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Research Article

2-D difference gel electrophoresis of the lung squamous cell carcinoma *versus* normal sera demonstrates consistent alterations in the levels of ten specific proteins

Most lung cancers are diagnosed too late for curative treatment to be possible, therefore early detection is crucial. Serum proteins are a rich source of biomarkers and have the potential to be used as diagnostic and prognostic indicators for lung cancer. In order to examine differences in serum levels of specific proteins associated with human lung squamous carcinoma, immunodepletion of albumin and five other high-abundant serum proteins followed by 2-D difference gel electrophoresis (DIGE) analysis and subsequent MS was used to generate a panel of proteins found to be differentially expressed between the cancer and normal samples. Proteins found to have increased abundance levels in squamous cell carcinoma sera compared to normal sera included apolipoprotein A-IV precursor, chain F; human complement component C3c, haptoglobin, serum amyloid A protein precursor and Ras-related protein Rab-7b. Proteins found to have lower abundance levels in squamous cell carcinoma sera compared to normal sera included alpha-2-HS glycoprotein, hemopexin precursor, proapolipoprotein, antithrombin III and SP40; 40. The data presented here demonstrate that high-abundant protein removal combined with 2-D DIGE is a powerful strategy for the discovery of potential biomarkers. The identification of lung cancer-specific biomarkers is crucial to early detection, which in turn could lead to a dramatic increase in survival rates.

Keywords:
Biomarker / Difference gel electrophoresis / Immunodepletion / Serum / Squamous
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1 Introduction

Over 1 million people are diagnosed with lung cancer each year [1]. Most lung cancer is diagnosed too late for curative treatment to be possible. Efforts at early detection and treatment have had little success to date and hence the overall prognosis remains poor. In the majority of those diagnosed with lung cancer, the disease has already metastasised at the time of diagnosis. Lung cancer comprises broadly of two groups, small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). NSCLC comprises more than 80% of lung cancers including squamous cell carcinoma, adenocarcinoma and large cell carcinoma, with squamous carcinoma being the most common type.

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Abbreviations: AHSG, alpha-2-HS glycoprotein; **Apo-AIV,** apolipoprotein A-IV precursor; **ATH III,** antithrombin III

A major factor in the high mortality of lung cancer patients is the presence of metastatic tumours in approximately two-thirds of patients at time of diagnosis [2]. Detection of cancer in these patients at earlier stages would increase survival rates dramatically. The identification of lung cancer-specific biomarkers is critical to early detection. The pathophysiology of lung squamous cell carcinoma development is complex and incompletely understood. Genetic alterations involved in the pathogenesis of lung cancer produce proteins involved in cell growth, invasion/metastasis, differentiation, cell cycle processes, apoptosis and angiogenesis. Discovering these mechanisms and pathways will undoubtedly lead to new ways in dealing with prevention, early detection and therapy, for example, expression profiling can subclassify lung adenocarcinoma in terms of predicting length of survival [3].

A number of potential biomarkers have recently been identified, but currently, no satisfactory biomarkers are available to screen for lung cancer due to low specificity and sensitivity [4, 5]. Lung cancer biomarkers have the potential to be involved in patient screening, monitoring of cancer progression, treatment response and as a predictive factor for

recurrence [6]. No single biomarker with 100% sensitivity and 100% specificity for lung cancer is likely to be discovered. Employment of panels of biomarkers with different individual sensitivities and specificities is likely to be more powerful. Panels of biomarkers from many different sources including the proteome, peptidome, fragmentome and degradome will undoubtedly enhance the ability to diagnose specific cancers at an early stage.

In order to examine differences in serum protein levels associated with human lung squamous carcinoma (hLSC), a proteomics-based approach was initiated involving immunodepletion of serum samples, 2-D difference gel electrophoresis (DIGE) analysis and subsequent MS to generate a panel of factors found to be differentially expressed between the cancerous and noncancerous samples. 2-D DIGE is a powerful and widely used method for the analysis of complex protein mixtures and together with powerful fractionation methods, such as immunodepletion, low-abundant proteins will be detectable and could prove to be a rich source of biomarkers.

2 Materials and methods

2.1 Serum samples

The patient group studied comprised eight individuals with NSCLC. All patients were treated at St. Vincent's University Hospital (SVUH), Dublin, between 2002 and 2003 and approval to conduct this study was granted by the SVUH Ethics Committee. A pooled sample, consisting of equal amounts of each of the 16 experimental samples, was made and used as a pooled internal standard. Blood samples (10 mL) were collected preoperatively in glass tubes without additive (10 mL BD Vacutainer No Additive, BD) and allowed to clot at room temperature for 120 min. Serum was separated by centrifugation at 1500 rpm for 15 min at room temperature. Aliquots of serum (1 mL) were taken and stored at -80°C until ready for use. The time from collection to frozen storage was no more than 3 h.

2.2 Removal of high-abundance proteins from serum samples

Serum samples were processed using a Multiple Affinity Removal Spin Cartridge (Agilent Technologies), which selectively removes albumin, IgG, IgA, antitrypsin, transferrin and haptoglobin from the serum sample. Samples were processed according to the manufacturer's instructions. For each sample, a low-abundance fraction was collected and concentrated using 5000 Da molecular mass cut-off spin concentrators (Agilent Technologies). Samples were subsequently cleaned prior to labelling using a 2-D Cleanup Kit (Bio-Rad). The protein pellets were resuspended in ice-cold 20 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS pH 8.5 buffer. Protein quantification was performed using the Quick Start Bradford Protein Assay (Bio-Rad) absorbance at 595 nm using BSA as a protein standard. Approximately 90% of total serum protein is removed by this method.

2.3 DIGE labelling

Samples were labelled with *N*-hydroxy succinimidyl ester-derivatives of the cyanine dyes Cy2, Cy3 and Cy5 following a standard protocol [7]. Typically, 50 μg of lysate was minimally labelled with 200 pmol of either Cy3 or Cy5 for comparison on the same 2-D gel. Labelling reactions were performed on ice in the dark for 30 min and then quenched with a 50-fold molar excess of free lysine to dye for 10 min on ice. A pool of all samples was also prepared and labelled with Cy2 to be used as a standard on all gels to aid image matching and crossgel statistical analysis. The Cy3 and Cy5 labelling reactions (50 μg of each) from each lysate were mixed and run on the same gels with an equal amount (50 μg) of Cy2-labelled standard.

2.4 Protein separation by 2-DE and gel imaging

A 24 cm linear immobilised pH gradient (IPG) strips, pH 4–7, were rehydrated in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer, 50 mM DTT) overnight, according to the manufacturer's guidelines. IEF was performed using an IPGphor apparatus (GE Healthcare) for a total of 40 kV/h at 20°C, 50 mA. Strips were equilibrated for 20 min in 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 1% w/v SDS containing 65 mM DTT and then for 20 min in the same buffer containing 240 mM iodoacetamide. Equilibrated IPG strips were transferred onto 18×20 cm^2 12.5% uniform polyacrylamide gels poured between low fluorescence glass plates. Strips were overlaid with 0.5% w/v low-melting-point agarose in running buffer containing bromphenol blue. Gels were run using the Ettan Dalt 6 apparatus (GE Healthcare) at 2.5 W/gel for 30 min then 100 W in total at 10°C until the dye front had run off the bottom of the gels. All the images were collected on a Typhoon 9400 Variable Mode Imager (GE Healthcare). Statistics and quantitation of protein expression were carried out in Decyder software (GE Healthcare).

2.5 Spot digestion and MS analysis

Excision of protein spots, trypsin digestion and protein identification by MS analysis using an Ettan MALDI-TOF Pro instrument from GE Healthcare was performed according to an established methodology. Preparative gels containing 300 μg of protein were fixed in 30% v/v methanol, 7.5% v/v acetic acid overnight and washed in water, and total protein was detected by poststaining with SyproRuby dye (Molecular Probes) for 3 h at room temperature or Colloidal Coomassie (Sigma) for 2 h at room temperature. Excess dye was removed by appropriate destaining/washing methods. SyproRuby-stained gels were imaged using a Typhoon 9400 Variable Mode Imager (GE Healthcare) at the appropriate excitation

and emission wavelengths for the stain. The subsequent gel image was imported into the BVA module of DeCyder software and was matched to images generated from DIGE analysis. Spots of interest were selected and confirmed using this software for subsequent picking using an Ettan Spot Picker. Gel plugs were placed into a presilconised 1.5 mL plastic tube for destaining, desalting and washing steps. The remaining liquid above the gel plugs was removed and sufficient ACN was added in order to cover the gel plugs. Following shrinkage of the gel plugs, ACN was removed and the protein-containing gel pieces were rehydrated for 5 min with a minimal volume of 100 mM ammonium bicarbonate. An equal volume of ACN was added and after 15 min of incubation the solution was removed from the gel plugs and the samples then dried down for 30 min using a vacuum centrifuge. Individual gel pieces were then rehydrated in digestion buffer (12.5 ng trypsin *per* μL of 10% ACN 40 mM ammonium bicarbonate) to cover the gel pieces. More digestion buffer was added if all the initial volume had been absorbed by the gel pieces. Exhaustive digestion was carried out overnight at 37°C. After digestion, the samples were centrifuged at 12 000 × *g* for 10 min using a bench top centrifuge. The supernatant was carefully removed from each sample and placed into clean and silconised plastic tubes. Samples were stored at −70°C until analysed by MS. For spectrometric analysis, mixtures of tryptic peptides from individual samples were desalted using Millipore C-18 ZipTips (Millipore) and eluted onto the sample plate with the matrix solution (5 mg/mL a-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA v/v). Mass spectra were recorded using the MALDI-TOF instrument operating in the positive reflector mode at the following parameters: accelerating voltage 20 kV; and pulsed extraction: on (focus mass 2500). Internal and external calibration was performed using trypsin autolysis peaks at m/z 842.50, m/z 2211.104 and Pep4 mix respectively. The mass spectra were analysed using MALDI evaluation software (GE Healthcare), and protein identification was achieved with the PMF Pro-Found search engine for peptide mass fingerprints. Results were also confirmed using MASCOT, an alternate search engine to identify proteins by PMF.

Lower-abundant proteins not identified by MALDI-TOF were digested with tryspin and analysed by 1-D LC-MS using the Ettan™ MDLC system (GE Healthcare) in high-throughput configuration directly connected to a Finnigan™ LTQ™ (Thermo Electron). Samples were concentrated and desalted on RPC trap columns (Zorbax™ 300SB C18, 0.3 mm × 5 mm, Agilent Technologies, and the peptides were separated on a nano-RPC column (Zorbax 300SB C18, 0.075 mm × 100 mm, Agilent Technologies) using a linear ACN gradient from 0 to 65% ACN (Sigma) over 60 min. All buffers used for nano-LC separation contained 0.1% formic acid (Fluka) as the ion pairing reagent. Full scan mass spectra were recorded in profile mode and tandem mass spectra in centroid mode. The peptides were identified using the information in the tandem mass spectra by searching against the Swiss-Prot database using SEQUEST™.

2.6 Statistical analysis

Two-sided, Student's *t*-tests were used to analyse differences in protein levels between squamous cell carcinoma and normal disease free samples. A *p*-value of less than 0.05 was considered statistically significant.

2.7 1-D gel and immunoblot analysis

Protein (8 μg) was loaded onto 12% NuPAGE Bis–Tris gels (Invitrogen) and electrophoretically separated using a MOS/SDS buffer. The samples were run according to the manufacturer's instructions and were stained using colloidal Coomassie blue (Sigma). Electrophoretic transfer of proteins to Hybond-ECL NC membranes (GE Healthcare) was carried out using a Bio-Rad Transblot SD cell (Bio-Rad). Proteins were transferred for 50 min at 0.34 mA. Efficiency of transfer was evaluated using Ponceau-S-Red staining of NC membranes, followed by destaining in PBS (50 mM sodium phosphate, 0.9% w/v NaCl, pH 7.4). Membranes were blocked for 1 h in 5% w/v fat-free milk powder in PBS containing 0.5% Tween 20. Membranes were then incubated overnight at 4°C with the primary antibody, serum albumin (ab10241); haptoglobin (ab14248) and actin (ab1801) (Abcam). NC replicas were subsequently twice washed for 10 min in blocking solution and then incubated with corresponding peroxidase-conjugated secondary antibody for 1 h at room temperature. NC membranes were washed twice for 10 min in blocking solution and rinsed twice for 10 min in PBS. Visualisation of immuno-decorated 1-D bands was carried out using an ECL kit (GE Healthcare).

3 Results

3.1 Clinical specimens

Sera from eight male patients with squamous cell lung carcinoma (Table 1) were analysed in this study. The patients age in range from 61 to 79 years. The tumour ranges in size from 2.1 to 7 cm. Eight healthy male disease free samples were used in the control group with an age range of 26–56 years.

3.2 Immunodepletion of high-abundant serum proteins

The serum proteome constitutes a highly complex array of circulating proteins, and is a rich source of potential diagnostic and prognostic biomarkers. The immunodepletion technique involves using multiple affinity removal columns which contain affinity-purified polyclonal antibodies to rapidly remove more than 99% of targeted proteins (albumin, Ig G, Ig A, α-1-antitrypsin, transferrin and haptoglobin) with minimal nonspecific removal of other proteins. The result of immunodepletion on a colloidal Coomassie blue-stained 1-D

Table 1. Clinical information for patients diagnosed with squamous cell carcinoma of the lung

Specimen no.	Diagnosis	Gender	Type	Size	Grade	Smoking	Metastasis
1	56	Male	Sq cell Ca	5.4	3	Yes	None
2	65	Male	Sq cell Ca	6.6	3	Yes	None
3	64	Male	Sq cell Ca	3.7	3	Yes	Yes, to peribronchial lymph nodes
4	74	Male	Sq cell Ca	2.1	2	Yes	None
5	62	Male	Sq cell Ca	7	3	Yes	Yes, three mediastinal lymph nodes
6	58	Male	Sq cell Ca	2.4	2	Yes	None
7	60	Male	Sq cell Ca	6	2	Yes	Yes, to peribronchial lymph nodes
8	76	Male	Sq cell Ca	2.5	3	Yes	None

Sq cell Ca, Squamous cell Carcinoma.

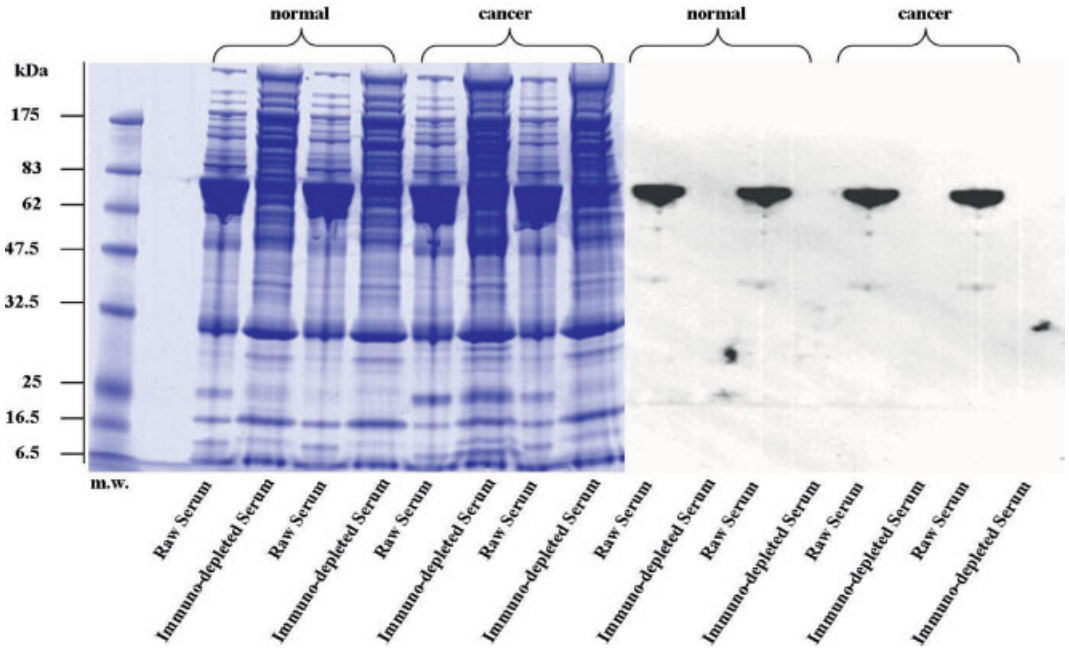

Figure 1. Colloidal Coomassie blue-stained 1-D gel of raw and immuno-depleted serum showing an increased number of visible proteins that were previously masked due to the presence of high-abundance proteins. Western blot analysis of raw and immuno-depleted serum using an antibody to albumin showing the effectiveness of the immuno-depletion column in removing selected high-abundance proteins.

gels where removal of high-abundant proteins like albumin allow evaluation of lower-abundant proteins previously masked due to the overpowering presence of the immuno-depleted proteins (Fig. 1). Recent papers have also confirmed the marked increase in detectable proteins due to immuno-depletion [8, 9]. Western blot analysis of raw and immuno-depleted serum using an antibody to albumin also shows the effectiveness of the immuno-depletion column (Fig. 1).

3.3 DIGE analysis of the immunodepleted serum proteome

Fifty micrograms of protein from each sample was labelled with Cy2, Cy3 or Cy5. Eight individual samples of squamous cell lung carcinoma serum and eight samples of normal serum were labelled with Cy3 and Cy5 respectively. All 16 samples employed in the experiment were used in the Cy2 labelled internal pooled standard. Samples were combined and separated by 2-DE. For DeCyder image analysis, the differential in-gel analysis mode of DeCyder was first used to merge the Cy2, Cy3 and Cy5 images for each gel and to detect spot boundaries for the calculation of normalised spot volumes/protein abundance. At this stage, features resulting from nonprotein sources, namely dust particles and scratches, were filtered out. The analysis was used to rapidly calculate abundance differences between samples run on the same gel. The biological variation analysis mode of DeCyder was then used to match all pairwise image comparisons from difference in-gel analysis for a comparative crossgel statistical analysis. Operator intervention was required at this

point to set landmarks on gels for more accurate crossgel image superimposition. Comparison of normalised Cy3 and Cy5 spot volumes with the corresponding Cy2 standard spot volumes within each gel gave a standardised abundance. This value was compared across all gels for each matched spot and a statistical analysis was performed (Fig. 2).

3.4 3-D images, statistics and MS

Examples for the evaluation by DeCyder of alterations in spot intensities using the DIGE system are displayed in Fig. 3. To show visually alterations in corresponding spot intensity proportions, selected spots are displayed as 3-D images. Figure 4 also displays the associated graph views of standardised log abundances of the selected spots among analysed gel replicates. The differentially expressed proteins included in the table all had a *p*-value of less than 0.05. Mass spectra were recorded using the Ettan MALDI-TOF Pro instrument from GE Healthcare operating in the positive reflector mode at the following parameters: accelerating voltage 20 kV; and pulsed extraction: on (focus mass 2500). Internal and external calibration was performed using trypsin autolysis peaks at m/z 842.50, m/z 2211.104 and Pep4 mix respectively. The mass spectra were analysed using MALDI evaluation software (GE Healthcare), and protein identification was achieved with the PMF Pro-Found search engine for peptide mass fingerprints. Lower-abundant proteins not identified by MALDI-TOF Samples were digested with tryspin and analysed by 1-D LC-MS using the Ettan™ MDLC system (GE Healthcare) in high-throughput configuration directly connected to a Finnigan™ LTQ™ (Thermo Electron). Proteins found to have higher abundance levels in squamous cell carcinoma sera compared to normal sera included apolipoprotein A-IV precursor (Apo-AIV), chain F; human complement component C3c, haptoglobin, serum amyloid A protein precursor and Ras-related protein Rab-7b while proteins found to have lower abundance levels included AHSG, hemopexin precursor, proapolipoprotein, antithrombin III (ATH III) and SP40; 40 (Table 2).

3.5 Western blot analysis

Raw serum from normal and cancer samples were electrophoretically separated and transferred to NC before probing with an antibody to haptoglobin. The results show an increase in abundance levels of haptoglobin in cancer samples compared to normal. These results from Western blot analysis were in good agreement with results from the 2-D DIGE data (Fig. 5).

4 Discussion

Lung cancer is the leading cause of cancer-related mortality in both men and women. The prevalence of lung cancer is second only to that of prostate cancer in men and breast cancer in women. Early detection of lung cancer would greatly improve the survival rates. Serum biomarker have

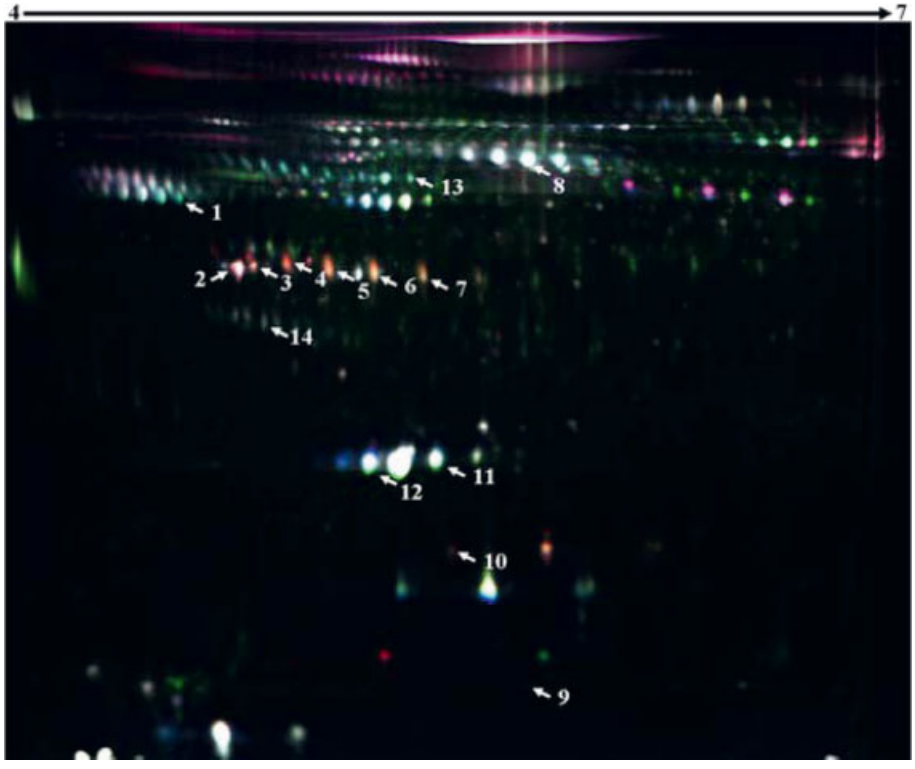

Figure 2. 2-D DIGE image of Cy 2, Cy 3 and Cy 5 labelled squamous cell carcinoma and normal serum proteins. Protein differences were analysed using 2-D PAGE (2-D DIGE) to resolve proteins based on their p*I*, in this case a range of 4–7 was employed and their molecular weight to generate a protein expression map (PEM).

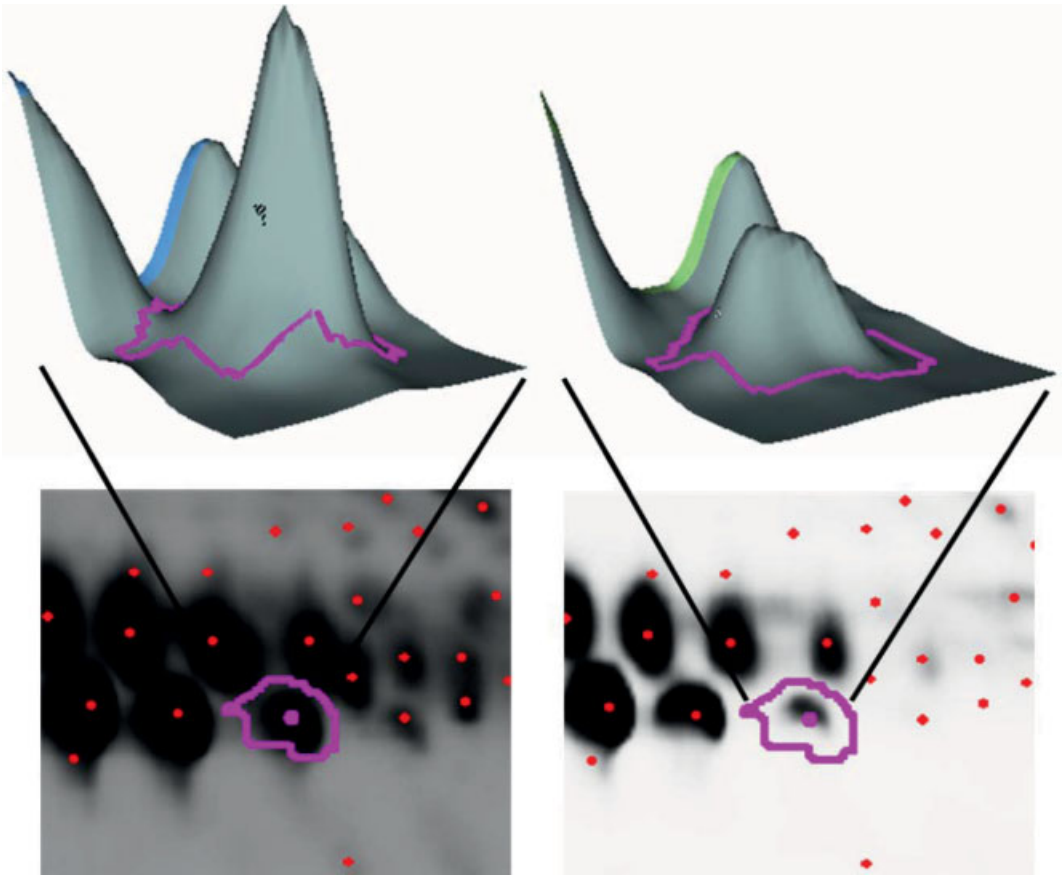

Figure 3. 3-D image of AHSG. Images were generated using the BVA module of DeCyder software and visually show the abundance levels for AHSG are lower in the cancer samples compared to normal.

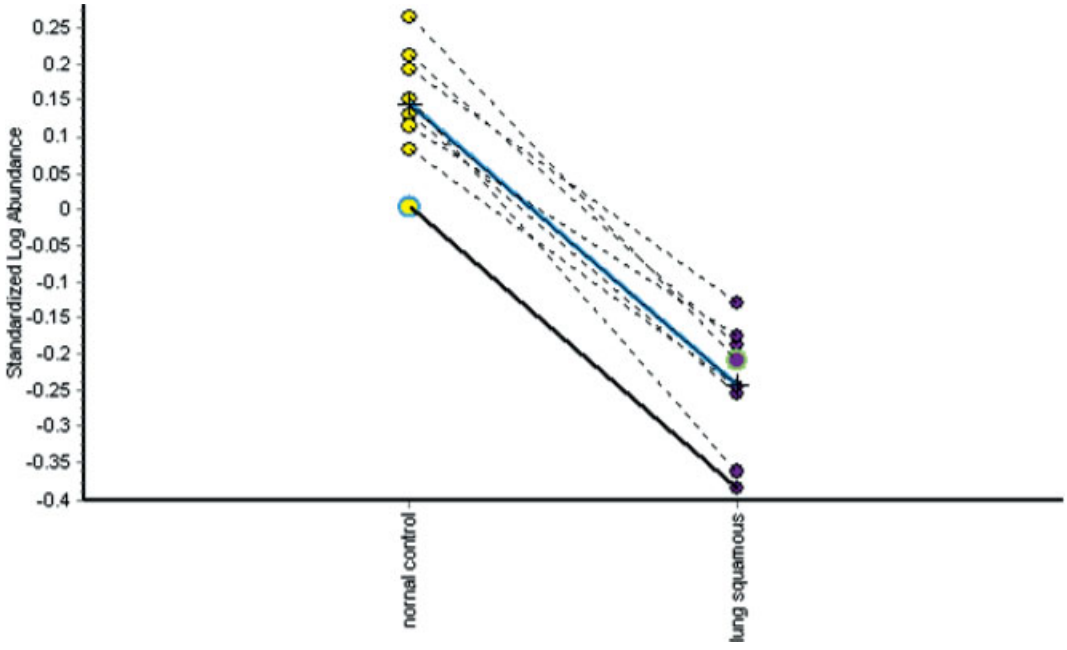

Figure 4. Statistical analysis of AHSG. Statistics were generated using the BVA module of DeCyder software and show an average of 2.43-fold lower abundance levels for AHSG in the cancer samples compared to normal.

Tabletpdel 2. Table of proteins found to have higher or lower abundance levels in squamous cell carcinoma sera compared to normal sera

Spot number	Protein identification	Gene index number	Average ratio	p*I*	MW (Da)	Method of identification
1	AHSG	gi|30851645|	−2.43	5.4	40 110	MALDI-TOF
2	Apo-AIV	gi|114006|	3.04	5.3	45 350	MALDI-TOF
3	Chain F; Human complement component C3c	gi|78101274|	1.58	4.8	40 210	MALDI-TOF
4	Haptoglobin	gi|1212947|	4.42	6.3	38 950	MALDI-TOF
5	Haptoglobin	gi|3337390|	3.9	6.1	38 730	MALDI-TOF
6	Haptoglobin	gi|3337390|	3.81	6.1	38 730	MALDI-TOF
7	Haptoglobin	gi|3337390|	3.58	6.1	38 730	MALDI-TOF
8	Hemopexin precursor	gi|386789|	−1.3	6.6	52 270	MALDI-TOF
9	Serum amyloid A protein precursor	gi|134167|	6.35	6.28	13 532	LC-MS/MS
10	Ras-related protein Rab-7b	gi|50401122|	7.81	6.31	22 511	LC-MS/MS
11	Proapolipoprotein	gi|178775|	−1.51	5.4	28 940	MALDI-TOF
12	Proapolipoprotein	gi|178775|	−1.53	5.4	28 940	MALDI-TOF
13	Antithrombin	gi|37682619|	−1.51	6.3	53 040	LC-MS/MS
14	'SP40; 40'	gi|338305|	−1.3	5.7	37 000	LC-MS/MS

Listed are the protein identities obtained from MALDI-TOF/LC-MS analysis, gene index number, average ratio, theoretical MW and theoretical p*I*. All proteins listed in the table were found to have a statistically significant *p*-value of less than 0.05.

great potential to facilitate the early detection and for monitoring of treatments/recurrence in patients with lung cancer in a noninvasive manner. The analysis of the serum proteome in patients with squamous cell carcinoma of the lung could identify potential biomarkers and provide a greater understanding of the pathophysiology.

The concentration of haptoglobin in the sera of patients with squamous cell carcinoma of the lung was found to be greater than that of the disease free control group. Haptoglobin was elevated beyond the normal capacity of the immunodepletion column and thus appeared in the flow-through fraction, with an average ratio of 3.9-fold greater concentration in the patients with squamous cell carcinoma of the lung. Due to the haptoglobin levels being greater that the capacity of the column which might have distorted the result, Western blot analysis was performed on raw serum from both the normal and cancer samples. This analysis confirmed a significant elevation in the abundance level of haptoglobin in the cancer samples compared to normal samples.

The full range of biological functions of haptoglobin in cancer is not well understood at present [10]. Intact haptoglobin is a glycoprotein mainly secreted by the liver. Haptoglobin combines with free plasma hemoglobin, preventing iron loss and renal damage. Many papers have shown increased abundance of haptoglobin in lung cancer patients. Maciel *et al.* [11] found that haptoglobin was found in greater levels in serum from adenocarcinoma lung cancer patients. Bharti *et al.* [12] findings suggested that serum levels of alpha-haptoglobin and HGF may serve as useful serum tumour biomarkers in SCLC.

Other proteins which we found to be of higher abundance in squamous cell carcinoma sera compared to normal sera included Apo-AIV, chain F; human complement component C3c, serum amyloid A protein precursor and Ras-related protein Rab-7b. Apolipoprotein is a carrier of lipids and regulates many cellular functions. The level of serum apolipoprotein was reported to be differentially expressed in colorectal cancer and adenocarcinoma lung cancer [11, 13]. Complement components are important mediators of inflammation and contribute to the regulation of the immune response. We found chain F; human complement component C3c to be 1.53-fold increased in squamous cell carcinoma sera. Two recent reports have shown that elevated levels of C3a were found in the ascitic fluids of ovarian cancer patients and in patients with chronic hepatitis C/HCV-related HCC [14, 15].

The serum amyloid A family comprises a number of differentially expressed apolipoproteins including acute-phase SAA1 and SAA2. The serum level of this apolipoprotein increases in a wide range of different disease conditions including reported increased levels in lung cancer [16, 17]. Howard *et al.* showed that using ELISA, SAA was present at 286 ng/mL in the serum of lung cancer patients *versus* 34.1 ng/mL in the serum of individuals without cancer. Benson *et al.* reported that serum amyloid A in carcinoma of the lung was significantly elevated.

Rab7, a member of the Ras oncogene family, is a small Rab GTPase that regulates vesicular traffic from early to late endosomal stages of the endocytic pathway. Northern blot analysis shows that Rab7b mRNA is expressed in lung cells [18]. Rab-7b was found in this study to be substantially elevated (7.81-fold on average) in the cancer serum samples.

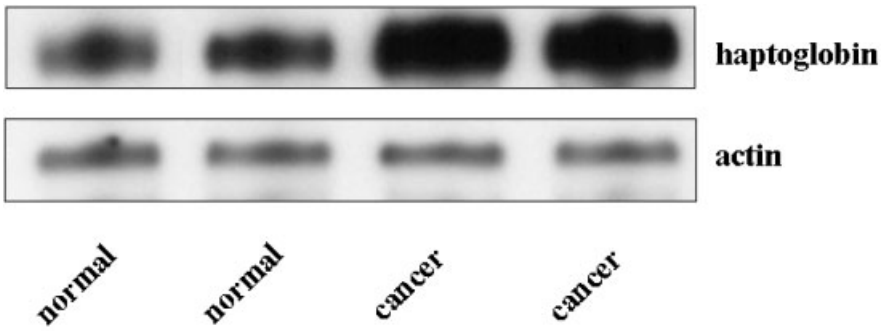

Figure 5. Western blot analysis of raw serum from normal and cancer samples using antibodies to actin (loading control) and haptoglobin. The results confirm the increase in abundance levels of haptoglobin in cancer samples compared to normal. This is a representative blot for analysis on all samples performed in triplicate.

Proteins found with lower abundance levels in squamous cell carcinoma sera compared to normal sera included AHSG, hemopexin precursor, proapolipoprotein, ATH III and SP40; 40. Alpha-2-HS glycoprotein (AHSG), is a glycoprotein present in the serum, is synthesised by hepatocytes and is important in blocking transforming growth factor-beta1 signal transduction, which is associated with tumour progression. AHSG has been observed to be depleted in certain tumours compared with normal tissue [19, 20]. Kawakami *et al.* [21] reported that AHSG was differentially expressed in the sera of hepatocellular carcinoma (HCC) patients who had undergone curative radiofrequency ablation treatment and may be a candidate biomarker for the development of diagnostic and therapeutic tools. Hemopexin is a serum glycoprotein that binds haeme and transports it to the liver for breakdown and iron recovery and has been reported to be overexpressed in nipple aspirate fluid (NAF) from patients with early stage breast cancer [22].

Proapolipoprotein, which is hydrolysed by the signal peptidase and propeptidase, through which apolipoprotein is generated, was recently reported to be increased in breast cancer serum patients using 2-D differential gel electrophoresis [23, 24].

ATH III is a plasma protein synthesised in the liver. Normal plasma levels are 115 to 160 mg/L. Reduced serum ATH III levels together with elevated fibrinogen, vWF, and D-dimer were found to be associated with poor survival outcome in ovarian cancer. The author suggests that fibrinogen, vWF, ATH III and D-dimer levels be used together as prognostic markers for disease outcome especially in patients with advanced ovarian cancer within 36 months of disease [25].

SP-40; 40 (serum protein 40 kDa; 40 kDa) or clusterin is an 80 kDa disulphide-linked, heterodimeric glycoprotein. Rodriguez-Pineiro *et al.* [26] have recently shown that serum clusterin and some of its isoforms could have a potential value as colorectal tumour markers and are interesting subjects for biomarker studies. Zhang *et al.* [27] reported that a loss of clusterin both in serum and tissue correlates with the tumorigenesis of esophageal squamous cell carcinoma.

Some of our results are consistent with recently published material on serum biomarkers in lung cancer. Elevated levels of haptoglobin and serum amyloid A protein have previously been reported in studies on lung cancer. It is not surprising that some of the proteins identified in this study have been found to be potential biomarkers for other cancers and are more likely indicators of local inflammatory reaction that is found in lung cancer. However, proteins highlighted in this study like AHSG and Ras-related protein Rab-7b have the potential to be more specific. By increasing the number of potential biomarkers discovered and developing our understanding their role in the pathophysiology of squamous cell carcinoma of the lung, such strategies have enormous potential in influencing the development of new treatments and more precise screening for this disease.

This study also highlights the effectiveness of 2-D DIGE in identifying proteins of differing abundance levels between normal and cancer specimens. Undoubtedly there are limitations associated with this proteomics approach. However with the continually development of immuno-depletion columns and alternate fraction methods 2-D DIGE will be an important platform in discovering potential biomarkers. Together with complementary proteomics approaches that allow researchers dig deeper into the serum proteome, the establishment of accurate panels of biomarkers for specific diseases is certainly achievable.

Serum is a highly complex bodily fluid that contains proteins ranging in concentrations over at least nine orders of magnitude. 2-D DIGE coupled to fractionation of serum has proved to be successful in the discovery of potentially useful biomarkers. The chances of discovering a single marker highly specific and sensitive for a particular cancer seem unattainable. However, panels of biomarkers from the proteome in combination with markers from the peptidome, fragmentome and degradome will undoubtedly enhance the ability to diagnose specific cancers at an early stage.

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