# Proteomics reveals differentially regulated pathways when comparing grade 2 and 4 astrocytomas

Denildo C. A. Verissimo[1,2]☯, Amanda C. Camillo-Andrade[1]☯, Marlon D. M. Santos[1], Sergio L. Sprengel[2], Simone C. Zanine[2], Luis A. B. Borba[2], Paulo C. Carvalho[1]*, Juliana de S. da G. Fischer[1]*

1 Laboratory for Structural and Computational Proteomics—Carlos Chagas Institute, Fiocruz Paraná, Curitiba, PR, Brazil, 2 Clinical Hospital of the Federal University of Paraná, Curitiba, Paraná, Brazil

☯ These authors contributed equally to this work.
* paulo@pcarvalho.com (PCC); julifr@gmail.com (JSGF)

Abstract

Astrocytic tumors are known for their high progression capacity and high mortality rates; in this regard, proteins correlated to prognosis can aid medical conduct. Although several genetic changes related to progression from grade 2 to grade 4 astrocytoma are already known, mRNA copies do not necessarily correlate with protein abundance and therefore could shadow further comprehension about this tumor's biology. This motivates us to seek for complementary strategies to study tumor progression at the protein level. Here we compare the proteomic profile of biopsies from patients with grade 2 (diffuse, $n = 6$) versus grade 4 astrocytomas (glioblastomas, $n = 10$) using shotgun proteomics. Data analysis performed with PatternLab for proteomics identified 5,206 and 6,004 proteins in the 2- and 4-grade groups, respectively. Our results revealed seventy-four differentially abundant proteins ($p < 0.01$); we then shortlist those related to greater malignancy. We also describe molecular pathways distinctly activated in the two groups, such as differences in the organization of the extracellular matrix, decisive both in tumor invasiveness and in signaling for cell division, which, together with marked contrasts in energy metabolism, are determining factors in the speed of growth and dissemination of these neoplasms. The degradation pathways of GABA, enriched in the grade 2 group, is consistent with a favorable prognosis. Other functions such as platelet degranulation, apoptosis, and activation of the MAPK pathway were correlated to grade 4 tumors and, consequently, unfavorable prognoses. Our results provide an important survey of molecular pathways involved in glioma pathogenesis for these histopathological groups.

Introduction

Gliomas are the most common primary brain tumors, accounting for more than 70% of all primary central nervous system (CNS) neoplasms. They are known for their high progression

capacity and for leading to increased mortality rates. Astrocytic tumors are neuroepithelial tumors originating from the supporting glial cells of the CNS [1]. They can occur from the first decade of life and manifest in any location of the central nervous system (CNS) [2].

The worldwide incidence of tumors of the nervous system in 2020 was about 300,000 new cases (17th position among registered types of cancer) and presented mortality of 241,037 as per Word Health Organization (WHO) (12th position) [3]. In Brazil, according to Cancer National Institute (INCA) data, the estimated incidence for 2022 is more than 11,000 new cases, and the mortality in 2020 was 9,309, following the relatively high global rates.

Proteomics was previously used by Gollapalli *et al.* to analyze various grades of glioma tissues. The authors pinpointed several metabolic pathways such as glycolysis, TCA-cycle, electron transport chain, lactate metabolism, and blood coagulation pathways that were altered in gliomas. Most of the more abundant proteins in gliomas were correlated with redox reactions, protein folding, pre-messenger RNA (mRNA) processing, antiapoptotic, and blood coagulation [4].

Although several genetic changes related to progression from grade 2 to grade 4 astrocytoma are already known [5], mRNA copies do not necessarily correlate with protein abundance. Here we use shotgun proteomics to shortlist pathways altered during the progression of astrocytes tumors. To achieve this, we compared proteomic profiles of biopsies from patients with grade 2- (diffuse, $n = 6$) versus grade 4 astrocytoma (glioblastomas, $n = 10$). Our results provide an important survey of molecular pathways involved in astrocytoma pathogenesis for these histopathological groups.

Materials and methods

Materials

Qubit$^®$ Protein Assay Kit (cat. no. Q33212) and RapiGest^{TM} SF acid-labile surfactant (cat. no. 186001861) were acquired from Invitrogen and Waters, respectively. Sequence grade modified trypsin (V511A) was purchased from Promega. All other laboratory reagents were bought from Sigma-Aldrich (St. Louis, MO), unless stated otherwise.

Methods

Patients and ethics aspects. All methods were performed in accordance with the relevant guidelines and regulations of Brazil. This study was approved by the Ethics Committee of Oswaldo Cruz Foundation and the Federal University of Clinical Hospital of Parana under the numbers CAAE: 63056316.8.0000.5248 and 63056316.8.3001.0096, respectively. Informed written consent was obtained from all subjects and/or their legal guardian(s) before participating in the study. All participants were provided with detailed information about the study, its objectives, and the use of their data for research purposes. No minors participated in this study. All data were fully anonymized before analysis to ensure participant privacy.

After being collected by a neurosurgeon belonging to the staff of the Clinical Hospital of the Federal University of Paraná, the tumor samples were immediately put on dry ice and then stored in a -80°C freezer until used. This study includes sixteen patients with astrocytoma; six diagnosed with grade 2 and ten with grade 4, respectively. The characteristics of the patients included in this study are presented in Table 1.

All astrocytomas grade 2 and all grade 4 were classified as NOS (Not Otherwise Specified) according to histopathological features and patient's history in accordance with WHO CNS5 specifications.

Sample preparation. The set of sixteen tissue samples was pulverized under liquid nitrogen, following a previously described technique [6]. Protein extraction was then carried out

Table 1. Characteristics of the patients included in this study, indicating the age, gender, and histopathological diagnosis.

Patient ID	Age	Gender	Tumor grade
11	19	Male	2
31	34	Female	2
48	18	Male	2
95	18	Male	2
106	27	Female	2
113	58	Male	2
25	75	Female	4
26	41	Male	4
59	64	Male	4
39	55	Male	4
53	72	Female	4
69	77	Female	4
46	47	Female	4
108	50	Male	4
112	74	Male	4
29	57	Female	4

https://doi.org/10.1371/journal.pone.0290087.t001

using a solution of 0.1% RapiGest (w/v) in 50 mM triethylammonium bicarbonate (TEAB). Subsequently, centrifugation at 18,000 x g at 4°C for 15 minutes took place, and protein quantification was performed with the Qubit 3.0 fluorometric assay in accordance with the manufacturer's protocol. A total of 100 µg protein from each sample was subjected to reduction with 10 mM dithiothreitol (DTT) at 60°C for 30 minutes. After cooling the samples to room temperature, they were incubated in the absence of light with 25 mM iodoacetamide (IAA) for 30 minutes. Lastly, the samples were digested over 20 hours with high sequence grade modified trypsin, utilizing a 1:50 (Enzyme/Substrate) ratio at 37°C [7].

Desalting and sample quantification. The enzymatic reaction was terminated by adding trifluoroacetic acid (0.4% v/v final) to the samples, and peptides were incubated for an additional 45 minutes to decompose RapiGest. The samples were then centrifuged at 18,000 x g for 15 minutes to remove any remaining insoluble components. Following this step, peptide quantification was performed using the Qubit 3.0 (Invitrogen) fluorometric assay, as per the manufacturer's guidelines. Each sample was desalted and concentrated employing Stage-Tips (STop and Go-Extraction TIPs), based on the method established by Rappsilber et al. [8].

Mass spectrometry acquisition. Peptides were analyzed via LC-MS/MS (Liquid chromatography with tandem mass spectrometry) using an UltiMate 3000 (Thermo Fisher®) ultrahigh-performance liquid chromatography (UHPLC) system connected to an Orbitrap FusionTM LumosTM mass spectrometer (Thermo, San José). Peptide mixtures were loaded onto a 75 mm i.d., 30 cm long column packed in-house with 3 µm ReproSil-Pur C18-AQ resin (Dr. Maisch) at a flow rate of 250 nL/min and subsequently eluted at a flow rate of 250 nL/min from 5% to 40% ACN in 0.1% formic acid over a 140 min gradient [9]. The mass spectrometer was configured in data-dependent acquisition (DDA) mode, allowing automatic switching between full-scan (MS) and MS/MS (MS2) acquisition. Survey MS spectra (from m/z 300–1,500) were captured in the Orbitrap analyzer at a resolution of 120,000 at m/z 200. The most intense ions obtained within a 2s cycle time were selected, excluding unassigned or 1+ charge state ions. These ions were sequentially isolated and fragmented using higher-energy collisional dissociation (HCD) with a normalized energy of 30. Fragment ions were analyzed at a

resolution of 15,000 at 200 *m/z*. General mass spectrometric conditions included a spray voltage of 2.5 kV, no sheath or auxiliary gas flow, ion transfer tube temperature at 250˚C, predictive automatic gain control (AGC) enabled, and an S-lens RF level of 40%. The mass spectrometer scan functions and nLC solvent gradients were controlled using the Xcalibur 4.1 data system (Thermo, San José). Each biological replicate underwent two technical replicates.

Peptide spectrum matching (PSM).   Data analysis was conducted using the PatternLab for proteomics software, which is freely accessible at http://www.patternlabforproteomics.org [10, 11]. Homo sapiens sequences were retrieved on June 6th, 2020, from Swiss-Prot and subsequently utilized to generate a target-decoy database, which included a reversed version of each sequence along with 104 common mass spectrometry contaminants. The Comet 2016.01 rev. 3 search engine was employed to identify the mass spectra [12].

The search parameters took into account fully and semi-tryptic peptide candidates with masses ranging from 550 to 5,500 Da, up to two missed cleavages, 40 ppm for precursor mass, and bins of 0.02 m/z for MS/MS. Fixed and variable modifications considered were carbamidomethylation of cysteine and oxidation of methionine, respectively.

Validation of peptide-spectrum match.   Evaluation of PSM validity was performed using the Search Engine Processor (SEPro) [13]. The identifications were organized by charge state (2+ and $\geq$ 3+) and tryptic status, generating four distinct subgroups. A Bayesian discriminator was created for each group using XCorr, DeltaCN, DeltaPPM, and Peaks Matches values. The identifications were sorted in a non-decreasing order based on the discriminator score. A cutoff score allowed a false-discovery rate (FDR) of 2% at the peptide level, determined by the number of decoys [14].

This procedure was executed independently for each data subset, resulting in an FDR not influenced by charge state or tryptic status. Moreover, a minimum sequence length of five amino acid residues and a protein score exceeding three were imposed. Lastly, identifications diverging by over 10 ppm from the average were excluded. The implementation of these postprocessing filters led to FDRs at the protein level being less than 1% for all search outcomes [15].

Relative protein quantification.   The quantification was carried out according to the PatternLab's Normalized Ion Abundance Factors (NIAF) as a relative quantification strategy. We remind that the NIAF is equivalent to the NSAF [16] but applied to the extracted ion chromatogram (XIC) [17].

Quantification was conducted using PatternLab's Normalized Ion Abundance Factors (NIAF) as a strategy for relative quantification. It is worth noting that NIAF is analogous to the NSAF [16] but applied to the extracted ion chromatogram (XIC) [17].

Pinpointing differentially abundant proteins and data analysis.   We employed PatternLab's TFold module [18] to pinpoint proteins exhibiting differential abundance between grades 2 and 4 groups. Our proteomic comparison considered only proteins identified with a minimum of two unique peptides (peptides mapping to a single database sequence) and a q-value $\leq$ 0.1. Proteins present in at least two biological replicates were considered for TFold analysis. This method is concisely outlined in our bioinformatics protocol [10, 11]. Protein pathways were mapped using Reactome [19].

Results and discussion

Protein identification in grade 2 and grade 4 astrocytomas

Here we use shotgun proteomics to shortlist alterations in the protein pathways when comparing biopsies of grade 2 versus grade 4 astrocytomas. The higher number of grade 4 astrocytoma (ten) is in line with its greater prevalence in the population [20].
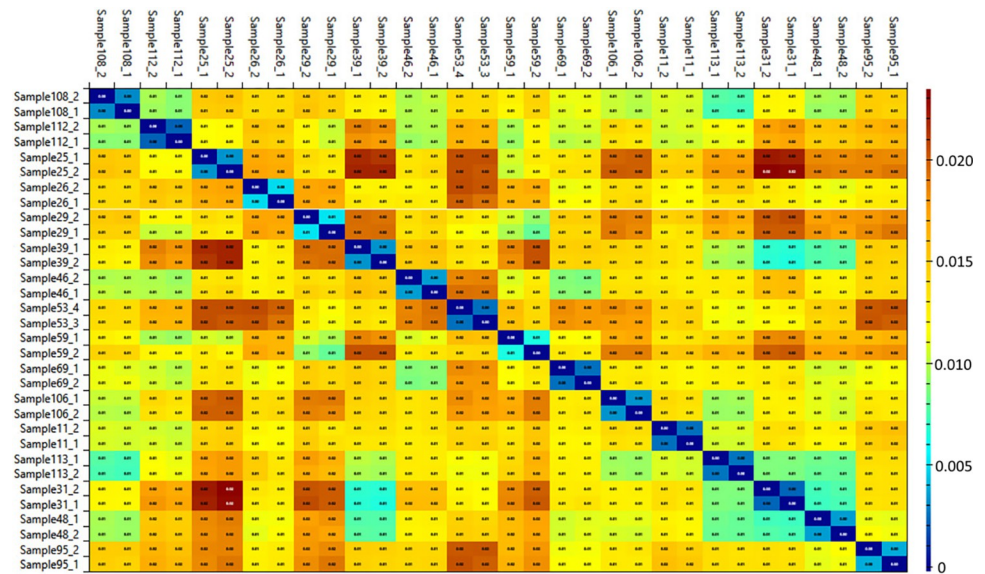
Fig 1. Heatmap comparing grade 2 and 4 astrocytomas studied. The colors represent the similarity between samples. The closer to zero (blue), the greater the similarity.

https://doi.org/10.1371/journal.pone.0290087.g001

For the group of grade 2 astrocytomas, we identified 52,133 peptides mapping to 5,816 proteins (5,206 with maximum parsimony). In the grade 4 group, we identified 62,759 peptides mapping to 6,686 proteins (6,004 with maximum parsimony).

We evaluated the reproducibility of our technical replicates with the RawVegetable program [21]; a heatmap is presented in Fig 1.

Our heatmap's lower-right corner presents a greater color uniformity, indirectly reflecting less proteomic variability among the grade 2 tumors.

We used PatternLab's Venn diagram module to list proteins uniquely identified in each group of tumors studied (Fig 2).

The Ven Diagram shows that 748 proteins were identified only in the grade 4 astrocytoma, while 174 proteins were exclusively for the grade 2 astrocytoma. As both tumor types originate from the same cell type, most proteins are common (2,658 proteins). The complete list of proteins found in each condition is available in S1 Table.

Differentially abundant proteins between grade 2 and grade 4 astrocytomas

The differentially abundant proteins between grade 2 and 4 were analyzed with the TFold module of PatternLab for Proteomics. The abundance of each protein was statistically evaluated under the criteria of a variable fold change and satisfying a q-value (Fig 3). The seventy-four differential abundant proteins list is available in S2 Table.

We used the Reactome (reactome.org) platform [19] to list the enriched metabolic pathways, for each grade tumor, under the light of differentially abundant proteins (blue dots in Fig 3) (Table 2).

Proteins exclusively identified in the grade 4 astrocytomas

Among the proteins identified only in the grade 4 astrocytomas, we highlight Rho GTPase-activating proteins, Cathepsin and S-100.

The Rho GTPase family is involved in critical cellular functions such as cell polarity, vesicular traffic, cell cycle, and transcriptome dynamics [22]. Due to this relationship with cancer,
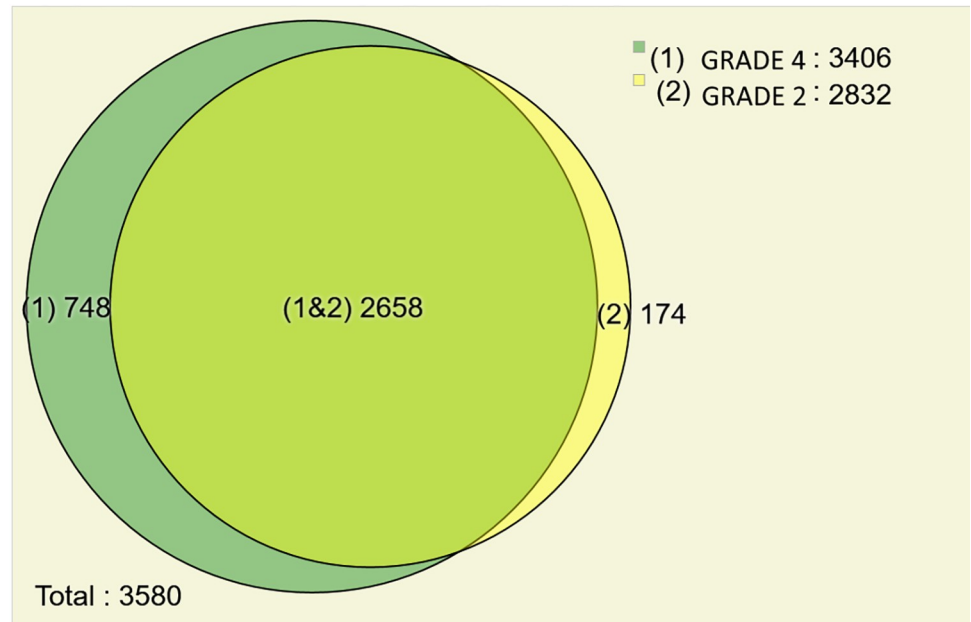
Fig 2. Venn diagram of proteins identified in the grade 2 and 4 astrocytomas. We pinpointed 748 and 174 proteins exclusive of the grade 4 and grade 2 astrocytomas, respectively.
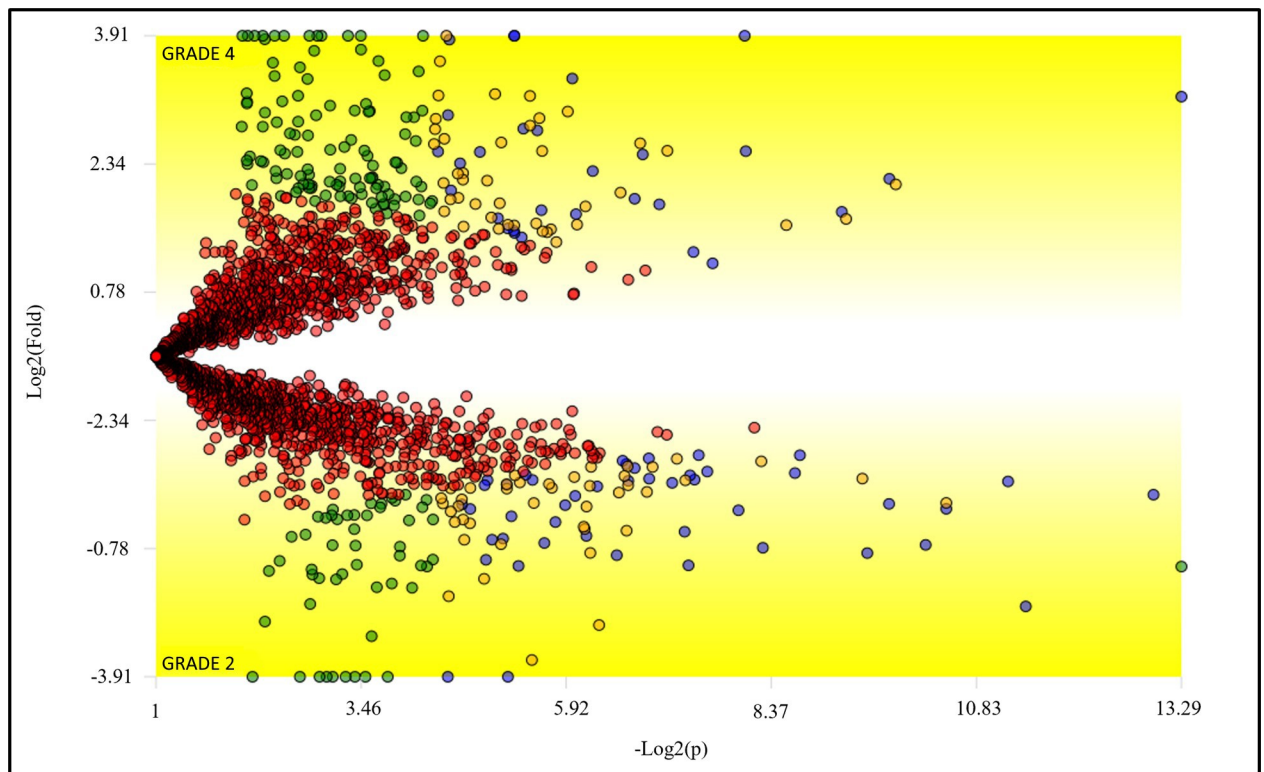
Fig 3. Differentially abundant proteins between grade 2 and grade 4 astrocytomas according to PatternLab for proteomics TFold module. Each "dot" represents a protein. The blue dots represent the 74 proteins that meet both statistical analyses plus the Benjamini Hochberg FDR correction between the two groups and are differential abundant. Orange dots indicate the 84 proteins that passed the t-test criterion but not the fold change criterion. The red dots represent not differentially abundant proteins in either of the two criteria. The green dots indicate the 165 proteins that have passed the fold change criterion. However, the t-test indicates that its differential abundance was random.

Table 2. Enriched metabolic pathways for grades 2 and 4 astrocytomas.

Grade 2	Grade 4
ECM proteoglycans	Integrin cell surface interactions
Pyruvate metabolism and Citric Acid (TCA) cycle	Fibronectin matrix formation
Mitochondrial protein import	Peroxisomal protein import
Metabolism of carbohydrates	Oncogenic MAPK signaling
Metabolism of lipids	Platelet Degranulation
Degradation of GABA	Integrin Signaling
Diseases associated with glycosaminoglycan metabolism	Programmed cell death and apoptotic execution phase
Axon guidance–NCAM1 signaling	Axon guidance–L1CAM interactions

MAPK–Mitogen Activated Protein kinase; GABA—Gamma-Amino Butyric Acid.

https://doi.org/10.1371/journal.pone.0290087.t002

this pathway may be an important therapeutic target [22]. Kwiatkowska *et* al. silenced RhoG using siRNA and showed marginal effects on GBM cell proliferation, but it significantly inhibited cell survival in colony formation assays [23].

According to Fortin Ensign *et* al., the study of aberrant Rho GTPase signaling in grade 4 astrocytoma such as glioblastoma is thus an essential investigation of cell invasion as well as treatment resistance and disease progression [24]. Our results corroborate with the literature and further suggest its potential therapeutic use.

Another protein exclusively identified in grade 4 astrocytoma is cathepsin. Cathepsins are cysteine proteases present in lysosomes and activated at acid pH. These proteins were shown to attenuate the antitumor immune response and act in the degradation of the extracellular matrix, cell invasion, and stimulation of mitosis [25]. This class of proteins was identified in glioblastomas, representing important activity in tumor progression and invasion, besides being indicated as possible therapeutic targets [26].

S100 proteins comprise a group of 21 proteins involved in several intra- and extracellular actions such as calcium homeostasis, regulation of protein phosphorylation, transcription factors, cytoskeleton component dynamics, inflammatory response, growth, and cell differentiation [27]. The biology of this family is complex and multifunctional, contributing to tumorigenesis through its mediation in phenomena such as cell proliferation, metastasis, angiogenesis, and immune evasion, and some protein subtypes are already considered a therapeutic target of drugs in clinical trials. [28]. Lu *et* al. demonstrated that S100 A12 was upregulated in tissues of glioma patients, and the expression was correlated to WHO stage and tumor size. Besides, the authors showed that knockdown of S100 A12 inhibits the proliferation, migration, and invasion of glioma cells by regulating cell apoptosis and EMT [29].

Proteins identified exclusively in the grade 2 astrocytomas

Among the proteins identified only in the grade 2 astrocytoma we highlight the Heat shock protein β-8, Long-chain-fatty-acid—CoA ligase, and Voltage-gated potassium channel.

Identified initially as heat stress-responsive proteins, acting as molecular chaperones (helper proteins), heat shock proteins (HSPs) have the role of maintaining intracellular functions in response to a wide variety of perturbations (physical or chemical) or even under alterations in physiological conditions, preventing cell death [30]. These proteins have been reported to be more abundant in cancer as a response to internal stress developed by the tumor cells. Some examples of stress causes are the breakdown of exacerbated protein synthesis or the presence of mutant proteins and hypoxia, nutrient deprivation, and acidosis [31].

Thus, in tumors, HSPs promote resistance to apoptosis, activation of the cell stroma, inducing progression, angiogenesis, and tissue invasiveness [32]. Research on these proteins is still preliminary in gliomas. HSPs have also been correlated to survival time and prognosis for both good and bad [33].

Fatty acids are essential nutrients and function as essential building blocks of the body and participate in energy metabolism and cellular signaling mechanisms. However, given the growing number of overweight and obese individuals, excess fatty acid metabolism has been increasingly associated with metabolic disorders and carcinogenesis [34]. The overexpression of Long Chain Fatty Acid Ligase (ACSL) has been reported in several types of cancers, such as human hepatocellular carcinoma, colon adenocarcinoma [35], and breast cancer, inferring a role in the carcinogenesis of these neoplasms [36]. In gliomas, this molecule was related to cell survival, especially in extracellular acidic environments [37], which is compatible with our results, being more frequent in grade 2 tumors, with better prognosis.

Differential abundant proteins in the grade 2 group

We used Reactome platform to help understand the role of the differential abundant proteins in each group. We found proteoglycans pathway, composed of hyaluronan and proteoglycan link protein, versican core protein, brevican core protein, and tenascin-R to be differential abundant in the grade 2 astrocytomas.

Hyaluronic acid (HA) is one of the main components of the brain extracellular matrix (ECM), constituting a three-dimensional extracellular mesh by connecting to binding proteins and proteoglycans [38, 39]. In tumors, its concentration is increased and acts as a stimulator for cell proliferation, mobilization, and invasion. In addition to inducing the production of numerous factors related to migration, such as the CD44 antigen, PTEN (phosphatase and tensin homolog), and matrix metalloproteinases, HA facilitates the spatial expansion of the tumor and assists in cell adhesion to occupied spaces through interactions with their binding molecules [40].

The HA-binding proteins and proteoglycans increased in the grade 2 samples could stabilize the proteoglycan aggregates from their natural dissociation, and protected their degradation under conditions of tissue catabolism activation [41].

Versican protein, along with Brevican protein, tenascin-R, AH-binding proteins, and the proteoglycans discussed above, are all more abundant in the grade 2 samples and constitute the basis of the HA-related complex commonly present in the ECM of mature brains [40, 42]. The Brevican protein, after its proteolysis by ADAMTS4, binds to fibronectin in cells leading to the production of integrins, inducing tumor motility and invasion [43, 44]. In addition to participating in ECM formation, tenascin-R and Brevican protein also functions in the modulation of regulatory and inhibitory synapses [45].

In agreement with our results, tenascin-R and other proteins of the tenascin family are more closely related to grade 2, showing decreasing levels of concentration in grade 4 gliomas [46, 47].

Regarding cell adhesion molecules, NCAM pathways were differentially activated in the grade 2 group through the overexpression of NCAM1 and PRN (Major Prion Protein). Researches published in the international literature indicate that the negative regulation of NCAM may contribute to the invasion of gliomas, promoting cell breakdown and secretion of proteases [48, 49], and their positive regulation (hyperexpression) is one of the determining factors for better prognosis of grade 2 tumors. Furthermore, the increased expression of this protein opens the possibility of a possible target of grade 2 to immunotherapies anti-NCAM [50].

We also identified a predominance of expression of citric acid cycle proteins (Krebs cycle), including isocitrate dehydrogenase type 2 (IDH-2), and oxidative phosphorylation, including NADP, in the grade 2 tumors, a fact also reinforced by the over-expression of mitochondrial import protein pathway (ADP/ATP translocase 1).

Besides glucose, other sources of acetyl-CoA for the Krebs Cycle include the breakdown of free fatty acids and ketone bodies in a fasting state. Based on this information, Schwartz *et al.* advocated in favor of a ketogenic diet for patients with gliomas [51]. That way induces increased mitochondrial metabolism in these tumors and potentially increases survival. Complementary clinical studies are still necessary to determine this approach's therapeutic efficacy.

We also observed a pathway related to the degradation of gamma-amino butyric acid (GABA), which is metabolized in the mitochondrial matrix to succinate by the action series of two enzymes, 4-aminobutyrate aminotransferase and semialdehyde succinate dehydrogenase (SSADH) [52], proteins differentially abundant identified in the grade 2 group of our work.

Decreased levels of GABA are associated with increased levels of its metabolic by-product 4-hydroxybutyrate (GHB). This by-product induces GBM stem cells' differentiation and infiltrates deep glioma in less aggressive cells [53]. As such, it can be hypothesized that the intensification of GABA degradation is closely related to the lower aggressiveness of gliomas, as demonstrated in the findings of our work and the increase in the incidence of epilepsy in this class of tumors.

Abundant proteins in the grade 4 group

The L1CAM pathway, related to cell adhesion molecules, was differentially activated in the grade 4 group through the overexpression of moesin and tubulin. Such hyperactivation is related to a worse prognosis of gliomas as it favors cell proliferation and motility [54, 55]; this is consistent with the behavior of glioblastomas. The inhibition of L1CAM through targeted therapies has shown promising results in inhibiting proliferation and cell invasion by suppressing the growth of gliomas [56, 57].

The fibronectin and fibrinogen gamma chain was also found as spotlights in cell surface integrins interactions and fibronectin matrix formation. Concerning gliomas, as early as 1985, increased levels of fibronectin were detected in the plasma of patients with glioblastoma [58]. In 1997, Ohnishi *et al.* demonstrated the promotion of fibronectin in glioma cell invasion and migration through chemotactic activity [59]. Over the years, the relationship between fibronectin activity and mechanisms of induction of malignancy in gliomas has become even more solid, especially in cell adhesion, differentiation, proliferation, and chemoresistance, as well as their possible mechanisms. Reductions in sox-2 and nestin levels and increased levels of glial fibrillary acidic protein (GFAP) and β-tubulin were also found in glioma lineages, indicating fibronectin-driven cell differentiation.

Additional studies demonstrated that fibronectin facilitated cell proliferation, as evidenced by increased Ki-67 levels, along with the activation of phosphorylated ERK1/2 and cyclin D1. Furthermore, fibronectin was found to inhibit apoptosis mediated by the p53 pathway and enhance the expression of P-glycoprotein [60]. More recent works also evidence this action by activating the PI3K / AKT signaling pathway [61].

In 2019, Golanov *et al.* used brain tissue samples taken during malignant glioma resection surgeries and showed that astrocytes and neurons could produce fibrinogens, albeit only produced in the presence of blood-brain barrier breakdown [62]. In addition to the fibrinogen gamma chain's role in the hemostasis mechanism, it also interacts with TLR-4 (Toll-Like Receptor 4), which is important in innate immunity and neuroinflammation are typical of grade 4 tumors [63, 64]. The greater presence of fibrinogen chains in grade 4 gliomas was also corroborated in another important work on proteomic analysis in different grades of glioma,

showing a positive correlation with increasing grade of gliomas, thus offering insight into the molecular basis behind its aggressive nature [4].

Our results also showed an increased abundance of Ras GTPase activating proteins (GAPs), as also found by Ren et al. [65] in the study of glioma proteomics. As mentioned earlier, this class of proteins is responsible for the inactivation of Ras. Considering the extensive literature demonstrating the hyperactivation of the Ras pathway in gliomas [66–70], our findings of the hyperactivation of its inactivation pathway make us hypothesize a mere biochemical compensation or even a failure of this inactivation mechanism, stimulating the hyperactivation of the genes of Ras GTPases.

Our results showed a significant increase in cytoplasmic isocitrate dehydrogenase type 1 (IDH-1); this has been associated with an overexpression of the non-specific lipid transfer protein, which are members of the complex peroxisomal import protein. These findings support the predominance of cytoplasmic metabolism in grade 4 tumors [71, 72].

Concerning the mechanisms of cell death, in addition to the overexpression of lamin-B1 and BAX protein, denoting a clear performance of apoptosis in grade 4 tumors, we found a differential abundance of Vimentin, recently cited as part of an inhibitory mechanism and responsible for the change in survival achieved in the treatment of cancer patients [73]. We also identified a statistically significant peak of histone H1.5 in this same group. Caspase-mediated changes in this histone family are considered a marker for early apoptosis [74]. Our findings are consistent with those previously reported in the literature [65].

We also found the hyperactivation of platelet degranulation pathways differentially present in the grade 4 tumors group by identifying statistically significant hyperexpression of the H4 heavy chain of inter-alpha-trypsin inhibitor, filamin-A, transgelin-2, and calumenin; these findings are consistent with previously published literature [65].

Platelets possess multifaceted roles in cancer progression, including the ability to safeguard circulating tumor cells from immune surveillance, fostering an environment that supports cell survival. Additionally, they can stimulate invasive characteristics in tumor cells, enhancing their metastatic potential. Platelets also facilitate the transfer of adhesive molecules, which play a crucial part in establishing interactions between tumor cells and the endothelial lining of blood vessels. This interaction aids in the formation of early metastatic niches, further driving the spread of cancer throughout the body [75]. These interactions favor the spread of these cells and cancer-associated thrombogenesis [76].

In 2007, Brockmann *et al.* reported preoperative thrombocytosis as an independent risk factor associated with shorter survival in patients with glioblastoma [77] caused by the growth and development of this tumor [78].

In addition to the relationship with platelet degranulation pathways, calumenin is present in biological functions such as cell adhesion and extracellular matrix organization, all of which are strongly correlated with the epithelial-mesenchymal transition. Its overexpression was related to more aggressive glioma phenotypes (such as wild-type HDI), with worse prognosis, and maybe a strong candidate for a target molecule, depending on future studies [79].

In view of all this, some studies have pointed to a possible antitumor effect of the use of antiplatelet agents, such as aspirin, clopidogrel, and ticagrelor [80, 81], of 10-protein-rich platelets induced by interferon-gamma [82] and the use of genetic engineering promoting platelet production as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) vectors [83].

Final considerations

Considering the results taken together, the identified alterations in extracellular matrix organization, cell division signaling, and energy metabolism offer valuable insights into the

mechanisms contributing to the distinct characteristics of these histopathological groups. For example, our study highlights the importance of GABA degradation pathways in grade 2 astrocytomas and their implications for understanding neurotransmitter signaling in glioma pathogenesis. Investigating the relationship between these pathways and tumor cell behavior may help elucidate why grade 2 astrocytomas are associated with a better prognosis. In contrast, the increased presence of platelet degranulation, apoptosis, and MAPK pathway activation in grade 4 astrocytomas suggests a more aggressive tumor phenotype. Understanding how these processes influence tumor growth and invasion could facilitate the identification of therapeutic targets or intervention strategies for grade 4 astrocytomas.

Our study also emphasized the significance of dysregulated energy metabolism in astrocytoma pathogenesis by pinpointing differences in energy metabolism pathways between grades 2 and 4 astrocytomas, which may contribute to their distinct growth rates and invasiveness. Investigating the specific metabolic alterations and their impact on tumor cell proliferation, survival, and migration could reveal novel therapeutic targets to hinder tumor progression.

In all, exploring the interplay between differentially abundant proteins and molecular pathways may provide a more holistic understanding of the complex cellular processes driving astrocytoma development and progression. Further studies examining these interactions to shortlist regulatory nodes are key to better therapeutic targeting to disrupt tumor growth and spread. Future studies should focus on these relationships and the underlying mechanisms, paving the way for more effective and personalized treatment strategies for patients with astrocytomas.

In our study, we acknowledge the limitation of not incorporating all WHO CNS5 guidelines for astrocytoma classification due to the unavailability of certain molecular marker tests, such as IDH mutation status and 1p/19q codeletion, in vast majority of Brazilian public services. This fact is also foreseen in the 5 who classification with the suffix NOS (not otherwise specified). Despite these limitations, our research provides valuable information on the proteomic differences between grade 2 and grade 4 astrocytomas based on their histopathological features and patients' natural history. We recognize that the integration of molecular markers in astrocytoma classification could offer a more accurate diagnosis and improved understanding of tumor biology, leading to better patient stratification and tailored treatment approaches. We encourage future research to build upon our findings and incorporate the WHO CNS5 guidelines for a more comprehensive analysis of astrocytomas and their underlying molecular pathways.

Conclusion

This work contributes to data on the differentiation of proteomes from grades 2 and 4 astrocytomas. We identified seventy-four differentially abundant proteins between the grades 2 and 4 astrocytomas by following strict statistical criteria. We shortlisted characteristic proteins from each histological group and thus correlating some with a greater malignancy for this type of tumor. We then used Reactome to list distinctly activated molecular pathways in both groups and corroborated our results with the literature and observations from clinical and surgical practice. We found significant differences in the extracellular matrix organization related to tumor invasion and signaling for cell division, which, together with marked contrasts in energy metabolism, determine factors in the speed of growth and dissemination of these neoplasms. The GABA degradation pathways, more present in the grade 2 group, were consistent with a better prognosis already reported. Other functions such as platelet degranulation, apoptosis, and MAPK approach activation were more related to grade 4 tumors, consistent with a worse prognosis. In all, our results mark a step forward, in the understanding at the molecular level, of these histopathological groups.

Supporting information

S1 Table. Complete list of proteins identified in grade 2 and 4 astrocytomas. The table provides the full list of proteins identified in each tumor grade, including the 748 proteins exclusive to grade 4 astrocytomas, the 174 proteins exclusive to grade 2 astrocytomas, and the 2658 proteins common to both tumor types.
(XLSX)

S2 Table. Differentially abundant proteins between grade 2 and 4 astrocytomas. The table contains the list of 74 differentially abundant proteins identified using the TFold module of PatternLab for Proteomics. The abundance of each protein was statistically evaluated based on variable fold change and q-value criteria.
(XLSX)

Acknowledgments

Author Contributions

Conceptualization: Denildo C. A. Verissimo, Luis A. B. Borba, Paulo C. Carvalho, Juliana de S. da G. Fischer.

Data curation: Denildo C. A. Verissimo, Amanda C. Camillo-Andrade, Marlon D. M. Santos, Sergio L. Sprengel, Simone C. Zanine, Luis A. B. Borba.

Formal analysis: Denildo C. A. Verissimo, Marlon D. M. Santos, Simone C. Zanine, Paulo C. Carvalho.

Funding acquisition: Paulo C. Carvalho.

Investigation: Denildo C. A. Verissimo, Amanda C. Camillo-Andrade, Sergio L. Sprengel, Simone C. Zanine, Luis A. B. Borba, Juliana de S. da G. Fischer.

Methodology: Denildo C. A. Verissimo, Amanda C. Camillo-Andrade, Marlon D. M. Santos, Simone C. Zanine.

Project administration: Denildo C. A. Verissimo, Luis A. B. Borba, Paulo C. Carvalho, Juliana de S. da G. Fischer.

Resources: Paulo C. Carvalho.

Software: Marlon D. M. Santos, Paulo C. Carvalho.

Supervision: Luis A. B. Borba, Juliana de S. da G. Fischer.

Validation: Denildo C. A. Verissimo, Paulo C. Carvalho.

Visualization: Denildo C. A. Verissimo, Amanda C. Camillo-Andrade, Marlon D. M. Santos, Sergio L. Sprengel, Simone C. Zanine.

Writing – original draft: Denildo C. A. Verissimo, Paulo C. Carvalho.

Writing – review & editing: Amanda C. Camillo-Andrade, Marlon D. M. Santos, Sergio L. Sprengel, Simone C. Zanine, Luis A. B. Borba.

References

1. Diwanji TP, Engelman A, Snider JW, Mohindra P. Epidemiology, diagnosis, and optimal management of glioma in adolescents and young adults. Adolesc Health Med Ther. 2017; 8:99–113. https://doi.org/10.2147/AHMT.S53391 PMID: 28989289

2. Aster JC KV. Robbins & COTRAN Patologia—Bases Patológicas das Doenças. 9a. Rio de Janeiro, RJ: Elsevier; 2016. 2696 p.

3. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. Acta Neuropathol (Berl). 2016 Jun; 131(6):803–20. https://doi.org/10.1007/s00401-016-1545-1 PMID: 27157931

4. Gollapalli K, Ghantasala S, Atak A, Rapole S, Moiyadi A, Epari S, et al. Tissue Proteome Analysis of Different Grades of Human Gliomas Provides Major Cues for Glioma Pathogenesis. Omics J Integr Biol. 2017 May; 21(5):275–84. https://doi.org/10.1089/omi.2017.0028 PMID: 28481733

5. Ohgaki H, Kleihues P. Genetic alterations and signaling pathways in the evolution of gliomas. Cancer Sci. 2009 Dec; 100(12):2235–41. https://doi.org/10.1111/j.1349-7006.2009.01308.x PMID: 19737147

6. Wippel HH, Santos MDM, Clasen MA, Kurt LU, Nogueira FCS, Carvalho CE, et al. Comparing intestinal versus diffuse gastric cancer using a PEFF-oriented proteomic pipeline. J Proteomics [Internet]. 2017 Oct [cited 2017 Oct 16]; Available from: http://linkinghub.elsevier.com/retrieve/pii/S1874391917303524 PMID: 29032071

7. Camillo-Andrade AC, Santos MDM, Fischer JSG, Swinka BB, Bosquetti B, Schuck DC, et al. Proteomics reveals that quinoa bioester promotes replenishing effects in epidermal tissue. Sci Rep. 2020 Dec; 10(1):19392. https://doi.org/10.1038/s41598-020-76325-6 PMID: 33173110

8. Rappsilber J, Ishihama Y, Mann M. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal Chem. 2003 Feb 1; 75 (3):663–70. https://doi.org/10.1021/ac026117i PMID: 12585499

9. Hahne H, Pachl F, Ruprecht B, Maier SK, Klaeger S, Helm D, et al. DMSO enhances electrospray response, boosting sensitivity of proteomic experiments. Nat Methods. 2013 Oct; 10(10):989–91. https://doi.org/10.1038/nmeth.2610 PMID: 23975139

10. Carvalho PC, Lima DB, Leprevost FV, Santos MDM, Fischer JSG, Aquino PF, et al. Integrated analysis of shotgun proteomic data with PatternLab for proteomics 4.0. Nat Protoc. 2015 Dec 10; 11(1):102–17. https://doi.org/10.1038/nprot.2015.133 PMID: 26658470

11. Santos MDM, Lima DB, Fischer JSG, Clasen MA, Kurt LU, Camillo-Andrade AC, et al. Simple, efficient and thorough shotgun proteomic analysis with PatternLab V. Nat Protoc [Internet]. 2022 Apr 11 [cited 2022 Apr 19]; Available from: https://www.nature.com/articles/s41596-022-00690-x https://doi.org/10.1038/s41596-022-00690-x PMID: 35411045

12. Eng JK, Hoopmann MR, Jahan TA, Egertson JD, Noble WS, MacCoss MJ. A deeper look into Comet—implementation and features. J Am Soc Mass Spectrom. 2015 Nov; 26(11):1865–74. https://doi.org/10.1007/s13361-015-1179-x PMID: 26115965

13. Carvalho PC, Fischer JSG, Xu T, Cociorva D, Balbuena TS, Valente RH, et al. Search engine processor: Filtering and organizing peptide spectrum matches. Proteomics. 2012 Apr; 12(7):944–9. https://doi.org/10.1002/pmic.201100529 PMID: 22311825

14. Barboza R, Cociorva D, Xu T, Barbosa VC, Perales J, Valente RH, et al. Can the false-discovery rate be misleading? Proteomics. 2011 Oct; 11(20):4105–8. https://doi.org/10.1002/pmic.201100297 PMID: 21834134

15. Yates JR 3rd, Park SKR, Delahunty CM, Xu T, Savas JN, Cociorva D, et al. Toward objective evaluation of proteomic algorithms. Nat Methods. 2012 May; 9(5):455–6. https://doi.org/10.1038/nmeth.1983 PMID: 22543378

16. Zybailov B, Mosley AL, Sardiu ME, Coleman MK, Florens L, Washburn MP. Statistical analysis of membrane proteome expression changes in Saccharomyces cerevisiae. J Proteome Res. 2006 Sep; 5 (9):2339–47. https://doi.org/10.1021/pr060161n PMID: 16944946

17. Neilson KA, Ali NA, Muralidharan S, Mirzaei M, Mariani M, Assadourian G, et al. Less label, more free: Approaches in label-free quantitative mass spectrometry. PROTEOMICS. 2011 Feb 1; 11(4):535–53. https://doi.org/10.1002/pmic.201000553 PMID: 21243637

18. Carvalho PC, Yates JR 3rd, Barbosa VC. Improving the TFold test for differential shotgun proteomics. Bioinforma Oxf Engl. 2012 Jun 15; 28(12):1652–4. https://doi.org/10.1093/bioinformatics/bts247 PMID: 22539673

19. Fabregat A, Sidiropoulos K, Garapati P, Gillespie M, Hausmann K, Haw R, et al. The Reactome pathway Knowledgebase. Nucleic Acids Res. 2016 Jan 4; 44(D1):D481–487. https://doi.org/10.1093/nar/gkv1351 PMID: 26656494

20. Miller KD, Ostrom QT, Kruchko C, Patil N, Tihan T, Cioffi G, et al. Brain and other central nervous system tumor statistics, 2021. CA Cancer J Clin. 2021; 71(5):381–406. https://doi.org/10.3322/caac.21693 PMID: 34427324

21. Kurt LU, Clasen MA, Santos MDM, Souza TACB, Andreassa EC, Lyra EB, et al. RawVegetable–A data assessment tool for proteomics and cross-linking mass spectrometry experiments. J Proteomics. 2020 Aug; 225:103864. https://doi.org/10.1016/j.jprot.2020.103864 PMID: 32526479

22. Bustelo XR, Sauzeau V, Berenjeno IM. GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. BioEssays News Rev Mol Cell Dev Biol. 2007 Apr; 29(4):356–70. https://doi.org/10.1002/bies.20558 PMID: 17373658

23. Kwiatkowska A, Didier S, Fortin S, Chuang Y, White T, Berens ME, et al. The small GTPase RhoG mediates glioblastoma cell invasion. Mol Cancer. 2012 Dec; 11(1):65. https://doi.org/10.1186/1476-4598-11-65 PMID: 22966858

24. Fortin Ensign SP, Mathews IT, Symons MH, Berens ME, Tran NL. Implications of Rho GTPase Signaling in Glioma Cell Invasion and Tumor Progression. Front Oncol. 2013; 3:241. https://doi.org/10.3389/fonc.2013.00241 PMID: 24109588

25. Nomura T, Katunuma N. Involvement of cathepsins in the invasion, metastasis and proliferation of cancer cells. J Med Investig JMI. 2005 Feb; 52(1–2):1–9. https://doi.org/10.2152/jmi.52.1 PMID: 15751268

26. Strojnik T, Kavalar R, Trinkaus M, Lah TT. Cathepsin L in glioma progression: comparison with cathepsin B. Cancer Detect Prev. 2005; 29(5):448–55. https://doi.org/10.1016/j.cdp.2005.07.006 PMID: 16183211

27. Donato R. Intracellular and extracellular roles of S100 proteins. Microsc Res Tech. 2003 Apr 15; 60 (6):540–51. https://doi.org/10.1002/jemt.10296 PMID: 12645002

28. Bresnick AR, Weber DJ, Zimmer DB. S100 proteins in cancer. Nat Rev Cancer. 2015 Jan 23; 15(2):96–109. https://doi.org/10.1038/nrc3893 PMID: 25614008

29. Lu C, Liu J, Yao M, Li L, Li G. Downregulation of S100 calcium binding protein A12 inhibits the growth of glioma cells. BMC Cancer. 2020 Dec; 20(1):261. https://doi.org/10.1186/s12885-020-06768-7 PMID: 32228516

30. Richter K, Haslbeck M, Buchner J. The heat shock response: life on the verge of death. Mol Cell. 2010 Oct 22; 40(2):253–66. https://doi.org/10.1016/j.molcel.2010.10.006 PMID: 20965420

31. Ciocca DR, Arrigo AP, Calderwood SK. Heat shock proteins and heat shock factor 1 in carcinogenesis and tumor development: an update. Arch Toxicol. 2013 Jan; 87(1):19–48. https://doi.org/10.1007/s00204-012-0918-z PMID: 22885793

32. Seclì L, Fusella F, Avalle L, Brancaccio M. The dark-side of the outside: how extracellular heat shock proteins promote cancer. Cell Mol Life Sci CMLS. 2021 May; 78(9):4069–83. https://doi.org/10.1007/s00018-021-03764-3 PMID: 33544155

33. Sun H, Zou HY, Cai XY, Zhou HF, Li XQ, Xie WJ, et al. Network Analyses of the Differential Expression of Heat Shock Proteins in Glioma. DNA Cell Biol. 2020 Jul 1; 39(7):1228–42. https://doi.org/10.1089/dna.2020.5425 PMID: 32429692

34. Tang Y, Zhou J, Hooi SC, Jiang YM, Lu GD. Fatty acid activation in carcinogenesis and cancer development: Essential roles of long-chain acyl-CoA synthetases. Oncol Lett. 2018 Aug; 16(2):1390–6. https://doi.org/10.3892/ol.2018.8843 PMID: 30008815

35. Cao Y, Dave KB, Doan TP, Prescott SM. Fatty acid CoA ligase 4 is up-regulated in colon adenocarcinoma. Cancer Res. 2001 Dec 1; 61(23):8429–34. PMID: 11731423

36. Tsai CH, Sheen-Chen SM, Huang CY, Chen YJ, Chi SY, Ko SF, et al. An evaluation of fatty acid-CoA ligase 4 in breast cancer. Anticancer Res. 2014 Mar; 34(3):1239–43. PMID: 24596366

37. Mashima T, Sato S, Sugimoto Y, Tsuruo T, Seimiya H. Promotion of glioma cell survival by acyl-CoA synthetase 5 under extracellular acidosis conditions. Oncogene. 2009 Jan 8; 28(1):9–19. https://doi.org/10.1038/onc.2008.355 PMID: 18806831

38. Kochlamazashvili G, Henneberger C, Bukalo O, Dvoretskova E, Senkov O, Lievens PMJ, et al. The extracellular matrix molecule hyaluronic acid regulates hippocampal synaptic plasticity by modulating postsynaptic L-type Ca(2+) channels. Neuron. 2010 Jul 15; 67(1):116–28. https://doi.org/10.1016/j.neuron.2010.05.030 PMID: 20624596

39. Mawrin C, Seidenbecher C. Glioma and Extracellular Matrix. In: Sedo A, Mentlein R, editors. Glioma Cell Biology [Internet]. Vienna: Springer Vienna; 2014 [cited 2022 Apr 12]. p. 271–92. Available from: http://link.springer.com/10.1007/978-3-7091-1431-5_10

40. Ferrer VP, Moura Neto V, Mentlein R. Glioma infiltration and extracellular matrix: key players and modulators. Glia. 2018 Aug; 66(8):1542–65. https://doi.org/10.1002/glia.23309 PMID: 29464861

41. Rodriguez E, Roughley P. Link protein can retard the degradation of hyaluronan in proteoglycan aggregates. Osteoarthritis Cartilage. 2006 Aug; 14(8):823–9. https://doi.org/10.1016/j.joca.2006.02.008 PMID: 16584896

42. Arslan F, Bosserhoff AK, Nickl-Jockschat T, Doerfelt A, Bogdahn U, Hau P. The role of versican isoforms V0/V1 in glioma migration mediated by transforming growth factor-β2. Br J Cancer. 2007 May; 96 (10):1560–8.

43. Held-Feindt J, Paredes EB, Blömer U, Seidenbecher C, Stark AM, Mehdorn HM, et al. Matrix-degrading proteases ADAMTS4 and ADAMTS5 (disintegrins and metalloproteinases with thrombospondin motifs 4 and 5) are expressed in human glioblastomas. Int J Cancer. 2006 Jan 1; 118(1):55–61. https://doi.org/10.1002/ijc.21258 PMID: 16003758

44. Lu R, Wu C, Guo L, Liu Y, Mo W, Wang H, et al. The role of brevican in glioma: promoting tumor cell motility in vitro and in vivo. BMC Cancer. 2012 Dec; 12(1):607. https://doi.org/10.1186/1471-2407-12-607 PMID: 23253190

45. Gottschling C, Wegrzyn D, Denecke B, Faissner A. Elimination of the four extracellular matrix molecules tenascin-C, tenascin-R, brevican and neurocan alters the ratio of excitatory and inhibitory synapses. Sci Rep. 2019 Dec; 9(1):13939. https://doi.org/10.1038/s41598-019-50404-9 PMID: 31558805

46. Tenascins Chiquet-Ehrismann R., a growing family of extracellular matrix proteins. Experientia. 1995 Sep; 51(9–10):853–62.

47. Chiquet-Ehrismann R, Orend G, Chiquet M, Tucker RP, Midwood KS. Tenascins in stem cell niches. Matrix Biol J Int Soc Matrix Biol. 2014 Jul; 37:112–23. https://doi.org/10.1016/j.matbio.2014.01.007 PMID: 24472737

48. Maidment SL, Rucklidge GJ, Rooprai HK, Pilkington GJ. An inverse correlation between expression of NCAM-A and the matrix-metalloproteinases gelatinase-A and gelatinase-B in human glioma cells in vitro. Cancer Lett. 1997 Jun 3; 116(1):71–7. https://doi.org/10.1016/s0304-3835(97)00171-7 PMID: 9177460

49. Wang Z, Dai X, Chen Y, Sun C, Zhu Q, Zhao H, et al. MiR-30a-5p is induced by Wnt/β-catenin pathway and promotes glioma cell invasion by repressing NCAM. Biochem Biophys Res Commun. 2015 Sep 25; 465(3):374–80.

50. Jensen M, Berthold F. Targeting the neural cell adhesion molecule in cancer. Cancer Lett. 2007 Dec 8; 258(1):9–21. https://doi.org/10.1016/j.canlet.2007.09.004 PMID: 17949897

51. Schwartz K, Chang HT, Nikolai M, Pernicone J, Rhee S, Olson K, et al. Treatment of glioma patients with ketogenic diets: report of two cases treated with an IRB-approved energy-restricted ketogenic diet protocol and review of the literature. Cancer Metab. 2015 Dec; 3(1):3. https://doi.org/10.1186/s40170-015-0129-1 PMID: 25806103

52. Malaspina P, Roullet JB, Pearl PL, Ainslie GR, Vogel KR, Gibson KM. Succinic semialdehyde dehydrogenase deficiency (SSADHD): Pathophysiological complexity and multifactorial trait associations in a rare monogenic disorder of GABA metabolism. Neurochem Int. 2016 Oct; 99:72–84. https://doi.org/10.1016/j.neuint.2016.06.009 PMID: 27311541

53. El-Habr EA, Dubois LG, Burel-Vandenbos F, Bogeas A, Lipecka J, Turchi L, et al. A driver role for GABA metabolism in controlling stem and proliferative cell state through GHB production in glioma. Acta Neuropathol (Berl). 2017 Apr; 133(4):645–60. https://doi.org/10.1007/s00401-016-1659-5 PMID: 28032215

54. Mohanan V, Temburni MK, Kappes JC, Galileo DS. L1CAM stimulates glioma cell motility and proliferation through the fibroblast growth factor receptor. Clin Exp Metastasis. 2013 Apr; 30(4):507–20. https://doi.org/10.1007/s10585-012-9555-4 PMID: 23212305

55. Zeng J, Xi SY, Wang F, Liao HD, Yang YZ, Hu WM. L1CAM High Expression Associates with Poor Prognosis in Glioma but Does Not Correlate with C11orf95-RELA Fusion. BioMed Res Int. 2020; 2020:1353284. https://doi.org/10.1155/2020/1353284 PMID: 32509846

56. Bao S, Wu Q, Li Z, Sathornsumetee S, Wang H, McLendon RE, et al. Targeting Cancer Stem Cells through L1CAM Suppresses Glioma Growth. Cancer Res. 2008 Aug 1; 68(15):6043–8. https://doi.org/10.1158/0008-5472.CAN-08-1079 PMID: 18676824

57. Liu H, Song Z, Liao D, Zhang T, Liu F, Zheng W, et al. miR-503 inhibits cell proliferation and invasion in glioma by targeting L1CAM. Int J Clin Exp Med. 2015; 8(10):18441–7. PMID: 26770450

58. Sawaya R, Cummins CJ, Smith BH, Kornblith PL. Plasma fibronectin in patients with brain tumors. Neurosurgery. 1985 Feb; 16(2):161–5. https://doi.org/10.1227/00006123-198502000-00006 PMID: 3974826

59. Ohnishi T, Arita N, Hiraga S, Taki T, Izumoto S, Fukushima Y, et al. Fibronectin-mediated cell migration promotes glioma cell invasion through chemokinetic activity. Clin Exp Metastasis. 1997 Sep; 15 (5):538–46. https://doi.org/10.1023/a:1018422926361 PMID: 9247256

60. Yu Q, Xue Y, Liu J, Xi Z, Li Z, Liu Y. Fibronectin Promotes the Malignancy of Glioma Stem-Like Cells Via Modulation of Cell Adhesion, Differentiation, Proliferation and Chemoresistance. Front Mol Neurosci. 2018; 11:130. https://doi.org/10.3389/fnmol.2018.00130 PMID: 29706869

61. Liao YX, Zhang ZP, Zhao J, Liu JP. Effects of Fibronectin 1 on Cell Proliferation, Senescence and Apoptosis of Human Glioma Cells Through the PI3K/AKT Signaling Pathway. Cell Physiol Biochem Int J Exp Cell Physiol Biochem Pharmacol. 2018; 48(3):1382–96. https://doi.org/10.1159/000492096 PMID: 30048971

62. Golanov EV, Sharpe MA, Regnier-Golanov AS, Del Zoppo GJ, Baskin DS, Britz GW. Fibrinogen Chains Intrinsic to the Brain. Front Neurosci [Internet]. 2019 [cited 2020 Nov 21];13. Available from: https://www.frontiersin.org/articles/10.3389/fnins.2019.00541/full PMID: 31191233

63. Trotta T, Porro C, Calvello R, Panaro MA. Biological role of Toll-like receptor-4 in the brain. J Neuroimmunol. 2014 Mar 15; 268(1–2):1–12. https://doi.org/10.1016/j.jneuroim.2014.01.014 PMID: 24529856

64. Zuliani-Alvarez L, Marzeda AM, Deligne C, Schwenzer A, McCann FE, Marsden BD, et al. Mapping tenascin-C interaction with toll-like receptor 4 reveals a new subset of endogenous inflammatory triggers. Nat Commun. 2017 17; 8(1):1595. https://doi.org/10.1038/s41467-017-01718-7 PMID: 29150600

65. Ren T, Lin S, Wang Z, Shang A. Differential proteomics analysis of low- and high-grade of astrocytoma using iTRAQ quantification. OncoTargets Ther. 2016 Sep;Volume 9:5883–95. https://doi.org/10.2147/OTT.S111103 PMID: 27713642

66. Bredel M, Pollack IF. The p21-Ras signal transduction pathway and growth regulation in human high-grade gliomas. Brain Res Brain Res Rev. 1999 Apr; 29(2–3):232–49. https://doi.org/10.1016/s0165-0173(98)00057-5 PMID: 10209234

67. da Fonseca CO, Linden R, Futuro D, Gattass CR, Quirico-Santos T. Ras pathway activation in gliomas: a strategic target for intranasal administration of perillyl alcohol. Arch Immunol Ther Exp (Warsz). 2008 Aug; 56(4):267–76. https://doi.org/10.1007/s00005-008-0027-0 PMID: 18726148

68. Ding H, Roncari L, Shannon P, Wu X, Lau N, Karaskova J, et al. Astrocyte-specific expression of activated p21-ras results in malignant astrocytoma formation in a transgenic mouse model of human gliomas. Cancer Res. 2001 May 1; 61(9):3826–36. PMID: 11325859

69. Fan Z, Bittermann-Rummel P, Yakubov E, Chen D, Broggini T, Sehm T, et al. PRG3 induces Ras-dependent oncogenic cooperation in gliomas. Oncotarget. 2016 May 3; 7(18):26692–708. https://doi.org/10.18632/oncotarget.8592 PMID: 27058420

70. Jeuken J, van den Broecke C, Gijsen S, Boots-Sprenger S, Wesseling P. RAS/RAF pathway activation in gliomas: the result of copy number gains rather than activating mutations. Acta Neuropathol (Berl). 2007 Aug; 114(2):121–33. https://doi.org/10.1007/s00401-007-0239-0 PMID: 17588166

71. Jose C, Bellance N, Rossignol R. Choosing between glycolysis and oxidative phosphorylation: A tumor's dilemma? Biochim Biophys Acta BBA—Bioenerg. 2011 Jun; 1807(6):552–61. https://doi.org/10.1016/j.bbabio.2010.10.012 PMID: 20955683

72. Wang HY, Tang K, Liang TY, Zhang WZ, Li JY, Wang W, et al. The comparison of clinical and biological characteristics between IDH1 and IDH2 mutations in gliomas. J Exp Clin Cancer Res. 2016 Dec; 35 (1):86. https://doi.org/10.1186/s13046-016-0362-7 PMID: 27245697

73. Chakraborty S, Kumar A, Faheem MM, Katoch A, Kumar A, Jamwal VL, et al. Vimentin activation in early apoptotic cancer cells errands survival pathways during DNA damage inducer CPT treatment in colon carcinoma model. Cell Death Dis. 2019 Jun 13; 10(6):1–16. https://doi.org/10.1038/s41419-019-1690-2 PMID: 31197132

74. Ohsawa S, Hamada S, Yoshida H, Miura M. Caspase-mediated changes in histone H1 in early apoptosis: prolonged caspase activation in developing olfactory sensory neurons. Cell Death Differ. 2008 Sep; 15(9):1429–39. https://doi.org/10.1038/cdd.2008.71 PMID: 18483489

75. Plantureux L, Mège D, Crescence L, Dignat-George F, Dubois C, Panicot-Dubois L. Impacts of Cancer on Platelet Production, Activation and Education and Mechanisms of Cancer-Associated Thrombosis. Cancers. 2018 Nov; 10(11):441. https://doi.org/10.3390/cancers10110441 PMID: 30441823

76. Mezouar S, Frère C, Darbousset R, Mege D, Crescence L, Dignat-George F, et al. Role of platelets in cancer and cancer-associated thrombosis: Experimental and clinical evidences. Thromb Res. 2016 Mar; 139:65–76. https://doi.org/10.1016/j.thromres.2016.01.006 PMID: 26916298

77. Brockmann MA, Giese A, Mueller K, Kaba FJ, Lohr F, Weiss C, et al. Preoperative thrombocytosis predicts poor survival in patients with glioblastoma. Neuro-Oncol. 2007 Jul; 9(3):335–42. https://doi.org/10.1215/15228517-2007-013 PMID: 17504931

78. Nolte I, Przibylla H, Bostel T, Groden C, Brockmann MA. Tumor–platelet interactions: Glioblastoma growth is accompanied by increasing platelet counts. Clin Neurol Neurosurg. 2008 Apr 1; 110(4):339–42. https://doi.org/10.1016/j.clineuro.2007.12.008 PMID: 18243523

79. Yang Y, Wang J, Xu S, Shi F, Shan A. Calumenin contributes to epithelial-mesenchymal transition and predicts poor survival in glioma. bioRxiv. 2020 Jul 6;2020.07.05.188318.

80. Cao Y, Nishihara R, Wu K, Wang M, Ogino S, Willett WC, et al. Population-wide Impact of Long-term Use of Aspirin and the Risk for Cancer. JAMA Oncol. 2016 Jun 1; 2(6):762–9. https://doi.org/10.1001/jamaoncol.2015.6396 PMID: 26940135

81. Santilli F, Boccatonda A, Davì G. Aspirin, platelets, and cancer: The point of view of the internist. Eur J Intern Med. 2016 Oct; 34:11–20. https://doi.org/10.1016/j.ejim.2016.06.004 PMID: 27344083

82. Li YT, Nishikawa T, Kaneda Y. Platelet-cytokine Complex Suppresses Tumour Growth by Exploiting Intratumoural Thrombin-dependent Platelet Aggregation. Sci Rep. 2016 Apr 27; 6:25077. https://doi.org/10.1038/srep25077 PMID: 27117228

83. Vizcaíno JA, Côté RG, Csordas A, Dianes JA, Fabregat A, Foster JM, et al. The PRoteomics IDEntifications (PRIDE) database and associated tools: status in 2013. Nucleic Acids Res. 2013 Jan; 41(Database issue):D1063–1069. https://doi.org/10.1093/nar/gks1262 PMID: 23203882