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Proteomic Identification of Biomarkers in the Cerebrospinal fluid (CSF) of Astrocytoma Patients

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Abstract

The monitoring of changes in the protein composition of the cerebrospinal fluid (CSF) can be used as a sensitive indicator of central nervous system (CNS) pathology, yet its systematic application to analysis of CNS neoplasia has been limited. There is a pressing need for both a better understanding of gliomagenesis, and the development of reliable biomarkers of the disease. In this report, we used two proteomic techniques, two-dimensional gel electrophoresis (2-DE) and cleavable Isotope-Coded Affinity Tag (cICAT), to compare CSF proteomes in order to identify tumor and grade specific biomarkers in patients bearing brain tumors of differing histologies and grades. Retrospective analyses were performed on 60 samples derived from astrocytomas WHO grade II, III and IV, schwannomas, metastatic brain tumors, inflammatory samples and non-neoplastic controls. We identified 103 potential tumor-specific markers; of which 20 were high-grade astrocytoma-specific. These investigations allowed us to identify a spectrum of signature proteins that could differentiate

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Significance: The measurement of alterations in the protein composition of the cerebrospinal fluid (CSF) can be used as a sensitive indicator of central nervous system (CNS) pathology, yet its systematic application to analysis of CNS neoplasia has been limited. There is a pressing need for both a better understanding of gliomagenesis, and the development of reliable biomarkers of the disease. Our investigations allowed us to identify a spectrum of signature proteins that could differentiate between low (AII) and high-grade (AIV) astrocytoma that warrant further validation as potential new markers for diagnosis, prognosis and disease follow-up. These candidate biomarkers may also have functional properties that play a critical role in the development and malignant progression of human astrocytomas, thus possibly representing novel therapeutic targets for this highly lethal disease.

Author contributions

Project concept and experimental designs were developed by EGVM and FWK. All experiments were performed by FWK with the following exceptions; Mass spectrometry and cICAT were done by MSR. BJS performed the statistical analysis, JP provided expertise with proteomic analyses; JJO, MDG, AG, SK and GYG all provided CSF samples. FWK and EGVM interpreted the results and wrote the manuscript. All authors read the manuscript.

between low (AII) and high-grade (AIV) astrocytoma, which may represent new diagnostic, prognostic and disease follow-up markers when used alone or in combination. These candidate biomarkers may also have functional properties that play a critical role in the development and malignant progression of human astrocytomas, thus possibly representing novel therapeutic targets for this highly lethal disease.

Keywords

cerebrospinal fluid; central nervous system; brain tumor; glioma; proteomics; two-dimensional gel electrophoresis; cleavable isotope-coded affinity tag (cICAT); mass spectrometry; biomarker discovery

Introduction

Diffusely infiltrating astrocytomas (WHO grade II – IV) are the most common primary brain tumors, with glioblastoma being the most aggressive subtype. Patients with glioblastoma have a life expectancy of less than one year even after surgery, chemo- and radiation therapies.^(1,2) Therefore, there is a pressing need for both a better understanding of gliomagenesis, and the development of reliable biomarkers of the disease.

Genetic alterations that drive glial cell transformation and malignant progression result in tumor-specific changes in protein expression. The identification of individual proteins or protein clusters expressed in neoplastic tissue could uncover critical mediators of tumor progression, and identify surrogate markers for diagnosis, prognosis and therapeutic response.⁽³⁾ Proteome profiles reflect the biological phenotype of individual tumors more accurately than transcriptome analyses, since changes in gene expression do not always correlate with protein expression.^(4,5) Moreover, proteomic analyses can detect post-translational modifications, and different isoforms which may specifically affect disease progression.⁽⁶⁾

In this study we examined the CSF as a potential reservoir of proteins secreted during brain tumor development to determine whether CSF from specific types of brain tumors have a characteristic proteomic profile that might contain important biomarkers or give insights into the process of gliomagenesis. CSF was used because of its proximity to the lesions in question and because of its relative ease of acquisition.⁽⁷⁾

In addition, normal CSF protein concentration is 100–400-fold lower than serum (CSF: < 1,000 proteins, Plasma: > 10,000 proteins)⁽⁸⁾, facilitating detection of disease specific protein alterations in the CSF. These potential advantages have spurred the investigation of new techniques for the rapid screening and accurate identification of proteins secreted into the CSF by tumor and stromal cells.

Two-dimensional gel electrophoresis (2-DE) in combination with mass spectrometry (MS) has identified numerous normal CSF proteins as well as potential protein markers for mainly neurodegenerative diseases.^(9,10,11) In contrast, there has only been a single study examining the CSF proteome of a small set of brain tumor patients, leading to identification of two potential useful diagnostic markers.⁽¹¹⁾

Here, we surveyed the CSF protein profiles from 60 patients with grade II–IV astrocytoma, non-astrocytic brain tumors and non-tumoral controls, using two independent proteomic techniques: 2-DE and cICAT. We found critical differences in the protein expression profiles of tumoral versus control CSF and identified a spectrum of signature proteins for low and high-

grade astrocytoma that may yield new prognostic markers and provide insight into glioma biology.

Experimental Section

Source of CSF and Sample Preparation

Detailed information and the sources for the 73 CSF and cyst fluid samples used as the training and validation sets are listed in Supplemental Table 1. Samples were collected after informed consent using Institutional Review Board approved protocols. Collection of retrospective samples for the training set was initiated at multiple sites to maximize the number of samples, as gliomas, especially AII and AIII, are rare tumors. Also, in addition to the inherent biases in retrospective analyses, since collection of CSF is not routine for CNS tumor operations, we cannot exclude that we introduced a bias in selecting patients that were amenable to peri-operative CSF collection. A separate validation set of samples consisting of 4 5 Controls (C), 1 AII, 1 AIII, and 6 AIV was collected prospectively for 6 months after assembly of the initial bio-panels. Because of the infeasibility of collecting CSF from healthy individuals, patients with headaches, hydrocephalus or other benign neurological conditions were used as non-tumor control samples. These control (C) samples were also within normal limits for protein content, biochemical and microbiological testing, and cytopathologic examination. Samples showing evidence of blood contamination were not used in the study. All samples were centrifuged at $750 \times g$ at 4°C to remove cells and debris and stored in aliquots at -70°C until use. Samples (1 ml) were concentrated five-fold using Centricon 3 kD filtration (Millipore, MA, USA) and partially depleted of IgG and albumin using Proteoprep Blue Albumin Depletion kit (Sigma, Mo, USA). Sample proteins were precipitated using 15% trichloroacetic acid (TCA), re-suspended in denaturing buffer (8 M urea, 4% CHAPS, 100 mM protease inhibitor cocktail (Roche, Mannheim, Germany)), followed by determination of protein concentration using an RCDC assay (BioRad; CA, USA).

Two-Dimensional Polyacrylamide Gel Electrophoresis (2-DE)

CSF samples were analyzed in duplicates or triplicates using 2-DE as described.⁽¹²⁾ The first dimension was performed on an IPGphor system using Immobiline Dry strips (pH 3–10NL and 4–7; 13 cm.) (Amersham Biosciences, NJ, USA) rehydrated overnight with 200 μg of CSF proteins (Total run= 130,000 Vh). The second dimension was run on 12.5% polyacrylamide gels with 2% SDS using the Protean II xi system (BioRad, CA, USA). Silver Stain Plus kit (BioRad, CA, USA) was used to visualize protein spots and the gels were analyzed using Melanie (EXPASY SwissPro 2-D database; <http://au.expasy.org/melanie/>) and Image Master (Amersham Biosciences) softwares.

In-Gel Digestion of Proteins and MALDI-TOF/TOF-MS Analysis

Protein spots of interest were excised from the gel, destained using SilverOUT kit (GenoTech, MO, USA) and digested overnight with 150 ng of sequencing grade trypsin (Promega, WI, USA). The resulting peptides were extracted using Montage In-gel digestion and peptide extraction kit (Millipore, MA, USA), spotted onto target plates, overlaid with alpha-cyanocinnaminic acid matrix (Agilent, DE, USA) and analyzed using a model 4700 Proteomics Analyzer (Applied Biosystems, CA, USA). Combined MS and MS/MS spectra from each spot were processed using GPS Explorer V2.0 (Applied Biosystems, CA, USA) with MASCOT (Matrix Science, MA, USA) which led to the identification of 210 proteins (Confidence interval >85%), of which 130 were found differentially expressed by biostatistical analysis in at least one of the comparisons.

Biostatistical Analysis of 2-DE Results

The arithmetic mean of the protein expression within the two to three 2-DE replicates was used to run a global F-test in the ANOVA setting. After ensuring that the groups were indeed different, the following pair wise comparisons were performed to find grade-specific proteins: non-tumoral controls versus AII, AII versus AIII, AII versus AIV and AIII versus AIV CSF samples. A separate comparison of AIV CSF samples versus AIV cyst-fluids was also performed. For the pair wise comparisons, the multiple testing problem was corrected using the Bonferroni correction (by multiplying each p-value by four). Proteins were declared significant using the False Discovery Rate (FDR) framework, controlling FDR at 5%.⁽¹³⁾ This framework controls for the multiple testing problem arising from performing many statistical tests (a test for each protein).

Cleavable Isotope-Coded Affinity Tag (cICAT) Analysis

cICAT technology uses stable isotope tags (^{12}C and ^{13}C), in combination with liquid 2D liquid chromatography-tandem mass spectrometry, to compare the levels of proteins in two related samples by analyzing cysteine-modified, isotopically labeled peptides thereof.⁽¹⁴⁾ For our analyses, due to cost considerations we compared pools of normal and glioma CSF samples, with three different equal volume CSF samples per pool analyzed. Two pools consisting of three different samples each were made for each category (6 total samples/category) and analyzed in two independent cICAT analyses (Applied Biosystems, CA, USA). Briefly, the control and glioma samples were separately labeled with light and heavy reagent, mixed in equal total protein ratio, and digested overnight with trypsin (trypsin:protein ratio=1:100, w/w). Next, the peptides were desalted in a single desalting step using a strong cation-exchange cartridge, and the cICAT-modified, cysteine-containing peptides were enriched/purified using a monomeric avidin column (Applied Biosystems). The biotin tag was cleaved off by treatment with 15% trifluoroacetic acid (TFA) and the sample was dried and reconstituted in 10% formic acid. After this processing, a portion of the sample was analyzed using an Ultimate nanoHPLC-MS/MS (Dionex/LC Packings, CA, USA) using a Vydac C18 silica column (5 μm , 300 $\text{\AA}$, 75 $\mu\text{m} \times 150 \text{ mm}$) interfaced to a QSTAR XL mass spectrometer (Applied Biosystems, CA, USA). The MS/MS data was processed using ProICAT software for protein identification and quantification. Only proteins with a ProtScore >1.0 (Confidence Interval >85%) were considered. Two cICAT comparisons using two separate sets of pooled CSF samples were performed in triplicate to identify low-grade-(AII) and high-grade- (AIV) specific proteins. Proteins were considered differentially expressed if multiple peptides generated concordant cICAT ratios in both analyses.

Western Blot Analysis

Electrophoresis and blotting were performed using the BioRad Criterion system on TCA precipitated CSF or cyst-fluid proteins. Antibodies used: anti-actin (1:1000)⁽¹⁵⁾, anti-FGF-14 (Santa Cruz, CA, USA; sc-16812; 1:500), anti-SPARC (Santa Cruz; sc-13324; 1:500), anti-VEGF-B (Santa Cruz; 1:500), anti- β -2-microglobulin (Abcam, MA, USA; Clone B2M-01; 1:250), anti- β -defensin-7 (Immunodiagnostik, Germany; AE1109.1; 1:100) and anti- β -defensin-6 (Novus Biologicals, CO, USA; ab14364; 1:100). Transthyretin (TTR) (Santa Cruz; sc-13098; 1:1000) was used as a loading control.

Biomarker Panel Construction

Only proteins found to be significantly differentially expressed, based on pair wise comparisons with an FDR of 5% (Table 2), were considered for the construction of the biomarker panels (Fig. 5). Proteins with lower p-values or those selectively present in a particular astrocytoma grade were given precedence since they were the strongest hits in the data set. The expression profile of these proteins was further compared to that from CSF of

patients with other primary brain or metastatic brain tumor to determine marker specificity to astrocytoma. Two panels were constructed; one with 31 proteins (Fig. 5A) and one restricted to the top 13 biomarkers from the larger panel (Fig. 5B). This smaller panel represents the minimum number of proteins needed to correctly identify astrocytoma grade. Each panel was assessed for its ability to correctly classify the tumor grade for subjects in the training set consisting of normal, AII, AIII, and AIV groups using k-nearest neighbors method with $k=1$. Still, an even better assessment of a panel is gained from testing an independent validation test set of data. To this end, each panel was tested blindly (compared to the nearest neighbor from among the original data) on a set of 11 new prospective CSF samples, collected over a period of 6 months.

RESULTS

Proteomic Analysis of the CSF

To establish protein profiles in the CSF, we used two methods. Two-dimensional gel electrophoresis (2-DE) provided a semi-quantitative evaluation of the relative protein abundance between samples. Since hydrophobic proteins and proteins <15 kDa or >100 kDa, as well as very basic or acidic proteins do not resolve well using 2-DE, we also used cleavable isotope-coded affinity tag analysis (cICAT) as a complementary technique to refine the identification of proteins.⁽¹⁶⁾

To create expression profiles by 2-DE, CSF samples were divided into control (C) and three staged tumor groups (AII, AIII and AIV). AII were considered low-grade, while AIII and AIV are high-grade astrocytomas. Each individual sample was analyzed in duplicate or triplicate by 2-DE. Silver stained protein spots were analyzed with ImageMaster software to establish semi-quantitative expression levels and excised from the gel for identification using TOF/TOF MS/MS analysis.

We observed consistent differences in the expression profiles of CSF from AII–IV patients versus non-tumoral, primary or metastatic brain tumors controls (Table 1). The number of protein spots in each group correlated directly with malignancy grade (Fig. 1). We identified an average of 86 proteins in non-tumor controls (range 76–90), 101 in AII (85–104), 136 proteins in AIII (123–148) and 175 in CSF or cyst fluids from AIV patients (168–198) (Figure 2A) and classified them into several groups based on their function (Table 2 and Fig. 2B). FGF-14 and PEDF, provide a demonstration of the specificity and rigorousness of the methodology (Fig. 3).

In pair wise comparisons between the normal control group versus AII, AII versus AIII, AII versus AIV, and AIII versus AIV groups, we found 29, 66, 132 and 89 proteins, respectively, to be significantly differentially expressed between the groups (Figure 2A). Using a false discovery ratio (FDR) of 5%, less than 2/29, 4/66, 7/132 and 5/89 proteins would be expected to be false positives. A total of 130 individual proteins were found differentially expressed through these comparisons (Table 2). Among these 130 proteins, 32% (41/130) were found in all groups while the remaining were differentially expressed (Figure 2D).

cICAT analyses were performed to compare between control and AII, and control and AIV CSF samples. Due to similarities in 2-DE profiles of AIII and AIV patients and the cost of cICAT analyses, we focused only on AII and AIV groups, and pooled 3 patients' samples for each group. The same non-tumoral control pool was used to compare to AII or AIV pools. Each analysis was run in triplicate and the entire experiment was repeated twice using different samples (6 samples total per control and tumor type). Only proteins with cICAT ratios of >1.1 or <.9 were analyzed, leading to identification of 53 proteins. Thus, half as many proteins were identified by cICAT versus 2-DE (53 versus 130) (Figure 2C). Still, there was a high

concordance between the techniques, as 30 of the 53 proteins identified by cICAT (57%) overlapped with the 130 found by 2-DE analysis (Table 1 and Figure 2C), while the remaining 23 proteins were found uniquely by cICAT (STables 3–4).

Effect of Patient Characteristics on biomarker discovery

On review of STable 1, one can see that there is variability in the demographic character, disease type and treatments used within each study group. This is unavoidable as brain tumors are rare and CSF infrequently available, thus it is quite difficult to collect CSF from large numbers of patients with homogeneous clinical parameters. As an example, the age range in the control group was 26 to 85 years and underlying diagnosis included headache, CSF leak, hydrocephalus, and arterio-venous malformation. Similarly in the high-grade study group, some individuals were early in their therapy and had only undergone diagnostic surgery, while others had advanced disease, were receiving radiation therapy, chemotherapy and possibly had more than one prior surgery (STable 1). A few of the patients appeared to be in the midst of tumor progression while others had stable disease. Another variable was the relationship of the tumor to the CSF containing spaces. In some individuals, the lesions were adjacent to or communicated with ventricles. In others, the lesion was deeper within the cerebral parenchyma and away from ventricular or sub-arachanoid space.

A second limitation of our study was the difference in methods of sample analysis, as 2-DE was performed on each individual sample while pooled samples were used for cICAT experiments. This pooling of samples in the cICAT experiments might have resulted in some of the identified discrepancies in the number of differentially expressed proteins found by the two-proteomic methods. Lastly, the 19 AIV samples included eleven cyst-fluids from tumor space, thus adding another variable to our analyses. To address the reliability of the assay, biomarkers discovered in the 11 AIV samples derived from fluid-filled tumor cysts were compared with those resulting from the 8 CSF samples from the same group. The distribution and levels of biomarkers identified in CSF and tumor cyst fluid were similar providing a strong internal control of reliability (STable 5; last column).

In spite of these points of heterogeneity, high intra-group correlations were found, attesting of the rigor of the proteomic techniques used and the consistency of the expression changes in CSF (Table 1 and STable 1). Our analyses led to the identification of only 200 out of 1,000 proteins possibly present in the CSF thus indicating that we observed only the most abundant of proteins with the most drastic changes in expression levels. Future validation studies on a large set of prospective samples will be needed to detect more subtle, intra-group differences that may reflect differences in stage of treatment and other clinical parameters.

Verification of Proteomic Results by Western Analyses

To confirm the presence and to independently quantify CSF proteins found differentially expressed by 2-DE and/or cICAT, we performed western blots on eight selected proteins, based on implied role in gliomagenesis and availability of antibodies. These consisted of SPARC and FGF-14 (growth/migration), VEGF-B (angiogenic), tau (microtubule-associated), and several immune-related molecules including β -2-microglobulin, β -defensins-6 and 7, and secreted attractin. Transthyretin, a pre-albumin found abundantly in the CSF, was used as a control. The relative expression levels of these proteins were found to correlate closely with our proteomic screen. Representative blots depicting grade-specific expression are shown (Fig. 4).

A differentially expressed protein was the secreted protein with acidic and cysteine-rich domains (SPARC) or osteonectin, a 45 kDa secreted glycoprotein.⁽¹⁷⁾ SPARC was detected at low levels in most non-tumoral control and AII CSF, and was up-regulated in the majority

of the high-grade samples by 2-DE (Table 1). Western blot analyses showed detectable levels of SPARC in only 30% (3/10) of non-tumoral controls and 33% (2/6) of AII patients. In contrast, consistent high levels were seen in samples from 86% (6/7) of AIII and 90% (18/19) of AIV astrocytoma patients (Fig. 4A). Tumor expression studies have previously shown an increase in SPARC production in high-grade gliomas^(18,19), corroborating our findings and providing a likely source for the SPARC present in the CSF. However, due to high variability between individuals, SPARC does not appear to be a good astrocytoma marker.

Similarly we found significant differential expression of FGF-14 between AIII/IV CSF samples and controls by 2-DE. FGF-14 is a member of the large fibroblast growth factor family that is normally found in the developing as well as the adult CNS.⁽²⁰⁾ Interestingly, two separate spots were identified as FGF-14 by 2-DE analysis in AIII and AIV samples (Fig. 3A). By western analysis (Fig. 4B), we found low levels of the larger 35 kD form (FGF-14L) in CSF from 70% (7/10) non-tumoral controls, and 100% (6/6) AII and still higher levels in 100% (7/7) of AIII samples. Low levels of a smaller, previously unreported, 27 kD form (FGF-14S) was apparent in 43% (3/7) of AIII samples and became the dominant form in 100% (10/10) of AIV samples.

Tau is another interesting protein due to its up-regulation in many CNS disorders.⁽⁹⁾ In our 2-DE analyses, tau was found to be expressed at low to medium levels in control and AII samples while its expression increased in AIII/AIV. Western blot analysis confirmed this trend, but showed high sample variability within each group, suggesting it is not a reliable marker (Fig. 4B).

High expression of VEGF-B was found predominantly in high-grade astrocytoma samples by both 2-DE and cICAT (Table 1). Multiple VEGF isoforms, a key tumor angiogenic factor, are known to be up-regulated in various forms of malignancies including gliomas.⁽²¹⁾ Using western blot analysis we saw no expression in 100% of the controls (5/5), low to moderate levels in 75% (3/4) of AII and 80% (4/5) of AIII, and high expression in 100% (8/8) of tested AIV samples (Fig. 4B).

Amongst proteins with increased secretion into the CSF in AIV samples was attractin, a large 175 kDa glycoprotein. Attractin has been implicated to be a major mediator of glioma cell motility and hence is of interest.⁽²²⁾ In addition to confirmation by both 2-DE and ICAT (Table 1), western blot analysis demonstrated 100% (19/19) of AIV cases analyzed to express attractin (Fig. 4C). However, attractin's expression was not exclusively in AIV, as we detected expression in 57% (4/7) of the AIII, and 33.3% (2/6) of the AII patients. In contrast, only 10% (1/10) controls showed detectable levels, a predicted result since secreted attractin is specifically excluded from the CNS.⁽²³⁾

Another interesting finding from our study was differential expression of several immune response related proteins in CSF from astrocytoma patients versus controls. Increased levels of secreted β -2-microglobulin (β 2m), an 11.5 kD plasma protein which is also an obligatory component of the HLA class I antigen presentation complex, were found with increased malignancy by both 2-DE as well as by cICAT, whereas β 2m was absent in the control CSF (Table 1). Western blot analysis confirmed detectable levels of β 2m in 33.3% (2/6) of AII, 100% (7/7) of AIII and 100% (19/19) of AIV samples, while none of the normal controls (0/10) were positive (Fig. 4A). In contrast, β -defensins, multiple complement components, and IgG variants were found up-regulated mostly in CSF from low grade but not high grade gliomas by cICAT and 2-DE (Table 1). For example, β -defensin-6, a 7 kD anti-microbial peptide, was observed to be elevated in most AII CSF by 2-DE, and strongly up-regulated (AII: C ratio >10) in both low and high-grade samples by cICAT.

Western blot analysis confirmed substantial β -defensin-6 in 100% (6/6) of AII and 80% (4/5) of AIII samples. Low but detectable levels were also found in 80% (8/10) non-tumoral controls

and 11% (2/19) of AIV samples (Fig. 4A). Similar results were obtained for β -defensin-7, which was found by both 2-DE and western to be up-regulated in AII samples.

Overall, despite the semi-quantitative nature, potential biomarkers identified by 2-DE and cICAT analyses were largely confirmed either through being detected independently by each of these proteomic techniques and/or further verification via western blot analysis. In some cases, 2-DE and cICAT failed to detect low levels of protein, likely representing the higher sensitivity of western blot analysis. Cleavable-ICAT also proved less reliable than 2-DE for semi-quantitative assessment, as quantification in cICAT is disrupted by overlapping MS spectrum peaks, a problem that might be overcome by new versions of this technology.⁽²⁴⁾

Construction and Assessment of a Biomarker Panel

To examine whether a subset of the differentially expressed proteins of Table 1 could be used to build a biomarker panel that would be able to recognize astrocytoma of different grades, we selected proteins with restrictive expression to one group (C, AII, AIII or AIV) or expressing a likely FDR of 5% or less between groups. We excluded proteins expressed differentially in CSF from other primary and metastatic brain tumors or in representative samples of infectious CNS diseases. The metastatic proteins fell in two categories (Table 1; last three columns). The profiles of proteins in CSF from lymphoma patients resembled those of AII CSF while metastatic brain tumor CSF profiles were closely related to AIII and AIV CSF profiles. Primary and metastatic brain tumor samples had too much heterogeneity leading to an insufficient training set to use for the biomarker panel construction and assessment. However, using these two groupings we were able to differentiate between tumor versus astrocytoma-specific proteins thus allowing us to design two potential panels for distinguishing C, AII, AIII and AIV. The first panel contains all 31 proteins for which a differential pattern of expression between the groups could be discerned (Fig. 5A). The second panel contains the minimal number of proteins necessary for the pattern of expression to correlate most strongly with diagnostic grade (Fig. 5B). For example, the AII profile is defined by β -defensins 6 & 7; while AIII CSF is distinguished by high levels of GP2 and MPHOSPH6 related protein. Finally, CSF from AIV patients contains high levels of many unique proteins including attractin, IL17E, NCF-1 and the short form of FGF-14 allowing for easier classification. These proteins were chosen as they were most consistently seen differentially expressed within the grade they represent. Both potential protein panels were cross-validated using k-nearest neighbors (kNN) to determine how well they could correctly classify astrocytoma grade based on the 2-DE data. Using the kNN algorithm (k=1), all subjects in the original training set of data were correctly classified with a misclassification rate of zero.

Next, we wanted to verify whether the two potential panels would indeed be able to correctly identify a set of prospective C, AII-AIV samples. Therefore, we sought to test the panels blinded on an independent test set consisting of 13 total samples (4-5 C, 1 AII, 1 AIII and 6 AIV) collected over a period of six months. Evaluation of the panels on this independent test set of data resulted in 13 out of 13 (100%) of the samples correctly classified using both the 31-protein and the 13 protein panels leading to a misclassification rate of 0. Even if our validation set was admittedly limited in size, these data suggest that these potential biomarker panels perform well in distinguishing between the differing astrocytoma grades and non-tumor controls, thus providing proof-of-principle evidence for the use of biomarker panels in the clinical assessment of brain tumors in the future. CSF is routinely drawn in neurology clinics for diagnostic purposes and in the near future, protein arrays might permit the rapid and simultaneous analysis of multiple proteins in small volumes. Such protein arrays could be developed into a clinical test that would be amenable for routine prognostic and disease follow-up evaluations.

Discussion

Proteomic Analysis to Identify Biomarkers of Gliomagenesis in the CSF

In this study, we used two complementary proteomic techniques (2-DE and cICAT) to analyze potential glioma biomarkers. We identified 153 differentially expressed proteins in the 60 CSF or cyst fluid samples analyzed; 30 detected by both techniques, 100 by 2-DE alone and 23 by cICAT alone. This allowed us to define a biomarker panel including 21 astrocytoma-specific proteins (1 AII, 2 AIII and 18 AIV), 13 of which correlated strongly with astrocytoma grade, and in a reverse analysis could be applied to the samples used here to diagnose grade with a 100% correlation to histological grading.

In addition, the potential biomarkers were compared against published CSF studies to ensure the specificity of our selected proteins to astrocytoma patients.^(9,11) Over eighty percent of the proteins selected for the final biomarker panel were absent from the “normal” control CSF used in other studies, and only one (apolipoprotein) was found differentially expressed in the CSF from other CNS disorders.^(9,10) However the panel still needs further expansion and modification to allow differentiation between different forms of brain tumors in addition to astrocytoma, before it can be used for diagnostic purposes. Regardless, this novel biomarker panel can immediately be tested on prospective CSF and plasma sample sets towards translation to the clinic. These future applications will be facilitated by the advent of novel protein chip technologies such as the immobilization of panel-specific antibodies as protein arrays. These arrays can then be incubated with small volumes of CSF for rapid detection and quantification. The ease of testing CSF compared with surgical biopsy, together with obtaining reliable numerical results as compared to observer-biased immunohistochemical techniques make this approach a priority.

Validation of Previously Identified, and Discovery of New Biomarkers

Our screen was validated by the identification of proteins already known to be associated with astrocytoma development as the result of microarray studies. These include pro-angiogenic factors VEGF-B and basic FGF⁽²⁵⁾; the blood clotting factor VIII;⁽²⁶⁾ immune response-related factors like β -2-microglobulin;⁽²⁷⁾ and bone morphogenesis proteins such as osteonectin/SPARC.⁽²⁸⁾

Amongst new biomarkers, we found high levels of FGF14 in AII-IV. FGF14 has been detected throughout CNS development and functions in neuronal signaling, axonal trafficking, and synaptosomal activity.⁽²⁹⁾ Expression of related FGF family members such as FGF-4, 5 and 6 can transform NIH3T3 cells while others act as mitogens in astrocytes, mammary cell carcinoma and keratinocytes.^(30–32) FGF-14 is normally observed as a 35kD protein due to post-translational modifications; however, our proteomics approach allowed us to also discover a smaller, previously unreported, ~27kD form specifically expressed in CSF from patients with AIV. It will be important to determine the precise biological function of this smaller form and whether it may be causal in the augmented malignancy associated with transition to AIV.

We also observed, for the first time, elevated levels of the secreted form of attractin in CSF from AIII and IV patients by both 2-DE and cICAT analyses. Attractin is a member of the CUB family of cell adhesion and guidance proteins existing in secreted (175 kDa) and transmembrane (~200 kDa) forms due to alternative splicing.⁽³³⁾ Transmembrane attractin is abundant in the brain where it is critical for maintenance of normal CNS architecture. It is a critically up-regulated gene in axonal regeneration, enabling correct path_finding following spinal cord Wallerian degeneration,⁽³⁴⁾ and its absence results in a juvenile-onset neurodegeneration.⁽³⁵⁾ In contrast, secreted attractin is an abundant serum glycoprotein, which is normally absent in the CNS and un-detectable in the CSF.⁽²³⁾ High levels of secreted attractin

interferes with neuron differentiation, altering neuron-extracellular matrix interactions, perhaps through regulation of chemokines.⁽²³⁾ Secreted attractin thus appears to have potential as a reliable biomarker for malignant astrocytoma as it is found almost exclusively in the CSF from high-grade astrocytoma patients. A recent study confirmed attractin as being highly expressed in high grade astrocytoma and further revealed that secreted attractin is a pro-migratory factor for glioma cells, suggesting its potential importance as a therapeutic target to antagonize the spread of the diffusely infiltrating astrocytomas.⁽²²⁾

Immune Response to Neoplastic Disease in Low-Grade Glioma Patients—

Another remarkable finding of our screen was the marked increases in the levels of several immune response proteins found in the AII but not AIII–AIV CSF samples. These included complement proteins which are known to be secreted secondary to inflammatory responses as would be expected due to the infiltration of macrophages and lymphocytes into the tumor microenvironment or the *in situ* activation of microglia.⁽³⁶⁾ It is known that astrocytic tumors secrete various chemokines, which are likely responsible for the immune cell infiltrates observed in these tumors.^(37,38) The presence of complement proteins in AII CSF may reflect an active host anti-tumor response. While the CNS has classically been considered an immunologically privileged site, it has become clear that strong immune responses do occur in the CNS.⁽³⁹⁾ The strength of this response may decrease in high-grade astrocytoma due to tumor-mediated immunological suppression through secretion of proteins like TGF- β 2, PGE-2 and IL-10 as well as high levels of steroids due to their use in high-grade patients as part of therapy.^(38,39)

Humoral response proteins like β -defensins were also increased in the AII CSF, while they were undetected in the majority of the non-tumoral controls. Known as multifunctional anti-microbial peptides, β -defensins are found in the plasma and may be involved in inducing cell proliferation and chemotactic properties in a variety of cell types.⁽⁴⁰⁾ Their detection in the low-grade tumor CSF could originate from leakage from plasma, secretion by the tumor cells or be related to an immune response against the tumor cells. A plasma origin is unlikely as the blood brain barrier is intact in low-grade astrocytomas (AII) and β -defensins were absent in the high grade (AIII, AIV and metastatic) samples despite the compromised blood-brain barrier found in the later (high grade) patients. Certainly, CSF compartments are connected to cervical lymphatics allowing immune response proteins from the lymphatic system to access the CNS.^(40,41)

Lastly, we found β 2m, another serum protein, elevated in the CSF with increasing grade of gliomagenesis. β 2m is an integral component, of MHC class I expression, is synthesized in the CNS, and plays a critical role in neural development.⁽⁴²⁾ Many tumors, including brain tumors, are known to down regulate class I MHC expression to avoid immune surveillance.⁽⁴²⁾ Although the extent of this surveillance for CNS-localized malignancies is unclear, the fact that β 2m is up-regulated in the context of MHC class I down-regulation suggests that it may be a positive feedback mechanism or pro-tumorigenic function.^(43,44) The latter hypothesis is supported by recent findings about β 2m tumor promoting activities in prostate cancer through activation of osteogenic proteins,⁽⁴⁵⁾ some of which, including SPARC, are also seen in our analysis. The proteins we identify do not exclude the possibility of anti-glioma immune reactions in the CNS. Analysis of the stage-specific tumor products may provide insight into designing therapeutic interventions that enhance and prolong such an immune reaction.

Origin of CSF Biomarkers

The most important question concerns the origin of novel or irregularly expressed proteins in the CSF as a result of neoplastic disease. Several proteins and protein fragments involved in metabolism, signal transduction, protein synthesis and modification and even some DNA

binding proteins were observed in the CSF. These are not normal CSF components and their presence may be due to aberrant expression by neoplastic cells, release from necrotic tumor areas, expression by reactive CNS structures, leakage from another body compartment, or their presence may be non-specific.^(8,42) A case in point here is the high expression of plasma-localized complement-related proteins and $\beta 2m$, which immediately suggest leakage across the blood-brain barrier as an origin. However, the complement-related proteins were only identified in the low-grade tumors, while attractin, beta-fibrinogen and many others were more specific to high-grade astrocytomas. This argues against the possibility of these particular proteins originating from the plasma. We of course do not exclude that some of the markers identified in AIV may have leaked from the plasma as the result of blood-brain barrier disruption in high-grade astrocytoma. These proteins are equally important to study as they are markers of blood-brain barrier disruption and may also play a paracrine role in brain tumor biology. Other possibilities than tumor cell origin for the identified biomarkers include glioma-induced neuronal damage or tumor-derived factor induced stimulation of non-transformed glial and stromal cells.^(46–48)

Conclusion

In summary, our comprehensive proteomic analysis of WHO grade II-IV astrocytoma revealed 21 potential CSF biomarkers for astrocytoma. We provide proof of principle evidence that these proteins could be assembled in biomarker panels that can differentiate between astrocytoma of grade II, III and IV. Biomarkers in general may become invaluable in the near future for early detection and diagnosis, or as surrogate markers for prognosis and disease follow-up. The biomarkers in the current panels can be immediately evaluated as potential prognostic indicators in astrocytoma patients in prospective studies, for example, the biomarkers could be evaluated as 1) monitors of response to therapy, tumor recurrence, time to progression or survival, or 2) time to progression and prediction of tumor transformation to a higher grade. Diagnostic use is clearly premature till enough samples can be collected and analyzed to evaluate the possibility of constructing a larger biomarker panel distinguishing all types of brain tumors. Perhaps the immediate impact of these candidate biomarker proteins will be their study as potential participants of the disease process as has already been demonstrated for attractin.⁽²²⁾ The association of function with grade-specific expression would open up new therapeutic avenues for exploration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AII

AIII and AIV, astrocytoma WHO grade II, III and IV respectively

$\beta 2m$

	β-2-microglobulin
MBT	metastatic brain tumor
PBT	primary brain tumor
I	inflammatory
CSF	cerebrospinal fluid
CF	cyst-fluid
Stable	supplemental table

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Figure 1. Representative 2-DE gels of CSF

Albumin and IgG depleted and TCA precipitated CSF samples from patients with A) non-tumoral control (#5) B) astrocytoma WHO grade IV (#27) were analyzed by 2-DE analysis using IEF strips pH 4–7 and 12.5% SDS-PAGE. Spots were visualized by silver staining and spot patterns were analyzed using ImageMaster software. Each sample was run in triplicate.

Figure 2. Analysis of CSF using proteomic methods

A) Average number of proteins found in CSF samples from each study group. Error bars indicate range of proteins found within the group; B) Functional categories and number of proteins identified within each functional category in the CSF samples analyzed by 2-DE and ICAT analysis; Low-grade = AII and high-grade = AIV CSF, and C) Ven diagram showing differentially expressed proteins found by 2-DE (dark grey); cICAT (light grey) and both (white) in the CSF from glioma patients compared to non-tumoral controls. D) Ven diagram showing distribution of the 130 differentially expressed proteins found in control and each astrocytoma grade samples.

Figure 3. Examples of differentially expressed proteins found by 2-DE and identified by MS/MS analysis

A) Differential expression of and FGF-14 on enlarged sections of representative 2-DE gel of control and AIV CSF. Solid circles highlight detected proteins by silver stain. B) Representative spectrum from one of the 15 peptides found from silverstained 2-DE gel spots for PEDF identified by MS/MS. The spectrum shown, identifies a tryptic peptide (LQSLFDSPDFSK) for PEDF.

Figure 4. Verification of 2-DE and cICAT results by western blot analysis

Differential expression of SPARC, beta-2-microglobulin (β 2m), beta-defensin-6 (β -def-6), beta-defensin-7 (β -def-7), Attractin (Atrn), FGF-14L (35 kd), FGF-14S (27 kd), VEGF-B and Tau were verified in the CSF from AII- AIV patients compared to non-tumoral controls. Transthyretin (TTR) was used as a loading control. Representative results (A, B, C) are shown on different patient samples of a total of 10 controls, 6 AII, 7 AIII and 19 AIV samples analyzed. Sample numbers are indicated above each lane.

A.

Protein Name	C	Access. No.	AII:C	AIV:C	Control	AII	AIII	AIV-CSF	AIV-CF	PBT	MBT	I
NFKB2		gil633222										
NPDC1		gil20149617										
Lipoprotein Gln I		prfj740525A										
Complement component 3		gil40786791	2.10									
Complement C3 precursor		gil116594	2.10									
Complement component 4A		gil57209752	2.78	1.32								
Complement C4B precursor		gil1314244	2.78	1.32								
IgG gamma-chain		gil553378	2.21									
SAP precursor		sptjP02743										
Peroxiredoxin 2 Isoform b		rfljNP_859427.1										
Beta-defensin 7		sptjP46165										
Beta-defensin 6		gil22506646	>10	>10								
GP2		trm Q60844										
Like MPHOSPH6		trm Q8NHT4										
PRO2619		gil11493459										
Unnamed protein		gil21754832										
ACIDA		gil46403719										
Beta-2 microglobulin		gil4757826	1.66	5.70								
Beta-fibrinogen		gil76826840	1.46	>10								
HP KIAA0542		sptjP46165		>10								
Fibrinogen Fragment D		pdbj1FZA_B		6.70								
APO A-IV precursor		gbjAAA51748.1	1.66	3.09								
SPARC-like 1		gil21359871										
Lamin A		gil27436947										
Attractin		gil21450863		>10								
IL17E		gil37183370										
NCF-1		gil42657778										
Transition protein 1		gil688356										
COX Vic		gil30582853										
BNIP1		gil15012107										
FGF-14S		gil2494463										

B. **C****Figure 5. Potential biomarker panels differentiate between astrocytoma grades using CSF**

A) Panel of 32 proteins that can be used to differentiate between AII-IV patients, non-tumoral controls, and other forms of brain tumors. Black = no expression, yellow = low expression, orange = moderate expression and red = high expression level as determined from ImageMaster analysis of 2-DE gels. C = Biomarker class; white = control specific, light blue = AII, green = AIII and pink = AIV biomarker.

B) Restricted panel containing top 13 proteins from panel A. white = control, light blue = AII, green = AIII and purple = AIV biomarkers. Vertical axis represents relative level (0–4) of each protein as estimated from 2-DE.

Table 1

Protein Name	MW	pI	Access. No.	ATI-C	ATI-V	ICAT	Control	ATI	ATI-II	ATI-III	ATI-CSF	ATI-CF	PBT	MBT	I
Cell Structure and Motility:															
Actin filament 3b	5	9.1	gi 25866109												
Actin filament 3c	5	9.1	gi 25866110												
Actin filament 3d	5	9.1	gi 25866111												
Actin filament 3e	5	9.1	gi 25866112												
Actin filament 3f	5	9.1	gi 25866113												
Actin filament 3g	5	9.1	gi 25866114												
Actin filament 3h	5	9.1	gi 25866115												
Actin filament 3i	5	9.1	gi 25866116												
Actin filament 3j	5	9.1	gi 25866117												
Actin filament 3k	5	9.1	gi 25866118												
Actin filament 3l	5	9.1	gi 25866119												
Actin filament 3m	5	9.1	gi 25866120												
Actin filament 3n	5	9.1	gi 25866121												
Actin filament 3o	5	9.1	gi 25866122												
Actin filament 3p	5	9.1	gi 25866123												
Actin filament 3q	5	9.1	gi 25866124												
Actin filament 3r	5	9.1	gi 25866125												
Actin filament 3s	5	9.1	gi 25866126												
Actin filament 3t	5	9.1	gi 25866127												
Actin filament 3u	5	9.1	gi 25866128												
Actin filament 3v	5	9.1	gi 25866129												
Actin filament 3w	5	9.1	gi 25866130												
Actin filament 3x	5	9.1	gi 25866131												
Actin filament 3y	5	9.1	gi 25866132												
Actin filament 3z	5	9.1	gi 25866133												
Actin filament 3aa	5	9.1	gi 25866134												
Actin filament 3ab	5	9.1	gi 25866135												
Actin filament 3ac	5	9.1	gi 25866136												
Actin filament 3ad	5	9.1	gi 25866137												
Actin filament 3ae	5	9.1	gi 25866138												
Actin filament 3af	5	9.1	gi 25866139												
Actin filament 3ag	5	9.1	gi 25866140												
Actin filament 3ah	5	9.1	gi 25866141												
Actin filament 3ai	5	9.1	gi 25866142												
Actin filament 3aj	5	9.1	gi 25866143												
Actin filament 3ak	5	9.1	gi 25866144												
Actin filament 3al	5	9.1	gi 25866145												
Actin filament 3am	5	9.1	gi 25866146												
Actin filament 3an	5	9.1	gi 25866147												
Actin filament 3ao	5	9.1	gi 25866148												
Actin filament 3ap	5	9.1	gi 25866149												
Actin filament 3aq	5	9.1	gi 25866150												
Actin filament 3ar	5	9.1	gi 25866151												
Actin filament 3as	5	9.1	gi 25866152												
Actin filament 3at	5	9.1	gi 25866153												
Actin filament 3au	5	9.1	gi 25866154												
Actin filament 3av	5	9.1	gi 25866155												
Actin filament 3aw	5	9.1	gi 25866156												
Actin filament 3ax	5	9.1	gi 25866157												
Actin filament 3ay	5	9.1	gi 25866158												
Actin filament 3az	5	9.1	gi 25866159												
Actin filament 3ba	5	9.1	gi 25866160												
Actin filament 3bb	5	9.1	gi 25866161												
Actin filament 3bc	5	9.1	gi 25866162												
Actin filament 3bd	5	9.1	gi 25866163												
Actin filament 3be	5	9.1	gi 25866164												
Actin filament 3bf	5	9.1	gi 25866165												
Actin filament 3bg	5	9.1	gi 25866166												
Actin filament 3bh	5	9.1	gi 25866167												
Actin filament 3bi	5	9.1	gi 25866168												
Actin filament 3bj	5	9.1	gi 25866169												
Actin filament 3bk	5	9.1	gi 25866170												
Actin filament 3bl	5	9.1	gi 25866171												
Actin filament 3bm	5	9.1	gi 25866172												
Actin filament 3bn	5	9.1	gi 25866173												
Actin filament 3bo	5	9.1	gi 25866174												
Actin filament 3bp	5	9.1	gi 25866175												
Actin filament 3bq	5	9.1	gi 25866176												
Actin filament 3br	5	9.1	gi 25866177												
Actin filament 3bs	5	9.1	gi 25866178												
Actin filament 3bt	5	9.1	gi 25866179												
Actin filament 3bu	5	9.1	gi 25866180												
Actin filament 3bv	5	9.1	gi 25866181												
Actin filament 3bw	5	9.1	gi 25866182												
Actin filament 3bx	5	9.1	gi 25866183												
Actin filament 3by	5	9.1	gi 25866184												
Actin filament 3bz	5	9.1	gi 25866185												
Actin filament 3ca	5	9.1	gi 25866186												
Actin filament 3cb	5	9.1	gi 25866187												
Actin filament 3cc	5	9.1	gi 25866188												
Actin filament 3cd	5	9.1	gi 25866189												
Actin filament 3ce	5	9.1	gi 25866190												
Actin filament 3cf	5	9.1	gi 25866191												
Actin filament 3cg	5	9.1	gi 25866192												
Actin filament 3ch	5	9.1	gi 25866193												
Actin filament 3ci	5	9.1	gi 25866194												
Actin filament 3cj	5	9.1	gi 25866195												
Actin filament 3ck	5	9.1	gi 25866196												
Actin filament 3cl	5	9.1	gi 25866197												
Actin filament 3cm	5	9.1	gi 25866198												
Actin filament 3cn	5	9.1	gi 25866199												
Actin filament 3co	5	9.1	gi 25866200												
Actin filament 3cp	5	9.1	gi 25866201												
Actin filament 3cq	5	9.1	gi 25866202												
Actin filament 3cr	5	9.1	gi 25866203												
Actin filament 3cs	5	9.1	gi 25866204												
Actin filament 3ct	5	9.1	gi 25866205												
Actin filament 3cu	5	9.1	gi 25866206												
Actin filament 3cv	5	9.1	gi 25866207												
Actin filament 3cw	5	9.1	gi 25866208												
Actin filament 3cx	5	9.1	gi 25866209												
Actin filament 3cy	5	9.1	gi 25866210												
Actin filament 3cz	5	9.1	gi 25866211												
Actin filament 3da	5	9.1	gi 25866212												
Actin filament 3db	5	9.1	gi 25866213												
Actin filament 3dc	5	9.1	gi 25866214												
Actin filament 3dd	5	9.1	gi 25866215												
Actin filament 3de	5	9.1	gi 25866216												
Actin filament 3df	5	9.1	gi 25866217												
Actin filament 3dg	5	9.1	gi 25866218												
Actin filament 3dh	5	9.1	gi 25866219												
Actin filament 3di	5	9.1	gi 25866220												