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Proteomic Cluster Analysis of Malignant Gliomas in Humans

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Abstract

Background: Gliomas are the most frequently observed primary brain tumors. These tumors comprise a variety of different histological tumor types and malignancy grades. Oligodendrogliomas typically contain a rich network of branching capillaries. Approximately 50%-80% of oligodendrogliomas demonstrate a combined loss of chromosomes 1p and 19q. Oligodendrogliomas differ from neurocytomas in that they show a diffusely infiltrating pattern of spread that precludes surgical cure.

Methods: We evaluated extracted proteins from tumors and normal brain tissues for protein purity by the Bradford test and spectrophotometry. We separated proteins by two-dimensional gel electrophoresis. The spots were analyzed and compared using statistical data and MALDI-TOF/TOF. Protein clustering analyses were performed on the list of proteins deemed significantly altered in oligodendroglioma tumor tissues.

Results: On each analytical two-dimensional gel, we observed an average of 1328 spots. A total of 157 exhibited up-regulation of expression levels, whereas the remaining 276 spots had decreased expression in astrocytoma tumors relative to normal tissue. The results demonstrated that functional clustering and principal component analysis had considerable merit in aiding the interpretation of proteomic data.

Conclusion: Clustering methodology is a powerful data mining approach for initial exploration of proteomic data. The clustering results depend on parameters such as data preprocessing, between-profile similarity measurement and the dendrogram construction procedure.

Keywords: Cluster, 2D-DIGE, Glioma, Proteomics, Oligodendroglioma

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Introduction

Proteomic studies rely heavily on a number of different techniques such as sample processing, high-performance liquid chromatography (HPLC), mass spectrometry (MS), and bioinformatics which enable the identification and quantitation of thousands of proteins. Originally, the protein variants (spots) were

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separated by two-dimensional (2D) gel electrophoresis.[1] It was expected that the combination of 2D gel electrophoresis and MS would allow the identification of thousands of proteins.[2, 3]

In the past genome era, the field of proteomics sparked great interest in the pursuit of protein/peptide biomarkers in complex biological systems.[4-6] The National Cancer Institute-Food and Drug Administration (NCL-FDA) clinical proteomics program was formed in the late 1990s with the intent to develop and apply novel technology to improve the ability to understand cancer biology.[7,8] Clinical neuroproteomics has aimed to advance our understanding of disease and injury that affect the central and peripheral nervous systems through the study of protein expression and the discovery of protein biomarkers to facilitate diagnosis and treatment.[9] The application of neuroproteomics specifically to gliomas is appealing since there has been very limited progress in treatment of malignant gliomas in the last 25 years.[10]

Gliomas are the most frequent primary brain tumors and include a variety of different histological tumor types and malignancy grades.[11] Primary tumors of the central nervous system (CNS) account for approximately 2% of human malignancies.[11, 12] The World Health Organization (WHO) classification divides gliomas into three main subgroups (astrocytomas, oligodendrogliomas and mixed oligoastrocytomas) and differentiates between four malignancy grades (WHO grades I-IV).[13,14]

Gliomas are composed of neoplastic cells that bear morphological resemblance to oligodendroglial tumors. Typically, oligodendroglioma cells exhibit a round nuclei and a clear cytoplasm that confer a fried-egg appearance resulting in an overall honeycomb histological pattern. Oligodendrogliomas typically contain a rich network of branching capillaries.[15] Approximately 50%-80% of oligodendrogliomas demonstrate a combined loss of chromosome 1p/19q. Although several identified genes have been studied, no firm candidate oligodendroglioma gene has been identified.[16-18] Deletions at 9p and 10q that involve mutation of p16ink4a, phosphatase and tension (PTEN), and epidermal growth factor receptor (EGFR) pathway deregulation by mutation/amplification appear to be associated with tumor progression towards grade III anaplastic forms.[19, 20] Loss of heterozygosity at 17p and Tp53 mutation are characteristic features of mixed tumors with a predominant astrocytoma component versus those with major oligodendroglioma components that display allelic losses at 1p/19q.[15, 21] Oligodendrogliomas generally do not show features of neuronal differentiation[22] but they may, on occasion, express neuronal antigens, suggesting that their relationship to neurocytomas may extend beyond simple histological resemblance.[23] Analysis of differentially expressed proteins using biased and unbiased bioinformatics techniques can identify motifs such as metabolism and invasion/migration that suggest fundamental differences between the tumor types.[24]

The first exploration of proteomic data has involved their clustering which enables samples or proteins to be grouped blindly according to their expression profiles.[25] In 1989, a national conference on clustering of health events convened to discuss empirical observations of disease clusters advances in statistical methods for analyzing cluster data, and risk perception and legal issues.[26] The goal of cluster analysis is to identify similar groups.[27] We have to decide on the criterion to be used for measuring similarity or distance.

In the present study, we investigated change in protein expression in the human oligodendroglioma tumors in order to obtain an understanding of data and specific software molecular diagnosis of oligodendroglioma tissue. Proteins from tumor and normal brain tissues were extracted and evaluated by proteomic tools (2D gel). After providing cluster and principal component analysis (PCA) of the spots, we monitored their alterations by statistical data and specific software. By using different proteomic approaches, we identified multiple differentially expressed oligodendroglioma proteins, of which

Table 1. Identification of differential expression of proteins between tumor and non-tumor brain tissues by MALDI-TOF/TOF.

Protein hits	Protein name	*P*-value ratio	Experimental MW
gi306785	G protein beta subunit	1.128e-006	38061
gi6680045	GBN1 protein	7.340e-005	38151
gi28336	Mutant beta-actin	2.253e-004	42128
gi340021	Alpha-tubulin	1.978e-004	50804
gi37492	Alpha-tubulin	2.253e-004	50810

some could be investigated further as potential surrogate markers for oligodendroglioma tumors.

Materials and Methods

Patient samples

Tissues were obtained following informed consent and Institutional Review Board approval from patients scheduled for tumor resection. All participants signed a written informed consent. The oligodendroglioma tumors were surgically removed at hospitals in Tehran and classified by a team of neuropathologists according to the guidelines of the WHO classification of tumors of the CNS. Non-tumor brain tissues were obtained from normal areas (either grey or white matter) of the brain tissues removed from patients who underwent non-tumor epileptic surgery.

Tissue and sample preparation

A total of eight tumor samples and four normal brain tissues were snap-frozen immediately after surgery in liquid nitrogen and stored at -80ºC until use for proteomic analysis. To obtain tissue extracts, the samples were broken into suitable pieces and homogenized in lysis buffer II that consisted of lysis buffer I [7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% 100×Bio-Lyte 3/10], dithiothreitol (DTT), 1 mM ampholyte, and protease inhibitor on ice. Cell lysis was completed by subsequent sonication (4×30 pulses). The samples were then centrifuged at 20000 g at 4°C for 30 min to remove insoluble debris. The supernatants were combined with 100% acetone and centrifuged at 15000 g. Next, the supernatants were decanted and removed (three times). Acetone (100%) was added to the protein precipitant and kept at -20°C overnight. The samples were then centrifuged again at 15000 g and the precipitant was allowed to incubate for 1 h at room temperature. The protein samples were dissolved in rehydration buffer [8 M urea, 1% CHAPS, DTT, ampholyte pH 4, and protease inhibitor]. Protein concentrations were determined using the Bradford test and spectrophotometry. The protein extracts were then separated and used for 2D gel electrophoresis.

Two-dimensional (2D) gel electrophoresis

The isoelectric focusing for first-dimensional electrophoresis was performed using 18 cm, pH 3–10 immobilized pH gradient (IPG) strips. The samples were diluted in a solution that contained rehydration buffer, IPG buffer, and DTT to reach a final protein amount of 500 µg per strip. The strips were subsequently subjected to a voltage gradient according to the manufacturer's instructions. Once focused, the IPG strips were equilibrated twice for 15 min in equilibration buffer I [50 mM tris-Hcl (pH 8.8), 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), and DTT] and equilibration buffer II. The 2D SDS-PAGE was carried out using 12% PAGE. Following SDS-PAGE, the gels were stained using the coomassie blue method overnight.

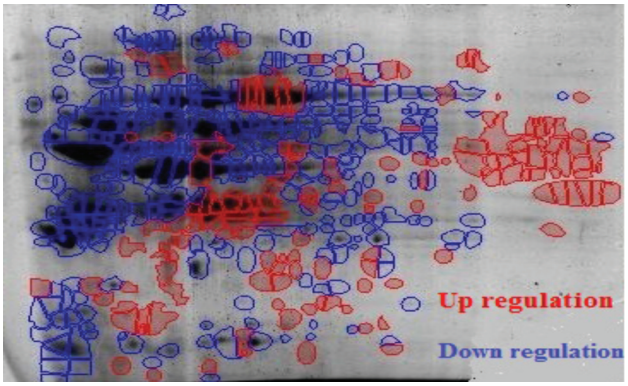

Figure 1. Red spots indicate up-regulation and blue spots down-regulation in the oligodendroglioma tumor relative to normal tissue.

Image analysis

The gel images were analyzed by Progenesis Samespots software to identify spots that differentially expressed between tumor and control samples based on their volume and density. The spots were carefully matched individually and only spots that showed a definite difference were defined as altered.

Statistical analysis

The student's t-test was used to rank proteins found altered in the oligodendroglioma tumor compared to normal tissue according to statistical probability. We have chosen the t-test to create a hierarchy because it is easily understood by a number of different target audiences and is currently a common practice in the majority of proteomics analyses. Protein clustering analyses were performed on the list of proteins deemed significantly altered in oligodendroglioma tumors (P<0.05).

Arithmetic cluster analysis was performed for the two groups. Arithmetic cluster analysis employs correlation analysis to define if alterations in the levels of one individual protein are associated with alterations in the levels of a second protein across all samples (oligodendroglioma and normal tissues). Arithmetic correlation algorithms are integral to the Progenesis Samespots software (Nonlinear Dynamics v. 3.0, 2008). Multiple areas on correlation coefficients between protein features have been calculated by Progenesis Samespots and the information visually represented in the form of a dendrogram.

Mass spectrometry (MS) analysis

The identity of differentially expressed proteins (P<0.05 and >2-fold) was established using

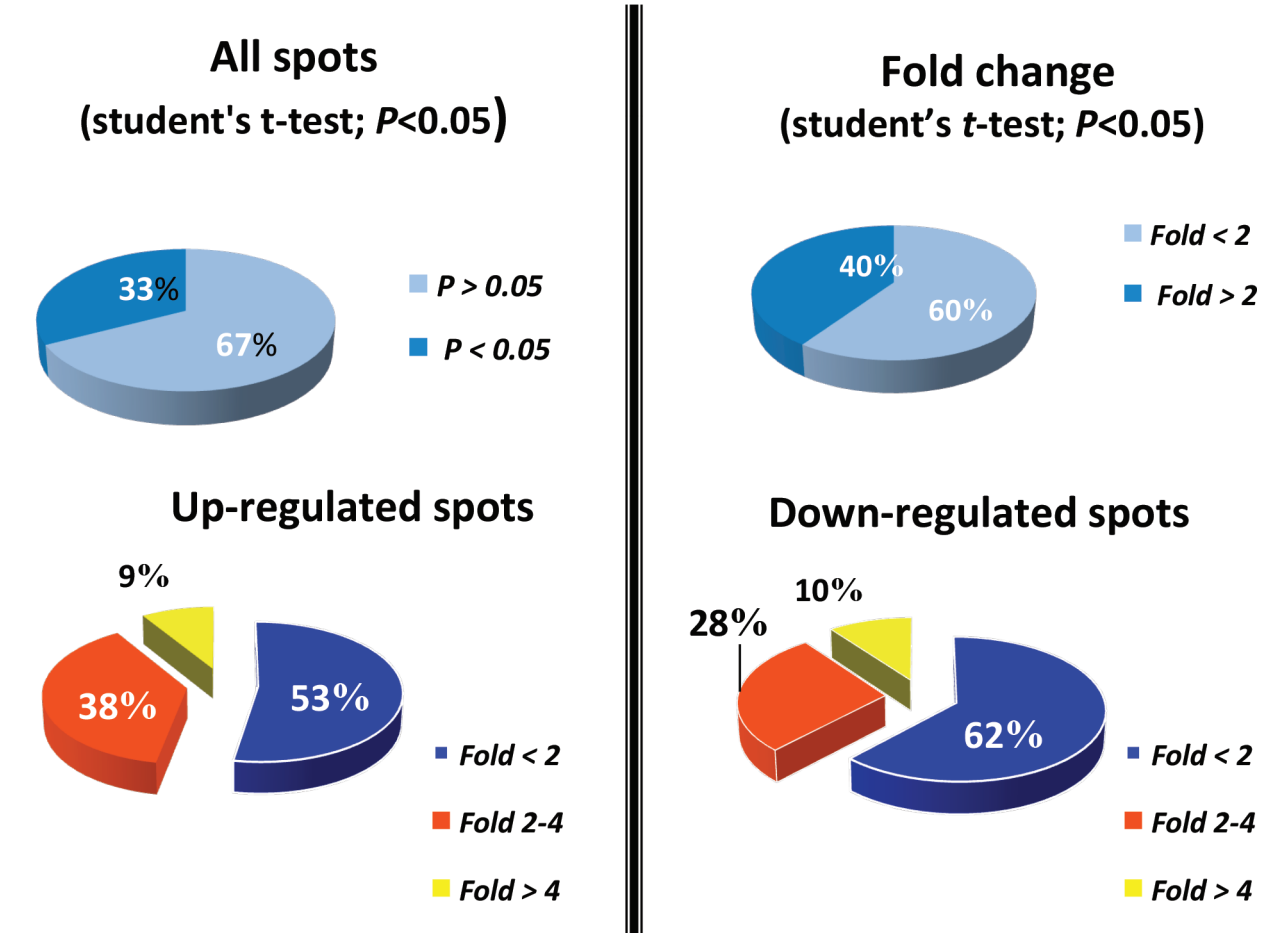

Figure 2. Categorization of change in protein expression (up- and down-regulation) shown in figure 1 of the 36% up-regulated (red) and 64% down-regulated spots (blue).

MALDI TOF TOF MS. The samples were analyzed by MALDI-TOF/TOF. Gel digestion was performed as mentioned and MS analysis was performed as previously described.

Results

We used 2D gel electrophoresis to identify proteins expressed in oligodendroglioma tumor and non-tumor samples. The spots were separated according their isoelectric pH and molecular weights. On each analytical 2D gel, we observed an average of 1328 spots that corresponded to proteins according to nonlinear Progenesis Samespots software. The representative set of overlaid 2D-difference gel electrophoresis (2D-DIGE) images is given in figure 1. The first-dimension analysis was performed with a broad pH range (pH 3–10) and IPG using strips of 18 cm. The total number of protein features was matched and analyzed between gels in the control group and tumor group. There were 433 spots (approximately 33% of the total detected spots) matched across all gels. In software analysis, a total of 433 differentially expressed spots satisfied the statistical parameters (t-test and one-way ANOVA; $P<0.05$).

Among these, 175 spots exhibited differences in expression level (>2-fold). A total of 433 spots showed statistically significant differences (student's t-test; $P<0.05$) in the gel, from which 157 spots exhibited up-regulation in expression levels and the remaining 276 spots had decreased expression in the oligodendroglioma tumor tissue relative to normal tissue. Up-regulation is shown as red and down regulation as blue in the imaging gel (Figure 1). Of the 157 up-regulated spots, 83 were between 1.1- and 2-fold, 60 spots were between 2- and 4-fold, and 14 spots exhibited over a 4-fold increase in expression level (Figure 2). Of the 276 down-regulated spots, 165 were between 1.1- and 2-fold, 74 were between 2- and 4-fold, and 27 spots exhibited over a 4-fold

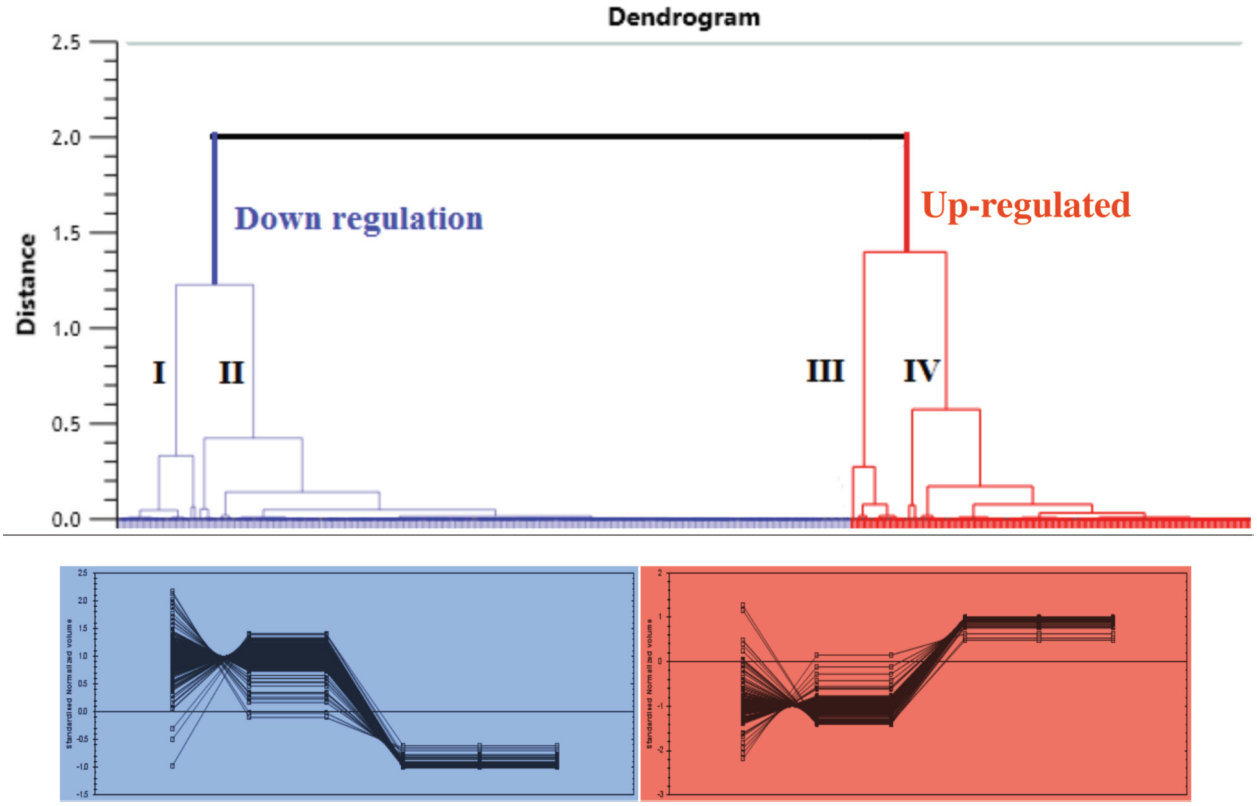

Figure 3. Arithmetic cluster analysis. Protein dendrogram of 433 proteins differentially altered ($P<0.05$) in oligodendroglioma tumors from two groups (up- and down-regulated). This dendrogram clearly indicates the cluster of 157 spot proteins found up-regulated (right branches, in red) and 276 spot proteins that down-regulated (left branch, in blue) in oligodendroglioma tumors.

reduction in expression level (igure 2).

Different proteins (4 spots of the total spots) were identified using the data obtained from MALDI-TOF/TOF in conjunction with a search of the NCBI databank. Mass spectometry analysis results revealed that those 4 spots represented a total of up-regulated proteins with $P<0.05$ and >2-fold. These proteins were G protein beta subunit, guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1, beta-actin, and alpha-tubulin.

Cluster analysis

The total number of protein features were matched and analyzed between tumor and normal tissues. Spot statistical analysis are commonly used nonlinear Progenesis Same spots software. Arithmetic cluster analysis was performed on this list of 343 spot proteins. Arithmetic cluster analysis explores how one individual protein level correlates with a second individual protein level across different samples. Protein levels that are tightly correlated suggest that the proteins may be regulated or involved in the same biological pathway. A clear cluster analysis (dendrogram) with several distinct subgroups of proteins has been generated (Figure 3).

Two main groups reflected the 157 spot proteins that increased (red) and 276 spot proteins that decreased (blue) in expression level in oligodendroglioma tissues relative to normal tissues.

The total up-regulated protein spots showed two main subgroups (I and II) where subgroup II involved two branches (red in Figure 3). The total down-regulated spot proteins showed two main subgroups (III and IV) where subgroup IV involved two branches (blue in Figure 3). Subgroups II and IV had the highest numbers of protein spots.

PCA was performed on all the spot proteins. The results showed two main groups, up- (red) and down-regulated (green) as seen in figure 4.

The guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1, G protein beta subunit, beta-actin, and alpha-tubulin represented a total of up-regulated proteins ($P<0.05$; >2-fold) by MS analysis results (Table 1).

Discussion

Proteomics combines technologies from several disciplines in an attempt to explain the structural, functional and interactive properties of proteins in cells, tissues and body fluids. The ultimate goal is to identify the interactive pathways of proteins for diagnostic purposes or as candidates for intervention.[28-30] Knowledge about the molecular biology of cancer, including CNS tumors continues to increase. A dynamic classification for

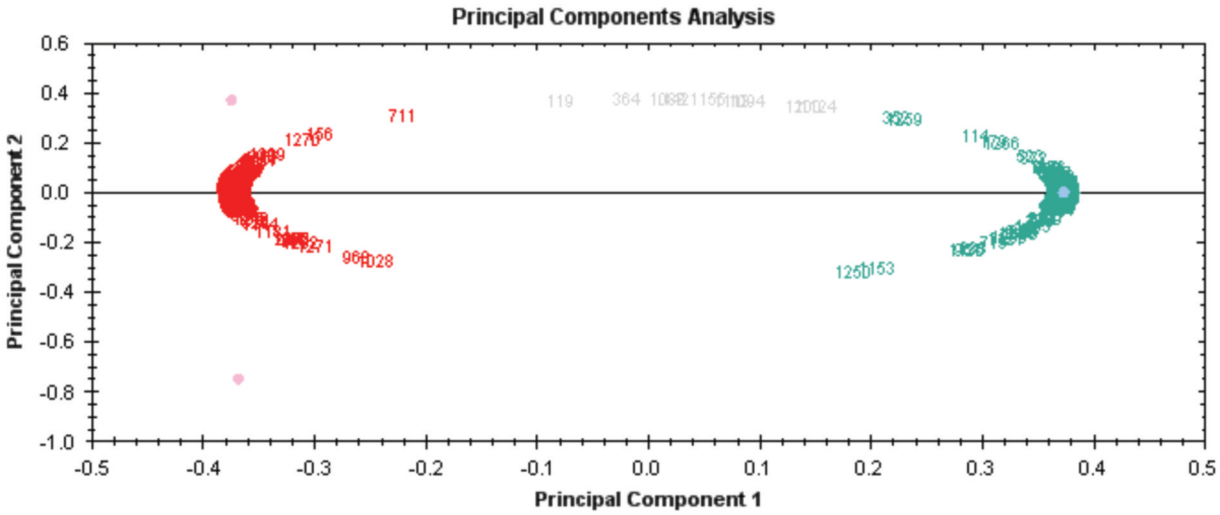

Figure 4. Principal component analysis (PCA) was performed on all the spot proteins. The PCA of 157 spot proteins was up-regulated (right) and 276 spot proteins were down-regulated (left) in the oligodendroglioma tumors.

tumors enables the integration of newly discovered markers to help determine prognosis and likelihood of therapeutic response.[9,10,31] Different methodologies can be combined. In proteomic studies methodologies more commonly used involve protein extraction from the sample, separation by one-dimensional or 2D electrophoresis, liquid chromatography, ionization, fragmentation, peptide analysis and detection, and data analysis.[1,32] These may influence data and show differences between sample groups that have no true biological meaning.[33]

The separation of proteins is a core component in proteomic studies. A range of techniques can be used to separate proteins in a tissue extract. However, gel based electrophoresis, particularly 2D-DIGE, is the most widely used approach for separating individual proteins in a tissue extract. The 2D-DIGE separates proteins according to their isoelectric point and molecular mass.[3,29,34] Hundreds of proteins can be separated and quantified from one tissue sample on a single gel depending on the technical conditions employed, solubilization, protein loading, and quality of the stain used.[3,35] High-resolution 2D-DIGE can resolve up to 5000 proteins simultaneously, with the capability to detect and quantify <1 ng of protein per spot. This technology is limited due to low-throughput, labor intensive, time consuming. It is problematic in detecting proteins that are basic in charge or smaller than 10000 Da.[36, 37] This lower molecular weight range may contain cleaved proteins or peptides that are aberrantly shed or secreted from response to a disease.[29, 38] Proteomic studies in gliomas remain limited in number and are characterized by lists of proteins found to be either up- or down-regulated in tissue specimens compared to the normal brain.[37, 39]

Biostatistics is essential to ensure the collection of robust, meaningful data and the results withstand the most rigorous statistical analyses at the level of the resulting clinical/analytical matrix.[33,40] This includes the determination of both false positive and false negative rates, which are critical for evaluating the success of the biomarker.[9] Although there are commercial software packages for 2D gel image analysis, considerable human intervention is needed for spot identification and matching. Moreover, the comparison of the quantitative features is either based on the simple t-test or relies on external as statistical tools for analysis.[41] The visual analysis of a 2D-DIGE image series intends to identify proteins that change their expression and reflect or cause certain biochemical and biomedical conditions of an organism. However, this requires high throughput analysis tools and the major challenge is to obtain both robust and reliable algorithmic solutions that work automatically, or at least need slight user interaction.[41, 42]

Hierarchical clustering methodology is a powerful data mining approach for the first exploration of proteomic data. It enables samples or proteins to be grouped blindly according to their expression profiles. Nevertheless, the clustering results depend on parameters such as data preprocessing, between-profile similarity measurements, and the dendrogram construction procedure.[25, 43] There have been numerous studies conducted on the clustering of protein sequences whose main objectives were to help classification and prediction of biological functions as well as recognition of new interpretation patterns among them. Among these, the most important ones include the protein sequences related to cancers. Most of the methods used have included the graphical and hierarchical clustering whose efficiency has been proven in numerous studies.[27,44] This property plays an important role in their method. However, intergal cross-validation can be used to assess the degree to which each single data type alone can reproduce the integrated cluster membership. Both ANOVA and clustering were higher than technical or biological variables which validated our analysis.[26,45] Simple statistics (student's t-test) was first applied to test the hypothesis that individual mean protein concentrations were different in glioma compared to control tissues. The student`s t-test was also used to rank proteins found altered in glioma tissues compared to control brain tissues according to statistical

probability. Arithmetic cluster analysis was performed on dataset two.[46]

The methodological problems that pertain to investigations of community cancer clusters fall into several categories. First, a false perception of a cluster may result from failure to consider changes in population size over time and the inability to account for migration in and out of the community. A separate problem is boundary of a shrinkage, defined as bias in defining the boundary of a cluster.[27,47] Additional problems that limit our ability to investigate cancer clusters include absence of data that pertain to relevant current or post-environmental exposures, low statistical power of most analyses which stem from small population sizes,[48] the need to consider perception issues in situations where cluster investigations are highly publicized, and vague definitions of disease such as brain tumors that often include dissimilar conditions characterized by a different pathogenesis and histologic features and, likely, a different etiology.[49]

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Conflict of Interest

No conflict of interest is declared.

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