

RESEARCH ARTICLE

Proteomic Profiling of Serum from Stage I Lung Squamous Cell Carcinoma Patients

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Abstract

Objectives: This study employed proteomic profiling to identify specific tumor markers that might improve early diagnosis of lung squamous cell carcinoma. **Methods:** Serum samples were isolated from 30 patients with stage I lung squamous cell carcinoma and 30 age- and gender-matched healthy controls, and proteomic profiles were obtained by matrix-assisted laser desorption/ionization time of flight mass spectrometry. **Results:** Three highly expressed potential tumor markers were identified in the sera of stage I lung squamous cell carcinoma patients, with molecular weights of 3261.69, 3192.07, and 2556.92 Da. One protein peak with molecular weight 3261.69 Da was chosen as the candidate biomarker and identified as a fibrinogen alpha chain through a search of the IPI, NCBI or SWISS-PROT protein databases. **Conclusion:** As a potential tumor biomarker, fibrinogen alpha chain may be applicable for the early diagnosis and prognosis of lung squamous cell carcinoma patients.

Keywords: Lung cancer - squamous cell carcinoma - fibrinogen alpha chain - tumor biomarker

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Introduction

Lung cancer is one of the most common malignancies worldwide and is the leading cause of cancer death in most countries. The incidence and mortality of lung squamous cell carcinoma has continued to increase in recent years (Kikuchi et al., 2007). Although progress has been made in understanding the underlying mechanism of carcinogenesis of this disease, definitive diagnostic and prognostic protein biomarkers are still unavailable. Therefore, it is important to identify these biomarkers for the early prevention and treatment of lung squamous cell carcinoma.

Proteomic profiling is a promising approach for screening potential tumor biomarkers. The development and application of proteomics depend on the following: First, advances in mass spectrometry (MS) and genomic sequencing that facilitate the identification and relative quantization of small amounts (femtomole) of nearly any single protein; Second, new methods in gel electrophoresis that allow the detection of subtle changes in protein expression, including posttranslational modifications; Third, automation and miniaturization that permit high-throughput analysis of clinical samples; and finally new bioinformatics and computational methods that support the analysis and interpretation of the abundant data generated by proteomics experiments. Clinical proteomics is a technology that has great potential to improve our knowledge of lung cancer by identifying patterns of protein expression. Because these protein expression

profiles can reveal broad pathologic processes such as altered proteolytic processing or glycosylation that may not have been evident with other technologies or reflect complex patterns that may serve as new diagnostic tools. The biological complexity of lung squamous cell carcinoma indicates that individual tumor markers have limited diagnostic sensitivities and specificities. Analysis of combinations of several tumor markers offers the possibility of enhanced diagnostic accuracy (Villanueva et al., 2006). High throughput proteomic profiling of serum has become popular with recent advances in MS (Diamandis et al., 2005), such as matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS. Especially, ProteinChip technology coupled with MALDI-TOF-MS technology is much faster, has a high-throughput capability, needs very little amounts of sample, and can analyze complex biological mixtures directly. Therefore, in this study we performed proteomic profiling of serum samples from stage I lung squamous cell carcinoma patients and established a model based on the proteomic pattern for stage I lung squamous cell carcinoma and the model enabled the reliable discrimination of lung squamous cell carcinoma cases from healthy controls.

Materials and Methods

Subjects

Thirty patients diagnosed with stage I lung squamous cell carcinoma were enrolled in this study from the Department of Thoracic Surgery, First Affiliated Hospital,

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Medical School of Xi'an Jiaotong University. Thirty controls were enrolled from qualified age-and gender-matched healthy volunteers. In addition, 22 patients with stage I lung squamous cell carcinoma and 19 healthy volunteers were enrolled for a blind test. All the subjects gave written informed consent and the study protocol was approved by the Ethics Committee of Xi'an Jiaotong University.

Serum samples

Five milliliter blood samples were collected from patients and healthy controls into sterile glass tubes. The serum was separated by centrifugation for 20 min at 4000 rpm, distributed into 100 µl aliquots, and stored frozen at -80 °C within one hour of collection. Specimens underwent no more than two freeze-thaw cycles before MS analysis.

Proteomic analysis

Serum samples were applied to 600 µm MB-WCX Anchorchip arrays (CIPHERGEN Bruker Daltonics, Germany). The Anchorchips were rinsed 3 times with 10 mL Milli-Q water in a conical tube and then SPA (a saturated solution of sinapinic acid in 50% acetonitrile and 0.5% trifluoroacetic acid) was applied twice to each Anchorchip array, letting the surface air dry between each sample application. Anchorchips were stored frozen in the dark at -24 °C until MALDI analysis. To minimize bias, serum samples from age- and gender-matched pairs of lung cancer patients and healthy controls were assayed on the same Anchorchip.

Anchorchips was placed in the Protein Biological System Autoflex-IIC MS reader (CIPHERGEN Bruker Daltonics, Germany) and TOF spectra were generated with the laser intensity set at 160, detector sensitivity at 8 and the optimization mass range 0.80 to 15 kDa, high mass at 20 kDa. To minimize bias, the mass spectrometer was calibrated weekly using an all-in-one peptide molecular mass standard (CIPHERGEN Biosystems, Sigma, USA).

To identify peaks, spectra were normalized to the total ion current in the m/z range over 800–15000 after top-hat baseline and Savitsky-Golay smoothing subtractions. Peak detection was performed using CIPHERGEN ClinProTools 2.1 software. Biomarker Wizard version 3.1 was used to identify corresponding peaks in each spectrum (peak clusters). The signal to noise ratio was 5 for the first pass and 2 for the second pass. Minimum peak threshold was 30%.

One protein peak (molecular weight: 3261.69 Da) was chosen as the candidate biomarker, whose peptide mass fingerprint map (PMF) was obtained by high-performance liquid chromatography (HPLC) and LCQ Deca XP liquid chromatography (LC)-MS. With BioworksBrowser 3.3.1 SP1 (SEQUENT) software, the amino acid sequence of the peptide was identified and the International Protein Index (IPI), National Center for Biotechnology Information (NCBI) or SWISS-PROT protein databases were used to search for the amino acid sequence of the peptide.

Statistical analysis

All the results were expressed as mean ± standard

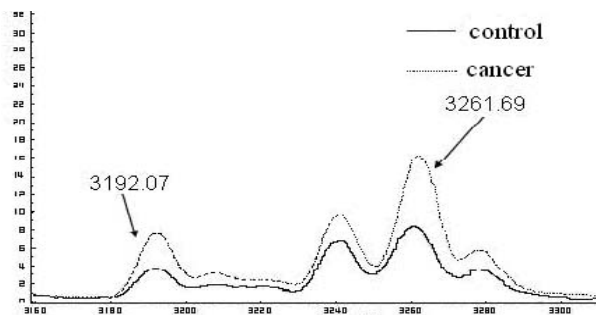


Figure 1. Spectra of Two Highly Expressed Protein Peaks in Stage I Lung Squamous Cell Carcinoma. X-axis: the molecular weight of the peaks; y-axis: intensity

Table 1. Sensitivity and Specificity of the Diagnosis of Lung Squamous Cell Carcinoma

Group	n	Blind test		
		Accuracy	Sensitivity	Specificity
Normal	19	94.7% (18/19)	—	94.7%
Lung squamous cell carcinoma	22	95.5% (21/22)	95.5%	—

deviation (SD). Student's t-test was performed for group comparison using SPSS 13.0 statistical software. Probability (P)-values <0.01 were considered statistically significant. Sensitivity was calculated as the ratio of the number of correctly classified diseased samples to the total number of diseased samples. Specificity was calculated as the ratio of the number of negative samples correctly classified to the total number of true negative samples.

Results

After comparisons with the protein profiles from the healthy control group, three highly expressed protein peaks were identified in the sera of patients with stage I lung squamous cell carcinoma, with molecular weights 3261.69, 3192.07 and 2556.92 Da (P <0.001). The spectra of two peaks are shown in Figure 1.

Using the MALDI software program, approximately 77 peaks/spectrum were detected in the 1-13 kDa mass range. Three highly expressed protein peaks were identified with molecular weights 3261.69, 3192.07, and 2556.92 Da. The model for the diagnosis of lung squamous cell carcinoma was constructed to discriminate between lung squamous cell carcinoma patients and healthy individuals by using these three masses as tumor marker patterns. To evaluate this model we performed a blind test for serum samples from 22 lung squamous cell carcinoma patients and 19 healthy volunteers. The results showed that the test based on this model achieved a sensitivity of 95.5% and a specificity of 94.7% (Table 1).

By HPLC and LTQ Orbitrap XL-MS analysis, the amino acid sequence of peptide 3261.69 Da was identified as "SSSYSKQFTSSTSYNRGDSTFESKSYKMA". Based on the IPI, NCBI and SWISS-PROT protein databases, this peptide was identified as a fragment of fibrinogen alpha chain.

Discussion

Lung cancer is divided clinically into small cell lung

cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for approximately 80% of all lung cancers, and is further divided into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Most patients with NSCLC are diagnosed at an advanced stage, thus have a poor prognosis, and the overall 5-year survival rate is only 15% (Jemal et al., 2004). Therefore, earlier detection of NSCLC is important to reduce lung cancer-related mortality. The current common approaches for the diagnosis of lung cancer in clinic are largely based on X-ray and computed tomography, which are not sensitive enough to detect small lesions (Takashima et al., 2003). In many cases, the definite diagnosis of lung cancer is based on pathological examination of the biopsy, an invasive surgical procedure that is not suitable for all kinds of lung cancer (Adams et al., 2012). For these reasons, progress on the prevention, diagnosis, and treatment of lung cancer may largely depend on the discovery of specific tumor markers that can distinguish and characterize lung cancer of different subtypes and stages (Huang et al., 2006).

Proteinchip technology coupled with MALDI-TOF-MS, or as it is often termed surface-enhanced laser desorption/ionization (SELDI)-TOF-MS, is a rapid, high-throughput tool for biomarker discovery. Due to the complex mechanism underlying tumorigenesis, individual tumor markers have limited diagnostic sensitivities and specificities. Thus the analysis of combinations of several biomarkers offers the possibility of enhanced diagnostic accuracy for cancer, including lung squamous cell carcinoma. Serum proteomic patterns by their complexity could reflect the underlying pathological state of cancer patients. A previous study (Petricoin et al., 2002) employed hydrophobic proteinchip arrays to screen serum samples from ovarian cancer patients and generated a discriminator pattern consisting of five protein masses of 534, 989, 2111, 2251, and 2456 Da, which yielded 94% accuracy in differentiating ovarian cancer from benign ovarian disease and healthy women, and a similar classification pattern consisting of nine protein masses for prostate cancer was reported (Adam et al., 2002). Our results in this report were derived from 101 samples, including 52 stage I lung squamous cell carcinoma patients and 49 healthy controls. We established the diagnosis model based on three masses representing 3261.69, 3192.07 and 2556.92 Da as a tumor marker pattern to discriminate lung squamous cell carcinoma patients from healthy controls. When this model was evaluated by a blind test that enrolled independent subjects, it yielded a sensitivity of 95.5% and a specificity of 94.7% for the diagnosis of lung squamous cell carcinoma. Taken together, our data and previous results from other groups demonstrate that biomarker patterns based on multiple protein masses provided higher sensitivity and specificity than any single biomarker currently used in the clinic (Kulpa et al., 2002). The establishment and further validation of these patterns help form a solid foundation for the prospective “molecular diagnosis” technology that will have great potential for clinical application.

In addition, we identified a differentially expressed peptide (molecular weight 3261.69 Da) with the amino acid sequence ‘SSSYKQFTSSTS

-NRGDSTFESKSYKMA’. An IPI database search revealed that this peptide was derived from two homologous protein isoforms: fibrinogen α -E (isoform 1 of fibrinogen α chain, 866 amino acid-residues, molecular weight 94,973 Da) and fibrinogen α (isoform 2 of fibrinogen α chain, 644 amino acid-residues, molecular weight 69,757 Da). Fibrinogen is a glycoprotein which contains 2964 amino acid residues. It has a hexamer composition of three chains (α , β , γ) linked through two disulfides (Brummel et al., 1999). As a coagulation factor I, fibrinogen functions to accelerate platelet aggregation and is directly involved in the clotting process. In recent years accumulating evidence shows that fibrinogen is related to the development and metastasis of malignant tumors (Castelli et al., 2006). The plasma fibrinogen is increased in patients with lung squamous cell carcinoma. Although lung squamous cell carcinoma cells did not synthesize fibrinogen, they can stimulate liver cells to produce abundant fibrinogen (Rijken et al., 2006).

We postulate that the increase and abnormal activation of fibrinogen lead to blood coagulation in patients with lung squamous cell carcinoma, which further induces the aggregation, adhesion, invasion and metastasis of cancer cells. Therefore, detection of fibrinogen may help enable the early diagnosis of malignant tumor, the judgment of early diagnosis and the assessment of treatment effectiveness. Further in-depth investigation of fibrinolysis and coagulation in patients with lung squamous cell carcinoma will be exceedingly beneficial not only to monitor the pathological condition, but also to develop effective approaches for diagnosis, prevention and treatment in order to prolong the survival of lung cancer patients.

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