Original Article

Differentially expressed glycosylated patterns of α-1-antitrypsin as serum biomarkers for the diagnosis of lung cancer

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Received 5 August 2014; Revised 19 October 2014; Accepted 21 October 2014

Abstract

Lung cancer is the most common malignancy worldwide. Thus, there is a critical need for diagnostic biomarkers with adequate sensitivity and specificity for lung cancer detection. Glycans in glycoproteins are significantly altered in cancer, and may serve as a tool for identifying potential diagnostic biomarkers. Recent studies have reported changes in α-1-antitrypsin (A1AT) glycosylation in lung cancer serum, tissue and cell lines. In this study, a lectin microarray was used to detect glycosylation changes in serum A1AT from patients with lung adenocarcinoma (ADC), squamous cell lung cancer, small-cell lung cancer (SCLC) and benign pulmonary diseases. Differentially expressed glycosylated patterns of A1AT were identified by lectin arrays and were confirmed by lectin-based enzyme-linked immunosorbent assay (ELISA). We found that galactosylated A1AT could distinguish non-small-cell lung cancer (NSCLC) from benign pulmonary diseases (AUC = 0.834); fucosylated A1AT showed exceptional capability in distinguishing ADC from benign diseases (AUC = 0.919) or other lung cancer subtypes (AUC = 0.844), and A1AT containing poly-LacNAc could detect SCLC from benign diseases (AUC = 0.905) or NSCLC (AUC = 0.707). The present study indicates that glycosylated patterns of A1AT may serve as potential biomarkers for detection of lung cancer. Further studies in larger sample sizes are necessary to validate the clinical utility of these markers.

Key words: α-1-antitrypsin, glycosylation, lectin-based ELISA, lectin microarray, lung cancer

Introduction

Lung cancer is the most common malignancy worldwide (Pirozynski 2006; Smith et al. 2011). It is also the leading cause of cancer-related deaths in both men and women. In China, there are ~540,000 new cases of lung cancer per year, and over 480,000 deaths annually (Chen et al. 2010). The main types of lung cancer are small-cell carcinoma (SCLC) and non-small-cell carcinoma (NSCLC). The most common types of NSCLCs include squamous cell lung cancer (SCLC) (30%), lung adenocarcinoma (ADC) (32%) and large-cell carcinoma (10%). SCLCs account for <20% of lung cancer cases. Despite our progress in the understanding of tumor biology and advancement in treatment strategies, the 5-year survival rate for patients with lung cancer is <15%. One of the main reasons for this dismal prognosis is the fact that the majority of patients with lung cancer are diagnosed during advanced stages of cancer and miss the opportunity for surgical resection.
Currently, testing for early lung cancer detection in asymptomatic individuals is not recommended by any medical/scientific organization (Smith et al. 2011). None of the current diagnostic methods, including chest X-ray, computed tomography and/or sputum cytology, have been able to detect lung cancer early enough and decrease lung cancer mortality (Pastorino et al. 2003; Pirozynski 2006). Serum biomarkers are one of the most important tools in screening for lung cancer, and can be used for early detection, staging, predicting the response to various therapeutic strategies and monitoring the course of the disease and recurrence after treatment. Serum-based biomarker testing is available and convenient. To date, several potential biomarkers have been proposed for the detection of lung cancer, including carcinoembryonic antigen, cytokeratin 19 (CYFRA21-1), neuron-specific enolase and carbohydrate antigen 125 (Bharti et al. 2007). However, their role in the clinical detection of lung cancer is limited owing to their low specificity and sensitivity (Bharti et al. 2007; Li et al. 2012, 2013). Therefore, it is necessary to further investigate novel serum biomarkers for the diagnosis and screening of lung cancer.

Glycan structures of serum glycoproteins have been reported to change significantly during the occurrence and progression of cancer (Zhao et al. 2006; Saldova et al. 2008, 2011). Thus, altered glycosylation patterns may serve as potential diagnostic biomarkers. α-1-Antitrypsin (A1AT) is a serum glycoprotein with three potential glycosylation sites (Carrell et al. 1982). Increased A1AT levels have been found in a variety of cancers, including lung, prostate and breast cancer, but some benign pulmonary diseases can also have elevated A1AT levels (Chen et al. 1992; El-Akawi et al. 2008, 2010). In recent years, a number of glycosylation changes have been reported in serum A1AT of lung cancer patients (Rho et al. 2009; Narayanasamy et al. 2011; Wen et al. 2012). Therefore, specific glycosylated alterations in serum A1AT may be important in the development of diagnostic biomarkers for lung cancer.

In the present study, a lectin array strategy was applied to detect lectin-specific glycosylation changes in the single serum glycoprotein A1AT in lung cancer and benign pulmonary disease. Differentially expressed glycopatterns of A1AT in serum were identified using lectin microarrays to distinguish patients with ADC, SQLC and SCLC from those with benign conditions. We also performed a lectin-based ELISA to verify these aberrant glycopatterns and determine their diagnostic value as potential biomarkers for lung cancer.

**Results**

**Purification of A1AT from patients sera**

Age- and gender-matched serum samples from ADC, SQLC, SCLC and benign controls (n = 12, for each group) were pooled. To extract the A1AT from the sera, the epoxy-coated magnetic particles conjugated to an A1AT-specific antibody were used. To confirm the efficiency of the affinity purification, purified samples were analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by silver staining (Figure 1A). The protein extracted from the serum presented one apparent band between 46 and 58 kDa. The band matched the molecular weight of A1AT, which is 51 kDa as previously reported (Carrell et al. 1982). In order to identify A1AT in eluted samples, western blotting analysis was performed (Figure 1B). Compared with the crude serum and washing fraction, A1AT was visible in the eluted sample after A1AT affinity purification, suggesting that the protein fraction eluted by 0.5% SDS was A1AT.

**A1AT glycosylation changes detected by lectin microarrays**

The glycopatterns of serum A1AT from four groups were detected using lectin microarrays. The images are shown in Figure 2A and the normalized fluorescent intensities (NFIs) are summarized in Supplementary data, Table VII. The results of the lectin arrays revealed that the patterns of A1AT glycans in ADC, SQLC or SCLC were different compared with those in the benign control. The normalized data of the three lung cancer groups were compared with the control group based on the fold changes and according to the following criteria: fold change >1.5-fold or <0.67-fold in the pairwise comparisons indicated up- or downregulation of the kind of glycan, and the P-value must be <0.05 (Qin et al. 2012). Twelve lectins that showed differences in glycan expression between the lung cancer and benign control groups are shown in Table I. The fluorescent intensities of 9 lectins, 4 lectins and 7 lectins indicated significant differences between ADC and control, SQLC and control, SCLC and control, respectively. For instance, rGal and αGalNAc binder BS-1 showed significantly increased NFIs in all three lung cancer groups ADC, SQLC and SCLC. A high-Mannose, Manα1-6(Manα1-3)Man binder ConA and a Galβ1-4GlcNAc, Siaα2-3Gal, Galβ1-3GlcNAc, Siaα2-3 binder MAL-I showed decreased signals in ADC and SCLC patients, while a Galβ1-3GlcNAc, Terminal GalNAc binder BPL showed decreased signals in ADC and SQLC patients. Moreover, Fucα1-6GlcNAc (core fucose) and Fucα1-3Galβ1-4GlcNAc binders AAL showed increased NFIs in ADC patients, but decreased NFIs in SCLC patients. A multivalent Sia and (GlcNAc)α1-2Man binder WGA and a high-Mannose, Manα1-3Man binder GNA showed increased NFIs in SQLC patients. Branched (LacNAc)α1-2Man binder PWM showed increased NFIs in SCLC patients.
Further statistical analysis was performed on lectins, which showed only significantly increased NFIs in one kind of lung cancer in comparison with the benign control. The NFIs of the lectins from the three lung cancer subtypes were compared with each other (Figure 2B). Briefly, the difference in AAL signals was significant between ADC and the other lung cancer subtypes, SQLC ($P = 0.038$).
and SCLC (P = 0.008). Our data also indicated that a significant difference (P < 0.001) existed in PWM signals between SCLC and other lung cancer subtypes, ADC or SQLC. Meanwhile, WGA and GNA showed significant differences in SCLC compared with ADC (P < 0.001 and P = 0.044), whereas SQLC exhibited no significant difference when compared with SCLC (P = 0.051 and 0.085).

From the lectin microarray analysis, we identified three candidate biomarkers: galactosylated A1AT (identified by BS-I) could be used for detection of lung cancer, fucosylated A1AT (identified by AAL) could be used for detection of ADC, and A1AT containing poly-LacNAc, which was identified by PWM, could be used for detecting SCLC.

Validation of glycosylation changes of A1AT by lectin-based ELISA

Three lectins (BS-I, AAL and PWM) were selected and subsequently used to validate the results of the lectin microarrays. BS-I, AAL- and PWM-based ELISAs were performed to confirm the presence of these candidate biomarkers in the crude sera of 28 ADC, 23 SQLC, 19 SCLC patients and 25 benign control individuals (Figure 2C). According to the results of BS-I lectin microarray, the levels of galactosylated A1AT were increased in the sera of the patients with the three types of lung cancer. Statistically significant differences were observed between the ADC and control samples (P < 0.001), between the SQLC and control samples (P = 0.001), but not between the SCLC and the control samples (P = 0.366). The serum levels of galactosylated A1AT, which was detected as an upregulated glycosylation pattern in the SCLC samples by lectin array analysis, exhibited no significant difference between the samples from the patients with SCLC and the controls. The discrepancy between the lectin microarrays and ELISA data might be attributed to differences in the treatment of the samples and detection methods. The AAL-based ELISA, consistent with the lectin array analysis, showed that the A1AT fucosylation levels were significantly higher in the ADC samples compared with the samples of the other three groups, SQLC, SCLC and control (P < 0.001). In addition, the levels of fucosylated A1AT containing poly-LacNAc were significantly increased in the SCLC samples compared with the ADC (P = 0.005), SQLC (P = 0.007) and control samples (P < 0.001), which is in concordance with the results of the lectin array.

Receiver operating characteristic analysis for the candidate biomarkers

Receiver operating characteristic (ROC) curve analysis was performed for each of the three A1AT glycopatterns to evaluate the possibility of galactosylated A1AT, fucosylated A1AT and A1AT containing poly-LacNAc as a biomarker of NSCLC, ADC and SCLC respectively, as shown in Figure 3. The serum levels of galactosylated A1AT had an AUC of 0.834 with a specificity of 76% at a sensitivity of 80.4% for distinguishing NSCLC from benign diseases (Figure 3A). The serum levels of fucosylated A1AT resulted in an AUC of 0.919 with a specificity of 88% at a sensitivity of 82.1% for differentiating ADC from benign diseases, and an AUC of 0.844 with a specificity of 69% at a sensitivity of 85.7% for differentiating ADC from other lung cancer subtypes (Figure 3B). The serum levels of fucosylated A1AT containing poly-LacNAc had an AUC of 0.905 with a specificity of 88% at a sensitivity of 88% for differentiating ADC from benign diseases (Figure 3C). The A1AT containing poly-LacNAc had an AUC of 0.905 with a specificity of 88% at a sensitivity of 88% for differentiating ADC from other lung cancer subtypes.

Correlation of serum biomarker levels with lung cancer stage

Linear regression analysis of the ELISA data was performed to ensure the correlation of serum glycosylation A1AT levels with lung cancer stage. We segregated NSCLC patients using their disease stages based on the American Joint Committee on Cancer staging system (Table II). Among the 28 ADC samples measured in the lectin-based ELISA, 2 were at Stage I, 4 at Stage II, 7 at Stage III and 15 at Stage IV. Moreover, among the 23 SQLC samples, 1 was at Stage I, 3 at Stage II, 12 at Stage III and 7 at Stage IV. The results in Figure 4 revealed that the serum levels of fucosylated A1AT correlated strongly with the ADC stage. The Spearman correlation coefficient (r) for fucosylated A1AT was 0.481 (P = 0.009). The serum galactosylated A1AT levels were not correlated with the NSCLC stage (r = 0.260, P = 0.065) as shown in Figure 4. As for SCLC, samples of SCLC were divided

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<table>
<thead>
<tr>
<th>Table I. Glycopatterns of serum A1AT</th>
<th>Fold change</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Lectin</td>
<td>Specificity</td>
<td>ADC/ benign</td>
<td>SQLC/ benign</td>
</tr>
<tr>
<td>AAL</td>
<td>Fucot-6 GlcNAc(core fucose), Fucot-3(Galβ1-4)GlcNAc</td>
<td>1.65*</td>
<td>–</td>
</tr>
<tr>
<td>RCA-120</td>
<td>β-Gal, Galβ1-4GlcNAc(type II), Galβ1-3GlcNAc (type I)</td>
<td>2.23***</td>
<td>–</td>
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<tr>
<td>STL</td>
<td>Trimmers and tetramers of GlcNAc, core (GlcNAc) of N-glycan, oligosaccharide containing GlcNAc and MurNac</td>
<td>0***</td>
<td>–</td>
</tr>
<tr>
<td>BS-I</td>
<td>αGal and αGalNAc</td>
<td>3.06***</td>
<td>2.04***</td>
</tr>
<tr>
<td>ConA</td>
<td>High-Mannose, Man0−6(Mano 1-3)Man, terminal GlcNAc</td>
<td>0**</td>
<td>–</td>
</tr>
<tr>
<td>DSA</td>
<td>β-D-GlcNAc, (GlcNAcβ1-4)n, Galβ1-4GlcNAc</td>
<td>0***</td>
<td>–</td>
</tr>
<tr>
<td>ACA</td>
<td>Galβ1-3GalNAcα-Ser/Thr(T)</td>
<td>0*</td>
<td>1.68**</td>
</tr>
<tr>
<td>WGA</td>
<td>Multivalent Sia and (GlcNAc)n</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PWM</td>
<td>Branched (LacNAc)n</td>
<td>0**</td>
<td>–</td>
</tr>
<tr>
<td>MAL-I</td>
<td>Galβ1-4GlcNAc, Sia2-3Gal, Galβ1-3GlcNAc, Sia2-3</td>
<td>0**</td>
<td>–</td>
</tr>
<tr>
<td>GNA</td>
<td>High-Mannose, Man0-3Man</td>
<td>–</td>
<td>1.81**</td>
</tr>
<tr>
<td>BPL</td>
<td>Galβ1-3GalNAc, terminal GalNAc</td>
<td>0*</td>
<td>0*</td>
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</tbody>
</table>

*, no significant difference.

**Showing significant differences between lung cancer groups and benign control group detected by lectin microarray. The NFIs of each lectin were compared based upon fold changes. **P < 0.05; ***P < 0.01; ****P < 0.001.
into the limited disease stage (LD; 4 samples) and the extensive disease stage (ED; 15 samples). Statistically significant differences were not observed in the serum levels of A1AT containing poly-LacNAc between the LD and ED samples ($P = 0.665$).

Taken together, our results suggest that the serum levels of galactosylated A1AT, fucosylated A1AT and A1AT containing poly-LacNAc could be used as potential biomarkers for NSCLC, ADC and SCLC, respectively.
Characteristics of the patients

<table>
<thead>
<tr>
<th>Patient category</th>
<th>ADC  n = 28</th>
<th>SQLC  n = 23</th>
<th>SCLC  n = 19</th>
<th>Benign  n = 50</th>
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<tbody>
<tr>
<td>Characteristics/total number</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (year)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>62</td>
<td>63</td>
<td>59</td>
<td>58</td>
</tr>
<tr>
<td>Range</td>
<td>40–82</td>
<td>39–82</td>
<td>35–76</td>
<td>36–83</td>
</tr>
<tr>
<td>SD</td>
<td>11.5</td>
<td>11.4</td>
<td>10.3</td>
<td>11.9</td>
</tr>
<tr>
<td>Gender</td>
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<td></td>
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</tr>
<tr>
<td>Male</td>
<td>15</td>
<td>19</td>
<td>13</td>
<td>33</td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>4</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever</td>
<td>11</td>
<td>18</td>
<td>13</td>
<td>28</td>
</tr>
<tr>
<td>Never</td>
<td>17</td>
<td>5</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td>Stagea</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>4</td>
<td>3</td>
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<tr>
<td>III</td>
<td>7</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>15</td>
<td>7</td>
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</tbody>
</table>

ADC, lung adenocarcinoma; SQLC, squamous cell lung cancer; SCLC, small-cell lung cancer.

aPatients with SCLC were divided into the LD stage (4 samples) and the ED stage (15 samples).

Fig. 4. Linear correlation of galactosylated A1AT serum levels with NSCLC stage and fucosylated A1AT serum levels with ADC stage. For each biomarker, the Spearman correlation coefficient (r) was given along with the probability that levels of the biomarkers were correlated with lung cancer stage.

Discussion

Glycosylation is one of the most common posttranslational modifications of secreted proteins and plays a significant role in cell–cell interactions, cell adhesion, malignant transformation and metastasis, which are important aspects in the development of cancer (Arnold et al. 2008). The identification of novel serum glycomarkers has become a topic of increasing interest, and may lead to improvements in the sensitivity and specificity of the detection and diagnosis of many cancers, such as breast, stomach, colon, pancreatic and hepatocellular carcinoma (Okuyama et al. 2006; Kyselova et al. 2008; Comunale et al. 2010; Bones et al. 2011; Park et al. 2012). In addition, the use of advanced glycochemistry techniques are aiding the discovery of glycosylated biomarkers for detection of various diseases. Lectin microarray, a high-throughput glycome technique, provides a rapid method to observe multiple, distinct carbohydrate structures simultaneously, and does not require the release of N- or O-linked glycans before analysis, which guarantees that the real state of protein glycosylation in the sample is reflected (Pilobello et al. 2005; Yu et al. 2012). Lectin-based ELISA is a fast and uncomplicated technique to detect the differences based on the glycosylation patterns of a specific protein in a complex biological sample like serum or tissue lysate, rather than the total protein level (Peter et al. 1998; Saeland et al. 2012).

Human A1AT is a serum glycoprotein composed of 394 amino acids, with three potential glycosylation sites. It is an acute-phase response protein, which can inhibit serine proteinase. A1AT genetic deficiency accompanied by the early onset of degenerative lung disease is common in European populations. Some recent studies have found elevated serum levels of A1AT in lung cancer (El-Akawi et al. 2008, 2010). However, since benign pulmonary diseases can elevate A1AT levels, A1AT lacks both the sensitivity and specificity to serve as an effective screening tool to differentiate lung cancer from benign conditions. A number of changes in A1AT glycosylation have been reported in lung cancer serum, tissue and cell lines. Using ConA-affinity chromatography and nano-LC-MS/MS analysis, Wen et al. (2012) found that the amount of A1AT bound to ConA columns was significantly lower in the sera of ADC patients than that of the healthy controls. Furthermore, a study by Kataoka et al. (1993) demonstrated that in LC-2/ad cells, an ADC cell line, derived A1AT showed decreased affinity to ConA when compared with the plasma A1AT of healthy individuals. Since A1AT can inhibit natural killer (NK) cell activity (Laine et al. 1990), and ConA non-reactive glycopatterns of A1AT inhibit NK cell activity more efficiently (Lejeune et al. 1989), tumor-derived A1AT may modulate the host NK cell activity in favor of the tumor cells. In this study, ConA reactive A1AT was also found to be down-regulated in ADC compared with benign controls. Thus, the aforementioned reasons could imply that mannose downregulated A1AT in ADC patient’s serum may be secreted by ADC cells and play an important role in the development of ADC.

In this study, we mainly focused on analyzing the glycosylation changes in serum A1AT in lung cancer. For the first time, we have identified that galactosylated A1AT can discriminate NSCLC from benign pulmonary diseases (AUC = 0.834) with a sensitivity of 80.4% and a specificity of 76% using lectin microarrays and BS-I-based ELISA analysis. We also examined the fucosylation changes in serum A1AT by AAL-based ELISA and found that fucosylated A1AT was increased in ADC. Consistent with our results, Wen et al. (2012) found that A1AT showed increased fucosylation during lung adenocarcinoma progression by western blotting and AAL staining. In another study, increased fucosylation levels were observed in the
is the major cause of the majority of deaths caused by cancer. Serum biomarkers for lung cancer have been premeditated in the hope of achieving the early detection of the disease. We compared the glycosylated A1AT serum levels between lung cancer samples at early stage and benign controls. The serum levels of galactosylated A1AT identified by BS-I were significantly increased in I/II stage NSCLC samples compared with the benign samples ($P = 0.018$). The serum levels of A1AT containing poly-LacNAc identified by PWM were significantly higher in limited stage SCLC samples compared with those in the benign samples ($P = 0.010$). Statistically significant difference was not observed between ADC and benign samples in the levels of fucosylated A1AT identified by AAL ($P = 0.056$). However, we found that the serum levels of fucosylated A1AT correlated strongly with the ADC stage. This marker might be a valuable prognostic index. The sample size at early stage is relatively small (6 for ADC, 4 for SCLC and 4 for SCLC), we will collect more early stage samples and conduct further investigation to confirm the diagnostic values of these biomarkers for detecting lung cancer at its early stage in the future.

There are still some limitations in this study. The data in our study need to be confirmed using larger groups of patients to determine whether these glycan-specific patterns of A1AT are truly reliable markers. Future studies will determine whether a combination of these A1AT glycosylation marker measurements with other potential biomarkers can significantly improve the sensitivity and specificity of current markers used for the diagnosis of lung cancer. We performed a lectin-based ELISA in this study, in which lectins were coated on the 96-well plate. The galactosylated A1AT discordance between our lectin microarray and ELISA results might be attributed to the usage of pooled samples in lectin array experiments and individual samples in ELISA, as well as the steric constraints in the lectin-based ELISA. Follow-up studies should be performed to improve the present method and attempt antibody-coated lectin ELISA to determine the optimal approach for detection of glycosylated A1AT in serum in future (Wang et al. 2009). Furthermore, the detailed molecular mechanisms of producing differential glycosylated structures of A1AT remain unknown. Further experiments need to be performed to focus on the glycosylation pathway in the development of lung cancer and to determine the association between specific glycosylation changes and malignant biological behavior.

In summary, we applied a glycomics strategy to identify and confirm differentially expressed glycosylation patterns of A1AT for the detection of lung cancer. Our results suggest that changes in the serum levels of glycosylation A1AT could be effectively used for detecting lung cancer. Finally, galactosylated A1AT, fucosylated A1AT and A1AT containing poly-LacNAc were identified as the most promising candidates for biomarkers to diagnose NSCLC, ADC and SCLC, respectively.

Materials and methods

Sample collection

The study methodologies conformed to the standards set by the Declaration of Helsinki; the protocols for this study were approved by the Institutional Review Boards of both Xi’an Jiaotong University and Northwest University. Informed consent was obtained from all patients, and clotted blood samples were obtained at the First Affiliated Hospital of Xi’an Jiaotong University from patients with a new diagnosis of lung cancer ($n = 28$ for ADC, $n = 23$ for SCLC, $n = 19$ for SCLC) and from patients with benign pulmonary disease ($n = 50$) between August 2012 and April 2013.
All lung cancer cases were obtained and confirmed histologically before treatment. The study excluded the patients who were diagnosed for any other primary cancer and lung cancer as their secondary primary cancer. Samples from patients with benign pulmonary diseases consisted of pneumonia, benign pulmonary nodule and tuberculous pleuritis. Patients with pneumonia were diagnosed by clinical symptoms and roentgenographic examination showed alveolar consolidation. The diagnosis of benign pulmonary nodule and tuberculous pleuritis was based on biopsy results of the nodules and pleura.

Serum samples were immediately separated from the clotted whole blood by centrifugation at 1500 x g for 10 min at 4°C. After centrifugation, the samples were divided into 100 μL aliquots in cryotubes and stored at −80°C until use. Epidemiologic and clinical data from the lung cancer patients and benign controls were collected by questionnaire and/or from individual medical case record/reports. The characteristics of all study participants are summarized in Table II.

Affinity purification of A1AT from serum

For affinity purification of A1AT, sera from 12 age- and sex-matched patients of each ADC, SQCLC, SCLC and benign pulmonary disease was, respectively, pooled. The procedure for affinity separation of A1AT is described as follows (Yang et al. 2012). Epoxy-coated magnetic particles were purchased from Shaanxi Lifegen Co (Xi’an, China). One hundred micrograms of goat anti-human A1AT polyclonal antibody (Abcam, Cambridge, MA) was dissolved in 600 μL of coupling buffer (20 mM boric acid buffer, pH 7.4). One milligram of epoxy-coated magnetic particles was washed three times with the coupling buffer, and then the antibody was coupled to the epoxy-coated magnetic particles by incubation under gentle shaking for 2 h. The magnetic particles were then blocked for 30 min in blocking buffer (1%, w/v, BSA, 2%, v/v, ethanolamine, pH 8.8). Fifty microliters of the pooled serum sample were incubated with the antibody-conjugated magnetic particles for 1.5 h in 600 μL of binding buffer (0.1 M Tris–HCl, 0.15 M NaCl, pH 7.4) under gentle shaking. The magnetic particles were washed five or six times with cleaning buffer (0.2%, v/v, Tween-20 in binding buffer) until the uncaptured proteins were removed from the supernatant. The captured proteins were then eluted with 300 μL of elution buffer (0.5%, w/v, SDS). The concentration of purified A1AT was measured by absorbance at 280 nm with the NanoDrop 2000 (Thermo Scientific, Waltham, MA). Experimental procedures above were performed at room temperature (RT).

SDS–PAGE and western blotting analysis

The purified protein was analyzed by SDS–PAGE and subsequent western blotting. For SDS–PAGE, samples were boiled for 4 min at 100°C, mixed with 5x loading buffer, and then loaded onto a 3% polyacrylamide stacking gel and a 10% resolving gel. After electrophoresis, the gel was stained directly with silver nitrate. For western blotting, the proteins were transferred onto a polyvinylidenefluoride membrane (Millipore, Bedford, MA). After transfer, the membrane was blocked with 5% (w/v) skim milk (Becton Dickinson, Franklin Lakes, NJ) in TBST (10 mM Tris–HCl, 150 mM NaCl, 0.2%, v/v, Tween-20, pH 7.6) at 4°C overnight. The membranes were then incubated with 2000-fold diluted mouse monoclonal anti-A1AT (Abcam) under gentle shaking at RT for 1 h. After washing three times with TBST, the membrane was incubated with 4000-fold diluted horseradish peroxidase (HRP)-labelled Goat Anti-Mouse IgG (Xianfeng Biotech, Xi’an, China) for 1 h, followed by three additional washings. The blots were visualized with a DAB HRP color development kit (Xianfeng Biotech).

Lectin microarrays

Thirty-seven lectins purchased from Vector Laboratories (Burlingame, CA), Sigma-Aldrich (St. Louis, MO) and Calbiochem (Darmstadt, Germany) with different specificities were spotted on homemade epoxysilane-coated slides according to a protocol (Jian et al. 2009; Qn et al. 2012) with Stealth micro spotting pins (SMP-10B) (TeleChem, Atlanta, GA) using a Capital smart microarroayer (CapitalBio, Beijing, China). The sugar-binding specificities of lectins and the layout of the lectin microarrays are summarized in Supplementary data, Table S1 and Figure S1. The concentration of each lectin was 1 mg/mL in the manufacturer’s recommended buffer containing 1 mM of the appropriate monosaccharide. Each lectin was printed in triplicate per block with triplicate blocks on one slide. BSA and BSA-conjugated with Cy3 were used to validate the feasibility of the lectin microarray. The slides were incubated in a humidity-controlled incubator at 50% humidity overnight to immobilize the lectins. After incubation, the slides were blocked with blocking buffer (2%, w/v, BSA, pH 7.4) for 1 h, and then washed twice with PBST (0.2%, v/v, Tween-20 in PBS, pH 7.4), followed by a final washing in PBS. The slides were dried by centrifugation at 600 rpm for 5 min before use.

A1AT labelling and incubation

Purified A1AT was concentrated using a Microcon YM-3 (Millipore) to 50 μL in 0.1 M sodium carbonate buffer (pH 9.3), and labelled with Cy3 fluorescence dye (GE Healthcare, Buckinghamshire, UK). Labelled proteins were separated from the excess free dye by Sephadex G-25 columns (GE Healthcare) according to the manufacturer’s instructions. Next, 4 μg (Qn et al. 2013) of Cy3-labeled proteins was diluted to 700 μL with hybridization buffer (2%, w/v, BSA, 500 mM glycine and 0.1%, v/v, Tween-20 in PBS, pH 7.4), and the resultant Cy3-labeled protein solution was applied to the lectin microarrays. After incubation in the chamber at 37°C for 3 h in a rotisserie oven set at 4 rpm, the slides were washed twice with PBST for 5 min each, once with PBS for 5 min and dried by centrifugation at 600 x g for 5 min. Finally, the slides were scanned at 70% photomultiplier tube and 100% laser power settings by a Genepix 4000B confocal scanner (Axon Instruments, Union City, CA).

Microarray data acquisition and analysis

The acquired images were analyzed for Cy3 detection at 532 nm by GenePix 3.0 software (Axon Instruments). The net intensity value of each spot was calculated by subtracting the average background value, and the values, which were less than the average background ±2SD were removed from each data point. The median of the effective values of each lectin was normalized by the sum of the medians of all of the lectin effective values in one block. Each group of pooled sera was observed by three repeated microarrays and the normalized medians of each lectin from nine repeated blocks were averaged and its SD was calculated. The normalized data of the ADC, SQCLC, SCLC groups and benign control group were compared with determine the relative change in serum A1AT glycosylation levels.

Lectin-based ELISA

Serum samples from 28 ADC, 23 SQCLC, 19 SCLC and 25 benign control patients were run on lectin-based ELISAs. BS-I, AAL and PWM, which were selected based on the lectin microarray analysis, were used to detect A1AT differentially expressed glycopatterns. Lectin-based ELISAs were performed as previously described (Narayanasamy et al. 2011; Wu et al. 2012; Wu et al. 2013). One hundred microliters of 3 μg/mL BS-I, AAL or PWM diluted in 0.05 M sodium carbonate.
buffer (pH 9.6) was added to each well of a 96-well plate and incubated at 4°C overnight. The coated plate was then blocked with 3% (w/v) BSA in PBST (0.1%, v/v, Tween-20 in PBS, pH 7.4) at RT for 1 h, followed by washing with PBST. Serum samples were diluted 80-fold with 1% (w/v) BSA in PBST, and then 100 μL of sample was applied to each well. After 1 h incubation at RT, the plate was washed five times with PBST. Subsequently, 100-fold diluted biotinylated anti-human A1AT polyclonal antibody (Abcam) in PBST was added for 1 h at RT. After additional washing, 2000-fold diluted HRP-conjugated streptavidin (Sigma-Aldrich) was added to the plate and incubated for 30 min. After washing the plate five times, TMB working solution (Sigma-Aldrich) was added to each well, followed by stop solution (2 M sulfuric acid). To determine the concentration of A1AT bound to a specific lectin, the absorbance of the plate was measured at 450 nm.

Statistical analysis

All statistical analysis was done with SPSS 20 software. Differences between two arbitrary datasets were evaluated using one-way analysis of variance (ANOVA) or Mann–Whitney test to each lectin signal, and $P$-values of $<0.05$ were considered statistically significant. ROC curves were constructed by plotting sensitivity versus 1-specificity for every possible cutoff score and area under the ROC curve (AUC) was calculated. The linear relation between the lung cancer stage and the marker level was assessed using Spearman rank correlation coefficient.

Supplementary data

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

Authors’ contributions

Y.L. and T.M. conducted the ELISA; H.Y., L.G., P.S., X.L., H.R. and S.Z. were involved with sample collection and processing; Y.L., A.T., L.J., Z.L. and M.C. designed the study, obtained the fund and conducted the microarrays. Y.L. and A.T. prepared the manuscript.

Funding

This study was supported by Research and Development Project of Science and Technology of Shaanxi Province (Grant No. 2013K12-05-03).

Acknowledgement

The authors are thankful to all the subjects involved in this study.

Conflict of interest statement

None declared.

Abbreviations

A1AT, α-1-antitrypsin; ADC, adenocarcinoma; AFP, α-fetoprotein; ANOVA, analysis of variance; ED, extensive disease; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; LD, limited disease; NK, natural killer; NSCLC, non-small-cell lung cancer; ROC, receiver operating characteristics; RT, room temperature; SCLC, small-cell lung cancer; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SQLC, squamous cell lung cancer.

References


