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Differential proteome profiling of pleural effusions from lung cancer and benign inflammatory disease patients

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ABSTRACT

The pleural effusion proteome has been found containing information that directly reflects pathophysiological status and represents a potential diagnostic value for pulmonary diseases. However, the variability in protein composition between malignant and benign effusions is not well understood. Herein, we investigated the changes of proteins in pleural effusions from lung adenocarcinoma and benign inflammatory disease (pneumonia and tuberculosis) patients by two-dimensional difference gel electrophoresis (2D-DIGE). Twenty-eight protein spots displayed significantly different expression levels were positively identified by MALDI-TOF-MS representing 16 unique proteins. Five identified protein candidates were further validated and analyzed in effusions, sera or tissues. Among them, hemopexin, fibrinogen gamma and transthyretin (TTR) were up-regulated in cancer samples. The effusion concentration of serum amyloid P component (SAP) was significantly lower in lung cancer patients than in benign inflammatory patients, but no differences were found in sera samples. Moreover, a Jumonji C (JmjC)-domain-containing protein, JMJD5, was observed to be down-regulated in malignant effusions, lung cancer tissues and cancer cells. These results shed light on the altered pleural effusion proteins as a useful and important complement to plasma or other routine clinical tests for pulmonary disease diagnosis.

1. Introduction

A pleural effusion is the abnormal accumulation of fluid between the two layers of pleura resulting from the disruption of the homeostatic forces that control the flow into and out of the area. In clinical practice, pleural effusions are classified as transudates or exudates, according to the biochemical characteristics of the fluid. Transudative effusions, usually caused by a limited number of recognized clinical conditions such as heart failure and cirrhosis, can be separated effectively from exudative effusions by using Light's criteria. While for further testing of exudates, malignant and benign effusions are sometimes a diagnostic challenge. Pleural effusions secondary to malignancy or infection (e.g. tuberculosis) usually have similar biochemical profiles or differential cell counts (DCC).

It has been known that pleural effusion, which is usually closer to the affected organ and thus more specific for lung disorders than other body fluids, always contain plasma proteins as well as proteins released by inflammatory, epithelial or even cancer cells [1–3]. Some of them may have potential diagnostic value [4]. Proteomics can simultaneously identify multiple proteins for disease-specific biomarker discovery and allow for deep insights into disease mechanisms. Using the proteomic approach, Tyan et al. analyzed the global protein composition of the human malignant pleural effusion [5,6], and Hsieh et al. further studied the differentially expressed proteins between malignant and transudative pleural effusions [7]. However, no comprehensive proteomic comparisons have been made between malignant and benign exudates. Since parapneumonic and tuberculous effusions are the principal differential diagnoses for malignancy in most developing countries [8], it's necessary to explore the difference in protein composition among these effusions. Meanwhile, like plasma proteome, high-abundant proteins such as albumin and immunoglobulin should be removed from effusion samples in order to visualize the low-abundance proteins that may be of great interest (e.g. biomarkers). Herein, the aim of this study was to identify proteins differentially expressed in malignant effusions from lung adenocarcinoma and benign inflammatory effusions from pneumonia and tuberculosis by using two-dimensional difference gel electrophoresis (2D-DIGE) after depletion of abundant proteins.

2. Materials and methods

2.1. Study subjects

The study protocol was approved by the Clinical Ethics Committee of the Sir Run Run Shaw Hospital. All patients enrolled in the study

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gave their consent. A total of 20 patients with pleural effusion were included, of whom 10 malignant effusions from lung adenocarcinoma patients, 10 benign effusions from tuberculosis (TB) (n = 6) and pneumonia (PN) (n = 4). Each lung adenocarcinoma diagnosis was verified by fluid cytological or cancer histopathological analysis. Patients with other primary sites of malignancies or without any lung parenchymal tumor were excluded. The diagnosis of TB and PN was based on a clinical evaluation of pleural effusions or a pleural biopsy.

Ten blood samples from pulmonary disease patients (4 lung adenocarcinoma, 3 TB and 3 PN) were collected when the initial diagnoses were confirmed. Four healthy volunteers were enrolled from the employees at the Sir Run Run Shaw Hospital and served as control subjects. All volunteers have no medical history of malignancy, TB or recent respiratory tract infection.

Pleural fluids and blood samples were collected in sterile tubes without adding any additives from each patient prior to any therapy. After centrifugation at 1000 rpm for 10 min at 4 °C, cell-free supernatants were stored in aliquots at −70 °C until further analysis.

The lung cancer and normal parenchymal tissue samples were taken from the resected pathological specimens from 10 lung adenocarcinoma patients undergoing curative lung surgery. In terms of TNM stages, the patients comprised 3 cases of stage I, 2 cases of stage II, 4 cases of stage III and 1 case of stage IV. After surgical resection, these lung tissues were immediately frozen to −70 °C.

2.2. Protein preparation and Cy-dye labeling

Pleural effusion samples were processed using the ProteoPrep Blue Albumin and IgG Depletion Kit (Sigma; St. Louis, Missouri, USA), which selectively removes albumin and IgG from the sample. Samples were processed according to the manufacturer's instructions. After further precipitation and purification using 2-D Clean-up Kit (GE Healthcare BioSciences; Little Chalfont, UK), protein extracts were solubilized in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris base, 35 μg/mL PMSF, 0.5 μg/mL leupeptin and 2 μg/mL aprotinin. Protein concentration was determined using 2-D Quant Kit (GE Healthcare).

Protein samples (50 μg) were labeled with CyDye DIGE fluors (400 pmol) (GE Healthcare) following the manufacturer's instructions. CyDye labeling (Cy3 or Cy5) was randomized between the 20 effusion protein samples (10 from lung adenocarcinoma, 6 from TB and 4 from PN) to rule out dye-bias. A pooled internal standard containing equal quantities of all protein extracts was labeled with Cy2. Labeling was performed for 30 min on ice in the dark, after which the reactions were quenched with the addition of 10 mM lysine for 10 min on ice in the dark. The quenched 50 μg Cy3- and Cy5-labeled samples were then combined and mixed with 50 μg of the quenched Cy2-labeled internal standard, after which an equal volume of 2× sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% DTT) supplemented with 2% Pharmalyte pH 3–10 was added.

2.3. 2-D gel electrophoresis

Immobilized drystrips (pH 4–7, 24 cm, GE Healthcare) were rehydrated at 50 V for 12 h in the rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.4% DTT, 0.5% IPG buffer pH 4–7, 0.002% bromophenol blue) using an Ettan IPGphor II IEF system (GE Healthcare). Once rehydration was complete, IEF was carried out as follows: 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 1000–8000 V for 30 min and 8000 V was applied until the total Vh reached 67.0–70.0 kVh at 20 °C. After IEF separation, the strips were equilibrated for 15 min in an equilibration buffer (100 mM Tris–HCl, 6 M Urea, 30% glycerol, 2% SDS and 0.002% bromophenol blue) containing 0.5% DTT, then equilibrated again for another 15 min in the same buffer, except that DTT was replaced with 4.5% iodoacetamide. Second dimension SDS electrophoresis was carried out on 12.5% slab gels using the ETTAN DALT II electrophoresis system (GE Healthcare) at 15 °C. SDS-PAGE was run at a constant power of 2.5 W/gel for 15 min, and switched to 8 W/gel until the bromophenol blue frontier reached the bottom of the gel. Gels were scanned while still between the glass plates at 480/530 nm (Cy2), 540/595 nm (Cy3), and 635/680 nm (Cy5) using an Ettan DIGE Imager (GE Healthcare). Post-silver staining was performed as previously described [9].

2.4. DIGE analysis

After the images were scanned, Decyder software (version 6.5; GE Healthcare) was utilized for a differential gel analysis. Both the Differential In-gel Analysis (DIA) and the Biological Variation Analysis (BVA) modules were used and no manual editing of the data was performed. 30 protein-spot maps from 10 gels were matched and average abundance changes were calculated. The Decyder program uses pixel intensity and performs Student's *t*-tests and one way analysis of variance (ANOVA) to determine whether a spot undergoes a statistically significant change ($P<0.05$) during transit. The validity of these changes was then confirmed by manual inspection of the gels. To better evaluate the results, the false discovery rate (FDR) and the q value were also determined by the significance analysis of microarrays (SAM) method (SAM version 3.05). In this analysis, the FDR was set at 10%.

2.5. Protein identification by MS

Spots of interest were chosen from the gels and put through in-gel digestion as we previously described [9]. Peptides were then dissolved in 0.1% TFA, and 1 μL of the mixture was mixed with an

Table 1
Identification of the differentially expressed proteins in lung adenocarcinoma patients as compared with tuberculosis patients.

Index no.	Ca/TB ratio	*P* value[a]	q value[b]	MW (kDa)[c]	p*I*[c]	Mascot search results				
						Peptide matches[d]	Cov (%)[e]	Score	Description (protein name)	RefSeq no.
827	1.52	0.001	0.00	52.4	6.55	12/24	32	137	Hemopexin precursor	NP_000604
876	2.20	0.002	0.00	52.4	6.55	6/12	21	75	Hemopexin precursor	NP_000604
890	2.34	0.004	0.00	52.4	6.55	12/20	38	141	Hemopexin precursor	NP_000604
979	5.15	0.033	0.00	51.4	7.95	23/35	45	217	Fibrinogen beta chain	NP_005132
1201	−1.21	0.012	0.01	49.3	6.27	1[f]	4	100	Apolipoprotein J precursor (clusterin)	NP_001164609
1232	−1.35	0.037	0.06	49.3	6.27	2[f]	6	126	Apolipoprotein J precursor (clusterin)	NP_001164609
1313	−3.01	0.026	0.02	52.4	7.23	9/14	17	100	Alpha1 (III) collagen	NP_000081
1942	2.12	0.001	0.00	20.3	5.16	10/19	55	166	Transthyretin	NP_000362
2252	−3.08	0.006	0.01	47.2	5.51	18/52	50	149	Jumonji domain containing 5	NP_001138820

[a] Based on the unpaired two-tailed Student's *t*-test.
[b] Determined by the SAM method.
[c] MW(kDa) and p*I*: theoretical.
[d] Number of peptides that matched those from the nrNCBI database entry and number of peptides searched.
[e] Percentage of the protein sequence covered by the matching peptides.
[f] Number of unique peptide sequences for proteins identified by MALDI-TOF/TOF MS.