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REVIEW ARTICLE

Glioma Pathophysiology: Insights Emerging from Proteomics

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Abstract

Proteomics is increasingly employed in both neurological and oncological research to provide insight into the molecular basis of disease but rarely has a coherent, novel pathophysiological insight emerged. Gliomas account for >50% of adult primary intracranial tumors, with malignant gliomas (anaplastic astrocytomas and glioblastoma multiforme) being the most common. In glioma, the application of proteomic technology has identified altered protein expression but without consistency of these alterations or their biological significance being established. A systematic review of multiple independent proteomic analyses of glioma has demonstrated alterations of 99 different proteins. Importantly 10 of the 99 proteins found differentially expressed in glioma [PHB, Hsp20, serum albumin, epidermal growth factor receptor (EGFR), EA-15, RhoGDI, APOA1, GFAP, HSP70, PDIA3] were identified in multiple publications. An assessment of protein–protein interactions between these proteins compiled using novel web-based technology, revealed a robust and cohesive network for glioblastoma. The protein network discovered (containing *TP53* and *RB1* at its core) compliments recent findings in genomic studies of malignant glioma. The novel perspective provided by network analysis indicates that the potential of this technology to explore crucial aspects of glioma pathophysiology can now be realized but only if the conceptual and technical limitations highlighted in this review are addressed.

INTRODUCTION

In 1990 the Human Genome Project formally began its epic 13-year task to elucidate the entire human genome, which we now know harbors approximately 20 400 genes. The impact has been enormous, with new avenues being opened into the diagnosis of disease, gene therapy, and earlier detection of genetic predispositions. Now, nearly 20 years on, the Human Proteome Project [HPP; coordinated by The Human Proteome Organization (HUPO)] is instigating a plan to identify one protein (excluding post-translational modifications, isoforms and splice variants) for each of the estimated human genes. This is of particular importance as genes are life's "blueprint," proteins represent the "bricks and mortar" from which it is built (70) and modifications or mutations at the level of the genome manifest as aberrations of the proteome.

The utility of proteomics (the large-scale study of multiple proteins) in cancer biology and central nervous system (CNS) disorders is currently one of the most dynamic research areas in medicine (4, 41, 73, 89). The pursuit for disease biomarkers, insights into biological pathways and translating proteomic findings into novel therapies has led to a proliferation of journals addressing aspects of these topics. The generation of large volumes of proteomic data, characterized by long lists of proteins, has also resulted in analogous studies on criteria for quality control and standards required for proteomic studies (20, 46). The need for caution in translating the plethora of proteomic data into functional significance has also been addressed. A sobering paper by Petrak and colleagues (2008) (58) demonstrated that, by analyzing the findings from 169 proteomic studies published in the journal *Proteomics* during the period 2004–2006, there were many commonly identified proteins found differentially expressed irrespective of the nature of the background disease. The ubiquity of these proteins was considered to arise from cellular stress responses and the limitations of two-dimensional gel electrophoresis (2DGE). Moreover, two other key areas of caution are the successful application of mass spectrometry (MS) and statistical analysis to ensure that proteins are correctly identified. HUPO have highlighted that most errors in MS based analyses arise not because the present technology is unable to identify specific proteins but because software programmes often misidentify them (70).

The application of proteomics specifically to gliomas is appealing since there has been very limited progress in treatment of malignant glioma in the last 25 years (2). Proteomic studies in gliomas remain limited in number and are characterized by lists of proteins found to be either up- or downregulated in tissue specimens compared to normal brain (19, 28). The utility of proteomics in glioma has recently been the subject of two reviews (10, 82) but neither of these articles explored in any detail the specific proteins found to be different in gliomas. The importance of attempting to derive some coherence from the glioma proteomic literature is

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perhaps even more pressing given the recent publication from The Cancer Genome Atlas Research Network (76). This comprehensive study outlined several pivotal, aberrant genetic pathways found in glioblastoma (GBM) and may provide some direction and focus for proteomic studies in human GBMs. Proteomic data needs to be presented in a way that either confirms or refutes the genetic data. Experiences from other types of malignant tumor (24, 44, 87) suggest that it is highly unlikely that a single GBM biomarker will be identified. However, functional analysis of proteomic data may provide insights into differentially regulated biological pathways that underpin aspects of the pathophysiology of this highly malignant tumor (13). In glioma (and in many other neurological diseases and cancer), a glut of proteomic data have been generated but there has been no unitary approach to establish whether key proteins and/or signaling pathways have been identified. In an attempt to evaluate the current utility of glioma proteomic data, we have performed a systematic review of glioma proteomics to date across multiple publications. Indeed, on cursory evaluation of the data there appears a complete lack of coherence as the validity, reproducibility and comparability of these studies is bedevilled by methodological, analytical and statistical differences. Whether such disparate data can then translate into diagnostic, predictive or prognostic biomarkers and novel therapies is a key question.

In this article we demonstrate a novel and fresh perspective, synthesized from the published glioma proteomic literature. We have performed a systematic review of all the proteins identified in glioma proteomic studies, highlighting where there is commonality between the datasets. We address the robustness and reproducibility of the data and whether any substantive conclusions can be drawn between and from this published series. In particular, we have determined if any coherence can be drawn from the noticeable disparate data published to date, to highlight some putative biological pathways. We have listed all the differentially expressed proteins described so far in human gliomas and have constructed a hierarchy of the most frequently described proteins. We have examined if there is any differential clustering of these proteins to certain parts of the 2D gel from which the proteins were identified to determine if the listed proteins are merely a reflection of 2DGE limitations (58). Finally, we have used sophisticated bioinformatics software to perform functional analyses to determine putative common biological pathways that incorporate these proteins. This analysis will determine whether such an approach adds value to a simple proteomic list. Additionally the findings are compared with biological pathways highlighted as abnormal in glioma, recently described in a genomic study published by The Cancer Genome Atlas Research Network (76). We also discuss how proteomic methodology may be best applied to glioma research in the future.

METHODS

Systematic review of human glioma proteomic literature

Using the search terms "glioma," "glioblastoma" and "proteomics" we identified 10 peer-reviewed articles reporting differential protein expression via a range of proteomic technologies (see Search Strategy and Selection Criteria). From each publication the sample size (ie, the number of tumors, "n"), sample origin, tumor pathology [utilizing the World Health Organization (WHO) system], age range of subjects, method of tissue analysis, use of "control" tissue, differential expressed proteins, function (where documented) and protein accession number were extracted. A comprehensive spreadsheet was constructed containing all of the aforementioned data (see Tables 1 and 2).

Critical appraisal of differentially expressed proteins in glioma: their location on 2D gels

In proteomic analysis, the same proteins are repeatedly identified as altered in a range of biological conditions (58). This reflects in part their functional roles (specifically the involvement of a single protein in numerous diverse cellular processes) and technical issues involving the separation and identification of proteins with particular molecular weights and isoelectric charge (pI). For example, in our own laboratory, 100% of the proteins significantly altered in a study of apoptosis induced by staurosporine, were of medium/high molecular weight (32.9–94.3 kDa) and of low pI (4.7–6.4) (71).

The extent to which proteins of particular molecular weights or charge disproportionately contribute to proteomic analysis of glioma, based on 2DGE, was examined. To determine if the proteins found differentially expressed in our systematic review were positioned randomly on a 2D gel or clustered to one part of the gel (which might indicate technological bias), an *in silico* gel was constructed that was divided into four quadrants. The *x*-axis was assigned a linear scale as the distribution of proteins in IPG strips in the first dimension of 2DGE is predominantly linear. In contrast a logarithmic scale was chosen for the *y*-axis because this most closely resembles the pattern of vertical protein migration in gels during the second dimension of 2DGE. The quadrants were defined as: I (30–100 kDa and 3–6.5 pH), II (30–100 kDa and 6.5–10 pH), III (10–30 kDa and 6.5–10 pH) and IV (10–30 kDa and 3–6.5 pH).

Critical appraisal of differentially expressed proteins in glioma: protein classification and protein–protein interactions

To ascertain whether the proteins found differentially expressed in glioma (listed in Table 2) were involved in common biological functions, protein accession numbers were entered into Protein Analysis Through Evolutionary Relationships (PANTHER) Classification System (http://www.pantherdb.org). PANTHER categorized all the proteins found altered in glioma into prespecified protein functional classes.

To gain further insight into whether functional protein–protein interactions existed amongst the list of proteins found altered in glioma, proteins were investigated using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Mountain View, CA, USA, http://www.ingenuity.com). IPA is web-based software that comprises a huge knowledge database [Ingenuity Pathways Knowledge Base (IPKB)] of biological and chemical information extracted from the literature. The accession numbers of all proteins found differentially regulated in GBM (WHO grade IV glioma) compared with control brain, were entered into the application and after critical assessment of the protein list, a non-prejudicial protein interaction network (showing direct interactions and associations only) was generated. The network was scored and ranked according to the inclusion of as many of the proteins that were inputted as possible.

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Table 1. Overview of the glioma proteomic literature. Abbreviations: 2DGE = two-dimensional gel electrophoresis; GBM = glioblastoma; AA = anaplastic astrocytoma; AO = anaplastic oligodendroglioma; oligo = oligodendroglioma; DNET = dysembryoplastic neuroepithelial tumor; MALDI-TOF = matrix-assisted laser desorption ionization—time of flight; nano LC = nanoliquid chromatography; SELDI-TOF = surface enhanced laser desorption ionization—time of flight.

Author/reference	n	Pathology who grade	Age range	Sample origin	Method	Control
Chakravarti *et al* (5)	94	56 GBMs 13 AAIII 25 low-grade gliomas	Not specified	US	Western blot analysis	Lysates from 16 week old fetuses
Hiratsuka *et al* (28)	5	2 GBM 2 Grade III 1 Grade I	Not specified	JAPAN	2DGE and MALDI-TOF	Same patients from which the tumors were taken.
Hobbs *et al* (29)	4	4 GBM	Not specified	US	SELDI-TOF-MS	None
Schwartz *et al* (67)	18	4 GBM 2 Oligo Grade II 2 AO Grade III 2 Embryonal carcinoma 1 Pheochromcytoma 1 DNET 1 Gemistocytic astrocytoma, grade II	3 days–80 years (includes control and tumor samples)	US	MALDI-MS	Temporal lobe specimens from epilepsy surgery.
Iwadate *et al* (35)	85	52 GBM 13 AA Grade III 10 Astrocytomas (Grades I and II) 10 normal brain	Not specified	JAPAN	2DGE and MALDI-TOF	Tissue from patients undergoing surgery or for epilepsy.
Furuta *et al* (19)	13	6 GBM (Primary)s 7 progressive GBMs	Not specified	US	2DGE	None
Odreman *et al* (54)	20	10 Grade II 10 GBM	Not specified	ITALY	2DGE, LC-ESI-MS and Western blot analysis.	"peri-lesional tissue" from all patients
Schwartz *et al* (68)	127	29 Grade II glioma 22 Grade III glioma 57 GBM	Not specified	US	MALDI-TOF	19 patients undergoing surgery for "non-neoplastic disease".
Chumbalker *et al* (11)	27	2 Grade I 1 Grade II 14 Grade III 10 GBM	3–65 years (includes control and tumor samples)	INDIA	2DGE and MALDI-TOF	Tissue from patients undergoing surgery for epilepsy.
Mustafa *et al* (52)	20	10 GBM 10 controls	Control: 24 weeks–76 years Tumor: 30–57 years	HOLLAND	Nano-LC prior to MALDI-TOF/TOF	samples from different patients with a variety of CNS conditions.

This table summarizes details of the clinical samples and methodology used in each of the publications identified from the literature that use proteomics to study glioma, in accordance with our "Search Strategy and Selection Criteria" (samples size "n"; sample origin, tumor pathology utilising the World Health Organization grading system, age range of subjects, method of tissue analysis and origin of "control" tissue).

Methodology: search strategy and selection criteria

A comprehensive review of the published literature was performed by searching PubMed, using the search items "proteomics," "gliomas" and "glioblastomas," to identify all peer-reviewed articles reporting differential protein expression via a range of proteomic technologies (eg, MALDI-TOF) up to October 2008. Papers were also identified from the authors' own files, and references from relevant articles. Only papers published in English were reviewed. All studies that were selected pertained to in vivo samples taken from human gliomas. Eleven studies were identified. One was discarded because of an eminent senior scientist disassociating himself from the publication (38).

RESULTS

What proteomic data have been generated in human glioma and where does this data originate from?

General comment

A total of 99 differentially expressed proteins were identified from 10 peer-reviewed articles (5, 11, 19, 28, 29, 35, 52, 54, 67, 68). These selected papers vary considerably in terms of sample size, tumor type, age range and control tissue used (see Table 1). The number of tumor specimens analyzed across the series of publications ranges from 4 to 127. In addition, a broad age range of

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Table 2. List of proteins highlighted in our systematic review of the glioma proteomic literature.

Protein name	Accn No.	Upregulated	Downregulated	Reference
Prohibitin (PHB)	P35232	Gliomas (5), GBM (28)	Grade III (11)	(5, 11, 28)
Alpha B crystallin (CRYAB/HSP20)	P02511	Grades I and II (28), GBM (19)	Grade III (67)	(19, 28, 67)
Serum albumin	NP_004468	Gliomas (5), Grade III and GBM (11)		(5, 11)
Epidermal growth factor receptor (EGFR)**	P00533	GBMs (54), GBMs (primary) (68)		(54, 68)
Phosphoprotein enriched in astrocytes (EA-15)	Q15121	Grades II and III (5)	Gliomas (29)	(5, 29)
RhoGDP dissociator inhibitor (RhoGDI)	P52565		Grade III (11, 11), GBM (11, 11)	(11, 11)
Apolipoprotein A1 (APOA1)	P02647	Gliomas (5), GBM (67)		(5, 67)
Glial fibrillary acidic protein (GFAP)	P14136	Grade II (67), Grade III (11)		(11, 67)
Heat shock 70 kDa protein 5 (HSP 70/HSPA5)	P11021	GBM (28)	Grade III (11)	(11, 28)
Protein disulfide isomerase 3 (PDIA3)	P30101	Grade I (28), Grade II (67)		(28, 67)
ADAMTS-19	19171152	GBM (secondary)		(68)
Beta-actin	P02570	GBMs		(67)
Cadherin related tumor supressor	Q14517	GBMs (secondary)		(68)
Fibronectin	P07589	Gliomas		(52)
hTRT**	CAE75638	GBMs		(54)
Calcyclin	P06703	GBMs		(29)
CREB1	P16220	GBMs		(28)
CDK4**	CAG4703	GBMs		(54)
CDKN1A	P38936	Grades I and II		(28)
Cyclin E1**	NP_001229	Gliomas		(54)
E2F-1	NP_005216		In 55% gliomas	(54)
HNRPA3	P51991	GBMs (secondary)		(68)
Transcription factor BTF3	Q13890	GBMs		(67)
WNT II protein precursor	20532422	GBMs (secondary)		(68)
SIRT2	NP_085096		Gliomas	(5)
Copine I	NP_003906	Gliomas		(5)
CRMP-4	NP_001378	Gliomas		(5)
Dynein light chain 2	Q96FJ2	LTS group		(29)
Ezrin	P15311	Grade III and GBMs		(28)
NAPG	NP_003817		Grade III	(11)
Neurocalcin delta	NP_114430	Gliomas		(5)
Protein kinase C gamma	P05129	Grade III and GBMs		(28)
Tropomodulin 2	NP_055363		Grade III	(11)
Tropomycin	NP_003281		GBMs	(11)
Tropomycin 4	NP_003281	Grade III	GBMs	(11)
Tubulin specific chaperone A	O75347	GBM		(29)
beta tubulin	gil223486		GBMs	(11)
Profilin 2	NP_444252		Gliomas	(5)
Vimentin	NP_003371	GBM		(11)
Tenascin X precursor	P22105	GBMs (primary)		(68)
Alpha-internexin	Q16352	GBM		(67)
Adenosine deaminase	1001165a		Grade III	(11)
ATP synthase	P06576		GBMs	(11)
ATPase	NP_001686		Grade III	(11)
Beta synuclein	NP_003076		Grade III	(11)
Calpactin 1 light chain	P08206	GBM		(29)
cAMP depended protein kinase	P17612	Grade II		(67)
Catechol-O-methytransferase	NP_000745	Gliomas		(5)
Cathepsin D precursor	P07339	Grade III and GBMs		(28)
Dihydropteridine reductase	P09417	Grade II		(67)
DUOX2	NP_054799	GBMs (secondary)		(68)
Enolase 1	AHH27725	GBMs (primary)		(68)
ERK kinase 1	Q02750	Grade III and GBMs		(28)
Fatty acid binding protein 5	Q01469	Grade III		(29)
Fatty acid binding protein 7	NP_001437	Gliomas		(5)
Glutamate dehydrogenase 1	P00367	Grades I and II		(28)
Glutathione S transferase	P09488	Grade III and GBMs		(28)
Glutathione S transferase P	P09211	Grades I and II		(28)

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Table 2. *Continued.*

Protein name	Accn No.	Upregulated	Downregulated	Reference
human mitochondrial aldehyde dehydrogenase	gil6137677		GBMs	(11)
Nucleolar GTP binding protein 1	Q9BZE4	Grade III and GBMs		(28)
Peroxiredoxin 1	Q06830	GBMs		(67)
Peroxiredoxin 6	P30041	GBMs		(67)
Phosphoglycerate mutase 1	P18669	Grade III and GBMs		(28)
Phosphopyruvate hydratase	P06733	Grades I and II		(28)
Phosphoserine phosphatase	NP_004568	Gliomas		(5)
Plasminogen activator inhibitor 1	P05121	Grade III and GBMs		(28)
RAB3A	P20336	Grade III and GBMs		(28)
Rac 1	P15154	GBMs		(28)
Rho A	P06749	GBMs		(28)
Tyrosine tryptophan mono-oxidase	NP_036611		Grade III	(11)
Ubiquitin carboxy-terminal hydrolase L1	gil4185720		GBMs	(11)
Ubiquitin protein	P09936	Grade II		(67)
Annexin A5	P08758	GBMs		(67)
Annexin II	P07355	Grade III and GBMs		(28)
Annexin IV	P09525	Grade III and GBMs		(28)
Annexin V	gil999926	Grade III		(11)
T complex protein I	P48643	Grade II		(67)
Colligin 2	P50454	Gliomas		(52)
HSP 27	P04792	Grades III and IV		(28)
HSP 60	P10809		Grade III	(11)
UCH-L1	P09936		Gliomas	(5)
Oncogene DJ1	NP00116849		Grade III	(11)
p14**		GBMs		(54)
p16		GBMs		(54)
p53	P04637	GBMs		(54)
Centrosome associated protein 350	18378735	GBMs (primary)		(68)
ERCC6	15834617	GBMs (secondary)		(68)
Eukaryotic initiation factor 4A	P04765	Grade III and GBMs		(28)
PTEN	NP_000305	GBMs		(54)
Tumor suppressor pRB	P06400		GBMs	(54)
Fribrinogen fragment D	2781208	GBMs		(67)
alpha-antitrypsin	P01009	GBMs		(11)
Ferritin	AAH16715	Grade III		(11)
Hemopxin	NP_000604	Gliomas		(5)
Synaptosomal associated protein 25	NP_003072		Grade III	(11)
Transthyretin	NP000362	Gliomas		(5)
Sorcin	NP_003121		GBMs	(11)
Unamed protein	16552261	GBMs (primary)		(68)

This all the proteins (99 in total) that were reported in the literature as altered in expression level in glioma. This data was extracted from the publications summarized in Table 1. It is important to be aware of two main limitations of the data: (1) the certainty of protein identification varies across studies (some studies report the probability of a definitive protein match and others do not); (2) the validation of identified proteins varies across studies (perhaps due to availability of adequate antibodies for validation of identified proteins by other methodologies such as immunocytochemistry, yet validation is essential to confirm true differences in expression). The proteins presented in this table are: (i) identified by name and accession number (Accn No.); (ii) shown as up-regulated or down-regulated in expression level in tumor compared to control tissue; and (iii) shown as to which grade of glioma their altered expression level is reported in. In addition, the data collated is cross-referenced to the article(s) from which the data was extracted. The 10 proteins found altered in two or more publications (PHB, CRYAB, serum albumin, EGFR, EA-15, RhoGDI, APOA1, GFAP, HSP70, PDIA3) are shown in bold at the top of the table, and proteins marked ** are proteins that have been shown in previous studies to predict poor patient survival.

samples was used in several studies (for example, Schwartz *et al* (67) used tissue from 3-day- to 80-year-old patients), whereas others do not stipulate an age range.

Spectrum of proteomic methodology

Proteomic methodology also varies between papers from simple Western blot analysis to 2DGE ± matrix assisted laser desorption ionization—time of flight (MALDI-TOF) MS, surface enhanced laser desorption ionization—time of flight (SELDI-TOF) MS or nanoliquid chromatography (Nano LC) with tandem mass spectroscopy. In addition the choice of protein stain, for example Coomassie stain (11), Silver stain (19, 35) or Sypro Ruby (28) [three stains with very different dynamic ranges; see (81)] varies together with the inclusion criteria of the size of proteins studied (ie, 20–120 kDa).

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Differences in control tissue

There is also no consistency with control tissue used, since three studies use specimens from epilepsy patients (11, 35, 67), whereas others utilize "normal" peri-lesional tissue from tumor patients (28, 54) or "normal" tissue from patients with a variety of other CNS conditions (52, 68). These differences give rise to very different experimental comparisons as epileptic tissue may be gliotic and peritumoral tissue may be infiltrated by tumor cells (such as GBM, oligodendroglioma or astrocytoma tumor cells).

Differences in comparisons of glioma proteomic data

Some of the studies exclusively investigate differential protein expression in GBMs versus control (52), whereas others pool different grades of tumor and compare these pooled mixed gliomas versus controls (5, 11, 28, 35, 67) or compare "low-grade" gliomas to that of "high-grade" tumors (54). Moreover Furuta *et al* (19) compare primary GBMs (GBMs that arise *de novo*) with secondary GBM (GBMs that progress from lower grade gliomas). These differences in experimental design almost certainly contribute to the disparate lists of proteins generated in glioma proteomics.

Differences in the purification of samples

Methodological variation in tissue sample preparation also exists. The majority of the proteomic studies use whole cell lysates generated from gross dissection of the control and tumor specimens (5, 11, 28, 29, 35, 54). However, Furuta *et al* (19) strived for purer populations of tumor cells by employing selective tissue microdissection prior to 2DGE. Selective tissue microdissection was based on a 10-μm-thick tissue section (adjacent to the section used for microdissection) stained for hematoxylin and eosin to identify areas not compromised by inflammation, necrosis or stromal or endothelial proliferation. Mustafa *et al* (52) also employed a purification step and specifically microdissected glioma blood vessels for investigation by MS. The proteome identified by Mustafa *et al* (52) was therefore confined to glioma blood vessels and not representative of all proteins altered in glioma tumors.

Differences in statistical methodology

Statistical analysis in glioma proteomic studies to date, range from almost nothing to complex bioinformatic paradigms (13). Moreover, the complex statistical paradigms that are used are diverse and include methodology such as hierarchical cluster analysis (35, 67), symbolic discriminate analysis (SDA) and weighted flexible compound covariate method (WFCCM) (68).

Proteins identified as differentially expressed in gliomas

Despite differences in experimental design, methodology and analysis all of the proteins reported as differentially regulated in glioma tumors were collated (see Table 2). Out of these 99 proteins, 10 proteins [PHB, CRYAB, serum albumin, epidermal growth factor receptor (EGFR), EA-15, RhoGDI, APOA1, GFAP, HSP70 and PDIA3] were found altered in multiple proteomic studies of glioma (see Table 2). The most commonly identified proteins were Prohibitin (PHB) and Alpha B crystalline (CRYAB), which were found differentially expressed in three distinct studies. Prohibitin is a highly conserved, multifunctional protein, which is found localized to mitochondria and nuclei (47, 48). Overexpression of prohibitin has been reported in human bladder tumors (3), prostate cancer (77) and thyroid carcinomas (17), suggesting that this protein may be widely involved in tumorigenesis. Prohibitin has also been shown to control Ras-Raf signaling, a major signaling pathway involved in cell growth and malignant transformation (60, 61). In gliomas, prohibitin has been reported to be both up- and downregulated [(11, 28, 35); see Table 2], a finding that in the first instance seems unclear. However, it is thought that prohibitin may exert different roles in tumorigenesis, having either a permissive action on tumor growth or a tumor suppressor role, depending on the cellular context and/or stage of tumorigenesis (47, 48).

CRYAB is a constitutively expressed molecular chaperone and member of the evolutionary conserved heat shock protein superfamily. CRYAB has also been found regulated in several different types of cancer (9, 30, 55, 56) and may reflect a general cellular stress response to tumorigenesis.

Additional comments about the top 10 proteins identified in glioma

The up-regulation of serum albumin and apolipoprotein A1 in gliomas is thought to reflect the ability of both these proteins to pass into the interstitium of malignant gliomas because either the blood brain barrier has broken down and/or the tumor capillaries have no BBB (69). It is also reassuring to find both EGFR and GFAP among the top 10 proteins identified in glioma. It has been widely recognized that EGFR is amplified and can be mutated in malignant gliomas (42) and the upregulation of GFAP is considered a fundamental and diagnostic ICC feature of glioma (15). In contrast, the remaining proteins from the list of top 10 have a range of different functional roles and it is not known whether or how they play a role in gliomagenesis. It is important to note that RhoGDI, has been frequently reported as regulated in proteomic studies regardless of the biological process and species studied (58).

Comments about the entire list of proteins found differentially expressed in gliomas (the 99 proteins)

In total, eight out of the total 99 differentially expressed proteins identified in glioma (Table 2) have been reported by Petrak *et al* (2008) (58) as proteins commonly identified in proteomic studies regardless of experiment, tissue or species (these proteins are: RhoGDI, vimentin, ATP synthase, cathepsin D precursor, enolase 1, peroxiredoxin 1, annexin 4 and HSP27). Aside from these nonspecific findings, however, several important, key putative players in glioma pathophysiology are listed. In particular tumor suppressor proteins p53, phosphatase and tensin homolog (PTEN) and p14 have been identified. Furthermore ferritin, previously reported as regulated in malignant gliomas (31), has been identified.

A critique of the proteins of interest in glioma with respect to 2DGE

To determine whether the differentially expressed proteins (the 99 proteins listed in Table 2) reflected any kind of preferential detec-

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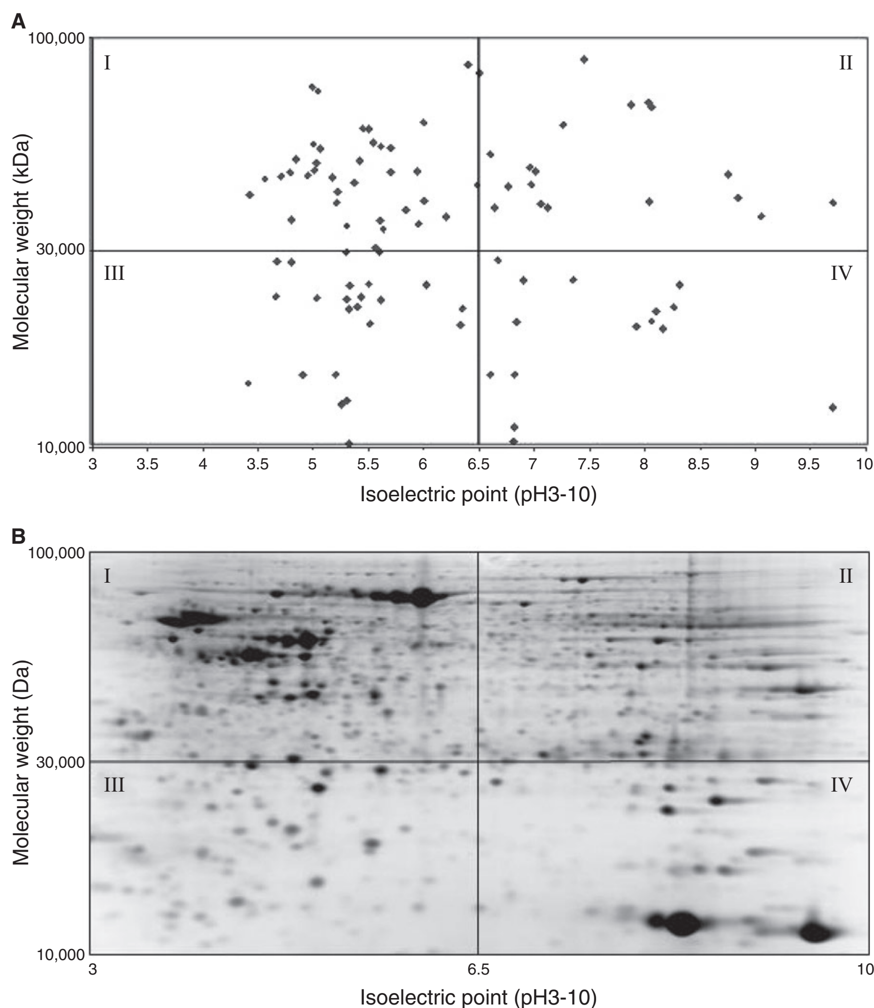

Figure 1. A. Location on two-dimensional (2D) gels of proteins putatively altered in glioma. **B.** The distribution of altered proteins in A mirrors the total identifiable protein distribution on a representative 2D gel of human brain tissue. The 99 proteins found altered in glioma proteomic studies (excluding seven proteins with molecular weights above 100 kDa), plotted on a virtual gel. The *y*-axis of the virtual gel A has a logarithmic scale of 10 to 100 kDa and the *x*-axis has a linear scale of 3–10 pH units to mimic the pattern of protein migration during 2D gel electrophoresis. The proteins altered in glioma are distributed across the gel in a similar fashion (% of proteins per gel quadrant) to total number of proteins in glioma. A representative 2D gel image of a human glioma specimen showing distribution of all proteins (visualized as spots on the gel) is shown in "B".

tion from a certain area of the 2D gel, we located the position of each candidate protein on a virtual gel. Seven proteins, with molecular weights above 100 kDa were excluded from the plot. Ninety-three proteins were plotted along a logarithmic scale of 10kDa to 100 kDa and against a linear scale of 3 to 10 pH units. Proteins were found distributed (see Figure 1) in the numbered gel quadrants as follows: 39 proteins in quadrant I [42%: 95% confidence intervals (CIs) 32–52%], 19 proteins in quadrant II (20%: 95% CI 13–30%), 14 proteins in quadrant III (15%; 95% CI 9–23%) and 21 proteins in quadrant IV (23%: 95% CI 15–32%). The percentage distribution and 95% CIs of differentially expressed proteins, is not dissimilar to the percentage distribution of the total number of proteins detected on a 2D gel for a malignant glioma. A representative and randomly selected GBM gel (generated from our laboratory) had an overall protein distribution in the four quadrants as follows: 246 proteins in quadrant I (34%: 95% CIs 30–36%), 223 proteins in quadrant II (30%: 95% CI 27–33%), 135 proteins in quadrant III (18%: 95% CI 16–21%) and 131 proteins in quadrant IV (18%; 95% CI 15–21%) (Figure 1).

Making sense of the individual protein findings: functional insight?

To investigate whether a common biological function characterized the list of 99 proteins found altered in glioma (Table 2), we performed PANTHER analysis on the 99 proteins collated (see the section "Critical appraisal of differentially expressed proteins in glioma: protein classification and protein-protein interactions"). PANTHER categorized all the proteins (according to prespecified functional groups) into 23 different functional groups (for example, cell structure and motility, cell cycle, intracellular protein trafficking, cell proliferation and differentiation, and signal transduction). Five proteins constituted the most proteins found in any of these functional groups. However, no clear or specific biological process was highlighted (Figure 2).

In a further attempt to make some functional sense of the proteomic data generated in glioma, we investigated protein–protein interactions between the differentially regulated 99 proteins using network analysis [Ingenuity Pathway Analysis; (IPA)]. When all these 99 proteins implicated to play a role in glioma

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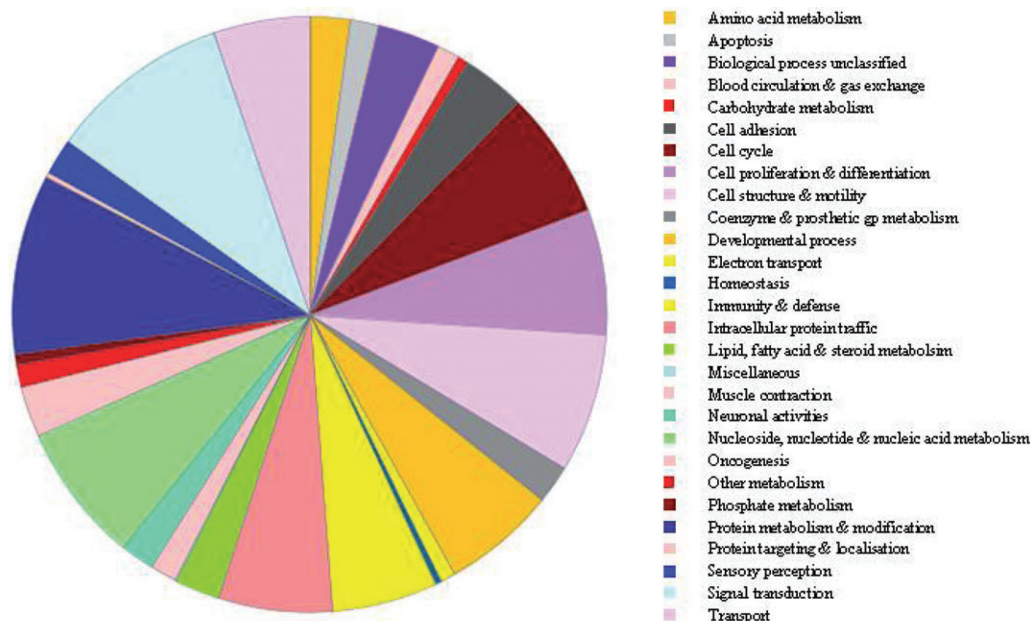

Figure 2. Biological functions of the 99 proteins putatively altered in glioma according to Protein Analysis Through Evolutionary Relationships (PANTHER) classification. A pie chart of the 23 functional groups assigned by PANTHER to categorize the proteins putatively altered in glioma. No clear or specific biological processes are highlighted.

pathophysiology were entered into IPA, 8 different protein–protein interaction networks were generated, containing a multitude of indirect protein–protein interactions. No functional coherence was gained from this preliminary IPA analysis. Such a lack of coherence was perhaps not surprising since low-grade tumors and high-grade tumors display distinct behavior and are likely to generate quite different protein–protein interaction responses.

However, when the 58 proteins found specifically altered in GBM (WHO grade 4 glioma) were entered into IPA a highly significant primary network was generated (score 57, refer to http://www.ingenuity.com for score details). This network (Figure 3) contains 28 of the proteins that were entered and shows a multitude of direct interactions or associations between all of these molecules. TP53 and RB1 are at the core of this proteomic network which is reassuring given that both genes have been known for some years to be fundamental to gliomagenesis (12, 65). Two other genes frequently described as abnormal in classical molecular studies of GBM are PTEN and EGFR (43, 79) and both these proteins are also central to the functional network generated. Consequently the proteomic network complements recent findings by The Cancer Genome Atlas Research Network (76) who found that GBMs harbor frequent genetic alterations in core components of the RB, TP53 and RTK pathways. In conclusion, the resultant network appears extremely robust and cohesive in terms of "key" players/signaling pathways integral to GBM pathophysiology.

DISCUSSION

Genetic analysis has dominated glioma research for nearly two decades. Genetics has provided important molecular information (related to therapy response and prognosis) that has further classified tumors within the same histological subtype but has not translated into improved patient outcomes (2). Primary and secondary GBM are indistinguishable histologically but distinct subgroups have been defined genetically (EGFR amplification and loss of PTEN in primary GBM; and overexpression of PDGF, EGF2 and mutations of p53 in secondary GBM). It is now being increasingly recognized that proteomics (the study of multiple proteins simultaneously) is a critical and powerful approach to complement and extend genetic studies, since proteins are the "workhorses" of the cell. The importance of studying protein expression levels and protein dynamics has further been emphasized by multiple discrepancies reported in the literature between the expression levels of genes and proteins in differential analyses of gliomas (L. Salford, pers. comm.).

Proteomics has been adopted in numerous clinical fields of research and has generated enormous amounts of data (63, 73). There has been general criticism however, that proteomic data has not yet yielded significantly novel insights into basic disease mechanisms and translation into either biomarkers or clinical benefit. This is perhaps due to the limitations of some high-throughput proteomic methodologies, a lack of interpretative tools and immaturity of proteomic analysis compared to genetic analysis (13). Brain tumor proteomics in particular is qualitatively and quantitatively well behind studies of breast, ovarian, colorectal and many other cancers (16, 33). At present, the glioma *in vivo* proteomic literature is where many studies in systemic cancers were about 5–7 years ago and consequently suffers from the same shortcomings (32).

Differences in analytical methodology between all the glioma proteomic studies raise multiple questions concerning reproducibility, validity and/or comparability of the data. Publications to date have employed a variety of methods with respect to statistical analysis, different protein-staining techniques and have set a

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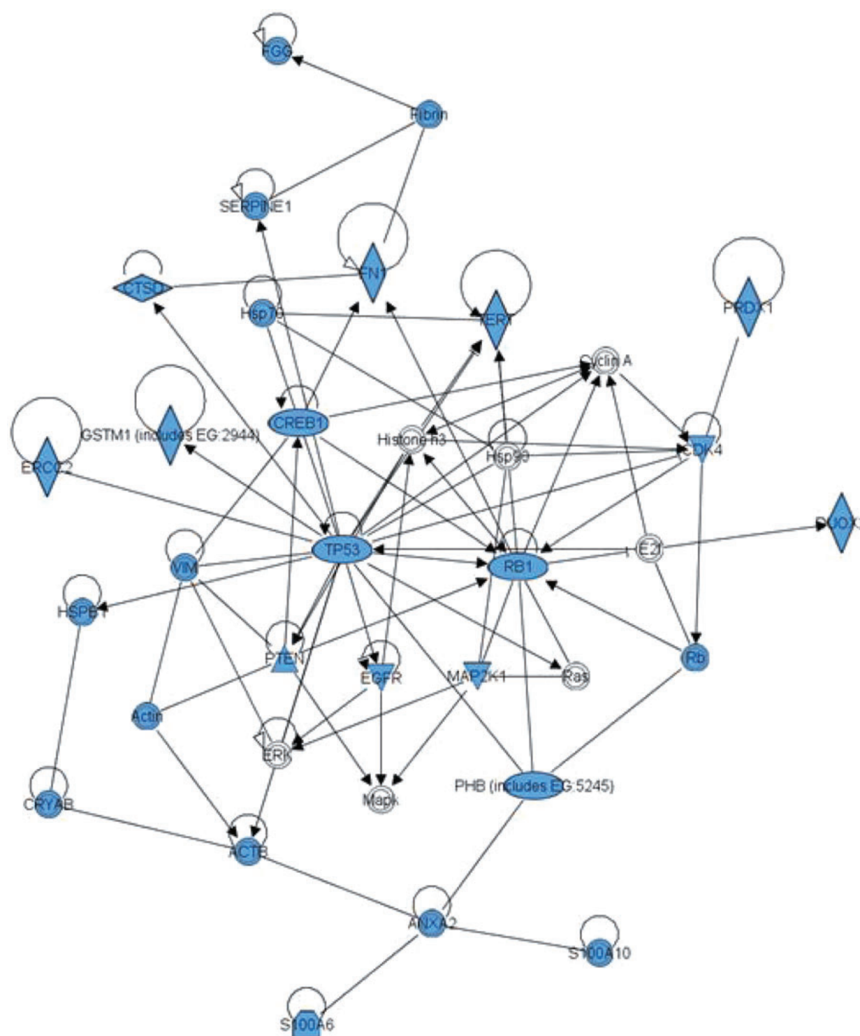

Figure 3. Half of proteins specifically altered in glioblastoma form a coherent network centred on TP53/RB1/PTEN/EGFR. Of the 99 proteins altered in glioma, 58 proteins were reported as altered in glioblastoma. A coherent functional network can be created from 28 of these 58 proteins using Ingenuity Pathway Analysis (IPA). Visual representation of the functional network containing the 28 proteins (in blue) is presented. Each node (blue shape) represents a protein and its association with other proteins, is represented by a line (edge). Nodes have different shapes to represent different molecule types (horizontal diamonds = peptidases, vertical diamonds = enzymes, and circles = "other"; see Ingenuity Systems for detailed node information). Solid lines represent direct interactions between proteins. Direct interactions are defined as those where two proteins make direct physical contact with each other with no intermediate step. Direct interactions may include chemical modifications, for example phosphorylation, but only if there is evidence that the protein can cause the chemical modification directly. The evidence for interactions is obtained from putatively peer-reviewed publications in "high quality" journals. It should be noted with caution that the evidence (accessible online from IPA) varies markedly in quantity and pertinence for each interaction. This protein–protein interaction network is highly connected with a multitude of direct interactions between proteins altered in glioblastoma.

variety of different inclusion criteria for determining which proteins are "key" in glioma pathophysiology. Questions are also raised with regard to the appropriateness of the "control" tissue or group used in these studies. Such methodological considerations may explain why proteins such as PTEN, p53, pRB, tenascin, ferritin, cathepsin, GFAP and EGFR, all of which have been considered fundamental in glioma pathophysiology (27, 45, 79, 90), have been so infrequently described in modern proteomic studies.

The failure of modern high-throughput proteomic studies to identify other differentially regulated proteins that have been recognized in neuropathological studies using immunocytochemistry or Western blotting is another cause for concern. Some candidate proteins identified in gliomas using classical techniques but not modern proteomics are listed in Table 3. The failure to identify these proteins may reflect their molecular weight, low abundance and/or subcellular location. For example, a protein abundance needs to be >100 ng/g (protein/wet weight tissue) for easy detection on 2D gels, it is a challenge to accurately identify proteins smaller than 30 kDa using MALDI-TOF MS, and identifying proteins in the membrane bound subcellular fraction is difficult. Even more disconcerting from the translational viewpoint is that several proteins not recognized in high-throughput proteomic studies have either translated into novel therapies such as EGF, TGF and VGF receptor antagonists or provide prognostic information about the response of GBMs to therapy with temozolomide (25, 45).

One commonality and perhaps pitfall of the glioma high-throughput proteomic studies reviewed is the use of whole cell lysates. Proteomics of whole cell lysates selects for high abundance proteins with molecular weights between 30 and 100 kDa. Although between 600 and 1000 proteins may be identified, analysis of subcellular fractions may provide insights that are more relevant and focused to glioma pathophysiology. Since dysregulation of cell proliferation and normal apoptosis are two fundamental processes sustaining malignant glioma, evaluation of proteins in the nuclear matrix fraction, chromatin binding fraction and intermediate filaments may be useful (1, 37). Using nanoliquid chromatography-mass spectroscopy (NLC-MS MS) up to 2000 proteins can be identified in each of these subnuclear fractions; some of which are unique to one compartment whereas many are common to all three compartments. Energy metabolism is another fundamentally dysregulated process in malignant glioma (66). Proteomic evaluation of the mitochondrial fraction may thus provide

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Table 3. List of some proteins found to be biologically important in the pathophysiology of malignant glioma either *in vivo* or *in vitro* using classic protein research techniques such as Western blotting and/or immunohistochemistry but not identified using high-throughput modern proteomic techniques.

Protein	~MW (kDa)	Reference
Growth factors		
Epidermal growth factor	6	Helseth *et al* (26)
Epidermal growth factor receptor VIII	145	Wikstrand *et al* (83)
Platelet-derived growth factor	31	Nister *et al* (53)
Vascular endothelial growth factor	45	Cheng *et al* (8)
Hepatocyte growth factor	82	Moriyama *et al* (51)
Insulin-like GF	250	Gammeltoft *et al* (21)
PI3 kinase	110	Chakravarti *et al* (7)
Apoptosis proteins		
MDM2	55	Reifenberger *et al* (62)
Bax	21	Krajewski *et al* (39)
Bcl2	53	Krajewski *et al* (39)
Survivin	16	Chakravarti *et al* (6)
DNA repair		
MGMT (06methyl guanine transferase)	22	Hegi *et al* (25)
Poly ADP ribose polymerase	116	Wharton *et al* (80)
Other		
Aquaporin 4	32	Saadoun *et al* (64)

mechanistic insights into aberrations of energy metabolism and apoptosis. Protein alterations associated with mitochondrial dysfunction are increasingly becoming a focus of cancer research in general (18, 22). Proteomic analysis of the glioma secretome and exosome may also provide insights into how gliomas attenuate immunological responses and signal to adjacent tumor cells. Moreover, further insights into all these areas may be facilitated by the recent development of mouse glioma models (34). Proteomic studies of these tumors may provide clues into specific mechanistic pathways that may have parallels in humans. Already proteomic studies of melanoma in mice have provided insights into protein pathways in humans (88).

The elucidation of genetic alterations in GBM has greatly advanced our understanding of the molecular basis of this brain tumor. Amplification mutations of EGFR (present in 45% of patients) and PDGFRA (present in 13% of patients) are thought to provide an autocrine drive to tumor growth (76). Disruption of cell cycle regulation (involving p53, CDKN2A, TP53 and RB) is a frequent genetic alteration in GBMs, as it is in tumors of the breast, colon and pancreas (72, 85). Disorder signaling (involving NF1, AKT and particularly PI3K and PTEN) are also frequently observed in GBM. While the genetic advances have underpinned mechanistic models of tumor genesis, proliferation, differentiation and growth, rarely does genetics dictate the clinical course of the disease [the increased survival of patients with mutated isocitrate dehydrogenase 1 and of TERT overexpression in high-grade tumor are notable exceptions (57, 78)]. The extent to which genetic alterations in GBM reflect alterations in protein levels is often poorly defined. Crucially, our network analysis of alterations in GBM (although originating from an amalgamation of proteomic data from different sources) has generated a highly interactive cluster of proteins centred round TP53 (deleted in 35% of patients) and RB1 (deleted in 78% of patients), consistent with key pathways implicated in genetic studies (76). The network offers confidence in the proteomic data generated in glioma. Future network analysis may also prove to be a useful tool for identifying novel proteins, beneath the detection limits of technology, which functionally interact with proteins found altered in major proteomic studies.

A major limitation of contemporary network analyses, however, to understand the molecular mechanisms of tumor progression, is their static nature. Biological systems are highly dynamic with proteins able to translocate between organelles and subcompartments conditional on environment, stimuli and the presence/absence of other proteins such as scaffolding molecules (86). The development and application of more advanced network algorithms, encompassing the concept of dynamic interactions [such as Seeded Bayesian networks (14)] will undoubtedly transform our understanding of pathophysiological mechanisms and provide an excellent platform for the generation of novel hypotheses and experimental verification.

Newer proteomic technologies with higher throughput, quicker identification, and the capability of detecting smaller and lower abundance proteins, will also be invaluable for the future of proteomics in glioma. Technologies such as LC-MS/MS, stable isotope labeling with amino acids in cell culture (SILAC) and isotope-coded affinity tag (ICAT) (40, 74) (new, fast, sensitive and accurate technologies) are increasingly being favoured over labor-intensive 2DGE studies and are revolutionizing progress in cancer proteomics. Antibody tissue arrays and reverse-phase protein lysate arrays are also proving to be popular, providing systematic approaches for investigating the regulation of major signaling pathways (36, 84). Moreover, a number of phosphoproteomic strategies have been developed for investigating the complexity and dynamics of protein signaling (49). Such phosphoproteomic studies are beginning to yield novel insight into established signaling pathways, such as the EGFR pathway, in response to stimuli and time (50). Additionally technological advances are now facilitating the study of glycosylation of proteins, which is the commonest post-translational modification of proteins (75).

The utility of proteomics in glioma research has clearly emerged from this critical analysis and systematic review. Proteomics allied to genetic analysis can now be applied to address important clinical questions. Parallel proteomic analysis in glioma may provide important insight into why genetic abnormalities too rarely impact on clinical decisions and prognosis. The interplay between proteins and genes may elucidate the role of stem cells in glioma etiology (59), the molecular mechanisms which make glioma stem cells resistant to therapies, the signals involved in tumor progression (from WHO II gliomas to higher grade gliomas), and the intracellular events associated with chemoradiotherapy in recurrent malignant gliomas. Focusing on the synaptic proteome may also provide insights into how peritumoral brain is functionally altered by the proximity of a glioma (23) and why lower grade gliomas cause seizures whereas higher grade tumors cause focal neurological deficits that are responsive to glucocorticoids (81). The potential of proteomics is now being realized in glioma research. The

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continuing advances in proteomic technology, interpretative network analyses and in particular, correlation with genetic data will advance our knowledge of glioma pathophysiology and ultimately improve the treatment of this terrible disease.

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CONFLICTS OF INTEREST

We have no conflicts of interest.

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