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Review

Glioma proteomics: Status and perspectives

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ABSTRACT

High grade gliomas are the most common brain tumors in adults and their malignant nature makes them the fourth biggest cause of cancer death. Major efforts in neuro-oncology research are needed to reach similar progress in treatment efficacy as that achieved for other cancers in recent years. In addition to the urgent need to identify novel effective drug targets against malignant gliomas, the search for glioma biomarkers and grade specific protein signatures will provide a much needed contribution to diagnosis, prognosis, treatment decision and assessment of treatment response. Over the past years glioma proteomics has been attempted at different levels, including proteome analysis of patient biopsies and bodily fluids, of glioma cell lines and animal models. Here we provide an extensive review of the outcome of these studies in terms of protein identifications (protein numbers and regulated proteins), with an emphasis on the methods used and the limitations of the studies with regard to biomarker discovery. This is followed by a perspective on novel technologies and on the potential future contribution of proteomics in a broad sense to understanding glioma biology.

Contents

1. Introduction . . . 1824
2. Potential benefit of glioma proteomics . . . 1824
3. Commonly applied proteomics technologies . . . 1825
   3.1. Gel-based proteomics . . . 1825
   3.2. LC-MS/MS-based proteomics . . . 1825
4. In search for protein signatures and biomarkers for gliomas . . . 1826
5. Perspectives: emerging technologies . . . 1834
   5.1. Antibody-based proteomics and data validation at the protein level . . . 1834

Abbreviations: CSF, cerebrospinal fluid; CNS, central nervous system; GBM, glioblastoma multiforme; 2DE, two-dimensional gel electrophoresis; WB, Western blot; IHC, immunohistochemistry; PMF, peptide mass fingerprint; 2D-DIGE, 2 dimensional differential gel electrophoresis; PF2D, 2D chromatographic protein separation (pI and RP); IP-RP-HPLC, ion-pair reversed phase chromatographic separation; PCA, principal component analysis; PLS, partial least squares; TMA, tissue microarray; n.i., not indicated; Buffer A, generic buffer containing 7M Urea, 2M Thiourea, 4% CHAPS; Buffer B, generic buffer containing 8–9M Urea, 40mM Tris–Base, 4% CHAPS, 40mM DTT

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5.2. Reverse phase protein lysate arrays (RPPA) . . . 1834
5.3. Top–down proteomics . . . 1834
5.4. Targeted proteomics (SRM) . . . 1834
5.5. MALDI mass spectrometry imaging (MALDI MSI) . . . 1835
5.6. Metabolomics . . . 1835
6. Conclusion . . . 1835
Acknowledgements . . . 1835
References . . . 1835

1. Introduction

While malignant brain tumors comprise only a small percentage of all adult tumors at a rate of 4–5 in 100,000 adults per year, their malignant nature makes them the fourth biggest cause of cancer death [1]. Major efforts in neuro-oncology research are needed to reach similar progress in patient survival to what has been achieved in other cancers over the last 10–15 years.

Following the classification system of the World Health Organization (WHO), tumors of the brain are classified according to their histological characteristics, that is, whether they display features of i.e. neural, glial or meningeal cells [2]. Thus glioma refers to primary brain tumors containing astrocytic, oligodendrocytic or ependymal elements. This review focuses on protein profiling of astrocytic glial tumors, which are the most common and include the most aggressive primary brain tumors in adults. They can be grouped in two major categories: (i) The non-malignant, more circumscribed growing astrocytoma including pilocytic astrocytoma (grade I tumor). These are rare tumors, appear in young adults and are normally cured by resection. (ii) The more common group of diffusely infiltrating astrocytomas: diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma multiforme (grade IV). These tumors generally affect the adult population, have a tendency for recurrence and malignant progression and are incurable with current treatment options. Their prominent infiltrative growth is a major challenge for efficient eradication. Glioblastoma (GBM) representing the most malignant of primary brain tumors is very heterogeneous and is characterized by high proliferation rates, hyperplasia, necrotic areas and extensive new blood vessel formation. Despite aggressive treatment (resection combined with radiotherapy and chemotherapy) median survival time for GBM patients is still only 12–14 months. Molecular and genetic analyses reveal numerous mutations, gene expression alterations and chromosomal abnormalities, most of which lead to the disruption of cell-cycle arrest [3]. The pathways that are commonly disrupted in GBM are the Ras/MAPK pathway, the PI3K/Akt, the retinoblastoma and the p53 pathway [4]. Epidermal growth factor receptor (EGFR) gene amplification and/or mutations are prominent in primary GBM. Unfortunately, improved insight into genetic alterations was not hitherto paralleled by the development of successful therapeutic options. The most recent advent in GBM treatment is the combination of cytotoxic drugs with agents that interfere with tumor angiogenesis, including antibodies against vascular endothelial growth factor (VEGF) and tyrosine kinase inhibitors [5,6].

2. Potential benefit of glioma proteomics

In addition to the urgent need for novel efficient drug targets against malignant gliomas, the identification of glioma biomarkers will provide a much needed contribution to diagnosis, treatment decision, prognosis and assessment of treatment response. Current diagnosis is based on classical histopathological examination, a challenging task considering the heterogeneity of the disease and the inherent subjective nature of histopathological grading. Discrepancies in the diagnosis of glial neoplasms are reportedly large, ranging from 23 to 43% [7,8]. Although the discordance is higher when the first diagnosis is not performed by a neuropathologist, discrepancies remain even between expert neuropathologists [9]. This clearly impacts on treatment decision and on the outcome and interpretation of clinical studies. In addition, with novel treatment modalities being tested and entering the clinics (e.g. targeted therapies), a faster and objective assessment of therapeutic efficacy is crucial. Tumor monitoring is currently performed with non-invasive imaging techniques such as magnetic resonance imaging (MRI) where the detection of small differences in tumor size and behaviour remain a challenge [10]. Moreover, MRI images face difficulties to differentiate between radiological damage and recurrence, and more recently, with the application of anti-angiogenic drugs, to distinguish between reduction of objective tumor volume versus loss of contrast enhancing area [11]. Thus the availability of molecular biomarkers or signature protein patterns identified from plasma or tumor biopsies, has the potential to improve routine diagnosis and monitor the pharmacological response of a therapeutic intervention thereby directly impacting on treatment decision. In addition, the study at the proteomic level of clinically relevant model systems, that model disease progression, tumor adaptation and treatment responses, will uncover novel target molecules for further therapeutic validation. Using such models in focused well designed studies, highly sensitive proteomics technologies have the potential to reveal the Achilles heel of glioma cells.

Over the past 5–6 years several groups have attempted whole proteome analysis of gliomas at different levels: analysis of patient biopsies or bodily fluids, analysis of glioma cell lines or animal models. Here we provide an overview of the outcome of these studies, with an emphasis on the methods used and the limitations of the studies with regard to biomarker discovery. This is followed by a perspective on what proteomics technologies in a broad sense can contribute to glioma diagnosis and treatment in the future.

3. Commonly applied proteomics technologies

Proteomics directly addresses the functional effectors of cellular and disease processes. Importantly there is far more information in proteins beyond their primary (amino acid sequence) and secondary structure (three-dimensional form of local segments). Post-translational modifications such as glycosylation, phosphorylation or acetylation, and protein processing such as ubiquitylation or partial proteolysis, may more adequately reflect disease status and treatment response than expression per se. All this information renders proteins the primary source for biomarker and therapeutic target identification. Current proteomics techniques are facing limitations in terms of their capacity to analyze the entire proteome of a tissue or biological fluid in a single reaction [12]. In bodily fluids like serum, plasma or cerebrospinal fluid (CSF), protein concentrations vary over more than ten orders of magnitude and the presence of high abundant proteins invariably masks the detection of low abundant proteins [13]. The strategy of many researchers in the field is thus oriented towards either combining two or more complementary technical approaches and/or analysing the subproteome of interest [14]. Many techniques for de-complexion of the proteome, enrichment or depletion of particular subproteomes and separation techniques for proteins/peptides have emerged in parallel with the development of mass spectrometry (MS) of high capacity, resolution and accuracy. Here we provide a short overview of commonly used proteomics approaches (Fig. 1). Excellent in depth reviews on mass spectrometry are available elsewhere [15,16].

3.1. *Gel-based proteomics*

The two-dimensional gel electrophoresis (2DE)-based proteomics separates the proteins on a polyacrylamide gel, by their isoelectric point (pI) and their molecular weight. Proteins are quantified by staining prior (e.g. CyDyes) or after the separation (e.g. Silver stain, Sypro Ruby, Coomassie stains), in-gel digested and identified by MS. In differential gel electrophoresis (2D-DIGE) spectrally distinct fluorescent labels (CyDyes: Cy2, Cy3 and Cy5) are chemically linked to proteins via a lysine residue, providing very good sensitivity and linear quantitation. Such labels can reliably visualize between 500 and 800 proteins (or more in highly experienced labs), of which roughly about two thirds can be identified. 2DE gels allow for the detailed analysis of post-translational modifications, however they have poor resolving power for high molecular weight and hydrophobic proteins, and difficulties in quantifying co-migrating proteins.

3.2. *LC-MS/MS-based proteomics*

In liquid chromatography (LC)-based proteomics, mixtures of proteins are specifically digested to peptides by proteases, separated by one or more dimensions of LC, and coupled to automated MS/MS [17]. Proteins are identified on the basis of one or more identified peptide sequences. The separation and analysis of tryptic peptides rather than proteins is also referred to as a bottom–up approach. The most common two-dimensional LC separation applied combines strong cation exchange (SCX) chromatography with reverse phase (RP) chromatography coupled with automated MS/MS, first described as multidimensional protein identification technology (MudPit) [18,19]. The fractions separated by RP are injected to the mass spectrometer via online electrospray ionization (ESI) or spotted to the MALDI target plates for analysis by MS/MS [20,21]. In a combined gel- and chromatography-based approach, proteins in a mixture are digested and separated on an immobilized pH gradient gel (isoelectric focusing — IEF). 'Focused' peptides are extracted from gel pieces and subjected to LC-MS/MS. In label-free LC-MS based proteomics relative protein quantitation can be achieved by quantitative mass spectrometry using measurements of mass spectral peak intensities, peptide and spectral counts. Of these, spectral counts, the number of MS/MS events observed for a peptide in the mass spectrometer, show the highest technical reproducibility [22,23].

Additional MS based quantitation methods are based on labeling proteins or peptides prior to the MS analysis. These include:

Stable isotope labeling of amino acids in cell culture (SILAC): Metabolic labeling using SILAC [24] allows for multiplexing up to 3 labels/samples (e.g. deuterium, 13C, 15 N). This approach is normally applied to cultured cells, where stable isotope containing amino acids are added to the cell culture medium and are metabolically incorporated in the proteins. More recently the application of this method *in vivo* to animal models has been reported [25]. After protein digestion, isotope labeled peptides are identified and quantified in the MS spectra as precursor ion pairs (or

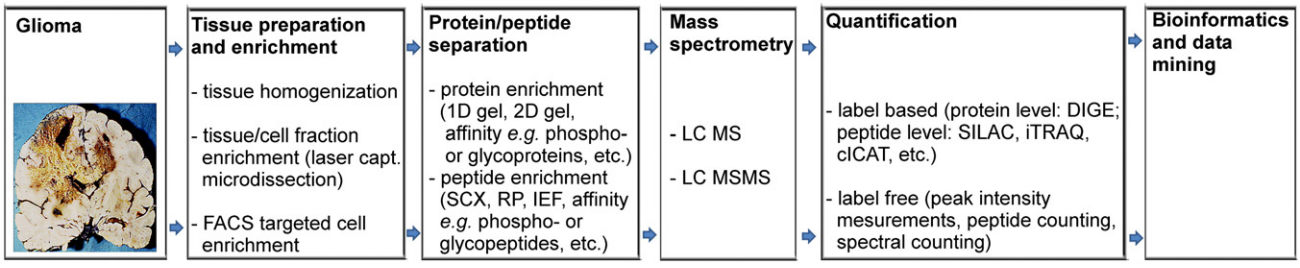

Fig. 1 – Diagram of common approaches to glioma proteomics. Different possibilities are depicted with regard to tissue preparation, protein/peptide enrichment and separation, mass spectrometry, quantification and data analysis.

triplets) differing in mass by known amount and their relative abundance is measured by comparing peak intensities or areas.

Isotope-coded affinity tags (ICAT): ICAT tags are biotin-tagged chemicals that are added onto the cysteine residues in proteins [26]. The biotin tag allows isolation of ICAT labeled peptides by affinity chromatography, leading to a reduction of sample complexity. Following enrichment of ICAT-labeled peptides, the sample is separated by (multidimensional) separation techniques and analysed by either ESI MS/MS or MALDI MS/MS [21]. More recently cleavable ICAT (cICAT) reagents containing an acid-cleavable biotin group are preferred since they facilitate the interpretation of the MS/MS spectra.

Isobaric tags for relative and absolute quantification of peptides (iTRAQ): iTRAQ tags are differential chemical labels of identical mass (isobaric) that are chemically linked to amine residues on peptides (N-terminal amine and lysine side chain amine) after protein digestion [27]. The differentially labeled intact peptide masses are indistinguishable, but produce diagnostic fragment peaks in MS/MS mode that provide relative quantitative information on proteins. An advantage of this approach is the possibility to label up to 8 different samples per run and to achieve higher sensitivity and high accuracy in protein identification due to the higher number of matching peptides per protein [28].

SELDI-TOF-MS (surface enhanced laser desorption ionization-time of flight) is a potentially powerful high throughput technique to identify protein signatures. Complex protein/peptide mixtures are fractionated on a chip array based on chromatographic separation. Chip surfaces are either chemically (hydrophobicity, charge) or biochemically modified (high affinity protein–protein interaction). After application of a matrix, a protein chip reader will generate a mass profile by MS analysis. This technique usually generates distinctive biomarker panels for healthy and disease status, but lacks the direct identification of the protein peaks.

4. In search for protein signatures and biomarkers for gliomas

Table 1 provides an in depth overview of glioma-related proteomics studies published between 2004 and 2009 (source PubMed) and the proteins identified therein. The vast majority of studies until 2008 have applied gel-based proteomics, generating rather low numbers of protein identifications (up to 200), with the majority of them being medium to high abundance proteins (Table 1). In recent years the trend goes towards more sophisticated methods e.g. capillary IEF, cICAT or iTRAQ labeling coupled to LC-ESI-MS/MS or LC-MALDI TOF/TOF or the combination of analytical methods [29–31]. Such techniques lead to a vast increase in protein identifications (several thousands) and thus herald the potential for novel discoveries. Due to the high workload these studies were limited so far in sample number and require extensive validation and/or increased throughput. For protein or peptide profiling of biofluids such as serum, plasma or cerebrospinal fluid (CSF), SELDI-TOF is the most widely applied technique (Table 1).

Because of easy retrieval and patient follow up, serum biomarkers are the ultimate aim of most biomarker discovery studies. Based on gene expression data or ELISA of serum samples, a number of potential serum biomarkers for glioma have been reported before the proteomics era (recently reviewed by Somasundaram et al. 2009 [32]). Proteins that are increased with tumor grade include GFAP, IGF-binding protein 2 (IGFBP2), IGFBP5, PBEF1/NAmPRTase (Nicotinamide phosphoribosyltransferase), plasminogen activator inhibitor-1 (PAI-1), Cathepsin-D, YKL-40, MMP9 and low MW Caldesmon (l-CaD), while the protein AHSG is found decreased with glioma grade (see [32] and references therein). Although some of these candidates show prognostic value, the majority may not be useful as single markers and all require large scale validation before translation into the clinic. In addition to serum, changes in CSF composition accurately reflect pathological processes in the CNS including tumor growth [33]. Because CSF is in proximity to the tumor, many tumor-related proteins may be secreted directly into the CSF and thus represent an easily accessible source for biomarker discovery (see Table 1). Another interesting and potentially more powerful approach to obtain a highly concentrated and tumor selective proteome is the analysis of interstitial tumor fluid collected by microdialysis, an *in vivo* correlate of the tumor secretome [34].

Due to the complexity and large dynamic range of protein levels in biofluids, direct analysis of tumor tissue represents an attractive alternative for the identification of tumor specific proteins. This is apparent in the published reports, where the majority used glioma cell lines or patient biopsies as the starting material (Table 1). So far only few (three) studies have analysed animal models [30,35,36], although such models, when clinically relevant i.e. reflecting human disease phenotype and heterogeneity, have several advantages. They allow controlled study design, reproducible sampling and follow-up of tumor progression over different time points. Another major advantage of an animal model is the possibility to collect several tissue samples (tumor, control tissue) and body fluids (plasma, CSF) from the same animal.

In our laboratory we applied 4-plex iTRAQ technology linked to LC-MALDI on a human/rat xenograft glioblastoma model to identify differential protein expression in highly infiltrative, non-angiogenic brain tumors compared to fully angiogenic tumors. With this technology we were able to identify over 7000 proteins (C.I. > 95%) in membrane enriched fractions, to our knowledge the largest available dataset in glioma proteomics [30]. In depth analysis of the data is still in progress. By generating a restricted dataset based on isoform- and species-specific protein identification, we were able to separate the tumor from the stromal compartment at the bioinformatics level (generating 3000 proteins). From these, about 300 proteins were regulated at least 1.5 fold in angiogenic *vs.* non-angiogenic phenotype ($p < 0.05$). Interestingly the data point at enhanced intercellular crosstalk and increased metabolic activity adopted by tumor cells in the angiogenic glioma phenotype [30].

Despite the considerable number of studies performed (Table 1) and the detection of some proteins in more than one

Table 1 – Summary table of publications on glioma proteomics between 2004 and 2009.

	Reference	Sample type	Sample treatment	Protein extract preparation	Proteomic analysis	Proteins detected / identified (IDs) / differentially expressed (diff.)	Identified differentially expressed proteins, highlighted	Comments
Cell lines								
1	Vogel et al. 2005 [53]	glioma cell lines : (U87, U251, U118, A172); compared to GBM tumors ($n=8$);	/	50,000 cultured cells, trypsinized; 50,000 cells from unstained serial sections of tumor	2DE, IEF: pH4–7, 11 cm, 2nd dim: 8–16% Tris–HCl	500 / 220 diff.	160 up (gained) and 60 down (lost) in cultures compared to tumors: DNA polymerase ε catalytic subunit A, hHYD, LRP-1, a-Actin 3, CAPG, AKAP-9, Calgizzarin, **Anx**, LASP-1, PDI, PKM1/2, **S100A6***, **GFAP***	Cell line / tumor comparison
2	Billecke et al. 2006 [54]	LNZ308 glioma cells	effect of chemotherapeutic agents: cisplatin, BBR3464, BBR3610	cells scraped in lysis buffer A, buffer exchange prior PF2D	PF2D, MALDI MS/MS	n.i. / 500 diff.	1 Triose-phosphate isomerase* (TIM)	Development of metacomparison software tool to compare differential PF2D chromatographic profiles
3	Zhou et al. 2006 [55]	2 glioma cell lines (U251, A172), distinct in vivo and in vitro tumor forming ability	/	cells, trypsinized, lysed in buffer B	2DE: IEF: pH3-10. 13 cm, 2nd dim: 12.5% SDS-PAGE, silver staining. MALDI TOF MS: PMF	n.i. / 46 diff.	18 proteins identified: Transketolase, Prohibitin, PR48, PARP, CBR, CypA , Grfb, DJ-1, **Cathepsin-D***	/
4	Trog et al. 2006 [56]	U87 glioma cells	effect of irradiation (RT), chemotherapy (CT) with temozolomide, or combined radiochemotherapy (RCT)	cells lysed in buffer B	2DE: IEF: pH4–7, 7 cm 2nd dim:12.0% SDS-PAGE, silver staining (2 replicates) Nanoelectrospray MS/MS	163-229 / 6–10 diff.	**Vimentin***: 50% up after CT and combined RCT, 70% down after RT alone; **TIP47*** down after CT, RT and RCT ; **RhoA GTPase*** 100% up in CT, lost in RT, 30% down in RCT	/
5	Shim et al. 2006 [57]	U87 glioma cells expressing 3 different PTEN clones	wildtype PTEN, mutant PTEN C124G (no catalytic activity), mutant PTEN G129E (lipid phosphatase deficient)	cells lysed in buffer B	2DE: IEF: pH3–10, 4–7, 4.5–5.5, 5.5–6.7; 18 cm 2nd dim:9–16% SDS-PAGE, silver staining. MALDI-TOF-MS: PMF	>1200 / 50 diff.	**Enolase**, **Vimentin**, **Cathepsin-L** preproprotein, SRC family associated phosphoprotein, cofilin, Glutathion S-transferase chain A, dihydropyrimidinase-like 2 protein, BANP isoform b, HSPA8, beta-actin	Vimentin observed as 3 MW forms (53, 41 and 35 kDa)
6	Ngo et al. 2007 [58]	U251 (1p+/+), A172 (1p+/–) glioma cell lines, anaplastic oligodendroglioma	Nitrosourea	cell lysate cleaned of nuclear fraction, 2D-DIGE compatible buffer	2D-DIGE: IEF pH 3–10, 24 cm MALDI MS/MS	n.i. / 29 diff.	19 unique proteins identified: **stathmin***, **α-enolase**, DJ-1	/

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Table 1 *(continued)*

	Reference	Sample type	Sample treatment	Protein extract preparation	Proteomic analysis	Proteins detected / identified (IDs) / differentially expressed (diff.)	Identified differentially expressed proteins, highlighted	Comments
Cell lines								
7	Puchades et al. 2007 [59]	U87 glioma cells with or without wildtype p53	cytotoxic drug SN38 (metabolite of Irinotecan CPT-11)	cells lysed in buffer B	2DE: IEF: pH5–8, 17 cm IPG 2nd dim: 8–16% PAGE, Sypro Ruby MALDI-TOF: PMF Linear ion trap FT-ICR MS	n.I. / 4	Galactokinase 1, GRP78, caspase 14, **galectin-1***: down in TP53 cells treated with SN38	Validation of high galectin-1 expression in high grade tumors and glioma cell lines (WB, IHC). High galectin 1 levels correlate with poor patient survival (microarray data).
8	Seyfried et al. 2008 [60]	astrocyte cell line (C8-D30) compared to astrocytoma cell line (CT-2A), not invasive but highly angiogenic.	/	membrane fraction (sucrose gradient centrifugation), 1D gel prior to tryptic digestion for LC-MS/MS	Label free quantitative LC-MS/MS LTQ linear ion trap	618 IDs (at least one peptide/protein) / 25 diff.	25 membrane proteins (13 up, 12 down) in CT-2A compared to AC. Up: **CSPG4*** (also called NG2), **IFITM1***, and **IFITM3***	NG2 associated with glioma proliferation, metastasis and angiogenesis
9	Bian et al. 2008 [61]	CHG-5 astrocytoma cell line, sHG-44 anaplastic astrocytoma cell line, U87 glioma cell line	nordihydroguaiaretic acid (Nordy), a lipoxygenase inhibitor	cells lysed in buffer B	2DE: IEF: pH3–10,13 cm 2nd dim: 8–16% gradient PAGE, CBB-R250 staining (triplicates) MALDI-TOF: PMF	n.i. / 10 diff. (common to the 3 cell lines)	6 down: PAG-A, ASF-3, beta galactoside binding lectin, **EIF-5A***, **cofilin-1***; 4 up: GST-pi*, glyceraldehyde-3-phosphate dehydrogenase, **α- enolase***, **cyclophilin***	/
10	Hill et al. 2009 [62]	U87 serum free conditioned medium ($n=3$)	dB-cAMP in serum free medium	secreted N-linked glycoproteins (hydrazide capture enriched)	Label free quantitative nano-LC-MS (Q-TOF Ultima, CAP-LC) Peptide identification by LC-MS/MS (MDLC chromato, LTQ IT)	>150 unique glycopeptides / 35 diff.	up: FSTL1*, **Cathepsin-L***, NBL1*, TFP12* down: **Tenascin C***, IGF2R*	Validated by WB and DNA microarrays
11	Koncarevic et al. 2009 [63]	GBM cell lines: NCH89, NCH82	platinum based drugs (TPCs)	cells lysis in 4 M urea buffer pH8, whole cell lysate	2DE, IEF: pH 4–9, 17 cm 2nd Dim: 12% PAGE. Silver stain (5 replicates) MALDI-TOF/TOF	1097 / 124 diff.	TrxR*, GR*, **p53** activation* (p53_S15Phos), **AnxA1, vimentin, α- enolase**, SOD, **EIF-5A**	Comparative proteomics and DNA microarray analysis. Hypusination of EIF-5A (mature form) reduced after TPC treatment
CSF/plasma								
12	Liu et al. 2005 [64]	Serum (total $n=105$): control, astrocytoma grade I–IV	/	whole serum	SELDI-TOF-MS	n.i.	/	Extensive bioinformatic analysis. Cross-validation. Discriminate-cluster analysis between GBM and healthy, between high and low grade

13	Khwaja et al. 2006a [65]	CSF (total $n=32$): CNS neoplasms ($n=10$), CNS inflammatory disease ($n=12$), Control group ($N=10$)	/	patient lumbar punction	SELDI-TOF-MS (SAX-2 chip), 1D-GE, MALDI-TOF/TOF, MALDI-TOF (triplicates)	average of 16 peaks (size range 3-200 kDa) in SELDI spectra from Neoplastic CSF	**Carbonic anhydrase** (CA) (isoform not identified)	CA candidate marker of neoplastic disease detectable in CSF. High expression correlates with poor prognosis in astrocytomas.
14	Khwaja et al. 2006b [66]	CSF (total $n=60$): astrocytoma grade II vs IV	/	patient lumbar punction. Sample concentration (3 kDa filtration), partial depletion of IgG and albumin	2DE, IEF: pH 3–10NL, 13 cm 2nd Dim: 12.5% PAGE Silver stain. cICAT (on 6 pooled samples) MALDI- MS/MS	n.i.	**Attractin*** (>95% confidence in all ICAT analyses)	Validation on brain tumor specimen ($n=108$). Functional assays (migration, scratch): CSF-derived attractin enhances migration of glioma cells (LNZ308, U87, LN229)
15	Khwaja et al. 2007 [67]	CSF (total $n=73$): astrocytoma grade II–IV, schwannomas, brain metastases	/	sample concentration, partial depletion of IgG and albumin, resuspended in buffer A.	2DE, IEF: pH 3–10, pH4–7 13 cm 2nd dim: 12.5% PAGE. Silver stain. MALDI-MS/MS cICAT, 2D-LC-MS	210 / 130 diff., overlap 30 prot. (2DE) 53 diff (cICAT)	103 tumor-specific markers, 20 high grade specific: SPARC*, FGF14*, **VEGF-B***, tau*, β2-microglobulin*, **attractin***	Signature proteins to distinguish CSF from control, low and high grade astrocytomas
16	Zhang et al. 2007 [68]	Serum (total $n=140$): astrocytoma grade I–II ($n=30$) and III–IV ($n=43$), healthy control group ($n=56$)	/	sample in buffer A, diluted prior to SELDI in 50 mM NaAc, pH4.0.	SELDI-TOF-MS chip : weak cation exchange (WCX)	47 peaks (size range 2–20 kDa)	7 serum markers deregulated	Discriminiation astrocytoma vs normal tissue with a sensitivity of 84.6%, selectivity 86.4%; two peaks significantly different in high grade
17	Petrik et al. 2008 [69]	Serum (total $n=200$): control, grade II–IV astrocytoma	/	whole serum, prior surgery	SELDI-TOF-MS, CM10 chip. Identification by Ciphergen Biosystems tandem MS	192 peak clusters	B-chain of α2-Heremans-Schmid glycoprotein (AHSG, fetuin A) decreased with increasing tumor grade	Patient follow up for 2 years. Prognostic power validated in different cohort of GBM patients ($n=72$)
19	Schuhmann et al. 2010 [70]	CSF (Total $n=24$): GBM vs normal control	/	lumbar puncture, CSF water diluted, pH adjusted	RP-LC MALDI-MS Identification by nanoESI-qTOF-MS/MS Differential peptide display	over 6000 peptides/ over 2000 peptide IDs/ 4 peptides diff.	elevated in GBM: C-terminal peptides of α-1-antichymotrypsin, osteopontin, transthyretin, N-terminal albumin peptide	Peptide display technology
Patient biopsies								
20	Iwadate et al. 2004 [71]	Astrocytomas (total $n=85$): GBM ($n=52$), anaplastic astrocytomas ($n=13$), grade II astrocytomas ($n=10$); normal brain tissue ($n=10$)	/	Tissue lysis in buffer A	2DE: IEF: pH3–10 N, 7 cm 2nd dim:12.5% SDS-PAGE, silver staining MALDI TOF MS: PMF	350 / 37 diff.	6 up in low grade: **PDI A3, αB-crystallin, enolase***, Glutamate dehydrogenase I, Phosphopyruvate hydratase; 19 up in high grade: 8 small G-proteins: RalA, Rab3B, nucleolar GTP-binding proteins, CREB* (in grade IV, not II), GRP78*, RhoA*, Rac1*	Hierarchical cluster analysis of proteomics data allowed patient stratification

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Table 1 *(continued)*

	Reference	Sample type	Sample treatment	Protein extract preparation	Proteomic analysis	Proteins detected / identified (IDs) / differentially expressed (diff.)	Identified differentially expressed proteins, highlighted	Comments
Patient biopsies								
21	Furuta et al. 2004 [72]	Astrocytomas (total *n*=30): Primary (*n*=6) and secondary (*n*=7) GBM ; astrocytomas grade II (*n*=5) and grade III (*n*=2); For large tumors multiple samples from 2–3 biopsy sites	/	Manual (laser free) tumor cell microdissection from 10 μm thick unstained sections, yielding 50,000 cells per sample, lysed in buffer B	2DE: IEF: pH4–7. 11 cm 2nd dim: 8–16% gradient SDS-PAGE gels, Silver staining. NanoLC-MS/MS (QSTAR)	n.i. / 11 diff.	In primary GBM: **Tenascin** precursor, **Enolase-1**, Centrosome associated protein 350, **EGFR***. In secondary GBM: ERCC6, DUOX2, Wnt-11 precursor, Cadherin-related tumor suppressor homolog precursor, ADAMTS-19; hnRNP A3*	/
22	Schwartz et al. 2004 [73]	Gliomas of different grades versus normal brain tissue (total *n*=20); normal (5), grade II (3), grade III (3), grade IV (4)	/	frozen tumor section, 12 μm thick unstained	MALDI-MS profiling, 25 μm diameter laser spot; Data range: *m/z* 2000–21,000	>200–400 signals (peaks) in mass profile	/	MALDI for direct proteomic tissue profiling
23	Chumbalkar et al. 2005 [74]	Astrocytoma (total *n*=27) of different grades. Comparison tumor fragment vs tumor periphery, tumor vs epilepsy control.	/	Tissue lysed in buffer A, dilution in buffer B for IEF.	2DE: IEF: pH4–7. 17 cm, 2nd dim: 12% gradient SDS-PAGE gels, Sypro staining (duplicates). MALDI-TOF : PMF ESI-QTOF-MS/MS peptide analysis	n.i. / 72 diff. (>2x difference), 29 common to multiple samples	Rho-GDP dissociation inhibitor, **GFAP***, prohibitin*, **αB-crystallin**, HSP70, HSP60, **Vimentin**	Detection of truncated forms (presumably proteolytic fragments) of glial filament proteins GFAP and vimentin.
24	Iwadate et al. 2005 [75]	Gliomas (*n*=93)	cytotoxic drugs	Tissue lysis in buffer A	2DE, silver stain, MALDI-TOF MS: PMF	n.i. / 41 diff.	signal transduction proteins associated with in vitro chemosensitivity	/
25	Wang et al. 2005 [31]	GBM (*n*=1)	surgically resected tumor covered with polyethyleneglycol and polyvinylalcohol, snap frozen	Laser free microdissection, 100,000cells/sample from HE stained 10 μm tissue sections (50 μg proteins). Cells lysed in buffer B	Capillary IEF-nRP-LC (cIEF), IEF: 84 cm long, pH 3–10 14 or 28 unique cIEF peptide fractions analysed by RP ESI MS/MS (QTOF).	1820 IDs (at least one peptide/ protein)	**Nestin, AnxA1** (16 and 18 unique peptides, respectively)	Methodologically interesting for limited sample amounts.
26	Odreman et al. 2005 [76]	Fibrillary astrocytomas (*n*=10, grade II) and GBM (*n*=10)	/	Tissue homogenized in lysis buffer A	2DE, IEF: pH 3–10, 4–9,18 cm 2nd dim: 10 or 14% PAGE. Silver stain. LC-ESI-MS/MS (ion trap)	85 / 15 diff.	up in grade IV: **peroxiredoxin 1* and 6***, α-internexin*, BTF3*; low in gr. IV: **PDI A3***, UCHL1*; **PKA***	/

27	Schwartz et al. 2005 [77]	Gliomas (total $n=162$); non neoplastic control group ($n=19$), grade II ($n=29$), grade III ($n=22$), GBM ($n=57$); U118MG cell line	/	frozen tumor section, 12 μm thick unstained	MALDI-profiling, Protein identification : 2D-LC and MALDI MS/MS or ESI-IT	300–500 prot peaks in range m/z 2000–70,000	6 candidate biomarkers: calcyclin, calpactin I light chain, tubuline- specific chaperone A, (up in gr. IV); astrocytic phosphoprotein PEA 15* (up in gr.II & III); fatty acid binding protein 5 (up in gr.III); 2 unique markers (dynein light chain 2 and calcyclin) discriminated between 2 surviver groups.	Direct MALDI profiling. Protein profile analysis with 2 independent supervised methods (SDA, WFCCM). Correlation with glioma grade and patient survival.
28	Jiang et al. 2006 [43]	Glioma (total $n=82$): grade II astrocytomas (8), oligodendrogliomas (7), oligoastrocytomas (3), anaplastic astrocytomas (10), oligodendrogliomas (11), oligoastrocytomas (6) and GBM (37)	/	Tissue grinded in liquid nitrogen and lysed in buffer C, extracts spotted on PVDF coated glass array (serial dilutions and triplicates)	Reverse phase protein array (RPPA): Hypridization with 46 antibodies.	46 / 18 diff.	Cluster analysis: cluster of 12 proteins up in GBM, cluster of 6 proteins decreased in GBM. Best discriminators between GBM and other gliomas: IkappaB, **EGFR**pTyr845, **AKT**pThr308*, **PI3K***, BadpSer136*, **IGFBP2***, **IGFBP5, MMP9*, VEGF, pRB**, Bcl-2, c-Abl.	NFkB and EGFR pathway, PI3K and AKT survival pathway, IGFBP2 and IGFBP5 invasion pathway. Follow up on IGFBP2 see [78–80]
29	Li et al. 2006 [81]	Astrocytoma grade II–IV ($n=10$) vs normal brain tissue ($n=4$)	/	Manual microdissection (laser free) of 100,000 cells from non stained serial 10 μm tumor sections	2DE: IEF: pH4–7. 12 cm, 2nd dim: 8–16% Tris–HCl gel, Silver staining. Nano-LC-MS/MS peptide identification	500 / 17 IDs (2DE)	Up in GBM: Ki-67*, N-CoR*, IRS-2*.	/
30	Khalil 2007 [82]	Gliomas (total $n=50$): grades I–IV, control samples	/	buffer B	2D-DIGE, pH3–10, 24 cm MALDI TOF MS: PMF LC-MS/MS	211 / 91 IDs diff.	Up in GBM: Alb protein, **peroxiredoxin 4**, SH3 domain binding glutamic acid-rich-like protein 3 Down in GBM: aldolase C fructose-biphosphate, creatine kinase, B chain dihydrolipoyl-glutathione lyase, lucine aminopeptidase, Mu- crystallin homolog, NADH-UO24, neurofilament triplet L, septin 2, **stathmin**, vacualor ATP synthase subunit E	/

(continued on next page)

Table 1 *(continued)*

	Reference	Sample type	Sample treatment	Protein extract preparation	Proteomic analysis	Proteins detected / identified (IDs) / differentially expressed (diff.)	Identified differentially expressed proteins, highlighted	Comments
Patient biopsies								
31	Mustafa et al. 2007 [83]	Blood vessels from GBM ($n=10$) and normal brain samples ($n=10$)	/	Laser microdissection of blood vessels, 8 μm cryosections mounted on polyethylene naphtalate-covered glass slides, frozen in RapiGest buffer	Direct MALDI-FTMS nano-LC-MALDI-TOF/TOF (1 sample)	MALDI-FTMS: 700–1100 monoisotopic peaks nano-LC: 189 identifications	16 differentially detected peptides in glioma vessels by MALDI- FTMS confirmation by nano-LC analysis: acidic calponin 3, fibronectin*, colligin 2 (HSP47)*	Follow up study on colligin 2 [84]
32	Li et al. 2008 [85]	GBM ($n=4$) vs normal brain cortex ($n=4$)	/	Manual microdissection, cells were digested with trypsin and filled with ampholytes in a CIEF capillary.	Capillary IEF-nRP-LC-MS/MS 12 unique cIEFpeptide fractions analysed by RP and quadrupole-TOF-MS.	104 IDs	Up in glioma: SMC5*, BS69*, prothymosin alpha*, WHSC1*, **Ki-67**	Validation on 94 glioma and 3 control brain samples. WHSC1 staining increases with glioma grade.
33	An et al. 2009 [86]	GBM ($n=10$) vs normal tissues cell lines: U87, HEK293T; F3 neural stem cells	/	tumor lysed in buffer A	2DE : IEF : pH3–10. 2nd dim:9–18% gel, CBB 250G stain MALDI-TOF: PMF	n.i. / 99 diff.	16 up in glioma, including **AnxA2***, TIMP-1*, COL11A1*, Bax*, CD74*, TNFRSF8* and SPTLC2*	Comparative proteomics and microarray anaylsis. AnxA2 identified in both, functional studies suggest involvement in gliotropism
34	Melchior et al. 2009 [28]	GBM ($n=1$)	/	MS compatible trifluorethanol extraction buffer	Bottom–up: peptide 2D-LC, MALDI- TOF/TOF (2 replicates) Semi-top down: IP-RP-	2660 IDs total / 1401 IDs (min. 2 peptides) /	**Calnexin**, neurofascin, transmembrane protein 65, gamma- glutamyltransferase 5, mitochondrial import	Technically interesting and innovative study. Complementarity of bottom–up and semi-

					HPLC on protein and peptide level, MALDI-TOF/TOF (3 replicates)		receptor subunit TOM22 homolog, adipocyte plasma membrane-associated protein	top down approach: 34% overlap between both methods.
35	Park et al. 2009 [87]	GBM ($n=3$), grade II and grade III gliomas	Tumor samples collected with intraoperative imaging from different areas (according to CE-MRI)	lysis in buffer A.	2DE: IEF: pH3–10, 18 cm 2nd dim:10% PAGE gel, silver stain MALDI-TOF: PMF	n.i. / 12 IDs	Ubiquitin carboxyl-terminal esterase L1 (UCHL1)* up in grade II than grade III/IV; transthyretin (TTR)* down in grade II	/
Animal models								
36	Wibom et al. 2006 [35]	BT4C rat glioma model in BDIX rats ($n=24$)	Radiotherapy, time study ($n=3$ / timepoint)	sequential homogenization buffers	SELDI-TOF-MS	77 diff. peaks between treated and untreated	/	Extensive multivariate analysis. PCA and PLS suggest radiation-treatment-induced changes and temporal effects
37	Goplen et al. 2006 [34]	GBM xenografts in nude rats ($n=2$)	non-angiogenic tumor vs necrotic/angiogenic tumor	tumor lysate in buffer B	2DE, pH3–10, 13.5% SDS PAGE, Silver stain MALDI-TOF MS: PMF	n.i.	up in non-angiogenic phenotype: Protein disulfide isomerase A6 precursor (**PDI A6**)	Migration assay suggest PDI A6 involvement in invasion
38	Rajcevic et al. 2009 [29]	GBM xenografts in nude rats ($n=4$)	Non-angiogenic vs angiogenic phenotype	Membrane proteins	iTRAQ, 2D-LC, MALDI-TOF/TOF	>7000 protein IDs (min. 2 peptides)	Panel of 60 proteins increased in angiogenic GBM; panel of 6 proteins increased in non-angiogenic GBM. Validated upregulation in GBM compared to low grade glioma: **Calnexin, AnxA2, AnxA5**	Largest number of reported IDs. Validation using high content TMAs. Increased metabolic activity, cell–cell interaction in angiogenic glioma phenotype

The table includes analyses of cell lines, animal models, patient biopsies and biological fluids (CSF or serum). The techniques employed and the main results (number of proteins and identified regulated proteins) are described. Proteins in bold were identified more than once or have been previously associated with glioma. *Proteins validated by alternative methods. *The authors apologize for any publications that may have been missed.*

study, there are unfortunately hitherto no clear cut biomarkers or target molecules available. A major drawback of many studies is the low sample number and the lack of subsequent validation experiments on a large number of samples. Most studies provide proof of principle of the technology but little or no follow up of the significance of the identified candidates. Validation of differential expression is however mandatory not only on the starting material but, more importantly, on a large number of blinded samples independent of the original analysis. For example, we applied high density glioma tissue microarrays (TMAs) to confirm the increased expression of Calnexin, AnnexinA2 and AnnexinA5 in high grade gliomas [30]. However even though these proteins show significantly elevated expression in high grade gliomas over a large number of samples, their significance to determine tumor type or treatment response in an individual sample remains to be addressed. Thus retrospective validation in e.g. repository tumor banks followed by prospective screening in relevant patient material (serum, CSF, biopsies) are necessary if identified proteins or protein profiles are to be of use in the clinical setting [37]. Nevertheless several proteins or protein families were identified on more than one occasion (cathepsins, annexins, Calnexin, Enolase, EIF-5a, Vimentin, GFAP, IGFPB2, and αB-crystallin) and these warrant further investigation. Some of the proteins may indeed be correlated to glioma grades, others may be functionally involved in glioma development. Bioinformatics-based meta-analysis of available datasets may be one way to separate the wheat from the chaff. This would lead to a selected protein set of interest that could be further exploited by selected reaction monitoring (SRM) (see below). To this aim data sharing in a standardized format and mandatory deposition of proteomic data related to glioma as generally suggested for clinical proteomics would be of great value [38].

5. Perspectives: emerging technologies

5.1. *Antibody-based proteomics and data validation at the protein level*

As mentioned above the validation of proteomic data by non-proteomic methods is crucial for the reliability of the data. Validation at the protein level is normally performed using antibodies against target proteins. The accessibility of quality antibodies has been a nuisance in the first two decades of the proteomics era. The problem has been challenged by the Human Proteome Organization (HUPO) initiative Human Protein Atlas based in Sweden. The Human Protein Atlas project (www.proteinatlas.org) was established to allow for a systematic exploration of the human proteome using antibody-based proteomics. This is accomplished by combining high-throughput generation of affinity-purified (mono-specific) antibodies with protein profiling in a multitude of tissues and cells assembled in tissue microarrays. The main objective of the project is to produce specific and validated antibodies to human target proteins. As reported by the leader of the project Mathias Uhlen at the HUPO 2009 World Congress [39], the new version 5.0 of the Human Protein Atlas contains 8000 validated antibodies targeting 6800 genes corresponding to approximately one third of the protein-encoded genes in humans. The antibodies have been generated to regions of low homology [40] and the long-term objective is to generate paired antibodies towards the protein targets with separate and non-overlapping epitopes [41]. The project of antibody generation will have a crucial impact on the data validation at the protein level in the future whereas it is already used by the Uhlen group as a discovery tool to find potential biomarkers for cancer diagnostics.

5.2. *Reverse phase protein lysate arrays (RPPA)*

High throughput screening of protein samples with specific antibodies can be performed on reverse phase protein microarrays (RPAs), where protein lysates are spotted onto a glass or membrane coated glass slide [42]. In essence similar to a Western blot, this technique allows for the simultaneous screening of several hundred samples with very little sample and antibody requirement. It is particularly useful for validation studies and pathway analysis. Application to glioma samples has been reported focusing on phosphoproteins of relevant signaling pathways [43].

5.3. *Top–down proteomics*

MS-based proteomics analysis at the protein level (top–down approach) is still in its infancies although it heralds some advantages over peptide analysis. In physiological conditions most proteins are modified by one or more types of post-translational modifications. These changes have an impact on protein function, localization, interactions and turnover. Moreover, multiple sequence variants can occur and proteins can associate into functional complexes. With the generation of tryptic peptides, we risk the loss of valuable information, as critical details regarding the extent and interrelationships of these important features are lost [44]. With Fourier-transform (FT) mass spectrometry along with the recent development of a novel mass spectrometer (Orbitrap) and new dissociation methods such as electron transfer dissociation, exciting new areas of proteomic application have been made possible. Although bottom–up proteomics (analysis of proteolytic peptide mixtures) remains the workhorse for proteome analysis, middle– and top–down strategies (analysis of longer peptides and intact proteins, respectively) should allow more complete characterization of protein isoforms and post-translational modifications [45]. Such information will have an important impact in protein target selection for anti-cancer therapy.

5.4. *Targeted proteomics (SRM)*

One limitation of currently employed shotgun proteomics is poor reproducibility which results in the identification of only partially overlapping sets of proteins from substantially similar samples, a problem that is partially inherent to MS where the selection of analysed peptides is automatic and largely stochastic. This is overcome in the approach of selected reaction monitoring (SRM) – also called multiple or targeted reaction monitoring – where a predefined set of peptides is analysed in new generation mass spectrometers

such as triple quadrupole MS [46]. High sensitivity and precise quantitation can be achieved by this novel technology which may emerge as a new standard for accurate quantitation of specific sets of biomarkers across multiple samples [12].

5.5. *MALDI mass spectrometry imaging (MALDI MSI)*

MALDI imaging is a new technology that allows for direct mapping of peptides and proteins on tissue sections with a lateral resolution of 30–50 μm [47]. A MALDI matrix is deposited on the section and upon irradiation of the sample discrete spots are desorbed and analysed in MS, with a detectable mass range between *m/z* 400 and 30,000. Image acquisition can be performed in a profiling mode, where a specified number of spots throughout the tumor section are compared, or in an imaging mode, where a detailed molecular image of the entire tissue section is achieved. The addition of the anatomical dimension to glioma proteomics certainly represents a powerful tool, yet tissue preparation, sensitivity and reproducibility of the technique are still under improvement [48].

5.6. *Metabolomics*

The latest "-omics" approach is metabolomics, the global quantitative assessment of endogenous metabolites within a given tissue. Directly reflecting protein function, metabolite analysis is undoubtedly highly complementary to current proteomics analyses. It can be performed *in vivo* in a localized manner by nuclear magnetic resonance (NMR) spectroscopy, allowing to determine a restricted number of metabolites within certain tumor areas, as was already tried to differentiate gliomas grades [49]. Alternatively for more sensitive and large scale analysis gas chromatography (GC)-MS and LC-MS based technology is used for the measurement of metabolites [50,51]. In view of the importance of the tumor metabolism and the presumed capacity of glioma cells to switch metabolism in response to changes in the microenvironment [52], the large scale determination of tumor metabolites is likely to generate crucial information on glioma grades and treatment response e.g. after anti-angiogenic therapy.

6. Conclusion

Initial proteomics studies aiming at the identification of biomarkers and molecular targets for glioma were mostly small scale classically stained gel-based approaches. In recent years more large scale approaches adopting MS/MS-based proteomics are reported. These generate large amounts of data that require extensive validation and follow-up analysis, they can also be used for comparative meta-analyses of different studies. No proteomics technique is currently able to reveal the complete human proteome, therefore the choice of the technique should be guided by the specific research question and ideally a combination of complementary techniques should be applied. Novel technologies are now starting to be applied to glioma proteomics and will undoubtedly improve the sensitivity and reproducibility of the results. It should be kept in mind though that an analysis will only be as good and as relevant as the starting material from which it was derived.

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