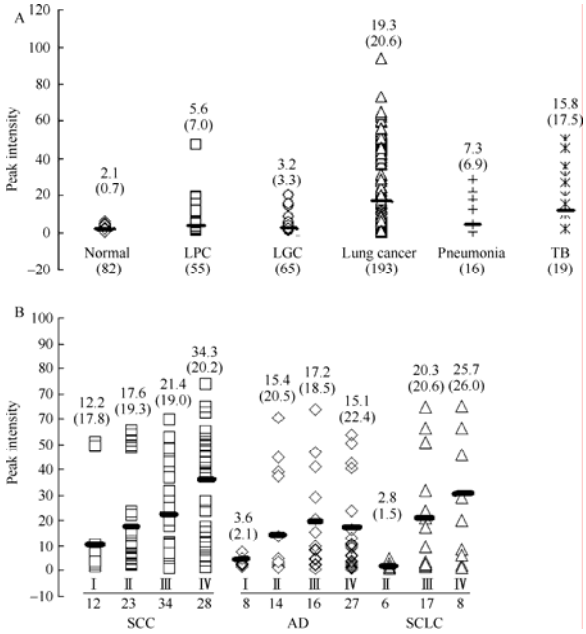


FIG. 1. Distribution of the peak intensity of biomarker 11.6kDa in patients with lung cancer, pneumonia, pulmonary tuberculosis, laryngopharyngeal carcinoma (LPC), laryngeal carcinoma (LGC), and normal individuals (A); and in patients with different subtypes of lung cancer including squamous cell carcinoma (SCC), adenomcarcinoma (AD), and small cell lung carcinoma (SCLC) (B). Mean peak intensities (±SD) are tabulated at the top of each column. Numbers at the bottom of the table show the number of samples studied.

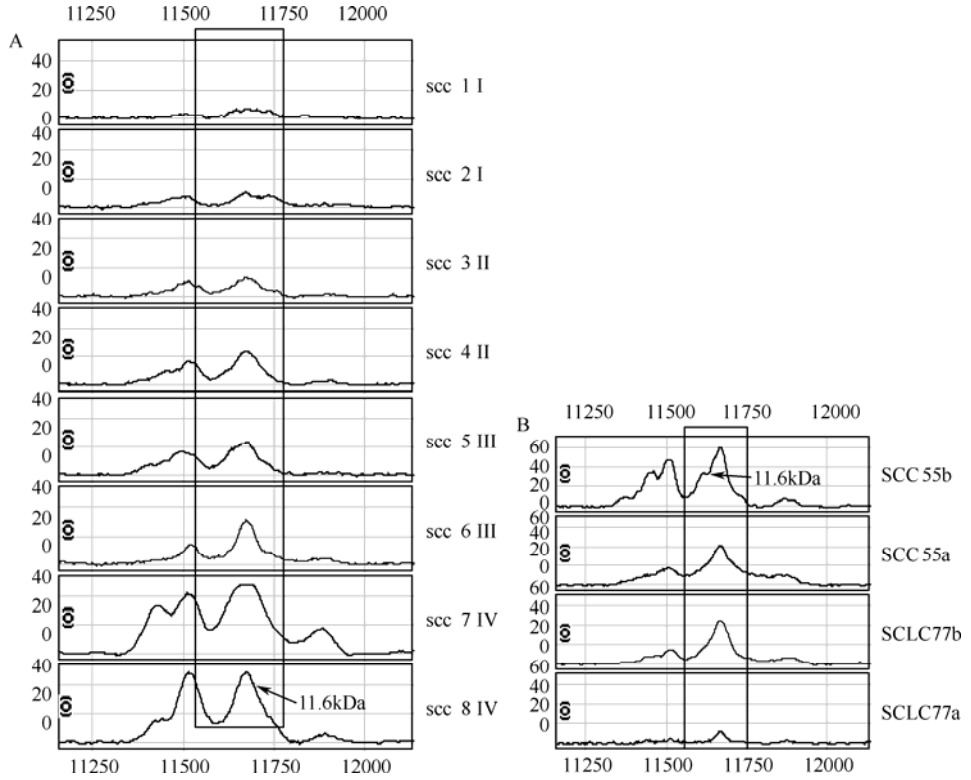


FIG. 2. A: Correlation of biomarker 11.6kDa elevation to the clinical stages of lung cancer. The spectra of 8 squamous cell carcinoma patients at stages I-IV were shown form top to bottom. Two patients in each stage were selected; B: decreased 11.6kDa biomarker in lung cancer patients after either operation or chemotherapy. The top two spectra were from one squamous lung cancer patient (No.55, IV stage, T4N1M0) one week before (55b) and one week (55a) after the operation. The bottom two spectra were from a patient with small cell lung cancer (No.77, IIIb stage, T2N3M0) before (77b) and after (77a) 2 cycles of chemotherapy with vinorelbine and cisplatin combination. The molecular mass range of 11.25-12kDa is illustrated in both spectra.

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[29], elevation of 11.6kDa biomarker was found to be correlated with the clinical stage of lung cancer. The intensity of 11.6 kDa peak was higher in stages III and IV than in stage I ($P$<0.038 and 0.002, respectively) using multiple comparisons of dependent variables (one-way ANOVA). To define more precisely 11.6 kDa peak elevation in lung cancer patients, a cutoff value for the biomarker was set at 3. According to the cutoff value, 11.6kDa elevation was found in 5 (25%), 17 (38.63%), 33 (49.25%), and 40 (62.29%) of lung cancer patients at stages I-IV, respectively, but not in any of the 82 normal individuals (0%). Figures 1B and 2A demonstrate that the level and percentage of the biomarker possessing the histological specificity were much higher in patients with squamous cell carcinoma than in patients with adenocarcinoma and small cell lung carcinoma. To validate the relationship of 11.6kDa biomarker with disease progression, a special comparison experiment was settled to analyze any possible changes of the screened biomarker after medical treatments. Twenty pairs of lung cancer sera were available before and after treatment and comparative analyses were performed with protein chip. It was interesting to find that either operation or chemotherapy could markedly decrease 11.6kDa biomarker in most patients, 6 of 7 patients on chemotherapy showed a dramatically decreased level of 11.6kDa biomarker, and similar results were found in 11 of the 13 patients after operation (Fig. 2B). This illustrated that the elevation of 11.6kDa biomarker was very likely correlated with the progression of lung cancer and subtypes of lung cancer. In addition, it showed that 11.6kDa biomarker was also elevated in 8 patients (53%) with pneumonia, 3 (42%) with pulmonary tuberculosis, 11 (20%) with laryngeal carcinoma and 5 (7%) with laryngopharyngeal carcinoma. Though the level of 11.6kDa biomarker was also elevated in patients with laryngeal and laryngopharyngeal carcinomas, it could not be down-regulated in most patients with

laryngopharyngeal carcinoma after radiotherapy. In the reproducibility test, the 22 peaks with good resolution and better ratio of signal/noise were chosen. The average coefficient of variance (CV) based on five normal pooling human sera for intensities of 22 peaks was lower than 20%. The results showed few variations on day-to-day sampling or chip variations.

*A Set of New Detectable Peptide Markers Generated by Trypsin Digestion in 11.6kDa Positive Patients*

The lack of ability to directly sequence biomarkers on chips is the major obstacle that limits the widespread application of SELDI, particularly when the studies are research-aimed. The ordinary way to identify any biomarkers discovered by SELDI is partial purification of biomarkers followed by trypsin digestion and spectrometric analysis. However, this strategy is not efficient for identification of all biomarkers and time-consuming. A new strategy was developed in the present work to identify biomarkers directly on chips. One hundred sera from patients with different subtypes of lung cancer were collected (including 45 patients with squamous cell carcinoma, 37 with adenocarcinoma, 18 with small cell lung carcinoma, and 40 normal individuals) and digested by trypsin prior to the chip loading. The peptide profilings were measured on ProteinChip Reader PCS 4000 to find the tryptic peptide biomarkers which discriminated the lung cancer patients from normal individuals. Four of new peptide biomarkers (with the molecular masses of 1550Da, 1612Da, 1912Da, and 2178Da) occurred stably in most lung cancer patients were recognized as 11.6kDa peak positive according to the non-digested screen. None of the new markers was detectable in any of normal individuals (Fig. 3). Two of the peptide biomarkers, 1912Da and 2178Da, were selected for analysis on ABI Q-Star quadrupole tandem mass spectrometer equipped with Ciphergen

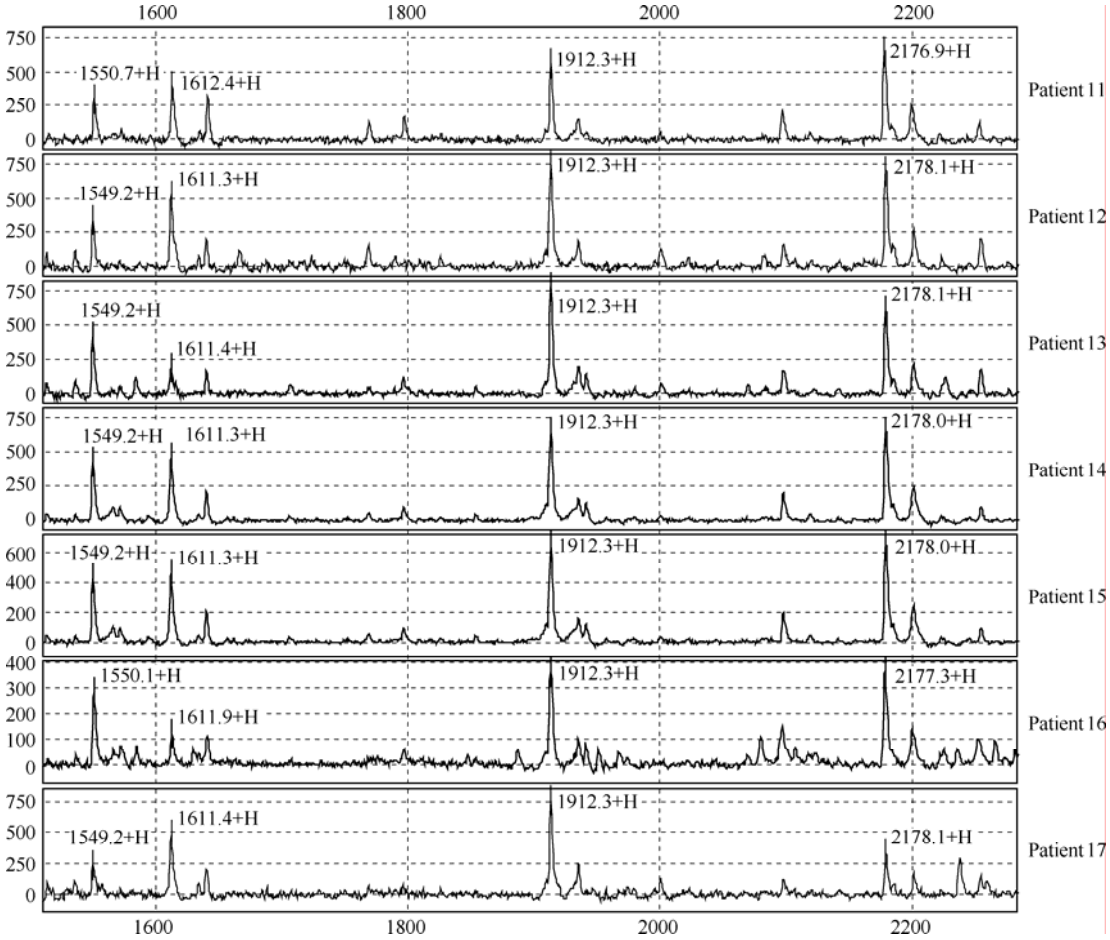


FIG. 3. A set of new peptides biomarkers generated from the sera with 11.6kDa peak by trypsin digestion. The words labelled on the peaks express the m/z of the peptides elevated in lung cancer patients compared with normal individuals. Spectra of four patients (No.11-14) with squamous cell carcinomas, two patients (No.15, 16) with adenocarcinomas and one patient (No.17) with small cell lung cancer are shown from the top to bottom, respectively. The molecular mass range of 1500-2250Da is illustrated.

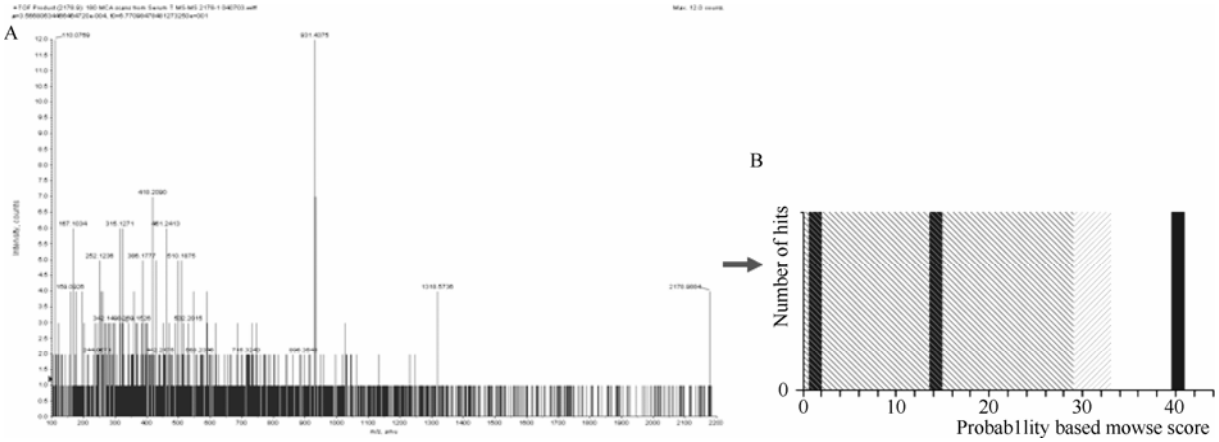


FIG. 4. Identification of the MS/MS fragmentation of 2178Da peptide (A) and the searching result of the fragment ions assigning to the protein SAA (B). The mowse score is 40 (individual ions scores >33 indicate identity or extensive homology, $P<0.05$).

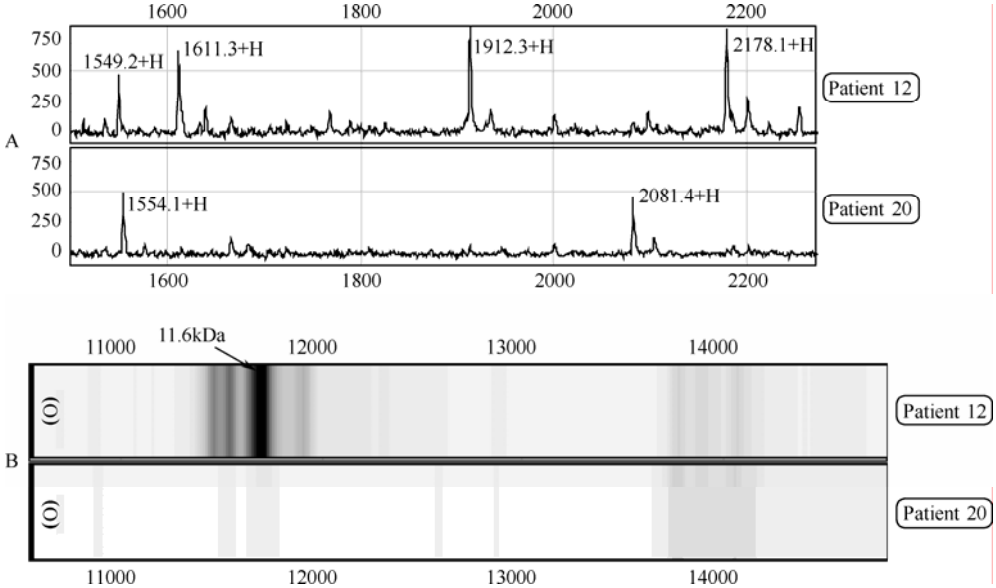


FIG. 5. Spectra of sera from two squamous cell carcinoma patients before and after trypsin digestion. A: a set of new tryptic peptides including 1611Da, 1912Da, and 2178Da occurred in patient 12 with 11.6kDa peak, but not in patient 20 without 11.6kDa peak. B: spectra in gel view of sera from patients 12 and 20 with or without 11.6kDa peak are shown, respectively. A molecular mass range of 1500-2250Da and 10-15kDa are illustrated, respectively.

ProteinChip Interface PCI 1000 (Fig. 4). The MS/MS ion fingerprints were entered into a mass spectrometry Internet search engine database, Mascot[16]. 2178Da peptide biomarker came out as a 18 amino acid residue signal peptide cleavage of SAA (MW 11 682.7Da). The other two peptides 1550Da and 1611Da, according to the database predication, were different tryptic fragments of SAA. However, 1912Da was not identified.

*11.6kDa Protein Further Confirmed As SAA*

11.6kDa marker was shown as SAA. The confidence of assignment for SAA was calculated by the Mascot automatically on the Internet and mowse score of 2178Da was 40 ($P<0.05$) (Fig. 4). The new set of digested peptides database searching was further confirmed by ProteinChip Array and 2DE as well as MS. In a much larger group, 80 sera from lung cancer patients with or without 11.6kDa were subjected to trypsin digestion and analyzed on NP20 protein chips with Ciphergen PCS 4000 ProteinChip Reader. 2178Da occurred 100% in patients with 11.6kDa biomarker whose peak intensity was above 3, whereas none of them was generated in patients without 11.6kDa protein (Fig. 5A-B). At the same time, we partially purified 11.6kDa protein from the serum of lung cancer patients by SDS-PAGE and the

corresponding band was excised from the gel and identified using tandem MS. 11.6kDa protein was again resolved as SAA. In addition, the same strategy was further applied to 11.6kDa biomarker analysis in patients with pneumonia, pulmonary tuberculosis, laryngeal carcinoma, and laryngopharyngeal carcinoma, and the same identification was achieved repeatedly.

## DISCUSSION

In recent years, the search for new biomarkers of human cancers has become more successful because of new high-throughput techniques in the field of proteomics. SELDI-TOF-MS is one of the most promising new proteomic tools for the detection of biomarkers, because of its reliable high-throughput capability, high speed, few sample requirements, and ability to analyze complex biological mixtures directly. The efficacy of SELDI in discovery of biomarkers has been proven in cancers of lung[30], pancreas, breast, prostate, and ovary[4,7-9], as well as in other diseases[31]. However, the disadvantage of SELDI is that it cannot directly identify of the biomarkers on chips. SELDI is not widely used in the mechanism research. In the present study, one biomarker which may have potential value for diagnosing and monitoring progression of lung cancer was identified using a new strategy as described in the result section. Its efficacy is consistent with Yip's report[32]. There is no doubt that this technique is applicable to many other studies and can facilitate the on-chip identification.

SAA is an acute phase reactant, whose level in blood is elevated in response to trauma, infection, inflammation and neoplasia[33-35]. Although SAA has been considered to be a acute-phase protein existing as various isoforms in a molecular mass of 11-14kDa, it was reported that SAA is significantly higher in patients with metastatic disease than in those with limited disease[36] and may act as a potential useful biomarker to monitor relapse, metastasis and prognosis in some cancers[1,20]. Recently, the relationship between SAA and lung cancer has been reported by Khan and Yip[21,32]. It has been shown that SAA level in patients with lung cancer is higher than that in those with squamous cell carcinoma and other diseases, but is not correlated with tumor size or clinical stage. However, our results suggest that SAA is a more important biomarker to monitor relapse, metastasis and prognosis in some cancers since it is one of the few biomarkers that are highly correspondent with the progression of lung cancer and can be effectively decreased after medical treatments.

Obstructive pneumonia most likely occurs in lung cancer patients, especially in the late stage, and results in elevation of SAA in patient serum due to infection or inflammation of lung. Analysis of the levels of SAA in 53 of 214 patients with obstructive pneumonia, showed no significant relation of SAA with obstructive pneumonia. On the other hand, SAA elevation was also detected in 8 (53%) patients with pneumonia and 3 (42%) with pulmonary tuberculosis. One patient with obsolete TB had a lower level of SAA than other TB patients. An increase of SAA has been reported in SARS patients at the period of extensive pneumonia, which is gradually decreased with the gradual recovery of the patients, but is further increased with superimposed multiple bacterial infections[30]. It is likely that SAA in pulmonary inflammation may be temporarily elevated and recovered soon after the elimination of infection, which may represent a primary difference between benign and malignant diseases of lung. The real situation is more complicated. For example, in a small number of laryngopharyngeal carcinoma patients, the increased level of SAA do not fall back after radiotherapy, suggesting that increased SAA is caused by some reasons, such as occasional infection or trauma during the radiotherapy. Our results, together with other reports, suggest that the elevation of SAA in different tissues may mainly depend on the pathological state of disease progression and tissue specificity. In spite of the lower sensitivity and specificity of SAA, it can serve as a useful serum biomarker for monitoring the progression of lung cancer in combination with other new biomarkers, such as LDH and Hsp90 which are significantly associated with the progression of lung cancer. Thus, to establish a multiple biomarker protein chip or a nanowire sensor array using more specific antibodies, such as SAA, LDH, and Hsp90α[37] for monitoring the progression of lung cancer can be expected.

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