Original Article

Haptoglobin is a serological biomarker for adenocarcinoma lung cancer by using the ProteomeLab PF2D combined with mass spectrometry

You-Kang Chang¹, Yu-Heng Lai², Yen Chu³, Ming-Cheng Lee⁴, Chun-Yao Huang⁵, Semon Wu⁴,⁶

Departments of ¹Radiation Oncology, ⁴Research, ⁵Pulmonary and Critical Care, Taipei Tzu Chi Hospital, The Buddhist Tzu Chi Medical Foundation, Taipei, Taiwan; Departments of ²Chemistry, ⁶Life Science, Chinese Culture University, Taipei, Taiwan; ³Division of Thoracic and Cardiovascular Surgery, Chang Gung Memorial Hospital, Taipei, Taiwan. *Equal contributors.

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Abstract: Identification of serological biomarker is urgently needed for cancer screening, monitoring cancer progression, treatment response, and surveillance for recurrence in lung cancer. Therefore, we try to find new serological biomarker that has more specificity and sensitivity for lung cancer diagnostics. In this study, the 2-D liquid phase fractionation system (PF2D) and mass spectrometry approach has been used for comparison the serum profiles between lung cancer patients and healthy individuals. Eight proteins were identified form PF2D and subsequently by mass spectrometry. Among these proteins, haptoglobin (HP) and apolipoprotein AI (APOA1) were chosen and validated with turbidimetric assay. We found that HP levels were significantly higher and APOA1 levels were significantly lower in lung cancer patients. However, after the participants were stratified by gender, the expression trends of HP and APOA1 in lung cancer patients existed only in men, which is gender specific phenomenon. HP, APOA1 and carcinoembryonic antigen (CEA), used for distinguishing lung adenocarcinoma, had a sensitivity of 64%, 64% and 79%, respectively. Area under the ROC curve (AUC) of HP, APOA1 and CEA were 0.768, 0.761 and 0.884, respectively. When restricted to male subjects, HP, APOA1 and CEA showed sensitivity of 89%, 73% and 100%, respectively. AUC of HP, APOA1 and CEA were 0.929, 0.840 and 0.877, respectively. Therefore, our results showed that combined with PF2D system and mass spectrometry, this is a promising novel approach to identify new serological biomarkers for lung cancer research. In addition, HP may be a potential serological biomarker for lung adenocarcinoma diagnostics, especially in male subjects.

Keywords: 2-D liquid phase fractionation system, mass spectrometry, biomarker, haptoglobin

Introduction

Lung cancer is the leading cause of cancer mortality in many countries, claiming more than 7,700 lives in 2013 in Taiwan [1]. The prognosis of patients with lung cancer is generally poor, with an overall 5-year survival rate for patients after receiving treatment [2]. Furthermore, the proportion of lung adenocarcinomas is increasing globally [3], which is progressed and characterized to early development of metastases [4]. Therefore, developing serological biomarkers for early detection of lung cancer is necessary to improve patient survival. Serological biomarkers are favorable for the early diagnosis because of its easily acquired by non-invasive methods. Current plasma tumor markers, such as carcinoembryonic antigen (CEA), Neuron-specific enolase, tissue polypeptide antigen, chromogranin, carcinoembryonic antigen 125, carcinoembryonic antigen 19-9, cytokeratin 19 fragment, and progastrin-releasing peptide, have limited sensitivity and specificity for early diagnosis [5]. Therefore, finding novel serological biomarkers with higher specificity and sensitivity is crucial for lung cancer diagnosis.

Comprehensive analysis of protein expression patterns in human serum, urine and other body fluids might facilitate to understand the complexity of tumor development and progression.
Comparing differential expression patterns of proteins among clinical groups helps identify useful biomarkers for further diagnostic and therapeutic applications [11]. Previously, many proteomic approaches have been attempted to analyze protein by multi-dimensional liquid chromatography system [12-14]. According to the experience, we used ProteomeLab PF2D (Beckman Coulter, Inc., Fullerton, CA), which is based on advances in technology by using chromatographic to separate proteins in liquid phase, to identify novel serological biomarkers for human lung cancer. Two dimensions were involved in the PF2D system to separate proteins. The first dimension, using a chromatofocusing column, it separates sample according to pH. And subsequently, nonporous reversed-phase HPLC column is used in the second dimension. Compared to the proteomic profiles that are generated by the PF2D, each sample can be analyzed by using DeltaVue software. DeltaVue allows easy pairwise comparisons of proteomic profiles by using graphical interface and is used to identify and interpret peaks that show different expressions. In this study, we demonstrated an analytical system combining ProteomeLab PF2D system with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to find a novel serological biomarker of lung adenocarcinoma.

Materials and methods

Subjects

Sixty-three study subjects, including 42 lung adenocarcinoma patients and 21 health volunteers, were enrolled for analysis. Healthy volunteers were recruited during routine health examinations (aged 44-78 years; median 60 years; 13 females and 8 males), and the patients (aged 44-77 years; median 60 years; 26 females and 16 males) were recruited after pathological diagnosis of lung adenocarcinoma. Blood samples of patients were obtained during surgery or before systemic chemotherapy. With the Ethics Committee of the Taipei Tzu Chi General Hospital approval, a total of 15 mL of fasting venous blood samples from healthy volunteers were collected. Venous blood samples were drawn from an antecubital vein with a 21-gauge needle. Plasma was obtained by centrifuging the blood at 3000 × g for 15 minutes at 4°C. Immediately after centrifugation, the plasma samples were frozen and stored at -80°C prior to analysis.

PF2D analysis

Before processing on the PF2D system, samples were depleted albumin and IgG with ProteoPrep® Blue Albumin and IgG Depletion Kit (Sigma, St. Louis, MO, USA) and then balanced by using a PD-10 G25 medium column (Amersham Biosciences, Piscataway, NJ, USA) with started buffer in pH 8.5. Then the Two-Dimensional Separation of Proteins was performed as manual.

1st-Dimension analysis (Chromatofocusing): Before injecting onto a new or previously stored HPCF column, flush column with 100% distilled water and equilibrate column with 100% Start Buffer. Column is washed with 100% Start Buffer to remove excess material before injecting 2.0 mL of plasma that has been balanced. During injection, fractions were collected by time based on the method. UV absorbance returns to baseline to monitor whether the washing is complete. During elution, pH value begins to decrease, and fractions will be collected at specific pH intervals. At 110 minutes time point, the pH of the eluent should be at pH 4.0±0.1. According to the manual, elution should be done within a minimum of 15 and a maximum of 18 fractions for the best mapping results.

2nd-Dimension analysis (Reversed Phase): When the 1st-dimension separation has completed, 200 µL of fractions from the 1st-dimension were then automatically started and injected on the 2nd dimension. After the sample has been applied to the column from the FC/I module, a gradient of 0-100% with 0.08% trifluoracetic acid (TFA) in acetonitrile is continued for 30 minutes. At the end of each 2nd-dimension section, equilibration of columns with initial mobile phase 0.1% TFA in water is started.

Data analysis

The UV absorbance data of each 2nd-dimension Chromatofocusing fraction were analyzed with ProteoVue™ from the PF2D Software Suite for protein mapping.

In solution protein digestion

Analytical significant peak fractions were collected and transferred into the eppendorfs. The
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collected fractions were dried and rehydrated with 25 mM NH₄HCO₃, and concentrated with 50 mM dithioerythritol at 37°C for 1 h. The fractions then were alkylated by 100 mM iodoacetamide in the dark at room temperature for one hour following by a thorough process of washing and drying. Finally, the dried gel was incubated with 10 ml of 25 mM NH₄HCO₃, containing 0.05 mg/ml trypsin, at 37°C overnight. Peptides were extracted with 100 ml of 50% acetonitrile/5% TFA and dried in SpeedVac. Peptide extract was resuspended with 1 ml 1% formic acid first, and then added 9 ml solutions containing 50% acetonitrile/0.1% formic acid into it for mass spectrometric analysis or stored at -80°C until used.

Protein Identification by MALDI-TOF MS

The peptide mixtures were equally mixed with a-cyano-4-hydroxycinnamic acid (Agilent Technologies Co. Ltd., USA) and spotted onto stainless steel MALDI sample target plates. Mass spectra were obtained from the ABI 4800 Proteomics Analyzer MALDI-TOF/TOF MS/MS (Applied Biosystems, Framingham, MA, USA). Data acquisition and spectral processing were carried out using Analyst and BioAnalyst™ software from Applied Biosystems.

Database searching

Spectra were processed using MASCOT software (Matrix Science, London, UK) to search for mass fingerprints for peptides, and MS/MS data was analyzed in the Swissport database, with accuracy of mass within 100 ppm. Only protein that was identified with a score greater than P < 0.05 was considered to be positive.

Biochemical measurements

Plasma apolipoprotein A1 (APOA1), heptoglobin (HP) and CEA levels were quantified by using turbidimetric assay on the Hitachi-7600 analyzer (Hitachi Inc., Tokyo, Japan). Commercial kits for APOA1 and HP were purchased from Roche Systems Inc. (Minneapolis, MN, USA), and CEA was from Johnson & Johnson Company (New Jersey, USA), respectively. To evaluate the potential of HP and APOA1 as cancer markers for lung adenocarcinoma, we performed Receiver Operating Characteristic (ROC) curve analysis [15]. Youden index (J) were used for optimizing cut-off point. J is the maximum vertical distance between the ROC-curve and the diagonal reference line and is defined as J = maximum (sensitivity) + (specificity) - 1. The Youden index allows optimization of cut-off point under the assumption, which sensitivity and specificity are equally weighted [16].

Statistical analysis

The clinical characteristics of the continuous variables are expressed as means ± SD and tested by two-sample t-test or ANOVA. A value of P < 0.05 using two-sided tests was considered statistically significant.

Results

Differential protein expression profiling of plasma samples from lung cancer patients and healthy controls by 2D liquid phase separation

For the PF2D analysis, plasma samples from six lung adenocarcinoma patients or six healthy
control subjects were pooled, individually. Each pool was injected two times into the 1st dimension chromatofocusing column in a randomized order. Each pl fraction was injected two times into the 2nd dimension reversed phase column. A total of the 15 pl fractions were analyzed by reversed phase chromatography. Samples from lung adenocarcinoma and healthy controls were then compared with the DeltaVue software. Eight peaks within four pl fractions in the 2nd dimension showed significant differences between lung adenocarcinoma and healthy control samples (Figure 1). These four peaks, which were eluted at 16.9 min (peak #1) in pl fraction 5.59-5.29, respectively, were consistently decreased in the lung cancer sample fraction. Quantitative analysis of 8 peak areas carried out by using the integration tools of the Karat chromatography software, which showed significant differences between lung adenocarcinoma patients and healthy controls (Table 1).

Table 1. Quantitative analysis of peak area differences using the 32 Karat chromatography software

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Peak area (lung cancer)</th>
<th>Peak area (health control)</th>
<th>Peak ratio (lung cancer/health control)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>2323472±77544</td>
<td>1042774±27937</td>
<td>2.23</td>
<td>0.021</td>
</tr>
<tr>
<td>#2</td>
<td>4103695±6988</td>
<td>994102±24209</td>
<td>4.12</td>
<td>0.001</td>
</tr>
<tr>
<td>#3</td>
<td>1176343±141890</td>
<td>325230±36670</td>
<td>3.62</td>
<td>0.046</td>
</tr>
<tr>
<td>#4</td>
<td>335559±34717</td>
<td>761525±2135</td>
<td>0.44</td>
<td>0.035</td>
</tr>
<tr>
<td>#5</td>
<td>1721202±141137</td>
<td>4236379±192640</td>
<td>0.40</td>
<td>0.006</td>
</tr>
<tr>
<td>#6</td>
<td>186642±2006</td>
<td>166337±51992</td>
<td>0.11</td>
<td>0.015</td>
</tr>
<tr>
<td>#7</td>
<td>3377047±4192</td>
<td>1172247±217988</td>
<td>2.89</td>
<td>0.044</td>
</tr>
<tr>
<td>#8</td>
<td>2350835±14391</td>
<td>5524962±108342</td>
<td>0.43</td>
<td>0.013</td>
</tr>
</tbody>
</table>

Table 2. The identified proteins from eight major different peaks

<table>
<thead>
<tr>
<th>Peak</th>
<th>Protein name</th>
<th>PI</th>
<th>Mr</th>
<th>Sequence coverage</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>Fibrinogen beta chain</td>
<td>8.54</td>
<td>56588</td>
<td>45%</td>
<td>377</td>
</tr>
<tr>
<td>#2</td>
<td>Haptoglobin</td>
<td>6.13</td>
<td>45873</td>
<td>7%</td>
<td>172</td>
</tr>
<tr>
<td>#3</td>
<td>Fibrinogen alpha chain</td>
<td>5.70</td>
<td>95668</td>
<td>24%</td>
<td>245</td>
</tr>
<tr>
<td>#4</td>
<td>Apolipoprotein A-I</td>
<td>5.56</td>
<td>30759</td>
<td>71%</td>
<td>616</td>
</tr>
<tr>
<td>#5</td>
<td>Transthyretin</td>
<td>5.52</td>
<td>15993</td>
<td>25%</td>
<td>150</td>
</tr>
<tr>
<td>#6</td>
<td>Transthyretin</td>
<td>5.52</td>
<td>15993</td>
<td>48%</td>
<td>261</td>
</tr>
<tr>
<td>#7</td>
<td>Ig alpha-1 chain</td>
<td>6.08</td>
<td>38501</td>
<td>17%</td>
<td>165</td>
</tr>
<tr>
<td>#8</td>
<td>Apolipoprotein A-I</td>
<td>5.56</td>
<td>30759</td>
<td>71%</td>
<td>424</td>
</tr>
</tbody>
</table>

Validation of the identified proteins with a turbidimetric assay

Each aliquot of different peaks was collected, performed in-solution digested with trypsin, and identified by MALDI-TOF-TOF MS. Eight proteins were identified from total eight fractions in the 2nd dimension (Table 2). These proteins included: fibrinogen beta chain in peak #1, HP in peak #2, fibrinogen alpha chain in peak #3, APOA1 in peak #4 and #8, transthyretin in peak #5 and #6, serotransferrin in peak #5, and Ig alpha-1 chain and Ig alpha-2 chain in peak #7.
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Discrimination between the lung adenocarcinoma group and the healthy control group

About 35% to 60% of patients with NSCLC expressed increasing level of CEA, which showed higher sensitivity in lung adenocarcinoma and advanced stages [17-19]. Therefore, we compared CEA with HP and APOA1 to evaluate the potential of being cancer biomarker. In ROC curve analysis (Table 3), the area under the ROC curve (AUC) was 0.768 for serum HP (P = 0.001, 95% confidence interval (CI) = 0.653-0.884), 0.761 for serum APOA1 (P = 0.001, 95% CI = 0.644-0.877) and 0.884 for the CEA (P < 0.001, 95% CI = 0.803-0.966). With a cut-off level of 183.85 mg/dL, serum HP had a sensitivity of 64%, specificity 95%; with a cut-off level of 146.25 mg/dL, serum APOA1 had a sensitivity of 64%, specificity of 86%; with a cut-off level of 2.87 ng/mL, serum CEA had a sensitivity of 64%, specificity of 86%. On the other hands, when focusing on male subjects, AUC of serum HP was 0.929 (P < 0.001, 95% CI = 0.849-1.009); APOA1 was 0.840 (P = 0.001, 95% CI = 0.715-0.965); CEA was 0.877 (P < 0.001, 95% CI = 0.770-0.985). With a cut-off level of 183.85 mg/dL, serum HP showed 89% of sensitivity and 92% of specificity; with a cut-off level of 147.65 mg/dL, serum APOA1 showed 73% of sensitivity and 85% of specificity; with a cut-off level of 8.87 ng/mL, serum CEA showed 100% of sensitivity and 73% of specificity.

Discussion

In this study, we used a proteomic approach with two-dimensional chromatography to separate intact proteins on PF2D platform, and identified the major changes of serum protein expression in lung adenocarcinoma patients and healthy controls by Mass spectrometry. Eight proteins were identified from total eight fractions in the 2nd dimension, including the fibrinogen beta chain, HP, fibrinogen alpha chain, APOA1, transthyretin, serotransferrin, Ig alpha-1 chain and Ig alpha-2 chain (Table 2). Some of these proteins were confirmed as secreted proteins or cancer markers in previous studies, including fibrinogen [20, 21], transthyretin [22-24], serotransferrin [24, 25] and Ig [26], which were consistent with our study.

We found that HP levels in lung adenocarcinoma patients were significantly higher than in healthy controls, while APOA1 levels in lung adenocarcinoma group were significantly lower. After the participants were stratified by gender, the differences in expression levels of both proteins were statistically significant in male subjects only but not in female subjects. ROC curve analysis was performed to evaluate the applicability of HP and APOA1 as a cancer marker for distinguishing between the lung adenocarcinoma group and healthy control group. CEA, one of the current cancer markers used clinically, was compared with HP and APOA1. The AUC of CEA, which was calculated based on whole population to diagnose lung adenocarcinoma, was higher than the AUC of HP and APOA1; however, the AUC of HP, which was calculated based on gender, was higher than the AUC of CEA and APOA1.

HP is an acute phase plasma glycoprotein, which is functioned as binding of free hemoglobin and preventing oxidative stress [27]. HP is generated from the liver. Increased amount of HP in blood plasma has been observed in infection, inflammation, and various malignant diseases, including lung cancer [28], bladder cancer [29], malignant lymphoma [30], breast can-
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Table 3. Sensitivity and specificity of haptoglobin, apolipoprotein A-I and CEA for discriminating between the lung adenocarcinoma group and the healthy control group

<table>
<thead>
<tr>
<th>Marker</th>
<th>Study population</th>
<th>Cut-off level</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haptoglobin</td>
<td>M+F</td>
<td>183.85 (mg/dL)</td>
<td>64%</td>
<td>95%</td>
<td>0.768 (P = 0.001, 95% CI = 0.653-0.884)</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>183.85 (mg/dL)</td>
<td>89%</td>
<td>92%</td>
<td>0.929 (P &lt; 0.001, 95% CI = 0.849-1.009)</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>M+F</td>
<td>146.25 (mg/dL)</td>
<td>64%</td>
<td>86%</td>
<td>0.761 (P = 0.001, 95% CI = 0.644-0.877)</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>147.65 (mg/dL)</td>
<td>73%</td>
<td>85%</td>
<td>0.840 (P = 0.001, 95% CI = 0.715-0.965)</td>
</tr>
<tr>
<td>CEA</td>
<td>M+F</td>
<td>2.87 (ng/dL)</td>
<td>79%</td>
<td>86%</td>
<td>0.884 (P &lt; 0.001, 95% CI = 0.803-0.966)</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>8.87 (ng/dL)</td>
<td>100%</td>
<td>73%</td>
<td>0.877 (P &lt; 0.001, 95% CI = 0.770-0.985)</td>
</tr>
</tbody>
</table>

Abbreviation: M+F, both sexes; M, male; AUC, area under the ROC curve.

In our findings, we confirmed that HP is a potential serological biomarker for lung cancer diagnostics, especially in male subjects. Circulating HP, and other plasma proteins showed minor and/or transient changes in women treated by oral administered androgen derivatives [37]. Increasing of HP in high-grade squamous intraepithelial lesions and invasive cervical cancer was observed, which is probably resulted from the intense immune suppressor effect on HP with steroid hormones ingestion [38]. In our study, serum level of HP in male subjects was higher and the AUC of HP was higher than AUC of other markers, which may be subjected to lung adenocarcinoma diagnosis. These findings suggested that androgen may play a role on serum level of HP and further investigation is needed.

APOA1, the major protein component of the antiatherogenic high-density lipoprotein particles, participates in efficient free-cholesterol efflux from peripheral tissues, including atherosclerotic lesions [39]. Apolipoproteins are acute phase reactants and associated with oxidative functions. Protein kinase, Akt, can be activated by apolipoproteins, block apoptosis, and promote growth factor-mediated cell survival, which suggested a potential tumorigenic effect on apolipoprotein [40]. APOA1 was reported to be down-regulated in ovarian [24, 41], breast cancer [42], and pancreatic cancer [43]; however, it was up-regulated in head and neck [44], and breast cancer [45]. In our study, APOA1 in the serum of lung adenocarcinoma patients were significantly down-regulated. Down regulation of APOA1 in cancer patients suggests the possibility that they are byproducts of the host response to the tumor [43], since they are acute phase reactants.

Conclusion

In our study, combined with PF2D system and MS, we developed a promising approach to identify novel serological biomarkers for lung cancer diagnosis. Moreover, HP may be a potential serological biomarker for lung adenocarcinoma diagnostics, especially in male subjects.

Acknowledgements

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Disclosure of conflict of interest

None.

Authors’ contribution

You-Kang Chang, Yu-Heng Lai and Semon Wu wrote the paper and conceived and designed
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the experiments; You-Kang Chang and Yu-Heng Lai analyzed the data; Yen Chu and Chun-Yao Huang collected and provided the sample for this study; Ming-Cheng Lee performed the ELISA.

Abbreviations

PF2D, 2-D liquid phase fractionation system; HP, Haptoglobin; APOA1, apolipoprotein Al; CEA, carcinoembryonic antigen; AUC, area under the ROC curve; TFA, trifluoroacetic acid; ROC, Receiver Operating Characteristic.

Address correspondence to: Semon Wu, Department of Life Science, Chinese Culture University, #55, Hwa-Kang Road, Yang-Ming-Shan, Taipei 11114, Taiwan. Tel: +886-2-2861-0511 Ext. 26234; Fax: +886-2-2862-3724; E-mail: semonwu@yahoo.com.tw

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