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Identification of differentially expressed proteins in human malignant pleural effusions

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Abstract

Higher protein concentration in malignant pleural effusions than in transudative effusions is a marker for clinical diagnosis. However the variability of protein compositions between these two forms of pleural effusions is not well understood.

To compare the protein compositions, we studied the proteomic profiles in 14 malignant and 13 transudative pleural effusions by two-dimension gel electrophoresis. Protein spots with differential expression were identified by matrix-assisted laser desorption/ionization quadrupole time of flight (MALDI-Q-TOF) and liquid chromatography-tandem mass spectrometry (LC/MS). Targeted proteins were further examined in all samples by ELISA assay and Western immunoassay.

Two-dimension gel electrophoresis revealed seven spots with reduced expression in malignant pleural effusions. Four of the abnormal spots were identified as fibrinogen γ-chain precursor, two as fibrinogen β-chain precursor, and one as pigment epithelium-derived factor (PEDF)**.** ELISA and Western immunoassay showed PEDF levels were significantly lower in malignant than in transudative pleural effusions.

We have demonstrated that proteomic technologies may help to discover proteins with potential functions. By applying these technologies, the level of PEDF, a potent antiangiogenic factor, was found to be significantly lower in malignant than in transudative pleural effusions. This finding allows for further exploration of the role of PEDF in mediating malignant pleural effusions.

Key words: angiogenesis, lung cancer, tumour markers

Abbreviations: PEDF, pigment epithelium-derived factor; **IEF**, isoelectric focusing; **DTT**, dithiothreitol; **2-DE,** two-dimensional gel electrophoresis; **SDS-PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis; **TFA**, trifluoroacetic acid; **PMF**, peptide mass fingerprinting; **CHCA**, cyano-4-hydroxycinnamic acid; **EPC-1**, early population doubling level cDNA-1; **VEGF**, vascular endothelial growth factor; **b-FGF**, basic fibroblast growth factor.

Introduction

A pleural effusion is an abnormal accumulation of fluid in the pleural space resulting from the disruption of homeostatic forces that control the flow into and out of the area [1]. The clinical impact of a pleural effusion depends on its volume and composition. A diagnostic thoracentesis is usually necessary in order to determine the cause of effusions [2]. The development of malignant pleural effusions in patients with advanced malignant neoplasms may result in serious symptoms including cough, chest pain, shortness of breath and fatigue. Several mechanisms of malignant pleural effusion formation had been mentioned. The spread of malignant cells to the visceral and parietal pleural may be associated with vascular invasion and lymphatic obstruction [3]. This results in disruption of the normal resorptive flow of fluid from the parietal to the visceral surface. Increased capillary permeability due to the inflammatory process also contributes the pleural fluid accumulation.

Despite the mechanisms of malignant pleural effusions being well documented, identifying the composition of various types of pleural effusion may help for the development of clinical diagnosis and perhaps specific therapy. New technology is yielding a wealth of information, allowing the generation of enormous amounts of data from a single experiment. These new techniques, for example, allow the identification of a wide range of proteins even in a small sample. Thus, with these new techniques, it would be interesting to compare the protein composition of malignant pleural effusions with that of normal pleural fluid. However, the latter is present in minute amounts, making it difficult to obtain for examination. Transudative pleural effusions form because of increases in hydrostatic pressure or decreases in osmotic pressure across an intact pleural endothelial barrier with consequently low numbers of cells and small amounts of protein [4]. For this reason, it seems likely that the protein composition of transudates approximates that of normal pleural fluid. This study was therefore

designed to identify major proteins differentially expressed in malignant and transudative pleural effusions, using a proteomic approach based on MALDI-Q-TOF mass spectrometry.

Material and Methods

Patients

The study was approved by the Institutional Review Board of Mackay Memorial Hospital and written informed consent was obtained from all participants. A total of 27 patients with pleural effusion were included, 14 with a cytologically positive malignant pleural effusion and 13 with a transudative effusion. The malignant effusions were due to lung cancer in nine patients (8 adenocarcinoma and 1 small cell carcinoma), metastatic tongue cancer in two, metastatic esophageal cancer in one and metastatic breast cancer in two. The transudates were attributed to congestive heart failure in 10 patients, nephrotic syndrome in one and liver cirrhosis in two.

About 150 to 200 ml of pleural fluid was collected by echo-guided aspiration from each of the participants. The effusion was centrifuged at 1000 rpm for 10 min at 4°C and the supernatant was stored in aliquots at -70°C until further analysis.

Protein preparation

Total proteins of transudative and malignant effusions were precipitated from each sample with a 2-D Clean-UP Kit (Amersham Biosciences, Uppsala, Sweden) before proceeding for proteomic studies. Briefly, 100 μL of pleural effusion was mixed with 300 μL of precipitant and incubated on ice for 15 min. Another 300 μL of coprecipitant was then added to the mixture and centrifuged at 8000 x g for 10 min. The pellet was resuspended with 500 μL coprecipitant and centrifuged for 5 min. The final product was obtained from the pellet remaining after centrifugation at 8000 x g for 10 min.

Precipitated proteins were prepared for Sypro Ruby staining or fluorescence (CyDye) labeling before processing to two-dimensional gel electrophoresis (2-DE). For Sypro Ruby staining, 1 mg of precipitated protein was resuspended with DeStreak Rehydration Solution (Amersham Biosciences, Uppsala, Sweden) to 350 μL prior to isoelectric focusing (IEF). For CyDye labeling, precipitated proteins were resuspended with dissolving solution (4% 3-[(3-Cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS), 30 mM Tris-HCl pH 8.5, and 7 M urea), and labeled with fluorescent cyanine dyes developed for fluorescence difference gel electrophoresis (DIGE) (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's protocol. Briefly, protein (50 μg) was labeled with 1 μL amine reactive cyanine dye (400 pmol/μL) freshly dissolved in anhydrous dimethyl formamide. The labeling reaction was incubated at 4°C in the dark for 30 min. The reaction was terminated by addition of 10 nmol lysine. Immobiline DryStrip rehydration buffer (7 M urea, 2 M thiourea, 2 mg/mL Dithiothreitol (DTT) and 1% pharmalytes) was added to make up the volume to 350 μL prior to IEF.

Two-dimensional gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis was used to separate protein compositions into a 2-dimensional array, which can be used to identify differentially expressed proteins between malignant pleural effusions and transudative pleural effusions. This was performed using IEF in the first direction (18 cm pH 3-10 and pH 4-7 Immobiline DryStrip, IPG-phor, Amersham Biosciences, Uppsala, Sweden) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for the second direction (Ettan DALTsix Large Vertical System, Amersham Biosciences, Uppsala, Sweden). Isoelectric focusing was run following a step-and-hold stepwise incremental voltage program: 30 V for 18 hr., 500 V for 30min., 1000 V for 30 min., 1500 V for 30 min.,

2000 V for 30 min., 3000 V for 30 min., 6000 V for 1 hr., and 8000 V for 10~13 hrs; adding up to a total power of 80000 volt–hour. After IEF, the strips were subjected to two-step equilibration in buffers containing 6 M urea, 2% w/v SDS, 30% w/v glycerol and 50 mM Tris-HCl (pH 8.8) with 1% w/v DTT for the first step and 2.5% w/v iodoacetamide for the second step. The strips were then transferred onto SDS-PAGE gels (1.0 mm thick gradient 12% *T*) and run at 15°C. For samples with Sypro Ruby staining, the gel was washed in 10% methanol/7% acetic acid for approximately 1 hr. and placed in Sypro Ruby for at least 3 hrs. Samples studied by DIGE were labeled with CyDyes before proceeding to 2-DE.

Image acquisition and analysis of 2-DE gels

The pattern of protein expression in each 2-DE gel was studied by image analyzer after image was acquired by a scanner Typhoon™ 9400 Imager (Amersham Biosciences, Uppsala, Sweden). The density of each protein spot on the scanned image represents the level of protein expression. Quantification of spot density is then determined by a specific image analyzer.

For Sypro Ruby images, the gels were scanned at an excitation wavelength of 610/30 nm to visualize all protein spots present in the gel. Images were cropped to remove areas extraneous to the gel image using ImageQuant™ V5.2 (Amersham Biosciences, Uppsala, Sweden) prior to analysis. The density of each differentially expressed spot was quantified by PDQuest Image Analysis software V7.0 (Bio-Rad).

For CyDye-labeled images, the Cy3 images (green fluorescence, malignant effusion) were scanned using a 532 nm laser and a 580 nm band pass (BP) 30 emission filter, the Cy5 images (red fluorescence, transudative effusion) using a 633 nm laser and a 670 nm BP30 emission filter, and the Cy2 images (blue fluorescence, internal control) using a 488 nm laser and an emission filter of 520 nm BP40. The resolution for

all gels was set to 100 μm. After acquisition, images were merged and analyzed by a differential analysis software DeCyder™ V5.0 (Amersham Biosciences, Uppsala, Sweden). In the merged image, a protein with similar levels in malignant and transudative effusions will appear as yellow fluorescence (green + red), by contrast a protein with higher level in malignant effusion will appear as light green or as orange if it expresses with higher level in transudative effusion. The fluorescent intensity of each spot was digitalized and converted into a 3-D image allowing both data and visual comparison between different spots.

In-Gel Tryptic Digestion

To identify the protein of interest, the protein spots with differential expression are firstly removed and then digested by proteases into peptides. The protein spots on 2-DE were manually excised from the gel and cut into pieces. Each gel piece was dehydrated independently with acetonitrile for 10 min., vacuum dried, rehydrated with 55 mM dithioerythreitol in 25 mM ammonium bicarbonate, pH 8.5, at 37°C for 1 hr., and subsequently alkylated with 100 mM iodoacetamide in 25 mM ammonium bicarbonate, pH 8.5, at room temperature for 1 hr. The pieces were then washed twice with 50% ACN in 25 mM ammonium bicarbonate, pH 8.5 for 15 min. each time, dehydrated with acetonitrile for 5 min., dried, and rehydrated with a total of 100 ng of sequencing grade, modified trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate, pH 8.5, at 37°C for 16 hrs. Following digestion, each vacuum of tryptic peptides were extracted with 50% ACN containing 5% formic acid for 15 min. with moderate sonication. For optimal extraction, the procedure was repeated again then the extracted solutions were pooled in one vacuum and evaporated to dryness under vacuum. For MALDI-MS analysis, dry peptide samples were redissolved in 0.1% trifluoroacetic

acid (TFA) and purified by C18 Zip-Tip (Millipore, Billerica, MA, USA) according to the manufacturer's instruction manual.

MALDI-Q-TOF analysis for protein mass fingerprinting (PMF) analysis of 2-DE protein spots and sequence database search

The final protein recognition and confirmation was performed by matching the peptides identified by MALTI-Q-TOF. The sequences of eluted peptides were firstly identified by subjecting the samples to MALDI-Q-TOF analysis [5]. Briefly, tryptic peptides from 2-DE protein spots were subjected to MALDI peptide mass fingerprinting using a MALDI-Q-TOF mass spectrometer (M@LDI™; Micromass, Manchester, UK) operated in reflectron positive ion mode. Samples were spotted onto a 96-well MALDI target plate using a saturated matrix solution of a-cyano-4-hydroxycinnamic acid in 50% ACN/1% TFA. Mass spectra were acquired for the mass range of 800 to 3000 Da and automatically processed by the mascot for PMF searches against the SWISS-PROT database. The ion masses were submitted to NCBInr database using the Mascot ions search for mass matching and verification of protein identity. The identification of protein based on MS/MS analysis of one peptide was considered sufficient if the Mascot score was above the significance level. Positive identification of proteins required at least five matching peptide masses with 50 ppm or better mass accuracy.

Direct nanoLC-nanoESI-MS/MS (LC-MS/MS) analysis for protein identification and sequence database search

If any of the proteins identified by MALDI-Q-TOF appear to have potential for further study, they will be subjected to re-confirmation by a higher precision method employed by LC-MS/MS. The method of LC-MS/MS was adopted from a previous study [5]. Briefly, LC-MS/MS analyses were performed on an integrated

nanoLC-MS/MS system (Micromass, Manchester, UK). After data acquisition, the individual MS/MS spectra acquired for each of the precursors within a single LC run were output as a single MASCOT-searchable peak list (.pkl) file. The peak list files were used to query the SWISS-PROT database using the MASCOT program. Only significant hits as defined by MASCOT probability analysis were initially considered.

Western blot analysis

The expression of any selected protein will be further studied in each pleural effusion by Western blot and Enzyme-Linked Immunosorbent Assay. Each protein sample was mixed with an electrophoresis buffer containing 2% SDS and 5% β-mercaptoethanol and boiled for 10 min. To ensure equal loading of different samples, protein quantification was done according to Bio-Rad protein assay (Bio-Rad, Laboratories, Inc. Hercules, CA, USA). Briefly, 800 μl of each sample was added with 200 μl of Bio-Rad Protein Assay Dye Reagent Concentrate. All samples were assayed in duplicate. After incubation at room temperature for at least 5 min., samples were quantified by measuring absorbance at 595 nm. Proteins (5 μg) were then separated by electrophoresis on a 10% SDS-polyacrylamide gel. The fractionated proteins were electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad, Laboratories, Inc. Hercules, CA, USA). The membranes were blocked for at least 2 hrs in 5% bovin serum albumin, 0.1% Tween 20 in Tris-buffered saline (TBST) and then incubated with primary antibody, diluted 1:1000, for 1hr. After washing in TBST, the membranes were incubated with peroxidase-conjugated secondary antibody for 1 hr., and proteins were detected using the enhanced chemiluminescence detection system (PerkinElmer Life and Analytical Sciences, Boston, USA). All protein bands were analyzed by a densitometry using the Multi Gauge Ver. 2.2 software (Fujifilm, Tokyo, Japan).

Results

Protein profiles of malignant and transudative pleural effusions

The laboratory characteristics of both pleural effusions are shown in Table 1. The protein profiles of 27 effusion samples were analyzed by 2-DE with pH values in the range of 3-10 for a greater protein separation. Figure 1A shows the representative 2-DE images of proteins from pleural effusions. About 643 spots can be visualized by PDQuest image analysis software in gels stained with Sypro Ruby. By comparing protein profiles from 2-DE gels of malignant and transudative effusions, seven protein spots were found with significant degree of differential expression (Fig. 1B).

Identification of protein spots differentially expressed in malignant and transudative pleural effusions

Protein profiles were compared in gels stained with Sypro Ruby (Fig. 1B) and CyDye (Fig. 2) images, seven spots consistently expressed higher levels in transudative effusions than in malignant effusions. The seven protein spots were excised and in-gel digested with trypsin, followed by MALDI-Q-TOF analysis. The sequence coverage of the identified proteins, by peptide mass fingerprinting, ranges from 3% to 15% depending on the size of proteins and the amount present. These spots were identified as fibrinogen beta, gamma chain precursor, and pigment epithelium-derived factor (PEDF), and summarized in Table 2. The locations of these seven differentially expressed protein spots were marked with numbers in one representative gel shown in Figure 1.

PEDF levels in pleural effusions

The identification of spot 27 as PEDF was further confirmed by nanoflow LC-MS/MS. Because PEDF is the most potent natural anti-angiogenesis factor, a decrease in PEDF concentration suggests a possible essential role in meditating malignant pleural effusion. Therefore, to evaluate the clinical significance of PEDF in

pleural effusions, the levels of PEDF were studied in all samples by Western blot analysis and ELISA. Briefly, PEDF concentrations were determined using a Chemikine™ Pigment Epithelium-Derived Factor (PEDF) Sandwich ELISA Kit (Chemicon International, Hampshire, UK). In order to measure total PEDF, samples were treated with urea (8 M) and diluted to 1:500 in assay diluents. PEDF levels were quantified by enzyme immunoassays according to the manufacturer's protocol (Fuji Chemical Industries, Toyama, Japan). Figure 3 shows that PEDF expression in Western blot analysis was significantly lower in all malignant pleural effusions, irrespective of cancer type, than in the transudative effusions. Because of substantial variation in protein quantities and fluid volumes among different forms of pleural effusions, the concentration of PEDF measured by ELISA may not correctly reflect actual lost or gain of PEDF in the effusions. That is even though the proportion of PEDF is actually decreased in malignant effusions, no matter how the mechanisms are, the concentrations measured by ELISA may give opposite results. Therefore PEDF level was adjusted according to the total protein in each pleural effusion. Figure 4 shows that the normalized concentration of PEDF was also significantly decreased ($p<0.0001$) in the malignant effusions (1.76±0.26 ng/mg of protein, n=14) than in the transudative effusions (4.33±0.52 ng/mg of protein, n=13).

Discussion

While we might have expected global proteomic analysis to demonstrate de novo proteins in malignant pleural effusion compared with transudates, by contrast our study demonstrated at least seven major spots, identified as PEDF, fibrinogen beta, and gamma chain precursor, with decreased expression in the malignant effusions. In a previous proteomic analysis using LC-MS, 44 proteins were identified from 242 spots in pleural effusions secondary to lung cancer [6]. PEDF was not identified in that study.

Our results suggest that PEDF is not identified possibly because the level is relatively low in malignant pleural effusions. Another study, using similar techniques, compared the proteomic profiles of exosomes isolated from different forms of human malignant pleural effusions. PEDF was identified in the breast-cancer-associated malignant pleural effusions, but not in lung-cancer- and mesothelioma-associated malignant pleural effusions [7]. The findings that exosomes isolated from lung-cancer- and mesothelioma-associated malignant pleural effusions did not contain PEDF were compatible to our results. Despite PEDF was found in exosomes of breast-cancer-associated malignant pleural effusions, it was not determined whether the level of PEDF was increased or decreased than in normal effusions.

PEDF, cloned from a human eye cDNA library, shares a sequence and structural homology with members of the serine protease inhibitor (serpin) gene family, but it does not appear to inhibit any known proteases [8]. It has been found that the early population doubling level cDNA-1 (*EPC-1*) gene, widely expressed in mammalian tissues and cells, encodes PEDF [9]. PEDF was initially identified as a neuronal differentiation factor produced by cultured human retinal pigment epithelial cells [10]. Subsequently, it was discovered that it is indeed the most potent natural inhibitor of angiogenesis [11]. PEDF blocks the stimulatory activity of multiple inducers of angiogenesis [11], partly by inducing endothelial cell apoptosis [12]. It is up-regulated during cell cycle phase G0 in young, but not senescent, cultured fibroblasts [13], inhibits microglial growth [14], and is neurotrophic for cerebellar granule cells [13].

A current direction in cancer research is to identify and modulate specific events in tumorigenesis. Angiogenesis is one such event known to be fundamental to the development, growth, and metastasis of cancers and is one of the characteristics that differentiate tumor from host tissue [15,16]. Mounting evidence suggests that

angiogenesis is regulated by a net balance between positive (angiogenic) and negative (angiostatic) factors produced by tumors and other cells [17-19]. Tumors are capable of releasing angiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF), which induce the growth of a capillary network surrounding the tumor [20]. One of the mechanisms underlying the formation of pleural metastasis and effusion is the induction of new, more permeable vessels by angiogenic factors. Aside from its angiogenic function, VEGF is also a key mediator in the formation of pleural effusions by inducing vascular permeability [21]. In patients with malignant pleural effusion, the level of pleural VEGF is significantly higher than in patients undergoing cardiac surgery [22]. In addition, blockage of VEGF activity has been shown to reduce the accumulation of malignant ascites and pleural effusion, indicating the important role of angiogenic factors in mediating malignant pleural effusion [23]. Thus, development of antiangiogenic therapies is of great interest in managing patients with malignant pleural effusion.

By contrast, tumors may also produce angiogenic inhibitors, including angiostatin, thrombospondin, and endostatin [18,24,25]. Recently PEDF has been reported to be a significant predictor of prognosis in various cancers, such as ductal pancreatic adenocarcinoma [26], hepatocellular carcinoma [27], and prostate tumors [28]. However, unlike other anti-angiogenic factors of which serum and pleural level are elevated in patients with cancer [29,30], the level of PEDF is decreased both in pleural effusions and the blood of patients with cancer [26]. This observation suggests PEDF may serve as a possible candidate molecules in the treatment of cancer. In fact, in vivo gene transfer of PEDF by adenovirus has been shown to inhibit tumor angiogenesis and growth in a syngeneic murine model of thoracic malignancies [31]. The mechanism by which PEDF is down-regulated in cancer patients remains undetermined. One can

postulate that the decrease is due either to degradation of the end-product (PEDF) or to suppression at the transcriptional level of the *EPC-1* gene. Recently, PEDF was shown to be a substrate for matrix metalloproteinase type 2 and 9, both of which have been implicated to play a role in the abnormal accumulation of pleural effusions [32,33]. The other possibility is that we may discover proteins in the proteomic profiles if they are responsible of down-regulating PEDF at the transcriptional level. In fact, two fibrinogen precursor monomers were found with significantly decreased expression in our 2-DE. Fibrinogen also has potent antiangiogenic activity *in vitro* and *in vivo* [34,35]. It is unclear whether there is any interaction between fibrinogen and PEDF in the course of their antiangiogenic activity. Further studies are needed to provide additional information between fibrinogen and PEDF.

In conclusion, we have demonstrated that PEDF and fibrinogen precursors are expressed at lower levels in malignant pleural effusions than in transudates. It is hoped that this finding, as well as the many others being generated in proteomic studies, will eventually lead to better diagnostic markers or therapeutic targets. However, much work remains to be done to determine which, if any, of the many substances found in this type of study will eventually prove to have clinical applicability.

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Table 1: Laboratory characteristics of malignant and transudative pleural effusions

	Glucose (mg/dL)	Protein (g/dL)	LDH (IU/L)	WBC (cells/uL)	Lymphs (cells/uL)	Neuts (cells/uL)
Transudate (n=13)	197±27	1.38± 0.18	82±13	292±50	181±50	55±33
Malignant (n=14)	128±15	3.62± 0.13	477±99	878±240	379±107	72±22

Data are presented as means±SEM

Table 2: Selected spots from 2-DE gel identified by MULTI-Q-TOF and SwissPort database searching

Serial code	Spot No	MS/MS	Peptide sequence	Protein name	Accession No
P1	13	YES	MLEEIMKYEASILTHDSSIR IHLISTQSAIPYALR	Fibrinogen gamma chain precursor	AAK19752
P2	14	YES	AIQLTYNPDESSKPNMIDAATLK MLEEIMKYEASILTHDSSIR YLQEIYNSNNQK IHLISTQSAIPYALR		
P3	15	YES	AIQLTYNPDESSKPNMIDAATLK IHLISTQSAIPYALR		
P4	26	YES	IHLISTQSAIPYALR		
P5	27	YES	LAAAVSNFGYDLYR IAQLPLTGSMSIIFFLPLK	PEDF	AAK92491
P6	53	YES	EEAPSLRPAPPPISGGGYR KGGETSEMYLIQPDSSVKPYR	Fibrinogen beta chain precursor	AAA18024
P7	54	YES	AHYGGFTVQNEANKYQISVNK IRPFFPQQ		

Figure legends:

Figure 1. (A) A representative 2-DE gel of pleural effusion stained by Sypro Ruby. (B) Spot 13, 14, 15, 26, 27, 53 and 54 represent individual protein with different degrees of expression in transudative and malignant pleural effusions. These spots were specifically selected and shown in the 4 insets.

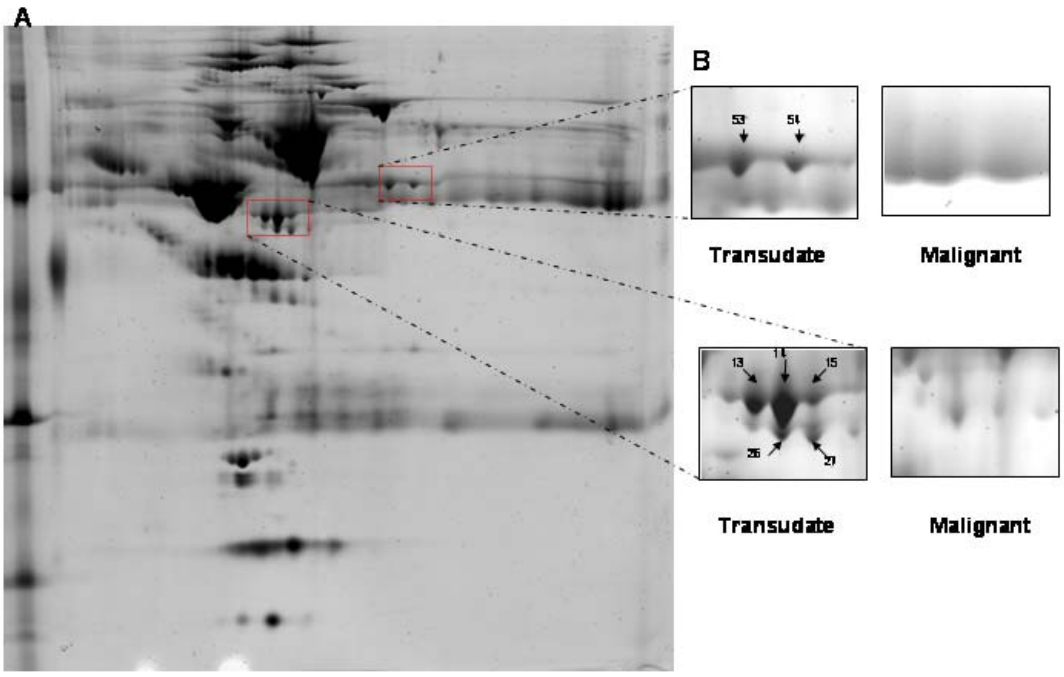

Figure 1.

Figure 2. CyDye-stained 2-DE gels of selected proteins from transudative and malignant effusions. Images were cropped from a representative 2-DE gel. Figures 2a to 2f are CyDye images. Figures g, h and i are 3D views of spots 27, 15, and 54. Figure 2c shows spots 13, 14, 15, 26 and 27 with orange color indicating higher expression levels in transudative effusion (Cy5). The same situation was observed in Fig. 2f. Spots 13, 14, 15 and 26 are fibrinogen gamma chain precursor; spots 53 and 54 are fibrinogen beta chain precursor; spot 27 is pigment epithelium-derived factor (PEDF).

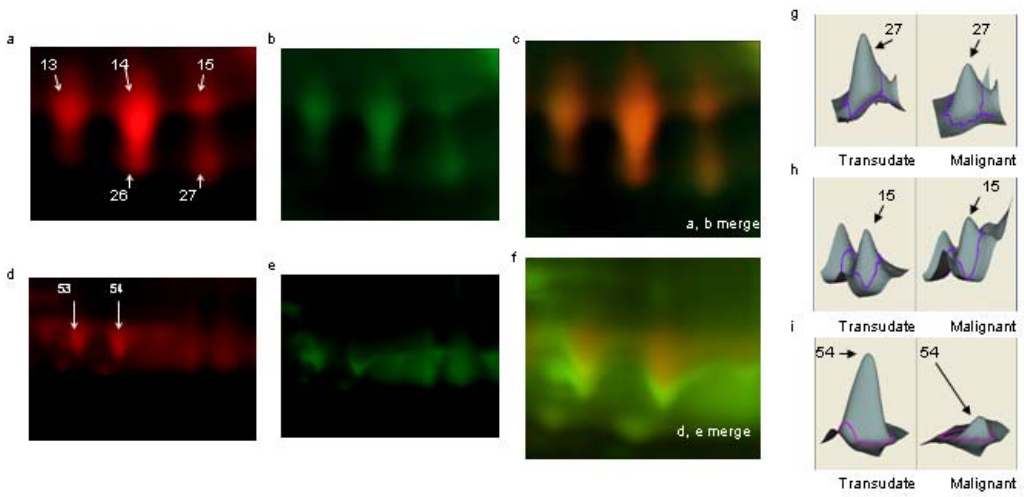

Figure 2.

Figure 3. Western blot analysis showed that the expression of PEDF were higher in transudative effusions (a) than in malignant effusions (b). Densitometry analysis of the Western blot also showed that PEDF was significantly higher in transudative effusions than in malignant effusions ($p<0.0001$, c).

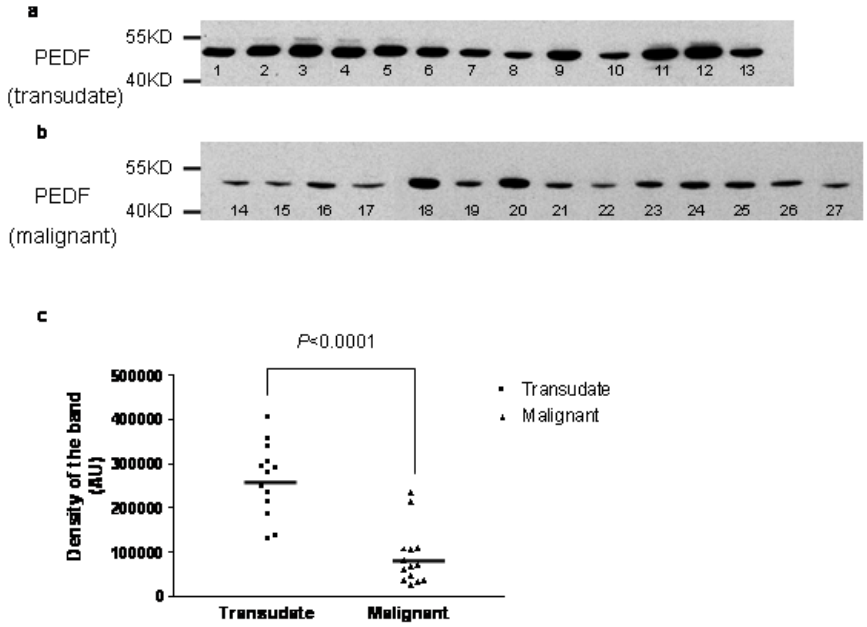

Figure 3.

Figure 4. The concentations of PEDF normalized by total protein were also significantly higher in transudative effusions than in malignant effusions ($p<0.0001$). Transudate:4.33±0.52 ng/mg (n=13); Malignant:1.76±0.26 ng/mg (n=14).

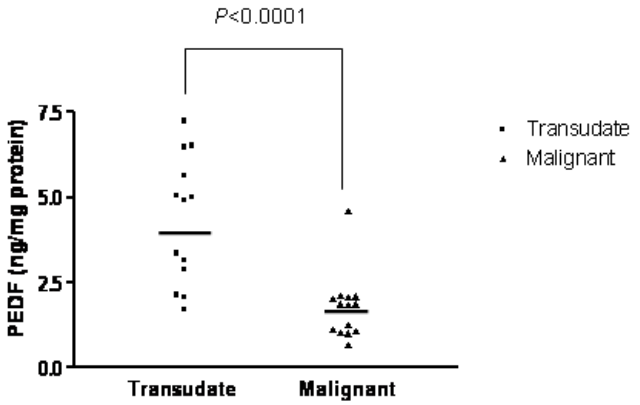
P<0.0001
PEDF (ng/mg protein)
7.5
5.0
2.5
0.0
Transudate
Malignant
Transudate
Malignant

Figure 4.