Proteomic-Based Prognosis of Brain Tumor Patients Using Direct-Tissue Matrix-Assisted Laser Desorption Ionization Mass Spectrometry

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Abstract

Clinical diagnosis and treatment decisions for a subset of primary human brain tumors, gliomas, are based almost exclusively on tissue histology. Approaches for glioma diagnosis can be highly subjective due to the heterogeneity and infiltrative nature of these tumors and depend on the skill of the neuropathologist. There is therefore a critical need to develop more precise, nonsubjective, and systematic methods to classify human gliomas. To this end, mass spectrometric analysis has been applied to these tumors to determine glioma-specific protein patterns. Protein profiles have been obtained from human gliomas of various grades through direct analysis of tissue samples using matrix-assisted laser desorption ionization mass spectrometry (MS). Statistical algorithms applied to the MS profiles from tissue sections identified protein patterns that correlated with tumor histology and patient survival. Using a data set of 105 glioma patients, two patient populations, a short-term and a long-term survival group, were identified based on the tissue protein profiles. In addition, a subset of 57 patients diagnosed with high-grade, grade IV, malignant gliomas were analyzed and a novel classification scheme that segregated short-term and long-term survival patients based on the proteomic profiles was developed. The protein patterns described served as an independent indicator of patient survival. These results show that this new molecular approach to monitoring gliomas can provide clinically relevant information on tumor malignancy and is suitable for high-throughput clinical screening. (Cancer Res 2005; 65(17): 7674-81)

Introduction

Primary central nervous system tumors present significant challenges for diagnosis and treatment. Gliomas are the most common primary brain tumor with ~25,000 new cases per year. Half of these tumors exhibit aggressive, infiltrative behavior, respond poorly to treatment, and are characterized as one of the more fatal human malignancies. Clinical diagnosis and treatment decisions for these tumors are based almost exclusively on tissue histology. Accurate glioma classification is therefore critical. Histologic diagnosis predominantly uses the WHO classification scheme (1), which classifies gliomas based on the principal cell type and assigns them a grade of I to IV by increasing degrees of malignancy. Current methods for diagnosing gliomas, however, are highly subjective and depend on the skill of the neuropathologist. Considerable diagnostic variability also occurs because of the heterogeneous and highly infiltrative nature of these tumors. Moreover, no reliable molecular markers are known to exist that accurately categorize tumors in a clinically relevant fashion. There is therefore a critical need to develop more precise, nonsubjective, and systematic methods to classify human gliomas based on molecular markers. Recent advances have used cDNA microarrays to identify tumor grade-specific gene patterns (2–8) and potential markers of survival (9–11). In addition, two-dimensional gel electrophoresis technology has been used to analyze known tumor biomarkers for grade-specific trends (12) and monitor protein changes relative to tumor grade and patient survival (13). Most of these approaches rely on histopathologic analysis for accuracy verification and are low throughput, and some require a foreknowledge of the markers of interest. Few efforts have focused on developing a grade-independent, high-throughput, patient prognostic tool applicable in a clinical setting.

We have developed a direct-tissue protein profiling approach to tumor analysis using matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) to correlate protein patterns obtained directly from tumor biopsies with patient survival trends. This technology is characterized by high mass measurement accuracy (100-200 ppm; ref. 14), high sensitivity (attomoles to femtomoles), and a potential for high throughput (prepared samples can be analyzed in ~5 minutes). The mass spectra acquired from tissue biopsies reflect a portion of the protein content within the tissue (i.e., ~300-500 proteins). This technology has been used previously for imaging protein localization within a tumor biopsy (15), monitoring protein changes in mouse prostate (16) and rat pituitary and pancreas (17), and identifying tumor and prognostic specific biomarkers for patients with lung carcinomas (18).

We report the identification of MALDI MS prognostic-specific protein patterns based on the analysis of 162 tissue biopsies from 127 patients. Protein patterns were identified that accurately classify glioma subtypes and distinguish patients into two prognostic groups, a short-term survival (STS) group and a long-term survival (LTS) group. In addition, we studied a well-characterized subset of patients with grade IV gliomas and identified protein patterns that predict differential survival.

Materials and Methods

Materials. The MALDI matrix compound 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), H&E, phenylmethylsulfonyl fluoride (PMSF),...
NaCl, and ammonium bicarbonate were purchased from Sigma Chemical Co. (St. Louis, MO). DMEM was from Life Technologies, Inc. (Rockville, MD) and fetal bovine serum (FBS) was from Gemini Bio-Products (Woodland, CA). T-PER extraction buffer was purchased from Pierce Biotechnology, Inc. (Rockford, IL). Sucrose, ammonium acetate, and ultrapure Tris were obtained from J.T. Baker (Phillipsburg, NJ). Sequencing-grade trypsinic acid (TEA) was from Burdick and Jackson (Muskegon, MI). High-performance liquid chromatography (HPLC)–grade acetoneitrile was purchased from EM Science (Merck, Darmstadt, Germany). Sequencing-grade trypsin was from Promega (Madison, WI) and anti-PEA-15 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Collecting and processing clinical material and patient information. Tissues were obtained, with informed consent and institutional review board approval, from patients undergoing tumor resection or other surgical procedures at Vanderbilt University Medical Center, Cleveland Clinic Foundation, and the NIH. A total of 162 tissue samples from 127 patients, including 19 patients undergoing resective surgery for nonneoplastic disease and 29 grade II, 22 grade III, and 57 grade IV glioma patients, were analyzed. Patient information was collected, including gender, age, treatment received before and after surgery, extent of surgery, current status (alive, alive with progressive disease, deceased, and cause of death), and survival from the time of original pathologic diagnosis. Samples were collected at the time of surgery, immediately snap frozen in liquid nitrogen, and stored at −80°C until analysis. Histopathologic diagnoses were made by a neuropathologist, blinded to the original clinical diagnosis, from subsequent H&E-stained sections according to the 2000 WHO classification and stored at

Both samples were separated in two dimensions, first by ion exchange chromatography followed by reverse-phase HPLC. The cell line supernatant was separated by anion exchange chromatography using a Hitrap Q HP anion exchange column (Amersham Biosciences, Uppsala, Sweden) and a NaCl gradient (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.45, 0.5, and 1 mol/L NaCl) based on the extraction solution. HPLC separation for selected fractions was achieved over a Vydac (Hesperia, CA) 194MM52 reverse-phase C4 column (5 mm particles, 2.1 mm × 25 cm) at 4°C using a linear gradient of 5% B to 25% B over 5 minutes, 25% B to 60% B over 5 minutes, and 60% B to 95% B over 20 minutes. Fractions were analyzed by MALDI MS for the markers of interest after each separation. HPLC fractions of interest were reconstituted in 0.1 mol/L ammonium bicarbonate and digested with trypsin (1:50, trypsin/protein, w/w; 37°C, pH 8.0, 1 hour). Digests were analyzed using either an Applied Biosystems 4700 MALDI TOF/TOF mass spectrometer or a ThermoLTQ ion trap mass spectrometer equipped with a Thermo Surveyor LC pump and a microelectrospray source (Thermo Electron, San Jose, CA).

MS and MS-MS spectra from MALDI TOF/TOF analysis were collected and the proteins were identified as described previously (23). The data were searched against the human National Center for Biotechnology Information database using the Mascot® database search algorithm. A significance cutoff score of 65 was used. Analysis on the ThermofLTQ mass spectrometer was done using one full MS scan followed by three MS-MS scans of the three most intense ions. MS-MS spectra were searched against the human database using Sequest (Thermo Electron) and the Sequest search outputs were filtered using a custom-designed software tool called Complete Hierarchical Integration of Protein Searches using the following filtering criteria: cross-correlation (Xcorr) value of >1.0 for singly charged ions, >1.8 for doubly charged ions, and >2.5 for triply charged ions. In addition, a RSp (ranking of preliminary score) value of <5 and a Sp value (preliminary score >350) were required for positive peptide identifications. A minimum of two peptide masses at a positive correlation and the m/z ratio detected and the molecular weight of the intact protein (including post-translational modifications) were also required for protein identification.

Immunohistochemistry. For immunofluorescence histochemistry, 18 µm thick sections were cut on a cryostat and incubated for 24 hours with PEA-15 antibodies (1:1,000). The sections were washed and incubated with Cy3-conjugated anti-mouse secondary antibodies (1:1,600; Jackson ImmunoResearch, West Grove, PA), washed, mounted, and coverslipped.

Results

Mass spectrometric profiling of human brain tissues. A total of 162 tissue samples from 127 patients (108 glioma and 19 nontumor patients) were collected and analyzed by MS. The general protocol is presented (Fig. 1). Tissue sections were coated with matrix droplets (typically 5-10 droplets were deposited on each section) and each droplet was directly analyzed by MALDI MS; serial sections were collected and stained with H&E for histopathology. Over 1,000 mass spectra, representing nontumor cell populations from nontumor patients or tumor cell populations from glioma patients, were used for comparative analysis. Between 300

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and 500 individual protein signals in the m/z range of 2,000 to 70,000 were detected. The spectra were processed and multiple spectra were averaged to generate one peak list per patient or tissue sample depending on the statistical analysis done. To determine the variability of our tissue profiling approach, the average intraclass agreement rate (protein pattern variation within a patient sample) was measured to be 91.3 ± 2.6% (95% confidence interval, 87.2-95.4%). This measurement suggests a relative spectrum-to-spectrum consistency within a given tissue sample.

Correlating protein pattern changes to glioma classification. Initial data analysis focused on verifying that direct-tissue MALDI MS analysis could be used for tissue classification. Supervised classification analysis was done to identify tumor classification-distinctive biomarker patterns verified by histology. Data were processed, averaged by biopsy, and grouped into one of four categories: nontumor tissue (26 samples), grade II tumor (35 samples), grade III tumor (28 samples), and grade IV tumor (73 samples). Nontumor tissue refers to samples collected from patients undergoing surgical resection for nonneoplastic processes. For statistical analysis, biopsies were separated into training and testing data sets, consisting of two-thirds and one-third of the samples per classification, respectively.

Pairwise comparisons were done on the training set to identify a subset of differentially expressed protein signals that best separated each classification: nontumor versus grades II, III, and IV biopsies; nontumor versus each individual tumor grade; grade II versus III; grade II versus IV; grade III versus IV; and grade II and III versus IV. Two independent methods were used for data analysis: SDA (21) and WFCCM (22). SDA uses genetic programming to build functions based on determined discriminatory signals, which distinguish sample classifications. WFCCM generates a model based on a linear combination of statistically determined discriminatory markers, which distinguishes sample groups.

For each analysis approach, a model was defined that best classified samples in the training data set. Based on the model, each patient was assigned a score using the expression, or signal intensity, of the determined biomarker signals; the accuracy of this classification scheme was verified on a blind data set (testing data set). The results from these analyses are summarized (Table 1). Classification and prediction accuracies are defined as the number of samples in the training and testing data sets, respectively, correctly classified. Biopsy protein patterns reflect a strong separation between tumor and nontumor tissues that extends to individual tumor grades. In all cases, nontumor tissues could be distinguished from gliomas with >92% classification accuracy. When comparing gliomas of different grades, the best separation was seen when comparing grade II and IV tumors (>93% classification accuracy), with slightly lower values for the grade III versus IV and grade II and III versus IV. The most difficult comparison was between grades II and III, which recapitulates the clinical situation. Accuracy limitations in protein profiling are due in part to the infiltrative nature of these tumors, the heterogeneous nature of the cells that comprise gliomas, and some methodologic limitations. Nonetheless, the results compare favorably with studies of interclass observer variability in pathology and neuropathology (24, 25).

Interestingly, both analyses exhibited similar abilities in segregating the individual classifications. Models from SDA and WFCCM did well in distinguishing nontumor from tumor and generally separating individual tumor grades but did poorly in segregating grade II from grade III. Although the marker patterns determined by SDA and WFCCM were distinct, 35% of the classification-specific markers determined using SDA were also selected by WFCCM. These results suggest that classification based on protein profiling may be independent of the statistical analysis technique used.

An independent agglomerative hierarchical clustering algorithm verified the statistically significant discriminator protein patterns, determined by WFCCM, in the training cohort for each of the classifications done. The results of three of these, nontumor versus tumor, nontumor versus grade IV tumor, and grade II versus grade IV tumor are shown (Fig. 2 A-C, respectively). Clustering patterns reflect the strong correlation between the MS protein profile and the tissue classifications.

Correlating protein pattern changes to glioma patient survival. Statistical analysis was then applied to the entire tumor data set of 108 glioma patients, with the spectra averaged by patient, to identify biomarker patterns that correlate to patient survival trends. Using WFCCM, a summary survival score was determined for each patient based on the statistically determined significant protein signals. Patients were then separated into short-term and
long-term prognostic groups using a sensitivity analysis according to their correlated protein and survival patterns (Fig. 3A). A proteomic pattern of 24 distinct MS signals distinguished patients based on survival trends from the time of pathologic diagnosis into two groups, STS (mean survival, <15 months) and LTS (mean survival, >90 months) groups. Seventeen of these markers were not determined as tumor- or tumor grade–specific discriminatory markers in the previous analyses. Several of these survival signal differences are displayed (Fig. 3C-E), including an overexpression of m/z 9,747 and 10,092 in the STS group and an overexpression of m/z 10,262 in the LTS group. Within the total tumor patient population, analysis identified 52 patients in the STS prognostic group and 56 patients in the LTS prognostic group, with P < 0.0001. Univariate analysis showed a positive correlation (P < 0.01) between advanced patient age, increasing tumor grade, and tumors with an astrocytic lineage and shorter survival trends. Taking these factors in account, a multivariate Cox proportional hazards model showed a strong correlation between MS protein pattern and patient survival after adjustment for patient age, gender, tumor subtype, tumor grade, extent of tumor resection, and use of radiation and chemotherapy treatments before and after surgery. Therefore, the protein pattern served as an independent indicator of patient survival. The key patient survival variables are shown in a modified model (Table 2).

Survival analysis of the glioma population from the time of surgery, in which the analyzed sample was resected, was also done with similar results (data not shown).

Glioblastoma multiforme, the most common and malignant form of glioma, is also one of the most rapidly fatal of all human malignancies; median survival after diagnosis for these tumors is measured in months. For patients with a glioblastoma multiforme, age, clinical performance status, and extent of surgical resection are the principal, well-validated prognostic variables. WFCCM analysis was used to determine whether protein patterns could further differentiate patients based on survival from the time of glioblastoma multiforme presentation. A proteomic pattern of two distinct MS signals was identified that segregated the patients into a STS group (average survival, 10.9 months) and a LTS group (average survival, 16.8 months; Fig. 3B). Neither of these signals was identified as a significant discriminatory marker in the previous analyses. A total of 28 of the 57 patients were classified into the STS group and 29 patients were classified into the LTS group (P < 0.0001). Although an independent correlation existed between increasing patient age and shorter survival trends, the protein pattern was a powerful, independent predictor of patient survival. A multivariate Cox proportional hazards model showed a strong correlation between MS protein pattern and patient survival after adjustment for patient age, gender, extent of tumor resection, histologic subtype, and use of radiation and chemotherapy treatments. A modified form of this model is presented (Table 2).

### Table 1. Results of pairwise biopsy comparisons based on histopathology

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Data set (no. biopsies training; testing set)</th>
<th>SDA</th>
<th>WFCCM</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>No. biomarkers determined</td>
<td>Classification accuracy (%)</td>
</tr>
<tr>
<td>NT/T</td>
<td>18/91/24/8/45</td>
<td>2</td>
<td>92</td>
</tr>
<tr>
<td>NT/TII</td>
<td>18/24/11</td>
<td>2</td>
<td>92</td>
</tr>
<tr>
<td>NT/TIII</td>
<td>18/17/8/11</td>
<td>4</td>
<td>94</td>
</tr>
<tr>
<td>NT/TIV</td>
<td>18/50/23</td>
<td>2</td>
<td>96</td>
</tr>
<tr>
<td>TII/TIII</td>
<td>24/17/11/24</td>
<td>2</td>
<td>76</td>
</tr>
<tr>
<td>TII/TIV</td>
<td>24/50/21/23</td>
<td>3</td>
<td>93</td>
</tr>
<tr>
<td>TIII/TIV</td>
<td>17/50/11/21</td>
<td>4</td>
<td>85</td>
</tr>
<tr>
<td>TIV/TIV</td>
<td>41/50/22/73</td>
<td>1</td>
<td>79</td>
</tr>
</tbody>
</table>

NOTE: Biopsies were separated with two-thirds of the samples in the training data set and one-third in the testing data set and the displayed pairwise classifications were done. NT, nontumor tissue; TII, grade II glioma; TIII, grade III glioma; TIV, grade IV glioma. A subset of biomarkers that best distinguished the two groups was determined and the classification accuracy for both the training data set (classification accuracy) and the testing data set (prediction accuracy) was measured.
increase in protein expression correlates well with the presence of a MS signal at m/z 15,035 collected from a consecutive grade II tissue section as opposed to the loss of this signal in the grade IV section (Fig. 4D).

Discussion

Direct-tissue profiling by MALDI MS has been used to analyze the protein patterns within human gliomas and correlate these patterns to tumor classification and patient survival. These data can be used to distinguish tumor tissue from nontumor brain tissue, define protein profiles specific to tumor grade, and identify differential patient survival patterns based on protein expression patterns.

Because the accepted standard for glioma classification is histopathologic grading, we initially sought to validate the MS approach by identifying grade-specific biomarkers that correlated to histopathologically determined classifications. Tumors were subclassified by two neuropathologists, blinded to the original diagnosis, and analyzed by MALDI MS without knowledge of the original classification. Only samples with coincident clinical diagnoses were included in the analysis. Based on two independent classification approaches, WFCCM and SDA, MALDI MS provided specific proteomic patterns that classified glial tumors and nontumor brain tissue with high accuracy and precision. Proteomic profiles were used to discriminate between normal brain tissue and gliomas >92% of the time, with individual classification accuracies between normal tissues and individual grades ranging from 92% to 100%. In addition, MALDI MS patterns were used to distinguish gliomas with high accuracy ranging from 76% to 97%. The most difficult distinction was between WHO grade II and III tumors, which mimics the clinical situation. Statistical analysis identified >100 potential, tumor-specific biomarkers. Validation of MS-based tumor classification using two different statistical techniques highlights the power of protein profiling for tissue characterization independent of the analysis approach.

WFCCM was also applied to identify MS-derived protein patterns that correlate to patient survival trends for all glioma patients and for a subgroup of patients with histologically confirmed glioblastoma multiforme. For all patients, standard treatment regimens were followed, including surgical tumor resection plus adjuvant radiotherapy and chemotherapy, as clinically indicated and tolerated. We show that a relatively small number of proteins can be used to distinguish between STS and LTS patients within the glioma patient population as a whole (P < 0.0001). Although these results are in line with previous clinical and pathologic studies, showing that the WHO grading system possesses discriminating predictive power, the protein pattern was an independent indicator of patient survival.

In addition, MALDI MS protein profiling was used to analyze a large group of patients with the most malignant form of glioma, glioblastoma multiforme, and found that the MS pattern from two
m/z signals could further stratify patients into a STS group and a LTS group \((P < 0.0001)\). For both analyses, the MALDI MS profile was the strongest determinant of survival in both univariate and multivariate analyses, stronger than most previously identified predictive variables, such as age, extent of resection, tumor grade, and use of adjuvant therapy. As expected, for the full glioma population, some overlap exists between grade-specific biomarkers and survival markers. Of the 24 discriminatory patient survival biomarkers for the entire glioma population, 17 were unique to the survival stratification. On the other hand, analysis of the glioblastoma multiforme population determined two unique markers that segregated the STS and LTS patients. These results suggest a novel approach to tissue classification based not on histopathologic features requiring visual analysis but on a molecular analysis of the protein patterns specific to the tissue sample.

Based on statistical analysis, several discriminatory proteins were identified, including calcyclin, dynein light chain 2, calpactin I light chain, astrocytic phosphoprotein PEA-15, fatty acid-binding protein 5 and tubulin-specific chaperone A. The MS signals from these proteins serve to discriminate gliomas from normal brain tissue and tumors of differing grade from one another; calcyclin and dynein light chain 2 also discriminated between glioma survival subgroups. These proteins are thought to be involved in several aspects of tumorigenesis. Calcyclin (S100A6), which plays a potential role in cell cycle progression and cell differentiation (26), is overexpressed in many tumors, especially at the margins of invasive cancers (27–30). Dynein light chain 2, a subunit of the microtubule-associated dynein motor complex, binds and sequesters Bim, a proapoptotic protein, to negatively regulate its apoptotic function (31). Calpactin I light chain (p11, S100A10) is expressed in many cancer cell lines (32, 33) and is thought to bind and stimulate plasminogen conversion to plasmin, a cell surface protease involved in tumor cell invasion and metastasis (34). PEA-15, an apoptosis inhibitor involved in several cell growth pathways (35, 36), is overexpressed in several tumor cell lines, including breast, larynx, cervix, and skin (36, 37), whereas studies have suggested overexpression of the fatty acid–binding protein 5 gene in prostate cancer tissue and cell lines (38, 39). Tubulin-specific chaperone A is a cofactor required for proper \(\beta\)-tubulin folding (40).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio (95% confidence interval)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade II, III, and IV gliomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS protein pattern</td>
<td>1.002 (1.001-1.003)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age</td>
<td>1.034 (1.006-1.062)</td>
<td>0.0177</td>
</tr>
<tr>
<td>Gender</td>
<td>0.926 (0.445-1.928)</td>
<td>0.8366</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.296 (0.107-0.814)</td>
<td>0.0184</td>
</tr>
<tr>
<td>Radiation</td>
<td>1.402 (0.561-3.500)</td>
<td>0.4697</td>
</tr>
<tr>
<td>Tumor grade</td>
<td>4.581 (1.440-14.575)</td>
<td>0.0100</td>
</tr>
<tr>
<td>Grade IV gliomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS protein pattern</td>
<td>1.014 (1.007-1.021)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Age</td>
<td>1.063 (1.026-1.021)</td>
<td>0.0006</td>
</tr>
<tr>
<td>Gender</td>
<td>1.551 (0.620-3.879)</td>
<td>0.3485</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.898 (0.327-2.467)</td>
<td>0.8349</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.799 (0.281-2.628)</td>
<td>0.6728</td>
</tr>
</tbody>
</table>

**NOTE:** Analysis shows a strong correlation between MS protein pattern and patient survival after adjustment for several patient variables, including patient age, gender, tumor grade, and use of radiation and chemotherapy treatments before sample resection.
Identification of these proteins was done in both a human glioblastoma cell line and a human glioblastoma tissue sample. These studies showed that, although cell lines are not ideal sources for protein identification, due to potential post-translational modifications and genomic mutations specific to the cell line, a positive correlation between the proteins identified from a cell line versus a tissue sample can exist. The identification of proteins from cell lines followed by further characterization of these proteins using traditional immunohistochemistry methods in intact tissues should serve as a valuable tool for protein identification and biomarker validation when resources are limited.

Our analysis has several potential limitations. A rank cutoff was used in WFCCM to determine the number of protein signals used in each classification. Therefore, the number of peaks reported is based not on the smallest or largest number of signals that could discriminate the classes but rather on an intermediate number based on statistical evidence. It may be possible to achieve a similar classification rate using a different subset of peaks. Although a variety of variables could lead to the misclassified samples, potential limitations include the diffuse cellular nature of the tumors as well as histopathologic inaccuracy. Furthermore, the tumors were not analyzed for genetic alterations suspected of playing a role in gliomagenesis, which may have prognostic significance, nor did we control for histologic homogeneity or require a specific treatment regimen. Although it may have been useful to focus this study on a homogeneous study population, the mixed nature of the tumors more faithfully corresponds to the clinical situation. Thus, this study represents an initial but important attempt to describe an unbiased molecular diagnostic tool that also possesses the power to predict overall outcome.

In summary, MALDI MS protein profiling has been used to determine protein expression patterns that distinguish primary gliomas from normal brain tissue and one grade of gliomas from another, with high sensitivity and specificity. In addition, we have shown that a small subset of protein signals can be used to predict survival of glioma patients as well as to identify differential survival patterns within a more homogenous population of glioblastoma multiforme patients. Additional studies are under way to enhance and expand the MALDI MS analysis of a larger spectrum of human gliomas as well as to identify differential survival patterns within a more homogenous population of glioblastoma multiforme patients. These studies showed that, although cell lines are not ideal sources for protein identification, due to potential post-translational modifications and genomic mutations specific to the cell line, a positive correlation between the proteins identified from a cell line versus a tissue sample can exist. The identification of proteins from cell lines followed by further characterization of these proteins using traditional immunohistochemistry methods in intact tissues should serve as a valuable tool for protein identification and biomarker validation when resources are limited.

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Fig. 4. Summary of proteins identified and PEA-15 immunohistochemical staining. A, MS and MS/MS analysis of separated and digested fractions of interest identified six discriminatory proteins summarized in the table. The label m/z designates the m/z ratio monitored during direct-tissue profiling, classification group identifies which tissue group overexpressed the marker (whether a grade II, III, or IV glioma or the STS or LTS group determined from survival analysis of the entire glioma patient population), source material specifies whether the protein was identified from the glioma cell line (cell) or tumor biopsy (tissue), and the Swiss-Prot accession no. is as stated. B-D, for identification validation, two human glioma samples, a grade II astrocytoma and a grade IV glioblastoma multiforme, were sectioned and analyzed to correlate MALDI MS protein profiling with PEA-15 immunohistochemical staining. Fluorescent immunohistochemical images collected from a grade II astrocytoma (B) and grade IV glioblastoma (C) after staining for PEA-15 are presented. D, MS profiles collected by direct-tissue MALDI MS analysis of serial glioma tissue sections are also displayed; the protein profiles from the grade II glioma (gray trace) and the grade IV glioma (black trace) are presented. The intensity of m/z signal 15,035, identified in glioma cell lines as PEA-15, is increased in the MS profiles obtained from grade II compared with grade IV gliomas. Images from serial tissue sections, stained with anti-PEA-15, corroborate this by demonstrating a stronger PEA-15 staining pattern in the grade II tumor specimen compared with the grade IV sample.

In summary, MALDI MS protein profiling has been used to determine protein expression patterns that distinguish primary gliomas from normal brain tissue and one grade of gliomas from another, with high sensitivity and specificity. In addition, we have shown that a small subset of protein signals can be used to predict survival of glioma patients as well as to identify differential survival patterns within a more homogenous population of glioblastoma multiforme patients. Additional studies are under way to enhance and expand the MALDI MS analysis of a larger spectrum of human gliomas as well as to identify additional known and suspected peptide/protein biomarkers. Once identified, characterization of the function and biological role of the specific proteins involved in the origin as well as the progression of gliomas may permit their use as diagnostic and prognostic markers. Because MALDI MS technology is capable of analyzing numerous samples, with analysis times of ~5 minutes per sample, this technology is amenable to high-throughput tissue screening in a clinical setting.

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