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Fucosylated glycoproteomic approach to identify a complement component 9 associated with squamous cell lung cancer (SQLC)

Arul Narayanasamy[a,1], *Jung-Mo Ahn*[a,1], *Hye-Jin Sung*[a], *Deok-Hoon Kong*[b], *Kwon-Soo Ha*[b], *Soo-Youn Lee*[c], *Je-Yoel Cho*[a,*]

[a] *Department of Biochemistry, School of Dentistry, Kyungpook National University, & ProtAnBio Daegu, South Korea*
[b] *Department of Molecular and Cellular Biochemistry, Vascular System Research Center, School of Medicine, Kangwon National University, Chuncheon, South Korea*
[c] *Department of Laboratory & Genetics, Samsung Medical Center, Sungkyunkwan University of Medicine, South Korea*

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ABSTRACT

Human lung cancer is a major cause of cancer mortality worldwide. Understanding the pathophysiological features and the development of novel biomarkers for diagnosis as well as treatment are major tasks. In the present study, sera from ten SQLC patients and healthy control (HEC) were collected and pooled, respectively. The pooled sera were depleted via an immunoaffinity method and further subjected to fucosylation enrichment. Enriched fucosylated glycoproteins were resolved by SDS-PAGE and subsequently analyzed by LC-ESI-MS/MS. From comparative proteomic analysis, we selected the C9 protein. C9 protein levels were validated by Western blot, protein arrays and the fucosylation levels of C9 by hybrid lectin ELISA (HLE) in the sera of 120 HEC and 118 SQLC patients. The C9 protein level was 6.4-fold higher in SQLC patients compared to HEC, as determined by Western blot analysis. The results were concurrently confirmed by a protein array that showed a C9 level significantly higher in SQLC patients, as compared to HEC, with a sensitivity of 53% and a specificity of 89%. C9 fucosylation levels were significantly higher in SQLC patients compared to HEC ($p<0.05$) when tested by HLE. These findings suggest that C9 and fucosylated form could serve as a useful marker for SQLC.

1. Introduction

Lung cancer is one of the most prevalent and life-threatening neoplasia in most parts of the world. It has an incidence of 1.2 million people worldwide and accounts for about 25% of all cancer deaths [1]. In Korea, the incidence and mortality associated with lung cancer are predicted to steadily increase in the future (Korea National Statistical Office). Lung cancer is classified into two main types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which constitutes about 80% of lung cancer cases. The major histological subtypes of NSCLC include lung adenocarcinoma (ADC), SQLC, and large cell carcinoma [2]. Accounting for 25% of lung cancers [3], SQLC is recognized by the histological features of intercellular bridging, squamous pearl formation, and individual cell keratinization [4]. About 30% of patients

* *Corresponding author at:* Department of Biochemistry, School of Dentistry, Kyungpook National University, 101, Dong In-Dong 2Ga, Jung-Gu, Daegu, 700-422, South Korea. Tel.: +82 53 420 4997; fax: +82 53 421 1417.
E-mail address: jeycho@knu.ac.kr (J.-Y. Cho).
[1] These two authors contributed equally to this work.

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who are diagnosed with NSCLC are able to undergo curative resection, whereas the remaining patients with the advanced disease are mainly treated with chemotherapy alone or in combination with a local modality [5].

Serum biomarkers for lung cancer have been premeditated in the hope of achieving the early detection of the disease, improving the diagnosis, predicting the response or monitoring the recurrence after treatments [6]. In the current clinical setting, a variety of serum markers are employed for NSCLC, although their utility is contentious due to their low sensitivity and specificity. Currently, several potential biomarkers are proposed for lung cancers, including enolase (ENO), carcinoembryonic antigen (CEA), cytokeratin 19 (CYFRA 21–1), squamous cell carcinoma antigen (SCCA), carbohydrate antigen 125 (CA-125), tissue polypeptide antigen (TPA) [7], and a fragment of the plasma kallikrein (KLKB1) [8]. However, none of these biomarkers have been integrated into clinical practice. Hence, while the discovery of clinical serum biomarkers in SQLC is required, larger outlook studies with clinically-relevant modeling and premises are required to address the usefulness of novel potential serum biomarkers in the management of SQLC patients.

Glycosylation is one of the most common post-translational modifications (PTM) of secreted proteins [9]. Glycoproteins play important roles in diseases, such as cancer and inflammatory disease [10]. Glycoprotein enrichment techniques provide a means not only to deplete abundant serum proteins but also to enrich for a functional subset of proteins. Specifically, fucosylation, which is the transfer of a fucose residue to oligosaccharides and proteins, is associated with a variety of events in carcinogenesis and cancer progression [11,12]. Our study indicates that fucosylated glycoproteins might be promising targets for SQLC biomarker discovery. Recently, proteomic technologies, such as mass spectrometry and protein chips, have improved proteomic-based biomarker discovery.

In this study, we searched SQLC serum biomarkers by label-free LC-ESI-MS/MS analysis using SQLC patient sera vs. healthy control (HEC) sera after albumin and IgG depletion and subsequently fucosylated glycoprotein enrichment. Among the identified proteins, we selected either up- or down-regulated proteins by comparative proteomic analysis. Among the up-regulated proteins, we selectively focused on the complement component 9 (C9) protein and validated the C9 protein as a potential serological marker for SQLC using Western blotting (WB), protein array, and hybrid lectin ELISA (HLE). Our results suggest that the elevated C9 and fucosylated form could be a useful marker for SQLC.

2. Materials and methods

2.1. Clinical serum sample collection

Serum samples from patients with SQLC (118), ADC (20), SCLC (20), breast cancer (BC; 20), hepatocellular carcinoma (HCC; 20), stomach cancer (STC; 20) and HEC (120) individuals were collected from Samsung Hospital (Seoul, Korea). The protocols for this study were approved by the Samsung Hospital Institutional Review Board (IRB) and included informed consent. We obtained serum samples from patients with a confirmed diagnosis of lung cancer and other solid cancer along with the clinical history data of the patients (Supplementary data 1). HEC serum samples were obtained with informed consent from 120 volunteers who received medical checkups at Samsung Hospital. All samples were stored at −80 °C until use. Among the samples described above, the samples for HLE, protein array and WB analysis were randomly selected, and some of the samples were used for two or three analyses.

2.2. Albumin and IgG depletion

For albumin and IgG depletion from 10 HEC and 10 SQLC pooled sera, which were randomly selected from 120 HEC and 118 SQLC samples, we used a ProteoPrep immunoaffinity albumin and IgG depletion kit (Sigma, USA) according to the manufacturer's instructions.

2.3. Fucosylated glycoprotein enrichment by AAL lectin column

Agarose-bound *Aleuria aurantia* lectin (AAL) was purchased from Vector Laboratories (Burlingame, CA). The extraction of fucosylated glycoproteins was performed as described with modifications [13]. Briefly, albumin/IgG-depleted samples (400 μl) were supplemented with 1.6 ml of lectin adsorption buffer (GALAB, Germany) and incubated at 4 °C for 16 h with AAL in a spin column (Bio-Spin; Bio-Rad). After unbound proteins were removed by washing with binding buffer, the fucosylated glycoproteins were eluted with 100 μl of AAL elution buffer (GALAB, Germany).

2.4. AAL blot analysis

Lectin blot analyses were performed as described previously [14]. Briefly, 5 μg of protein was subjected to 12% SDS-PAGE. After electrophoresis, the gels were transferred to nitrocellulose membranes (Whatman, Germany). The membranes were blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) at 4 °C overnight and then incubated with biotinylated AAL (1.0 μg/μl; Vector Laboratories, CA) at room temperature (RT) for 1 h. The membrane was then washed three times with Tris-buffered saline-Tween 20 (TBS-T) and incubated with diluted horseradish peroxidase-avidin (Biolegend, CA) at RT for 1 h. The membrane was again washed three times with TBS-T and developed using an ECL system (Amersham Biosciences, UK).

2.5. LC-ESI-MS/MS analysis

Following 1D SDS PAGE and coomassie brilliant blue (CBB) staining, protein bands were excised and subjected to in gel tryptic digestion as previously reported [8]. LC-MS/MS analysis was performed using an LTQ mass spectrometer (Thermo Electron, San Jose, CA, USA) equipped with NSI sources (San Jose, CA). Analysis conditions for mass spectrometry were the same as previously reported [8]. Tandem mass spectra were