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Identification of potential lung cancer biomarkers using an *in vitro* carcinogenesis model

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Abbreviations: CSC, cigarette smoke condensate; IPG, immobilized pH gradient; LDH, lactate dehydrogenase; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; MMP, matrix metalloproteinase; NSCLC, non-small cell lung carcinoma; P.E., pituitary extract; PGP9.5, protein gene product 9.5; PRX6, peroxiredoxin 6; SCC, squamous cell carcinoma; SCLC, small cell lung carcinoma; SPARC, secreted protein, acidic, cysteine-rich; TCTP, translationally controlled tumor protein; TIMP-2, tissue inhibitors of metalloproteinases-2; TPI, triosephosphate isomerase; 2-DE, two-dimensional electrophoresis

Abstract

Lung cancer is one of the deadliest and commonly diagnosed neoplasms. Early diagnosis of this disease is critical for improving clinical outcome and prognosis. Because the early stages of lung cancer often produce no symptoms, it is necessary to identify biomarkers for early detection, prognostic evaluation, and recurrence monitoring of the cancer. To identify potential lung cancer biomarkers, we analyzed the differential protein secretion from transformed bronchial epithelial cells (1198 and 1170-I) as compared to immortalized normal bronchial epithelial cells (BEAS-2B) and non-transformed cells (1799) all of which are derived from BEAS-2B and represent multistage bronchial epithelial carcinogenesis. The proteins recovered from the conditioned media of the cells were separated on two-dimensional gels. There was little difference between the secretome of the BEAS-2B and 1799 cells, whereas the patterns between the transformed 1198 and 1170-I cells and non-transformed 1799 cells were significantly different. Using mass spectrometry and database search, we identified 20 proteins including protein gene product 9.5 (PGP9.5), translationally controlled tumor protein (TCTP), tissue inhibitors of metalloproteinases-2 (TIMP-2), and triosephosphate isomerase (TPI), that were either increased or decreased simultaneously in conditioned media of both 1198 and 1170-I cells. Furthermore, levels of PGP9.5, TCTP, TIMP-2, and TPI were significantly increased not only in the conditioned media of both transformed cell lines when compared to those of BEAS-2B and 1799 cells, but also in plasmas and tissues from lung cancer patients when compared to those in normal controls. We suggest the PGP9.5, TCTP, TIMP-2, and TPI as promising candidates for lung cancer serum biomarkers.

Keywords: biological markers; lung neoplasms; proteomics; serum

Introduction

According to global cancer statistics, lung cancer is the most commonly diagnosed cancer (1.35 million new cases in 2002) and the most common cause of death from cancer (1.18 million deaths in 2002) (Chen *et al.,* 2003; Parkin *et al.,* 2005). Despite improvements in diagnostic and therapeutic procedures, high case fatality persists (ratio of mortality to incidence, 0.87) (Chen *et al.,* 2003; Parkin *et al.,* 2005). In Korea, the mortality rates from lung cancer are the highest since 2000 (21.4% in 2006), and the 5-year overall survival rate for patients receiving treatment is just 13.7% for past 5 years since 1998 (Korea National Cancer Center). The best chance for successful treatment is offered by surgical resection in the early stages. However, the majority of lung cancers have been reported to reach stages III/IV (32%/ 41%) and Regional/Distant stages (35%/42%) at the time of diagnosis, and thereby show poor prognosis (Fry *et al.,* 1999; Hoffman *et al.,* 2000; Jemal *et al.,* 2008). Detection of lung cancer at an early disease stage is therefore critical for successful clinical outcome such as improved prognosis and survival rate.

Serological markers can be exploited in cancer screening, monitoring of response to anticancer therapy and cancer progression, and surveillance of recurrence. Although several proteins such as carcinoembryonic antigen, neuron-specific enolase, tissue polypeptide antigen, chromogranin A, carbohydrate antigen 125, carbohydrate antigen 19-9, cytokeratin 19 fragment marker, and progastrin releasing peptide have been reported as putative serum biomarkers for lung cancer, they are not ideal for the detection of lung cancer due to their low specificity and/or sensitivity (Tarro *et al.,* 2005; Schneider, 2006). Identification of biomarkers with high specificity and sensitivity is essential for more effective lung cancer diagnosis.

The emergence of cancer-specific autocrine and paracrine signals originating from tumor cells often includes the supraphysiological expression of secretory proteins or their receptors (Sporn and Roberts, 1985; Welsh *et al.,* 2003; Park *et al.,* 2005). Proteins that are secreted from neoplastic cells into the extracellular microenvironment are taken-up into the bloodstream. The secreted proteins, whose serum levels increase in the relatively early stages of cancer development and correlate with cancer cell proliferation and/or protein overexpression, can be considered as potential serum biomarkers of cancer and new molecular targets for therapeutic intervention (Welsh *et al.,* 2003; Wu *et al.,* 2005). In general, serum is the preferred specimen for the early diagnosis of cancer because it provides several key advantages, including low invasiveness, minimal cost, and easy sample collection and processing (Veenstra *et al.,* 2005; Pan *et al.,* 2008). However, a major limitation in the identification of candidate cancer biomarkers by serum proteome analysis is that high-abundant proteins such as albumin and globulin may obscure the detection of low-abundant proteins. Thus, much interest is currently being focused on the proteins secreted from isolated cancer cells in order to identify potential diagnostic and therapeutic markers.

Search for cancer biomarkers using cell lines established from different individuals is complicated since the genetic background of the cell lines are not identical. In that case, the possibility cannot be excluded that the difference in secreted proteins originates from the difference in genetic backgrounds rather than from the malignancy itself. It is well known that most lung cancers arise from the bronchial epithelium (Vogelstein and Kinzler, 2002). Therefore, in this study, we employed the following cell lines; non-transformed (1799), transformed but non-tumorigenic (1198), and tumorigenic (1170-I) cell lines. Each of cell lines is derived from a single immortalized human normal bronchial epithelial cell line, BEAS-2B. They not only possess the same genetic background but also represent multistep bronchial carcinogenesis (Reddel *et al.,* 1988; Klein-Szanto *et al.,* 1992).

The differentially secreted proteins from these cell lines are expected to be applicable as biomarkers for early detection of lung cancer. Although identification of differentially expressed genes in the human lung carcinogenesis model using cDNA microarray techniques has been previously reported (Feng *et al.,* 2001; Lacroix *et al.,* 2006), this is the first time, to our knowledge, that the differential secretion of proteins has been investigated in this model. We successfully demonstrated that PGP9.5, TCTP, TIMP-2, and TPI were differentially secreted from both transformed 1198 and 1170-I cells, and were present in significantly high concentrations in plasmas and tissue samples of lung cancer patients. These results suggest that these four proteins are strong candidates for diagnostic markers of lung cancer and that secretome analysis of cell lines representing multistage human lung carcinogenesis is a valuable approach to the identification of novel and specific tumor biomarkers.

Materials and Methods

Cells, cell culture, and conditioned medium collection

An *in vitro* lung carcinogenesis model comprised of a series of immortalized normal (BEAS-2B), non-transformed (1799), transformed but non-tumorigenic (1198), and tumorigenic (1170-I) human bronchial epithelial cells was used in this study. BEAS-2B is a normal human bronchial epithelial cell line derived by immortalization using a hybrid of adenovirus and SV40 (Ad12SV40) (Reddel *et al.,* 1988). The 1198 and 1170-I cells were derived from BEAS-2B exposed *in vivo* to beeswax pellets containing cigarette smoke condensate (CSC), and 1799 cells were derived from BEAS-2B exposed *in vivo* to beeswax alone as control (Klein-Szanto *et al.,* 1992). The CSC induced *in vivo* phenotypic changes in BEAS-2B cells similar to the progressive changes that occur during human lung carcinogenesis. The BEAS-2B, 1799, 1198, and 1170-I cells were obtained from Dr. Y. H. Kim (Korea Univ., Seoul).

The BEAS-2B and 1799 cells were grown in Keratinocyte serum free media (Gibco BRL, Eggstein, Germany) supplemented with EGF (Gibco BRL), and pituitary extract (P.E., Gibco

BRL). The 1198 and 1170-I cells were cultured in the medium described above, except that 3% FBS (Gibco BRL) was added to the medium. Cells were cultivated at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed every 2 or 3 days. To prepare conditioned media, cells were cultured until reaching subconfluency and then washed 3 times with PBS. The cells were maintained for 24 h in fresh medium for medium conditioning. To remove cells and cell debris, the collected media were centrifuged for 10 min at 14,000 rpm and 4°C, and supernatants were used as conditioned media in the following study.

Lactate dehydrogenase (LDH) activity assay

To evaluate the extent of cell death that may occur during medium conditioning, LDH activities were analyzed in conditioned media. The assay was performed with LDH assay kit (Sigma Chemical Co., St. Louis, MO) according to the manufacture's protocol. The enzyme reaction was performed under dark conditions at room temperature and terminated by adding 1N HCl. The absorbance at wavelengths of 490 and 690 nm was measured using a Gemini microplate reader (Molecular Devices Co., Sunnyvale, CA). The LDH activities were determined by calculating absorbance difference and normalized by cell numbers.

Sample preparation for two-dimensional electrophoresis (2-DE)

Samples for proteomic analysis were prepared from the equivalent volume of conditioned media (normalized by cell number). Protein in the medium was concentrated using TCA precipitation. Briefly, the protein was precipitated by adding 100% TCA in an amount equivalent to 1/10 volume of conditioned medium. The mixture was incubated for 2 h at 4°C and thereafter centrifuged for 20 min at 14,000 rpm and 4°C. The protein pellet was resuspended in ice-cold acetone for 1 h and centrifuged under the same conditions as described above. The pellet was immediately resuspended and dissolved in lysis buffer [2 M thiourea, 7 M urea, 4% w/v CHAPS, protease inhibitors cocktail (Complete, EDTA-free, Roche Applied Science, Indianapolis, IN), 1% w/v DTT, and 2% v/v Immobilized pH gradient (IPG) buffer] for isoelectric focusing of 2-DE analysis.

Preparation of clinical specimens

This study was approved by the Institutional Review Board of Korea University College of Medicine (Approval No. 2005-1114-1).

Matched normal and tumor tissues from 11 lung cancer patients [6 squamous cell carcinomas (SCCs) and 5 adenocarcinomas] were obtained from Korea Lung Tissue Bank (Seoul, Korea) assigned and supported by Korea Science and Engineering Foundation in the Ministry of Science and Technology. Frozen tissues were homogenized in lysis buffer at 30,000 rpm on ice for 15 cycles of 5 s using a Tissue Tearor (BioSpec Products Inc., Bartlesville, OK). The tissue homogenates were next sonicated with Vibra-Cell Ultrasonic Processors (Sonics & Materials Inc., Newtown, CT) on ice for 6 cycles of 3 s each with 10 s-intervals and centrifuged at 14,000 rpm at 4°C for 10 min. The tissue lysates were then aliquoted and stored at -70°C until use.

Human plasma samples (treated with EDTA), which were collected from lung cancer patients and normal controls, were obtained from Korea University Anam Hospital (Seoul, Korea) and BioInfra (Seoul, Korea) and used for Western blot analysis and ELISA. Clinical information on the patients is summarized in Table 1. There were 23 SCCs (ages 51-84 years; 6 females and 17 males; clinical stage I-IV). The control group consisted of

Table 1. Clinical features of lung cancer patients who donated plasmas.

Case No	Gender	Age	Degree of differenciation	Clinical stage
1	Male	45	Moderately	Ib
2	Female	84	Moderately	IIb
3	Male	58	Moderately	IIb
4	Male	55	Well	IIIa
5	Male	69	N/A	IIIa
6	Male	59	Well	IIIa
7	Male	69	Moderately	IIIa
8	Female	63	Moderately	IIIb
9	Male	66	Poorly	IIIb
10	Male	54	N/A	IIIb
11	Female	63	Moderately	IIIb
12	Male	57	Moderately	IIIb
13	Male	51	N/A	IIIb
14	Male	63	N/A	IIIb
15	Female	65	N/A	IV
16	Male	51	N/A	IV
17	Female	70	N/A	IV
18	Female	71	N/A	IV
19	Male	63	N/A	IV
20	Male	70	Moderately	IV
21	Male	75	N/A	IV
22	Male	74	Moderately	IV
23	Male	69	Moderately	IV

N/A, data not available

17 healthy donors (ages 55-72 years; 5 females and 12 males). The EDTA-treated blood was centrifuged at 2,000 rpm for 10 min. The supernatants were recovered as plasma and stored at -70°C until use. Protein concentration was determined using the Bradford Method.

2-DE and protein identification

Isoelectric focusing, the first dimension of 2-DE was performed using a Multiphor II Electrophoresis System (Amersham Pharmacia, Uppsala, Sweden) as described in previous reports (Rabilloud, 1998; Choe and Lee, 2000; Gorg *et al.,* 2000; Zuo and Speicher, 2000; Lim *et al.,* 2006). IPG gel strips (Linear pH 4-7, 18 cm long) were rehydrated overnight (~16 h) at room temperature in rehydration buffer (2 M thiourea, 7 M urea, 2% w/v CHAPS, 0.3% w/v DTT, 2% v/v IPG buffer and bromophenol blue). Each sample protein dissolved in lysis buffer was applied to the IPG strip using cup-loading. The first dimension was performed at 53 kVhr using the following conditions at 20°C: 300 V for 5 h, 3,500 V for 5 h, and 3,500 V for 12 h. SDS-PAGE, the second dimension of 2-DE was performed using 10% gel (18 cm × 1.5 mm) at 15 mA/gel for 15 min and then 30 mA/gel until the front of the bromophenol blue dye reached the bottom of the gels as previously described (Rabilloud, 1998; Gorg *et al.,* 2000; Zuo and Speicher, 2000). Following electrophoresis, gels were visualized by silver staining (Gharahdaghi *et al.,* 1999; Yan *et al.,* 2000) and scanned in a flatbed densitometer (ImageScanner, Amersham Pharmacia). Spot detection and image analysis were performed by Progenesis (Nonlinear Dynamics Group, Newcastle upon Tyne, UK).

For peptide mass fingerprinting, protein spots of interest were excised from gels, destained and treated with 20 μl of trypsin (10-15 μg/ml) at 37°C for 16-24 h, and the proteins were extracted. Matrix-assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) was performed at Yonsei Proteome Research Center (Seoul, Korea) and Genomine, Inc. (Pohang, Korea). The peptide masses were measured as mono-isotopic masses. To identify proteins, the measured mono-isotopic masses of peptides were analyzed using multiple Web-based search programs such as Mascot (http://www.matrixscience.com), MS-Fit (http://prospector.ucsf.edu), and ProFound (http://prowl.rockefeller.edu/prowl-cgi/profound.exe) and public protein databases such as the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and Swiss-Prot/TrEMBL (http://www.expasy.org/sprot).

Western blot analysis and quantification

Protein samples were prepared as described in "Sample Preparation for 2-DE" and "Preparation of Clinical Specimens", separated on 10% or 12.5% SDS-polyacrylamide gel, and then transferred to nitrocellulose membranes using semidry blotting (Bio-Rad Laboratories, Inc., Hercules, CA). Blots were blocked overnight in TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% skim milk and then incubated for 2 h with primary antibodies (500 ×) against β-actin, GAPDH, Lamin B, PGP9.5, peroxiredoxin 6 (PRX6), secreted protein, acidic, cysteine-rich (SPARC), TCTP, TIMP-2, and TPI (Abcam, Cambridge, MA). Immunodetection was carried out with an enhanced chemiluminescent (ECL) kit (Amersham Pharmacia) according to the manufacturer's protocol. To determine protein abundance, band intensities were quantified using a flatbed densitometer (ImageScanner, Amersham Pharmacia) and image analysis software (LabWorks 4.6, UVP, Upland, CA). Quantitative analysis of TIMP-2 protein in human plasma was carried out using human TIMP-2 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Statistical analysis

Statistical analyses were performed by the unpaired *t*-test or the Mann-Whitney U test, depending on the outcome of the normality test using SigmaStat 2.0 (Systat Software, Inc., San Jose, CA).

Results

Optimization of medium conditioning

Because P.E. and FBS contain complex mixtures of proteins, their addition to the medium for conditioning may complicate the discovery of low-abundant secreted proteins. When the protein profiles of unconditioned media were examined, media supplemented with P.E. and/or FBS showed a large number of protein spots, in contrast to medium alone or medium supplemented only with EGF, as expected (data not shown). Although some components necessary for preparing complete medium, such as EGF, P.E., and FBS, can be omitted to increase the probability of identification of differentially secreted proteins, it may also result in increased cell death, and consequent contamination of the conditioned medium with intracellular proteins released from the dead cells. To determine the difference in death rate between cells

cultured in media from which some supplements were omitted, LDH activities were measured in the conditioned media (Figure 1). Medium supplemented only with EGF showed almost no difference in LDH activities of the four cell lines when compared to complete medium or medium containing P.E. and EGF. In contrast, death rates were high and also there was a significant difference in the rates of the four cell lines when cultured in the medium lacking all of P.E., FBS, and EGF. Taken together, these results indicate that medium containing only EGF was sufficient to maintain the viability of the cells in culture and was therefore used as the conditioning medium for the identification of differentially secreted proteins.

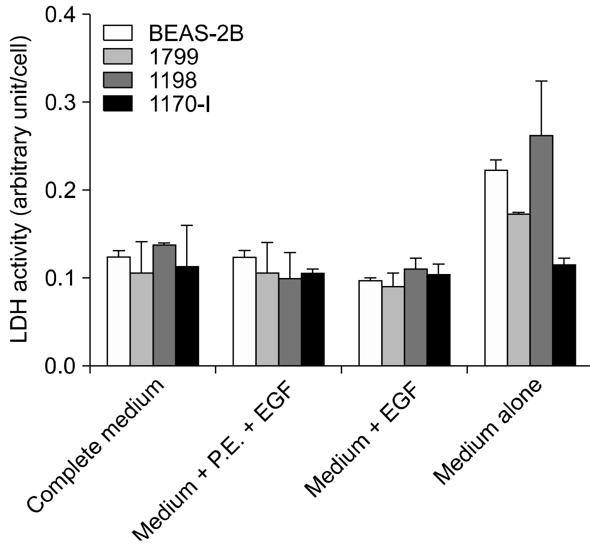
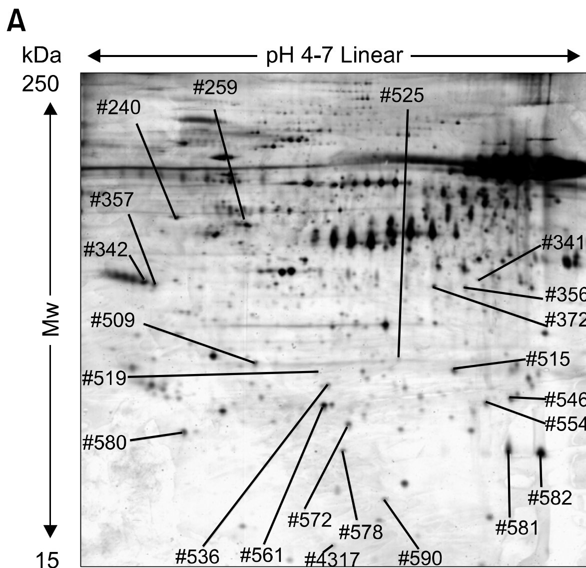


Figure 1. Optimization of medium conditioning. BEAS-2B, 1799, 1198, and 1170-I cells were cultured for 24 h in fresh media as indicated. Cells and cell debris were removed by centrifugation from the collected media, and supernatants were used as conditioned media. LDH activities were measured in the conditioned media and normalized by cell number. All of the data represent the means ± standard deviations obtained from four independent experiments. EGF and P.E. indicate epidermal growth factor and pituitary extract, respectively.

Identification of differentially secreted proteins in conditioned medium

Approximately 600 spots in the pH range 4-7 and molecular mass ranges of 15-120 kDa were detected in the conditioned media from BEAS-2B, 1799, 1198, and 1170-I cells (Figure 2). Proteomic patterns were similar between the conditioned media of BEAS-2B and 1799 (non-transformed) cells, and between those of 1198 (transformed, non-tumorigenic) and 1170-I (transformed, tumorigenic) cells. In contrast, the patterns obtained from the conditioned media from 1198 and 1170-I were markedly different from the patterns of BEAS-2B and 1799 cells (data not shown).



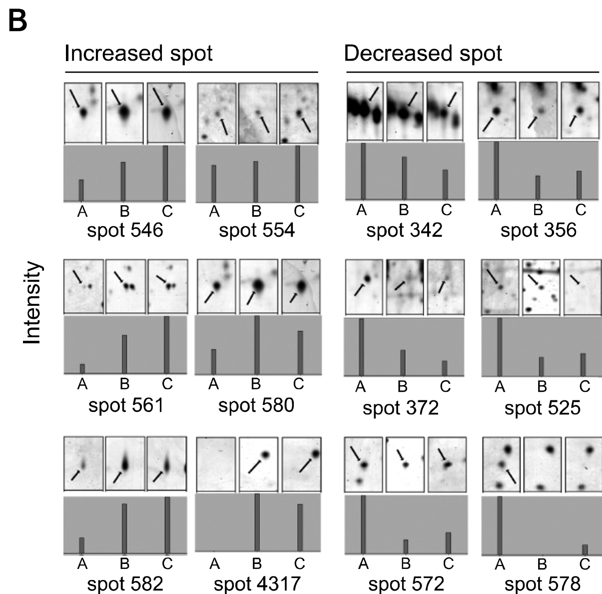


Figure 2. Representative 2-dimensional reference map of 1170-I-conditioned medium (A) and change in secretion pattern across 1799, 1198 and 1170-I cells (B). Proteins in the equivalent volume of conditioned media (normalized by cell number) were concentrated using TCA precipitation and separated on pH 4 to 7 linear IPG strips and 10% SDS-PAGE gels. Protein spots were visualized by silver staining, and spot intensities were measured by densitometer. Twenty proteins whose levels were changed by more than 1.5-fold were identified using MALDI-MS and annotated by spot numbers. Corresponding protein names were listed in Table 2. The map, spot images, and graphs represent one of five independent experiments with similar results. A, B, and C indicate 1799, 1198 and 1170-I cells, respectively.

Out of the secreted proteins whose levels were changed by more than 1.5-fold, 33 were increased and 38 were decreased in 1198-conditioned medium, and 38 were increased and 61 were decreased in 1170-I-conditioned medium compared with 1799-conditioned medium. Of those proteins that were identified as differentially secreted in the transformed 1198 and 1170-I cells, 16/32 were increased or decreased in both transformed cells.

To identify the proteins that were differentially secreted in the conditioned media of both 1198 and 1170-I cells (16 increased and 32 decreased), the protein spots of interest were excised from the silver-stained 2-DE gels and subjected to in-gel digestion. The peptide mass fingerprints (PMFs) were obtained by MALDI-MS and were then used to query the NCBI and Swiss-Prot/TrEMBL protein databases with Mascot, MS-Fit, and ProFound softwares. Ten increased and ten decreased proteins were successfully identified (annotated in Figure 2A). The data for all identified protein spots are shown in Table 2, together with their corresponding spot numbers, Swiss-Prot/TrEMBL accession numbers, gene and protein names, molecular weights and pI values, peptide matched sequence coverage, and fold changes as compared to the levels of each protein in the conditioned medium of 1799 cells. Proteins whose secretion was increased in both 1198 and 1170-I cells include glutathione-S-transferase omega 1, PGP9.5, PRX6, TCTP, TIMP-2, and TPI, and those whose secretion was decreased in the cells include plasma glutathione peroxidase, protein disulfide isomerase, proteasome activator subunit 1, proteasome activator subunit 2, and SPARC.

Validation of differentially secreted proteins in conditioned medium

To verify the reliability of proteomic analysis, the

Table 2. Protein identification of 20 differentially secreted proteins with recurring ≥ 4 times out of the 5 pairs of samples.

Spot No	[1]Accession No	Gene name	Protein name	Mw (kDa)	pI	Seq. coverage	[3]Fold change	
							1198	1170-I
#590	P00441	SOD1	Cu, Zn Superoxide Dismutase	16.01	5.7	35%	+ 2.1	+ 1.5
#4317	Q9Y3B8	REXO2	RNA exonuclease 2 homolog (REX2)	23.96	5.6	23%	+ [2]N/C	+ [2]N/C
#515	P78417	GSTO1	Glutathione-S-transferase omega 1	27.83	6.2	21%	+ 1.9	+ 1.7
#536	Q9Y696	CLIC4	Chloride intracellular channel 4	28.89	5.3	41%	+ 1.6	+ 2.0
#341	P34932	HSPA1L	Heat Shock Protein 70 (HSP70)	53.61	5.6	14%	+ 1.7	+ 2.3
#546	P30041	PRDX6	Peroxiredoxin 6 (PRX6)	25.13	6.0	43%	+ 1.6	+ 1.7
#580	P13693	TPT1	Translationally-controlled tumor protein 1 (TCTP)	19.75	4.8	30%	+ 2.0	+ 1.8
#554	P601074	TPI1	Triosephosphate isomerase 1 (TPI)	26.94	6.4	38%	+ 1.7	+ 1.9
#581, #582	P16035	TIMP2	Human tissue inhibitor of metalloproteinase-2 (TIMP-2)	22.42	6.5	26%	+ 2.1	+ 2.6
#561	P09936	UCHL1	Ubiquitin carboxyl-terminal esterase L1 (UCHL1), protein gene product 9.5 (PGP9.5)	25.15	5.3	48%	+ 3.5	+ 4.2
#572	P09211	GSTP1	Glutathione S-transferase P	23.39	5.4	24%	- 2.6	- 1.8
#509	O00299	CLIC1	Chloride intracellular channel 1	27.25	5.1	54%	- 1.9	- 1.9
#578	P22352	GPX3	Plasma glutathione peroxidase	16.75	9.2	47%	- [2]N/C	- 4.0
#240	P07237	P4HB	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide	57.50	4.8	25%	- 4.3	- 4.1
#525	Q06323	PSME1	Proteasome activator subunit 1 (PA28 alpha)	28.88	5.8	39%	- 2.7	- 2.1
#356	Q9UBS4	DNAJB11	DnaJ (Hsp40) homolog, subfamily B, member 11	40.77	5.8	16%	- 2.1	- 1.5
#519	Q9UL46	PSME2	Proteasome activator subunit 2 (PA28 beta)	27.51	5.4	34%	- 2.5	- 1.7
#342, #357	P09486	SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	35.47	4.7	29%	- 1.6	- 1.9
#372	P36952	SERPINB5	Serine (or cysteine) proteinase inhibitor	42.58	5.7	42%	- 3.6	- 2.3
#259	P68363	TUBA1B	Tubulin, alpha, ubiquitous	50.82	4.9	49%	- 2.9	- 4.3

[1] Accession number from Swiss-Prot/TrEMBL
[2] N/C (Not calculatable): the spots on one of the paired gels were too weak or non-detectable.
[3] Fold change in secretion compared with 1799

levels of PGP9.5, PRX6, SPARC, TCTP, TIMP-2, and TPI were examined in conditioned media by Western blot analysis (Figure 3). It was observed that the levels of PGP9.5, PRX6, TCTP, TIMP-2, and TPI were significantly higher in the conditioned media from both transformed 1198 and 1170-I cells than from BEAS-2B and 1799 cells. This finding is in agreement with the proteomic analysis results and was reproduced in whole cell lysates, with the exception that TIMP-2 was not detectable. In contrast, there was no difference in the levels of SPARC between the conditioned media of four cell lines, and SPARC was undetectable in the whole cell lysates. It is probable that the differential secretion of proteins is a secondary event resulting from a generalized increase in protein expression. To address this point, GAPDH levels were evaluated in the conditioned media and whole cell lysates of four cell lines, because overexpression of many glycolytic enzymes including GAPDH is observed in a variety of tumors including lung cancer and accompanied during malignant transformation (Tokunaga *et al.,* 1987; Pelicano *et al.,* 2006). Although GAPDH levels were markedly increased in the lysates of both transformed 1198 and 1170-I cells contrasted with BEAS-2B and 1799 cells, the protein was not detected in the conditioned media of four cell lines, indicating that the differential secretion of proteins observed in the present study is a tumor-specific phenomenon.

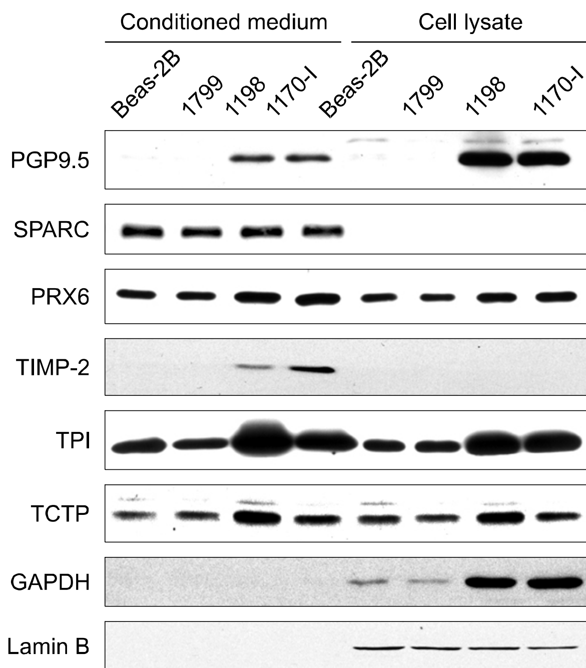


Figure 3. Immunoblot analysis of differentially secreted proteins in conditioned media and whole cell lysates. The conditioned media and whole cell lysates were prepared from BEAS-2B, 1799, 1198 and 1170-I cells. Proteins were separated on 10% or 12.5% SDS-PAGE gels, and visualized using ECL method after incubation with the primary antibodies against GAPDH, PGP9.5, PRX6, SPARC, TCTP, TIMP-2, and TPI. Lamin B proteins were used as an internal control to correct protein loading errors. Data are a representative of three or four independent experiments with similar results.

Validation in lung cancer patients' plasmas and tissues

Next, a comparative analysis of the levels of PGP9.5, PRX6, TCTP, TIMP-2, and TPI was performed in tissues (matched normal and tumor tissues of 11 patients) and plasmas of 23 lung cancer patients and 17 normal controls using immunoblot analysis and ELISA.

In the tissues, levels of PGP9.5, PRX6, TCTP, and TPI that were detected by immunoblot analysis were significantly increased, and TIMP-2 was once again not detected (data not shown). These findings are consistent with the results observed in whole cell lysates as described above (Figure 4). Plasma levels of the proteins were quantified using immunoblot densitometry or ELISA (Figure 5). The levels of PGP9.5, TCTP, TIMP-2, and TPI were statistically significantly increased in the plasmas of lung cancer patients compared to normal controls. However, the level of PRX6 in the plasmas of lung cancer patients was not different from normal control plasmas. Altogether, PGP9.5, TCTP, TIMP-2, and TPI are identified as potential serological markers for lung cancer to be used in the diagnosis and surveillance of lung cancer.

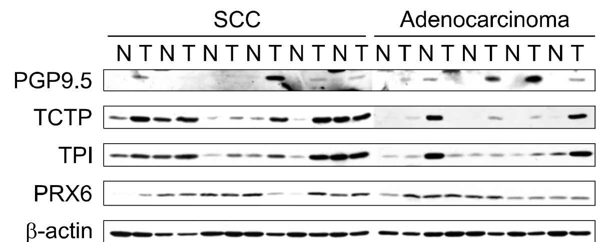


Figure 4. Immunoblot analysis of differentially secreted proteins in lung cancer tissues and adjacent normal lung tissues. Matched normal and tumor tissues obtained from 11 lung cancer patients [6 SCCs and 5 adenocarcinomas] were used. Proteins (20 to 50 μg) were separated on 10% or 12.5% SDS-PAGE gels and then transferred to nitrocellulose membranes. The membranes were incubated with the primary antibodies against β-actin, PGP9.5, PRX6, TCTP, and TPI. Proteins were visualized by ECL method. β-actin proteins were used as an internal control to correct protein loading errors. Data represent one of three independent experiments with similar results.

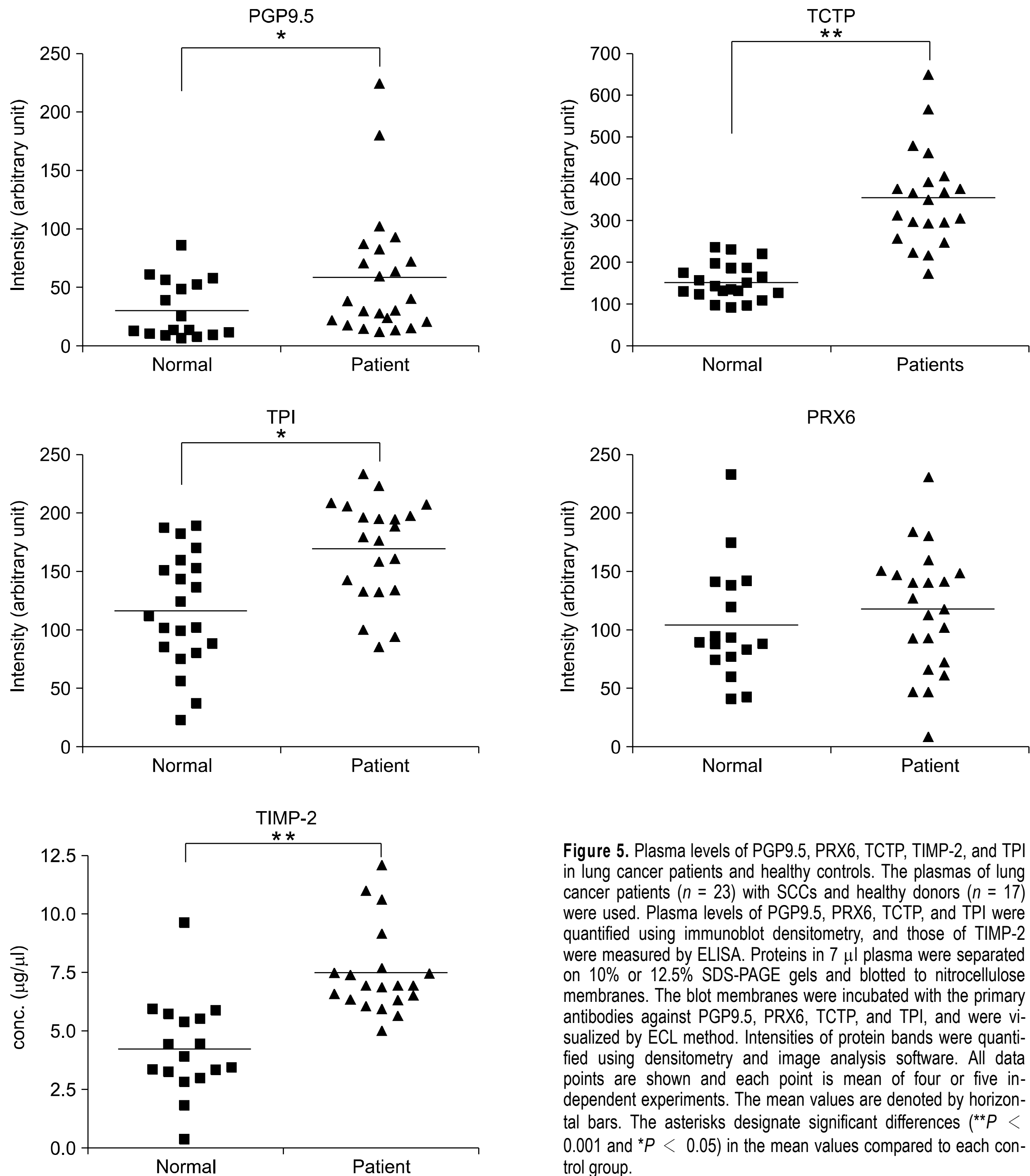


Figure 5. Plasma levels of PGP9.5, PRX6, TCTP, TIMP-2, and TPI in lung cancer patients and healthy controls. The plasmas of lung cancer patients (n = 23) with SCCs and healthy donors (n = 17) were used. Plasma levels of PGP9.5, PRX6, TCTP, and TPI were quantified using immunoblot densitometry, and those of TIMP-2 were measured by ELISA. Proteins in 7 µl plasma were separated on 10% or 12.5% SDS-PAGE gels and blotted to nitrocellulose membranes. The blot membranes were incubated with the primary antibodies against PGP9.5, PRX6, TCTP, and TPI, and were visualized by ECL method. Intensities of protein bands were quantified using densitometry and image analysis software. All data points are shown and each point is mean of four or five independent experiments. The mean values are denoted by horizontal bars. The asterisks designate significant differences (**$P < 0.001$ and *$P < 0.05$) in the mean values compared to each control group.

Discussion

To identify potential early biomarkers for lung cancer, we performed comparative secretome analysis in a series of non-transformed (BEAS-2B and 1799) and transformed (1198 and 1170-I) cell lines all derived from a single human bronchial epithelial cell line, BEAS-2B. Among the 48 protein spots (16 increased and 32 decreased spots) that were differentially secreted in the conditioned media of both transformed cell lines, 20 proteins (10 increased and 10 decreased proteins) were identified by PMF. Interestingly, these proteins can be classified into two groups based on their

change in secretion pattern across 1799, 1198 and 1170-I cells: one group showing a gradual increase or decrease across all three cell lines, and another group showing a marked change in 1198 cells compared to 1799 cells but not much difference between 1198 and 1170-I cells (Figure 2B). It is probable that proteins belonging to the latter group rather than the former are more promising biomarkers for early cancer detection, because secretion of the proteins is dramatically and similarly changed in both transformed cell lines irrespective of their tumorigenicity. Immunoblot analysis and ELISA in samples from tissues and plasmas of lung cancer patients as well as conditioned media demonstrated that four of these identified proteins, PGP9.5, TPI, TCTP, and TIMP-2, were specifically secreted at a high level from 1198 and 1170-I cells, and their levels in clinical specimens of lung cancer patients were significantly higher than those in normal controls, suggesting that the four proteins can serve as biomarkers for early detection of lung cancer. These results also demonstrate that our proteomic results are reliable.

PGP9.5 (ubiquitin COOH-terminal esterase L1, or UCHL1) is a ubiquitin hydrolase that is widely expressed in neuronal tissues at all stages of neuronal differentiation (Brichory *et al.,* 2001). Ubiquitination of cellular proteins for ubiquitin-mediated proteolysis is an important mechanism for cell cycle control. Increasing clinical evidence shows that alterations in the ubiquitination of cell-cycle regulators play a significant role in the etiology of many human malignancies (Reed, 2003; Nakayama and Nakayama, 2006), although whether PGP9.5 plays a pivotal role in the oncogenic transformation of human lung epithelial cells remains unclear. PGP9.5 protein and/or transcript were observed in both small cell lung carcinoma (SCLC) and non SCLC (NSCLC) cell lines (Abbona *et al.,* 1998; Hibi *et al.,* 1998, 1999; Chen *et al.,* 2002), and PGP9.5 expression was strongly associated with the pathological stage of the cancer in primary NSCLCs (Hibi *et al.,* 1999). Moreover, it has been reported that PGP9.5 autoantibody (9 cases) and/or antigen (2 cases) was detected in the sera of 64 lung cancer patients regardless of the histological types. PGP9.5 protein was also detected in the conditioned medium of A549 human lung adenocarcinoma cells (Brichory *et al.,* 2001; Huang *et al.,* 2006). These reports suggest that PGP9.5 may serve as a biomarker for lung cancer.

TCTP, a highly conserved protein in various species such as *Drosophila, S. cerevisiae, C. elegans,* zebrafish, mouse, and human (Hsu *et al.,* 2007) has been reported to have IgE-dependent histamine releasing activity (MacDonald *et al.,* 1995, 1996). It has been reported to function as a guanine nucleotide dissociation inhibitor on the translation elongation factor, eEF1A and act as a guanine nucleotide exchange factor for Ras homologue enriched in brain (Rheb), which is involved in mammalian target of rapamycin (mTOR) signaling (Cans *et al.,* 2003; Hsu *et al.,* 2007). It has been suggested that TCTP can be a target of tumor reversion, a process by which some cancer cells lose their malignant phenotypic characteristics such as rapid cell growth and proliferation, activation of anti-apoptotic pathways, malignant transformation and tumorigenicity (Tuynder *et al.,* 2002, 2004; Chen *et al.,* 2007). The level of TCTP protein is increased in various human tumor tissues including breast, larynx, liver, lung, ovary, prostate, rectum, skin, thyroid, and uterus (Tuynder *et al.,* 2002; Kuramitsu and Nakamura, 2006) and in the human SCLC cell line (Ziv *et al.,* 2006). Furthermore, TCTP protein levels were up-regulated in chemo-resistant sublines of melanoma cells (Sinha *et al.,* 2000). To our knowledge, detection of TCTP in the conditioned medium of human lung cancer cell lines and the serum of patients having solid tumors has never been reported, although TCTP was initially isolated as a secreted factor from U937-derived cultured supernatants (MacDonald *et al.,* 1995) and confirmed by subsequent studies.

Tissue inhibitors of metalloproteinases (TIMP-1, -2, -3 and -4) are endogenous inhibitors of metalloproteinase (MMP) activities. There is currently a great deal of interest in the MMP- independent, cell surface receptor-mediated, and cellular context-dependent functions of TIMPs. Of special interest are both pro- and anti-tumorigenic activities of TIMPs, that have an effect on a number of cellular processes, including cell growth and apoptosis, in a wide range of cells including cancer cells (Stetler-Stevenson, 2008). In contrast to our results on TIMP-2, it has been reported that TIMP-2 protein levels were significantly lower in the sera of patients with NSCLC than in normal control sera (Ylisirnio *et al.,* 2000; Suemitsu *et al.,* 2004), and that TIMP-2 abundance was decreased in human cancers both through genetic polymorphisms and epigenetic mechanisms involving hypermethylation of the TIMP-2 promoter (Stetler- Stevenson, 2008). The levels of TIMP-2 protein were significantly higher in the sera of patients with SCC than in those with adenocarcinoma (Suemitsu *et al.,* 2004), while its level was more frequently increased in adenocarcinoma tissues than SCC tissues (Thomas *et al.,* 2000), indicating the possibility that serum levels of TIMP-2 do not

correlate to its levels in the corresponding tumor tissues. This may explain why TIMP-2 protein is detected in the conditioned medium of lung cancer cells and serum of patients having lung cancers whereas it is undetectable in the corresponding cell lysates and cancer tissues. To date, there are only few reports addressing the level of TIMP-2 in the sera and/or plasmas of lung cancer patients and the potential value of TIMP-2 as a serological marker of lung cancer. Therefore, whether TIMP-2 level is increased in lung cancer patients' sera/plasmas needs to be further investigated in large sample-size study.

TPI, is a highly conserved glycolytic enzyme that catalyze the interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. An increase in TPI expression was observed in a spontaneous transformant of an immortalized but non-tumorigenic cell line derived from alveolar type II pneumocytes as well as in lung tumor tissues versus normal lung tissues from a chemical carcinogenesis mouse model (Peebles *et al.,* 2003; Kassie *et al.,* 2008). TPI was identified by comparative proteomic analysis as being significantly overexpressed in lung cancer tissues compared with adjacent normal lung tissues (Chen *et al.,* 2002; Kuramitsu and Nakamura, 2006). In the conditioned medium of A549 cells treated with Gefitinib (Iressa), an inhibitor of the EGF-R Tyrosine Kinase, decreased levels of TPI were observed when compared to vehicle-treated control cells (McClelland and Gullick, 2007). In addition, it has recently been reported that TPI autoantibody and/or antigen was detected in the sera of lung cancer patients, and the frequency of the autoantibody detection was significantly high in SCC lung cancer when compared to other types of lung cancer. (Li *et al.,* 2006; Nakanishi *et al.,* 2006; Okano *et al.,* 2006; Yang *et al.,* 2007).

In summary, this study produced results that are in accord with previous reports on PGP9.5 and TPI but in opposition to previous reports on TIMP-2. In the present study, we first demonstrated that TCTP is detected in the plasmas of lung cancer patients as well as in the conditioned medium of human lung cancer cells. Our results suggest that PGP9.5, TCTP, TIMP-2, and TPI are potential lung cancer biomarkers. Our finding also demonstrated that our approach, utilizing secretome analysis in multistep human lung carcinogenesis model, provides a reliable way to identify serological markers, even though large-sample investigation with quantitative analysis is necessary to validate whether the identified proteins are reliable and specific markers for early detection and prognostic evaluation of lung cancer.

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