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Proteomics Analysis of Brain Meningiomas in Pursuit of Novel Biomarkers of the Aggressive Behavior

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Abstract

The aim of this pilot study was to evaluate the use of advanced proteomics techniques to identify novel protein markers that contribute to the transformation of benign meningiomas to more aggressive and malignant subtypes. Multiplex peptide stable isotope dimethyl labelling and nano-LCMS was used to identify and quantify the differentially expressed proteins in WHO Grade I, II and III meningioma tissues. The proteins identified will help elucidate the process of transformation to malignancy and may contribute to improved diagnosis and treatment of these aggressive tumors.

Keywords: Meningioma; Anaplastic meningioma; Atypical meningioma; Proteomics; Biomarkers

Introduction

Meningiomas are the most common benign intracranial tumors and their first-line treatment is surgical removal if the lesion can be largely removed at sufficiently low risk. However, a subset of patients develops more aggressive tumors. According to the World Health Organization (WHO), meningiomas are classified as typical, atypical and anaplastic; up to 20% of patients may have atypical meningiomas and 1-3% may develop anaplastic or malignant subtypes [1]. These aggressive subtypes of tumors typically exhibit more rapid tumor progression, invasiveness and recurrence precluding complete surgical removal and requiring additional therapies of radiosurgery/radiotherapy and chemotherapy [2]. Occasionally, meningiomas have malignant transformation with distant metastases outside the central nervous system (CNS).

Extent of tumor resection has been shown to correlate with recurrence rate. In 1957, Simpson D described a grading system that has been expanded and validated over the decades [3-5]. WHO grade I tumors tend to have a direct inverse correlation between extent of resection and tumor recurrence. MiB (Ki67) level greater than 3%, helps predict recurrence rate in Simpson I-III meningiomas. MiB is not a criterion used for WHO II or WHO III meningiomas. Hence, additional biomarkers are necessary to elucidate the likelihood and mechanisms of tumor recurrence.

Most WHO I tumors harbor a few mutations [6,7] and can be categorized into groups expressing NF2, AKT-1, SMO, TRAF7, KLF4. WHO grade II and III tumors harbor a wider variety of mutations including (hTERT/telomerase, MADH2, MADH4, APM-1, DCC, CDKN2A, $p14^{ARF}$, CDKN2B, TP53, MEG3, ALPL, Notch, WNT, IGF and NDRG2 [8].

Few genetic and proteomics markers have been studied for meningioma subtypes with various aims [9-11] and their correlation to clinical behaviour and response to therapy is limited. While there is a notable overlap with some biomarkers found in other malignant neoplasms (glioblastoma, adenocarcinoma, squamous cell carcinoma and melanoma), the mechanisms that result in transformation from benign meningiomas to more aggressive subtypes are poorly understood. This study aims to better define biomarkers of transformation into aggressive tumors in patients with benign meningiomas using and proteomics analysis and may identify targets for future therapies.

Proteomics plays an important role in medical research, because of the link between proteins, genes and diseases [12]. Most current drugs are either proteins or they target specific proteins in the body [13]. Identifying unique protein expression associated with specific tumors is a very important and promising area in the field of clinical proteomics; hence proteomics analysis of brain tissues is an essential part of neuroscience research [14]. Although it faces many challenges, most importantly the difficulty of obtaining sufficient sample for mass spectrometry analysis, and protein purification methods has to be optimized for each type of cell or tissue [14-17].

Three tumor tissues (typical, atypical and anaplastic), and two controls (fresh cadaveric dura) were used for proteomics analysis. Multiplex peptide stable isotope labelling method was used to label all samples. With this method, all primary amines (the N terminus and the side chain of lysine residues) in a peptide mixture are converted to dimethylamines. The labelled samples are then mixed in equal ratios and analysed by liquid chromatography–mass spectrometry (LC/MS). The mass difference of the dimethyl labels is used to compare the peptide quantity across all samples. The advantages of this labelling method over others, besides allowing the comparison of multiple samples in a single experiment; it uses inexpensive reagents and is applicable to almost any sample (tissue/cell) [18].

Materials and Method

Samples

Three meningiomas, typical (I), atypical (II) and anaplastic (III) (Figure 1), that were resected at Providence Saint John's Health Center

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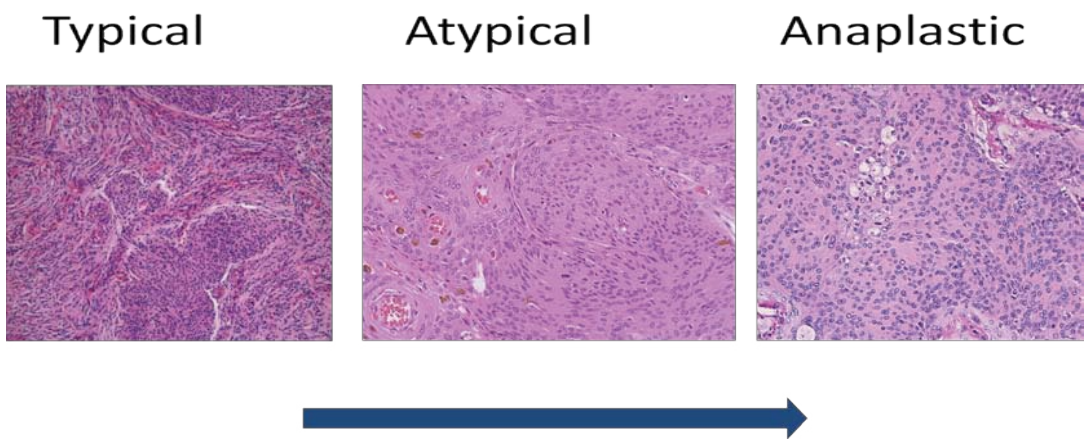

Figure 1: Histopathological progression of meningiomas from grade I (typical) to grade II (atypical) to grade III (anaplastic) subtypes. As the tumors become increasingly aggressive the cellularity increases, nuclear atypia formation and loss of cytoarchitecture.

by Drs. Barkhoudarian and Kelly, were selected from the John Wayne Cancer Institute brain tumor tissue bank. These tissues had been cryogenically preserved per standard protocol [19]. Dura mater was obtained from two cadaveric specimens, cryogenically preserved, and used as controls.

Protein extraction

Tissues homogenization was carried out with12 mM sodium lauryl sarcosine, 0.5% sodium deoxycholate, and 50 mM triethyl ammonium bicarbonate (TEAB). The samples were then centrifuged at 16,000 × g for 5 minutes and the supernatant was collected, heated at 95°C for 5 minutes and placed in a water bath sonicator for 5 minutes.

Protein concentrations

The total protein concentration of the samples was determined using BCA Protein Assay Kit (Pierce, Thermo Fischer Scientific). Bovine serum albumin was used to generate the standard curves.

Reduction, alkylation and trypsin digestion

Protein disulfides were reduced with 5 mM Tris 2-carboxyethyl phosphine, for 30 minutes at room temperature. Ten mM iodoacetamide was then added for alkylation, and incubation in dark for 30 minutes at room temperature. The protein solutions were diluted five-fold with 50 mM TEAB.

Trypsin was prepared in 50 mM TEAB, and added to the samples in (1:100) ratio then incubated for 4hrs at room temperature. This step was repeated twice. The peptide solutions were acidified with a final concentration of 0.5% trifluoroacetic acid (TFA), vortexed for 5 minutes. Detergents were removed by adding 1:1 (vol/vol) of ethyl acetate to the tryptic digests, vortexed for 5 minutes and centrifuge at 12,000 × *g* for 5 minutes at room temperature, supernatant were discarded. The tryptic peptides lyophilized before dimethy labelling.

Dimethyl labelling

The dimethyl labelling was carried out according to Boersema et al. [18], using in-solution dimethyl labelling protocol. The digested samples were reconstituted in 100 µL of 100 mM TEAB. Four microliters of 4% (vol/vol) formaldehyde isotopes (CH2O, CD2O and ^{13}CD2O) were then added to the samples to be labelled with light, intermediate and heavy dimethyl respectively, samples mixed and spun down. Four microliters of 0.6 M sodium cyanoborohydride (NaBH3CN) isotope was added for light and intermediate labelling and 0.6 M of sodium cyanoborodeuteride (NaBD3CN) isotope for heavy labelling. All samples were then placed on a bench mixer and incubated for 1 hr at room temperature.

The labelling reaction was quenched by adding 16 µL of 1% (vol/vol) ammonia and 8 µL of 5% (vol/vol) formic acid to acidify the samples for mass spectrometry analysis.

The brain tissues were labelled as follows: control 1 = light, control 2 = intermediate, meningioma samples (T1, TII and TIII) = heavy. The samples were grouped in 3 triplex per Table 1 below. The differentially labelled samples were then mixed in 1:1:1 ratios, and analysed by nanoLC-MS.

Chromatographic separation and nanoLC-MS

C18 and SCX stage tips were prepared in house. The stage tips were conditioned with 20 µL methanol and 20 µL of buffer containing [ammonium acetate (NH_4AcO) using gradient elution from 0.2 to 5%, 0.5% acetic acid (AcOH) and 30% of acetonirile (ACN)]. The same buffer was used for SCX fractionation and sample elution. The samples then dried in SpeedVac and reconstituted in acetonirile 3% (ACN) and 0.1 % Formic acid (FA).

Fractionated samples were analysed with an Eksigent 2D nanoLC mass spectrometer attached to a Thermo Orbitrap XL. Peptides were injected onto a laser-pulled nanobore 20 cm × 75 µm C18 column (Acutech Scientific) in buffer A containing (3% acetonitrile with 0.1% formic acid) and resolved using a 3 hour linear gradient from 3-40% buffer B containing (100% acetonitrile with 0.1% formic acid). The Orbitrap XL was operated in data dependent mode with 60,000 resolution and target auto gain control at 5e6 for parent scan. The top 12 ions above +1 charge were subjected to collision induced dissociation set to a value of 35 with target auto gain control of 5000. Dynamic exclusion was set to 30 seconds.

Data Analysis

The MS/MS spectra were analysed using MaxQuant software version 1.5.1.2 (Germany). The different dimethyl isotope labels were set as variable modifications on the peptide N termini and lysine

residues. Carbamidomethyl cysteine was set as a fixed modification while oxidized methionine was set as variable modification. Trypsin was set as a proteolytic enzyme, and maximum 2 missed cleavages were allowed, peptide tolerance 10 ppm, fragment ions tolerance 0.5 amu.

Results

Five brain tissues were used for this quantitative proteomic study, grouped per (Table 1) above to study the variability and consistency of protein expressions between; (i) the two controls: (ii) between the controls and tumor samples: (iii) across all three tumor samples [typical (I), atypical (II) and anaplastic (III)]. In total 649 proteins were identified from 15 MS runs. Protein abundances were derived from peptide abundances for multiple peptides. Protein abundances were calculated from the sum of all unique normalised peptide ion abundances for a specific protein on each run. The Supplementary Table 1, includes a list of protein names, their intensity in the controls (C), their intensity in the three phenotypes (I, II and III), the expression ratios of average controls (vs.) phenotypes I, II and III, as well as the expression ratios between all of the three phenotypes (I, II and III).

Table 1: Triplex samples for analysis. C1 and C2 = controls. S1, S2 and S3 = meningioma samples.

A	B	C
C1 + C2 + T1	C1 + C2 + TII	C1 + C2 + TIII

Our analysis and observation was focused on the proteins that showed up or down-regulation in one phenotype compared to the others and compare to the control, as those proteins could potentially be investigated as biomarkers for aggressive tumors, e.g. protein alpha-adducin, was expressed in C, TI and TII only, and it was up-regulated in TI by 3 fold compare to the control, however in TII was down-regulated by 0.25 compare to the control, and wasn't detected in TIII; hence the expression ratio for TI: TII was 11.6 (Supplementary Table 1). This may suggest that this protein is mainly present in the non-aggressive form of meningioma, or its representing gene (ADD1) may be switched off in the aggressive forms. Other proteins that showed similar pattern to alpha-adducin are summarized in (Table 2 and Figure 2).

Another intriguing observation of this data is the presence of some

Table 2: Selected protein expressions (intensities), in controls and meningioma tissues.

Protein name	Ave (C)	TI	TII	TIII
Apoptosis-associated protein	22612	0.01	391410	0.01
Transmembrane protein 109	863006	3104800	1602000	1143100
BTB/POZ domain-protein	326921	3116400	0.01	0.01
Beta-actin-like protein 2	36158500	94091000	12285000	14177000
ATP-dependent RNA helicase A	349960	426750	629360	859420
Protein SET	731725	1848400	2145600	15151000
Brain acid soluble protein 1	2273925	259030	690070	21530000
40S ribosomal protein S28	684885	3198000	2910100	6894400
Heterogeneous nuclear rib- K	3006850	3801700	4588700	25966000
Activated RNA polymerase II trans p15	1338271	2809900	6207100	12637000
Basal cell adhesion molecule	615190	452370	234410	870010
Lumican	58945833	24028000	4039000	3530400
Prolargin	90667333	51906000	3970600	9401300
Malate dehydrogenase,	4966816	2812100	1151600	1022800
Peroxiredoxin-2	6842683	8677400	4756600	2568400
Rab GDP dissociation inhibitor alpha	2722066	2395500	1308800	672160
Nucleolin	1565608	3120600	11362000	12647000
Stathmin; Stathmin-2	1482545	284420	1140500	6319600
Alpha-adducin	281103	846030	72814	0.01
Glutathione S-transferase P	904686	2566600	933570	330930
Myelin basic protein	177968	394030	29499	0.01
Synaptic vesicle membrane	596081	438400	355470	184350
Calnexin	1139366	0.01	4238500	5735800
Serine / arginine-rich splicing F2	97133	886340	1175300	2511700
Annexin A11	368523	0.01	321640	1155500
Transketolase	4291416	15510000	979660	1905700
Plasma protease C1 inhibitor	2377966	736090	270950	327160
Complement factor B	10816883	955230	408350	466770
S-phase kinase-associated prot-1	354538	114820	149220	510400
CD44 antigen	77701	0.01	1469000	2307700
Tenascin	12078016	3160100	238210	212320
Cofilin-1	6860700	9157100	8618400	18678000
Complement C4-A;B;Comp. C4 beta	15141850	2184300	1534700	489510
Rho GDP-dissociation inhibitor 2	319165	0.01	225460	335440
Protein canopy homolog 2	138681	567330	1771900	1879000
Protein disulfide-isomerase A3	5554233	10539000	15571000	43184000
Tumor protein D54	643386	0.01	989430	1940400
Alpha-enolase	27090833	35751000	15517000	7497900
Annexin A4	3241150	6722600	6252500	1376000

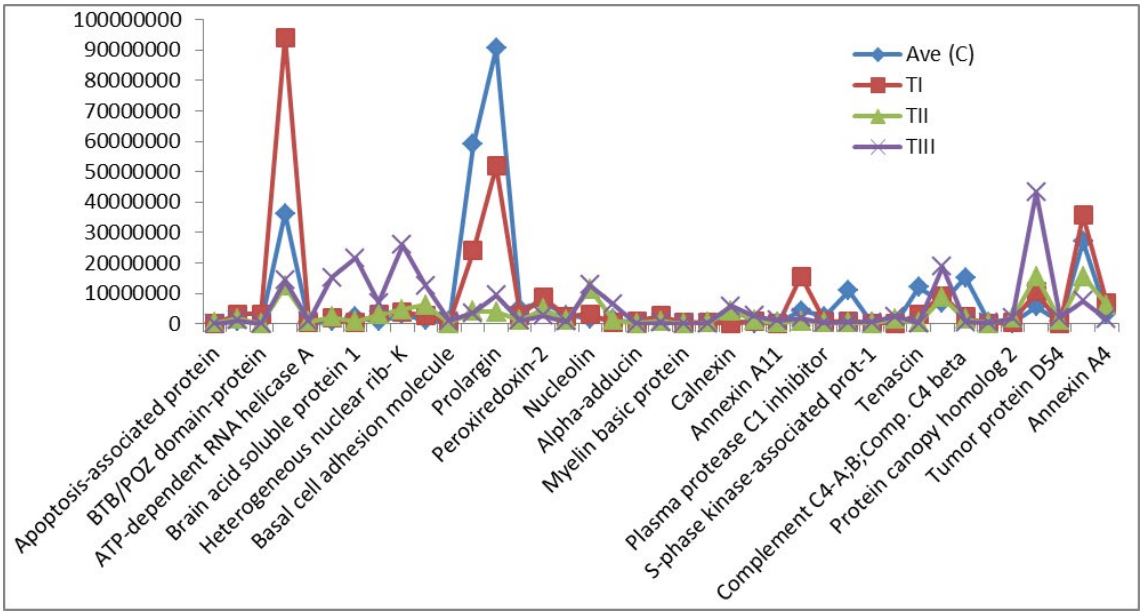

Figure 2: Chart of the expression levels of selected proteins from Table 2. Their intensities in Ave control (C) and meningioma tissues (I, II and III).

Table 3: Proteins expressed in anaplastic tumor tissues only.

Protein name
Junctional adhesion molecule B
Lysosome membrane protein 2*
Eukaryotic translation initiation factor 4B
Dihydrolipoyl dehydrogenase, mitochondrial
Chromobox protein homolog 1
Amyloid beta A4 protein
26S proteasome non-ATPase regulatory subunit 9
Double-strand break repair protein MRE11A
Splicing factor 1*
Yorkie homolog*
Mitochondrial import inner membrane trans9
Glucose-induced degradation protein 8 homolog
PRKC apoptosis WT1 regulator protein*
Heme-binding protein 2
Enhancer of rudimentary homolog
MARCKS-related protein*
Cell surface glycoprotein MUC18
Insulin-like growth factor II
Sorting nexin-1
Tumor protein D52*
Polyadenylate-binding protein-interacting protein 1
Chromobox protein homolog 1
ADP-sugar pyrophosphatase

*Tumour associated proteins

proteins in one subtype only compare to other subtypes and compare to the control. Twenty three proteins were detected in TIII only (Table 3 and Supplementary Table1), including tumor protein D52, lysosome membrane protein 2, splicing factor-1 and MUC18. These proteins are of importance in biomarker study of meningiomas due to their unique expression.

Conclusion

This data suggests the feasibility of identifying and quantifying the proteins in brain meningioma tissues for comparison studies. Due to rare clinical samples, only five brain tissues were used for this study. Larger numbers of specimen are required to conduct a large scale experiments to significantly obtain novel protein biomarkers that correlate with the aggressive tumors. Concurrent genomic and epigenomic analysis will also be helpful to assess post-transcriptional mechanisms. These biomarkers will be clinically utilized in future management of patients, to better identify aggressive tumors for closer surveillance and application of novel targeted therapies. Ultimately this may potentially reduce the need for major high-risk surgery in this patient population.

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Supplementary Information

http://www.omicsonline.org/0974-276X/JPB-09-s053.rar

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