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A STAT3 Gene Expression Signature in Gliomas is Associated with a Poor Prognosis

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Abstract: Gliomas frequently display constitutive activation of the transcription factor STAT3, a protein that is known to be able to mediate neoplastic transformation. STAT3 regulates genes that play a central role in cellular survival, proliferation, self-renewal, and invasion, and a cohort of STAT3 target genes have been found that are commonly coexpressed in human cancers. Thus, these genes likely subserve the transforming ability of constitutively activated STAT3. To determine whether the coordinated expression of STAT3 target genes is present in a subset of human gliomas, and whether this changes the biology of these tumors in patients, gene expression analysis was performed in four distinct human glioma data sets for which patient survival information was available. Coordinate expression of STAT3 targets was significantly associated with poor patient outcome in each data set. Specifically, patients with tumors displaying high expression of STAT3 targets had a shorter median survival time compared to patients whose tumors had low expression of STAT3 targets. These data suggest that constitutively activated STAT3 in gliomas can alter the biology of these tumors, and that development of targeted STAT3 inhibitors would likely be of particular benefit in treatment of this disease.

Keywords: brain tumors, signal transduction, gene expression, transcription factors

Introduction

Central nervous system malignancies remain among the most difficult tumors to treat, owing to both anatomic and biologic features. The intimate association with critical structures and the highly vascular and infiltrating nature of gliomas make complete surgical resection particularly difficult. Furthermore, these tumors often manifest an intrinsic resistance to cell death triggered by cytotoxic agents or radiotherapy. To enhance our approach to the treatment of this disease it would be valuable to understand the molecular abnormalities that underlie these tumors.

A number of mutations have been found to occur commonly in human gliomas (Holland, 2001; Maher, 2001). One recurrent finding is the activation of tyrosine kinases, particularly the epidermal growth factor (EGF) receptor. The EGF receptor may be constitutively activated as a result of overexpression or from structural mutations that render the catalytic domain continually activated (Bigner, 1990; Libermann, 1985). In addition, activation of the platelet-derived growth factor (PDGF) receptor can occur due to concomitant overexpression of both the receptor and the PDGF ligand (Guha, 1995). Another soluble factor that can promote mitogenesis of normal and malignant glial cells is interleukin (IL)-6 (Van Meir, 1990). IL-6 can display enhanced expression in human gliomas (Tchirkov, 2001) as well as murine models of glial tumors (Weissenberger, 2004). Although each of these soluble proteins can activate a number of signaling pathways, one transcription factor that plays a central role in transducing signals from EGF, PDGF, and IL-6 is STAT3 (Alvarez, 2006).

Under basal conditions, STAT3 is found in the cytoplasm. Once activated through phosphorylation of a unique carboxy-terminus tyrosine residue, STAT3 forms dimers, translocates to the nucleus, and binds to nine base pair sequences in the promoter regions of target genes thereby activating (or in some cases repressing) transcription (Darnell, 1997; Ihle, 1996). STAT3 targets include genes involved in cell cycle progression, survival, self-renewal, and invasion (Alvarez, 2005). Reflecting these physiological functions of STAT3 target genes, STAT3 has been found to be activated inappropriately in a

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wide array of human tumors, including gliomas (Frank, 2003). In fact, in at least some systems, activation of STAT3 is sufficient to lead to neoplastic transformation of cells (Bromberg, 1999). STAT3 activation is likely to be directly involved in the pathogenesis of CNS tumors as depleting STAT3 through RNA interference can lead to apoptosis in astrocytoma cell lines (Konnikova, 2003). The activation of STAT3 in human gliomas may be of particular clinical importance in that, through activation of pro-survival genes, constitutive STAT3 activation can confer resistance to ionizing radiation and cytotoxic chemotherapy in other tumor systems (Alas and Bonavida, 2003).

Although understanding specific target genes is an important approach for dissecting the mechanism by which a transcription factor can contribute to oncogenesis, much information can also be gleaned from analyzing global patterns of target gene expression (Alvarez and Frank, 2004). In fact, analyzing the coordinate expression of STAT3 target genes has proven to be a powerful approach to determine the presence of functionally active STAT3 in a cell (Alvarez, 2005). Given the central role that these genes play in the biology of a cell, identification of a STAT3 gene expression signature in a tumor may connote specific prognostic information. For example, it is known that STAT3 activation is associated with a decreased survival in acute leukemia and other tumors (Benekli, 2002). Furthermore, the presence of a STAT3 gene expression signature may identify tumors appropriate for molecular therapy specifically targeting this transcription factor (Darnell, 2002; Frank, 2006). Finally, recent evidence has suggested that grouping gliomas based on gene expression might be a better predictor of survival than histologic classification (Nutt, 2003).

To determine whether coordinate expression of STAT3 target genes carries prognostic information in human glial tumors, we analyzed expression of STAT3 target genes in primary gliomas, and examined the relationship of this pattern of gene expression to patient survival.

Materials and Methods

Datasets

To assess the relationship between expression of STAT3 target genes and survival in glioma, we analyzed four independent gene expression data sets for which information was available on both gene expression and patient survival. The use of these disparate data sources also allowed us to avoid artifacts arising from distinctions in institutional clinical assessment or differences in gene expression methodology on primary tumor samples. Specifically, we analyzed a cohort of 29 high grade gliomas with non-classical histology from several hospitals in Europe and North America (Nutt, 2003); a cohort of 74 grade III and IV gliomas from the University of California, Los Angeles (Freije, 2004); the BWH Glioma GeneChip Dataset (Chakravarti et al. manuscript in preparation), encompassing 29 gliomas, three quarters of which were glioblastoma multiforme; and, a cohort of 21 classic gliomas (Nutt, 2003).

Gene set enrichment analysis (GSEA)

To determine if STAT3 target genes were enriched in poor-outcome tumors, we performed gene set enrichment analysis (GSEA; Subramanian, 2005). Briefly, all genes for which expression data was available were ranked based upon differential expression between poor-outcome and good-outcome classes using the signal-to-noise metric. The distribution of STAT3 targets on this ranked list was then assessed using a Kolmogorov-Smirnov distribution test, which produces a normalized enrichment score (NES), a measure of the extent to which STAT3 targets are enriched in one class or the other. The normalized enrichment score for each analysis is shown in Figure 1. The statistical significance of this enrichment was evaluated by performing permutation testing using randomized class labels, thereby generating a nominal p value. To account for multiple hypothesis testing, the enrichment score was normalized, and a q value of the false discovery rate (FDR) was calculated (Reiner, 2003). A q value <0.25 is considered significant. Both the p value and the FDR q value are provided for each analysis.

STAT3 target genes were defined in three ways. First, analyzing genes directly responsive to activation of STAT3 in a cell culture system yielded approximately 100 STAT3 targets (Alvarez, 2005). Recognizing that only a subset of these genes was likely to be coexpressed in human tumors, coordinate expression of these 100 genes was analyzed in a dataset of 190 human tumors. This analysis revealed a group of 12 genes, known as a STAT3 signature, that showed strong
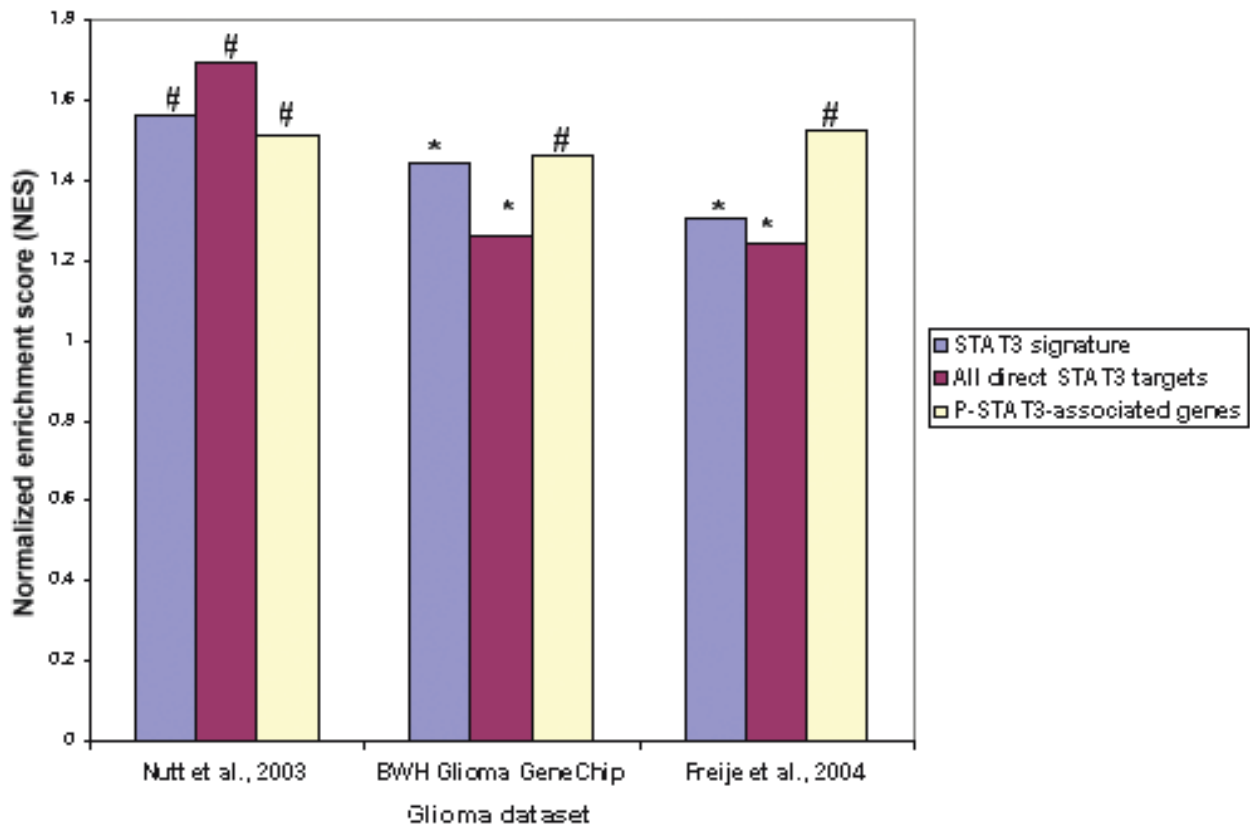
Figure 1. Glioma patients with a short survival have tumors with enhanced expression of STAT3 target genes. Gene set enrichment analysis was performed comparing the short survival and long survival cohorts of each of three independent data sets (Subramanian, 2005). Normalized enrichment scores were calculated, and statistical significance was determined by calculating a nominal p value by permutation testing, and, to account for multiple hypothesis testing, an FDR q value was determined. *, FDR q value <0.25; #, FDR q value <0.25 and p value <0.05.

co-association in these human tumors. The 12 genes in this group are: vascular endothelial growth factor (VEGF), protein tyrosine phosphatase (PTP)-CAAX1, kruppel-like factor (KLF)-4, exostosin (Ext)-1, Neimann-Pick C1 (NPC1), p21-activated kinase (Pak)-2, Mcl-1, JunB, Bcl-6, NF-IL3, calpain-2, and early growth response (Egr)-1. Finally, examining expression of STAT3 target genes in 96 primary human breast cancers displaying histological evidence of tyrosine phosphorylated STAT3, a subset of 300 STAT3 target genes was independently identified.

Hierarchical clustering
Hierarchical clustering was performed to separate gliomas on the basis of STAT3 target gene expression using dChip software.

Results
Given that STAT3 activation is a common and biologically important finding in gliomas, and that clustering gliomas based on gene expression provides powerful prognostic information, we sought to determine whether a STAT3 gene expression signature was associated with a distinct clinical outcome in this disease. We first investigated a well characterized cohort of 29 high grade gliomas that displayed histologic features which could not be classified definitively by experienced neuropathologists (Nutt, 2003). Patient survival data were available for each of these tumors allowing us to directly address the question of whether tumors that showed enhanced expression of STAT3 targets had a difference in clinical outcome independent of pathologic classification. To avoid discrepancies arising from delays in diagnosis or clinical assessment, patient survival from the time of diagnosis was divided into thirds, and gene expression profiles were compared between tumors from the third of patients who experienced the longest survival and from the third with the shortest survival. GSEA revealed prominent enrichment of STAT3 target genes in the poor prognosis group

of these gliomas, whether defined by the full complement of STAT3 targets ($p < 0.01$; FDR q = 0.02), the 12 gene STAT3 signature ($p < 0.01$; FDR q = 0.02), or the STAT3 target genes enriched in tumors with tumors with histochemical evidence of STAT3 phosphorylation (p = 0.06; FDR q = 0.03) (Fig. 1).

We next evaluated a cohort of 74 grade III and grade IV gliomas from patients treated at the University of California, Los Angeles (Freije, 2004). To allow clear assessment of outcome, patient survival was again divided into thirds, and gene expression profiles were compared between tumors from the third of patients who experienced the longest survival and from the third with the shortest survival. Once again, strong associations were seen for expression of the STAT3 target genes in the poor prognosis tumors using the gene sets defined by histologic evidence of STAT3 activation (p = 0.02; FDR q = 0.19), the 12 gene STAT3 signature (p = 0.18; FDR q = 0.18), and the complete set of STAT3 target genes (p = 0.18; FDR q = 0.16) (Fig. 1).

To further validate that STAT3 target gene expression was associated with a poor prognosis in gliomas, we analyzed a distinct set of 29 tumors (greater than 75% of which were glioblastoma multiformes) for which survival was bifurcated into greater than or less than two years (BWH Glioma GeneChip Dataset; Chakravarti et al. manuscript in preparation). Once again, a significant association was found with STAT3 target gene expression in the tumors of patients with short survival using the gene sets derived from the tumors with histochemical evidence of STAT3 phosphorylation (p = 0.02; FDR q = 0.19), the 12 gene STAT3 signature set (p = 0.12; FDR q = 0.09), and the full complement of STAT3 targets (p = 0.15; FDR q = 0.24) (Fig. 1).

We also evaluated an additional data set of 21 classic gliomas, comprising 14 glioblastomas and 7 oligodendrogliomas (Nutt, 2003). Although there was a notable enrichment of STAT3 signature genes in the tumors of patients with short survival, the small number of patients limited the statistical power. We thus took an alternative approach to address the correlation of STAT3 target genes with outcome in CNS tumors. Unsupervised hierarchical clustering of these gliomas was performed based upon the relative expression of STAT3 targets (Fig. 2A). In this manner, tumors were separated into two well-defined groups. Eight of these tumors displayed high expression of STAT3 targets (6 glioblastomas and 2 oligodendrogliomas) and 13 had relatively low expression (8 glioblastomas and 5 oligodendrogliomas). We then evaluated the survival of these two groups using Kaplan-Meier analysis. Patients whose tumors displayed high expression of STAT3 targets had a shorter median survival than the group without (Fig. 2B), indicating that enhanced expression of STAT3 targets correlates with poor outcome in these gliomas as well. When we performed hierarchical clustering using the STAT3 target genes enriched in tumors with histochemical evidence of STAT3 phosphorylation, the patients again segregated into two groups with significantly different survival (data not shown). Taken together, these analyses on data sets from disparate institutions and investigators indicate that a STAT3 gene expression signature is associated with a poor prognosis in malignant gliomas.

Discussion

The finding from these independent data sets that coordinate expression of well defined STAT3 target genes is associated with a worse prognosis likely reflects the physiologic function mediated by STAT3 and the genes it regulates. STAT3 target genes in general, and the 12 gene STAT3 gene expression signature in particular, include genes whose functions span the range of phenotypic abnormalities found in cancer (Hanahan and Weinberg, 2000). These include the promotion of cell cycle progression, (e.g. JunB and Egr-1) survival (e.g. Mcl-1), self-renewal, (e.g. Bcl-6 and Klf-4), and invasion and angiogenesis (e.g. vascular endothelial growth factor (VEGF)). Thus, it is not surprising that activation of STAT3, and elevated expression of the genes controlled by this transcription factor, may confer a more aggressive phenotype on gliomas cells. For example, a pro-survival gene such as Mcl-1 may mediate resistance to apoptosis, thereby rendering therapies such as radiation and chemotherapy less effective. It is also possible that other biological features of malignant glial cells are altered in such a way as to render these tumors more difficult to eradicate. For example, heightened expression of c-met may increase the mobility and invasiveness of gliomas, thereby making them more difficult to surgically extirpate (Abounader, 2001). Similarly, another STAT3 target gene, VEGF, may be of particular
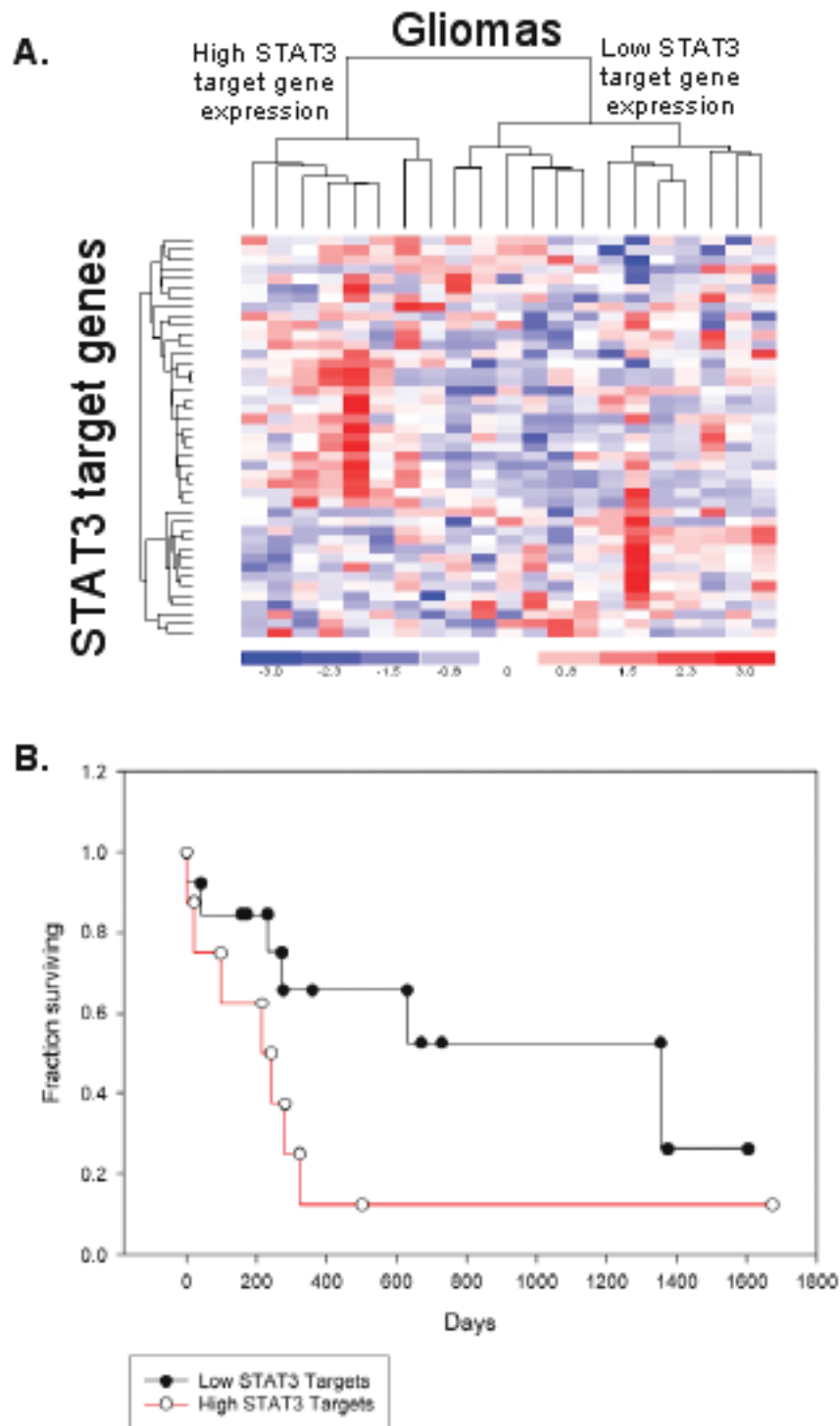
Figure 2. STAT3 target gene expression is associated with a shorter survival in gliomas. (**A**) Segregation of gliomas based on STAT3 target gene expression. Unsupervised hierarchical clustering was performed on a data set of 21 gliomas (Nutt, 2003) (http://www.broad.mit.edu/cgi-bin/cancer/datasets.cgi) based on expression of STAT3 targets using dChip software. Eight tumors displayed high STAT3 target gene expression and 13 displayed low expression. (**B**) Kaplan-Meier analysis of survival of gliomas based on expression of STAT3 target genes.

importance in enhancing the angiogenesis of gliomas, which are often highly vascular (Plate, 1992). It is also notable that while these STAT3 signature genes were initially defined in murine fibroblasts, they have been validated in human tumors of lineages as divergent as hematologic cells and glia (Alvarez, 2005). This suggests that fundamental aspects of STAT3 signaling are likely maintained across tissue types.

A recent study has suggested that STAT3 phosphorylation in glioblastomas and anaplastic astrocyotmas, as assessed by immunohistochemistry, correlates with expression of mutant EGF receptors (EGFRvIII), and was not a strong prognostic marker (Mizoguchi, 2006). However, the difference in findings between that study and the current one may reflect fundamental differences arising from the methodology used. In determining the activation state of a transcription factor such as STAT3, several methods can be utilized. STAT3 activation has been assessed in tumors most commonly by assessing the phosphorylation state of the protein (using immunohistochemistry or immunoblotting) or determining the presence of nuclear STAT3 dimers competent to bind to DNA (using electrophoretic mobility shift analysis). Identification of expression of STAT3 target genes complements these techniques, and provides a number of additional advantages. Since the effects of STAT3 in promoting malignant transformation in general, or the more malignant phenotype of gliomas as reported here, are mediated through its target genes, identification of a STAT3 gene expression signature is perhaps the most direct way to assess the functional activation of this protein. In addition, it is certainly possible that transient or intermittent STAT3 activation may occur which may limit the ability to detect activated STAT3 by biochemical means, whereas the more prolonged activation of expression of target genes may be more likely to be detected over a larger temporal window. Conversely, it is conceivable that STAT3 activation can occur in the setting of chromatin modifications or the presence of transcriptional repressors that will attenuate the expression of STAT3 target genes. The absence of expression of STAT3 target genes in this situation would appropriately exclude these tumors from therapeutic considerations that might otherwise apply to tumors displaying functionally activated STAT3.

We chose instead to use the presence of a STAT3 gene expression signature as a surrogate for STAT3 activation. This signature was identified in vitro and verified in various tumor types in vivo. This approach provides a sensitive and specific means of assessing the transcriptional activity of STAT3, independent of the biochemical approaches described above. Indeed, a recent study using an expression signature for the androgen receptor (AR) as an indicator of AR activation was able to identify inhibitors of androgen signaling, thereby validating this approach (Hieronymus, 2006). Furthermore, with the development of targeted signal transduction inhibitors for the treatment of gliomas and other tumors, the ability to identify biomarkers to allow patient selection and monitoring of pharmacodynamics becomes more important. Identification of heightened expression of STAT3 target genes in general, and perhaps selected STAT3 target genes in particular, may allow the identification of suitable biomarkers.

Given the nature of the data sets used in this study, it was not possible to obtain pathologic specimens to directly assess the phosphorylation status of STAT3. We have shown a high correlation between histological evidence of STAT3 activation (as measured by nuclear staining with an antibody to tyrosine-phosphorylated STAT3) and a STAT3 gene expression profile in breast cancer (Alvarez, 2005). As noted, the gene expression profile may be a more powerful predictor of STAT3 function, and the use of gene expression for glioma classification has been established as a powerful prognostic tool (Nutt, 2003). Nonetheless, it would be of interest to determine the correlation between histological activation of STAT3 and a STAT3 gene expression profile in a prospective validation study in gliomas. Similarly, very little clinical information was available with these data sets. A validation study with sufficient clinical data to allow a multivariate analysis of the predictive value of a STAT3 gene expression profile would also be desirable.

The finding of a STAT3 gene expression signature in gliomas can have several implications, and suggests further areas for study. First, the enhanced expression of STAT3 target genes may reflect the activation of specific tyrosine kinase pathways, such as those downstream of the EGF or PDGF receptors (Mizoguchi, 2006). Thus, gliomas displaying a STAT3 signature might be particularly sensitive to the use of an appropriate kinase inhibitor. Second, given the particularly poor prognosis of tumors with a STAT3 signature, and the possibility that STAT3 targets may promote invasion and

angiogenesis, a more aggressive therapeutic strategy may be warranted in patients with this subset of gliomas. Finally, as STAT3 inhibitors are developed, tumors expressing a STAT3 gene expression signature may be most appropriate for initial clinical trials (Frank, 2006).

Advances in the treatment of gliomas will likely arise from an enhanced understanding of the molecular pathogenesis of these tumors, and developing the means to target specific signaling abnormalities. The finding that STAT3 is activated commonly in gliomas, and that a STAT3 gene expression signature connotes a worse prognosis, reflects the likelihood that STAT3 target genes underlie key aspects of the biology of these tumors. Focusing on STAT3 and its target genes may provide critical information for the molecular characterization of gliomas and for the development of targeted therapeutic interventions.

Acknowledgements

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