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Protein profiling of formalin fixed paraffin embedded tissue: Identification of potential biomarkers for pediatric brainstem glioma

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Abstract

Little is known about the molecular characteristics of pediatric brainstem gliomas (BSG), which continue to have a dismal prognosis. Targeted molecular strategies are limited due to rarity of biopsy BSG specimen coupled with obstacles associated with the analyses of formalin-fixed paraffin-embedded (FFPE) autopsies. The objective of this study was to develop methodologies to successfully identify the proteome profile from these archived FFPE specimens. Peptides were extracted from both tumor and adjacent normal FFPE brainstem specimen and quantified using ¹⁸O proteolytic labeling strategy and LC-MS/MS analysis. The ingenuity pathway analysis software was used to elucidate interactions amongst differentially expressed proteins. We identified 188 proteins of which 54 (29%) were found up-regulated (1.5-fold) in BSG compared to normal sections. Of these, 15 (28%) proteins have previously been reported as potential biomarkers for supratentorial malignant gliomas, while the rest appear to be exclusive to pediatric BSG. Because the majority of differentially expressed proteins are unique to BSG, we conclude that pediatric BSG is distinct from supratentorial gliomas. To the best of our knowledge, this is the first proteome profile of pediatric BSG, which may facilitate discovery of novel therapeutic targets for early diagnostics and improving prognostics.

Keywords

Formalin fixed paraffin embedded; Ingenuity pathway analysis; Pediatric brainstem

1 Introduction

Pediatric brainstem glioma (BSG) is the most difficult childhood cancer to treat, largely due to the inoperability and diffuse nature of the lesion. Apart from conventional radiation therapy, which typically results in only transient symptomatic relief, other treatments have failed to improve outcome, including high doses (up to 78 Gy) of radiation [1] and the

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addition of chemotherapy that has been effective for other types of brain tumors [2–4]. In fact, for diffused gliomas, despite numerous clinical research trials over the last three decades investigating the use of experimental chemotherapy, radiation and biologic therapy, the overall survival rate of approximately 10% has remained unchanged [5, 6].

BSG predominantly occurs in children between the ages of six to nine years and accounts for 10–20% of all pediatric CNS tumors [7, 8]. The pons is the most common brainstem location of BSG; however, it can also arise in the cervicomedullary junction, midbrain, or the tectum. BSG are categorized into two main groups: diffusely infiltrative and focal [9, 10]. Diffusely infiltrative BSG spreads throughout the pons with occasional dissemination to other parts of the brainstem. Focal brainstem gliomas are, however, localized tumors typically occurring in the midbrain or medulla.

Recent advances in the treatment of other refractory cancers have been accomplished through drug targeting of identified biomarkers that are specific to the type of tumor. To date, however, there are no known biomarkers specific to BSG. Despite advancement of proteomics and genomics applications, little progress has been made towards understanding the molecular constituents of BSG primarily because of the relative lack of biological samples for conducting research studies of this kind. Because this tumor has a typical appearance on magnetic resonance imaging (MRI), and because it is often difficult to safely perform biopsies of BSG, obtaining tumor tissue for histological confirmation of the diagnosis is generally not clinically warranted. Thus, there is a lack of available fresh-frozen tissue for the molecular characterization of these tumors. Furthermore, the complexities associated with protein profiling of formalin-fixed paraffinembedded (FFPE) autopsy specimens have similarly limited performing analyses for biomarker discovery. New methodologies allowing for the identification of BSG-specific biomarkers are therefore greatly needed.

Formalin fixation has been long used to study molecular interactions. Histone-histone and histone-DNA complexes for example, were studied by formation and then reversal of formalin cross-links [11–13]. Cross-linking experiments have also been insightful into the interaction of anticancer drugs and DNA molecule [14, 15]. Formalin-fixed autopsy samples are an excellent resource for genomic and proteomic analyses. During the past decades, methods for reversing cross-links of FFPE samples have improved, specifically for antibody retrieval in immunohistochemistry assays [16–20]. Recent advances in proteomics field have also led to the successful extraction and analysis of peptides from FFPE tissues [21–23]. In an attempt to discover potential BSG markers for therapeutic targeting, as well as define the molecular signature of the tumor, we used FFPE pediatric BSG archival tissues for protein identification and quantification. Our analyses show the feasibility of profiling proteins from archival FFPE brain tissues and obtaining qualitative and quantitative molecular insights into BSG. Here, we have successfully conducted comparative protein profiling of normal *versus* BSG brainstem area obtained from the same FFPE patient sample and generated a list of 54 differentially expressed proteins. Thus, we present the first proteome profile of BSG, suggesting potential biomarkers and molecular signatures of disease progression for possible therapeutic intervention as well as describe a technique that may be utilized broadly for all FFPE cancer specimens.

2 Materials and methods

2.1 Human subjects

FFPE BSG autopsy samples were provided by the Children's National Medical Center. Institutional Review Board (IRB) was obtained to perform evaluations on these samples. All identifiers were removed from the samples prior to evaluation.

2.2 Removal of formalin and partial reversal of cross-links

Two FFPE BSG autopsy samples were analyzed. Hematoxylin and eosin (H&E)-stained FFPE sections (5 μ m thick), were examined by a neuropathologist using light microscopy to identify the area of the brainstem involved by tumor versus the adjacent normal areas. All the cases selected were astrocytoma. For each case, the best possible representative area of tumor was highlighted by the neuropathologist with a marker such that the identical area on the unstained slide could be located by overlapping the H&E-stained section with the unstained section. The unstained sections were all 7 μ m in thickness and were prepared on Superfrost Plus microscope glass slides (Fisher Scientific, Houston, TX). Ten slides were used to extract tumor and normal areas for each experiment. Slides were heated in a bench-top oven at 65°C for 60 min to remove excess wax. To ensure maximum wax removal, slides were washed for two rounds of xylene (5 min each), two rounds of 100% fresh ethanol (5 min each), two rounds of 95% ethanol (3 min each), two rounds of 90% ethanol (3 min each), two rounds of 75% ethanol (3 min each) and then rehydrated in water for 30 s.

Delineated areas of interest were then removed directly from the slides as explained here. To remove tissue sections, 10–15 μ L of extraction buffer (100 mM ammonium bicarbonate and 30% ACN) was directly deposited on marked tumor or normal areas. Hydrated tissue was then scraped off using the pipette tip, suctioned off the slide, and deposited into 1.5-mL polypropylene tubes (Eppendorf, Westbury, NY). The tumor tissue subsequently removed by pipetting resulted in the recovery of the most representative area of tumor for analysis. To reverse cross-links, tubes containing the tissue were placed in a bench-top oven at 95°C for 30 min followed by 2-min incubation on ice and 3 h at 65°C. Samples were immediately placed in vacuum centrifuge and completely dehydrated.

2.3 Proteolytic ^{18}O -labeling and peptide extraction

Differential labeling by stable isotope was performed using ^{18}O proteolytic labeling strategy [24]. Briefly, dehydrated tissue extracts were digested by trypsin either in ^{18}O -enriched (97%) water (Sigma-Aldrich, St. Louis, MO) or in ^{16}O -water. Two micrograms of horse cytochrome C (CYC; Sigma-Aldrich) was added in each sample as an internal standard. For data confirmation and validation, reverse labeling was also performed where normal extracts were digested in the presence of heavy ^{18}O and tumor extracts were digested in the presence of light ^{16}O -water. All digestions were performed overnight in a water bath set at 37°C in the presence of 4.5 μ g MS grade trypsin (Promega, Madison, WI). To ensure a complete digestion and incorporation of ^{18}O atoms in the C termini of tryptic peptides, additional trypsin (3.5 μ g) was added next day and digestion was continued for an additional 5 h.

2.4 Quality control of peptides extracted from FFPE tissue and of ^{18}O labeling

After digestion, samples were spun in a bench top centrifuge at maximum speed to pellet cellular debris. Supernatant was then removed and stored at –80°C until further processing. Two microliters of supernatants from each sample was desalted using C18 ZipTip micropipette tips (Millipore, Bedford, MA) following the manufacturer's User Guide. The peptides were eluted from the ZipTip in 10 μ L of ACN/0.1% TFA (70:30 v/v). Peptide solution (0.3 μ L) was mixed with 0.3 μ L of matrix solution (50 mM CHCA in ACN/0.1% TFA 70:30 v/v) and spotted on the MALDI plate. MS analysis was performed on a 4700 ABI TOF-TOF mass spectrometer (Applied Biosystems, Foster City, CA) in a positive reflectron mode. A mixture of standard peptides was used to externally calibrate the instrument. The high resolution of the ABI-4700 MALDITOF-TOF and its sensitivity helped verification of both the quality of extracted peptides and the incorporation rate of ^{18}O atoms at the C termini of each tryptic peptide. Inspected samples were then completely dried using a vacuum centrifuge, dissolved in 4 μ L of 0.1% TFA and immediately analyzed by LC-MS/MS as described below.

2.5 Nanoflow LC-MS/MS

Differentially labeled peptides from normal and tumor sections were mixed at a 1:1 ratio (4 μ L each) and an aliquot (6 μ L) was analyzed by LC-MS/MS using an LC-Packing system (DIONEX Ulti-Mate™ Capillary/Nano LC System, Dionex, Sunnyvale, CA) connected to a Linear IT (LTQ) mass spectrometer (Thermo Electron, San Jose, CA). Each sample was injected *via* an autosampler and loaded onto a C18 trap column (300 μ m $\times$ 1 mm, LC Packing) for 6 min at a flow rate of 10 μ L/min. The sample was subsequently separated by a C18 RP column (75 μ m $\times$ 15 cm, Vydac, Columbia, MD) at a flow rate of 300 nL/min. The mobile phases consisted of water with 0.1% formic acid (A) and 90% ACN with 0.1% formic acid (B), respectively. A 240-min linear gradient from 5 to 50% B was typically employed. Separated peptides were introduced into the mass spectrometer *via* a 10-mm silica tip (New Objective, Ringoes, NJ) adapted to a nanoelectrospray source (Thermo Electron). The spray voltage was set at 1.7 kV and the heated capillary at 160°C. The LTQ was operated in data-dependent mode in which one cycle of experiments consisted of one full-MS survey and subsequently three sequential pairs of intercalated zoom scans and MS/MS experiments. The targeted ion counts in the IT during full-MS, zoom scan, and MS/MS were 30 000, 3000 and, 10 000, respectively. Peptides were fragmented in the trap using CID with the collision gas (helium) pressure set at 1.3 mTorr and the normalized collision energy value set at 35%.

2.6 Protein database search

Protein identification was performed with the BioWorks 3.2 software (Thermo Electron, Waltham, MA). Briefly, each file was searched against the human Swiss-Prot database (version 52.0, release date of March 2007), which contained 15 945 protein sequences. Horse CYC FASTA sequence (Swiss-Prot ID P00004) was added manually to the Swiss-Prot library, with the following possible protein modifications: 16-Da shift for oxidized Met and 4-Da Cterminal modification (18 O incorporation). The acceptance criteria for peptide identification were set as follows: a DeltaCn (DCn).0.1, a variable threshold of Xcorr *versus* charge state (Xcorr = 1.9 for z = 1, Xcorr = 2.5 for z = 2, and Xcorr = 3.5 for z =3) and a peptide probability based score with a *p* value <0.001. False discovery rate (FDR) was determined at the protein level by searching a reversed FASTA format of human Swiss-Prot database using the same filtration criteria. FDR was calculated to be less than 3.2% using the following formula: $FDR = [2 \times (\text{Reverse Database Protein Hits})/(\text{Reverse} + \text{Forward Database Protein Hits})] \times 100$.

2.7 ZoomQuant analysis

To measure the intensity ratios of labeled and unlabeled peptide pairs, we used the ZoomQuant software developed by Halligan and colleagues [25]. The generated SEQUEST output files were loaded into the Epitomize filter to generate a colon-delimited text file that contained the list of identified proteins, the sequence and atomic composition of the identified peptides with scan number, charge state, *Xcorr*, and mass data. This file was then loaded into the ZoomQuant software along with a file containing zoom scans that were extracted from the corresponding raw data file using a visual basic RawBitz script. Zoom scan information was then matched to the SEQUEST-identified peptides, after which labeled and unlabeled pairs were detected based on their amino acid composition and theoretical masses. Zoom- Quant software takes into account partial (one 18 O molecule) as well as full (two 18 O molecules) incorporation of heavy oxygen. Ratios were determined from the peak areas of the isotope clusters of the labeled and unlabeled peptides. Obtained ratios were then normalized to the horse CYC ratios as described here.

2.8 Data normalization and assessment of peptide concentration

Horse CYC was used as the internal control. Two micrograms of purified horse CYC was added to each sample prior to proteolytic assay. Peptide concentration estimation was done using spectra generated by MALDI-TOF for both the normal and tumor regions prior to mixing the samples. Consequent to analyzing samples with MALDI-TOF, generated spectra were searched and the following three peptide peaks of CYC were selected: MIFAGIKK ($m/z = 907.8$), TGPLNLHGLFGR ($m/z = 1168.1$), and IFVQKCAQCHTVEK ($m/z = 1633.3$) for ^{16}O -labeled (Fig. 2A, Insets A₁, A₂, and A₃) and ^{18}O -labeled peptides (Fig. 2B, Insets B₁, B₂, and B₃). Relative peptide concentrations were estimated by dividing the total ion count value of all observed peaks by the average ion count of the three CYC peaks. These relative concentrations were then used to determine the normalization factor we used to adjust for the ZoomQuant (tumor to normal) ratios. Average fold change was calculated based on values generated by the two samples as well as reverse labeling experiments.

3 Results

3.1 Assessment of peptide extraction from (FFPE) tissue and of ^{18}O -labeling efficiency

We have implemented and optimized a method for the extraction of peptides from FFPE BSG samples (Fig. 1). This improved protocol includes three main steps, (i) peptide extraction, (ii) stable isotope labeling using enriched ^{18}O water, and (iii) proteome profiling. Each step of this process was checked for quality control (QC) to ensure the validity of the process.

Before mixing ^{18}O -labeled and unlabeled samples for proteome profiling, we checked both the yield of peptide extraction from FFPE tissue and the efficiency of ^{18}O incorporation during proteolysis (see Section 2). Each sample was spiked with 2 μg horse CYC before proteolysis. Following the completion of proteolysis, 2 μL of tryptic digested peptides from ^{16}O - (Fig. 2A) and ^{18}O -labeled peptides (Fig. 2B) were separately analyzed by MALDI-TOF (Fig. 2). The relative higher intensity of peaks with lower m/z in Fig. 2B compared to A is due to the interference of matrix background. Because the absolute peptide extract, including CYC, is lower in the tumor (Fig. 2B) compared to the normal (Fig. 2A), the relative intensity of the background seems higher. Peak 860.7 (Figs. 2A and B) is a good indicator of matrix noise, as it is not ^{18}O labeled (Fig. 2B). Generated spectra were inspected and three predominant CYC peptide peaks were selected (see Section 2) (Fig. 2, Insets A₁, A₂, A₃, B₁, B₂, and B₃). The relative amount of peptides extracted from normal and adjacent tumor FFPE brainstem regions was estimated using the CYC peaks as internal control (see Section 2). To account for protein concentration differences, protein ratios generated by ZoomQuant were normalized to the internal control (horse CYC) following ZoomQuant analyses (see Section 2). Furthermore, spectra generated by MALDI-TOF were inspected to assess the efficiency of ^{18}O labeling. Majority of inspected peaks (>95%) showed 4-Da shift due to incorporation of two ^{18}O molecules as well as 2-Da shift due to the incorporation of a single ^{18}O molecule. ZoomQuant software was used to account for single as well as double incorporation of ^{18}O as described below.

3.2 Proteome profiling and identification of differentially expressed proteins in BSG FFPE tissues

Once efficient peptide extraction and isotopic labeling were confirmed, labeled and unlabeled sample pairs were mixed at 1:1 ratio and subjected to LC-MS/MS analysis as described in Section 2. To increase proteome coverage, we increased the peptide elution time from the most commonly used 90 to 240 min. This significantly increased the number of identified peptides when compared to samples that were processed for only 90 min (data not shown). We performed two sets of experiments, one, in which peptides extracted from

normal sections were labeled with ^{16}O and tumor with ^{18}O and a reverse experiment, where peptides from tumour sections were labeled with ^{16}O while peptides from normal section were labeled with ^{18}O . Figure 3 shows a based peak chromatogram and a zoom scan of co-eluting pairs of labeled and unlabeled peptides with m/z of 795.8 and 793.8, respectively. These doubly charged ions show a 4-Da mass difference, indicating incorporation of two ^{18}O atoms. A low intensity peak, however, was detected between the two isotopic clusters of the peptide pairs, corresponding to species with single ^{18}O atom incorporation. We used ZoomQuant software, developed by Halligan and colleagues [25], to measure ratios between labeled and unlabeled peptides (Fig. 4). Briefly, after protein identification using SEQUEST search engine, both identified peptide list and the raw MS run were uploaded into the ZoomQuant software where zoom scans were extracted and ratios between labeled and unlabeled peptide pairs were measured (Fig. 4). Ratios were determined from the peak areas of isotope clusters of the labeled and unlabeled peptide pairs (Fig. 4) using the following equation $L/U = [A_1 + A_2]/A_0$ where A_0 represents the cluster area of the unlabeled peak, A_1 the cluster area of the peak with a single ^{18}O incorporation and A_2 with a double ^{18}O incorporation. Obtained ratios were then normalized to the ratios of internal standard (horse CYC) as described below.

3.3 Analyses of identified proteins

The combined analyses of both forward and reverse ^{18}O labeling identified and quantified 188 proteins. Initially, labeled and unlabeled peptide ratios (tumor to normal) levels were normalized to internal standard horse CYC and then relative expression levels of each protein was calculated (see Section 2). Our analyses showed that the expression levels of 134 proteins (71%) were not changed while 54 proteins (29%) showed up-regulation of 1.5-fold in tumor compared to the normal brain tissue and these are listed in Table 1. We then inspected this list for known markers of tumor formation, metastasis and cell invasion. We found that 15 (28%) of these 54 differentially expressed proteins have been previously reported as molecules with a potential role in tumor formation (Table 1A) and six of these proteins were indicative of angiogenesis or associated with various tumor types (Table 1B) while the remaining 33 could be potential novel components of pediatric BSG (Table 1C).

4 Discussion

We present the first proteome profile of childhood BSG, thus providing new insight into the molecular pathogenesis of this disease. BSG differentially expressed proteins can be potential biomarkers and molecular signatures of disease progression for possible therapeutic intervention. Importantly, because the etiology of pediatric brainstem glioma has remained unknown, primarily due to the lack of viable tissue for experimental purposes, we describe a technique that may be utilized to study BSG from existing archived specimens, as well as more broadly for all other cancers. Given that FFPE tumor samples are routinely archived and accessible throughout the world, our goal was to develop methodologies to extract and quantify peptides from FFPE samples.

In this study, the largest amount of cells recovered from tumor, were neoplastic astrocytes. However, because of the heterogeneous nature of astrocytomas, other cell types that are an integral part of the tumor, such as the proliferating endothelial cells of vessels and to a much lesser extent, reactive stromal macroglial cells, normal glial cells and neurons, were necessarily present as well. It is important to note that we did not use LCM to recover the cells. Our aim was to recover the most representative area of tumor with the entire intrinsic cellular components in order to obtain the most robust protein signature specific to brainstem astrocytomas given that tumors are molecularly highly heterogeneous and thus random LCM may likely give an erroneous signature based on sampling alone. The same

technique was used to recover the adjacent normal area of the brainstem not involved by tumor. The adjacent areas are composed of all the normal elements anatomically present in that specific area of the brain, including neurons, astrocytes, oligodendrocytes and macroglial cells.

Extracting intact proteins from FFPE samples involves removal of the paraffin wax and the reversal of formalin-induced cross-links. Wax removal is accomplished by a combination of heating, xylene and ethanol treatment. In 1991, Shi and colleagues [17] described a method for retrieval of antigens from FFPE tissues through microwave heating in the presence of metal solutions. The molecular mechanism of formalin fixation, wax removal and cross-link reversal of FFPE samples is well studied for antigen retrieval in immunohistochemical assays [16–20]. However, the majority of our BSG FFPE samples are between 10–25 years old. These tissue samples have been processed by different pathologists and possibly by different methods (*e.g.* fixation period from days to months). To address these issues, we combined and slightly modified existing protocols [16, 17, 20–23, 26, 27] and tested the feasibility of extracting proteolytically digested peptides for analysis and protein identification rather than extracting whole intact proteins. The concept of proteolytic digestion is that the enzyme (trypsin in this case) has access to cleavage sites on cross-linked proteins resulting in the easy release of peptides from the paraffin-embedded tissue. Here, we used a stepwise process in which de-waxed tissues were rehydrated in decreasing ethanol gradient followed by a prolonged tryptic digestion period (Fig. 1).

To ensure the validity of our findings we checked the yield of peptide extraction as well as the efficiency of ^{18}O labeling at each step of our methodology. Since normal and tumor areas were not equal in size, protein measurement for each fraction was required. However, concentration measurements were hindered by the fact that we primarily dealt with peptides and not proteins. Although there are methods by which peptide concentrations can be measured (*e.g.* modified Bradford assays), in our experience, these methods either lack sensitivity or require a large amount of peptides. To address this issue, we spiked each sample with 2 μg of horse CYC (Swiss-Prot ID P00004), which served as internal standard. We chose horse CYC because it generates several unique tryptic peptides that are different in mass from tryptic peptides of human CYC. Since tryptic digestion of horse CYC was performed simultaneously with tryptic digestion of FFPE tissue, we were able to both test for the efficiency of ^{18}O incorporation and measure the relative peptide concentration in each sample. Furthermore, to strengthen our approach, we performed reverse labeling experiments, in which tryptic peptides of normal brainstem sections were labeled with ^{18}O , and compared to ^{16}O -labeled peptides of tumor regions. Therefore, only proteins for which the expression levels were confirmed in both directions were retained.

Using this stringent methodology, we identified 54 proteins that were up-regulated in pediatric FFPE BSG when compared to the normal tissue. Our data agree with previous microarray gene expression profiling studies that have used either brain tumor cell lines or tissue from adult supratentorial gliomas [28–30]. We found that 15 out of the 54 differentially expressed proteins have been reported in these previous studies as potential tumor biomarkers. Among these, vimentin, peroxiredoxin-6, SOD1, annexin V, GFAP, and DRP-2 have been shown to be up-regulated in adult glioblastoma multiforme (GBM) [28–31] whereas tubulin beta-2C chain, neurofilament light polypeptide, and pyruvate kinase are known to be up-regulated in medulloblastoma cell lines [29, 32]. We also detected a high expression of angiogenesis-related proteins in BSG tumor compared to the normal tissue sections and these included hemoglobin subunits, which are an indication of angiogenesis, as well as periostin and mimecan precursors. Overexpression of periostin is known to enhance invasiveness and angiogenesis in oral cancer cell lines [33]. The remaining 33 proteins that were found to be uniquely up-regulated (1.5-fold) in pediatric BSG might be

considered as potential novel biomarkers of this disease. Indeed, our analysis of the 54 identified proteins using the ingenuity pathway analysis (IPA) software clustered 16 of these proteins in a single cancer-related pathway. IPA generates pathways based on curated publications taking into account known protein interactions. Perhaps the most intriguing BSG candidates in this pathway are α 3-collagen type VI precursor (2.3-fold up-regulated), α 1-collagen type VI (4.6-fold up-regulation), and a-spectrin (2.2-fold upregulated), which are excreted or membrane-associated proteins. Furthermore, these mentioned proteins are known to be involved in extracellular matrix remodeling and tumor progression [34, 35] (Fig. 5).

Our observation that 61% of identified proteins did not overlap with previous studies of gliomas and other brain tumors, implies that pediatric BSG has a distinct pathology compared to gliomas in other locations of the brain. Furthermore, the strengths of our study are in the direct analysis of pediatric BSG tissue, rather than surrogate cell lines or adult glioma tissue, and in our comparison of BSG to the normal brainstem of the same patient, versus a different individual, thereby eliminating two main confounding variables. Further investigation using this methodology with larger sample sizes will be necessary to confirm that these proteins are novel tumor biomarkers and potential therapeutic targets of childhood BSG.

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Abbreviations

BSG	brainstem glioma
CYC	cytochrome C
FFPE	formalin fixed paraffin embedded

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Figure 1.

Flowchart of the implemented methodology for the extraction and the analyses of peptides from BSG FFPE samples. The overall methodology consists of three major steps: (i) removal of paraffin by heating samples and a series of washes followed by extraction of tryptic peptides (information acquisition); (ii) addition of 2 mg horse CYC to each sample as internal control followed by ^{18}O or ^{16}O proteolytic labeling and control quality by MALDI-TOF analysis; (iii) in the last step, peptides extracted from the tumor and the normal regions are mixed and subjected to LC-MS/MS analysis.

Figure 2.

Analyses of FFPE extracted peptides using MALDI TOF-MS. Aliquots of ^{16}O - and ^{18}O -labeled peptides extracted from FFPE samples were separately analyzed by MALDI TOF-MS to verify the efficiency of peptide extraction (Panels A and B). Relative amount of extracted peptides in the presence of either ^{16}O (A), or ^{18}O (B) were estimated by dividing the TIC value of all observed peaks by the average ion count of the three CYC peptide peaks: MIFAGIKK ($m/z = 907.8$, Inset A₁), TGNLHGLFGR ($m/z = 1168.1$, Inset A₂), and IFVQKCAQCHTVEK ($m/z = 1633.3$, Inset A₃). Compared to ^{16}O (Insets A₁, A₂, and A₃) incorporation, labeling with ^{18}O resulted in a shift of molecular masses of the three CYC peptides (B, insets B₁, B₂, and B₃).

Figure 3. LC trace of a mixture of total extracted peptides from normal and control tissue. Shown is the LC trace of peptides eluted during a 4-h run. Peptides from tumor and normal areas were extracted, labeled by ^{18}O and ^{16}O , respectively, then mixed and analyzed by LC-MS/MS as described in Section 2. Inset shows a co-eluted labeled and unlabeled peptide pair generated from the same protein that is present equally in tumor and normal tissue.

Figure 4.

Schematic representation of ^{18}O -labeled and unlabeled peaks by ZoomQuant software. Shown are ^{18}O -labeled and unlabeled peaks for different peptides visualized by ZoomQuant software. A doubly charged peptide (GESGPSGPAGPTGARR) of collagen alpha 1(I) (Swiss-Prot ID P02452) (A) and a doubly charged peptide (LADVYQAE LR) of glial fibrillary acidic protein (Swiss-Prot ID P14136) (C) were measured to be 1.8- and 2.8-fold up-regulated, respectively, in tumor *versus* normal. Reverse experiments, in which normal tissue was labeled with ^{18}O , confirmed the observation (B and D). ZoomQuant software uses both single and double incorporation of ^{18}O atoms in a given peptide to calculate the

ratios between ^{16}O - and ^{18}O -labeled samples using the following equation: $L/U = [A_1 + A_2]/A_0$ where A_0 represents the cluster area of the unlabeled peak, A_1 the cluster area of the peak with a single ^{18}O incorporation and A_2 with double ^{18}O incorporation (D).

Figure 5.

Pathway generated by the Ingenuity software depicting interaction between proteins, whose expression was altered in BSG *versus* normal area. Shown is biological pathway generated by Ingenuity software depicting interaction of 16 proteins upregulated in BSG. Protein names are abbreviated and fold changes are shown where applied. Proteins highlighted in dark gray are up-regulated (shown as numerical values) in tumor when compared to the normal brainstem region (score of 45). Protein-protein interactions are shown as follows: indirect (--->), acts on (→), or (—) direct interactions.

Potential pediatric brainstem glioma biomarkers

Table 1

Protein	gi	SP_ID	Average FC	Standard error	Peptide count
A. Proteins previously associated with adult tissue/cell lines of supratentorial gliomas					
Glial fibrillary acidic protein, astrocyte	121135	P14136	2.86	1.00	25
Vimentin	55977767	P08670	2.64	1.10	17
Heat-shock protein beta-1	19855073	P04792	2.56	0.01	2
Peroxisredoxin-6	1718024	P30041	2.45	1.10	17
Tubulin beta-2C chain	55977480	P68371	2.41	0.10	2
Neurofilament light polypeptide	62511894	P07196	2.13	0.45	6
Dihydropyrimidinase-related protein 2 (DRP-2)	3122051	Q16555	2.07	1.10	3
Superoxide dismutase 2	134665	P04179	1.97	0.75	2
Annexin A5	113960	P08758	1.90	0.10	6
Glucose-6-phosphate isomerase	17380385	P06744	1.86	0.20	2
Fructose-bisphosphate aldolase C	113613	P09972	1.82	0.56	5
Serum albumin precursor	113576	P02768	1.74	0.02	7
Alpha crystalline B chain	117385	P02511	1.68	0.28	2
L-lactate dehydrogenase B chain	126041	P07195	1.67	0.20	2
Pyruvate kinase isozymes M1/M2	20178296	P14618	1.64	0.45	6
B. Proteins previously associated with angiogenesis or various tumor types					
Periostin precursor	93138709	Q15063	2.42	0.16	3
Hemoglobin subunit alpha	57013850	P69905	2.26	0.25	4
Hemoglobin subunit beta	56749856	P68871	1.98	0.95	2
Fibrinogen beta chain precursor	399492	P02675	1.96	0.43	1
Mimecan precursor	129078	P20774	1.71	0.13	2
Ferritin light chain	120523	P02792	1.66	0.50	2
C. Novel potential biomarkers associated with pediatric brainstem gliomas					
α1-Collagen type VI	125987811	P12109	4.56	1.90	2
Transgelin	3123283	Q01995	3.42	1.40	4
Collagen alpha-2(VI) chain precursor	125987812	P12110	2.92	1.40	3
Ras-related protein Rap-1A precursor	51338596	P62836	2.80	1.40	1

Protein	gi	SP_ID	Average FC	Standard error	Peptide count
ADAMTS-12 precursor	17366354	P58397	2.80	0.62	2
Spectrin beta chain, brain 1	116242799	Q01082	2.73	0.45	2
Keratin, type I cytoskeletal 10	547749	P13645	2.49	0.55	1
Myelin transcription factor 1-like protein	76789661	Q9UL68	2.46	1.20	2
Excitatory amino acid transporter 1	1169458	P43003	2.35	0.45	2
ATP synthase subunit beta, mitochondrial	114549	P06576	2.34	0.42	5
Creatine kinase B-type	125294	P12277	2.33	1.19	4
α3-Collagen type VI precursor	5921193	P12111	2.30	0.65	11
Heat shock protein HSP 90-α (HSP 86)	92090606	P07900	2.29	0.64	1
Collagen alpha-2(I) chain precursor	124056488	P08123	2.28	0.66	4
Spectrin alpha chain, brain	94730425	Q13813	2.23	0.30	3
Histone H2B type 1-L	7387743	Q99880	2.21	0.69	2
Tropomyosin alpha-4 chain	54039746	P67937	2.18	0.90	2
Myelin basic protein (MBP)	17378805	P02686	2.17	0.07	5
Protein S100-B	134138	P04271	2.16	0.45	2
Histone H2A type 2-A (H2A.2)	74757558	Q6F113	2.07	0.81	5
Tubulin beta-4 chain	75075849	Q4R4X8	2.03	0.90	6
Tubulin alpha-3/alpha-7 chain	135418	P05214	1.97	0.15	2
Transgelin-2	586000	P37802	1.97	0.34	2
Alpha-actinin-1	46397817	P12814	1.91	0.38	2
Actin, alpha skeletal muscle	55976646	P68139	1.89	0.64	4
Collagen alpha-1(I) chain precursor	124056487	P02452	1.85	0.60	4
2,3'-cyclic-nucleotide 3'-phosphodiesterase	1705945	P09543	1.70	0.01	2
Ferritin light chain	120523	P02792	1.66	0.51	2
Elongation factor 1-α 1	55584035	P68104	1.64	0.20	2
Tubulin alpha-6 chain	20455322	Q9BQE3	1.61	0.10	4
Plectin-1 (PLTN)	134044255	Q15149	1.61	0.01	2
Keratin, type II cytoskeletal 1	1346343	P04264	1.60	0.14	7
Glyceraldehyde-3-phosphate dehydrogenase	120649	P04406	1.60	0.31	4