Comparative Proteomic Profiling Using Two-Dimensional Gel Electrophoresis and Identification via LC-MS/MS Reveals Novel Protein Biomarkers to Identify Aggressive Subtypes of WHO Grade I Meningioma

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Abstract

Background Meningiomas represent the most common primary intracranial tumor. The majority are benign World Health Organization (WHO) Grade I lesions, but a subset of these behave in an aggressive manner. Protein biomarkers are needed to distinguish aggressive from benign Grade I lesions.

Materials and Methods Pooled protein lysates were derived from five clinically aggressive Grade I and five typically benign WHO Grade I tumors snap frozen at the time of surgery. Proteins were separated in each group using two-dimensional gel electrophoresis (2DGE) and protein spots of interest were identified using liquid chromatography–mass spectrometry (LC-MS). Potential biomarker candidates were validated using western blot assays in individual tumor samples and by tissue microarray (TMA).

Results Seven candidate biomarkers were obtained from the 2DGE and validated via western blot and TMA. Biomarker validation data allowed for the creation of predictive models using binary logistical regression that correctly identified 85.9% of aggressive tumors within the larger cohort of Grade I meningioma.

Conclusion Simple protein separation by 2DGE and identification of candidate biomarkers by LC-MS allowed for the identification of seven candidate biomarkers that when used in predictive models accurately distinguish aggressive from benign behavior in WHO Grade I meningioma.

Keywords

- meningioma
- proteomic
- two-dimensional gel electrophoresis
- mass spectrometry
- biomarker discovery

Introduction

Meningiomas are now the most common primary intracranial tumor¹ with results from autopsy studies suggesting

~8.2% of the general population may develop a meningioma at some point in their life.^{2,3} Currently, meningiomas are classified into three groups determined by the World Health Organization (WHO) based on histological criteria.⁴ Grade I

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tumors represent ~70% of meningiomas and are considered benign, whereas Grade II tumors represent ~30% and are associated with a poorer clinical outcome.⁴ WHO Grade III tumors are frankly malignant and represent 1% or less of meningiomas. Histological grading criteria were designed to predict tumor recurrence with 5-year recurrence rates of 7 to 25%, 29 to 52%, and 50 to 94% for WHO Grades I, II, and III lesions.⁴

Despite revisions of the WHO grading criteria over the past two decades, many studies have identified subgroups of benign tumors that do not behave in accordance with grade.^{5–12} These tumors rapidly recur after complete resection, or residual tumor beds demonstrate aggressive growth after subtotal resection. Clinically, these “aggressive” Grade I lesions behave in a similar manner to a higher grade lesion, yet remain classified as a Grade I lesion by the WHO grading histological criteria. The most recent WHO grading scheme, from 2007, addressed such criticism by identifying benign Grade I meningiomas with brain invasion as criteria for Grade II. A few studies have focused on WHO Grade I meningiomas that demonstrate a much more aggressive clinical course^{9,13} and attempted to define biomarkers that predict this clinical behavior. Most of these studies have focused on radiographic characteristics of aggression such as bone invasion which has not been clearly demonstrated to alter recurrence, and even fewer studies focus on recurrent Grade I tumors.^{10,11,14–16} Due to the prevalence of aggressive Grade I meningiomas, and an inability to identify these neoplasms, a need exists to identify additional biomarkers to improve diagnostic protocols.

In the present study, we address this challenge of biomarker discovery by defining several unique protein expression patterns associated with aggressive Grade I meningiomas via two-dimensional gel separation in combination with mass spectroscopy. We then validated the efficacy of these proteins as biomarkers in a tissue microarray comprised meningiomas across all grades. By defining the differences in the proteome of an aggressive subtype of meningioma, we hypothesize that the biomarkers we identified could have use in predicting clinically aggressive and recurrent meningiomas independent of grade.

Materials and Methods

Ethics Statement

The design of this study was reviewed and approved by the University of Washington Institutional Review Board. All patients provided informed written consent to have tissue samples stored and used for laboratory research purposes prior to undergoing surgical tumor resection.

Experimental Groups Definitions and Tissue Acquisition

Preset clinical criteria were defined for aggressive WHO Grade I meningiomas as follows: (1) Any patient undergoing gross total resection for histologically confirmed WHO Grade I meningioma that required repeat resection for recurrence within 2 years, with pathology at the second surgery con-

firmed a WHO Grade I diagnosis. (2) Any patient undergoing gross total resection of a WHO Grade I meningioma and then requiring stereotactic radiosurgery for a recurrence within 2 years, and demonstrating progression despite stereotactic radiosurgery (SRS). (3) Any patient undergoing more than two operations for a recurrent tumor. The second group of tumors designated as “nonaggressive” was from patients who underwent a complete Simpson Grade I resection at the time of initial operation, with no evidence of recurrence on imaging at a follow-up period of 5 years or more. A database of 822 patients who underwent an operation for a meningioma at the University of Washington, or Harborview Medical Center, was retrospectively reviewed to identify 24 such tumors that met our criteria for an aggressive Grade I.

Two-Dimensional Gel Electrophoresis

For each tumor specimen, ~100 mg of each tumor was cut over dry ice, placed in a homogenization buffer, mechanically homogenized, precipitated with acetone, and quantified using a bicinchoninic acid assay (Thermo Scientific). Equal amounts of protein from the five specimens in each group were pooled, yielding a total of 150 µg of protein. An isoelectric focusing (IEF) protein standard (Bio-Rad Laboratories) was spiked into the samples as a standard. The samples were then rehydrated on 24 cm, pH 3 to 10 immobilized pH gradient strips (Bio-Rad Laboratories) in reswelling trays for 24 hours, and then separated via IEF. The strips were then washed in 2% dithiothreitol (DTT) and 2.5% iodoacetamide and separated again via a 4 to 20% gradient Tris-HCL gel (Jule, Inc). Gels were then fixed in 10% acetic acid, 30% ethanol, and 40% water, and then stained with silver nitrate.

Image Analysis and Protein Spot Selection

After silver staining, the spots were imaged on a Versadoc Imaging Station (Bio-Rad Laboratories). The images were analyzed using Image Master Software (GE Lifesciences). The spots in each gel were normalized to the density and size and of IEF protein standard. Spots with differential ratios in density of 1.5:1 or greater were selected for further evaluation.

Liquid Chromatography and Mass Spectrometry

Selected spots were excised from the gel, washed and destained of silver, reduced in 10 mM DTT, and dehydrated with acetonitrile. The samples were digested for 18 hours with trypsin and the peptides extracted, dried in a vacuum centrifuge, and then resuspended in a trifluoroacetic acid solution. Each sample was run on an AB Sciex 4800 Plus MALDI TOF/TOF for peptide identification and the peptide data were analyzed using ProteinPilot v4.0 software (AB Sciex).

Western Blot Analysis

In total, seven proteins were selected for validation via western blot: gelsolin (Abcam: ab113229, 1:2,000), galectin-1 (Abcam: ab25138, 1:4,000), heterogeneous nuclear ribonucleoprotein K (HNRNPK) (Abcam: ab52600, 1:1,000), vimentin (Abcam: ab92547, 1:1,000), calreticulin (Abcam: ab22683, 1:1,000), eukaryotic translation initiation factor 3

(EIF3 β) (Abcam, ab133601, 1:1,000), and dimethylarginine dimethylaminohydrolase 1 (DDAH-1) (Abcam: ab108088, 1:2,000). Beta-actin staining was used as a standard in each western.

Each gel was imaged and quantitatively analyzed using the gel analysis tool in ImageJ (NIH). The intensity of each sample was standardized against beta-actin (see **►Supplementary Table 1**). Fisher's exact test was used to identify any significant differences in protein expression between Grade I and aggressive Grade I tumors. A one-way analysis of variance (ANOVA) was used to identify significant differences between all grades. A Tukey's test post hoc analysis was performed to identify which specific grades were statistically different.

Tissue Microarray

Meningiomas from patients with at least 5 years of follow-up were considered for use in the tissue microarray (TMA). To obtain a representative demographic population, only quantity of tissue was used as a final selection criterion; therefore, our array contained patients with prior radiation, embolism, different WHO grades, and varying clinical outcomes. We purposefully selected a series of three tumors that were sequentially resected from the same patient before and after radiation therapy to evaluate differences in our panel of biomarkers as a result of radiation on a patient-specific level.

A total of 84 meningioma samples were analyzed against the panel of proteins identified in the 2D gel. Thirty-eight Grade I, 20 aggressive Grade I, 24 Grade II, and 3 Grade III meningiomas were included. Of these tumors, 19 had had prior radiation.

Tumor samples were fixed in formalin and embedded in paraffin. Of the seven proteins in our panel, only EIF3 β was not validated in the TMA due to a lack of an effective antibody for immunohistochemistry. Three separate individuals independently ranked each array as exhibiting negative, weak-positive, weak-moderate positive, moderate-strong positive, or strongly positive staining. Cells which stained positively within the field of view were counted and divided by the total number of cells to determine the percentage of positive cells. In addition, localization of each protein within each cell was noted.

Statistical Analysis of Tissue Microarray

Each staining intensity was given a numerical value on a scale of 0 to 4 in preparation for statistical analysis, 4 being a strong positive stain. Statistical analysis was performed using SPSS Version 19 (IBM Corporation). A Fisher's exact test and an independent *t*-test were used to determine single variable changes. Of note, the single variable analysis revealed a significant increase of HNRNPK expression in relation to radiation treatment ($p = 0.050$, see **►Supplementary Table 2**), prompting us to control for radiation in all statistical models. Pearson's correlations, using the weighted values of protein expression, were used to identify proteins that exhibited direct or inverse relationships (see **►Supplementary Table 3**). Weighted protein expressions clustered using a hierarchical method via Cluster 3.0 (Eisen Laboratory, Stanford

University) and visualized as a heat map using Java Tree View (Baryshnikova Laboratory, Princeton University).

Four predictive models were constructed in SPSS using a binary logistical regression. The following variables were used as predictors: embolization prior to surgery, previous radiation, first/primary meningioma diagnosis, protein intensity (0–4 scale), percentage of specimen positively stained, weighted protein expression values, protein localization within the cell, and significant ($p < 0.05$) protein relationships identified using Pearson's correlations. Each model was optimized by removing insignificant variables as predictors until false-positive and false-negative predictions were minimized.

Results

Protein Identification and Western Validation

In total, 22 spots were identified as having $> 1.5:1$ difference in the magnitude of density (**►Fig. 1**). Of these spots, six contained either individual proteins or no more than two proteins, which were then used for further validation via western blot (**►Fig. 1C**). Fisher's exact test between five Grade I and five aggressive Grade I tumors revealed only a significant difference in the amount of gelsolin ($p = 0.003$), with aggressive Grade I tumors having the highest expression (**►Fig. 2A**). The one-way ANOVA used to analyze the western blots of all WHO grades found significant differences in the expression of galectin-1 ($p = 0.009$), DDAH-1 ($p = 0.003$), and gelsolin ($p = 0.011$) between aggressive Grade I tumors and other WHO grade tumors (**►Fig. 2B**). Post hoc Tukey's test showed that aggressive Grade I tumors had significantly higher expression of galectin-1 over Grade III tumors ($p = 0.006$) and trending significance over Grade I tumors ($p = 0.098$) (**►Fig. 2B**). Aggressive Grade I tumors also had higher expression of DDAH-1 over all other grades ($p < 0.05$) (**►Fig. 2B**). Gelsolin expression was found to be statistically different between Grades II and III tumors ($p = 0.007$) with higher expression in Grade III meningiomas (**►Fig. 2B**).

Tissue Microarray

Fisher's exact tests and one-way ANOVA revealed that no single variable, or localization of a protein within a cell, significantly predicted tumor recurrence or an aggressive Grade I tumor (**►Fig. 3**; **►Supplementary Table 2**). However, the Pearson's correlation analysis showed many strong correlations between the expression, or localization, of two proteins that were unique to recurrent tumors, tumors of higher grade, and aggressive Grade I tumors (**►Supplementary Table 3**). Gelsolin and galectin-1 were the only proteins which showed changes in cellular localization patterns to either the cytosol or nucleus; therefore, nuclear and cytosolic expression patterns were noted (see **►Fig. 4**).

The logistical regression model built to identify aggressive Grade I tumors within a cohort of Grade I tumors did succeed in correctly classifying Grade I and aggressive Grade I meningiomas ($p = 0.00002$) (**►Table 1**); 76.2% of the

Fig. 1 2D gel proteomic signatures of aggressive and benign WHO Grade I meningiomas. (A) Images taken of the whole proteome separation of both aggressive Grade I and benign Grade I meningiomas. (B) Differences in spot densities comparing aggressive Grade I and benign Grade I tumors. (C) List of proteins found in each spot, identified by MS. MS, mass spectrometry; 2D, two-dimensional; WHO, World Health Organization.

aggressive Grade I tumors were identified, and 91.4% of all Grade I tumors were correctly identified, yielding a total accuracy of 85.7%. This model relies on the localization and expression of calreticulin, gelsolin, galectin-1, vimentin, and HNRNPK to predict which tumors will act aggressively. The radiation controlled model proved to significantly predict Grade I and aggressive Grade I meningiomas ($p = 0.00039$) (►Table 1). This model succeeded in correctly predicting 97.1% of Grade I tumors and 76.9% of aggressive Grade I tumors. The radiation-controlled model incorporated the

same proteins as the model that included radiated tumors with the addition of DDAH-1. The logistic regression model created to predict recurrence across all grades succeeded in identifying 75.6% of nonrecurrent tumors and 83.7% of recurrent tumors for an overall accuracy of 79.8% ($p = 0.00003$) (►Table 1). This model relies on the expression and localization of vimentin, galectin-1, DDAH-1, gelsolin, and HNRNPK to predict tumor recurrence. The radiation-controlled model also proved to be significant ($p = 0.00029$) (►Table 1). This model successfully

Fig. 2 Western blot validation of proteins identified via 2D gel separation. The western blots of proteins with significant differences are shown with the corresponding beta-actin. All other proteins are displayed graphically. (A) Validation of the six identified biomarkers in individual meningiomas comparing benign grade I and aggressive grade I. (B) Validation of the six identified biomarkers in individual tumors across all grades. 2D, two-dimensional.

Fig. 3 Identification of protein expression patterns in the tissue microarray to reveal profiles to identify recurrent and aggressive meningiomas. (A) Graphic representation of each protein in relation to grade. This approach revealed no significant differences between grades. (B) Graphic representation of each protein in relation to recurrence. This approach revealed no significant differences between recurrent and nonrecurrent diseases. (C) Protein profiles identified by a clustering algorithm. This method revealed several relationships between proteins which were able to distinguish recurrent from nonrecurrent meningiomas.

predicted 94.6% of all nonrecurrent tumors and 74.1% of all recurrent tumors for an overall accuracy of 85.9%. This model differed from the radiation-included model in that it relies on the expression and localization of calreticulin and not DDAH-1 to predict recurrence.

Discussion

This study succeeded in identifying a small group of biomarkers that can identify a group of clinically aggressive Grade I meningiomas that the WHO grading criteria cannot identify. Using these same protein biomarkers, recurrent meningiomas can be identified regardless of grade. Finally, the identification of both aggressive Grade I and recurrent meningiomas using the biomarkers identified in this study can be applied to tumors of many clinical histories, including tumors that have been previously radiated, embolized, or that have recurred.

Through the development of logistic regression models, we have demonstrated the diagnostic capabilities of the biomarkers identified in this study. Further validation in a larger cohort of meningiomas could lead to the development of a diagnostic platform that improves upon the current WHO grading scheme. By developing a more sensitive predictive model, clinicians could provide better patient care by identifying tumors that may require adjuvant therapies in addition to surgery.

It is important to note that the predictive models created in this study were constructed from a population

of meningiomas that is not representative of the general distribution of meningiomas. Our tissue microarray cohort had a much higher prevalence of aggressive and recurrent meningiomas in comparison to other series of meningiomas. This could influence the magnitude of value placed on each predictive factor in the logistic regression models. Therefore, further validation of our panel of biomarkers in a larger series of meningiomas will help refine the predictive accuracy of the regression models presented in this study.

The presented predictive models rely on evaluating immunohistochemistry staining of six proteins: gelsolin, galectin-1, HNRNPK, calreticulin, vimentin, and DDAH-1. Each model relies on different expression profiles of these proteins. However, the expression and localization of galectin-1, gelsolin, and HNRNPK as predictive factors remain the same across each model. The reliance of each model on galectin-1, gelsolin, and HNRNPK suggests these proteins may function as universal markers of aggression and recurrence in meningiomas. Previous studies have shown that these proteins are involved in proliferation,^{17–19} cell motility,^{20–22} and gene transcription^{23–25} depending on their location inside the cell (► Fig. 5).

Changes in expression of galectin-1, gelsolin, and HNRNPK have been previously reported in other cancers, which further reinforces their potential as marker for oncogenesis.^{18,21,23,26,27} Of these three proteins, only galectin-1 has been reported as a biomarker for identifying meningiomas,²⁸ although it has not been studied across all grades of

Fig. 4 Representative staining from tissue microarray analysis. Examples of immunohistochemistry staining for each of six candidate biomarkers are shown in different WHO grades of meningioma. WHO, World Health Organization.

meningiomas until this study. Galectin-1 is a small lectin-binding protein with a variety of cellular functions.²³ Cytosolic galectin-1 has been linked to RAF and PI3K activation which can lead to proliferation.^{23,24} In the nucleus, galectin-1 interacts with Gemin4 to induce transcription.²³ HNRNPK is an messenger RNA (mRNA) processing protein that is a part of the spliceosome; overexpression can lead to tumor metastasis.^{18,29} Gelsolin has the highest binding affinity for actin of any known protein and is responsible for cytoskeletal remodeling and cell motility.²² When combined with PI(4,5) K, gelsolin inhibits caspase 9 to prevent apoptosis.^{22,30} Gelsolin also acts as a transcription initiator when cleaved by caspase 3.^{22,31}

Recent genetic sequencing studies have found mutations in AKT, smoothen, TRAF7, and KLF4 in meningiomas.³² Several of these genes have been known to interact with the biomarkers identified in our study. AKT interacts with HNRNPK to regulate mRNA processing.^{33,34} KLF4 also directly interacts with gelsolin to induce changes in the actin cytoskeleton.³⁵ These interactions suggest that our predictive model could be strengthened with the addition of genetic screening. This could be accomplished by correlating genetic mutations to alterations in the proteome. Moreover, the addition of genetics could provide a high-throughput method to expedite the diagnostic process, although studies into these potential correlations for diagnostic purposes have yet to be published.

Table 1 Predictive models derived from a tissue microarray utilizing six of the identified biomarkers

	Predicting a Grade 1.5 within a cohort of Grade I			Predicting a Grade 1.5 within a cohort of Grade I, excluding radiated tumors			Predicting recurrent tumors across all grades			Predicting recurrent tumors across all grades, excluding radiated tumors		
	Predictor	Beta	p-Value	Predictor	Beta	p-Value	Predictor	Beta	p-Value	Predictor	Beta	p-Value
Factors that predict aggressiveness or recurrence	First diagnosis	5.772	0.002	first diagnosis	6.633	0.021	First diagnosis	1.782	0.01	First diagnosis	2.505	0.023
	Hn-W	5.78	0.028	Hn-W	10.06	0.041	Gal-Cp-In	1.648	0.083	Gal-Cp-In	2.962	0.037
	Gel-Cp-In	8.065	0.058	Gel-Cp-In	11.125	0.075	Gel-Cp-W, Gal-Nu-W	0.411	0.015	Gel-Cp-W, Gal-Nu-W	1.148	0.021
	Vim-W, Cal-W	0.35	0.094	Cal-W, Gal-Cp-W	1.052	0.036	Gal-Cp-W, Hn-W	0.539	0.05	Cal-In	1.738	0.025
	Gel-Nu-W, Gal-Nu-W	1.13	0.085	Dd-%+	2.322	0.238	Vim-In	2.533	0.012	Vim-W, Hn-W	1.203	0.042
							Dd-W, Hn-W	0.628	0.025			
Factors that predict benign tumor prognosis	Gel-Cp-W	- 7.829	0.058	Gel-Cp-W	- 10.42	0.08	Gel-Cp-%+	- 0.039	0.003	Gel-Cp-%+	- 0.082	0.01
	Hn-In	- 6.416	0.019	Hn-In	- 10.06	0.04	Hn-In	- 2.102	0.024	Hn-In	- 2.346	0.064
							Gal-Cp-%+	- 0.032	0.062	Gal-Cp-%+	- 0.076	0.015
	Gel-Nu-%+	- 0.115	0.047	Gel-Nu-W, Hn-W	- 0.76	0.294	Vim-W, Gal-Cp-W	- 1.043	0.009	Vim-W	- 3.818	0.042
				Vim-In	- 2.91	0.095	Dd-W, Gel-Nu-W	- 0.867	0.039	Gal-Cp-W, Gal-Nu-W	- 0.818	0.035
				Gel-Cp-%+	- 0.068	0.097				Gel-Cp-In	- 1.059	0.28
	Constant	0.002	0.999	Constant	2.505	0.342	Constant	1.661	0.328	Constant	13.515	0.038
	Model significance: 0.00002			Model significance: 0.00039			Model significance: 0.00003			Model significance: 0.00029		
	Cox-Snell: 0.470			Cox-Snell: 0.487			Cox-Snell: 0.382			Cox-Snell: 0.418		
	Nagelkerke: 0.640			Nagelkerke: 0.707			Nagelkerke: 0.509			Nagelkerke: 0.562		

Abbreviations: Cal, calreticulin; Cp, protein localized to the cytoplasm; Dd, DDAH-1; Gal, galectin-1; Gel, gelsolin; Hn, HNRNP; In, intensity of stain rated on a 0 to 4 scale; Nu, protein localized to the nucleus; Vim, vimentin; W, weighted by multiplying intensity by the percentage of cells that stained positive; %+, percentage of cells stained positive.

Notes: Two models were created to identify aggressive Grade I meningiomas from a cohort of Grade I meningiomas, with and without radiated tumors. Two additional models were created to identify recurrent tumors across all grades, with and without radiated tumors. Each model proved to be very significant ($p < 0.001$). Each predictor is presented in the following format: "protein-localization/prevalence." Predictors based on two proteins are presented in the following format: "protein-location, protein-location."

Fig. 5 Schematic depicting a potential unified mechanism of oncogenesis relating each of the candidate biomarkers identified after literature review of protein-protein interactions.

Conclusion

Identifying aggressively behaving and recurrent subtypes of WHO Grade I meningioma is important to the neurosurgeon and neuro-oncologist treating patients harboring such tumors. Currently, the WHO grading system does not identify these aggressive outliers in what is an otherwise benign cohort of tumors. By using strict criteria for selecting clearly outlying tumors, we have been able to use simple protein separation techniques as a means of biomarker discovery. Validation of these markers with two independent methods and predictive modeling have allowed us to identify proteins that can predict a small cohort of WHO Grade I tumors that recur at a much higher rate than expected. While our results are based on a small sample size, further validation in a larger cohort may help clinicians use these markers to identify aggressively behaving WHO Grade I tumors in the future and better direct therapies.

Conflict of Interest

None of the authors has any conflict of interest to disclose.

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