An Approach to Studying Lung Cancer-related Proteins in Human Blood*

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Early stage lung cancer detection is the first step toward successful clinical therapy and increased patient survival. Clinicians monitor cancer progression by profiling tumor cell proteins in the blood plasma of afflicted patients. Blood plasma, however, is a difficult cancer protein assessment medium because it is rich in albumins and heterogeneous protein species. We report herein a method to detect the proteins released into the circulatory system by tumor cells. Initially we analyzed the protein components in the conditioned medium (CM) of lung cancer primary cell or organ cultures and in the adjacent normal bronchus using one-dimensional PAGE and nano-ESI-MS/MS. We identified 299 proteins involved in key cellular process such as cell growth, organogenesis, and signal transduction. We selected 13 interesting proteins from this list and analyzed them in 628 blood plasma samples using ELISA. We detected 11 of these 13 proteins in the plasma of lung cancer patients and non-patient controls. Our results showed that plasma matrix metalloproteinase 1 levels were elevated significantly in late stage lung cancer patients and that the plasma levels of 14-3-3 σ, β, and η in the lung cancer patients were significantly lower than those in the control subjects. To our knowledge, this is the first time that fascin, ezrin, CD98, annexin A4, 14-3-3 σ, 14-3-3 β, and 14-3-3 η proteins have been detected in human plasma by ELISA. The preliminary results showed that a combination of CD98, fascin, polymeric immunoglobulin receptor/secretory component and 14-3-3 η had a higher sensitivity and specificity than any single marker. In conclusion, we report a method to detect proteins released into blood by lung cancer. This pilot approach may lead to the identification of novel protein markers in blood and provide a new method of identifying tumor biomarker profiles for guiding both early detection and therapy of human cancer. ***Molecular & Cellular Proteomics 4:1480–1486, 2005.***

Lung cancer is the leading cause of cancer death worldwide. The 5- and 10-year patient survival rates remain very low at 14 and 8%, respectively (1), despite diagnostic imaging and therapy improvements over the past decade. The best therapeutic opportunity for surgical resection is in the early stage patients, but disease diagnosis is usually late, and prognosis is accordingly poor (2). Detection of lung cancer at an early disease stage is critical for successful clinical therapy, an improved prognosis, and increased survival rate.

Scientists have been seeking tumor-specific biomarkers for decades. Neuron-specific enolase, carcinoembryonic antigens, cytokeratin 19 fragments, and some other proteins are the most commonly used lung cancer markers (3). However, few of these markers are useful in a routine clinical setting, thus underscoring the need for new clinically relevant sources (4). Liotta *et al.* (5) state "it is time to rethink our approach to tumor biomarker discovery. The blood contains a treasure trove of previously unstudied biomarkers that could reflect the ongoing physiological state of all tissues" (5) including tumor tissues. Protein expression level meta-analysis of patients' blood serum holds promise for diagnostic tumor signature detection.

Each year millions of blood samples are collected for medical diagnosis, forming a vast clinical data resource. However, blood plasma is difficult to analyze because it comprises large amounts of albumin and a wide dynamic range of other heterogenic proteins (6). The human plasma proteome provides a tremendous opportunity for detecting, therapeutic monitoring, and deciphering basic cancer mechanisms. Mass spectrometry is a powerful high throughput analytical approach to cancer identification that yields comprehensive peptide and protein serum profiles. Mass spectrometry is efficient because it does not require large samples or arduous protein separations (7, 8). However, its clinical effectiveness has garnered doubt from the scientific community (8, 9). Proteome complexity reduction is key for proteomic analysis of human blood. Recent advances in mass spectrometry may acceler-

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ate disease protein marker identification by proteome complexity reduction.

Our study objective was to provide pilot data from a novel tumor cell protein detection method and to identify lung cancer-related protein blood profiles. Initially in this study we analyzed the proteins released into serum-free conditioned medium (CM)[1] from the tumor microenvironment with short time-cultured lung cancer and adjacent normal bronchial epithelial cells, one-dimensional (1-D) PAGE, and liquid chromatography-tandem mass spectrometry. We then analyzed select proteins from this dataset with ELISA in patient plasma. Using this system allowed us to investigate the diagnostic aspects of the *in vivo* tumor-host microenvironment. Moreover this method should be applicable for blood detection of other human solid tumors in organs such as liver, prostate, pancreas, and breast.

EXPERIMENTAL PROCEDURES

Samples—We collected all human samples, including blood, lung cancer tissue, and the adjacent normal bronchi, from patients in the Cancer Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences. These patients did not ingest any medication before surgery. We collected preoperative peripheral blood samples. We obtained healthy non-patient control blood samples from a "healthy screening" program at the Cancer Hospital. The ethics committees of the Cancer Institute (Hospital) of the Peking Union Medical College and Chinese Academy of Medical Sciences reviewed and approved our experimental procedures. We diagnosed and classified the tumor histologies according to the World Health Organization histological typing of lung tumors and staged them following the tumor node metastasis classification of malignant tumors as defined by the International Union Against Cancer.

We used 628 plasma samples for ELISA, including 419 lung cancer patients between the ages of 15 and 87 (median, 61) years old, 75 non-malignant lung disease patients between the ages of 28 and 74 (median, 53) years old, and 134 non-cancer control patients between the ages of 19 and 79 (median, 44) years old. The malignant and non-malignant lung cancer characteristics used for categorizing the different protein plasma levels are described in Supplemental Tables 1 and 2.

Cell Culture and CM Preparation—We purchased all reagents for cell culture from Sigma unless otherwise stated. Supplemental Table 3 describes the primary cell and organ culture sample characteristics.

For the primary lung cell cultures, we harvested lung cancer tissues and the adjacent normal bronchi from six lung cancer patients into washing buffer (L 15 medium containing 1% antibiotic) immediately after surgery and then transferred them to the laboratory within 30 min. We cultured the primary cells according to the procedures of Lu *et al.* (10). Briefly we washed and minced the tissues in washing buffer immediately after harvesting and digested them in MCDB 151 medium containing DNase I, collagenase type I, and hyaluronidase. We then seeded the cells in 100-mm culture plates in serum-free MCDB 151 medium with growth factors and pituitary extract maintained at 36.5 °C in a humidified atmosphere containing 4% CO_2.

We conducted the lung cancer and bronchial tissue organ cultures according to our previously published method (11). Briefly we chopped the fresh harvested tissues into small (2–3-mm^3) pieces and washed them in washing buffer. We transferred the tissue explants into 100-mm culture dishes and established them in the same serum-free medium as mentioned above. We placed the culture dishes in a culture box containing 50%O_2, 45%N_2, and 5%CO_2 at 36.5 °C on a shaker and agitated the dishes three to four times per minute to expose the explants continuously to the gas and medium.

Conditioned Medium Collection—In this study, we collected the CM after $\sim 3 \times 10^6$ cell growth or within 1 week on each 100-mm dish for cell culture or organ culture, respectively. After removing the spent medium, we rinsed the cultures three times with Hank's balanced salt solution and then incubated them in the pituitary- and serum-free MCDB 151 CM for 1 h at 36.5 °C. We washed the cultures two more times and then added 3 ml of fresh CM/dish. After 48 h of incubation, we collected and centrifuged the CM for 5 min at 4,000 $\times$ *g* at 4 °C to remove cell debris.

SDS-PAGE Separation and Liquid Chromatography-Tandem Mass Spectrometry Analysis—We dialyzed the CM supernatant (molecular mass cutoff, 3500 Da; Serva, Heidelberg, Germany) against distilled water for 24 h at 4 °C. We then lyophilized the dialyzed CM using a vacuum centrifuge (Savant). We dissolved the lyophilized proteins in sampling buffer and separated them with 10% SDS-PAGE. After separating, imaging, and documenting the protein separation gel (Fig. 1), we sliced it into 34 slivers manually from the loading well to the bottom. After in-gel digestion, we diluted the peptides directly into a quadrupole time-of-flight mass spectrometer (Micromass, Manchester, UK). Following the MS/MS analysis, we generated the peak list files with MassLynx3.5 (Micromass) and used these files to search against the UniProt (www.ebi.ac.uk) protein database through a local Mascot server.

Bioinformatic Analysis—We compared our CM protein dataset with three others including serum proteins reported in the literature (Seru_Lit; Ref. 12), a proteome database from serum analysis (Seru_MS; Refs. 12–15), and a total urinary proteome database (Urin_MS; Ref. 16). We used the SignalP 3.0 (www.cbs.dtu.dk/services/; Refs. 17 and 18) with Hidden Markov models to predict the potential signal peptide in the CM protein sequence and TMHMM 2.0 (19) to find the transmembrane helices (www.cbs.dtu.dk/services/).

ELISA Analysis—We collected venous blood preoperatively in sodium heparin-coated plastic tubes for preparation of plasma samples. We then centrifuged the tubes at 1,600 $\times$ *g* for 20 min, removed supernatants, and divided and stored them in aliquots at −80 °C until analysis. We measured the matrix metalloproteinase 1 (MMP1) and tissue inhibitor of metalloproteinases 1 (TIMP1) concentrations in human plasma with a commercially available ELISA kit (Oncogene, San Diego, CA) according to the manufacturer's recommendations.

We detected the plasma levels of fascin, CD98, ezrin, polymeric immunoglobulin receptor/secretory component (PIGR/SC), annexin A4, laminin γ-2 (LAMC2), 14-3-3 σ, 14-3-3 β, and 14-3-3 η by double antibody sandwich ELISA according to the published method (1, 20). The antibodies used for ELISA analysis are listed in Supplemental Table 4. Two independent researchers from our group performed all analyses blind and in duplicate.

Statistical Analysis—We used SPSS software, Version 11.5 (SPSS Inc., Chicago, IL) to calculate all statistical comparisons. All comparisons were two-tailed, and p values of <0.05 were considered significant. We used the independent sample t test to analyze protein plasma differences among the various groups.

RESULTS

Proteome Database of CM Proteins—We used proteomic techniques to analyze the CM samples harvested from the

[1] The abbreviations used are: CM, conditioned medium; PIGR/SC, polymeric immunoglobulin receptor/secretory component; LAMC2, laminin γ-2; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; hnRNP, heterogeneous nuclear ribonucleoprotein; 1-D, one dimensional.

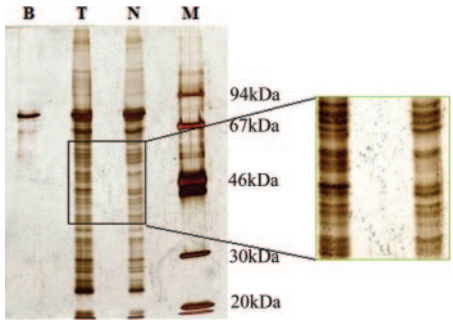

FIG. 1. **CM proteins separated by 10% 1-D PAGE and identified with silver staining.** *B*, blank; *T*, lung tumor tissue; *N*, normal bronchial; *M*, marker.

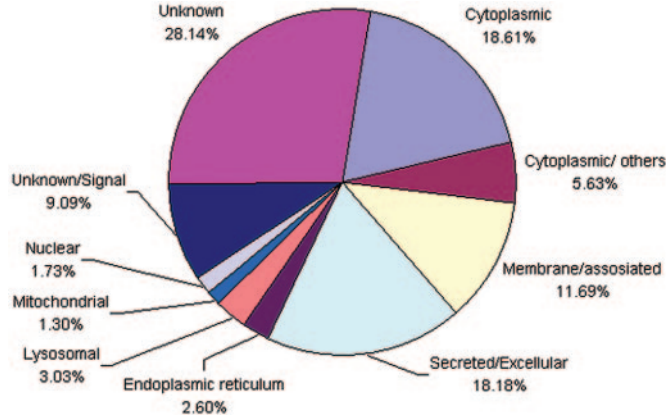

FIG. 2. **Subcellular location of the CM proteins.**

primary cultures of lung cancer cells and the adjacent normal bronchial epithelial cells of six lung cancer patients. After separating the proteins with 1-D SDS-PAGE (Fig. 1), digesting them in-gel, extracting the peptides, and analyzing them with nano-LC-ESI-MS/MS, we identified 231 distinct proteins (Supplemental Table 5). These proteins are involved in cell growth and/or maintenance, protein metabolism, catabolism, response to external stimulus, organogenesis, signal transduction, and cell adhesion activities.

Among these 231 proteins, 42 (18.18%) are extracellular or secreted proteins (such as α_1-antichymotrypsin, metalloproteinase inhibitor 1, serotransferrin, and plasminogen activator inhibitor-1), 56 (24.24%) are cytoplasmic and/or others (such as fibronectin, heat shock protein 90-α, L-lactate dehydrogenase B chain, and α enolase), and 27 (11.69%) are membrane-associated proteins (such as low density lipoprotein receptor, integrin αV, and CD98 antigen). Only 3.03% of the proteins are nuclear or mitochondrial (Fig. 2). The CM proteins span a broad range of molecular weights, and only a few proteins are very basic or acidic, whereas most are hydrophilic (data not shown) according to the isoelectric point distribution.

We collated two protein lists, one for tumor cell CMs and the other for adjacent normal bronchial epithelial cell CMs. Comparisons between the two lists showed that many proteins were detected differentially. We detected 76 proteins from the tumor cell samples, including annexin A4, CD98, PIGR/SC, and cathepsin B, and 35 proteins in the adjacent normal bronchial epithelial cell samples, including fascin, 14-3-3 η, and ezrin (Supplemental Table 5).

We analyzed two pairs of CM lung cancer and bronchus organ culture samples with the same proteomic technique and detected 117 proteins (Supplemental Table 6). Of these proteins, we found 68 to be unique to the organ culture samples (that is, we did not find them among the primary cell culture CM protein population). These potential organ tissue biomarkers include the lung and nasal epithelium carcinoma-associated protein 1 precursor, 1,4-β-*N*-acetylmuramidase C, antileukoproteinase 1, spermatogenesis-associated protein 7, and many hypothetical proteins or proteins of unknown function.

Measurement of MMP1 and TIMP1 Levels in Plasma of Patients and Healthy Controls—The plasma levels of MMP1 were significantly elevated in patients with lung cancer (n = 157; median = 1.82 ng/ml; range, from 0.01 to 44.05 ng/ml) compared with those in healthy controls (n = 30; median = 1.08 ng/ml; range, from 0.71 to 7.14 ng/ml; p = 0.001). The plasma level of MMP1 in the late stage (III and IV) patients (n = 80; median = 2.26 ng/ml; range, from 0.05 to 44.05 ng/ml) was significantly higher than that in the early stage (I and II) patients (n = 76; median = 1.60 ng/ml; range, from 0.01 to 7.42 ng/ml; p = 0.005). However, we found no significant difference between MMP1 levels in lung cancer patients and non-malignant lung disease patients (n = 58; median = 1.90 ng/ml; range, from undetectable to 44.05 ng/ml; p = 0.992; Fig. 3*a*).

The plasma TIMP1 levels were not correlated significantly with the different tumor node metastasis stages. The median plasma TIMP1 level was 131.25 ng/ml (n = 76; range, from 85.86 to 221.45 ng/ml) in early stage patients and 131.75 ng/ml (n = 50; range, from 76.74 to 223.62 ng/ml) in late stage patients (p = 0.330). Furthermore we found no significant difference between TIMP1 plasma levels in lung cancer patients (n = 127; median = 131.75; range, from 76.75 to 223.62 ng/ml) and in non-malignant lung disease patients (n = 30; median = 134.84; range, from 77.05 to 231.12 ng/ml; p = 0.353).

Measurement of Human Plasma Levels of CD98, Ezrin, Fascin, PIGR/SC, Annexin A4, LAMC2, 14-3-3 σ, 14-3-3 β, and 14-3-3 η—We used the ELISA absorbance at 450/570 nm to analyze semiquantitatively the plasma protein levels. We observed a significantly reduced CD98 plasma level among the lung cancer patients (n = 115; median = 0.562; range, from undetectable to 1.068) compared with that in the non-malignant lung disease patients (n = 24; median = 0.597; range, from 0.320 to 1.216; p = 0.035). Lung cancer levels were not statistically different from those of healthy controls (n = 51; median = 0.462; range, from 0.234 to 1.192; p = 0.517; Fig. 3*b*). Plasma CD98 levels in small cell lung cancer patients (n = 24; median = 0.452; range, from 0.280 to 0.844) were signif-

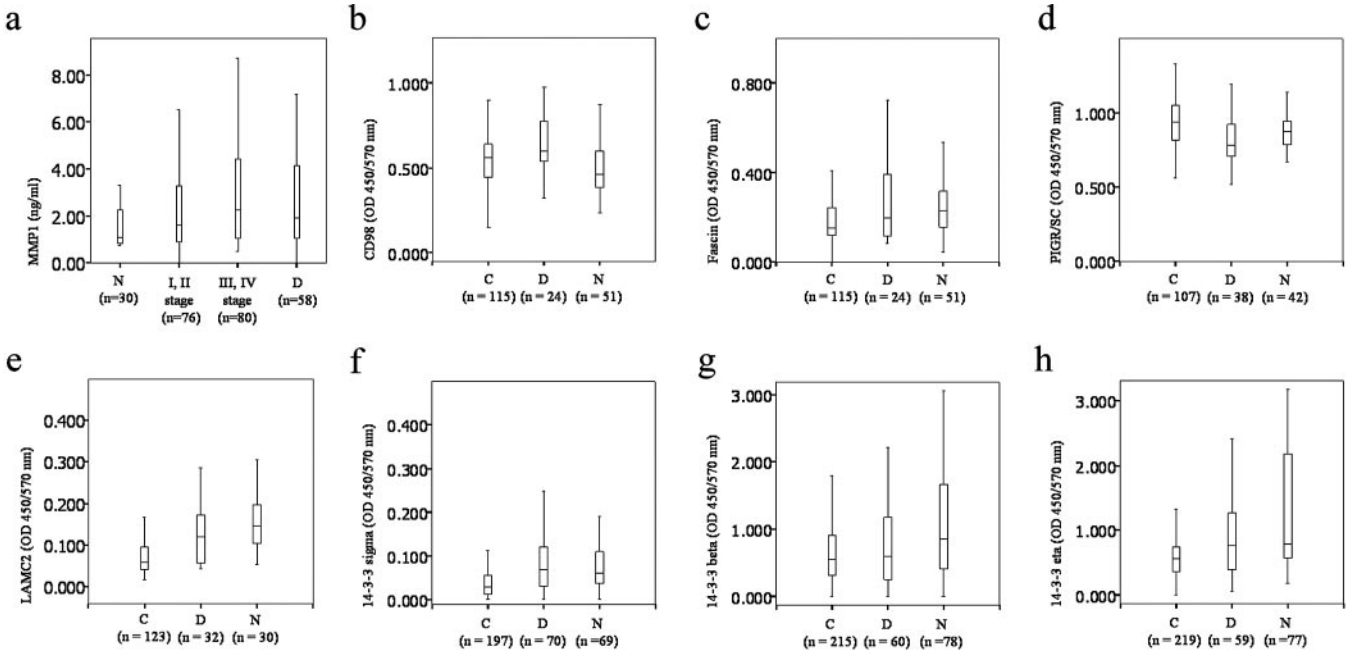

FIG. 3. **ELISA results.** *a*, the plasma levels of MMP1 were significantly elevated in patients with lung cancer compared to those in healthy controls. The plasma level of MMP1 in the late stage patients was significantly higher than that in the early stage. *b*, a significantly reduced CD98 plasma level among the lung cancer patients compared to that in the non-malignant lung disease patients. *c*, the fascin plasma level was significantly reduced in the lung cancer patients compared to the healthy controls. *d*, the PIGR/SC plasma level was significantly higher in the lung cancer patients than in the non-malignant lung disease patients. *e*, LAMC2 plasma levels were significantly lower among the lung cancer patients than they were among the non-malignant lung disease patients and the healthy controls. *f*, the 14-3-3 σ plasma levels in the lung cancer patients were significantly lower than those in non-malignant lung disease patients and those in the healthy controls. *g*, the 14-3-3 β plasma levels in the lung cancer patients were reduced significantly compared to those in the healthy controls. *h*, the 14-3-3 η plasma levels in the lung cancer patients were significantly reduced compared to those in the non-malignant lung disease patients and in the healthy controls. *C*, lung cancer patients; *D*, non-malignant lung disease patients; *N*, normal subjects.

icantly lower than those in non-small cell lung cancer patients ($n = 91$; median = 0.568; range, from undetectable to 1.068; $p = 0.011$). The fascin plasma level was significantly reduced in the lung cancer patients ($n = 115$; median = 0.154; range, from undetectable to 0.650) compared with the healthy controls ($n = 51$; median = 0.230; range, from 0.040 to 0.800; $p = 0.008$; Fig. 3*c*) but not compared with the non-malignant lung disease patients ($n = 24$; median = 0.197; range, from 0.080 to 1.620; $p = 0.075$). The PIGR/SC plasma level was significantly higher in the lung cancer patients ($n = 107$; median = 0.935; range, from 0.147 to 2.122) than in the non-malignant lung disease patients ($n = 38$; median = 0.779; range, from 0.516 to 1.989; $p = 0.013$). The mean PIGR/SC plasma levels were elevated among the lung cancer patients compared with the healthy controls ($n = 42$; median = 0.873; range, from 0.668 to 1.360) although not significantly ($p = 0.174$, Fig. 3*d*). LAMC2 plasma levels were significantly lower among the lung cancer patients ($n = 123$; median = 0.059; range, from 0.017 to 0.641) than they were among the non-malignant lung disease patients ($n = 32$; median = 0.119; range, from 0.044 to 0.494; $p = 0.008$) and the healthy controls ($n = 30$; median = 0.147; range, from 0.054 to 1.453; $p = 0.019$; Fig. 3*e*). We did not detect significant ezrin and annexin A4 plasma level differences among the lung cancer patients, non-malignant lung disease patients, or the healthy control patients ($p > 0.05$; data not shown).

The 14-3-3 σ plasma levels in the lung cancer patients ($n = 197$; median = 0.029; range, from undetectable to 1.648) were significantly lower than those in non-malignant lung disease patients ($n = 70$; median = 0.069; range, from undetectable to 0.618; $p < 0.001$) and those in the healthy controls ($n = 69$; median = 0.061; range, from undetectable to 3.447; $p < 0.001$; Fig. 3*f*). The 14-3-3 β plasma levels in the lung cancer patients ($n = 215$; median = 0.557; range, from undetectable to 2.140) were reduced significantly compared with those in the healthy controls ($n = 78$; median = 0.857; range, from undetectable to 3.063; $p < 0.001$). The median 14-3-3 β plasma levels in the lung cancer patients were also lower than those in the non-malignant lung disease patients ($n = 60$; median = 0.602; range, from undetectable to 2.586), although this difference was not statistically significant ($p = 0.187$; Fig. 3*g*). The 14-3-3 η plasma levels in the lung cancer patients ($n = 219$; median = 0.560; range, from undetectable to 2.343) were significantly reduced compared with those in the non-malignant lung disease patients ($n = 59$; median = 0.760; range, from 0.060 to 3.000; $p = 0.001$) and in the healthy controls ($n = 77$; median = 0.790; range, from 0.184 to 3.183; $p < 0.001$; Fig. 3*h*). We did not find any significant

plasma protein differences due to patient age among the lung cancer patients, non-malignant lung disease patients, or healthy control patients ($p > 0.05$; data not shown).

DISCUSSION

Currently available biomarkers, such as MMP1 and TIMP1, when used alone often misclassify early stage lung cancer with other diseases such as pneumonia and tuberculosis and thus have limited clinical application value. Proteins entering the blood stream from surrounding tumor tissues may provide a reliable source of undiscovered tumor biomarkers, although plasma concentrations are likely very low (5). Finding and measuring these proteins is difficult because the protein population in the blood plasma is large and diverse. In the present study we report pilot data from a method to detect tumor-related proteins released into simple chemical-defined medium by fresh lung cancer samples. From the serum-free conditioned medium, we identified 231 proteins produced by primary lung cancer and adjacent normal bronchus cell cultures and 117 proteins produced by lung cancer organ culture. As determined by gene clustering and custom annotation procedures, these proteins are involved in cell growth and/or maintenance, metabolism, catabolism, response to external stimulus, organogenesis, signal transduction, and cell adhesion.

It was essential in our study to determine whether we could detect these proteins in the plasma of lung cancer patients. Thus, we selected 13 proteins from our CM list and analyzed the plasma of both lung cancer patients and controls using ELISA. Of these 13 proteins, we detected 11 in the plasma of lung cancer and control subjects. Only nm23-H1 and hnRNP A2/B1 were not detected possibly because they were below the present sensitivity threshold. An extensive literature search revealed that 82 proteins were previously detected and reported in human plasma (Supplemental Table 5). For example, the serum levels of cathepsin B, α_1-antitrypsin and urokinase-type plasminogen activator are associated with tumor progression and prognosis (21–23). To confirm these findings, we need to identify and validate at least 200 additional human plasma proteins. We believe that this detection method will allow us to explore the expression patterns of many more proteins and other biological molecules in the highly complex human blood proteome.

Zhu *et al.* (24) observed MMP1 overexpression in tumor tissue and associated it with tumor invasion, metastasis, and poor prognosis. In our studies, the MMP1 plasma levels in the lung cancer patients were elevated significantly above that in the healthy controls. Furthermore the MMP1 levels in late stage patients were significantly higher than those in the early stage patients. However, neither MMP1 nor TIMP1 plasma levels in the lung cancer patients were significantly different from those in non-malignant lung disease patients. These findings suggest that the MMP1 overexpression and disproportionate MMP1/TIMP1 ratios may be late lung carcinogenesis events. Thus, MMP1 and TIMP1 may not be useful biomarkers for early diagnosis of lung cancer because of their inability to distinguish cancer from pneumonia or tuberculosis.

Fascin, a 55-kDa actin-bundling protein, plays a primary role in membrane protrusion development (25), which is associated with tumor cell motility. Recent data by Pelosi *et al.* (26, 27) demonstrate that fascin is overexpressed in invasive and more aggressive non-small cell lung cancer and may affect lung tumor cell differentiation. However, our data are contradictory. The ELISA analysis in our study revealed that the fascin plasma levels in the lung cancer patients are significantly lower than those in healthy subjects. We observed a similar phenomenon for LAMC2, which is considered a potential malignant and metastatic cancer invasion marker (28, 29). In our experiments the LAMC2 plasma levels in the lung cancer patients were significantly lower than in the non-malignant lung disease patients and in the healthy controls. One interpretation to reconcile these conflicting results is that the complex tumor microenvironment mechanism tethers fascin or LAMC2, preventing their release into the blood. The biological roles of these two proteins warrant further investigation.

We measured significantly higher protein plasma levels of PIGR/SC in the lung cancer patients than in the non-malignant lung disease patients. This finding may indicate that overexpression of PIGR/SC plays an important role in lung tumorigenesis. PIGR/SC is involved in the secretory immune system of the human embryo and fetus and is widely distributed in 4-week-old embryos. This protein is detectable later in the mucosa and glands of the digestive, respiratory, and urogenital tracts (30).

In the present study, we found that 14-3-3 σ, β, and η plasma levels in lung cancer patients were significantly lower than those in either the non-malignant lung disease patients or healthy subjects. The 14-3-3 family comprises seven highly conserved proteins (σ, β, ϵ, γ, ζ, η, and τ) that are broadly expressed in all eukaryotic cells and are associated with regulating cell proliferation, differentiation, apoptosis, maintaining cell cycle checkpoints, and aiding in cell adhesion and motility (31). For example, 14-3-3 σ may act as a G_2 checkpoint sentinel by preventing mitotic catastrophe after DNA damage (32). Chan *et al.* (32) determined a decrease in 14-3-3 σ protein expression using immunohistochemical analysis. Hypermethylation of 14-3-3 σ in the promoter region can result in decreased 14-3-3 σ gene expression in various human epithelial cancers of the lung and breast (33, 34). However, the biological functions of 14-3-3 β and η remain unclear. Our results point to the 14-3-3 protein family as a harbinger and potential propagator of human lung carcinogenesis. Furthermore to our knowledge, ours is the first observation of fascin, ezrin, CD98, annexin A4, 14-3-3 σ, 14-3-3 β, and 14-3-3 η in human plasma by ELISA.

Cancer is produced by the tumor-host microenvironment (5). Tumor growth and progression is not only dependent on

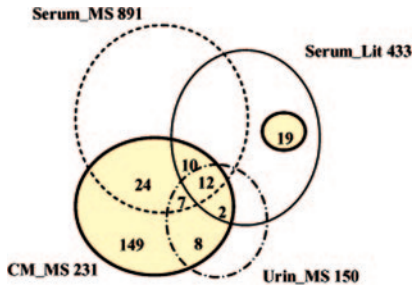

FIG. 4. **Venn diagram of proteins found in the CM of primary cell cultures and other datasets.** Only the proteins common to both CM and other datasets are shown (two-way, three-way, and four-way). *Numbers* represent the shared accessions by BLAST searching for the CM proteins *versus* those in the other three datasets.

the malignant potential of the tumor cells but also on the multidirectional interactions of local factors produced by the various cell types present in the local microenvironment (4). To elucidate the role of the tumor microenvironment on carcinogenesis, we analyzed short time (<1 week) organ-cultured lung cancer tissues and adjacent normal bronchus CM proteins. From these tissues we identified 117 proteins in the organ-cultured CM. Of these, 68 are distinct from the CM proteins of primary cultured cells, including lung and nasal epithelium carcinoma-associated protein 1 precursor and 1,4-β-*N*-acetylmuramidase C. Fresh cancer tissue organ cultures may provide further insight into the *in vivo* tumor microenvironment.

Proteome constituents of body fluids such as serum (plasma) and urine are measured routinely and extensively to identify potential protein markers for early disease diagnostics. Adkins *et al.* (13) and Pieper *et al.* (14, 35) identified 891 proteins while characterizing the serum proteome with numerous sample preparation methods and technique platforms. Anderson *et al.* (12) constructed a blood proteome database for these and other proteins including 433 "non-proteomic" serum or plasma proteins identified through literature searches. Nielsen *et al.* (17) characterized 150 non-redundant proteins in the urinary proteome. We cross-referenced the sequences and homologue searches from the CM proteins identified in the present study with the four published body fluid proteome protein databases. Only 12 of these CM proteins appear in all four datasets (Fig. 4).

It is likely that many factors, including the complexity of cancer types, stages, heterogeneity, and genetic background, will disallow any single biomarker to guide the diagnosis, prognosis, and treatment of all human cancers. Rather tumor biomarker profiling could lead us step by step to a final clinical application of cancer diagnosis and individualized treatment. To this end we combined several proteins from our dataset to predict the various disease aspects. We used the Tclass classification system for this analysis. The Tclass is an algorithm developed originally for gene expression profile-based tumor classification (36). The Tclass system uses Fisher's linear discriminant analysis and many feature selection procedures such as stepwise optimization and all possible feature combinations. Our preliminary results showed that the cross-validation cancer stage prediction accuracy when combining four proteins, CD98, fascin, PIGR/SC, and 14-3-3 η, was extremely high, reaching 86.2%, at a sensitivity of 95.56% and a specificity of 76.92%. These percentages are higher than the prediction accuracy of any single marker at a sensitivity of 85.79% and a specificity of 53.13% (data not shown).

The cancer biomarker detection system detailed herein should be applicable to any human solid tumor type and may provide a springboard for discovering other tumor-specific proteins. As new human blood proteins are identified, the tumor-related profile will become more robust to staging the various carcinomas.

Currently we and our colleagues are identifying human plasma proteins in the liver and gastric systems of cancer patients. We are building a new database to store and analyze cancer-related proteins and biological molecules of human solid tumors. The next challenge we face is raising antibodies in-house against the plethora of new proteins. With these new antibodies, we will design antibody chips for rapid analysis of protein levels in human blood samples and establish tumor marker profiles for cancer patients. We will also perform studies to determine the functionalities of the new proteins identified in this model.

In conclusion, we report pilot data and a method for detecting proteins released into the circulatory system by the tumor microenvironment. This method detects novel protein markers in blood and provides an approach to establish tumor biomarker profiles for early human cancer detection and therapy.

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