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Proteomic Analysis Reveals Drug Accessible Cell Surface N-Glycoproteins of Primary and Established Glioblastoma Cell Lines

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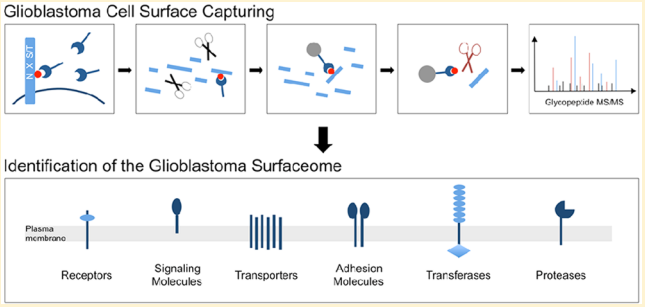
Supporting Information

ABSTRACT: Glioblastoma is the most common primary brain tumor in adults with low average survival time after diagnosis. In order to improve glioblastoma treatment, new drug-accessible targets need to be identified. Cell surface glycoproteins are prime drug targets due to their accessibility at the surface of cancer cells. To overcome the limited availability of suitable antibodies for cell surface protein detection, we performed a comprehensive mass spectrometric investigation of the glioblastoma surfaceome. Our combined cell surface capturing analysis of primary ex vivo glioblastoma cell lines in combination with established glioblastoma cell lines revealed 633 N-glycoproteins, which vastly extends the known data of surfaceome drug targets at subcellular resolution. We provide direct evidence of common glioblastoma cell surface glycoproteins and an approximate estimate of their abundances, information that could not be derived from genomic and/or transcriptomic glioblastoma studies. Apart from our pharmaceutically valuable repertoire of already and potentially drug-accessible cell surface glycoproteins, we built a mass-spectrometry-based toolbox enabling directed, sensitive, and repetitive glycoprotein measurements for clinical follow-up studies. The included Skyline Glioblastoma SRM assay library provides an elevated starting point for parallel testing of the abundance level of the detected glioblastoma surfaceome members in future drug perturbation experiments.



KEYWORDS: *glioblastoma, N-glycoproteins, cell surface accessible drug targets, cell surface capturing, N-glycoproteome SRM library*

INTRODUCTION

Glioblastoma (GBM) is a highly aggressive and infiltrating type of brain tumor, which shows an average post-treatment survival of approximately 15 months.[1] GBM therefore belongs to one of the most severe diseases of the human brain. Of all glia-derived tumors, the primary GBM presents the most frequent tumor of the human brain.[2] Histopathological examination of GBM is currently the main diagnostic procedure,[3] and only little supportive information for detailed classification[4] and targeted molecular therapy is available.[5] Despite advances in radiotherapy, chemotherapy, array-based screening tools, and neuroimaging, the GBM tumor biology is still poorly understood. There remains a significant medical need for effective therapy.[6] Currently, the main limitation is the lack of accessible and targetable molecular features in GBM.[5]

The cell surface proteome (surfaceome[7]) is the most accessible subproteome of the cell for affinity-based probes. The surfaceome mediates cellular interactions with the local microenvironment and further contributes to key functions of the plasma membrane such as the transport of information and biochemical molecules. In GBM, the surfaceome constitutes a pool of accessible proteins that can be used as direct molecular targets in drug therapy, as cell surface markers for delivery of drugs to the tumor tissue and as marker molecules for the monitoring of the disease. Due to its accessibility, the surfaceome represents the largest group of clinically used drug targets.[8] Currently used GBM drugs against cell surface proteins mainly target proteins of the receptor tyrosine kinase (RTK) and integrin signaling pathways.[5] Commonly targeted RTK in GBM are EGFR and PDGFRA[9,10] and are related to the regulation of cell growth. Integrins have been shown to play

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a major role in glioblastoma invasion[11] and are currently evaluated as targets for small molecule inhibitor drugs.[12]

Cell type characterization is commonly performed by the use of cell surface exposed protein markers.[13] Detailed characterization of the GBM surfaceome therefore has the potential to improve tumor-specific drug targeting by specific surface ligands, to improve the classification of the tumor phenotype in diagnostics, to ameliorate monitoring of the disease, and to define key players of GBM malfunction. Despite the importance of the GBM surfaceome in molecular therapy, the knowledge about cell surface protein expression in malignancies of the central nervous system is relatively limited.[14] The type and quantity of the GBM surfaceome is not yet defined and understood. Only a fraction of the GBM surfaceome can be currently investigated by antibody (AB)-based technologies due to general limitations in the availability of suitable antibodies that target the surfaceome. Mass spectrometry (MS)-based proteomics is an alternative approach, which can be applied for surfaceome discovery and quantification, independent of antibodies as affinity reagents. Current MS technology enables the unbiased, sensitive, and parallel investigation of complex protein mixtures.[15] MS further enables the study of post-translational modifications (PTMs). A common PTM of cell surface exposed proteins is protein glycosylation. The chemoproteomic cell surface capturing (CSC) technology[16] specifically targets cell surface exposed proteins that are N-glycosylated. The combination of CSC with AB-independent analysis by MS has demonstrated the possibility of generating sensitive and specific insight into the complex cell surface glycoproteome composition of eukaryotic cells.[16,17]

Here, we provide an extensive analysis of the potentially druggable GBM surfaceome. In order to reveal the most accessible and targetable proteins of GBM, we established a surfaceome map of GBM glycoproteins using the MS-based CSC approach. We further provide Skyline SRM libraries that can be applied for AB-independent and targeted analysis of the GBM surfaceome upon drug perturbation.

MATERIALS AND METHODS

Cell Culture

Four cell lines and four ex vivo cultures established from patients with primary (de novo) GBM were analyzed in the study. LN18 and LN229 cell lines[18,19] were kindly provided by Dr. Nicolas de Tribolet (Geneva, Switzerland). The T98G cell line was obtained from the American Type Culture Collection (ATCC number CRL-1690).[20] The U251MG cell line[21] was obtained from the Human Protein Atlas Project (Stockholm, Sweden).[22] Four ex vivo GBM cell cultures were established at the Department of Neurosurgery of the University Hospital Zürich (Switzerland) as previously described.[23] All procedures were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Canton Zurich. All cell lines were cultured in DMEM medium supplemented with 10% fetal calf serum, 0.292 mg/mL L-Glutamin (Gibco), 100 units/mL penicillin (Gibco), and 100 units/mL streptomycin (Gibco).

CSC Labeling and GBM Surfaceome Preparation

Equal amounts of cells ($\sim 10^8$) were used of each cell line for CSC analysis. The surface exposed GBM glycoproteome was labeled by the bifunctional linker molecule biocytin hydrazide (Biotium) in a two-step procedure.[24] First, oligosaccharides present on cell surface exposed glycoproteins were oxidized to *cis*-diols by sodium periodate (Sigma-Aldrich). A concentration of 1.25 mM sodium periodate was chosen for the experiments to preserve cell survival during oxidation. Second, the linker molecule biocytin hydrazide was attached to the oxidized oligosaccharide forming a covalent bond. Protein mixtures were generated by cold hypotonic lysis (10 mM Tris pH 7.5, 0.5 mM $MgCl_2$). Cell surface proteins were pre-enriched by ultracentrifugation at 35000 rpm (150,000*g*) at 4 °C for 1 h. The resulting membrane pellet (1000 μg) was subjected to detergent lysis by 400 μL of lysis buffer containing 100 mM ammonium bicarbonate/0.1% RapiGest, reduction by 5 mM TCEP (Pierce), and carbamidomethylation of free cysteines by 10 mM IAA (Pierce) before enzymatic digestion into peptides by trypsin (protease/protein ratio of 1:50, Promega). Samples were incubated on a rotor (Miltenyi Biotech) at 37 °C overnight. Trypsin was heat inactivated after digestion at 95 °C for 10 min. Further, the biotin labeled N-glycopeptides were affinity purified by streptavidin capture.[16] Enzymatic cleavage (500 units PnGaseF, New England Biolabs) of the carbohydrate chain specifically released the formerly N-glycosylated peptides from the affinity resin, introducing a MS-detectable mass shift of 0.984 Da (deamidation) at the cleavage site. Before C18 glycopeptide purification, remaining RapiGest was cleaved by acidification (pH = 2.7) at room temperature for 15 min. Ultra MicroTIP Columns (The Nest Group) with 3−30 μg capacity were used for desalting, and peptide mixtures were dried in a vacuum concentrator. Prior to analysis, peptides were solubilized in LC−MS grade water (Fisher Scientific) supplemented with 3% acetonitrile (Fischer Scientific) and 0.1% formic acid (Merck).

Flow Cytometry

CSC labeling of GBM cells was determined by direct immunofluorescence. A total of 500,000 GBM cells labeled with biocytin hydrazide were incubated in 100 μL of a 1:11 dilution of antibiotin-APC AB (Miltenyi Biotec) at 4 °C in the dark for 15 min. CSC labeling efficiency was determined by the analysis of 10,000 cells using flow cytometry (Accuri, C6) in combination with FlowJo software version 7.6.5 (Tree Star).

Confocal Microscopy

Cell surface specificity of the biotin label was determined by immunofluorescence. CSC-labeled GBM cells were grown on poly-L-ornithin/laminin-coated glass coverslips. After CSC labeling, GBM cells were fixed in 4% paraformaldehyde for 30 min. Further, cells were permeabilized in 0.5% NP40/0.5% BSA containing PBS solution for 5 min. After washing, permeabilized GBM cells were stained with 1:10 dilution of antibiotin-APC AB (Miltenyi Biotec) in 0.5% BSA containing PBS blocking solution for 60 min. Further, the cell nucleus was stained with 1 μg/mL DAPI (Merck) for 8 min and washed with blocking solution. Cover slides were mounted using Mowiol (Polysciences). Images were recorded with a confocal laser scan microscope Leica TCS SPE (Leica) with a 40 × 1.4 oil objective and filters for DAPI (excitation 405 nm; emission 450/40) and APC (excitation 635 nm; emission 660/20). Images were analyzed using LCS Lite (Leica) and Photoshop CS4 (Adobe) software.

Mass Spectrometric Analysis of the GBM Surfaceome

Peptide mixtures were analyzed in duplicates on a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific). Peptides (approximately 500 ng/injection) were loaded on a 10 cm reversed phase-high performance liquid chromatography

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column (75 μm diameter) packed with C18 material (Magic C18 AQ 3 μm; Michrom Bioresources) and eluted into the mass spectrometer over a linear gradient of 5−30% Buffer B (2% H_2O, 0.1% formic acid in acetonitrile) in Buffer A (2% acetonitrile, 0.1% formic acid in H_2O) for 60 min. The flow rate was set to 0.3 μL/min. During MS analysis, the peptide ion mass to charge range of 400−1600 was monitored in one high resolution (60000) MS1 scan followed by four MS2 fragmentation scans on the four most intense ions in collision induced dissociation mode. After one MS2 scan, precursor ion masses were temporarily excluded from fragmentation for 30 s. Only ions with charges 2, 3, and 4 were selected for MS2 fragmentation.

MS Data Search and Protein Identification

Raw data files were converted to the open mzXML data format[25] by the ReAdW algorithm (v4.3.1), and MS peptide spectra were searched by the SEQUEST against the human UniProt-SwissProt protein database (version 57.15)[26] extended by common protein contaminants and reversed sequences as decoy entries (in total 40,521 protein entries). Amino acid modifications were permitted for static carbamidomethylation of cysteine (+57.021 Da), variable deamidation of asparagine (+0.984 Da), and variable oxidation of methionine (+15.995 Da). Peptide mass tolerance was set to 0.04 Da. Search allowed for semitryptic peptides and one missed peptide cleavage. Statistical evaluation was based on a target-decoy search strategy and was performed by software tools derived from the Trans Proteomic Pipeline (TPP version 4.4).[27] A peptide and protein false discovery rate of 1% was determined using the nonparametric model in combination with the decoy option in Peptide Prophet. Further, accurate mass binning, N-glycomotif option and leave asterisked score values option were used in Peptide Prophet (-OAPglp). Peptide sequence and N-glycosite assignment information is available in Supplementary Table 1B and the associated Skyline library (Supporting Information).

Bioinformatic Surfaceome Analysis

Search results were filtered for peptides containing a deamidated asparagine present in the N-glycosylation motif consensus sequence (N-x-S/T). The alphabetically first UniProt-SwissProt identifier was used for peptide identifications that were leading to indistinguishable protein group identification. Proteins identified by a single MS spectrum over the whole data set were excluded from further analysis. CD protein annotation was obtained from the UniProt-SwissProt database (v57.15). Protein transmembrane domains were predicted using the PHOBIUS algorithm (v1.01).[28] Membrane-anchored proteins were assigned according to annotation of lipid moiety-binding regions in the UniProt/SwissProt knowledgebase.[26,29] Spectral counting was used to estimate approximate glycoprotein abundances.[30] Classical normalization by molecular weight could not be applied due to the specific enrichment for few distinct N-glycopeptides per protein by the N-glycocapture approach. Therefore, the share of glycopeptide spectrum identifications over all eight cell lines is reported for each protein as an approximate estimation of overall glycoprotein abundance.

GBM N-Glycopeptide Skyline Library for Preselection of Targeted SRM Assays

GBM surfaceome search results and related MS data were processed with the Skyline software (v1.1.1.3110).[32] The Skyline data file (including the 633 identified GBM N-glycoproteins and the corresponding GBM N-glycopeptides) and the associated GBM surfaceome spectral library files are provided as a public resource and are available for download in the Supporting Information.

GBM Surfaceome Spectral Library

A GBM surfaceome consensus spectral library including decoy entries was generated by SpectraST (v4.0).[31] Options for deamidated asparagines (-cg) and peptide probability (-cP0.75) providing approximately 1% FDR were used. A minimum of two replicate MS spectra was required for each consensus library entry. Ratio of decoy versus real size was set to one. The GBM surfaceome spectral library can be used to improve performance of proteomic database search and is available for download in the Supporting Information.

RESULTS

Cell Surface Capturing-Based Glioblastoma Surfaceome

The extraction and analysis of GBM cells is hindered by the fact that GBM is a diffusely infiltrating tumor of the cerebral hemispheres in the human brain.[33] In contrast, cell lines provide a homogeneous system, and we therefore analyzed the surfaceome composition of eight primary GBM cell lines by MS. The cell lines consisted of the four GBM cell lines LN18, LN229, T98G, and U251MG characterized elsewhere.[19−21] Further, we included four primary GBM lines in our study (GBM1−4) that were recently established at the University Hospital Zurich (Table 1). All GBM cell lines used in our study were defined as grade IV glioblastoma according to the World Health Organization (WHO) tumor grade definition.[3]

Table 1. Characteristics and Origin of Primary ex Vivo GBM Cells

cell line	age (years)	gender	WHO[a] tumor grade
GBM1	62	male	IV
GBM2	70	male	IV
GBM3	67	male	IV
GBM4	69	male	IV

[a]World Health Organization.

Cell surface exposed N-glycoproteins were identified by using the CSC technology[16] in combination with high-resolution MS workflows (Figure 1A). Glycostructures of cell surface exposed glycoproteins were labeled with a bifunctional linker molecule upon oxidation of *cis*-diols present in extracellular oligosaccharides. The two-step workflow confers specificity for only labeling proteins exposed at the cell surface of living cells at the time of labeling (Figure 1B and 1C). Labeled glycoproteins were further digested into peptides. Glycopeptides carrying the biotin label were affinity purified and enzymatically released from the affinity resin with PNGaseF and subsequently subjected to MS analysis. A high specificity for N-linked glycoproteins of approximately 80% was obtained over the whole data set in agreement with published literature.[16,34] In average, we identified 343 glycoproteins for each cell line (lowest: 284 in GBM2, highest: 394 in GBM3) using discovery-driven MS strategies. In total, 633 N-glycoproteins of the GBM surfaceome were identified in the analysis (Table 2 and Supplementary Table 1A).

Classical methodologies for the study of GBM are mainly based on antibodies as affinity reagents. The CD nomenclature defines a collection of high quality antibodies that target cell

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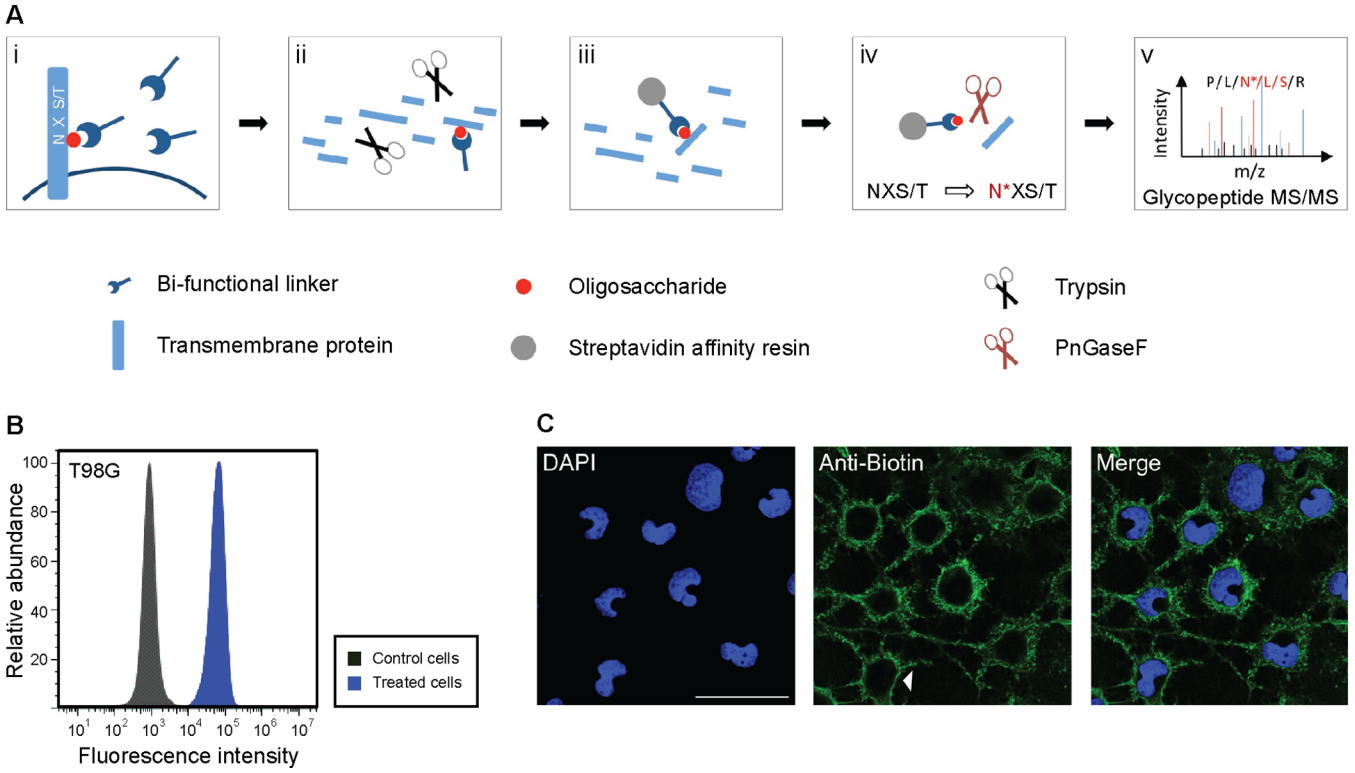

Figure 1. Identification of the glioblastoma surfaceome. (A) CSC workflow targeting GBM surface exposed glycoproteins: (i) attachment of biocytin hydrazide linker molecules to oxidized oligosaccharide structures located at N-X-S/T sequence motifs of glycoproteins exposed to the GBM cell surface; (ii) tryptic digestion of glycoproteins preserving the attachment of linker molecules to the glycopeptides; (iii) affinity purification of glycopeptides; (iv) enzymatic release of glycopeptides by PNGaseF assisted cleavage of oligosaccharide structures. Deamidation (N to D) introduces a glycopeptide mass shift of 0.984 Da; (v) MS analysis of glycopeptides followed by glycoprotein identification using database search. (B) Representative flow cytometry analysis (n = 3) of CSC labeling efficiency after oxidation of living GBM cells. Oxidized cell population efficiently binds the hydrazide linker molecule compared to a nonoxidized control population. Equal amounts of cells (10,000) were used for analysis. Similar results were obtained for three GBM cell lines. (C) Immunofluorescence staining of CSC labeling indicates plasma membrane specificity of the bifunctional linker molecule. DAPI: nucleus; biotin: antibiotin-APC AB; scale bar: 50 μm; arrow: membrane-specific staining.

Table 2. Number of N-Glycoproteins and CD-Annotated Proteins Identified in Four GBM Cell Lines and Four ex Vivo GBM Cultures

cell type	passage	glycoproteins	CD proteins	enrichment[b]
LN18	NA[a]	366	82	72.3
LN229	NA	377	79	71.8
T98G	NA	332	68	73.5
U-251MG	NA	286	69	87.8
GBM1	6	315	73	76.4
GBM2	4	284	77	94.0
GBM3	3	394	79	94.6
GBM4	5	393	85	92.4
total		**633**	**108**	**77.2**

[a]NA: not applicable. [b]Glycoprotein enrichment efficiency (%).

surface proteins.[13] To make use of these commercially available CD antibodies in the future, the GBM surfaceome was subdivided into CD- and non-CD glycoproteins to identify GBM cell surface molecules with an immediate value for clinical research. From the 633 identified glycoproteins, 108 were annotated as CD proteins (Supplementary Table 1A). As expected, the majority of the 633 identified glycoproteins are non-CD-annotated N-glycoproteins (83%), emphasizing the strength of the MS-based approach for GBM surfaceome characterization complementary to AB-based technologies (Figure 2).

Another advantage of the chemoproteomic CSC approach is the possibility to identify many different types of hydrophobic membrane proteins with high sensitivity. These proteins are difficult to detect by commonly applied gel-based approaches. Membrane proteins share the common characteristic of high hydrophobicity that is caused by the hydrophobic transmembrane protein domains. To further examine the different types of membrane proteins identified by our approach, we subjected the 633 identified GBM glycoproteins to a bioinformatics transmembrane-domain (TMD) analysis using the PHOBIUS algorithm.[28] The majority of glycoproteins identified (55%) specifically contained one TMD (Figure 3). Prominent examples of the single TMD category were members of the growth factor receptor family such as the GBM related epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) which represent two major drug targets in GBM.[5] Another 26% of the identified GBM glycoproteins belong to the multi-TMD category. Proteins of the multi-TMD group included G-protein coupled receptors with seven TMDs, which represent a large and pharmacologically important receptor family.[35] The remaining 19% of the identified glycoproteins consisted of glycosylphosphatidylinositol (GPI)-anchored membrane proteins and membrane-associated glycoproteins. Our CSC approach experimentally verified the occupancy of 1123 sites of N-linked glycosylation in GBM. Due to the nature of the labeling approach, identified N-glycopeptides are located at the

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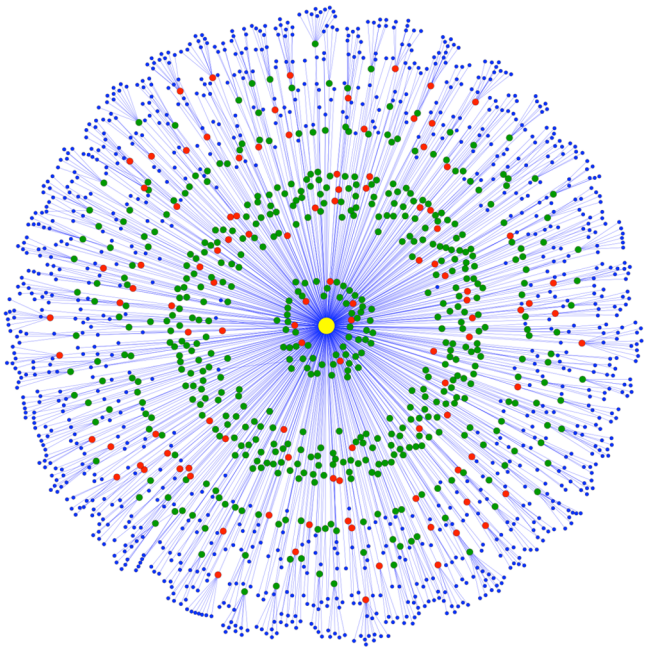

Figure 2. Representation of the glioblastoma surfaceome. The GBM surfaceome consists of 633 glycoproteins and 1123 unique N-glycosylation sites. Proteins identified with multiple N-glycopeptides are located toward the outer part of the figure. CD antibodies can currently target only 17% of the GBM surfaceome. Color code: GBM cell surface proteins (green) with CD annotation (red) and experimentally verified tryptic N-glycopeptides (blue) located around GBM cell (yellow center). Software: Cytoscape (version 2.6.3).[48]

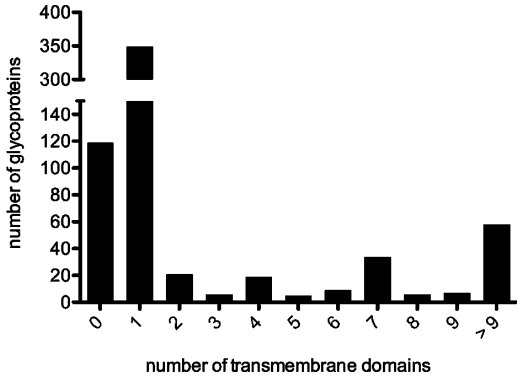

Figure 3. Transmembrane domain content of 633 GBM surface proteins. CSC analysis reveals GPI-anchored/membrane associated, single, and multi-TMD containing GBM glycoproteins. TMD prediction software: PHOBIUS (v1.01).[28].

extracellular domain of the membrane protein (Figure 4). Information about extracellular glycosylation site occupancy can be biologically important and aides in antigen design during AB development. It further improves topology prediction of membrane proteins in protein databases.[36]

Cell surface proteins provide the interface for cellular communication and cell interaction with the microenvironment. Different cell surface protein classes thus fulfill a variety of cellular functions. We grouped the GBM surfaceome into functional categories by the PANTHER protein classification system.[37] Of the 633 glycoproteins, 502 glycoproteins were functionally annotated. Six major categories were identified consisting of receptor proteins (32%), transporters (15%), signaling molecules (14%), transferases (12%), proteases (4%),

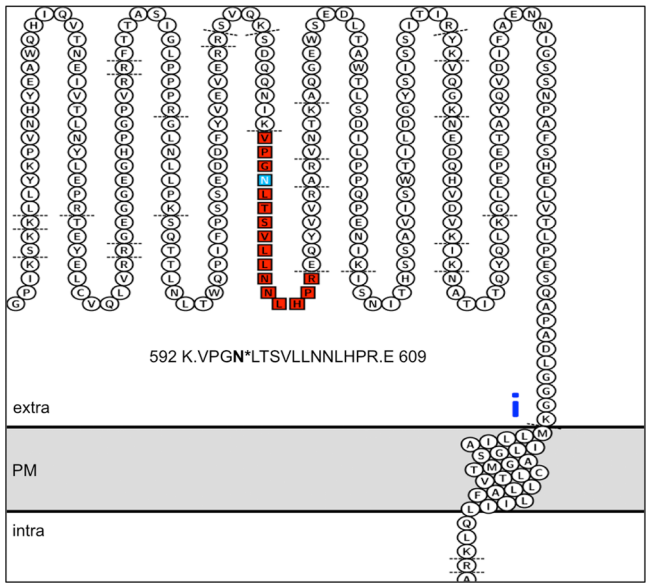

Figure 4. Experimental verification of extracellular N-glycosites in GBM. CSC targets N-glycopeptides located in the extracellular domain of glycoproteins. The angiopoietin-1 receptor CD202b (TIE2) is shown as a representative example. It is involved in the remodeling of the local tumor microenvironment and was recently identified as being increasingly expressed from low to high grade malignant glioma.[49,50] The glycopeptide 592-K.VPGN*LTSVLLNNLHPR.E-609 (red) verified the presence of CD202b (TIE2) in the CSC analysis of the GBM surfaceome. CD202b (TIE2) sequence and TMD derived from UniProt. Potential tryptic cleavage sites are indicated (dashed lines). PM: plasma membrane; N*: site of N-linked glycosylation; software: PROTTER (unpublished).

and cell adhesion molecules (4%) (Figure 5A). An overview of functional protein classes of the GBM surfaceome is provided in Supplementary Table 1C.

In addition to the functional characterization, the abundance of a protein and its common expression are of interest for drug target selection. Spectral counting was used to obtain an approximate estimate of glycoprotein abundance.[30,38] We then determined the relative amount of protein dedicated to a functional category on the GBM surface. On the basis of the estimation of protein abundance nearly 70% of the GBM surfaceome is made up of the six identified major functional protein classes (Figure 5B and C). The group of cell adhesion molecules was found to have a larger contribution on the GBM surfaceome based on protein abundance than expected before by the numbers of proteins that were classified in this category. It mainly consists of integrin proteins and might indicate their important role in GBM tumor biology as previously described.[39] The hyaluronate receptor CD44 is known to be highly expressed in GBM[40] and was the protein identified with the highest number of spectra in the eight GBM cell lines. The three proteins identified with most spectra for each functional category were CD44, CD166 (ALCAM), CD29 (ITGB1) for receptors; CD90 (THY1), CD109, FINC (FN1) for signaling molecules; GTR1 (GLUT1), AT1B1 (ATP1B1), AAAT (SLC1A5) for transporters; CD49c (ITGA3), CD49e (ITGA5), CD51 (ITGAV) for cell adhesion molecules; PTK7 (CCK4), PLXB2 (PLXNB2), EGFR (ERBB1) for transferases; and CD13 (ANPEP), ECE1, SEPR (FAP) for proteases.

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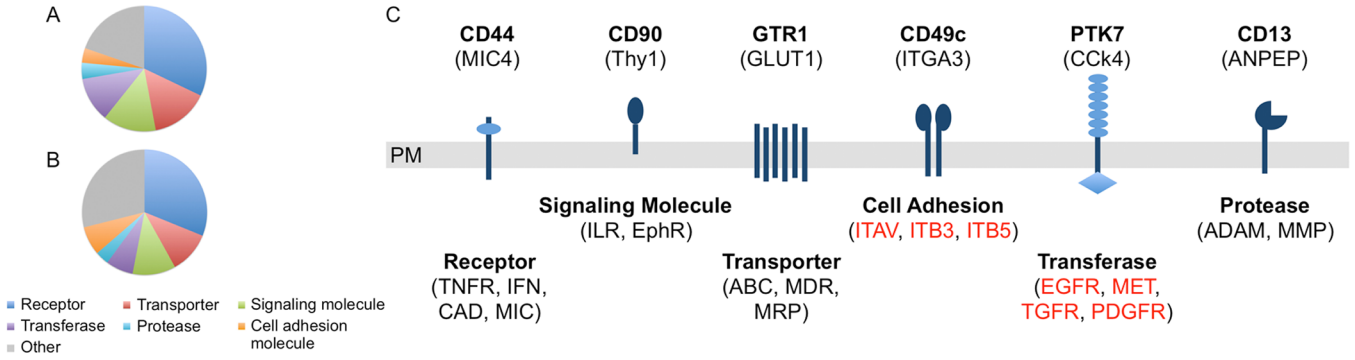

Figure 5. GBM surfaceome characterization by CSC. Functional GBM surfaceome categorization by PANTHER based on (A) number of proteins present in each functional group and (B) approximate protein abundance determined by spectral counting. (C) Visualization of the GBM surfaceome. Major protein groups are shown based upon their functional influence. The top protein identified with most spectra for each functional group is highlighted. Prominent GBM proteins and protein families identified are indicated below. Existing GBM drug targets are highlighted in red.

Cell surface proteins currently used in molecular targeted therapy of GBM are based on receptor tyrosine kinases (RTKs) and integrins.[5] In our surfaceome analysis, we identified family members belonging to both protein groups including their major drug targets, which are currently used in therapy or evaluated in clinical trial studies. Of the frequent RTK drug targets, we identified the EGFR and CD340 (HER2), CD140a (PDGFRA) and CD140b (PDGFRB), and MET. Of the 24 existing integrin chains, 16 were identified as members of the GBM surfaceome, including CD51 (ITGAV), CD61 (ITGB3), and ITB5 (ITGB5), which are known to form the GBM-related heterodimers $\alpha v\beta 3$ and $\alpha v\beta 5$. These heterodimers are promising targets in GBM therapy that are currently being evaluated in clinical trials.[41] In total, we identified 123 GBM glycoproteins that are annotated as general drug targets by DrugBank[42] and might represent additional targets for GBM molecular targeted therapy (Supplementary Table 1D). In summary, the CSC analyses revealed the cell surface composition of GBM including known and potential new drug targets and allowed the establishment of a functional surfaceome map.

As a complementary technology to monoclonal AB-based methodologies, MS-based proteomics enables the discovery and quantification of surfaceome proteins that are currently difficult to study due to the lack of suitable affinity reagents. Targeted measurements of selected proteins of interest by selected reaction monitoring (SRM)[43,44] has been emerging recently. Together with isotopically labeled peptide standards, it provides the possibility of absolute measurements of protein abundance at high sensitivity and specificity.[45] SRM measurements require the prior experimental determination of MS-suitable peptide and fragment ion masses as well as the measurement of the correct chromatographic elution time. The setup of such assays might vary depending on machine type and chromatographic system used. With our GBM surfaceome data set, we provide the necessary LC–MS information (target glycopeptides, glycopeptide transitions, chromatographic elution time) for the setup of individual glycoprotein assays targeting the GBM surfaceome in the form of a Skyline data file and a GBM Skyline spectral library[32] (Supporting Information).

DISCUSSION

We present an extensive GBM surfaceome analysis that provides detailed information about the quality and an approximate estimation of quantity of cell surface accessible GBM glycoproteins. The acquired GBM surfaceome map enables the rational selection and ranking of potential GBM drug targets based on their detectability and estimated abundance. CSC analysis revealed 633 distinct glycoproteins based on a set of eight GBM lines. The detailed characterization of the typical GBM surfaceome map highlights six major functional protein groups, which define but also limit cellular interaction capacities of GBM with the local tumor microenvironment. Among the identified glycoproteins, several clinically relevant GBM glycoproteins were detected, such as the RTK drug targets EGFR, CD340 (HER2), CD140a (PDGFRA), CD140b (PDGFRB), and MET and several integrin chains that constitute the drug targeted $\alpha v\beta 3$ and $\alpha v\beta 5$ heterodimers. The rediscovery of known drug targets demonstrate the potential of the presented strategy for future drug target discovery and potential drug repurposing. Furthermore, a large number of coexpressed cell surface exposed glycoproteins were identified with little prior information in the context of GBM and for which the clinical relevance can now be investigated.

The analysis of the GBM surfaceome using CSC technology in combination with quantitative MS technology enabled the approximate estimation of abundance ranges for cell surface exposed N-glycoproteins based on the spectral counting approach. Quantitative spatial information is important for the pharmaceutical industry, which is interested in developing affinity-based probes for drug delivery against selected proteins with high expression at the cell surface. Further, our GBM surfaceome data provide clinicians with initial cues about approximate glycoprotein abundances within the observed GBM surfaceome. Depending on the desired pharmaceutical targeting strategy, protein abundance estimates within the GBM surfaceome may aid in the prioritization of targets based on protein function, detectability, and abundance range. Quantitative information about protein abundance in a specific subcellular location is difficult to obtain by other technologies, such as transcriptomic approaches. Our data thus provides a basis for the selection of potential drug targets based on cell surface protein abundance in a particular location without claiming a detailed protein abundance ranking order.

The GBM surfaceome data further provides insights into GBM membrane protein topology. The specific identification of 1123 extracellular protein N-glycosylation sites enables the refinement of topology prediction algorithms such as PHOBIUS by adding the N-glycosylation information as

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specific constraint during the model building. Improvement of the ability to predict the orientation of multitransmembrane spanning proteins is a prerequisite for the targeted development of antibodies against cell surface exposed protein domains.

Affinity-based technologies are important tools for single cell level studies of GBM and molecular targeted therapy. Our data experimentally confirmed the cell surface expression of 108 CD annotated proteins. In addition, the GBM surfaceome data can be used for selection of antibodies from publicly available databases such as the Human Protein Atlas portal.[22] IHC validated HPA-ABs were found for 97 members of the GBM surfaceome (Supplementary Table 1E). This information can be immediately used for the selected testing and evaluation of antibodies in clinical screening studies of GBM samples of different WHO tumor grades.[3] Depending on the quality of the available antibodies, such an approach could also be extended to CyTOF technology, which now enables high multiparametric phenotyping of cancer cells on the single cell level.[46]

However, the value of identified GBM glycoproteins, which are not yet accessible by currently available AB-based probes, should not be neglected. This large pool of proteins, either not known or understudied so far by the research community, can now be investigated by using the provided Skyline[32] N-glycoprotein SRM assay library. SRM-based workflows[43,44] provide the technology to determine absolute quantities of selected proteins over a large number of samples. Our data provides preselected proteotypic glycopeptides of the GBM surfaceome and their fragment ions masses based upon their characteristics in the mass spectrometer. A Skyline library is provided as a public resource for the rapid selection of CSC-verified GBM glycopeptides. Such surfaceome-dedicated N-glycopeptide assays go in line with the recent HUPO initiative that aims to map the entire human proteome.[47] Usage of the provided SRM assays for the parallel investigation of proteins of clinical interest in larger patient cohorts might allow prequalification of glycoproteins as markers for disease monitoring in GBM model systems and ultimately patients.

■ ASSOCIATED CONTENT

Supporting Information

Proteomic GBM surfaceome MS data, protein annotation by PANTHER and protein cross-references to Drugbank and Human Proteinatlas. This material is available free of charge via the Internet at http://pubs.acs.org. Data available for download at Tranche (https://proteomecommons.org/tranche/): Skyline library and data files (I3z832lZF6lNQrYLSUj/gVhkF3LlBsre3pdWPRFMS3iKkvjrLzmLk6Js66m84WYGfaA44SemnPzNQ+87PEZ/WXTu294AAAAAAAADIg==); SpectraST data (ZRrw4shhsBEfoumktQTB5dKh0geG1wI8l6zgP9dfJ3ENAoMUkbnhDMYWMM2/+CY50Uh8mQw3g8U1fOgHVSfgKilWUUkAAAAAAAACpw==); MS RAW data (hF7ojMW+T0Q3Ys06cQ7Zkupm/PVRspafRjswYuo2Aoa+P9tZn/IG3cWtPtENHLtvVz5GJzQ4WzN/GY+RTzvIuyX8WlYAAAAAAAACUg==)

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Notes

The authors declare no competing financial interest.

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T.B. and B.W. conceived the project and wrote the manuscript. B.W. supervised the research. T.B. performed the experiments and analyzed the data. H.M., U.O., A.F., D.B., and A.H. assisted with proteomic experiments and data analysis. S.D. performed ex vivo cell culture. A.J. assisted in flow cytometry experiments and data analysis. E.L. provided human proteinatlas data. K.F., N.K., and R.A. assisted with study design and provided vital research reagents. K.F., E.L., M.U., and R.A. commented on the strategy and the manuscript and helped with data interpretation. All authors discussed the results and commented on the manuscript.

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