



Identification of Population-Specific Novel Protein Biomarkers and Possible Therapeutic Targets in Gliomas by Proteomics Approach

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Abstract

Objective To analyze the differential proteomic profile of gliomas in patients from South India and to identify novel protein glioma biomarkers and possible therapeutic targets to tailor the treatment to individual patients.

Material and Methods We have prospectively analyzed the differential proteomic profile of 34 patients with glioma imaging characteristics and compared them with that of normal brain tissue. This research was conducted at the Institute of Neurosurgery, Madras Medical College, in technical collaboration with the Indian Institute of Technology, Madras, over 1 year.

Statistical Analysis Biological variate analysis (I-ANALYSIS OF VARIANCE (ANOVA)) was used, with *p*-value less than 0.05 being significant.

Results Twenty proteins (10 upregulated and 10 downregulated) were differentially expressed in tumor tissue. The expression of three pro-apoptotic proteins was downregulated and the expression of three anti-apoptotic proteins was upregulated with statistical significance. The cellular functions of the 20 differentially regulated proteins were subjected to pathway analysis revealing significant alterations in heme biosynthesis, deoxyribonucleic acid (DNA) replication, fibroblast growth factor (FGF) signaling, and epidermal growth factor (EGF0 receptor signaling in glioma.

Conclusion KRT18, PRS4, and EF1A2 are anti-apoptotic proteins and are significantly upregulated in gliomas. EARS2, COX5A, and LSM3 are pro-apoptotic proteins, and are significantly downregulated in gliomas. This subverts the apoptotic pathways resulting in prolonged cell survival. This study's statistically significant dysregulation of these six proteins was unique, suggesting that they might be considered population-specific biomarkers and possible therapeutic targets for patients from South India. Abnormalities of heme biosynthesis at the proteomic level were identified in this study, which has not been very well studied previously.

Keywords

- ▶ glioma
- ▶ proteomics
- ▶ protein biomarkers
- ▶ dysregulation of proteins
- ▶ therapeutic targets

Introduction

Brain tumors are now more frequently diagnosed, coinciding with the introduction of modern, newer imaging technology. Brain tumors represent approximately 85% of central

nervous system (CNS) tumors, with gliomas being the most common.

The age-adjusted global annual incidence of gliomas for females is 17.2 per 100,000, and for males, it is 15.8 per 100,000.¹ A glioma cancer survey registry in India has

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revealed the incidence of gliomas (per 100,000 population) to be 5.8, 6.7, 3.5, 5.6, and 28.2% among males, and 6.3, 5.6, 7.5, 0, and 21.8% among females, in the cities of Mumbai, Bangalore, Chennai, Dibrugarh, and Trivandrum, respectively.²

Today's optimal care for gliomas, which includes gross total resection, radiation therapy, and concomitant or subsequent chemotherapy, has helped improve the median survival in low-grade gliomas from 44 months in 1999 to 57 months in 2010. The benefits have been even more modest in glioblastoma multiforme (GBM), with patients living for an average period of 15 months after diagnosis, despite the development of newer modalities of imaging, early identification of high-grade lesions, development of newer chemotherapy agents and technical advancements in radiation delivery.

This failure can be ascribed mainly to the one mutation—one drug formula we have followed so far. This tunnel vision—a strategy to block only one or two of the mutated or downregulated cancer signaling pathways—has led to the failure of various therapeutic agents.

Recently, many markers and characteristic signatures have been identified as factors with prognostic and predictive value in GBM using newer techniques of molecular and genetic profiling. Not one of them has been successful in clinical practice. The reasons are varied. Although the initiating and important step in the expression of genes is transcription, resulting in the formation of messenger RNA (mRNA), the stability of mRNA and the resultant protein determines the protein levels.³ Alternate splicing of a gene results in the formation of multiple proteins. So, three or more proteins can be encoded by a single human gene. Proteins undergo modifications after translation, which regulate their functions by altering their interactions, turnover, and location. Proteins, as the functional units of cells, influence the genesis of cancer by varying their synthesis rate, levels, and interactions. Defective signaling pathways involving an entire network of proteins result in cancer development and progression.³ Moreover, the huge costs involved in setting up and managing the infrastructure and logistics for genomic labs, and the issues of their availability and accessibility preclude their routine use in the management of gliomas in this part of the world.

Therefore, there is an overwhelming need to develop newer molecular diagnostic tools to identify and develop more potential protein biomarkers from biopsies, serum, or plasma⁴ for the early detection of tumor growth, recurrence, and therapeutic response, and also for the delivery of personalized, individualized oncologic cure through precision medicine. The methods of protein extraction and quantification are relatively more economical and more widely available when compared to genetic labs.

Glioma Proteomics

Proteomics deals with the analysis of proteins produced by a cell, tissue, or organism at a specific time and in a specified

environment. Differentiating the proteomic profile of brain tumors vis-a-vis normal brain tissue enables one to detect and monitor the pathological changes by the discovery of potential protein biomarkers for the diagnosis, prognosis, prediction, or assessment of response to treatment of CNS neoplasia, particularly gliomas. This also helps develop possible therapeutic targets in the population under study.

Proteomic analysis can be done using glioma biopsy specimens, cerebrospinal fluid (CSF) and tumor cyst fluid, plasma/serum, cell lines of glioma, and animal models.⁵ The latter sources, though easy to collect, have several limitations like low biomarker concentrations, biomarker dilution in the peripheral blood, the presence of the blood–brain barrier, and the presence, in plasma, of proteins from the whole body (not from the CNS alone). These factors restrict their use in the discovery of biomarkers.⁶

So, tumor tissue analysis provides a direct approach to discovering glioma-specific protein biomarkers.⁶ Even tissue sampling has its difficulties and limitations that preclude its routine use as a tool for clinical diagnosis. These include its invasive nature, the inadequate volume of tissue obtained to perform statistical analysis, and inherent sampling errors.

These limitations could be circumvented by other methods of sampling, and proteomics permits analysis with lesser quantity of tissue. The understanding of tumor biology and tumor characterization is enhanced by tissue-derived biomarkers.⁵

Most studies for glioma biomarker discovery have been done using tumor specimens. Studies conducted in 2004^{7,8} identified many grade-specific biomarkers that were dysregulated in astrocytoma specimens when compared to normal tissue. The use of brain tumor specimens and nontumoral brain tissue to identify differential protein expression patterns was demonstrated by Schwartz et al.⁷ Using gel-based and mass spectrometry-based techniques, many studies have reported such differential protein profiling involving α B-crystallin, glial fibrillary acidic protein (GFAP), and many other proteins including Ki-67.^{9,10} Newer proteins like TIMP-1 (tissue inhibitor of metalloproteinases 1), neurofascin, and UCHL-1 (ubiquitin carboxy-terminal hydrolase-1) with possible grade specificity, and the known glioma markers—AnxA2 and calnexin—have been the subject of further functional assay verification.¹¹

Eighty-five tissue specimens (52 glioblastoma, 13 anaplastic astrocytoma, 10 diffuse astrocytoma, and 10 normal brain samples) were subjected to proteomic analysis by Iwadate et al, and 57 protein spots with potential for differentiating tumors from the normal brain were identified.¹² Immunohistochemical analysis confirmed that grade IV tumors exhibited increased expression of four proteins—VREB1, GRP78, RhoA, and Rac1—and decreased expression of enolase when compared to lower-grade tumors. Odreman et al, in 2005, analyzed 10 grade II and 10 grade IV specimens and identified 15 proteins that showed differential expression in these two grades.¹³ The proteins

Peroxiredoxin 1 and 6, the transcription factor BTF3, and R-B-crystallin were more highly expressed in GBM, as against GFAP, protein disulfide isomerase A3, and the cAMP-dependent protein kinase's catalytic subunit, which showed higher expression in low-grade astrocytomas. Using 2 dimensional electrophoresis (2DE) and Matrix Assisted Laser Desorption Ionization- Time of Flight (MALDI-TOF) spectrometry, Hiratsuka et al found 11 proteins to be upregulated and 4 to be downregulated in glioma.¹⁴ Glioma downregulation of SIRT2 was confirmed by Northern blotting. High levels of SIRT2 caused malfunction of the microtubule network and prevented colony stabilization, demonstrating its possible tumor suppressor function. Ngo et al endeavored to show that glioma cells that differed in their chromosome 1p status expressed proteins differently.¹⁵ They revealed that decreased expression of stathmin, a microtubule-associated protein, was related to loss of heterozygosity and longer recurrence-free survival in anaplastic oligodendroglioma patients. Patients with 1p^{+/-} anaplastic oligodendroglioma tumors with less stathmin expression had improved outcomes on treatment with nitrosourea, possibly due to an increased mitotic arrest in cells caused by decreased levels of stathmin. This was also confirmed in mice carrying xenograft tumors, suggesting an inverse correlation between overall survival and stathmin levels.

Other proteins could also alter the sensitivity of gliomas to chemotherapy. Galectin 1 has high expression in glioma cell lines. Puchades et al, using control U87 glioma cells or p53 adenovirus-infected cells treated with SN38, found that downregulation of galectin1 by p53 was further enhanced by SN38 treatment, suggesting that galectin 1 could be targeted for therapy.¹⁰

But all the studies mentioned earlier have been conducted with patients from developed Western countries.

In our study, using tumor biopsy specimen and employing recent proteomic techniques, we have endeavored to investigate the differential proteomic profile for glioma in South Indian patients as compared to normal tissue and, in the process, identify novel glioma protein biomarkers and possible therapeutic targets to tailor the treatment to individual patients.

Material and Methods

We have prospectively analyzed the differential proteomic profile of 34 patients with glioma imaging characteristics and compared them with that of normal brain tissue. This research was conducted at the Institute of Neurosurgery, Madras Medical College, in technical collaboration with the Indian Institute of Technology, Madras, over 1 year.

Inclusion Criteria

Patients with imaging characteristics of glioma admitted to the Institute of Neurosurgery, Madras Medical College, and Rajiv Gandhi Government General Hospital and who consented to participate in the study.

Exclusion Criteria

- Patients not consenting for surgery.
- Patients not consenting to take part in the study.

Forty patients with glioma imaging characteristics who consented to be involved in this study were included. Six patients were excluded from the study due to insufficient RNA extraction from the tumor sample, leaving 34 samples for proteomic analysis. The patients' demographic data, relevant past medical and family history, imaging characteristics, and operative details were recorded.

The tumor tissue specimen was bisected, and one-half was used for histopathological examination. The other half of the tumor was taken for proteomic analysis. In liquid nitrogen, the portion for proteomic analysis was snap frozen and stored at -80°C within 15 minutes of surgical resection and then transported to the lab. Non-neoplastic matched reference samples were obtained from patients with severe diffuse axonal injury who underwent decompressive craniectomy. The tissues were deposited at the National Cancer Tissue Biobank, Department of Biotechnology, Indian Institute of Technology, Madras, where proteomic analysis was performed using 2DE and tandem mass spectrometry (►Fig. 1). Proteins that were dysregulated (up- or downregulated) in tumor tissue when compared to normal tissue were identified. A mixture of identical amounts of all the samples to be analyzed was used as an internal control, and this allowed accurate measurement of differential protein expression, irrespective of the number of matched gels, and random matching of normal tissue and tumor samples in each gel.

The statistical significance of the dysregulation status was analyzed with biological variate analysis with *p*-value less than 0.05 considered statistically significant.

Results

The protein expression data were analyzed in 34 patients. There were 22 males and 12 females in the study group. The mean age of the patients was 39.6 years, and the range was from 14 to 65 years.

The patients were classified as grades I to IV based on the WHO grading. There were 2 patients in grade I, 5 in grade II, 6 in grade III, and 21 in grade IV.¹⁶

Twenty proteins (10 upregulated and 10 downregulated) were found to be expressed differentially in tumor samples in comparison to normal brain tissue in the same gels, as shown in ►Figs. 2–4.

With reference to the available database for glioma, correlation analysis of 20 differentially expressed target proteins revealed that upregulation of 3 (KRT18, PRS4, and EF1A2) of the 10 upregulated proteins and downregulation of 3 (EARS2, COX5A, and LSM3) of the 10 downregulated proteins were statistically significant (►Table 1).

The cellular functions for the 20 differentially regulated proteins were subjected to pathway analysis revealing significant alterations in heme biosynthesis (25%), DNA

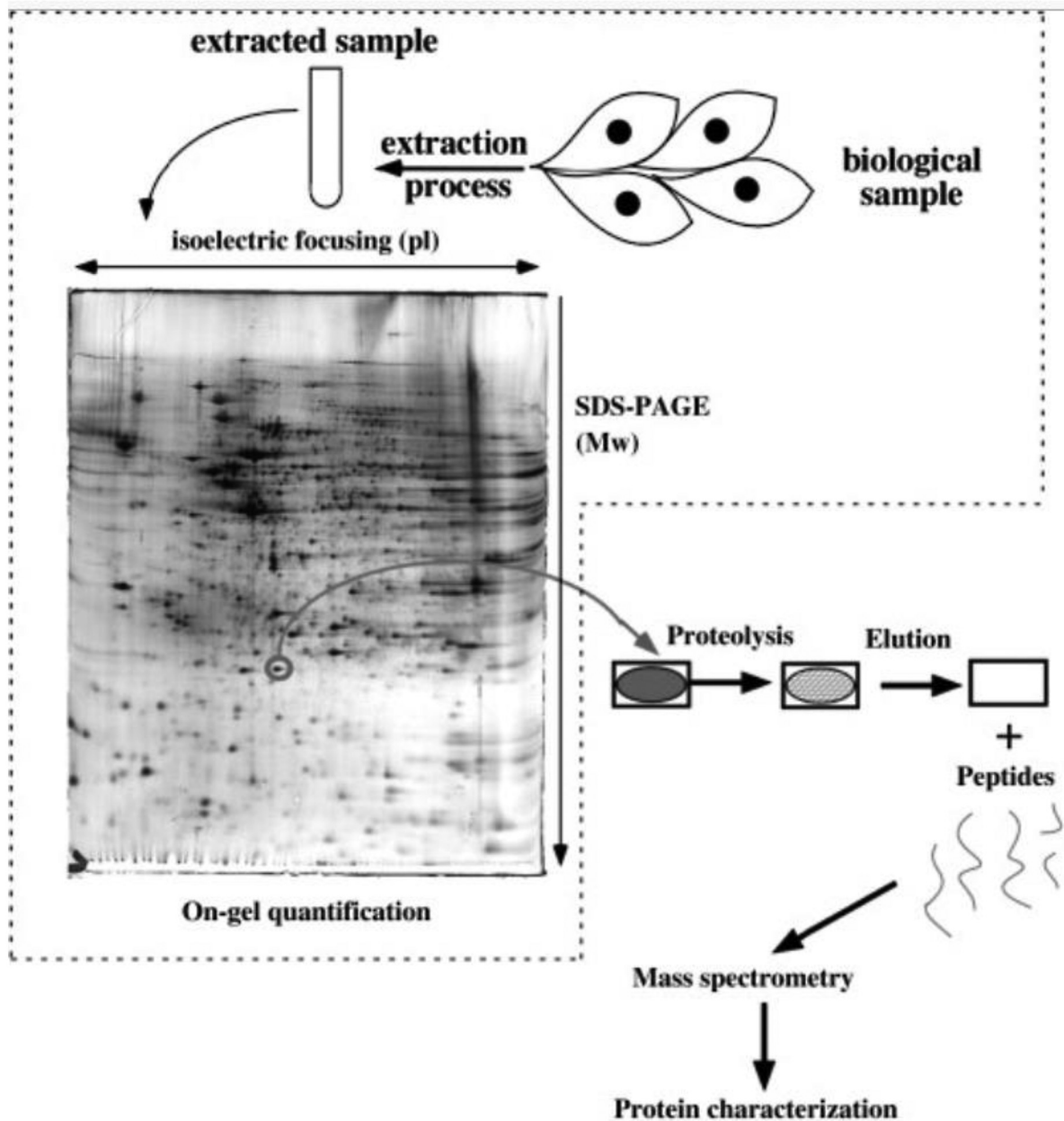


Fig. 1 Extraction of sample and identification of proteins.

replication (25%), FGF signaling (25%), and EGF receptor signaling (25%) in glioma.

Discussion

Our study used 2DE coupled with tandem mass spectrometry for proteomic analysis of glioma tumor samples and to identify differentially expressed protein signatures in glioma tumor samples. We identified 20 dysregulated proteins in glioma, which were validated by comparison with normal tissue specimens in the same gels.

Among 20 dysregulated proteins identified, 10 proteins, including phosphoglycerate phosphatase (PGP), Ras-related protein Rab-5C (RAB5C), KRT18, annexin A3 (ANXA3), 26S protease regulatory subunit 4 (PRS4), nitric oxide synthase

interacting protein (NOSIP), elongation factor 1-alpha 2 (EF1A2), NADH dehydrogenase iron-sulfur protein 4 (NDUS4), serine/threonine protein phosphatase 6 (PPP6), and aminoacyl tRNA synthase complex interacting multifunctional protein-1 (AIMP1), were upregulated in glioma.

In contrast, 10 proteins including notchless protein homolog 1 (NLE1), hemoglobin alpha-1 globin chain (HBA1), probable glutamate tRNA ligase (EARS2), proliferating cell nuclear antigen (PCNA), cytochrome-c oxidase subunit 5A (COX5A), uncharacterized protein (H7BZT4), GrpE protein homolog 1 (GRPEL1), pyridoxine 5'-phosphate oxidase variant (Q53FP0), LSM3, an arginase-2 (ARGI2), were downregulated in glioma in comparison to normal brain tissue samples.

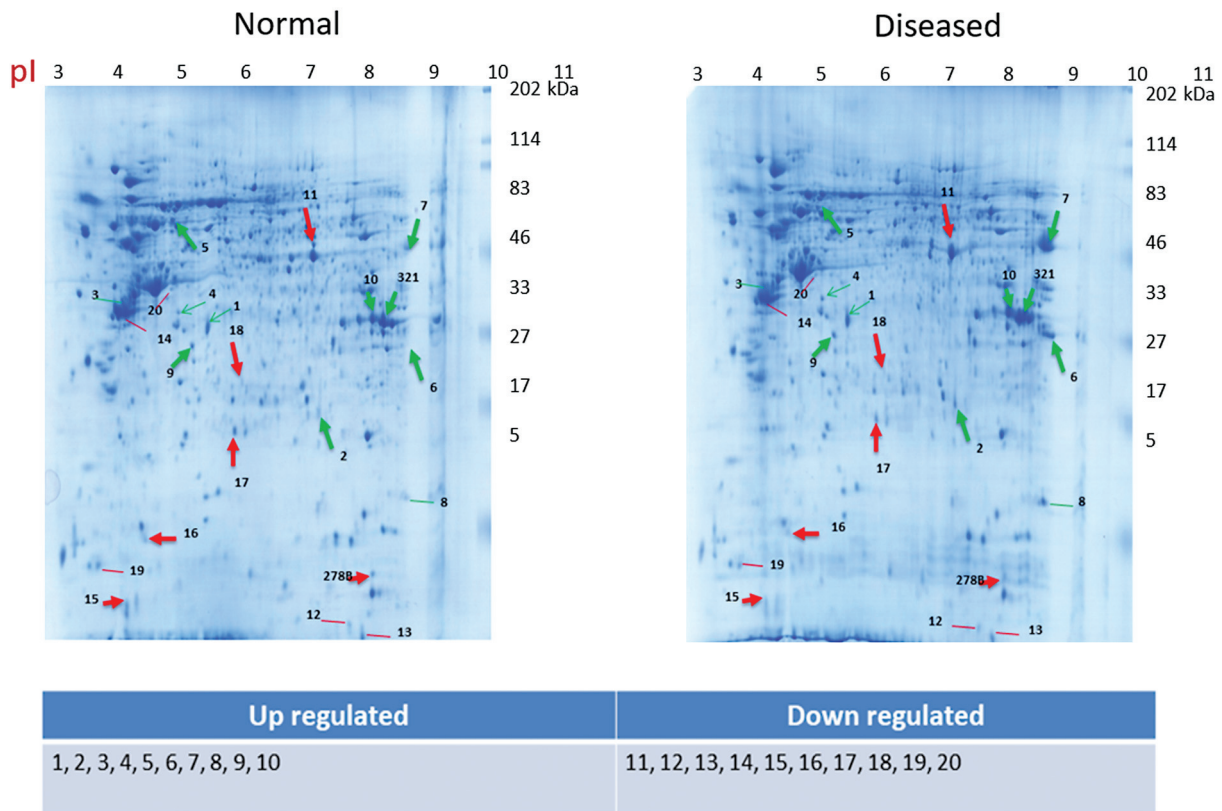


Fig. 2 Separation of proteins using two-dimensional (2D) electrophoresis. *Green arrows* indicate upregulated proteins and *red arrows* indicate downregulated proteins. *More prominent spots or darker spots* signify increased expression (upregulation), and *smaller or lighter spots* signify decreased expression (downregulation). For example, the spot for protein number 7 appears smaller and lighter in normal tissue when compared to the tumor tissue, implying upregulation in tumor tissue.

With reference to the available database for glioma, correlation analysis of the 20 differentially expressed target proteins revealed that upregulation of 3 (KRT18, PRS4, and EF1A2) of the 10 upregulated proteins and downregulation of 3 (EARS2, COX5A, and LSM3) of the 10 downregulated proteins were statistically significant (► **Table 1**), suggesting that these 6 proteins might be considered as population-specific biomarkers for the South Indian population because they have not been found to be statistically significantly dysregulated in any other study (► **Fig. 5**). These analyses highlight the importance of biomarker-based population screening and their potential to be used in future therapeutic strategies for better glioma management.

These results suggest that among the 20 dysregulated proteins modulating cell survival cascade, the statistically significant downregulation of pro-apoptotic proteins—EARS2, COX5A, and LSM3—and upregulation of anti-apoptotic proteins—KRT18, PRS4, and EF1A2—in glioma samples in comparison to matched normal tissue signify their action of promoting cell survival during tumorigenesis. Evasion of apoptosis, being a significant hallmark of cancer, can be used for targeted treatment to prevent tumor progression.¹⁷ Reduced expression of pro-apoptotic proteins and elevated expression of anti-apoptotic proteins result in prolonged cell survival by circumvention of the apoptotic pathways.

The cellular functions for the 20 differentially regulated proteins were subjected to pathway analysis, revealing significant alterations in heme biosynthesis, DNA replication, FGF signaling, and EGF receptor signaling in glioma. Although abnormalities of DNA regulation, EGFR signaling pathway, and FGF signaling pathway have been studied in detail previously, abnormalities of heme biosynthesis have not been so very well studied.

Conclusion

- This study has identified 20 dysregulated proteins modulating cell survival cascade in human brain gliomas. Out of these, six proteins (KRT18, PRS4, EF1A2, EARS2, COX5A, and LSM3) have not been previously reported as being statistically significantly dysregulated in brain gliomas, probably because they are unique to the test cohort, namely, South Indian population.
- KRT18, PRS4, and EF1A2 are anti-apoptotic and are significantly upregulated in gliomas.
- EARS2, COX5A, and LSM3 are pro-apoptotic and are significantly downregulated in gliomas.
- Abnormalities of heme biosynthesis have not been so very well studied previously. To our knowledge, this is the first

Up regulated

Spot ID	Accession	Description	Score	Coverage	MW [kDa]	calc. pI
1	A6NDG6	Phosphoglycolate phosphatase OS=Homo sapiens GN=PGP PE=1 SV=1 - [PGP_HUMAN]	34.89	41.12	34	6.14
2	P51148	Ras-related protein Rab-5C OS=Homo sapiens GN=RAB5C PE=1 SV=2 - [RAB5C_HUMAN]	36.83	59.72	23.5	8.41
3	I6L965	KRT18 protein (Fragment) OS=Homo sapiens GN=KRT18 PE=2 SV=1 - [I6L965_HUMAN]	72.74	56.72	42.1	5.1
4	P12429	Annexin A3 OS=Homo sapiens GN=ANXA3 PE=1 SV=3 - [ANXA3_HUMAN]	41.07	29.5	36.4	5.92
5	P62191	26S protease regulatory subunit 4 OS=Homo sapiens GN=PSMC1 PE=1 SV=1 - [PRS4_HUMAN]	96.38	54.55	49.2	6.21
6	Q9Y314	Nitric oxide synthase-interacting protein OS=Homo sapiens GN=NOSIP PE=1 SV=1 - [NOSIP_HUMAN]	54.55	46.18	33.2	8.82
7	Q05639	Elongation factor 1-alpha 2 OS=Homo sapiens GN=EEF1A2 PE=1 SV=1 - [EF1A2_HUMAN]	485.3	58.1	50.4	9.03
8	O43181	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial OS=Homo sapiens GN=NDUFS4 PE=1 SV=1 - [NDUS4_HUMAN]	51.35	61.14	20.1	10.3
9	O00743	Serine/threonine-protein phosphatase 6 catalytic subunit OS=Homo sapiens GN=PPP6C PE=1 SV=1 - [PPP6_HUMAN]	25.61	20.33	35.1	5.69
10	Q12904	Aminoacyl tRNA synthase complex-interacting multifunctional protein 1 OS=Homo sapiens GN=AIMP1 PE=1 SV=2 - [AIMP1_HUMAN]	44.04	55.77	34.3	8.43

Fig. 3 Spot ID, accession number, protein name, score, coverage %, number of covering peptides, molecular weight (calc MW), and calculated isoelectric point za (calc. pI) of the upregulated proteins.

study to discover heme biosynthesis abnormalities at the proteomics level.

- The usefulness of population screening for biomarkers and its potential use in future therapeutic strategies for enhanced and individualized glioma management is highlighted by this study.

Limitations of the Study

- Grade I gliomas that show different biological behavior could have been left out from the study.
- Grade-specific proteomic profiling for glioma grades II to IV was not performed in our study due to lesser number of samples of lower-grade gliomas. This would have improved the prognostication value of the study.
- The proteomic profile of the participants of our study was compared with that of the Western population. It would have been more relevant to have used participants from other parts of India for comparison.
- A larger cohort of participants would improve the statistical significance of the findings.

Future Directions

- Grade-specific proteomic profiling of gliomas will be a more useful diagnostic and prognosticating tool.
- Region-specific proteomic profiling would help better understand the differences in the biological behavior of these tumors.
- Many newer proteins dysregulated in glioma are being validated as brain cancer biomarkers through immunoblotting and immunohistochemical analysis. If they are successfully validated, their functional and biological characterization may allow them to be used as diagnostic and prognostic markers, as therapeutic targets, to predict responses to therapy, and also to identify new molecular targets for therapy. Further studies with larger cohorts are required to achieve this.

Ethical Approval Statement

The approval was obtained from the Institutional ethics committee.

Down regulated

Spot ID	Accession	Description	Score	Coverage	MW [kDa]	calc. pI
11	Q9NVX2	Notchless protein homolog 1 OS=Homo sapiens GN=NLE1 PE=1 SV=4 - [NLE1_HUMAN]	44.57	40.62	53.3	7.34
12	E9M4D4	Hemoglobin alpha-1 globin chain (Fragment) OS=Homo sapiens GN=HBA1 PE=3 SV=1 - [E9M4D4_HUMAN]	70.37	49	10.8	8.48
13	I3L166	Probable glutamate-tRNA ligase, mitochondrial OS=Homo sapiens GN=EARS2 PE=2 SV=1 - [I3L166_HUMAN]	125.6	18.75	12.4	10.42
14	P12004	Proliferating cell nuclear antigen OS=Homo sapiens GN=PCNA PE=1 SV=1 - [PCNA_HUMAN]	50.71	38.7	34.8	4.69
15	P20674	Cytochrome c oxidase subunit 5A, mitochondrial OS=Homo sapiens GN=COX5A PE=1 SV=2 - [COX5A_HUMAN]	587.71	61.33	16.8	6.79
16	H7BZT4	Uncharacterized protein OS=Homo sapiens PE=4 SV=1 - [H7BZT4_HUMAN]	241.71	34.74	10.8	5.5
17	Q9HAV7	GrpE protein homolog 1, mitochondrial OS=Homo sapiens GN=GRPEL1 PE=1 SV=2 - [GRPE1_HUMAN]	88.46	65.44	24.3	8.12
18	Q53FP0	Pyridoxine 5'-phosphate oxidase variant (Fragment) OS=Homo sapiens PE=2 SV=1 - [Q53FP0_HUMAN]	53.41	45.98	29.9	7.4
19	B2R5H5	cDNA, FLJ92476, highly similar to Homo sapiens LSM3 homolog, U6 small nuclear RNA associated (S. cerevisiae) (LSM3), mRNA OS=Homo sapiens PE=2 SV=1 - [B2R5H5_HUMAN]	149.4	66.67	11.8	4.7
20	P78540	Arginase-2, mitochondrial OS=Homo sapiens GN=ARG2 PE=1 SV=1 - [ARGI2_HUMAN]	58.83	57.06	38.6	6.47

Fig. 4 Spot ID, accession number, protein name, score, coverage %, number of covering peptides, molecular weight (calc MW), and calculated isoelectric point za (calc. pI) of downregulated proteins.

Table 1 Twenty differentially regulated proteins with name, statistical significance value, and regulation status obtained from the biological variate analysis

Sl. no.	Name	I-ANOVA/p-value	Regulation in tumor
1.	PGP	0.703	Up
2.	RAB5C	0.482	Up
3.	KRT18	0.012	Up
4.	ANXA3	0.135	Up
5.	PRS4	0.027	Up
6.	NOSIP	0.452	Up
7.	EF1A2	0.043	Up
8.	NDUS4	0.624	Up
9.	PPP6	0.675	Up
10.	AIMP1	0.171	Up
11.	NLE1	0.256	Down
12.	HBA1	0.335	Down
13.	EARS2	0.05	Down
14.	PCNA	0.685	Down
15.	COX5A	0.0056	Down

(Continued)

Table 1 (Continued)

Sl. no.	Name	I-ANOVA/ <i>p</i> -value	Regulation in tumor
16.	H7BZT4	0.189	Down
17.	GRPE1	0.815	Down
18.	Q53FPO	0.230	Down
19.	LSM3	0.00096	Down
20.	ARGI2	0.464	Down

Note: The proteins shown in bold fonts are statistically significantly dysregulated.

KRT 18

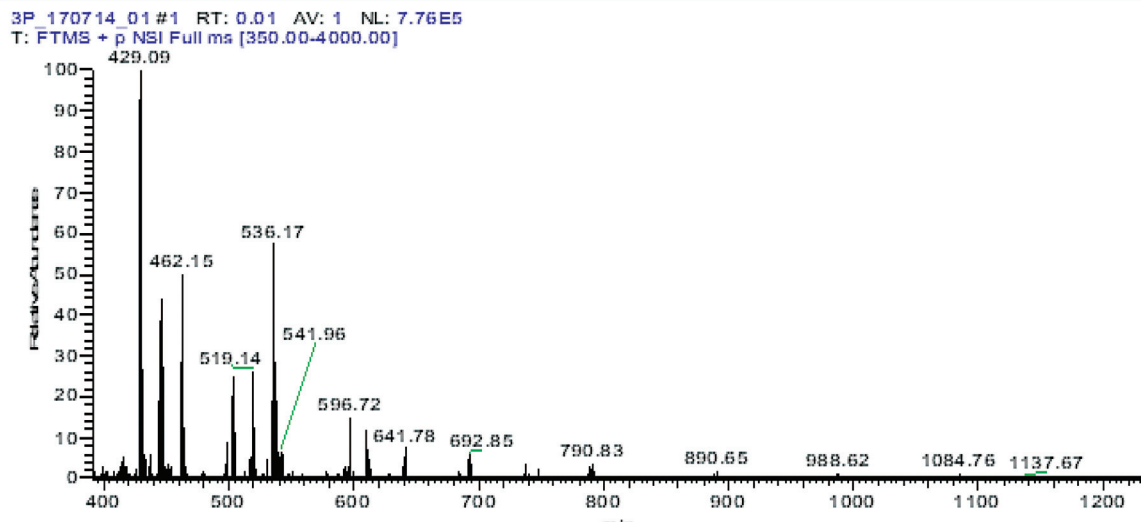
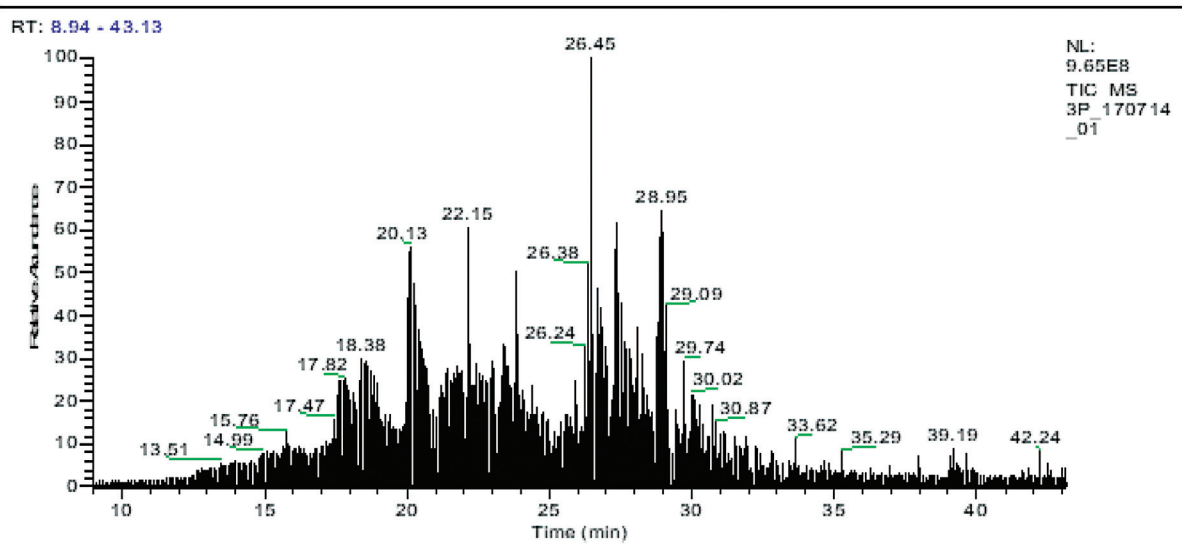


Fig. 5 Spot identification of protein KRT 18 showing good spectrum (as an example).

Conflict of Interest
None declared.

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