Protein Expression Profiling Identifies Macrophage Migration Inhibitory Factor and Cyclophilin A as Potential Molecular Targets in Non-Small Cell Lung Cancer

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ABSTRACT

Current diagnostic and therapeutic strategies for lung cancer have had no significant impact on lung cancer mortality over the last several decades. This study used a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) discovery platform to generate protein expression profiles in search of overexpressed proteins in lung tumors as potentially novel molecular targets. Two differentially expressed protein peaks at m/z 12,338 and 17,882 in the MALDI-TOF spectra were identified in lung tumor specimens as macrophage migration inhibitory factor and cyclophilin A, respectively. Overexpression of both proteins was confirmed by Western blotting, and cyclophilin A was localized to the tumor cells by immunohistochemistry. These data demonstrate the feasibility of using a MALDI-TOF platform to generate protein expression profiles and identify potential molecular targets for cancer diagnostics and therapeutics.

INTRODUCTION

Lung cancer continues to be a major public health issue with >170,000 new cases diagnosed in the United States each year alone. Despite extensive research efforts in genomics, drug discovery, and lung cancer screening, the overall 5-year survival remains ~14% (1). Thus, alternative diagnostic and therapeutic strategies are necessary for changes in outcome to be realized. Recent efforts to improve diagnostic capabilities, facilitate phenotypic stratification, and promote the discovery of new molecular targets for cancer therapies have focused on the spectrum of genetic changes of cancer using gene arrays (2–6). Given the disparity between gene transcription and protein expression, however, and the absence of information on post-translational modification, it is not apparent that genetic analysis alone will be sufficient to improve clinical outcomes.

As another means of addressing this challenge, we sought to determine whether protein expression profiling with a MALDI-TOF MS platform could be used as an alternative strategy in the search for new diagnostic and therapeutic targets. Because the phenotype of a disease is largely a reflection of its protein complement, we reasoned that a display of the expressed proteins within a tissue could form a pattern that could hold detailed information regarding the biology of the disease. For this study, we developed a mass spectrometry-based platform to generate protein expression profiles from lung tissue lysates in an effort to discover and identify novel molecular targets for lung cancer.

MATERIALS AND METHODS

Patients and Specimens. All patients at our institution who were to undergo pulmonary resection for lung cancer or have a lung biopsy for unknown lung disease were considered eligible for the study. After informed consent approved by the Institutional Review Board, patients with lung cancer had a sample of the tumor and adjacent normal lung removed from the gross specimen at the time of resection. Those patients with unknown lung disease and open biopsy had a sample of the abnormal lung tissue obtained for MALDI-TOF MS analysis.

After gross examination by a staff pathologist, tissue specimens were frozen in liquid nitrogen usually within 5 min after resection. Regions of specimens used to make tissue lysates were sectioned, stained with H&E, and examined to verify the histological diagnosis (i.e., tumor, normal lung, or other lung disease).

Lysate Preparation. Tissue specimens were minced, and blood contamination was reduced by end-over-end rotation for 30 min at 4°C in a conical centrifuge tube containing 10 ml of PBS. A portion of the washed tissue (~10 mg) was then placed into a microcentrifuge tube containing 70 μl of mammalian protein extraction reagent (Pierce, Rockford, IL), crushed with a plastic pestle, and shaken for 30 min at 4°C. Cellular debris was then removed by centrifugation at 16,000 × g for 20 min at 4°C. Total protein content in the supernatant fraction was estimated by a Bradford dye binding assay using BSA as standard (7).

MALDI-TOF Analysis. Cell lysate samples were prepared for MALDI analysis using a conventional dried droplet protocol. In this protocol, sinapinic acid was used as the MALDI matrix. The sinapinic acid matrix was prepared as a saturated, aqueous solution that contained 50% (volume for volume) acetonitrile and 0.1% (volume for volume) trifluoroacetic acid. A 1-μl aliquot of each cell lysate was mixed with 30 μl of the sinapinic acid matrix solution before depositing 2 μl of this mixture on the MALDI sample stage where the solvent was evaporated under ambient conditions.

All MALDI-TOF mass spectra were acquired on a Voyager DE Biospectrometry Workstation (PerSeptive Biosystems, Inc., Framingham, MA) in the linear mode using a nitrogen laser (337 nm). Mass spectra were collected in the positive ion mode using an acceleration voltage of 25 kV, a grid voltage of 23 kV, a guide wire voltage of 75 V, and a delay time of 225 ns. Each spectrum obtained was the sum of ~32 laser shots. The raw data in each mass spectrum were smoothed using a Savitsky-Golay smoothing routine before mass calibration using proteins of known mass as internal or external calibrants. S/Ns were evaluated using the standard software provided by the instrument manufacturer. All spectra used in this study were obtained from ~500 ng of total lysate protein.

Data Analysis. MALDI-TOF mass spectra from each sample were analyzed using a T2 test-based statistical pattern recognition approach to determine which parts of the spectra, or bins, were the most valuable in terms of their ability to separate the two sets of data (i.e., tumor or normal). The test yields a P for each bin, which is a measure of the probability that the means of the two groups of data in that bin are equal. Consquently, a low P indicates that the two means are not close to each other; hence, that bin has a reasonable capability of separating the sample sets. The lower the P, the more separable the data are in that particular bin.

Protein Identification. Partial purification of the proteins responsible for the ion signals at m/z 12,338 and 17,882 was accomplished by first performing preparative liquid phase isoelectric focusing (Rotofor; Bio-Rad, Hercules, CA) on seven pooled tumor lysates. The fractions from this separation technique containing the m/z 12,338 and 17,882 peaks were identified by MALDI-TOF MS, pooled, lyophilized, and subjected to further purification by reversed-
phase high-performance liquid chromatography on a C4 column (Vydac, Hesperia, CA). The high-performance liquid chromatography fractions containing the peaks of interest were then pooled, lyophilized, and subjected to two-dimensional gel electrophoresis using a pH 3–10 ReadyStrip immobilized pH gradient strip (Bio-Rad) for the first dimension and an 8–16% polyacrylamide gel for the second dimension. After silver staining, protein spots migrating between ~10 and 20 kDa were selected and subjected to peptide mapping experiments at the Protein Core Facility at the University of North Carolina at Chapel Hill using a Bruker Reflex III and an Applied Biosystems 4700 Proteomics Analyzer.

Immunoblotting. Tissue lysates were subjected to one-dimensional SDS-PAGE (10 μg of lysate protein/well) on a 15% polyacrylamide gel, and the proteins were electroblotted to poly(vinylidene difluoride) using standard methods. Blots were probed with a rabbit polyclonal antihuman CyP-A antibody (Upstate Biotechnology, Inc., Lake Placid, NY), and bound antibody was detected using horseradish peroxidase-conjugated secondary antibody and a chemiluminescent detection system (Pierce, Rockford, IL). The blots were then stripped of bound antibodies (Restore stripping solution; Pierce) and reprobed with a mouse monoclonal antihuman MIF antibody (R&D Systems, Minneapolis, MN). Before MIF immunoblot analysis, removal of anti-CyP-A antibodies was verified by incubation with horseradish peroxidase-conjugated antirabbit antibody and chemiluminescent detection.

Immunohistochemistry. Immunohistochemical analysis of acetone-fixed (~70°C, 30 s), 5–8-μm sections of lung tumor and normal lung tissue was performed as described previously (8). Briefly, polyvalent anti-CyP-A rabbit antibody (Upstate Biotechnology) and normal rabbit immunoglobulins were used at 13 μg/ml; incubation with biotinylated goat antirabbit IgG and horseradish peroxidase-conjugated Streptavidin (Zymed, South San Francisco, CA) was performed following manufacturer’s recommendations. Slides were developed with diaminobenzidine (Immuno-Pure Ultra-Sensitive ABC Staining Kit and Metal Enhanced 3,3-diaminobenzidine substrate Kit; Pierce), counterstained with hematoxylin, dehydrated, mounted, and scored by two independent observers. Analysis was performed at the Immunohistochemistry Shared Resource of the Duke Comprehensive Cancer Center.

RESULTS

MALDI-TOF Analysis of Tissue Lysates. For this study, we developed a mass spectrometry-based platform to generate protein expression profiles from lung tissue lysates in an effort to discover and identify novel molecular targets. We initially obtained 10 lung cancer specimens and the matched normal lung tissue specimens from the same 10 patients, which were frozen immediately after surgical resection. We then prepared tissue lysates from these specimens, analyzed the lysates by MALDI-TOF MS, and compared all 10 tumor lysate protein profiles to all 10 normal lung protein expression profiles. The most statistically significant differentially expressed peaks in the tumor as compared with the normal tissue lysates were found at m/z 12,338 and 17,882 (±0.1% mass accuracy).

To validate the above results on a larger sample size, we prepared lung tumor tissue/normal lung tissue lysate pairs from an additional 30 patients who had pulmonary resection. Twenty-four patients had radiological findings suspicious for malignancy and were diagnosed with non-small cell lung cancer, 4 patients had radiological findings suspicious for malignancy but were diagnosed with benign lung disease, and 2 patients had imaging findings suggestive of interstitial lung disease which were confirmed by pathology. Figs. 1 and 2 show 2 different patients both with CT and PET findings suspicious for lung cancer, although only the mass spectral data from patient 1 shows the peaks in the tissue lysates at m/z 12,338 and 17,882 consistent with malignancy (Fig. 1C).

Using a classification scheme based on our mass spectral analyses of the original set of 10 tumor/normal lysate pairs, we classified the blinded tissue lysates from these additional 30 patients (Fig. 3). Overall, both of the distinguishing ion signals were seen in 27 of 34
tumor lysates and in only 1 of 40 nonmalignant tissue lysates (79% sensitivity, 98% specificity). Ion signals at either m/z 12,338 or 17,882 were found in 30 of 34 tumor lysates and 3 of 40 nonmalignant lysates (88% sensitivity, 93% specificity). When examined individually, the peak at m/z 12,338 was seen in 30 of 34 tumor lysates and 3 of 40 nonmalignant lysates (88% sensitivity, 93% specificity). The peak at m/z 17,882 was found in 27 of 34 tumor lysates and 1 of 40 nonmalignant lysates (79% sensitivity, 98% specificity).

**Protein Identification.** A mass spectrometry-based peptide mapping strategy was used to identify the proteins producing the ion signals at m/z 12,338 and 17,882. Ultimately, several proteins in spots that had migration distances consistent with 12,338 and 17,882 kDa proteins were subjected to an in-gel tryptic digestion. The resulting peptides from each protein digest were sequenced by mass spectrometry. The identification of CyP-A was substantiated by MS/MS data on the following 7 tryptic peptides: (a) VSFELFADK; (b) FEDENFILK; (c) EGVMNIVEAMER; (d) VSFELFADKVPK; (e) VKEGMNIVEAMER; (f) SIYGEKFEDENFILK; and (g) HTGPGILSMANAGPNTNGSFFICTAK. These peptides spanned ~40% of CyP-A’s linear amino acid sequence. MIF was identified based on MS/MS data from one tryptic peptide with the sequence PMFIVNTNVPR and on mapping the mass of an additional tryptic peptide to the following linear sequence in MIF: ISPDRVYINYDMNAANVGWNSTFA.

The two proteins identified in this peptide mapping experiment, MIF and CyP-A, had reported molecular masses of 12,345 and 17,881 kDa and were consistent with those of the protein ion signals observed in our initial MALDI-TOF mass spectra of tumor (the error of our m/z measurements was typically ±0.1%).

**Fig. 2.** MALDI-TOF analysis of a 1.8-cm lung nodule suspicious for lung cancer by CT and PET imaging. In A, axial CT of a 46-year-old male demonstrated a 2.2-cm right lower lobe nodule (arrow). In B, further evaluation of this patient with FDG-PET was performed, and a coronal image showed increased FDG uptake (arrow), suggestive of a malignancy. In C, because of the concern for lung cancer, the patient was taken to surgery. Mass spectrum of the nodule lysate did not contain the peaks at m/z 12,338 and 17,882. This pattern was suggestive of a benign abnormality, Dirofilaris (dog heartworm).

**Fig. 3.** Comparison of S/Ns for the MIF and CyP-A peaks in MALDI-TOF spectra of lysates from all 40 tumor/normal pairs and six other lung diseases. This ratio was used to determine whether overexpression in the sample was present. A, plot of specimen number versus average S/N for the MIF peak in the 10 tumor/normal pairs from the training set (●), 24 additional tumor/normal pairs (○), and in six other lung diseases (□). B, CyP-A peak data, symbol key same as in A. The Y axis of each plot is displayed on a log scale for clarity. The error bars represent the SD associated with the S/Ns extracted from 10 different spectra.
Immunoblot Analysis and Immunohistochemistry. After protein identification, we verified by immunological techniques that MIF and CyP-A were overexpressed in lung tumor cell lysates. We performed immunoblot analyses on cell lysate proteins from a series of tumor/normal lung matched pairs, as well as from a series of other malignant and nonmalignant lung diseases. We found MIF to be more abundant in lung tumor cell lysates from five of seven matched tumor/normal pairs (Fig. 4A; Tumors 1, 4–6, 10) and three non-matched tumor lysates (Fig. 4A; Tumors 7–9). Higher levels of CyP-A expression were found in tumor lysates in six of seven matched tumor/normal pairs (Fig. 4B; Tumors 1, 3–6, 10) and three non-matched tumor lysates (Fig. 4B; Tumors 7–9). The level of MIF and CyP-A in the other lung disease tissues was approximately equivalent to the normal specimens, with the exception of the patient with hypersensitivity pneumonitis (Fig. 4, A and B; HP).

Tissue localization of CyP-A to the cytoplasm and nuclei of the epithelial tumor cells was demonstrated by strongly positive immunohistochemical staining of frozen lung tumor sections. These data confirmed that the malignant cells were responsible for CyP-A overexpression in the lysates and that this was not attributable to a surrounding host inflammatory response. The matched normal lung tissue was negative (Fig. 5). We did not similarly assess MIF by immunohistochemistry because a previous study reported cytoplasmic and nuclear overexpression of MIF in adenocarcinoma of the lung (9).

DISCUSSION

The discovery of new molecular targets for lung cancer diagnostics and therapeutics has the potential to significantly change the clinical approach and outcome of this disease. Current diagnostic techniques, including noninvasive imaging followed by pathologic examination of tissue, are still fundamentally based on anatomical and morphological analysis. Therefore, we have designed an alternative strategy and explored the possibility of using a MALDI-TOF MS-based platform as a means of generating protein expression profiles for the discovery of novel molecular targets. In theory, this approach has several advantages over other modalities used to identify differential expression of proteins (10, 11). MALDI-TOF analyses are capable of detecting small amounts of protein material, hence, the potential to detect low abundance proteins. MALDI-TOF exhibits excellent resolution of proteins of low molecular mass, and it is able to detect both very acidic and basic proteins, which can be particularly troublesome on two-dimensional gels. More importantly, this type of discovery technique makes absolutely no assumptions about known or unknown proteins, allowing the process to be completely independent of all presupposed hypotheses.

The current study demonstrates that it is possible to generate mass spectrometry profiles from small human tissue samples and that lung cancer tissue can be differentiated from normal lung and other lung diseases based on a MALDI-TOF-generated protein expression profile. Elucidation of MIF by this process helps validate our experimental approach, because we were able to identify this protein, which has been reported to be overexpressed in adenocarcinoma of the lung (9). Although initially described as a cytokine causing T-cell activation, subsequent reports have shown MIF to have a variety of cytokine activities in a spectrum of diseases (12). Additional studies are needed to elucidate MIF’s potential as a diagnostic or therapeutic target in cancer.
More importantly, however, the discovery of CyP-A overexpression in lung cancer demonstrates the power of this technology to elucidate novel molecular targets. CyP-A has never been reported in lung cancer. CyP-A is a member of the immunophilin family of proteins, typically studied for their binding of various immunosuppressive drugs, most notably Cyclosporin A, and their role in cellular signaling pathways (13, 14). CyP-A has also been shown to possess peptidyl prolyl cis-trans isomerase activity and thus may have a role in protein folding (15). Although CyP-A has numerous known activities, its role in cellular growth and differentiation, transcriptional control, cell signaling, and immunosuppression suggests that it could be involved in an important aspect of oncogenesis.

Although this study focused on these two most statistically significant differentially expressed proteins, many other differentially expressed proteins have yet to be explored. The MALDI mass spectra generated in this study were from unfractionated tissue cell lysates, and thus, spectra contained ion signals for only a small fraction of the thousands of different proteins that are expected to be present in such a sample. The particular subset of proteins that we observed was defined by the relative desorption/ionization efficiencies of each protein under the conditions of our MALDI analysis (16, 17). A number of factors has been noted to contribute to the relative desorption/ionization efficiency of proteins in complex mixtures, such as the protein’s relative abundance, relative ionization efficiency, and specific interactions with the MALDI matrix. We expect that the separation of the specimen proteins into less complex mixtures will result in more extensive representation of the expressed proteins and in turn yield spectra with higher information content.

Although there is a tremendous interest in proteomics, this is the first study to fingerprint lung cancer using MALDI-TOF MS. We identified two potential molecular targets, MIF, an overexpressed protein in lung cancer reported previously, and CyP-A, a protein not known previously to be overexpressed in non-small cell lung cancer. The process of protein profiling has the potential to expand the current repertoire of molecular targets, with the possibility for phenotypic stratification. This type of information should lead to more rationally designed diagnostic and treatment strategies, which will hopefully translate into improved patient outcomes.

REFERENCES

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