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Comparative Proteomic Analysis of Lung Cancer Cell Line and Lung Fibroblast Cell Line

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Abstract. *Lung cancer is the leading cause of cancer-related to death in both men and women. Protein biomarkers for lung cancer were investigated using the expression of proteins from lung cancer cell line (A549) and compared with those of normal lung fibroblast cell line (MRC-5). Two-dimensional gel electrophoresis of A549 and MRC-5 cells was carried out and followed by protein identification using nanoelectrospray tandem mass spectrometry. Most proteins over expressed in A549 cells were phosphoproteins such as lamin AC 70 kDa, aldehyde dehydrogenase, α-enolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and peroxiredoxin. Moreover, some proteins were expressed only in A549 cells such as heterogenous ribonucleoprotein A1, nuclear corepressor KAP1, transketolase and cytokeratin 18. Furthermore, the phosphoprotein chaperonin 60 was highly expressed in A549 cells. It is known to function in protein interactions and protein conformation. The over expression of this protein in cells may result in abnormalities of protein conformation and lead to early stage cancer. These proteins may be used as biomarker of lung cancer for early detection and clinical prognosis.*

Cancer is a disorder resulting from autonomous, uncontrolled cell growth and differentiation, with cells invading and disrupting other tissues, and spreading to other areas of body. Cancer affects the body by destroying the surrounding adjacent tissues and replacing normal functioning cells in distant sites. Lung cancer is the leading cause of cancer-related mortality in both men and women. It is the malignant transformation and expansion of lung tissue, and is the most lethal of all types of cancer worldwide, responsible for 1.3 million deaths annually. It is caused mainly by cigarette smoking, and mostly affects men, being the leading cause of death of men between the ages of 40 and 65. With increased smoking among women, lung cancer is now occurring more frequently in women. While there are potential biomarkers cited in scientific literature, no efficient diagnostic tool is currently in use for early detection of lung cancer.

In recent years, '-omics' analyses have been developed and promise to define 'fingerprints' of patterns in malignant cells. These analyses derive their power from the simultaneous measurement of the expression levels of multiple transcripts, protein products and/or protein modification (1). Since proteins play a central role in the life of an organism, proteomics is instrumental in the discovery of biomarkers, such as markers that indicate a particular disease. The scale of proteomic analysis varies according to its aim, such as determining the protein content of a whole organism, analyzing target proteins in a tissue, or in a cell. The direct evaluation of the proteins expressed in tumor cells offers information that cannot be obtained by the study of DNA alterations or RNA expression pattern. The most important characteristic that genomic or genome analyses cannot detect is post-translational modifications (PTM) of proteins, such as proteolytic processing, phosphorylation, or glycosylation.

Phosphorylation is one of the most common and best characterized post-translational modifications of cellular proteins (2). It plays the central role in many biological and biomedical phenomena. It has increasingly become the focus of cell biology because it is involved in gene expression,

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protein synthesis and signal transduction, which determines cell growth, cell division or differentiation and cancer. Phosphorylation acts as an on/off switch for many biochemical functions. It occurs by the reaction of protein kinases. In eukaryotic organisms, the typical acceptors for phosphorylation are the hydroxyamino acids serine, threonine and tyrosine. The identification of phosphoproteins is possible by many direct and indirect means, but the localization of the sites of phosphorylation remains a technical challenge because that of only few phosphoproteins is known (3).

By using proteomic approach as described above, protein expression of lung cancer cell line (A549) and normal lung fibroblast cell line (MRC-5) were studied and potential biomarkers were sought to be used in early diagnosis of lung cancer.

Materials and Methods

Cell cultures. The lung cancer cell line A549 was purchased from the American Type Culture Collection (ATCC). A549 cells were grown in RPMI 1640 (Gibco, Grand Island, NY, USA) containing 25 HEPES supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (125 ng/ml). Normal lung fibroblast cell line MRC-5, originally obtained from the ATCC, was kindly provided by Dr. Mammen Mammen and Dr. Ananda Nisalak of the Department of Virology, the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. MRC-5 was grown in DMEM (Gibco) with 10% FBS and the same antibiotics. Both cells were maintained in a humidified atmosphere, 95% air, 5% CO_2 at 37˚C.

Sample preparation. Both cell types were collected by gently washing with 0.25 M sucrose 3 times, prior to scraping with a rubber policeman in 3 ml of 0.25 M sucrose containing protease cocktail inhibitor (Sigma, St. Louis, MO, USA). Each sample was centrifuged at 3,000 rpm at 4˚C for 10 minutes and the supernatant was collected. The protein concentration of the cell supernatant was determined by Bradford's method (4).

Two-dimensional gel electrophoresis. Proteins were solubilized in standard lysis buffer (7 M urea (ICN Biomedicals, Inc, USA), 2 M thiourea (Sigma), 2% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (USB Corporation, Cleveland, OH, USA), 2% dithiothreitol (DTT) (USB Corporation), 2% Ampholine (Serva, Heidelberg, FRG) and 1X cocktail inhibitor (Sigma)). 350 μg of sample was mixed with rehydration buffer and applied to a 7 cm pH 3-10, immobilized pH gradient (IPG) (GE Healthcare, USA) strip. First dimensional separation was performed using 100 V for 30 minutes, 200-3,500 V for 1.5 hours and 3,500 V for 1-1.5 hours. The IPG strips were equilibrated in two steps of equilibration buffer. The first step employed 50 mM Tris HCl buffer (pH 6.8), 6 M urea, 30% glycerol, 1% SDS, and 1% DTT, while 2.5% iodoacetamide replaced DTT in the second step. Second dimensional separation was performed by using 12.5% SDS polyacrylamide gel (100 mm×80 mm×1.5 mm). Electrophoresis of the minigel was performed in a Hoefer system at 20 mA at room temperature for 2 hours (5, 6).

Protein spot detection and analysis. After separation, protein spots were visualized by Coomassie blue R-250 staining. The gels were scanned at 300 dots per inch by ImageScannerII (GE Healthcare, USA). Spots were detected and percent volumes were calculated with ImageMaster 6.0 (GE Healthcare).

Phosphoprotein detection. For phosphoprotein detection, after proteins were separated by 12.5% 2D PAGE, the gels were stained with Pro-Q Diamond phosphoprotein staining solution (Invitrogen-Molecular Probes, Carlsbad, CA, USA) followed the instruction manual provided by the manufacturer. The stained gels were visualized on a Typhoon™ imager (GE Healthcare). The phosphoprotein-stained gels were compared with the Coomassie blue R-250-stained gels.

Tryptic in-gel digestion. Protein spots were excised and transferred to 0.5 ml microfuge tubes. Fifty microliters of 0.1 M NH_4HCO_3 in 50% acetonitrile (ACN) were added. The gel was incubated three times for 20 minutes at 30˚C. The solvent was discarded and gel particles were dried completely by SpeedVac (Labconco, Kansas City, MO, USA). Reduction and alklylation was performed by swelling the gel pieces in 50 μl buffer solution (0.1 M NH_4HCO_3, 10 mM DTT and 1 mM EDTA) and incubating at 60˚C for 45 minutes. After cooling, the excess liquid was removed and quickly replaced by the same volume of freshly prepared 100 mM iodoacetamide in 0.1 M NH_4HCO_3 solution. The reaction was incubated at room temperature in the dark for 30 minutes. The iodoacetamide solution was removed and the gel pieces were washed with 50% ACN in water, three times for 10 minutes each, and the gel pieces were completely dried. Aliquots (1 μg trypsin/10 μl of 1% acetic acid) of trypsin (Pro-mega Corporation, WI, USA) were prepared and stored at –20˚C. Fifty microliters of digestion buffer (0.05 M Tris-HCl, 10% ACN, 1 mM $CaCl_2$, pH 8.5) and 1 μl of trypsin were added to the gel pieces. After incubating the reaction mixture at 37˚C overnight, the digestion buffer was removed and saved. The gel pieces were then extracted by adding 60 μl of 2% freshly prepared trifluoroacetic acid (TFA) and incubating for 30 minutes at 60˚C. The extract and the saved digestion buffer were finally pooled and dried.

Protein identification by LC/MS/MS. The trypsinized protein spots were identified by nanoflow liquid chromatography coupled with electrospray ionization (nano ESI MS/MS) quadrupole-time of flight tandem mass spectrometry (Q-ToF micro; Micromass, UK). The trypsinized peptides were concentrated and desalted on a 75 μm id × 150 mm C_{18} PepMap column (LC Packings, Amsterdam, the Netherlands). Eluent A and B were 0.1% formic acid in 97% water and 3% ACN, respectively. Six microliters of sample were injected into the nano-LC system and separation was performed using the following gradient: 0 min 7% B, 35 min 50% B, 45 min 80% B, 49 min 80% B, 50 min 7% B, and 60 min 7% B. MS/MS spectra produced by nano ESI MS/MS were automatically processes and sought against a nonredundant database using ProteinLynx Global SERVER (www.micromass.co.uk). For some proteins that were difficult to identify, the search was performed on www.matrixscience.com by Mascot searching tool.

Western blotting and immunodetection. Protein extracts from the lung cancer cell line (A549) and normal lung fibroblast cell line (MRC-5) were run using 12.5% SDS-PAGE in a Hoefer system at

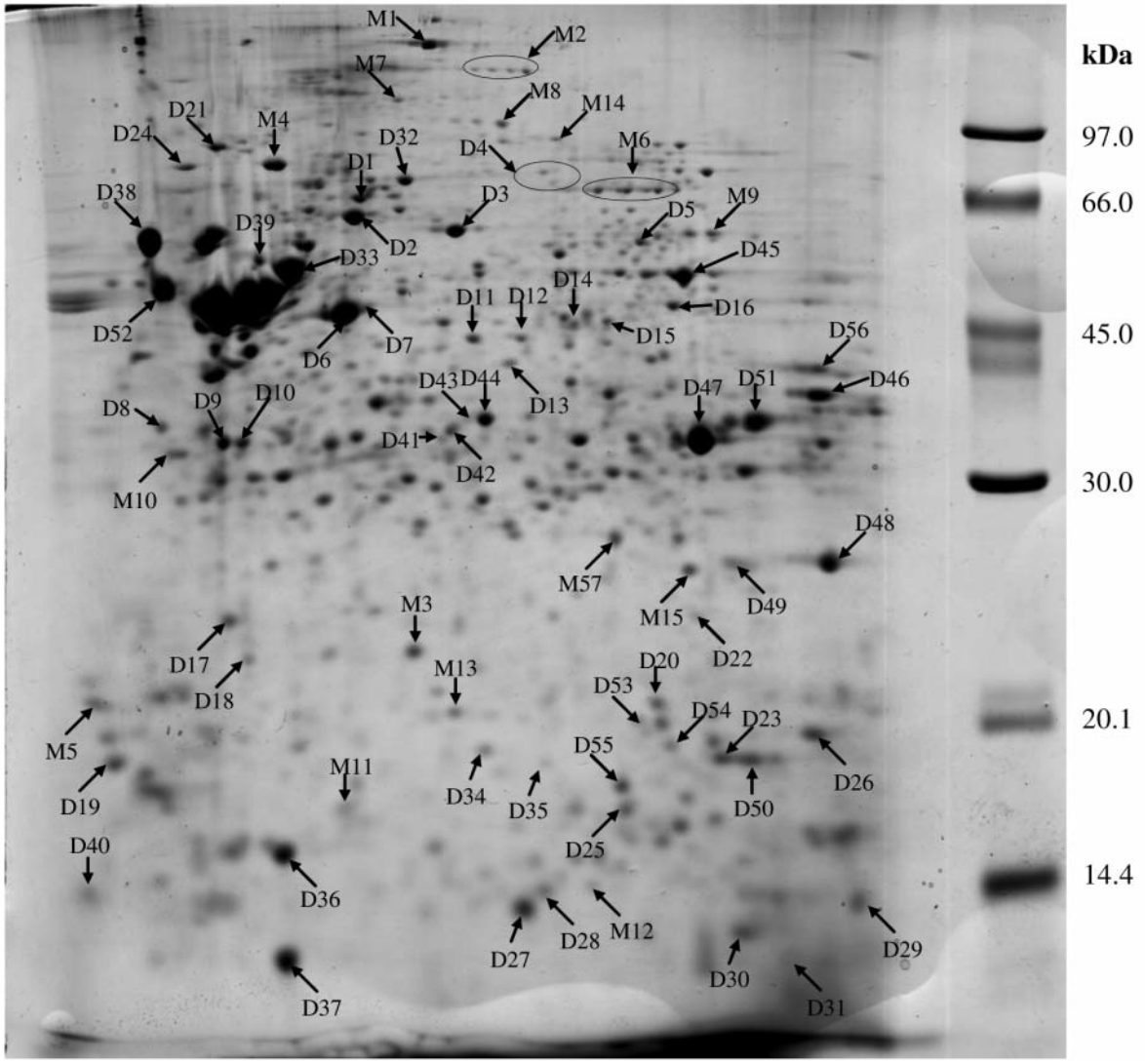

Figure 1. *The proteomic pattern of normal lung fibroblast cell line MRC-5. Spots marked D show proteins found in both MRC-5 and A549 cells, while those marked M show proteins found only in MRC-5 cells.*

10 mA at room temperature for 1.5 hours. The separated proteins were electrotransferred onto nitrocellulose membranes (Hybond ECL; GE Healthcare) at 100 V for 1 hour at 4˚C and incubated for 1 hour at room temperature with a blocking buffer consists of TBST (Tris-buffered saline, 0.1% Tween 20) and 5% non fat dry milk. The membranes were washed and incubated with monoclonal antibodies, anti-cytokeratin 8 (Anti-CK8) and anti-cytokeratin 18 (Anti-CK18) (Chemicon International, Temecula, CA, USA) and used at 1:1,000 dilution overnight. After washing with TBST, membranes were incubated with secondary antibody conjugated with horseradish peroxidase (HRP) (rabbit anti-mouse, IgG; Dako Cytomation, Glostrup, Denmark) at 1:5,000 dilution for 1 hour, washed and incubated for 15 seconds with enhanced chemiluminescence reagent (ECL) (Pierce, Rockford, IL, USA) and detected by high-performance film (Hyper-film ECL; GE Healthcare).

Results

Protein expression in lung cancer cell line A549 and normal lung fibroblast cell line MRC-5. The two dimensional gel electrophoresis of the proteins from the two cell types were performed five times. The representative protein patterns of A549 and MRC-5 cells are shown in Figures 1 and 2. The ImageMaster 6.0 program could detect an average of 634 and 693 spots in A549 and MRC-5, respectively. A total of eighty-three proteins from both cells were identified by ESI Q-Tof. Fifty seven proteins were expressed in both cells, while eleven and fifteen proteins were only expressed in A549 and MRC-5, respectively. Of fifty seven proteins, thirty four proteins were over expressed and twenty three proteins were under expressed

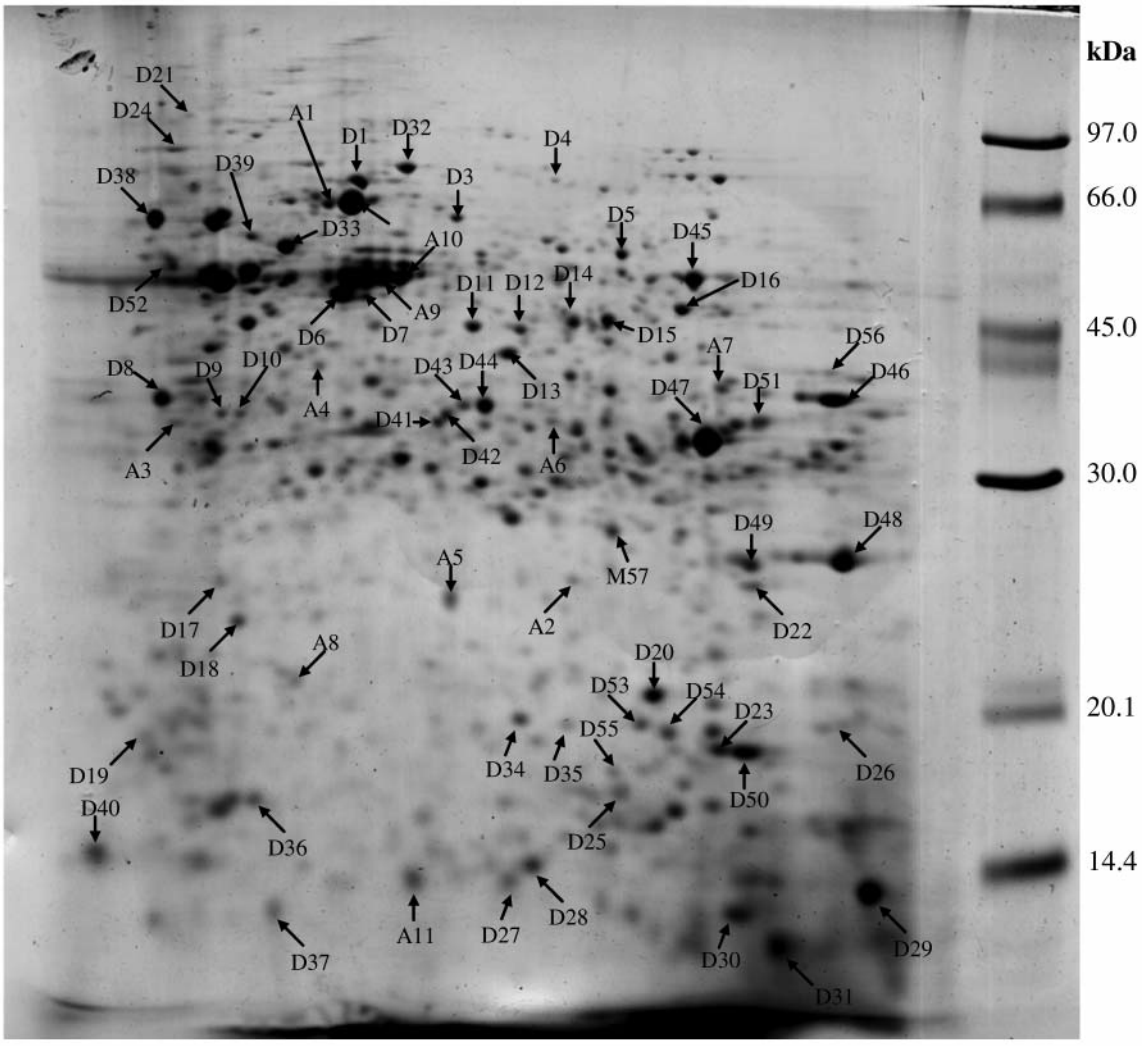

Figure 2. *The proteomic pattern of lung cancer cell line A549. Spots marked D show proteins found in both MRC-5 and A549 cells, while those marked A show proteins found only in A549 cells.*

in A549 by twofold. The proteins which were found in both gels have been marked as D series. The unique proteins in MRC-5 and A549 are marked M series and A series, respectively. The details of the differences in protein expression and protein identification between two cell lines will be discussed.

Protein identification using in-gel digestion and ESI Q-Tof. Protein spots were manually cut and subjected to digestion with trypsin as described in the experimental section. ProteinLynx Version 2.2 (Micromass) was used to generate searchable peak lists processing the data. Initial protein identification was made by correlation of uninterpreted tandem mass spectra to entries in SWISS-PROT using Global server (Version 2.0; Micromass). In addition, the amino acid sequence determination was obtained by Matrix science search, Mascot search engine (http://www.matrixscience.com). Molecular weight, pI and search result of each spots are shown in Tables I, II and III. The fold changes were calculated for percent volume by using ImageMaster 6.0 program. Moreover, the identified proteins were classified based on their function into various categories, namely chaperone/stress response, metabolism, cytoskeleton/mobility, binding/folding, protein synthesis/degradation, protection/ detoxification, signal transduction/transcription, unknown and other as shown in Figures 3-5.

Phosphoprotein detection. Phosphoproteins from MRC-5 and A549 cells were re-analyzed using Pro-Q Diamond phosphoprotein staining solution as described in the experimental section (data not shown). Only few spots from the staining appeared in both gels as marked in Table I. The

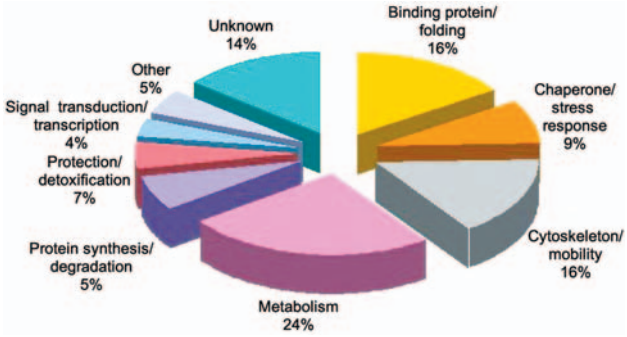

Figure 3. *Summary of functions of proteins which were found in both MRC-5 and A549 cells (D series).*

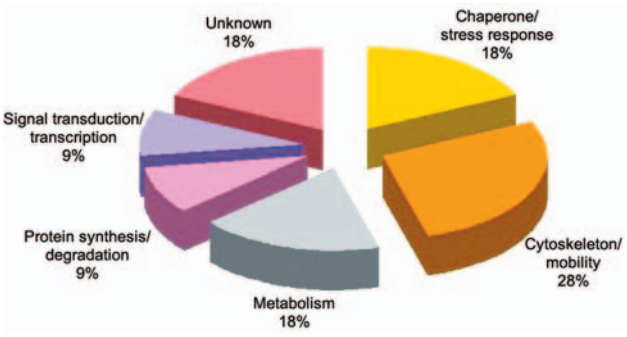

Figure 5. *Summary of protein functions of proteins which were found in A549 cells (A series).*

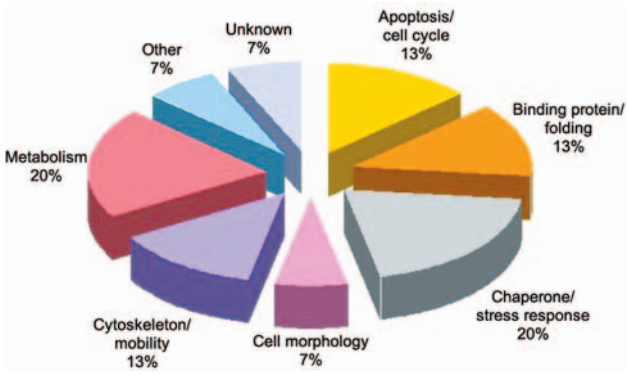

Figure 4. *Summary of functions of proteins which were found in MRC-5 cells (M series).*

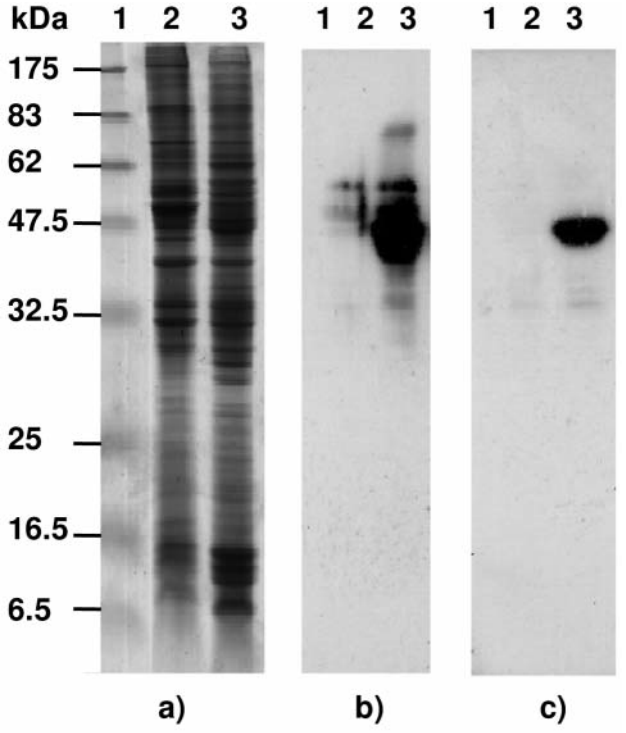

Figure 6. *The SDS-PAGE of MRC-5 and A549 cells were run and stained by Coomassie blue R-250 (a) and compared with 1-DE immunodetection of cytokeratin 8 (b) and cytokeratin 18 (c) lane1: Standard, lane 2: MRC-5 cells, lane 3: A549 cells.*

phosphoproteins were matched from SWISS-Prot database and the phosphopeptides were from mass spectrometry data. The results suggested that out of 83 proteins, 58 phosphoproteins were found in both gels.

Biomarkers studied by Western blotting and immunodetection. After proteins were separated by 2-DE technique and identified by mass spectrometry, cytokeratin 18 (CK18) and cytokeratin 8 (CK8) were found to be differentially expressed in the two cell types. Cytokeratin 18 was found to be expressed only in A549 (spot no. A8, A9 and A10), while cytokeratin 8 (spot no. D7) was found in both cell but 1.7 fold higher in A549. To confirm this result, 1-D immunodetection was used. The Coomassie blue R-250 stain 12.5% gel of MRC-5 and A549 cells (a) was compared with 1-DE immunodetection of cytokeratin 8 (b) and cytokeratin 18 (c) as shown in Figure 6.

Discussion

Comparative proteomics between lung cancer cell line A549 and normal lung fibroblast cell line MRC-5. From Table III, eleven proteins were only expressed in A549 while from Table I, fifteen proteins expressed two-fold higher in A549. For example, chaperonin 60 (Hsp60, spot no. A1, A2 and D2) and stress 70 protein (Hsp70, spot no. D1) were found highly expressed in A549. The heat shock proteins (Hsps) are molecular chaperones, mediating protein folding, transport and formation of protein structures for the ability of cells to survive external stress. Hsps were reported to be over expressed in the majority of tumor tissues from both small cell lung cancer and non-small cell lung cancer as compared to adjacent normal parenchyma/stroma by using

Table I. *The identification of protein spots which were found in both MRC-5 and A549 cells.*

Spot no.	Description	MW (Daltons)	pI	Phospho-staining	Phospho-peptides found	Phospho-proteins (SWISS-Prot)	Protein expression in		Protein function
							A549	MRC-5	
D1	75 kDa Glucose-regulated protein	73,734	5.9	-	-	√	3.0	–3.0	Chaperone/stress response
D2	60 kDa Heat-shock protein, mitochondrial	61,016	5.6	-	√	√	2.5	–2.5	Chaperone/stress response
D3	Protein disulfide isomerase A3	56,747	5.9	-	–	-	–3.9	3.9	Metabolism
D4	Lamin-A/C	74,095	6.6	-	–	√	–1.1	1.1	Cytoskeleton/mobility
D5	Aldehyde dehydrogenase 1	54,696	6.3	-	–	-	–1.9	1.9	Metabolism
D6	Beta-actin	41,710	5.1	√	-	√	–1.6	1.6	Cytoskeleton/mobility
D7	Keratin type II cytoskeletal 8	53,671	5.3	-	–	√	1.7	–1.7	Cytoskeleton/mobility
D8	Complement component 1 Q subcomponent-binding protein, mitochondrial	31,342	4.5	-	–	√	3.7	–3.7	Unknown
D9	Tropomyosin alpha-4 chain	28,504	4.5	-	–	√	–3.1	3.1	Cytoskeleton/mobility
D10	Tropomyosin alpha-3 chain	28,743	4.7	-	–	√	–2.0	2.0	Cytoskeleton/mobility
D11	Alpha-enolase	47,008	7.2	-	√	√	1.7	–1.7	Metabolism
D12	Alpha-enolase	47,008	7.2	-	–	√	1.7	–1.7	Metabolism
D13	Hemoglobin beta chain	15,988	6.9	-	–	√	3.8	–3.8	Binding protein/folding
D14	Non-POU domain-containing octamer-binding protein	54,197	9.4	-	–	√	–4.0	4.0	Binding protein/folding
D15	Unnamed protein product	65,980	7.6	-	–	-	3.2	–3.2	Unknown
D16	Alpha-enolase	47,008	7.2	-	√	√	1.7	–1.7	Metabolism
D17	Translationally-controlled tumor protein	19,582	4.6	-	–	√	1.1	–1.1	Binding protein/folding
D18	DNA-binding protein	35,801	8.9	-	–	√	2.0	–2.0	Binding protein/folding
D19	Unnamed protein product	59,492	5.2	-	–	-	–1.7	1.7	Unknown
D20	Heterogeneous nuclear ribonucleoprotein A1	38,822	9.3	-	–	√	2.8	–2.8	Protein synthesis/degradation
D21	Vimentin	53,681	5.0	-	√	√	–6.1	6.1	Cytoskeleton/mobility
D22	Peroxiredoxin 1	22,096	8.2	-	√	√	1.8	–1.8	Protection/detoxification
D23	Peptidyl-prolyl cis-trans isomerase A, cyclophilin	17,981	7.4	-	√	√	1.4	–1.4	Binding protein/folding
D24	78 kDa Glucose-regulated protein	72,377	5.1	-	√	√	–1.0	1.0	Chaperone/stress response
D25	Unnamed protein product	65,980	7.6	-	–	-	1.5	–1.5	Unknown
D26	Cofilin-1	18,490	8.2	-	√	√	–1.9	1.9	Cytoskeleton/mobility
D27	Protein S100-A11	11,732	7.0	-	–	√	–1.7	1.7	Binding protein/folding
D28	Glyceraldehyde-3-phosphate dehydrogenase	35,899	8.7	-	–	√	1.6	–1.6	Metabolism
D29	10 kDa Heat-shock protein, mitochondrial	10,576	9.4	-	–	√	1.3	–1.3	Chaperone/stress response
D30	Annexin A2	38,552	7.6	-	–	√	1.7	–1.7	Signal transduction/ transcription
D31	Unidentified protein	-	–	-	–	-	5.2	–5.2	Unknown
D32	Unidentified protein	-	–	-	–	-	1.3	–1.3	Unknown
D33	Vimentin	53,619	4.9	√	√	√	–3.3	3.3	Cytoskeleton/mobility
D34	Chlordecone reductase homolog	36,680	6.2	-	–	-	1.3	–1.3	Other
D35	Aldo keto reductase	35,997	7.7	-	–	-	3.0	–3.0	Protection/detoxification
D36	ADP-ribosylation factor-like 2	23,402	9.7	-	–	-	–2.5	2.5	Unknown
D37	Chain A, crystal structure of calcium free human S100aG	10,161	5.3	-	–	-	–3.2	3.2	Other
D38	Calreticulin precursor	48,111	4.1	√	-	-	–2.9	2.9	Chaperone/stress response
D39	Retinoblastoma-binding protein7	47,790	4.9	√	-	√	–1.7	1.7	Binding protein/folding
D40	Histone H2A.2	13,899	10.2	√	-	√	2.0	–2.0	Signal transduction/ transcription
D41	Alpha-enolase	47,079	7.0	√	-	√	3.3	–3.3	Metabolism
D42	Pyruvate kinase	57,841	7.6	√	√	√	1.5	–1.5	Metabolism
D43	Pyruvate kinase	57,841	7.6	√	-	√	2.7	–2.7	Metabolism
D44	Pyruvate kinase	57,841	7.6	√	√	√	1.4	–1.4	Metabolism
D45	Alpha-enolase	47,139	7.0	√	√	√	–1.2	1.2	Metabolism
D46	Heterogenous nuclear ribonucleoprotein A2/A1	35,984	8.7	√	-	-	1.5	–1.5	Protein synthesis/degradation

Table I. *continued*

Table I. *continued*

Spot no.	Description	MW (Daltons)	pI	Phospho-staining	Phospho-peptides found	Phospho-proteins (SWISS-Prot)	Protein expression in		Protein function
							A549	MRC-5	
D47	Glyceraldehyde-3-phosphate dehydrogenase	36,031	8.3	√	-	√	–1.0	1.0	Metabolism
D48	Peroxiredoxin 1	22,096	8.3	√	-	√	1.9	–1.9	Protection/detoxification
D49	Peroxiredoxin 2	22,096	8.3	√	-	√	1.8	–1.8	Protection/detoxification
D50	Peptidyl-prolyl cis-trans isomerase A, cyclophilin	17,870	7.8	√	√	√	2.3	–2.3	Binding protein/folding
D51	Glyceraldehyde-3-phosphate dehydrogenase	36,031	8.3	√	-	√	–2.3	2.3	Metabolism
D52	Calumenin	37,050	4.4	-	–	√	–3.1	3.1	Other
D53	Chain A, structure of lamin-A/C globular domain	13,360	8.9	-	–	-	3.5	–3.5	Cytoskeleton/mobility
D54	Transgelin	22,596	8.9	-	–	√	2.7	–2.7	Binding protein/folding
D55	R33729_1	11,326	7.0	√	-	-	–2.0	2.0	Unknown
D56	Chain A, aldolase A	39,264	8.4	√	√	√	–2.0	2.0	Metabolism
D57	Heterogeneous nuclear ribonucleoprotein H1	49,198	5.9	√	-	-	1.2	–1.2	Protein synthesis/degradation

Table II. *Identification of protein spots found only in lung fibroblast MRC-5 cells*.

Spot no.	Description	Phospho-staining	Phospho-proteins	MW (Daltons)	pI	Matched peptides	Protein function
M1	Collagen, type I, alpha1	-	-	138,926	5.7	23	Chaperone/stress response
M2	Collagen, type VI, alpha2	-	-	108,539	5.8	11	Chaperone/stress response
M3	Peroxiredoxin 2	-	-	21,795	5.4	9	Metabolism
M4	78 kDa Glucose-regulated protein	-	√	70,888	5.2	26	Binding protein/folding
M5	Ras-related protein Rab-18	-	√	22,963	5.1	4	Apoptosis/cell cycle
M6	Lamin A/C isoform 2	-	√	65,096	6.4	21	Cytoskeleton/mobility
M7	Chain A, crystal structure of human full length vinculin(residue1-1066)	-	-	115,928	5.8	20	Cell morphology
M8	Collagen, type VI, alpha2	-	-	108,539	5.8	11	Chaperone/stress response
M9	Pyruvate kinase	-	√	57,841	7.6	16	Metabolism
M10	Laminin binding protein	-	√	31,774	4.8	2	Binding protein/folding
M11	Unnamed protein product	-	√	59,492	5.2	4	Unknown
M12	Beta-2 microglobulin	-	-	12,791	5.8	2	Other
M13	Stathmin 1	-	√	17,292	5.8	6	Cytoskeleton/mobility
M14	ESP-2	-	-	53,071	5.9	4	Apoptosis/cell cycle
M15	Manganese superoxide dismutase (MnSOD)	-	-	24,720	8.3	6	Metabolism

standard immunohistochemistry. The investigations of Hsps as potential tumor biomarkers and therapeutic targets are promising in lung cancer (7).

Peroxiredoxin 1 (spot D22, D48) and peroxiredoxin 2 (spot D49) were 1.8, 1.9 and 1.8 fold higher in A549 compared to MRC-5. These proteins are the antioxidant enzymes involved in redox regulation of cells. Moreover, they may play important roles in eliminating peroxides generated during metabolism or might participate in the signaling cascades of growth factors and tumor necrosis factor by regulating the intracellular concentration of H_2O_2 (8). They are highly homologous members of the peroxiredoxin protein family and have been found to be elevated in several human cancer cells and tissues, and are involved in diverse cellular processes including cell survival, proliferation, and apoptosis. The elevation of peroxiredoxin 1 was presented in oral, esophageal, pancreatic, follicular thyroid, and lung cancers (9-14). Both peroxiredoxin 1 and 2 were also suggested to enhance the aggressive survival phenotype of cancer cells.

Table III. *Identification of protein spots found only in lung cancer A549 cells.*

Spot no.	Description	Phospho-staining	Phospho-proteins	MW (Daltons)	pI	Matched peptides	Protein function
A1	60 kDa Heat-shock protein, mitochondrial, Chaperonin	-	√	60,986	5.7	22	Chaperone/stress response
A2	60 kDa Heat-shock protein, mitochondrial, Chaperonin	-	√	60,986	5.7	5	Chaperone/stress response
A3	Heterogenous nuclear ribonucleoprotein A1 isoform a	-	√	34,175	9.51	2	Protein synthesis/degradation
A4	Nuclear corepressor KAP-1	-	√	88,479	5.52	2	Signal transduction/transcription
A5	Unnamed protein product	-	√	59,492	5.17	9	Unknown
A6	Peroxisomal enoyl-coenzyme A hydratase-like protein	-	-	35,793	8.16	6	Metabolism
A7	Transketolase	-	√	67,751	7.9	11	Metabolism
A8	Keratin type I cytoskeletal 18	-	√	47,305	5.27	22	Cytoskeleton/mobility
A9	Keratin type I cytoskeletal 18	-	√	48,003	5.39	17	Cytoskeleton/mobility
A10	Keratin type I cytoskeletal 18	-	√	47,305	5.27	26	Cytoskeleton/mobility
A11	Unnamed protein product	-	√	59,492	5.17	9	Unknown

Spot M9 was expressed only in MRC-5 as the mixture of pyruvate kinase and pyruvate kinase isozymes M1/M2 with MW/pI as 57,840/7.6 and 57,900/7.8 while spots D42, D43 and D44 expressed in both cells but were up-regulated in A549 cells for 1.5, 2.7 and 1.4 fold, respectively. The spots D42, D43 and D44 were matched to the same mixture of pyruvate kinase and pyruvate kinase isozymes M1/M2 but appeared at lower molecular weights (about 33,000-35,000 daltons) and lower pI (5.75-5.80). Pyruvate kinase is an enzyme involved in glycolysis which catalyzes the transfer of a phosphate group from phosphoenol pyruvate to ADP, yielding a pyruvate molecule and producing one molecule of ATP. There are several isoforms of pyruvate kinase. They are expressed in a tissue-specific manner (type L-PK, R-PK, M1-PK and M2-PK) (15). During multi-step carcinogenesis, the first step is the loss of the tissue-specific isoenzymes, *e.g.* L-PK in liver and kidney and M1-PK in human brain and muscle, followed by the subsequent expression of the M2-PK isoenzyme. This was reported in lung cancer, colon, breast and gastrointestinal cancer. The M2 isoenzyme in normal pulmonary tissue is in tetrameric form, lung tumor tissue produces a dimer (16). The term "tumor M2 pyruvate kinase" (tumor M2-PK) was used to describe this new enzyme.

Spots D11, D12, D16 and D45 were α-enolase or enolase 1, a multifunctional enzyme that, as well as its role in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance and allergic response, and is used as a diagnostic marker for many tumor types. Recently, enolase was reported to be a multifunctional protein and has three isoforms in mammalian cells. They are designated as a-(ENO1), h-(ENO3), and g-(ENO2) enolases. The expression of these isoforms is developmentally regulated. There was data strongly supporting a prognostic role of ENO1 in determining tumor malignancy of patients with NSCLC in a tissue-specific manner (17).

D23 and D50, cyclophilin, the intracellular receptor for cyclosporin A (CsA), a peptidyl-prolyl cis-trans isomerase (PPIase), was found to be over expressed in A549 cell line. Cyclophilin is involved in many biological processes, including apoptosis, inflammation, protein folding, tumors and virus infection. This protein was suggested to be a biomarker for small cell lung cancer (18).

D54, transgelin or SM22 was over expressed in A549 cell line. Transgelin, a dominant protein in smooth muscle cells was widely reported to be up-regulated in esophageal squamous cell carcinoma (19-20) and was found to be a novel biomarker for gastric adenocarcinomas (21).

The interesting proteins found only in MRC-5 cells (M series), such as spots M1, M2 and M8, were found to be collagens with high molecular weights ranging from 108 to 140 kDa. Spot M7 was matched to vinculin (22), which is involved in cell adhesion and may be involved in the attachment of actin-based microfilaments (23) to the plasma membrane, and thus may play an important role in cell morphology and locomotion. Spot M13 was matched to stathmin, which is a highly conserved 17-kDa protein. Its function as an important regulatory protein of microtubule dynamics has been characterized. There was a report of the role of stathmin in the regulation of the cell cycle, resulting in this protein being named oncoprotein 18 (op18). This protein can also cause uncontrolled cell proliferation when mutated and not functioning properly. If stathmin is unable to bind to tubulin, it allows for constant microtubule assembly and therefore constant mitotic spindle assembly. With no regulation of the mitotic spindle, the cell cycle is capable of cycling uncontrollably, resulting in unregulated cell growth characteristic of cancer cells (24).

The functions of proteins in the D, M and A series are summarized in Figures 3, 4 and 5, respectively. For the D series, the major type of uniquely expressed proteins is metabolism, and for the M series, the major types are metabolism and chaperone/stress response. On the other hand, cytoskeleton/mobility is the major type for the A series.

Conclusion

The comparison between the cancer cell line A549 and normal lung fibroblast cell line MRC-5 was studied by proteomic analysis, and two-dimensional gel electrophoresis of the two cell lines showed distinct differences. Chaperonin 60, peroxiredoxin 1, peroxiredoxin 2, pyruvate kinase/ pyruvate kinase isozymes M1/M2, transgelin, α-enolase, cytokeratin 8 and 18 may serve as potential biomarkers for lung cancer as these proteins were over expressed in only the cancer cell line.

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