

## Review

# Molecular Analysis of Glioblastoma

## Pathway Profiling and Its Implications for Patient Therapy

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### ABSTRACT

Technological advances in the ability to analyze patterns of gene expression and signal transduction pathway activation are improving our understanding of cancer. Previously unrecognized molecular subsets and pathway profiles that convey predictive and prognostic information about individual cancer patients are being identified. Patients with glioblastoma, the most common malignant primary brain tumor of adults, stand to benefit considerably from these advances. Recent data suggest that morphologically indistinguishable glioblastomas have distinct classes of causal oncogene activation, and that these subclasses may be targetable by oncogene/signaling pathway specific therapies. Oncogene/signaling pathway inhibitors show great promise for the treatment of patients with some types of cancer, but their clinical application for glioblastoma has been severely limited by an inability to determine which inhibitor is most likely to benefit a specific patient. Identifying biologically relevant molecular subsets of glioblastoma and detecting pathway profiles that can be used to guide patient therapy are likely to result in significant improvement in the survival of glioblastoma patients.

### INTRODUCTION

Cancer diagnosis is moving from a purely morphological classification of tumors to one that is based on molecular criteria. In light of the development of new pharmacologic pathway inhibitors for cancer therapy, this goal is now even more important for the purposes of treatment discovery and selection of appropriately matched patient subsets. Glioblastomas are the most common primary malignant brain tumor of adults, and are among the most lethal of all cancers.<sup>1,2</sup> The diagnosis of glioblastoma, which is based on a set of characteristic morphological and immunohistochemical features, is currently the “gold standard”. It is reproducible between pathologists at different institutions and it generally correlates with prognosis. Unfortunately, prognosis is almost universally dismal; the median survival of glioblastoma patients is one year from the time of diagnosis, and less than 20% survive two years.<sup>3</sup>

### PITFALLS OF PATHOLOGIC DIAGNOSIS

There is much about glioblastomas that standard pathologic analysis does not reveal. Morphology provides no clear indication of the underlying molecular lesions, and has yet to prove useful for determining the optimum therapy for an individual patient. Further, identical glioblastomas can have distinctive clinical presentations and associated molecular abnormalities<sup>2,4,5</sup> that suggest the presence of multiple subsets that cannot be detected by our current method of pathologic analyses. Some glioblastomas arise de novo as grade IV tumors (primary glioblastomas) while others develop from low-grade gliomas (secondary glioblastomas),<sup>4,6</sup> and these clinical differences are associated with distinct sets of non-overlapping molecular abnormalities. Epidermal growth factor receptor (EGFR) amplification and/or over-expression and PTEN deletion are common in primary glioblastomas.<sup>4,6</sup> In contrast, secondary glioblastomas usually contain TP53 mutations.<sup>4,6</sup> Widespread EGFR amplification is extremely rare in secondary glioblastomas, although individual cells may show amplification.<sup>7</sup> To date, these distinctions have yet to prove useful in guiding therapy. This is likely due to the relatively non-specific mechanism of cytotoxicity (i.e., DNA damage) employed by current glioblastoma therapy.

The development of targeted molecular therapies is making identification of molecular subsets more clinically relevant. Pathway/oncogene specific therapies that target the genetic lesions and their consequent signal transduction pathway consequences within cancer cells hold considerable promise for cancer therapy.<sup>8</sup> Paradoxically, the genetic alterations and deregulated signaling pathways that drive cancer cell behavior may also be their “Achilles

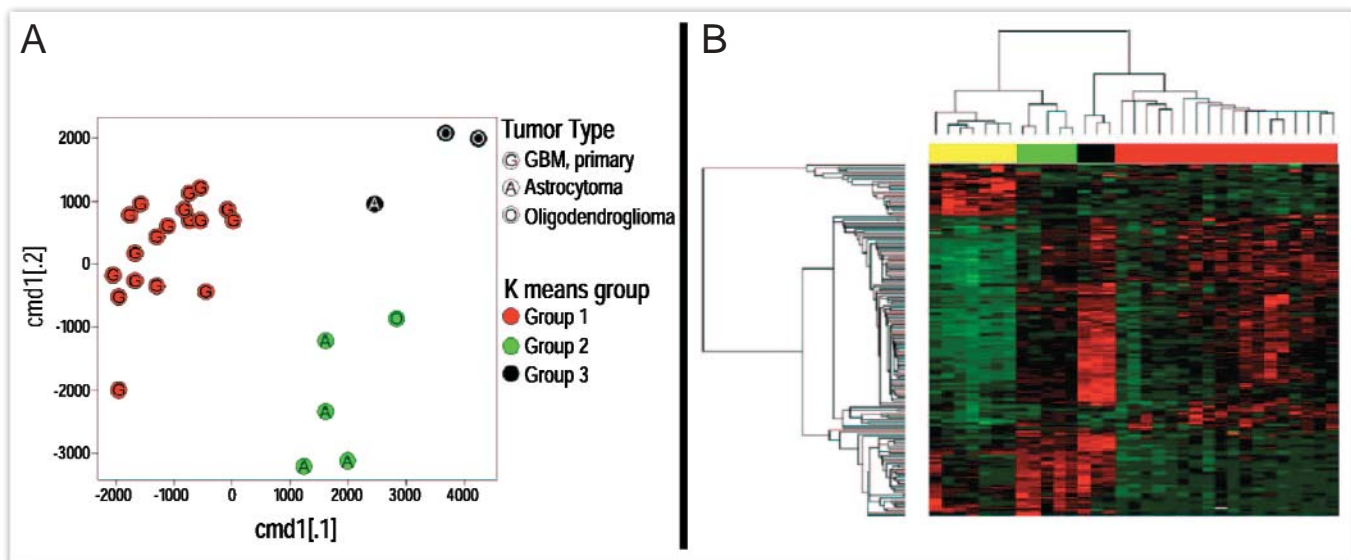


Figure 1. Different subtypes of gliomas have distinct gene expression profiles. The gene expression patterns of 35 gliomas of different type and grade, as well as 7 normal brain samples were analyzed. (A) Multidimensional scaling based on the expression of 12,355 probe sets demonstrates that distinct gliomas have distinct transcriptional profiles. Non-hierarchical k-means clustering demonstrates evidence for three distinct groups. (B) Hierarchical clustering based on the expression of 170 genes demonstrates that the relevant transcriptional differences between the subtypes can be characterized by a relatively modest number of genes. Figure adapted from reference 18.

Heel". Non-cancerous cells have "back-up" mechanisms that allow them to survive and even proliferate in the face of disruption of a single pathway. In contrast, cancer cells may become dependent upon on signaling through chronically activated pathways.<sup>2,9</sup> Pathway inhibitors have demonstrated dramatic success for the treatment of patients with some types of cancer. STI-571, an inhibitor of the Abl and c-Kit kinases is an effective, non-toxic therapy for patients with chronic myelogenous leukemia (which commonly bear constitutively active BCR-ABL fusion tyrosine kinases) and gastrointestinal stromal tumors (which often contain gain of function mutations of the c-KIT tyrosine kinase).<sup>8,10-12</sup> Patients may ultimately develop resistance to these drugs as single agents; however, these studies provide evidence that small molecule inhibitors can potentially significantly benefit cancer patients.

Clinical trials of pathway inhibitors will likely fail to demonstrate significant benefit if the relevant molecular subsets of tumors cannot be identified. In an illuminating study, Betensky and colleagues analyzed the results of a hypothetical randomized controlled trial and showed that unrecognized molecular heterogeneity, if it confers different risks to patients, can result in a clinical trial that is underpowered, and which fails to detect a truly effective new therapy for cancer.<sup>13</sup> Therefore, molecular analysis of glioblastoma has taken on a new urgency. Can we identify previously unrecognized subsets and/or predictive molecular markers and profiles that can be used to guide treatment decisions?

## GENE EXPRESSION PATTERNS DISTINGUISH SUBTYPES OF GLIOMAS

Gene expression is a critical determinant of protein expression and thus of biological function. Cellular behavior is dictated in large part by which of a large number of possible genes are being expressed. Through signal transduction cascades and transcriptional networks, alterations of one gene can impact a large number of genes and result in global effects on cell behavior. Therefore, identifying patterns of gene expression may provide enhanced information

about the biology of a tumor and may help identify subsets of a tumor type that might potentially respond to specific targeted therapies. Genomic methods such as cDNA microarray or oligonucleotide arrays coupled to analysis methods that correlate expression patterns with external parameters such as survival or response to therapy (supervised approaches) or that identify unique transcriptional patterns without any a priori knowledge of types, groups or outcomes (unsupervised approaches) allow for the detection of complex patterns of gene expression that distinguish previously unknown molecular subsets and identify clinically important gene expression signatures.<sup>14</sup> Studying expression differences between large numbers of genes provides not only a quantitative increase in data, but more importantly, a qualitative difference in the kind of data that can be obtained. Patterns of transcriptional activation may be more informative than are individual genes for identifying molecular subsets and developing predictive and prognostic "biomarkers".<sup>14</sup> This is not surprising; if the cell's biology is dictated by the complex interplay of many inter-related transcriptional networks, detection of patterns gives a deeper sense of the "structure" of the underlying biological program.

Many of the gene expression studies of cancer to date have demonstrated that morphologically different tumors have distinct transcriptional profiles, and that there are patterns of gene expression that correlate with increasing grade of malignancy, including in gliomas.<sup>15-17</sup> For example, low-grade astrocytomas, oligodendrogliomas and glioblastomas have distinctive global gene expression profiles, which are clearly separable from each other and from normal brain tissue.<sup>18</sup> These different types and grades of gliomas can be accurately distinguished from each other by a relatively small number of genes, which are heavily weighted towards genes encoding proteins involved in such critical processes as cellular proliferation, proteosomal function, energy metabolism and signal transduction.<sup>18</sup> Therefore, morphologically distinctive gliomas of different type and grade have different global patterns of gene expression,<sup>15-17,19,20</sup> which may be informative about their underlying biology.

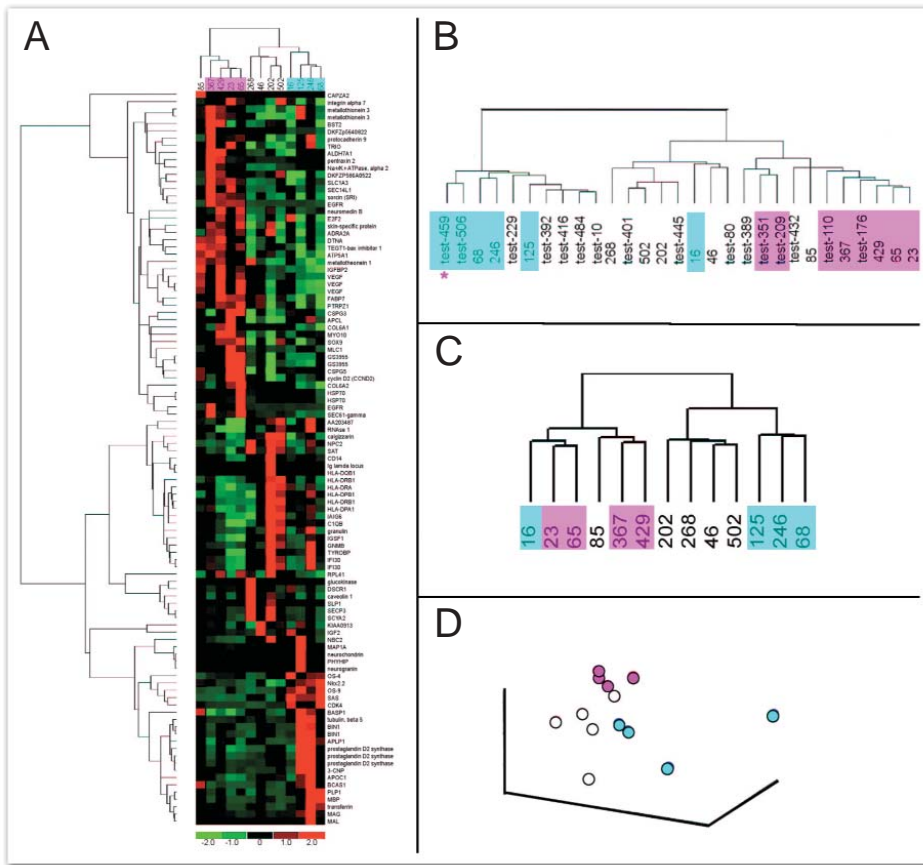


Figure 2. (A) Hierarchical clustering identifies 3 molecular subsets of primary glioblastomas. The differential expression of 90 genes can distinguish these subsets. One of the subsets is based on EGFR expression (red), one is based on over-expression of a contiguous set of genes on chromosome 12q13-15, and the third lacks either alteration. (B) This class predictor correctly classified additional independent glioblastoma samples into the correct molecular subset. (C) The global gene expression patterns of these 3 subsets are distinct, as demonstrated by hierarchical clustering. (D) Multidimensional scaling further demonstrates that these subsets have distinct global transcriptional profiles. Figure adapted from reference 29.

biological and transcriptional consequences of EGFR over-expression in glioblastoma have not been clarified. To address this issue, primary glioblastomas were stratified as being either EGFR protein expressing or EGFR protein negative and differences in the transcriptional profiles were analyzed (Fig. 2).<sup>29</sup> EGFR protein expressing glioblastomas had a globally distinctive pattern of gene expression relative to non-EGFR expressing primary glioblastomas, suggesting that they are a biologically relevant subset. Further, a relatively small number of genes (90 genes) could readily distinguish between EGFR expressing and EGFR negative

### IDENTIFICATION OF NOVEL MOLECULAR SUBSETS OF GLIOBLASTOMA BY GENE EXPRESSION ANALYSIS

primary glioblastomas, and this list of genes was highly enriched for signaling molecules, many of which could potentially provide therapeutic targets.<sup>29</sup> In line with these findings, another group has recently demonstrated that EGFR expressing glioblastomas have a distinctive transcriptional profile.<sup>30</sup> Not surprisingly, the EGFR negative primary glioblastomas were not a uniform subclass. At least two subsets of EGFR negative primary glioblastomas were detectable based on global patterns of gene expression, including one that was associated with over-expression of a set of contiguous genes on chromosome 12q13-15 (Fig. 2).<sup>29</sup> These data suggest that patterns of gene expression can uncover biologically relevant molecular subsets of morphologically identical glioblastomas. It remains to be seen whether this approach can meet the challenge of identifying clinically exploitable molecular subsets that will provide enhanced prognostic and predictive information and identify new therapeutic targets.

Gene expression profiling may not necessarily augment pathologic analysis when it comes to separating tumors of different type and grade. Morphologic analysis is already good at this. The real challenge for genomic methods is to provide clinically important information that standard pathologic analysis cannot detect. Gene expression profiling has the potential to identify biologically relevant subsets of tumors that can be used to guide treatment decisions, and to develop new therapeutic targets. Gene expression studies have uncovered previously unrecognized subclasses of a number of types of cancer, that have distinct molecular and/or clinical phenotypes or responses to therapy.<sup>21-27</sup> To date, relatively few studies have aimed at identifying relevant subsets of glioblastoma.

It has long been suspected that primary and secondary glioblastomas are not homogeneous groups, but rather contain clinically relevant, and as yet undetected, molecular subclasses. EGFR expression is common in primary glioblastomas, being detected in approximately two-thirds of cases.<sup>4-6,28</sup> It has been unclear whether EGFR expressing glioblastomas are a distinct molecular subset, and the

primary glioblastomas, and this list of genes was highly enriched for signaling molecules, many of which could potentially provide therapeutic targets.<sup>29</sup> In line with these findings, another group has recently demonstrated that EGFR expressing glioblastomas have a distinctive transcriptional profile.<sup>30</sup> Not surprisingly, the EGFR negative primary glioblastomas were not a uniform subclass. At least two subsets of EGFR negative primary glioblastomas were detectable based on global patterns of gene expression, including one that was associated with over-expression of a set of contiguous genes on chromosome 12q13-15 (Fig. 2).<sup>29</sup> These data suggest that patterns of gene expression can uncover biologically relevant molecular subsets of morphologically identical glioblastomas. It remains to be seen whether this approach can meet the challenge of identifying clinically exploitable molecular subsets that will provide enhanced prognostic and predictive information and identify new therapeutic targets.

### SIGNAL TRANSDUCTION PATHWAYS AS TARGETS

Transcriptional patterns are one important indicator of cellular activity; post-translational modifications of proteins, such as phosphorylation events, are another critical way in which cellular information

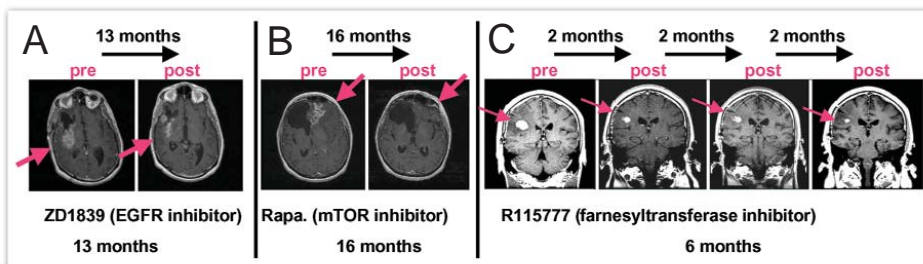


Figure 3. Clinical Response to Kinase Inhibitors. Some patients manifest clinical response to kinase inhibitors. (A) Patient treated with the EGFR inhibitor ZD1839 for 13 months, (B) patient treated with the mTOR inhibitor rapamycin for 16 months and (C) patient treated with FTI R115777 for 6 months. Tumor shrinkage (arrows) is detectable in all three patients.

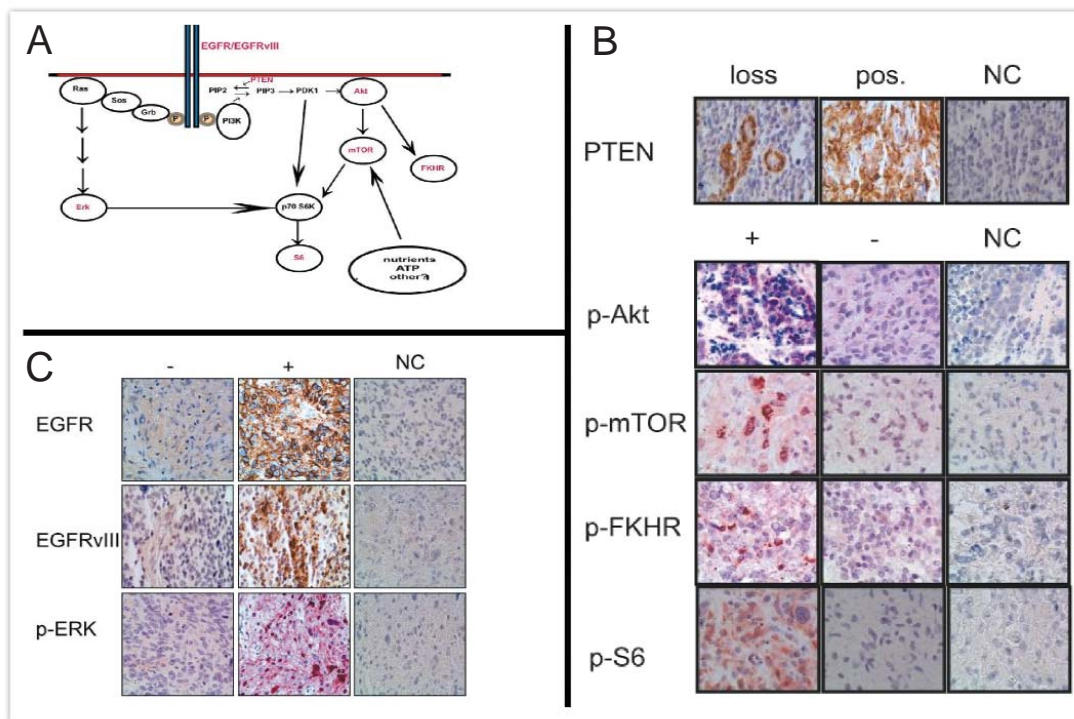


Figure 4. PTEN loss is associated with activation of the PI3K pathway in glioblastoma patients in vivo. (A). Schematic diagram of the signaling pathways (analyzed molecules are in red). (B) PTEN protein is lost in a subset of glioblastomas, as seen by retention of PTEN expression in the vascular endothelium with loss of PTEN in the tumor cells. The PTEN deficient tumors demonstrate phosphorylation of AKT, mTOR, FKHR and S6. (C) EGFR, EGFRvIII and p-ERK were also detected by immunohistochemistry. Figure adapted from reference 41.

is conveyed. Gene expression patterns provide indirect information about signaling pathways because pathway activation usually leads to gene transcription alteration. However, direct analysis of signal transduction cascades may provide additional information that can be used to identify clinically relevant molecular subsets. Over the past few years, deregulation of signal transduction pathways has emerged as a dominant theme in the development and progression of cancer,<sup>8,31,32</sup> including in glioblastoma.<sup>3,33</sup> In mouse genetic models, chronic activation of signaling pathways that are used during normal development can result in the formation of gliomas,<sup>3,34</sup> particularly in the presence of multiple pathway alterations or when combined with cell cycle abnormalities.<sup>3,34,35</sup> Chronic activation of the phosphatidylinositol 3-kinase (PI3K) and RAS/MAPK signaling pathways appear to be particularly potent in glioblastoma pathogenesis, and these pathways are commonly deregulated in human glioblastomas, often in combination.<sup>2,33,35</sup>

PI3K is a lipid kinase that promotes diverse biological functions including cellular proliferation, survival and motility.<sup>32</sup> The RAS/MAPK pathway plays a pivotal role in cellular proliferation, downstream gene transcription and cancer cell invasion.<sup>36</sup> PI3K and the RAS/MAPK pathways can become deregulated on the basis of oncogene activation and tumor suppressor gene losses that are common in glioblastoma. Up to 40% of glioblastomas contain alterations of the PTEN tumor suppressor gene, a negative regulator of PI3K signaling, which results in constitutive activation of the PI3K pathway.<sup>37</sup> Upstream of PI3K, EGFR overexpression and coexpression of the constitutively activated EGFRvIII variant, may also potentially lead to deregulated PI3K and RAS/ERK signaling.<sup>2,4-6,28,38,39</sup>

A variety of pathway inhibitors that can safely target the PI3K and RAS/ERK pathways in glioblastoma patients are now being

studied. In early clinical trials, mTOR inhibitors (which block downstream PI3K signaling), farnesyl transferase inhibitors (which block RAS signaling by preventing its farnesylation) and EGFR inhibitors all demonstrate promising results in a subset of glioblastoma patients (Fig. 3). The critical challenge is to determine which patients are most likely to benefit. Our understanding of the signaling events that regulate, and are regulated by, PI3K and RAS/MAPK signaling derives primarily from in vitro models,<sup>32,40</sup> and from the mouse genetic studies.<sup>34</sup> Dissecting the molecular events associated with PI3K and RAS/ERK pathway deregulation in cancer patients in vivo represents a critical extension of this work, and has important implications for the design of "smart" clinical trials with pathway inhibitors. Up to now, assessment of multiple nodes in this pathway in routinely processed patient biopsy samples has not been possible. However, development of phospho-specific antibodies that allow for detection of activated signaling molecules in paraffin-embedded biopsy tissues has enabled the analysis of the PI3K and RAS/MAPK pathways in glioblastoma biopsies.

## ACTIVATION SPECIFIC ANTIBODIES CAN BE USED TO ANALYZE THE PATHWAYS

In a study of 45 primary glioblastoma patients on a tissue microarray, immunohistochemical analysis using phospho-specific antibodies was performed, and hierarchical clustering and multidimensional scaling, as well as univariate and multivariate analyses, were used to dissect the PI3K and ERK pathways in vivo.<sup>41</sup> PTEN loss, which antagonizes PI3K pathway activation, was highly correlated with activation of the main PI3K effector AKT in vivo, and AKT activation was significantly correlated with phosphorylation of

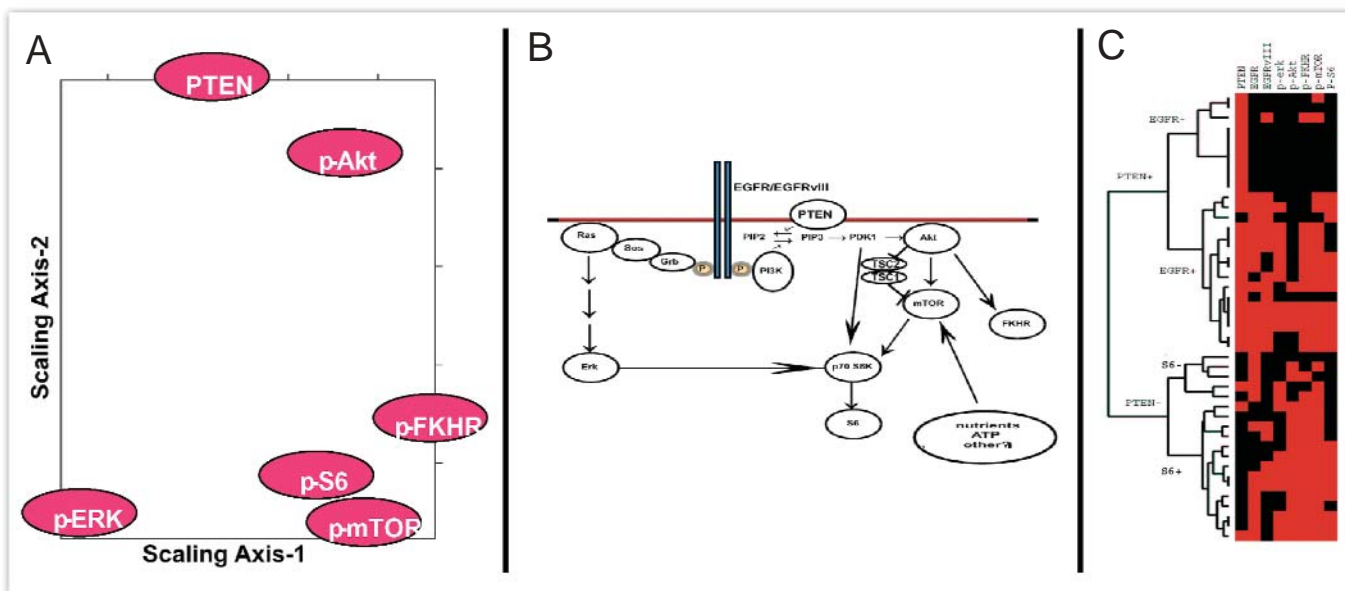


Figure 5 A) Multidimensional scaling visualizes the pattern of correlations between signaling molecules in a two-dimensional graph. This analysis does not assume any prior knowledge of relationships between these molecules. The pattern of correlations visualized based on the expression/activation status of the markers analyzed in 45 glioblastoma patients (A) recapitulates our understanding of the pathway structure based on *in vitro* data, as represented in a classic signaling diagram (B). C) Hierarchical clustering of patients based on expression/activation of these markers can be used to identify cancer clusters that are informative about the underlying biology and which may potentially be used to guide treatment decisions. Each row represents one of the 45 glioblastoma patients; each column represents a marker. Red refers to expressed (or activated form expressed), black means not expressed/activated. Two main subsets were identified, based on PTEN expression ( $p < 0.0000001$ ), and two subsets of PTEN expressing glioblastomas were found, based on EGFR expression ( $p = 0.00001$ ). EGFRvIII was co-expressed with EGFR in 88% of PTEN expressing glioblastomas, but only 26% of PTEN deficient glioblastomas ( $p = 0.003$ ), suggesting an important role for combined Ras and PI3K pathway deregulation. The patterns suggest that there may be: (i) a group of patients that are not well-suited for kinase inhibitors, (ii) a subset of patients that may require combined PI3K/ERK blockade, and (iii) a subset of patients who may be suited for single inhibitor therapy. Figure adapted from reference 41.

mTOR, FKHR and S6, which are thought to promote its effects. Expression of the mutant epidermal growth factor receptor EGFRvIII was also tightly correlated with phosphorylation of these effectors, demonstrating an additional route to PI3K pathway activation in glioblastomas *in vivo*. In contrast wild type EGFR overexpression correlated with ERK activation. These results were consistent with the presumed relationships between PTEN and other known components of the PI3K and ERK signaling pathways.<sup>41</sup>

In many ways, the kind of data that comes out of analysis of signal transduction pathways shares similarities with data gathered from gene expression profiles. As opposed to analyzing individual marker, both approaches seek to analyze larger numbers of inter-related markers in which analytic tools can be used to detect meaningful patterns. Therefore, many of the tools such as hierarchical clustering and multidimensional scaling that are commonly applied to the analysis of gene expression profiles may have relevance for identifying signal transduction profiles. Multidimensional scaling analysis, a form of principle component analysis, can be used to visualize the pattern of relationships between variables without assuming previous knowledge of their interactions. When multidimensional scaling was used to analyze the activation pattern of the signal transduction molecules in the 45 glioblastoma patients, a pattern of inter-relationships between these signaling molecules *in vivo* was demonstrated that reflects the current knowledge of the signaling pathway derived from *in vitro* experimental studies. This raises the possibility that this form of analysis might be applied to other data sets where the connectivity between the variables is less defined.<sup>41</sup>

Hierarchical clustering, another unsupervised analysis method, was also used to identify potentially meaningful molecular subsets. The 45 glioblastoma patients were clustered based on the expression and/or activation of the key signaling molecules detected by immunohistochemistry (EGFR, EGFRvIII, p-ERK, PTEN, p-AKT, p-mTOR, p-FKHR and p-S6). Two main subclasses of primary glioblastomas were detected, based on expression of PTEN protein. Within the PTEN-expressing glioblastomas, two additional subsets were detected, based on expression of EGFR. The PTEN-expressing, non-EGFR expressing tumors lacked either ERK or the PI3K pathway activation, suggesting that these patients may not be good candidates for PI3K and RAS/MAPK inhibitors. Hierarchical clustering also demonstrated that co-expression of the EGFRvIII mutant receptor with EGFR was significantly more likely to occur when PTEN protein was expressed than when PTEN protein was deficient ( $p = 0.003$ ). Because univariate analyses had demonstrated that EGFR expression is associated primarily with RAS/MAPK activation while EGFRvIII expression was associated with signaling downstream of AKT, these data suggest that EGFRvIII may be selected for in the absence of PTEN loss as a way to activate AKT signaling. This further suggests a critical role for combined chronic RAS and AKT pathway activation in the development and progression of glioblastomas.<sup>34</sup> These results suggest that phospho-specific antibodies can be used to detect the activation state of key signaling molecules *in vivo*, and that analytic tools such as multidimensional scaling and hierarchical clustering can help identify biologically meaningful patterns of pathway activation which can potentially be used to stratify patients for therapy with pathway inhibitors.<sup>41</sup>

## CONCLUSIONS AND FUTURE CHALLENGES

The success of pathway inhibitors in treating some cancer patients, including a subset of those with glioblastoma, have increased the importance of identifying predictive markers that can help guide treatment decision. Not surprisingly, gene expression profiling studies and signal transduction pathway analyses are showing that patterns of gene expression and protein expression/activation provide better insight into the underlying biology of tumors and may prove to be more useful predictors of therapeutic response. As more patients are treated with kinase inhibitors and studies of gene expression and signaling pathway activation are performed in these clinical trial patients, predictive profiles will begin to emerge. This is exciting, as it begins to point to a rational individualized way to treat glioblastoma patients. However, only time and further study will tell whether these approaches can be used to direct the most beneficial therapy to each patient. Further, the inherent intra-tumor molecular heterogeneity of glioblastomas poses a direct challenge for any attempt at molecular analysis and biomarker identification, as well as for any targeted therapy. In the future, it will be important to begin analyze meaningful patterns within this heterogeneity, so that synergistic combinations of targeted therapies can be successfully applied. The challenges are great, but these new molecular approaches are bringing hope to glioblastoma patients.

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