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MRM based validation of protein biomarkers across different grades of glioma tissues

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MRM based validation of protein biomarkers across different grades of glioma tissues

Introduction

Gliomas are tumors originating from the glial cells, accounting for ~28% of all CNS tumors. They are characterized by rapid proliferation rate and poor prognosis. Around 80% of the gliomas are malignant with a median survival of about 10-12 months. Based on the immunohistology, microscopic properties and mutational status, gliomas are classified into four grades. The current study was performed to develop MRM assays for validating candidate proteins arising from previous shotgun proteomics experiment and holds potential to distinguish between different grades of gliomas.

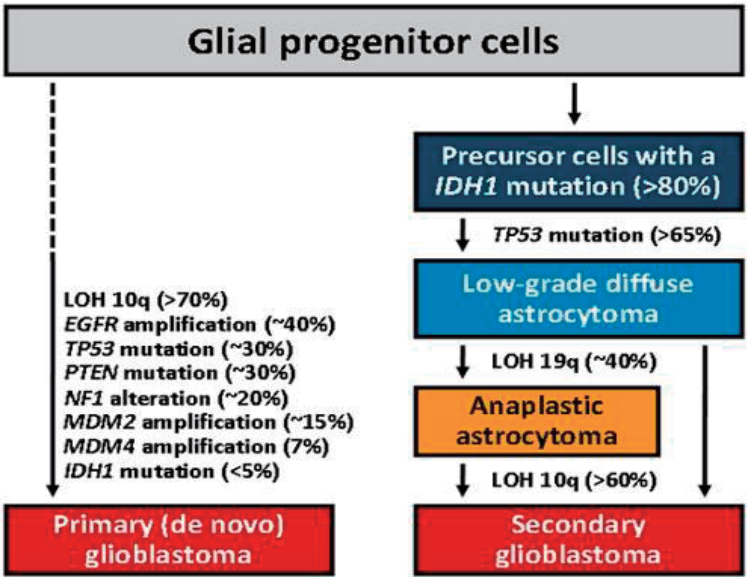

Figure1. Mutations associated with primary GBM and Secondary GBM. Adapted from Ohgaki H, et al [1].

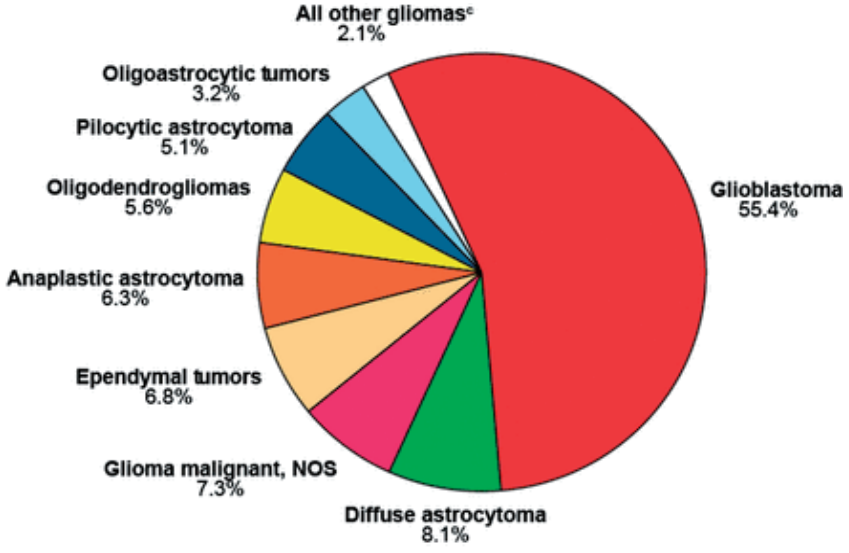

Figure2. Prevalence of gliomas (N=117,906) according to Central Brain Tumor Registry of the United States (CBTRUS) 2009-2013 [2]

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Method and Materials

Experimental strategy for proteomic analysis

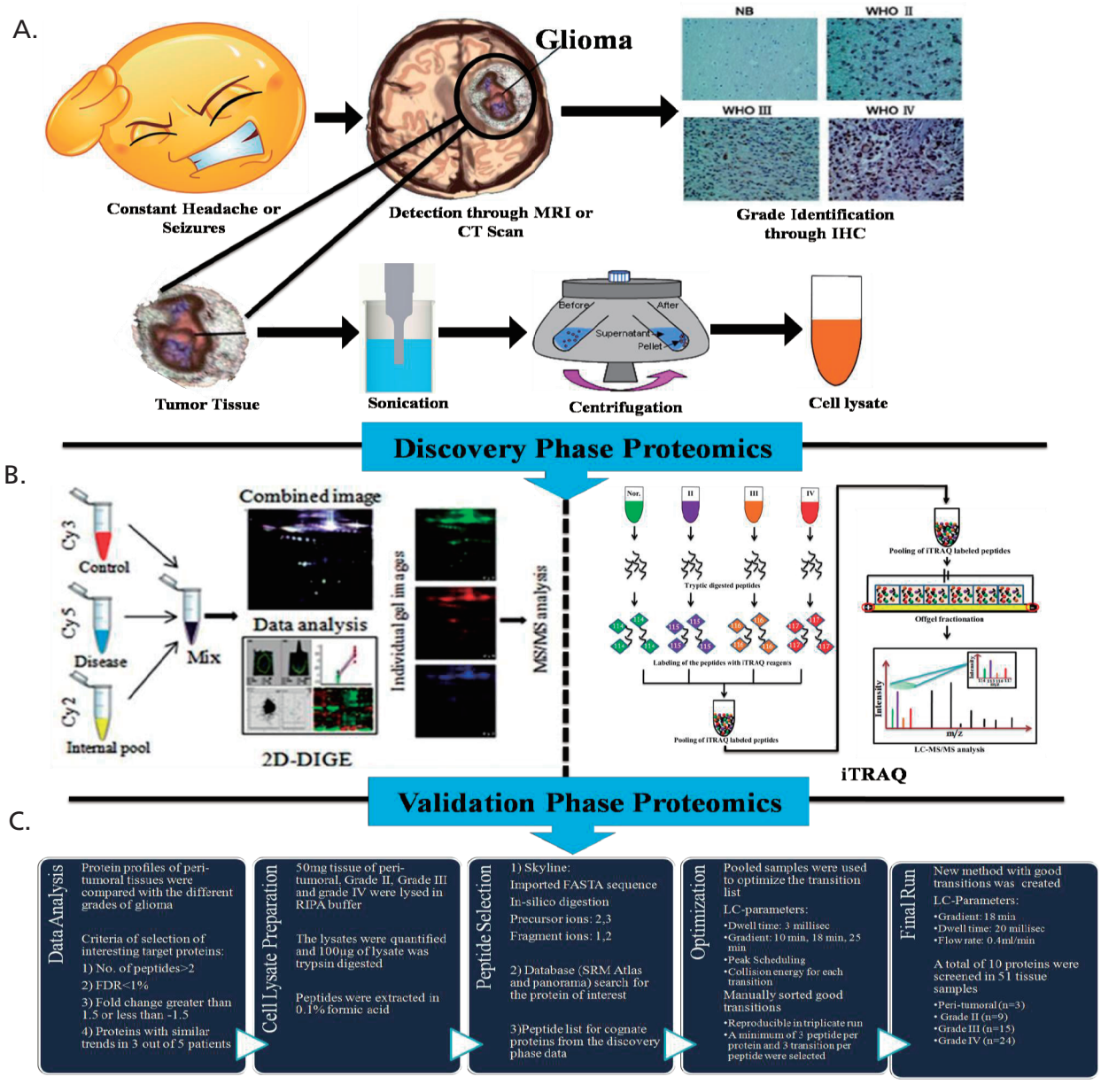

Figure 3. Schematic representation of experimental strategy of glioma tissue proteomic analysis. A) Diagnosis of glioma, grade identification and schematic of tissue lysate B) Comparative tissue proteomic analysis of different grades of glioma with peritumoral tissue using 2D-DIGE and iTRAQ-based quantitative proteomics approaches (C) Validation of certain interesting protein targets using MRM Assay.

MRM based validation of protein biomarkers across different grades of glioma tissues

LC/MS/MS analysis

Samples were analyzed using Ultra High Performance Liquid Chromatography (UHPLC) Nexera coupled with LCMS-8050 triple quadrupole system (Shimadzu Corporation, Japan). The details of analytical conditions are given in Table 1.

Figure 4. Nexera with LCMS-8050

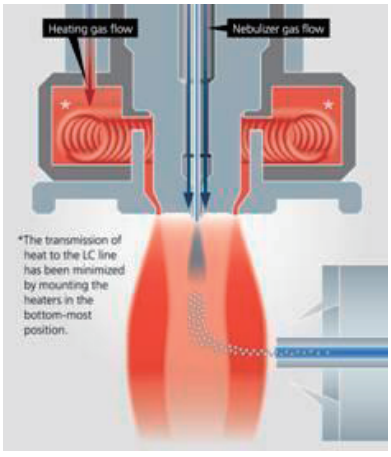

Figure 5. Heated ESI probe

LCMS-8050 triple quadrupole mass spectrometer by Shimadzu (shown in Figure 4), sets a new benchmark in triple quadrupole technology with an unsurpassed sensitivity (UFsensitivity), ultra fast scanning speed of 30,000 u/sec (UFscanning) and polarity switching speed of 5 msec (UFswitching). This system ensures highest quality of data, with very high degree of reliability.

In order to improve ionization efficiency, the newly developed heated ESI probe (shown in Figure 5) combines high-temperature gas with the nebulizer spray, assisting in the desolvation of large droplets and enhancing ionization. This development allows high-sensitivity analysis of a wide range of target compounds with considerable reduction in background.

Table 1. LC/MS/MS conditions

Column	:	Shim-pack XR-ODS II (75 mm L x 3 mm I.D.; 2.2 µm)
Guard column	:	Phenomenex Security Guard ULTRA cartridge
Mobile phase	:	A: 0.1 % formic acid in water
	:	B: 0.1 % formic acid in acetonitrile
Gradient program (B %)	:	0.01-1.5 min → 3 (%); 1.5-10.0 min → 3-60 (%); 10.0-11.0 min → 60-95 (%); 11.0-14.0 min → 95 (%); 14.0-14.1 min → 95-3 (%); 14.1-18.0 min → 3 (%)
Flow rate	:	0.4 mL/min
Oven temperature	:	40 °C
Injection volume	:	3 µL
MS interface	:	Electro Spray Ionization (ESI)
Nitrogen gas flow	:	Nebulizing gas 2 L/min; Drying gas 5 L/min
Zero air flow	:	Heating gas 15 L/min
MS temperature	:	Desolvation line 150 °C; Heating block 250 °C Interface 400 °C

Results

Discovery Phase proteomics

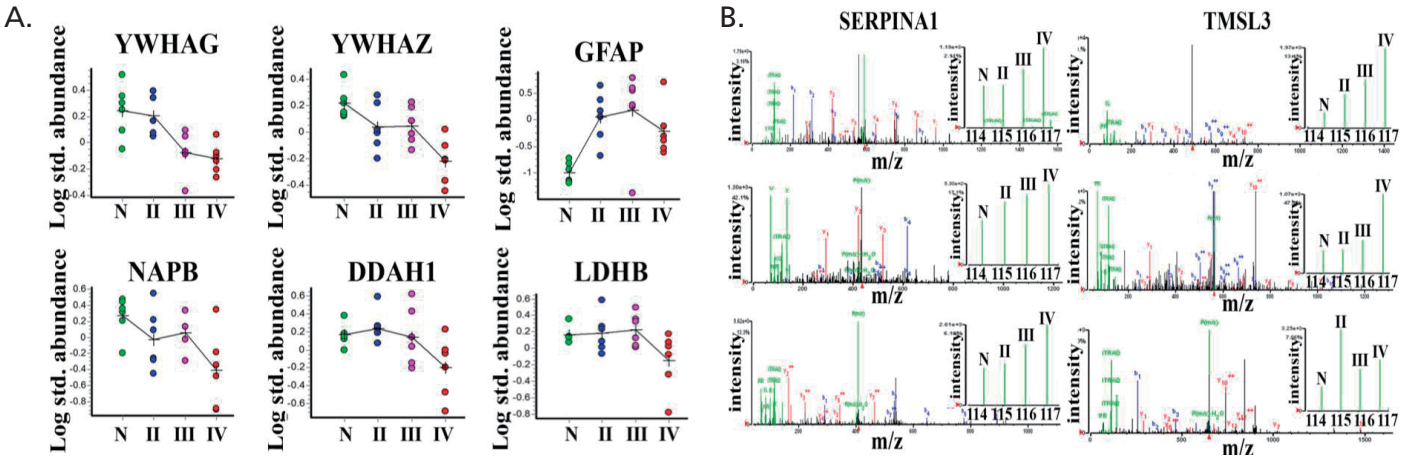

Figure 6. Differential abundance of a few proteins across different grades of glioma. A) Dot plots representing the expression values of proteins using 2D-DIGE in different grades of glioma. B) Representative spectra of two of the interesting target proteins showing differential expression across different grades of glioma

Statistical Analysis of Discovery Phase Data

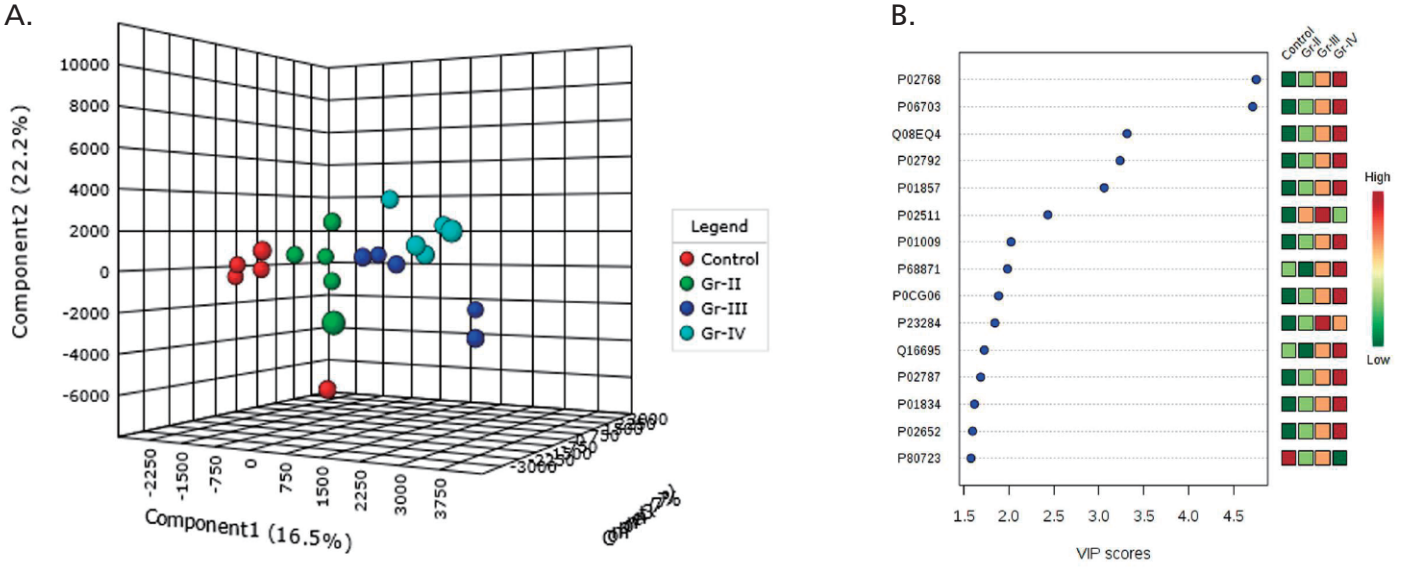

Figure 7. Statistical analysis to select a panel of proteins with ability to segregate grades of glioma. A) PLS-DA plot representing the segregation of different grades of glioma B) Dot plot representing the list of classifier proteins that could potentially differentiate between different grades of glioma [3].

MRM based validation of protein biomarkers across different grades of glioma tissues

Biological Pathways Altered in Glioma

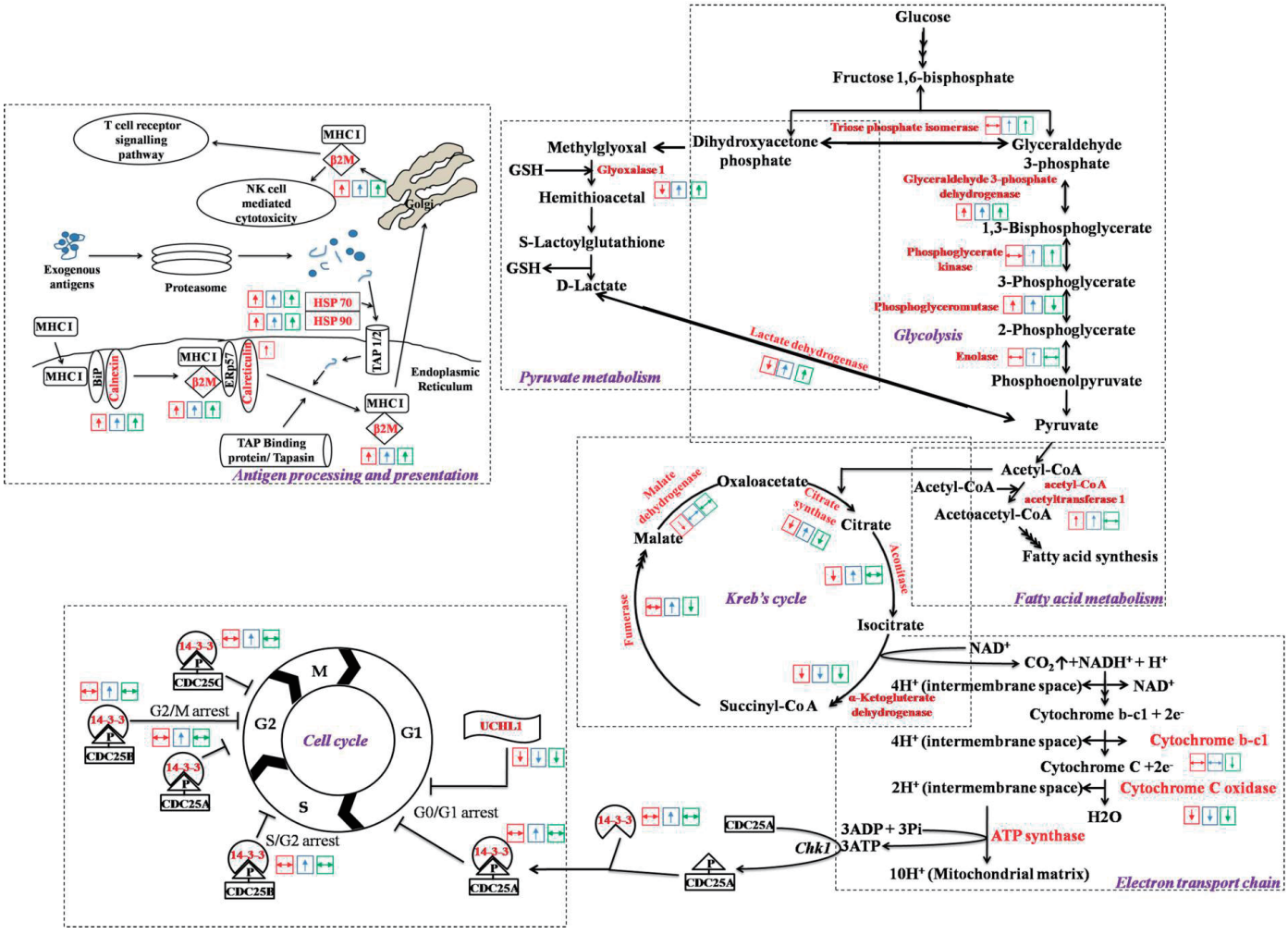

Figure 8. Significantly altered pathways in glioma. Proteins involved in pathways like antigen processing and presentation, pyruvate metabolism, fatty-acid metabolism, electron-transport chain and cell cycles showed differential abundance across different grades of glioma. Arrows in red, blue and green represents the trend of protein abundance in Grade II, Grade III and Grade IV glioma, respectively when compared to peri-tumoral tissues [3].

- Discovery Phase proteomics was performed using differential in-gel-electrophoresis (2D-DIGE) and gel free method (iTRAQ-LCMS/MS).
- Gel-free based proteomics approach identified a total of 171 proteins to be differentially altered in at least three out of five sets.
- Several metabolic pathways were found to be perturbed like integration of energy metabolism, carbohydrate, amino acid, pyruvate metabolism, neurodegenerative disorders, antigen processing and presenting pathways.
- Abundance of a few significantly altered proteins showed a positive correlation with increasing grade of gliomas.

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Validation Phase Proteomics

- From the discovery dataset, 10 differentially regulated proteins i.e., Protein S100-A6, Profilin 1, α-crystallin B chain, L-lactate dehydrogenase A chain, α-1-antitrypsin, Glial fibrillary acidic protein, Nucleolin, α-Synuclein, Brain acid soluble protein 1 and Synapsin 1 were selected for validation using targeted proteomics approach.
- A total of 51 samples comprising peri-tumoral (n=3), Grade II (n=9), Grade III (n=15) and Grade IV (n=24) tissues were analyzed on LCMS-8050. Each sample was run in triplicates.

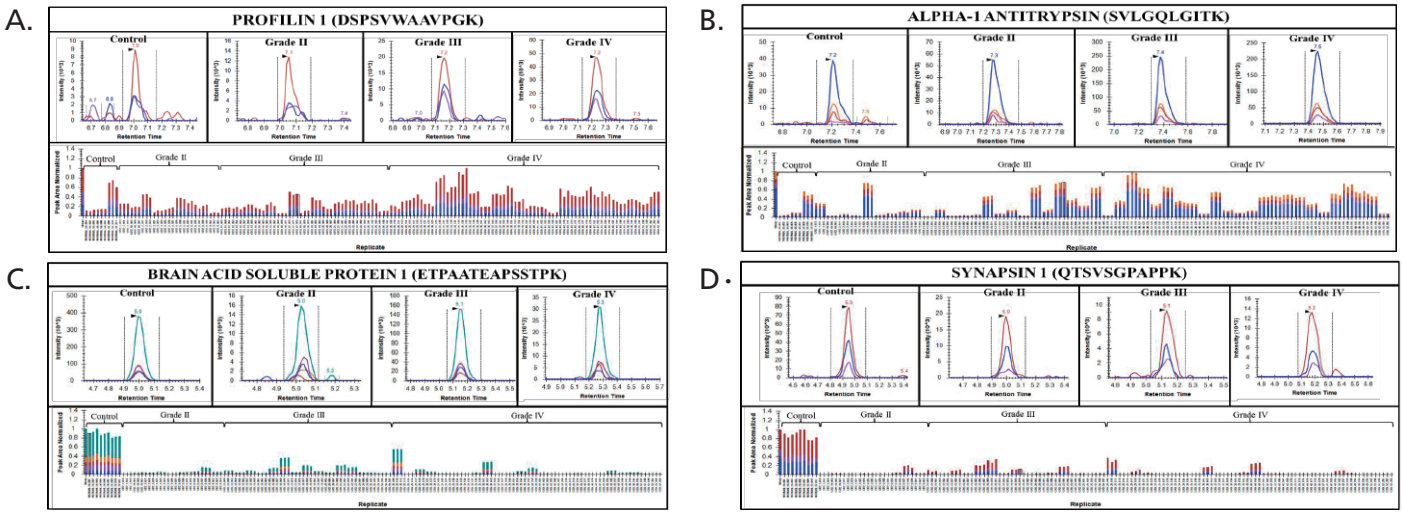

Figure 9. Representative spectra of some significantly altered proteins that were validated using multiple reaction monitoring (MRM) method. A & B) Represents peak areas for two of the up-regulated proteins i.e., Profilin 1 and Alpha-1 antitrypsin. C & D) Illustrative image for two down-regulated proteins (BASP 1 & Synapsin 1) in all the grades of tumor when compared to control samples

Statistical Analysis of Validation Phase Data

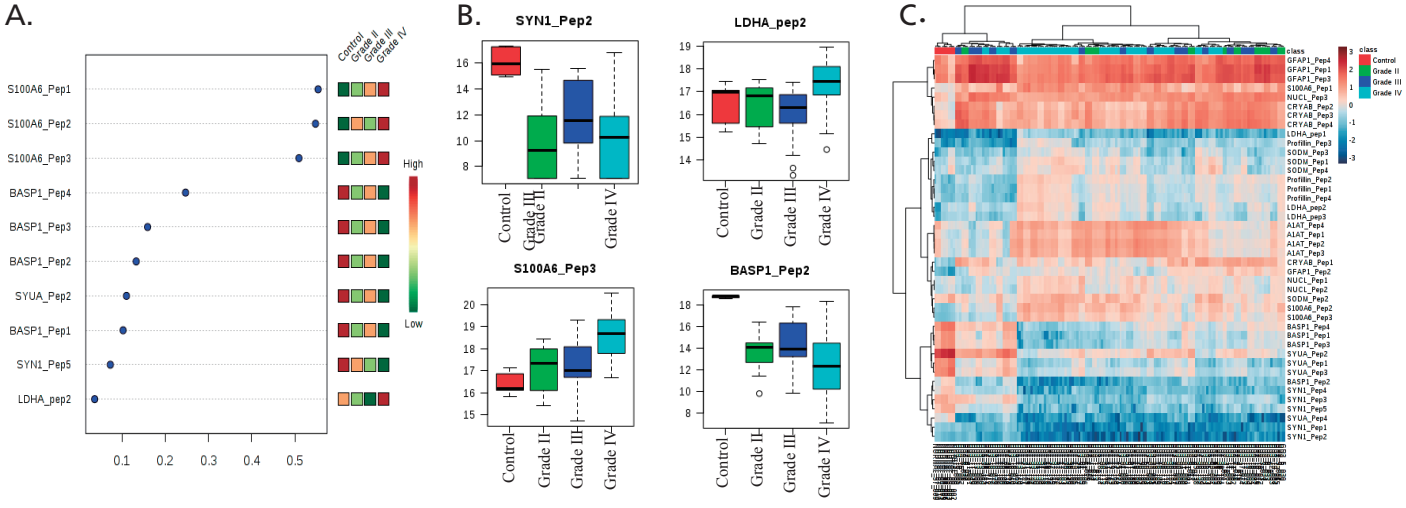

Figure 10. Cumulative peak areas for each peptide was subjected to statistical analysis using metaboanalyst 3.0 A) Dot plot representing the list of classifier peptides that could potentially differentiate between different grades of glioma. B) Box plots representing the peak areas for some of the significantly altered peptides across different grades of glioma. C) Heat-map representing the intensities of each peptides across all the glioma samples.

MRM based validation of protein biomarkers across different grades of glioma tissues

Conclusion

- This preliminary study was performed to study the pathophysiology of glioma.
- The study aimed to identify diagnostic and prognostic markers that could differentiate between grades of glioma.
- The validation of a few of the interesting candidates was performed using MRM approach. The results were in accordance to that of the discovery data obtained from shotgun proteomics experiments.
- Further validation on larger cohorts is needed to substantiate the findings.

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