

RESEARCH ARTICLE

Pathway alterations during glioma progression revealed by reverse phase protein lysate arrays

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The progression of gliomas has been extensively studied at the genomic level using cDNA microarrays. However, systematic examinations at the protein translational and post-translational levels are far more limited. We constructed a glioma protein lysate array from 82 different primary glioma tissues, and surveyed the expression and phosphorylation of 46 different proteins involved in signaling pathways of cell proliferation, cell survival, apoptosis, angiogenesis, and cell invasion. An analysis algorithm was employed to robustly estimate the protein expressions in these samples. When ranked by their discriminating power to separate 37 glioblastomas (high-grade gliomas) from 45 lower-grade gliomas, the following 12 proteins were identified as the most powerful discriminators: IB α , EGFRpTyr845, AKTpThr308, phosphatidylinositol 3-kinase (PI3K), BadpSer136, insulin-like growth factor binding protein (IGFBP) 2, IGFBP5, matrix metalloproteinase 9 (MMP9), vascular endothelial growth factor (VEGF), phosphorylated retinoblastoma protein (pRB), Bcl-2, and c-Abl. Clustering analysis showed a close link between PI3K and AKTpThr308, IGFBP5 and IGFBP2, and IB α and EGFRpTyr845. Another cluster includes MMP9, Bcl-2, VEGF, and pRB. These clustering patterns may suggest functional relationships, which warrant further investigation. The marked association of phosphorylation of AKT at Thr308, but not Ser473, with glioblastoma suggests a specific event of PI3K pathway activation in glioma progression.

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Abbreviations: EGFR, epidermal growth factor receptor; FDR, false-discovery rate; IGFBP, insulin-like growth factor binding protein; MMP9, matrix metalloproteinase 9; PI3K, phosphatidylinositol 3-kinase; pRB, retinoblastoma protein; VEGF, vascular endothelial growth factor

1 Introduction

Gliomas constitute the most common primary tumors of the central nervous system, with glioblastoma representing the most advanced stage of gliomas with a median survival of less than 1 year [1]. Glioblastoma is highly refractory to current therapies including surgical resection combined with pre- or postoperative chemotherapy, biotherapy, and/or radiotherapy. In patients with low-grade gliomas, such as

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oligodendroglioma, long-term survival is reachable after surgical resection and postoperative chemotherapy [1]. This variation in survival implies that there are important molecular distinctions among the different grades of glioma. Molecular and genetic studies have identified alteration of a number of genes that may play an important role in glioma progression. Among them are the amplification of epidermal growth factor receptor (EGFR), loss of heterozygosity (LOH) of chromosome 10 and mutation or deletion of PTEN, LOH of chromosome 9 and deletion of p16 genes, and mutation of p53 [2]. Recent genomic studies using cDNA microarrays have revealed a large number of gene expression changes, including the discovery of overexpression of insulin-like growth factor binding protein (IGFBP)2 in 80% of glioblastomas [2–5]. In contrast, parallel high-throughput proteomic analyses of gliomas have been lacking.

Inspired by the application of high-throughput DNA microarray techniques to comprehensively analyze the molecular basis of various diseases, the parallel analysis of protein function using a biochip format has been developed to provide high-throughput protein expression characterization [6]. One of the protein array technology platforms, the RP protein microarray, was developed for screening molecular markers [7]. The RP protein microarray involves a process of spotting cell extracts from a large number of biological samples on a coated glass slide and subsequently probing the array with a large number of antibodies [7]. The technology has shown promising results for monitoring the expression of disease-related proteins and for investigating the cellular effects of pharmaceutical agents [8–11].

To further understand the molecular mechanism of glioma progression at the protein level, we applied 46 antibodies to a protein lysate array composed of protein extracts from 82 different glioma tissues of either glioblastoma or lower-grade glioma. Each sample was spotted in triplicate with six twofold dilutions to increase the accuracy of the assay. Using a recently developed algorithm [12], we quantified the protein lysate array data and identified proteins or their phosphorylated forms that are differentially expressed between glioblastoma and lower-grade gliomas.

2 Material and methods

2.1 Samples

The frozen glioma tissue samples were obtained from the Brain Tumor Center Tissue Bank at The University of Texas M. D. Anderson Cancer Center. A total of 82 samples from 82 different patients were collected, including the following subtypes of gliomas: 37 glioblastomas (WHO grade IV), 8 low-grade astrocytomas (WHO grade II), 7 oligodendrogliomas (WHO grade II), 3 oligoastrocytomas (WHO grade II), 10 anaplastic astrocytomas (WHO grade III), 11 anaplastic oligodendrogliomas (WHO grade III), and 6 anaplastic oligoastrocytomas (WHO grade III), which pos-

sess mixed lineage features and are often called mixed gliomas. Patient survival data were obtained from the clinical database. The demographic characteristics of patients are listed in Table 1. The samples were taken from patients who had received no treatment before surgery. All studies were approved by the Institutional Review Board through an established protocol.

2.2 Lysate array construction

Protein isolation from glioma tissues has been described previously [13]. Briefly, the frozen tissue was ground in liquid nitrogen and lysed with protein lysis buffer (20 μ M Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40) freshly supplemented with 0.02 mM leupeptin. The concentrations of the lysate solution were determined using the Bradford assay according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA) and adjusted to 20 μ g/ μ L with lysis buffer. The lysate solution was then serially twofold diluted six times with lysis buffer. The serially diluted protein lysates were printed on PVDF-coated glass slides in triplicate using a robotic spotter (G3, Genomics Solutions) as described in [12].

2.3 Antibodies

We used 46 antibodies (described in Table 2). Among them, 31 antibodies were tested by Western blotting using two tumor tissues, and a single dominant band was detected. Others were shown to behave similarly in published results. The secondary antibodies, including anti-goat, anti-rabbit and anti-mouse antibodies were purchased from Vector Laboratories (Burlingame, CA, USA).

2.4 Detection of protein expression

Detection was conducted with a DakoCytomation catalyzed signal amplification system kit (CSA[™], DakoCytomation; Carpinteria, CA, USA) as described previously [12]. Briefly, endogenous biotin was blocked for 5 min using the biotin blocking kit, followed by application of protein block reagent for 10 min. Primary antibodies were diluted and incubated on slides for 2 h, and biotinylated secondary antibodies were incubated for 1 h. For signal amplification, the slides were incubated for 15 min with a streptavidin-biotin-peroxidase complex provided in the amplification kit, and for 15 min each with amplification reagents (biotinyl-tyramide/hydrogen peroxide, and streptavidin-peroxidase). Development of slides was completed using hydrogen peroxide. The slides were then allowed to air dry. Primary and secondary antibodies used in these studies were diluted 1:100–200 and 1:4000–10 000, respectively. Besides β -actin, which served as the positive control in each set of protein arrays, one negative control without any primary antibody was included in each set of experiments. The hybridized slides were scanned at optical resolution of 1200 dpi and saved as uncompressed TIFF files. After inverting the 16-bit-per-pixel grayscale

Table 1. Clinical information of the patient cohort in this study^{a)}

	GBM	LOA	OL	OA	AA	AO	AOA	Total
F/M	17/20	3/5	4/3	2/1	7/3	5/6	3/3	41/41
Age	51.3 ± 18.7	36.5 ± 19.0	36.0 ± 11.1	30.3 ± 5.7	35.8 ± 5.4	38.8 ± 11.2	36.5 ± 15.2	43.2 ± 17.0

a) GBM, glioblastomas; LOA, low-grade OA; OL, oligodendrogliomas; OA, oligoastrocytomas; AA, anaplastic astrocytomas; AO, anaplastic OL; AOA, anaplastic OA; F/M, female/male ratio.

Table 2. Antibodies used in the experiments

Company	Name of antibody
Cell Signaling Technology, Inc. Beverly, MA 01915	AKT, AKTpSer473, AKTpThr308, PTEN, PTENpSer380, RSK1/RSK2/RSK3, p90RSKpThr573, GSK3beta, BADpSer136, Cleaved Caspase 8 Asp374, Cleaved Caspase 9 Asp315, Puma, PDGFRβ, MAPK, mTOR, mTORpSer2481, CD11b, EGFRpTyr845
BD Biosciences Immunocytometry Systems San Jose, CA 95131	PI3K, Integrin α5, p16, pRB, Src Pan, Src pTyr529, NFκB (p65), cathepsin D
Santa Cruz Biotechnology, Inc. Santa Cruz, CA 95060	p53 (Do-I), BCL-2, Bax, p – PDGFRβ, Cdk7, Cdk4, Cyclin D3, VEGF, Tie2, IGFBP2, IGFBP3, IGFBP5, IκBα, EGFR VIII, c-Abl
GeneTex Inc. San Antonio, TX 78245	p14ARF, c-Myc
Invitrogen Corporation (Zymed Laboratories Inc.) Carlsbad, CA 92008	EGFR
Abcam Inc. Cambridge, MA 02139	MMP2, MMP9
Sigma Chem Co. St. Louis, MO 63178	β-Actin

image (that allows the same analysis approach as in cDNA microarray technology), the spots were segmented and quantified with ArrayVision (Imaging Research Inc., Catharines, Ontario, Canada).

2.5 Analysis of lysate array data

The lysate was printed in triplicate with six twofold dilutions, yielding 18 data points for each sample. The design of the glioma lysate-array contains 96 samples with a total of 1728 spots. The protein expression was quantified using the robust least squares method, described in [12]. On a log-log scale, the relative expression of two proteins is the distance between the two linear models. We have shown previously [12] that the estimated error rates for the robust least squares model are markedly smaller than that of other models.

The expression levels were normalized against β-actin measured on each production lot (24 slides in each lot) with the same spotting procedure on a 1728-spot protein array. The inter-slide variability was normalized using the quantile-to-quantile normalization method [14].

Guided by the most important clinical characteristics, which is survival time, we focused on finding features that distinguish glioblastomas from all other gliomas. Therefore, the classification is a two-class discrimination problem: glioblastoma *vs.* others. After normalization with β-actin, we selected the protein features with the highest discrimination power based on the ratio 'between sum of squares'/'within sum of squares' (BSS/WSS) [15]. We selected the subset corresponding to the critical value of the false-discovery rate (FDR), which is defined as the expected proportion of false positives among the declared significant results [14–18]. The source code is available upon request by contacting the authors.

3 Results

Survival times among different grades of gliomas vary, with the most significant difference existing between glioblastoma and lower-grade tumors. To characterize the 82 tumor samples used in this study, we performed a Kaplan-Meier survival analysis according to the patient sur-

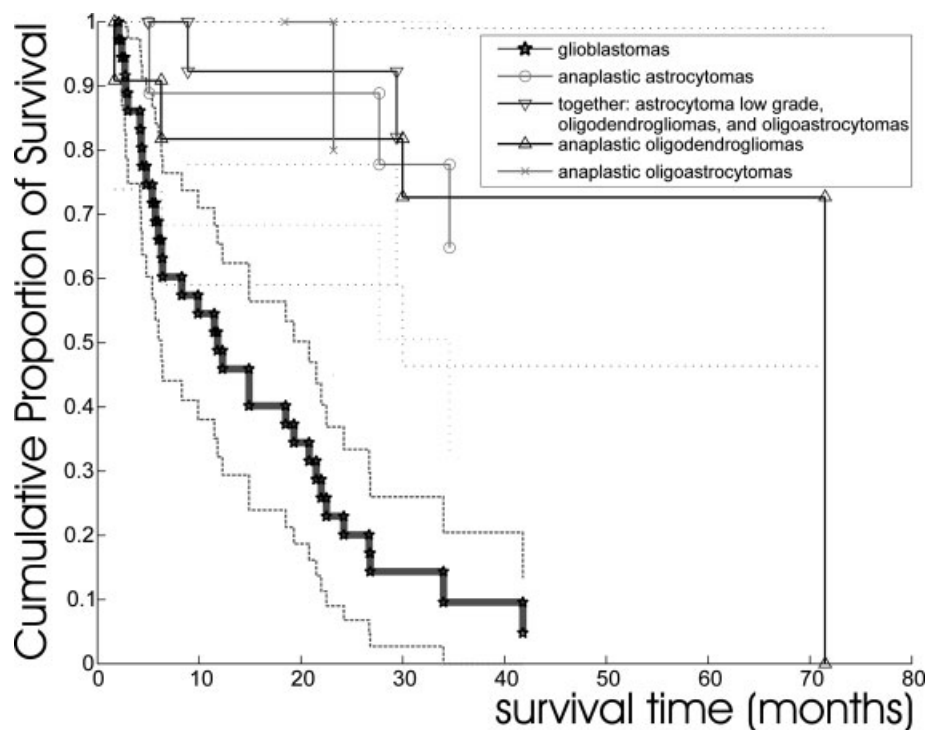


Figure 1. Kaplan-Meier survival analysis. The survival curves for patients show that there is a distinction between glioblastoma and lower-grade glioma. However, the survival difference between the subgroups of lower-grade gliomas in this sample set is not significant.

vival information from our clinical database. As shown in Fig. 1, among the 82 patients, there was a significant distinction in survival time between glioblastoma and all other gliomas combined. The difference in survival times among the other glioma subgroups in this patient cohort is not significant. Therefore, we reasoned that, for this study, the grouping scheme based on survival difference is likely to generate biologically meaningful information in the pathway analysis. Further subgrouping may result in misrepresentation due to insufficient number of samples in each subgroup.

Accumulating evidence from conventional molecular biology studies has produced an understanding that signal transduction pathways in cell growth, cell death, and metabolism, are disturbed in glioma progression. Therefore, to systematically survey a subset of proteomic changes in gliomas using a parallel protein lysate array platform, we first generated a list of proteins (Table 2) that have been previously implicated in oncogenetic pathways; some of the proteins have already been shown to be activated in glioblastoma through other types of assays [2].

Protein lysate arrays provide a high-throughput platform that allows simultaneous detection of a protein in a large number of samples with replicates and serial dilutions. However, the dot-blot nature of the assay demands that high quality antibodies be used to avoid high levels of nonspecific hybridization, which would produce an unacceptable level of noise and render the data uninterpretable. Therefore, we either tested the antibodies on a Western blot or searched the previously published results to make certain that the antibodies applied to the array detect a single dominant band on

a Western blot. Representative Western blots that tested two randomly selected glioma tissues are shown in Fig. 2. Forty-six antibodies passed the two criteria and were subsequently used in the hybridization experiments. A hybridization experiment without primary antibody was used as a negative control in each set of experiments. Although we did not routinely perform a preimmune antibody hybridization as negative control, a number of antibodies did not produce appreciable levels of signals and essentially functioned as preimmune negative controls.

After the hybridized arrays were imaged and quantified, all data were normalized against β -actin in a fashion similar to that routinely performed in Western blotting analysis. To diminish variability introduced by microarray production runs, each protein lysate array production lot (24 slides) was normalized against β -actin separately. The normalized data were then statistically analyzed to identify those feature proteins that distinguish glioblastoma from other gliomas in our sample set. The heat map of proteins (green for decreased in glioblastoma and red for overexpressed in glioblastoma) ranked in decreasing order of discriminative power of glioblastoma vs. other lower grades is shown in Fig. 3a.

Figure 3c illustrates the density estimates of discrimination values, using the kernel smoothing method, in two situations. The first distribution, marked in red, models the BSS/WSS ratio [15] (referred to in the following as discriminative value) obtained for each protein in the two-class classification problem when using the known, correct labels of patients. The second distribution is marked in yellow and models the discriminative values for each protein when the

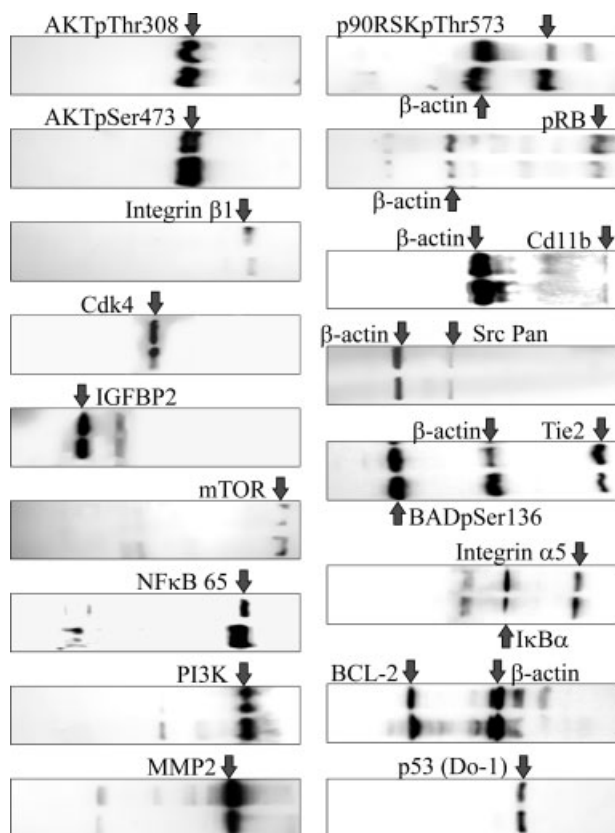


Figure 2. Quality evaluation of the antibodies used on protein lysate arrays. Antibodies were tested in a Western blotting assay to confirm that they preferentially detect a single band in two different glioma samples (two lanes). Some blots were sequentially probed with two or three different antibodies and the composite results are shown. Representative results are shown here.

labels of the patients are randomized, using an ensemble of 1000 runs. The ratio of the two cumulative distribution functions is utilized in estimating the FDR, which is illustrated in Fig. 3d. The critical value of the discriminative value is chosen as $c^* = 0.05$, below which the increase in the FDR becomes accelerated, as seen in Fig. 3d. The 18 proteins having the discriminative value larger than the critical point $c^* = 0.05$ are listed in Fig. 3b and represented as a heat map.

To gain insight into potential pathways and protein relationships, we also performed clustering analysis of the protein data. Hierarchical clustering of the 18 proteins revealed the existence of two distinct clusters, as seen in Fig. 3b. Cluster 1 corresponds to proteins that are typically overexpressed in glioblastoma cases, while the proteins in the second cluster are typically decreased in glioblastoma.

The feature proteins were ranked by the discrimination between glioblastoma and other glioma subtypes (Fig. 3). The proteins with high discriminative value and overexpressed in glioblastomas were determined to be: I κ B α , EGFRpTyr845, AKTpThr308, phosphatidylinositol 3-kinase (PI3K), IGFBP5, IGFBP2, matrix metalloproteinase 9

(MMP9), bcl-2, c-Abl, vascular endothelial growth factor (VEGF), BadpSer136, and retinoblastoma protein (pRB). Clustering analysis of overexpressed proteins in glioblastomas showed that I κ B α groups with EGFRpTyr845, PI3K with AKTpThr308, and IGFBP5 with IGFBP2. MMP9, Bcl-2, VEGF and pRB formed another cluster.

4 Discussion

4.1 General remarks

Glioblastoma poses a formidable challenge to the cancer research community. The extremely poor prognosis and the refractory nature with respect to all conventional therapies suggest a complex pattern of alterations likely involving multiple cellular pathways and molecular regulatory systems in the tumor cells. Thus, a comprehensive survey of protein expression and functionally important modification is particularly useful in providing insight into the workings of altered pathways during disease progression. We used the recently developed RP protein lysate array technology to investigate the expression levels of 46 proteins among 82 glioma tissue samples, focusing on identification of proteins that are significantly different between glioblastoma and lower-grade gliomas. Survival analysis of patients in this cohort showed a major difference between these two groups. Therefore, the difference in protein expression may very well be responsible for the distinct clinical phenotypes. We attempted to understand the relationship among some of the proteins, based on the fact they share some common patterns of expression (or were clustered together in the clustering analysis). These findings, in the context of known functions of the proteins and literature reports are discussed.

4.2 Nuclear factor- κ B/I κ B and EGFR pathways and their relationship

I κ B α was one of the proteins with expression that differed most between glioblastoma and other glioma subtypes in our analysis. I κ B α is the key regulator of nuclear factor-kappa B (NF- κ B), one of the most important transcriptional factors involved in cell growth, apoptosis, and immune response [19]. Most NF- κ B is localized in the cytoplasm through its interaction with I κ B. When I κ B is phosphorylated by activation of IKK, NF- κ B is released from the cytoplasm and enters the nucleus to act on its target genes, including I κ B itself. Therefore, a regulatory feedback loop exists between these two proteins, and an increase in I κ B is often considered a result of activation of NF- κ B [20]. In a recent study, we reported that NF- κ B was indeed activated in glioblastoma [21]. Thus, these results of the protein lysate array data from a large set of patient sample are consistent with our previous observation.

Our clustering analysis showed that I κ B clustered with phosphorylated EGFR. EGFR is critical for cell growth, differentiation, survival, and migration. Amplification and

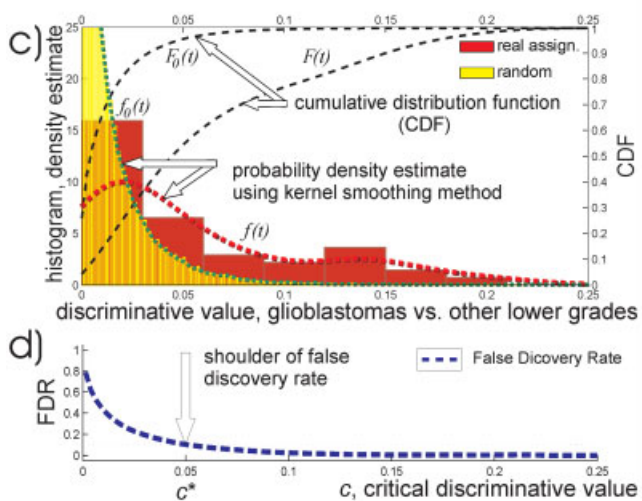
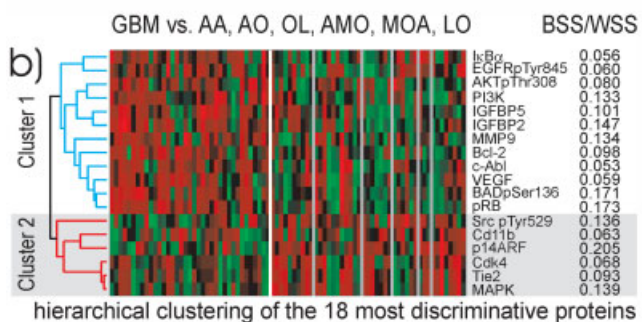
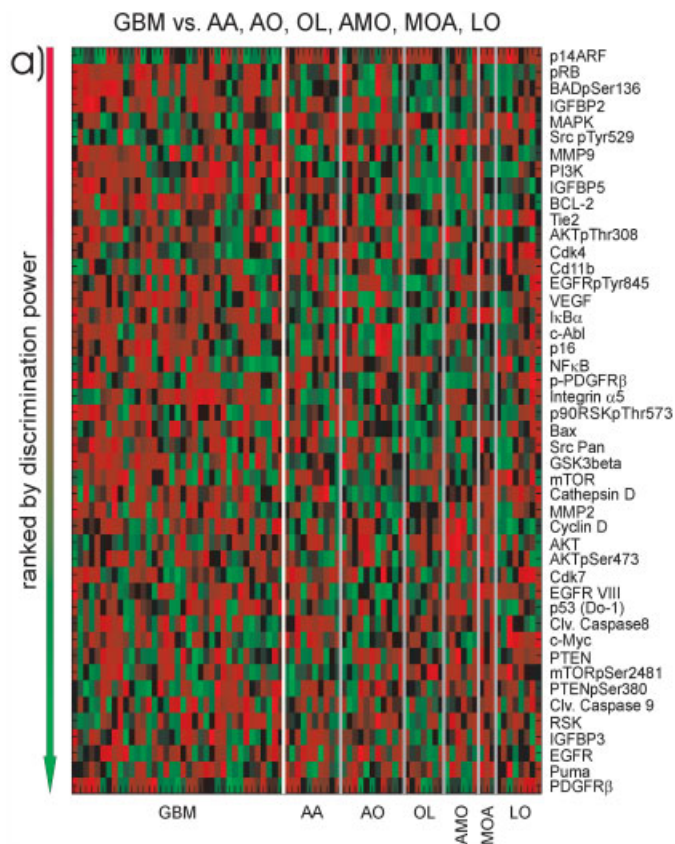


Figure 3. Quantitative analysis of protein expressions. To quantitate the protein expression, we used the robust least squares method that fits a linear model in the log-log space of dilutions. The protein levels were normalized against β -actin and then quantile-normalized. The protein expression levels are represented as a heat map (a and b), where the proteins are ranked in the decreasing order of their discriminative value when classifying glioblastomas vs. other lower grades. The 18 most discriminative feature proteins, having a discriminative value larger than the critical $c^* = 0.05$, are listed in (b), where two main clusters are obtained by hierarchical clustering. In cluster 1 the proteins are overexpressed for glioblastoma cases. Visually, FDR is proportional to the ratio of areas between the probability density estimates corresponding to random assignment (c, green curve) and to correct assignment (red curve). The location of the critical value of the discriminative value ($c^* = 0.05$), below which FDR starts increasing significantly, is shown in (d).

overexpression of EGFR genes occurs in 40% and 60% of glioblastomas, respectively [1]. A mutation in the EGFR gene that results in a shortened form of EGFR called EGFR VIII has been detected in approximately 20% of glioblastomas [22]. In our lysate array study, we also detected EGFR VIII in 12% of glioblastomas (data not shown), but because of its relatively low frequency of detection, EGFR VIII was not selected by our statistical analysis as distinguishing between the two glioma groups.

Tyrosine kinase receptors are often activated by phosphorylation, and the functional activation of the protein is a major switch in the growth pathway in the cells. Thus, it is not surprising that we found EGFRpTyr845 rather than EGFR as one of the top significant discriminators between glioblastoma and other subtypes of gliomas. Tyr 845 is highly conserved within the active loop of the kinase domain on the EGFR and phosphorylation of this residue is mediated by c-Src and dependent on EGF stimulation [23, 24]. Phosphorylation of EGFR on Tyr845 residues is necessary for the binding of EGFR to cytochrome c-oxidase subunit II (Cox II) [25]. After EGF stimulation, EGFR translocates to the mitochondrion, where it interacts with Cox II to regulate cell survival. Integrin proteins induce phosphorylation of EGFR on tyrosine 845, 1068, 1086 and 1173 residues [26], thus EGFR activation is also implicated in increased cell motility and invasion.

It is intriguing that I κ B α and EGFRpTyr845 formed a cluster in our analysis. Reports have shown that EGFR activates NF- κ B, and there is an NF- κ B regulatory element in the promoter of EGFR [27]. Thus, the clustering of the two proteins may reflect their mutual functional regulation in glioblastoma.

4.3 PI3K and AKT survival pathway

Our results showed that both PI3K and phosphorylated AKT are among the top feature proteins that distinguish glioblastoma from lower-grade gliomas. This is consistent with prior reports that activation of PI3k pathway including the AKT protein was more frequent in glioblastomas [28].

Our studies revealed that phosphorylation of AKT is a complex process. It has been observed that AKT is phosphorylated on two major residues: Thr308 and Ser473. Two antibodies are available that specifically recognize these sites. Our study showed that phosphorylation of Akt in gliomas occurs on both residues. However, phosphorylation of Thr308 is a discriminating event between glioblastomas and other low-grade gliomas, whereas phosphorylation of Ser473 is not. Many published studies do not specify which site is phosphorylated. However, a few investigations showed that the two sites of phosphorylation present a differential pattern due to distinct kinases. AKTpThr308 is phosphorylated by 3-phosphoinositide-dependent kinase-1 (PDK1) and AKTpSer473 by the putative kinase PDK2 [29, 30]. In response to insulin stimulation, AKTpThr308 increased, whereas AKTpSer473 was not significantly affected [31]. Thus, site-specific phosphorylation of AKT may represent two different switches. For gliomagenesis, phosphorylation of Ser473 may

represent an early event in cancer progression, and phosphorylation of Thr308 may represent a later event leading to glioblastoma. Thus, full Akt activation may require phosphorylation on both sites [32].

4.4 IGFBP2/IGFBP5 invasion pathway

As mentioned above, genomics studies coupled with tissue microarray experiments have shown that IGFBP2 overexpression is a signature event in glioblastoma. IGFBP2 is a promoter of glioma invasion, one of the most important phenotypes of glioblastoma [3, 33]. There are six members in the IGFBP family, and they have very different functions, especially those that are IGF independent [33]. Among these, IGFBP5 has been implicated in breast cancer metastasis [34, 35]. In this study, we showed that IGFBP5 is also overexpressed in glioblastoma and IGFBP2 and IGFBP5 are closely clustered. Thus, both proteins may contribute to glioma invasion and/or other common functions.

4.5 The other feature proteins

Several other proteins were identified as feature discriminators for glioblastoma. Angiogenesis is a key phenotype in glioblastoma, and thus the selection of VEGF as one of the feature proteins was expected. Resistance to apoptosis is another important phenotype. Therefore, Bcl-2 and BADpSer136 as two discriminators was also consistent with the phenotype. Bcl-2 is a survival protein and has been shown to be expressed in glioblastomas [36]. BAD is an apoptosis-promoting protein, but when phosphorylated, BAD becomes inactive and perhaps may even gain survival function [37]. Although BAD phosphorylation is believed to be a downstream event of AKT phosphorylation, intriguingly, we found that BADpSer136 did not cluster with AKTpThr308 and PI3K. This may mean that there are other upstream regulators of BAD phosphorylation. In support of this hypothesis, Scheid and Duronio [38] showed that activation of AKT alone was not sufficient to phosphorylate BAD and complete inhibition of PI3K/AKT did not abrogate the phosphorylation of BAD.

An interesting finding from our study is that c-Abl is also highly expressed in glioblastoma. In an earlier microarray experiment, we found that c-Abl mRNA expression is associated with poor survival, although those results were not published due to a small sample size of 25 patients. The present study, however, appears to support the earlier finding. Additional experiments should be carried out to pursue this observation because of the clinical implications. Imatinib (Gleevec), which inhibits Bcr-Abl in chronic myeloid leukemia and c-Kit in gastrointestinal stromal cancers, has been one of the most successful therapeutic agents used for targeted therapy [39]. A clinical trial with Gleevec in glioblastoma is ongoing [40], and it may be insightful to view the results of that trial through the prism of our findings.

In summary, our survey of 46 proteins and post-translationally modified isoforms in 82 glioma tissue samples has yielded several biologically relevant discoveries that further our understanding of glioma systems. Some of these findings provide confirmation for some previously proposed concepts. Others are novel and provide focus for further, in-depth, functional studies. The present glioma protein lysate array study demonstrates the utility of this proteomics discovery tool in advancing our understanding of glioma physiology.

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