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MALDI-MS derived prognostic protein markers for resected non-small cell lung cancer

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Abstract

Protein signals obtained directly from frozen lung tissue sections using MALDI-MS were used to predict nodal involvement and survival in resected non-small cell lung cancer (NSCLC). We have identified a list of these protein signals and further evaluated their prognostic values for NSCLC using immunohistochemistry (IHC). Kaplan-Meier analysis was used to assess the mortality risk associated with the prognostic protein IHC-staining intensities. The combined IHC scores of calmodulin, thymosin β 4, and thymosin β 10 were found to be correlated with NSCLC patient survival ($p = 0.004$). Furthermore, low cofilin-1 IHC-staining intensity was found to be correlated with a better outcome for patients with negative lymph node status ($p = 0.006$) while high cofilin-1 IHC-staining intensity was found to be correlated with a better outcome for patients with positive node status ($p = 0.034$). In conclusion, the prognostic protein signals selected using MALDI-MS can be identified and tested by IHC in formalin-fixed tissue samples. MALDI-MS-derived protein signals can be potentially translated to a conventional clinical setting to aid in the prognosis of patients with NSCLC at the molecular level.

Keywords

Biomarkers; Immunohistochemistry; MALDI-MS; Non-small cell lung cancer; Prognosis

1 Introduction

Lung cancer is the leading cause of cancer death in the United States for both men and women [1], but the survival rate of patients with lung cancer has not significantly improved in decades [1, 2]. The value of molecular markers for lung cancer has been emphasized with the development of targeted therapies, such as the epidermal growth-factor receptor inhibitors erlotinib and gefitinib [3]. However, the precise diagnosis and prognosis of lung

carcinomas using molecular bio-markers has been far behind that of hematopoietic malignancies. The complex behavior of heterogeneous lung carcinomas cannot be fully understood using light microscopy or through the analysis of individual genes or proteins. Comprehensive analysis of protein expression patterns is more likely to be required to improve our ability to understand the behavior of individual lung cancers at the molecular level.

We have previously shown that protein signals with diagnostic and prognostic value could be obtained directly from a cohort of surgically resected human non-small cell lung cancer (NSCLC) tissue sections using MALDI-MS [4]. In that study, a training cohort composed of 42 lung tumors and eight normal lung samples was correctly classified based on the selected proteomic signals. This class-prediction model was then applied to a blinded test cohort, including 37 lung tumors and six normal lung samples, and accurate classification of tumor *vs.* normal was achieved. Clinically occult nodal metastasis was identifiable with a 75% accuracy based on two selected protein signals. Furthermore, a proteomic indicator comprised of 15 MALDI-MS signals was obtained that was able to classify patients into good and poor prognosis groups.

Although the proteomic signals obtained by MALDI-MS can aid in lung-tumor classification and prediction of patient outcome, these protein signals themselves consist of various *m/z* values and anonymous protein identities. Identification of these protein signals is therefore necessary before attempting to translate findings into immunoassays that can be easily adapted to conventional clinical settings. Furthermore, the protein signals obtained from MALDI-MS were from fresh frozen tissue sections, which are not as widely available as formalin-fixed paraffin-embedded (FFPE) samples. In this study, the identification of several MALDI-MS-derived prognostic protein signals is described. Immunohistochemical staining was used to validate the prognosis values of these identified NSCLC protein markers using FFPE tissue microarrays.

2 Materials and methods

2.1 Chemicals

Sucrose and Tris were purchased from J. T. Baker (Phillipsburg, NJ). PMSF, sinapinic acid, ammonium hydrogen carbonate, puromycin, DTT and iodoacetamide were purchased from Sigma (St. Louis, MO). Formic acid (98–100%) was acquired from EM Science (Merck, Darmstadt, Germany). Sequencing-grade TFA was purchased from Burdick and Jackson (Muskegon, MI) and HPLC-grade ACN was purchased from Fisher Scientific (Fair Lawn, NJ). Bovine sequence-grade trypsin was purchased from Promega (Madison, WI). Peroxide, DAB reagent, and hematoxylin were obtained from Ventana Medical Systems (Tucson, AZ).

2.2 Patient characteristics and tissue microarray

The clinical characteristics of these patients are described in Table 1. Archived FFPE-tissue blocks and fresh frozen NSCLC tissues were selected from the Department of Pathology at Vanderbilt University and the Department of Veterans Affairs Medical Center in Nashville, Tennessee, between 1989 and 2002 as described earlier [5]. H&E-stained sections from each tissue block were reviewed by a board-certified pathologist with expertise in lung cancer (A.L.G.). Areas to be punched for array production were carefully marked, and cores 0.6 mm in diameter were taken from the selected area of each FFPE specimen and inserted into a recipient paraffin block. All tumors were punched in triplicate for a broader representation of each tissue. Sections of 5- μ m thickness were cut from the arrays and mounted on charged slides. The presence of the histological feature of interest was confirmed by examining every 15th section stained by H&E. Small-cell lung carcinomas were excluded from this

analysis. All clinical information is maintained in a regularly updated clinical database through the Specialized Program of Research Excellence in Lung Cancer at Vanderbilt University. Overall survival was determined by the time elapsed from the date of tissue resection to the time of death or last follow-up. The study was approved by the local Institutional Review Board.

2.3 Protein marker identification

The overall schematic representation of the protein marker identification process is illustrated in Supporting Information Fig. 1. Fresh frozen tissue sections (12 μm) were cut using a cryostat and mounted on an MALDI-MS target plate. After deposition of a sinapinic acid matrix solution, the tissue sections were analyzed using an Applied Biosystems Voyager MALDI mass spectrometer (Framingham, MA). The lung cancer tissues, which had the target protein signals, were homogenized, and a series of centrifugation steps were performed according to the procedures described previously [6]. The collected supernatant was then separated by a Vydac (Hesperia, CA) polymeric column (5- μm particle, 4.6 mm $\times$ 15 cm) at 40°C using a Waters Alliance HPLC system (Milford, MA). Solvent A contained 0.1% TFA in water and solvent B contained 0.085% TFA in ACN. The sample was eluted at a flow rate of 1 mL/min with a linear gradient started from initial condition 5% B for 5 min, followed by an increase from 5% B to 60% B over 70 min, and then a further increase to 95% B over the following 20 min, holding at 95% B for 10 min. Fractions were collected once per minute in Eppendorf Safe-lock microcentrifuge tubes (Brinkmann Instruments, Westbury, NY). After separation, all of the HPLC fractions were completely lyophilized using a Bentop 3.3/Vacu-Freeze Drier from VirTis Co (Gardiner, NY). Dried HPLC fractions were reconstituted in 10 μL of ACN/water/TFA (5/5/0.03, v/v/v). One microliter of sinapinic acid matrix solution (20 mg/mL, 5/5/0.03, v/v/v ACN/water/TFA) was mixed with 1 μL of each HPLC fraction and deposited on an MALDI target plate. Mass spectra were obtained using a Voyager DE-STR MALDI TOF mass spectrometer from Applied Biosystems (Framingham, MA). These fractions containing the target protein markers were digested using trypsin as previously described [6]. Next, the resulting tryptic peptides were further separated using nano-LC [7]. The separated peptides were directly analyzed using ESI-MS/MS using an IT LCQ XP Plus mass spectrometer (Thermo Finnigan, San Jose, CA). Protein identification was performed by searching the resulting MS/MS spectra against the human Refseq v.26 database using the SEQUEST algorithm [8] and the NCBI 36 (ENSEMBL) database using the Global Proteome Machine (GPM) [9], respectively. Search parameters were as follows: the mass tolerances were 3 Da for the parent monoisotopic ion and 0.4 Da for fragment mass ions. Full tryptic digestion was applied and no missed cleavage was allowed. Potential acetylation of N terminus, oxidation of methionine and carbamidomethylation of cysteine residues were added as variable modifications. Selected tryptic peptides were further analyzed using nano-ESI-MS/MS on an MDS Sciex QqTOF mass spectrometer (Concord, Canada) with higher mass accuracy and peak resolution. All of the MS/MS spectra corresponding to the targeted peptide sequences were manually verified.

2.4 Immunohistochemistry

Five-micron sections were cut from NSCLC-tissue micro-arrays and mounted on charged slides. The slides were deparaffinized and endogenous peroxidase was quenched on a Ventana Benchmark automated immunostainer (Ventana, Tucson, AZ). For the calmodulin antibody (Zymed, Carlsbad, CA), a 1:4000 dilution was applied to the slides without any further pretreatment for 32 min at 37°C. Similarly, the cofilin antibody (Cytoskeleton, Denver, CO) was applied to the slides using a 1:50 dilution. For thymosin β 4 and thymosin β 10 antibodies (BioDesign, Saco, ME), a citrate buffer antigen-retrieval step was applied to the slides using a Ventana Benchmark automated immunostainer. The dilutions for thymosin

$\beta 4$ (1:500) and thymosin $\beta 10$ (1:1000) were used. All additional immunohistochemical applications were performed by the Ventana Benchmark using a DAB reagent set. The slides were lightly counterstained with Mayer's hematoxylin. Finally, slides were dehydrated manually using alcohols of increasing concentration, placed in xylene and then coverslipped using Permunt. Each core was scored based on the immunohistochemical staining intensity 0 (absent) to 3 (strong) only for tumor cells. The slides were scored independently by two board-certified pathologists (A.L.G and S.E.M.). Discrepancies in scoring were reviewed together and a consensus score was determined. The highest staining intensity for triplicate cores of each patient was used to represent that patient's marker expression level.

2.5 Statistical analysis

The statistical analysis was focused on studying the association between the immunohistochemistry (IHC) staining intensity scores (0–3) generated from the identified protein markers and survival time. Kaplan-Meier survival curves were generated for all survival analyses. Cox regression was used to compute the hazard ratio and the 95% confidence interval. Cox models were also adjusted for age and gender. All p -values were based on two-sided comparisons, with $p < 0.05$ considered as significant. Our previous study identified a combined proteomic indicator including calmodulin, thymosin $\beta 4$, and thymosin $\beta 10$, and other signals that were statistically significantly associated with NSCLC patient outcome [4] based on the Weighted Flexible Compound Covariate Method (WFCCM) [10]. To be consistent, we followed the same procedure to generate a combined indicator including these three protein markers. Survival analysis was performed based on this indicator. The difference between the upper 50 and lower 50% of scores was assessed using a log-rank test. For cofilin-1 analysis, because approximately 60% of patients had an intensity of 3, we combined those of patients having intensity 0, 1 or 2 as one group to compare with the intensity 3 group for survival analysis using a log-rank test.

3 Results

3.1 Protein marker identification

Frozen human lung tumor tissues were first analyzed using MALDI-MS to confirm the presence of the targeted protein signals. Figure 1A illustrates two examples of the different protein profiles obtained from a human squamous cell lung carcinoma and a normal lung tissue. Singly charged protonated protein ions in the form of $[M+H]^+$ (in which M denotes the protein molecular weight) were predominantly formed. The labeled peaks correspond to the identified protein signals. The HPLC chromatogram (UV trace) of the lung tumor tissue homogenate fractionation is shown in Fig. 1B. The fractions containing the target protein signals were further analyzed using MS/MS. For example, the protein signal having an $[M+H]^+$ of 18 415 was detected in one of the HPLC fractions using MALDI-MS. Following the trypsin digestion of this fraction, the peptides [Ac-ASGVAVSDG-VIK], [EILVGQVGVQTVDDPYATFVK], and [YALYDATYETK] were found to correlate with cofilin-1. Acetylation (Ac-) at the N terminus of cofilin-1 was unambiguously confirmed. These three peptides constitute 26% of the cofilin-1 sequence. Figure 1C shows the MS/MS spectrum corresponding to the peptide [Ac-ASGVAVSDGVK], the amino acid sequence coverage of which is shown with fragmented peptide ions also observed in the spectra. The theoretical $[M+H]^+$ for cofilin-1 is 18 414.3, which is consistent with the observed signal at m/z 18 416 $\pm$ 1.5 measured directly from tissue. Thus, the protein marker was identified based on matching of amino-acid sequences and intact protein molecular weight.

Similarly, three other prognostic protein markers, thymosin $\beta 4$, thymosin $\beta 10$, and calmodulin were identified. All of these identified protein markers are listed in Table 2,

including protein name, Swiss-Prot accession number, marker type, peptide sequences found, protein sequence coverage, observed m/z values, theoretical m/z values and post-translational modifications. All of the MS/MS spectra corresponding to the identification of these proteins are shown in Supporting Information Fig. 2.

3.2 Immunohistochemistry on human tissue microarray

Lung cancers are epithelial tumors, which arise from the respiratory or alveolar epithelium. In this study, only non-small cell tumors were evaluated, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Neuroendocrine tumors, including small cell carcinoma were not evaluated. IHC analyses were performed on tissue microarrays for these four protein markers associated with prognosis. Examples of IHC with negative (score 0) and positive (score 3+) staining intensities for thymosin β 4, thymosin β 10, calmodulin and cofilin-1 are shown in Fig. 2. Immunohistochemical staining for these markers showed predominantly cytoplasmic expression, with thymosin β 4, calmodulin, and cofilin-1 each showing moderate to strong staining in a majority of tumors. Thymosin β 10 showed nuclear staining in some tumors in addition to cytoplasmic staining. In positive tumors, expression was usually diffuse throughout the tumor, with negative surrounding stroma.

3.3 Protein markers associated with NSCLC patient outcome

The Kaplan-Meier survival curves for the combined scores of thymosin β 4, thymosin β 10 and calmodulin are shown in Fig. 3A. There were 92 patients with 50 events in the upper group and 92 patients with 68 events in the lower group. Each event is defined as a patient's death. A significant difference in survival was observed between the groups ($p = 0.004$, HR = 0.58, 95% CI: 0.40, 0.84). The median patient survival was 22.8 (95% CI: 13.0, 32.5) months for the lower group and 44.7 (95% CI: 32.6, 56.7) months for the upper group.

The MS signal corresponding to cofilin-1 was previously selected as a nodal involvement predictor through MALDI-MS analysis [4]. In this study, 66 patients with 37 events had a cofilin-1 intensity of 0 to 2, and 109 patients with 77 events had cofilin-1 intensity of 3. The IHC scores (0 to 2 vs. 3) of cofilin-1 were found to be significantly associated with NSCLC patient survival ($p = 0.027$, HR = 1.51, 95% CI: 1.0, 2.2) (Fig. 3B). The median patient survival time for the cofilin-1 intensity 0 to 2 and intensity 3 groups are 44.5 months (95% CI: 27.3, -) and 28.6 months (95% CI: 19.5, 34.3), respectively.

Furthermore, we found that cofilin-1 IHC intensity and nodal status were associated with the survival, as shown by the Kaplan-Meier survival curves in Fig. 4. In the lymph node-negative patient group, patients with cofilin-1 intensity of 0 to 2 showed longer survival than those with intensity 3 ($p = 0.006$, HR = 1.80, 95% CI: 1.11, 2.9). Interestingly, in the lymph node positive patient group, the association was reversed and the patients with intensity 3 showed longer survival ($p = 0.034$, HR = 0.44, 95% CI: 0.2, 0.96). In the high cofilin-1 expressing group (score 3), nodal status had no effect on survival ($p = 0.211$), but in the low cofilin-1 group (score 0 to 2), nodal status had a significant effect on survival ($p < 0.001$, HR = 5.89, 95% CI: 2.62, 13.23).

To demonstrate the continuum data of MALDI-MS and IHC, the boxplot and dotplot comparison of thymosin β 10 with respect to patients with good and poor survival are shown in Fig. 5A. Both sets of data show the same trend, *i.e.* a higher expression of thymosin β 10 correlates with good survival. In addition, Fig. 5B shows that the MALDI-MS peak intensities for thymosin β 10 correlate with IHC staining intensities obtained from the same patients.

4 Discussion

Prognostic biomarkers for lung cancer may impact the management of this deadly disease. The rapid advancement of proteomic technologies has accelerated our capacity for cancer biomarker discovery. One such technique is direct tissue protein profiling using MALDI-MS, which has successfully discovered various proteomic patterns for brain cancer, lung cancer, breast cancer, kidney disease, and more [7, 11–14]. Translating these proteomic findings to clinically adaptable tests is the key factor for successful “bench-to-bedside” research. Here, we demonstrate that the NSCLC prognostic protein signals derived from MALDI-MS using fresh frozen tissues can be validated by IHC using FFPE samples. The sequence and the post-translational modifications of these protein markers were obtained. The prognostic values of the identified protein markers can be further tested using IHC in FFPE samples.

A combined proteomic indicator including thymosin β 4, thymosin β 10, and calmodulin was shown to be able to classify NSCLC patients according to good or poor prognosis. Thymosin β 4 is a potent regulator of actin polymerization [15] and has been previously reported to be highly expressed in lung cancer tissues compared with normal lung tissue using IHC [16]. Overexpression of thymosin β 4 in tumors has been suggested to stimulate lung tumor metastasis by activating cell migration and angiogenesis [17]. The gene expression of thymosin β 4 was also significantly associated with metastasis in NSCLC patients and it was suggested as a prognostic parameter for NSCLC [18]. Thymosin β 10 is also an actin sequestering protein that regulates actin dynamics, and its overexpression in human cancers has been reported previously [19]. Thymosin β 10 gene expression was suggested as a possibly useful tool in the diagnosis of thyroid neoplasms [20]. Calmodulin is known to be able to regulate the kinase activities of various proteins. The activation of death-associated protein kinase (DAPK), a cytoskeletal-associated cell death serine/threonine kinase by Ca^{2+} /calmodulin, is suggested to be linked to cytoskeletal alterations that occur during cell death [21]. Patients whose lung tumors exhibit hypermethylation of the DAPK promoter have a statistically significant lower probability of overall survival at 5 years after surgery than those without such hypermethylation [22].

Cofilin-1 was selected previously as a protein signal related to mediastinal nodal involvement for NSCLC [4]. In our study, cofilin-1 expression was found to be associated with NSCLC patient survival as well. Additionally, we found that the association of different cofilin-1 staining intensities with NSCLC patient outcome varied based upon their lymph node status. One possible explanation for this finding might be that node-positive patients have been more likely to receive chemotherapy, and high cofilin expression might be associated with benefit from chemotherapy. However, we had too few patients who received chemotherapy alone as their primary treatment to test the effect of cofilin-1 expression on chemotherapy benefit directly. Overexpression of cofilin in human NSCLC H1299 cells has been shown to enhance the cells' radiosensitivity by altering DNA repair capacity [23]. Cofilin-1 is one of the essential regulators of actin dynamics and plays important roles in cell motility, cytokinesis, transformation and other cellular processes [24]. As a key protein in chemotaxis of cancer cells, cofilin-1 is directly related to invasion, intravasation, and metastasis of mammary tumors [25]. Since one of the primary determinants of patient outcome after surgical resection is the development of distant metastasis, this correlation is consistent with its proposed role in hematogenous metastasis.

As a valuable discovery tool, MALDI-MS can find previously unknown protein markers that are associated with disease progression. MALDI-MS can detect proteins by precise molecular weight. Proteins with different iso-forms or PTM, which may have distinct biological functions, can be detected and separated in MS protein profiles. However,

MALDI-MS is not readily available as a diagnostic or prognostic tool in current clinical settings. Fresh frozen tissue sections are usually required for MALDI-MS-based protein profiling. The m/z values detected by MALDI-MS may represent fragments of the intact protein. Thus, increases in the fragment could potentially correspond to either increased or decreased intact proteins. Antibody-based assays are widely adapted in clinical settings as standard diagnostic and prognostic tools. Large supplies of FFPE-tissue samples are available and can be used to extensively study protein markers of interest using IHC. Our study demonstrates that prognostic protein signals selected using MALDI-MS can be identified and tested using IHC in FFPE tissue samples. In conclusion, MALDI-MS derived protein signals can potentially be translated to a conventional clinical setting to aid in the prognosis of patients with NSCLC at the molecular level. The steady advancement of MS-based technologies in conjunction with conventional methods such as IHC holds great promise for cancer protein marker discovery and clinical translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

FFPE	formalin-fixed paraffin-embedded
IHC	immunohistochemistry
NSCLC	non-small cell lung cancer

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Figure 1.

The identification of the MALDI-MS derived protein signal, cofilin-1, using HPLC and MS/MS. (A) Examples of different protein profiles obtained from fresh frozen lung tissues using MALDI-MS. (B) The HPLC chromatogram (UV trace) of the lung tumor tissue homogenate fractionation. (C) The MS/MS spectrum corresponding to the cofilin-1 peptide [Ac-ASGVAVSDGVK].

Figure 2.

Representative immunohistochemical staining for the protein markers in the human lung tumor FFPE tissue micro-array. (A, B) Thymosin β 4 intensity 0 and 3, respectively; (C, D) thymosin β 10 intensity 0 and 2, respectively; (E, F) calmodulin intensity 0 and 3, respectively; (G, H) cofilin-1 intensity 0 and 3, respectively. The magnification is 400x.

Figure 3.

(A) The Kaplan-Meier survival curves for patients with good and poor prognosis based on the upper and lower 50% combined scores for three protein markers (thymosin β 4, thymosin β 10 and calmodulin). (B) The Kaplan-Meier survival curves for patients with good and poor prognosis based on different cofilin-1 immunohistochemical staining intensities.

Figure 4.

The Kaplan-Meier survival curves for patients with good and poor prognosis based on different cofilin-1 immunohistochemical staining intensities and patients' lymph node status (E/N = number of events/number of patients).

Figure 5. The correlation of MALDI-MS peak intensity and IHC staining intensity. (A) Left panel: Boxplot of thymosin β10 MALDI-MS peak intensity (log10 transformed) related to patient survival; Right panel: Dotplot of thymosin β10 IHC staining intensity related to patient survival. (B) Examples of MALDI-MS peak intensities for thymosin β10, which correlate with IHC staining intensities obtained from the same patients. The magnification for IHC images is 400x.

Table 1

The clinicopathological data of the patients in the IHC analysis

Characteristic	Number of patients (%)
Sex	
Female	70 (37.2)
Male	118 (62.8)
Age (years)	
Range	34–99
Mean $\pm$ S.D.	65.3 $\pm$ 11.4
Median	66.5
History of smoking	
Current smoker	84 (44.7)
Ex-smoker	91 (48.4)
Never	11 (5.9)
Tumor stage	
IA	74 (39.4)
IB	56 (29.8)
IIA	6 (3.2)
IIB	21 (11.2)
IIIA	31 (16.5)
Histology	
Adenocarcinoma	84 (44.7)
Adenosquamous carcinoma	3 (1.6)
Large cell carcinoma	23 (12.2)
Squamous cell carcinoma	78 (41.5)
Nodal metastasis	
Negative	149 (79.3)
Positive	39 (20.7)
Pre-treatment	
Neoadjuvant chemotherapy	24 (13)

Summary of the identified protein markers

Table 2

Protein name	Swiss-Prot accession number	Marker types	Peptide sequences found	Protein sequence coverage (%)	Observed m/z (± 1.5)	Theoretical m/z	Post-translational modification
Thymosin $\beta 4$	P62328	Primary LT vs. NL and prognosis patient survival	[Ac-SDKPDMAEIEK]	26	4695	4964.3	N-terminal acetylation
Calmodulin	P62158	Prognosis patient survival	[Ac-ADQLTEEQIAEFK] [EAFSLFDKDGDTITTK] [EADIDGQGQVNYEEFVQMMTAK]	35	16 793	16 791.5	N-terminal acetylation; K ₁₁₅ trimethylation
Thymosin $\beta 10$	P63313	Prognosis patient survival	[Ac-ADKPDMGEIASFDK] [NTLPTK]	47	4939	4937.5	N-terminal acetylation
Cofilin	P23528	Nodal metastasis	[Ac-ASGVAVSDGVIK] [EILVGDVGQTVDDPYATFVK] [YALYDATYETK]	26	18 416	18 414.3	N-terminal acetylation