Glycosylated Alpha-1-acid glycoprotein 1 as a potential lung cancer serum biomarker

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ABSTRACT

Presently existing screening approaches for lung cancer are not being proving sufficient and sensitive, so a study was conducted to identify disease related biomarker proteins for diagnostic applications. A total of 100 lung cancer patients (88 non-small cell lung cancer and 12 small cell lung cancer) and 50 healthy controls were included in this study. Serum samples of patients and healthy controls were subjected to a series of proteomic approaches and as a result of two dimensional gel electrophoresis, a ~43 kDa protein was found to be differentially expressed compared to healthy controls. Quantitative profiling of two dimensional gels by Dymension software analysis displayed 3.58 fold increased expression of ~43 kDa protein in squamous cell carcinoma and 2.92 fold in case of adenocarcinoma. Mass spectrometric analysis resulted in identification of 8 differentially expressed proteins, out of which human Alpha-1-acid glycoprotein 1 was targeted for further validations. This candidate protein exhibited N-linked glycosylation at five amino acid residues; 33, 56, 72, 93, and 103 with significant score of 0.66, 0.78, 0.78, 0.53 and 0.66, respectively. Sandwich ELISA quantified high serum levels of Alpha-1-acid glycoprotein 1 in squamous cell carcinoma (2.93 g/l ± 1.22) and adenocarcinoma (2.39 g/l ± 1.13) when compared with healthy controls (0.83 g/l ± 0.21). One-way ANOVA analysis predicted highly significant variation of Alpha-1-acid glycoprotein 1, among all the study types (F-value 65.37, p-value 0.000). This study may prove as a non-invasive, cost effective and sensitive scheme for diagnosis of lung cancer, by passing the expensive and painful screening procedures.

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1. Introduction

Lung cancer, a leading cause of cancer mortalities all over the world; in both males and females (Lihong et al., 2014). Amongst 28% of total cancer mortalities 1.3 million deaths are attributed to lung cancer annually (Mascaux et al., 2010). In Asian population, especially in Pakistan male mortality due to lung cancer is elevated since last few years (GLOBOCAN, 2008). Lack of health education about smoking, radiation exposure and air pollution along with specific occupational toxicity may act as causative agent for this elevation.

Biomarker is defined as a molecule present in blood, tissues or other body fluids which acts as an indicator of a normal orabnormal process or disease (NCI, n.d.). Serum is a rich source of biochemical products which may serve as predictor of physiological or clinical status of patients (Steel et al., 2003).

Acute phase proteins like Alpha-1-acid glycoprotein 1 have been related to less prognostic outcomes in varied pathological states like cancer (Suarez Nieto et al., 1986). Over expression of Alpha-1-acid glycoprotein 1 has been reported in non-small cell lung cancer (NSCLC) patients (Kremer et al., 1988) and found exceptionally sensitive and specific predictor of lung cancer (Ganz et al., 1984). Considerable rise in Alpha-1 acid glycoprotein was observed in patients with active lung and gastrointestinal carcinomas compared to inactive disease (Ganz et al., 1983).

In this study, using different proteomic methodologies, differential lung cancer serum protein biomarkers have been identified and validated as compare to healthy control samples. This strategy will be helpful to study effective utility of identified proteins for timely diagnosis of disease and evaluation of therapeutic feedback.

2. Materials and methods

2.1. Sampling, serum preparation and protein quantification

Whole blood from healthy volunteers was collected from University of the Punjab, Lahore, while of patients from Allah Wali Oncology ward (Gulab Devi Chest Hospital, Lahore) according to approved Institutional Review committee protocol. Samples from patients were categorized in two groups: NSCLC (n = 88) and SCLC (n = 12). Amongst them NSCLC was further sub-divided into adenocarcinoma (n = 34), squamous cell carcinoma (n = 48) and large cell carcinoma (n = 6). Serum was separated and stored at $-80\,°C$ prior to assay. Total protein contents were estimated by Bradford method (Bradford, 1976).

2.2. One dimensional SDS-PAGE analysis of serum

SDS-PAGE (10%) was run according to method described by Laemmli (1970) and kept in fixative solution (30% ethanol, 10% acetic acid and 60% deionized water) for four hours. Staining was carried out overnight using Colloidal Coomassie stain G-250 (Sigma Chemicals Co., USA). After destaining, images were scanned by gel documentation system (SynGene, Gene snap).

2.3. 2DE (two dimensional gel electrophoresis) analysis of serum

On the basis of decreased incidence of small cell lung cancer as well as large cell type, only squamous cell carcinoma and adenocarcinoma were selected for further proteomic investigations (Siddiqui et al., 2010). 350 μl of rehydration buffer was used for IPG strips (18 cm,non-linear, pH 3–10, Serva) and 1–2 ml silicon oil (Fluka) was overlaid for preventing the dehydration of gel. Complete assembly was incubated at 20 °C overnight.

2.3.1. Isoelectric focusing (first dimension)

After rehydration, 500 μg of total serum proteins from both patients and healthy controls were resolved on IPG strips on flat bed equipment (Amersham, Scie plus, UK) to achieve 32 kV h.

2.3.2. SDS-PAGE (second dimension)

IPG strips were kept at room temperature until normalized and equilibration steps were carried out as recommended. Focused strips were run on 12% gel at recommended voltage followed by staining and destaining steps.

2.3.3. Image analysis

The gel images were scanned using image scanner (HP ScanJet 8200). 2D gel images of patient serum samples were compared with healthy controls by Dymension v 3.2.1 (SynGene, UK). The protein spots with expression intensity more than 1.5 fold (increase or decrease) were considered statistically significant for further proteomic analysis.

2.4. Tryptic digestion (In-gel) and mass spectrometric analysis (MALDI-TOF/TOF)

Labeled protein spots were picked, washed and incubated several times for removing stain and other contaminants in multiple steps. Finally, 400 ng (per spot) of trypsin (Promaga) was added and incubated on ice for 45 min followed by addition of 50 mM Ambic and then incubated at 37 °C overnight. Digested peptide extracts were obtained with trifluoro acetic acid for their mass spectrometric analysis.

Peptide mass fingerprinting was carried out on (Ultraflex III; BrukerDaltonics GmbH, Breman, Germany) MALDI-TOF/TOF mass spectrometer, by mixing 1:1 of tryptic digest and matrix (α-Cyano 4-hydroxy Cinnamic acid) followed by spotting on MALDI plate. Procedure was carried out according to the recommended instrumental protocol and resulting peak data was subjected to MASCOT search program for protein identification. The protein identity showing MASCOT score greater than 60 and sequence coverage 20% or more was accepted. MASCOT search parameters were set as follows: carbamidomethyl modification of Cysteine and possible oxidation of Methionine up to one missed cleavage, the enzyme was trypsin (Promaga, Cat# V5111). Protein identification databases were SwissProt and NCBInr, the specie selected for analysis was homo sapiens. Peptide mass tolerance used for search was less than 1.0 Dalton.

2.5. Detection of glycosylation

Glycosylation was predicted and confirmed in protein sequences of targeted protein using software; NetNGlyc 1.0 Server-CBS for prediction of N-linked glycosylation.

2.6. Immunological validation

Primary antibody of Alpha-1-acid glycoprotein 1 was raised in male albino mice in triplicate. The guidelines provided by the animal ethical committee were followed during whole immunization procedure. For each mouse, 50 μg of antigenic protein was mixed with PBS (phosphate buffer saline, pH 7.4) and inoculated subcutaneously using Freund's complete adjuvant. Three booster doses were prepared in incomplete Freund's adjuvant. After immunization, blood was drawn by cardiac puncture scheme and anti-serum was purified by using Pearl, IgG purification kit (G-Biosciences, Cat# 786-798) according to the manufacturer's instructions.

Western blotting was performed following the protocol of Towbin et al. (1979). 10 μg of serum proteins from all serum samples were resolved on 15% SDS-gels and gels were transferred to blotting membrane (8.5×7.5) (Nitrocellulose, 0.22 μm pore size (G-Biosciences, Cat# 786-018NC) allowing transfer of proteins for 1.5 h, at constant voltage of 18volts in a Semi Dry Transfer Cell assembly (Bio-Rad Trans-Blot Sd). The optimized dilution ratio (1:200) of primary antibody was applied to the blotting membrane and incubated at 4 °C overnight. Goat anti-mouse IgG, AP-conjugated secondary antibody (Cat# 786-R43, G-Biosciences) was added and incubated for 2 h at room temperature, followed by three washings with same washing buffer. Finally, the blot was developed by AP-substrate buffer and obtained desired signal intensity of the bands.

2.7. Densitometric analysis

The protein abundance in patient and healthy control samples was analyzed by Image J software (1.48v) for signal intensity measurements according to the recommended protocol of software.

2.8. Sandwich ELISA

Serum levels of targeted protein were quantified by sandwich ELISA using commercial ELISA kit (Antibodies-online.com, Cat # ABIN819742), following recommended protocol.

2.9. Data analysis

One-way ANOVA (SPSS version 19.0) was applied on 2D quantification data and ELISA data to compare and evaluate the statistical significance of targeted protein among study groups. p-value < 0.01 was considered to be significant in both cases. False discovery rate was also calculated. The histological types of patients