Emerging concepts in glioma biology: implications for clinical protocols and rational treatment strategies

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Glioblastoma multiforme (GBM), the most common primary central nervous system neoplasm, is a complex, heterogeneous disease. The recent identification of stem cells in murine tumor xenografts that were capable of recapitulating the tumor phenotype adds a new dimension of complexity to the already challenging treatment of patients with GBMs. Although specific cellular and genetic changes are commonly associated with GBM, the mechanism by which those changes occur may have a significant impact on treatment outcome. Of the many bioinformatics techniques developed in recent years, gene expression profiling has become a commonly used research tool for investigating tumor characteristics, and the development of rationally targeted molecular therapies has also accelerated following the initial success of specifically designed inhibitors in the treatment of malignancies. Despite these advances in research techniques and targeted molecular therapies, however, limited clinical impact has been achieved in the treatment of infiltrative malignancies such as GBMs. Thus, further extension in survival of patients with GBMs may require use of multiple analyses of tumors to develop tailored therapies that reflect the inter- and intratumoral heterogeneity of this disease. In this review, the authors briefly consider the potential use of expression profiling combined with mutation analysis in the development of treatment modalities to address the heterogeneity of this complex tumor phenotype.

KEY WORDS • glioblastoma multiforme • microarray • stem cell • targeted therapy

OVERVIEW

Glioblastoma multiforme is the most common primary central nervous system neoplasm, accounting for more than half of all such tumors. Despite advances in survival for many patients with malignancies, the prognosis for those with GBM remains poor, even with modern aggressive intervention, including resection, radiotherapy, and chemotherapy. The overall 2-year survival rate is 25% at best, and 5-year survival rates remain in the low single digits. Thus, it is clear that new treatments are needed to overcome the limitations of conventional therapy. Emerging evidence indicates that patient-specific therapies tailored to the unique biology of an individual’s GBM may be required to achieve significant improvements in clinical outcomes. In this article we briefly discuss emerging insights into glioma cell biology and common genetic mutations. In addition, we highlight how understanding the cell biology and gene expression profiles of each patient’s GBM could be useful in implementing more effective treatment.

ASPECTS OF GBM

Cellular Characteristics

The GBM is a diffuse and infiltrative tumor. Migrating GBM cells can be found several centimeters away from the margins of resection. Accordingly, although tumor recurrence predominantly occurs near the primary resection site, it can arise in distant regions of the brain. The GBM is composed of genetically and morphologically diverse cells, not only among different patients, but also among cells within the same tumor. Furthermore, any therapy that relies on cell division to kill tumor cells is complicated by the fact that only a fraction of glioma cells are actively mitotic during a given treatment window.

Mounting evidence indicates that, similarly to leukemia and breast cancer, GBM tumors are composed of stem cell–like precursor cells and also more differentiated tumor cells. The former, which we will call BTSCs, are endowed with many features of NSCs, including the ability to generate daughter cells with different phenotypes from a single mother cell, the capacity to differentiate into a diverse population of cells, and expression of NSC markers. Moreover, BTSCs have been isolated from human GBMs that can form tumors in the brains of mice that bear a morphological resemblance to the...
original human tumor. These BTSCs demonstrate immunoreactivity for CD133, a primitive progenitor cell marker, and appear to be the tumor-initiating cells not only in mouse xenografts but also potentially in spontaneous human GBM. Furthermore, when BTSCs were induced to differentiate in vitro, they retained the ability to form neurospheres when cultured in stem cell–supportive media, an ability that is unique to BTSCs. Thus, BTSCs appear to have the capacity to revert from a terminally differentiated state back to a more primitive state, a characteristic distinct from normal NSCs and consistent with cells found in other malignancies.

The ability of cells isolated from human tumors to self-renew and contribute to various cell types in both in vitro and in vivo tumorigenesis models establishes a potential mechanism for the complex heterogeneity characteristic of GBM and highlights the need for therapeutic strategies targeting not only malignant, more differentiated GBM tumor cells, but also GBM stem cells that likely contribute to the inevitable recurrence of this disease. The potential phenotypic differences between GBM stem cells and normal NSCs may be subtle, and may in fact be undetectable by currently used clinical tests. Additionally, the source of BTSCs may not be the tumor itself. In at least one study in which a spontaneous mouse model of GBM was used, it was found that tumor-initiating cells might migrate from a separate location within the brain, only to repopulate an area with a favorable microenvironment for tumor growth. Although the combination of invasiveness, the ability to become migratory, and complexity of cellular composition in human GBMs represents a formidable challenge for effective therapy, the identification of BTSCs provides a rational target for new therapies, because these cells appear to be the tumor-initiating component of GBM.

Complex Heterogeneity and Genetic Mutations

Numerous genetic mutations have been identified in GBM that have been postulated to contribute to gliomagenesis. Alterations in tumor suppressors are the genetic lesions most commonly found. Retinoblastoma protein, the \( RB-1 \) gene product, is present ubiquitously and at relatively constant levels throughout the body. In its dephosphorylated state, this protein functions as a tumor suppressor by sequestering molecules that normally promote progression through the cell cycle. Cell proliferative signals, such as RTK activation through ligand binding, in turn promote phosphorylation of RB protein in the nucleus. The progressive state of phosphorylation causes RB to release bound transcription factors such as E2F proteins, allowing them to drive progression of a cell through the mitotic cell cycle. Thus, RB plays a key role in modulating cell division, largely based on its level of phosphorylation. The \( RB \) gene is commonly mutated or deleted in primary human GBM specimens, thereby implicating RB-mediated cell cycle regulation in the progression of GBM.

Deletion or alteration of the \( p53 \) tumor suppressor is present in 25 to 30% of GBMs and has also been extensively studied in these tumors. The \( p53 \) gene is not commonly expressed at high levels in healthy tissues; its expression is induced by a variety of mechanisms usually implicated in the induction of DNA damage, like that caused by radiation exposure. When present in the cell, \( p53 \) promotes the transcription and translation of proteins that block progression through the cell cycle, allowing DNA damage to be repaired. Loss of \( p53 \) allows cells with DNA damage to progress through the cell cycle unchecked, perpetuating or compounding the DNA damage. Direct mutation, deletion, or loss of expression of \( p53 \) is commonly observed in GBMs, as in many other solid tumors.

Furthermore, changes in genes upstream of \( p53 \) in this pathway are also commonly found in tumors. Amplification or overexpression of \( MDM2 \) occurs in 10 to 15% of GBMs, resulting in a blunted or absent \( p53 \) response, and has in some studies been associated with poor prognosis in patients with GBM. The \( MDM2 \) binds to and sequesters \( p53 \), promoting its rapid degradation within the cell, thus preventing transcription of antiproliferative genes. The \( MDM2 \) is in turn regulated by another gene product, human p14ARF (called ARF in this paper); ARF antagonizes \( MDM2 \), releasing \( p53 \) inhibition to induce cell cycle arrest.

Although \( ARF \) is located in the \( INK4A \) genomic locus and shares exons 2 and 3 with \( p16INK4A \), it differs in structure and function due to a frameshift caused by splicing from an alternative exon 1. This second gene encoded by the \( INK4A \) locus, \( p16INK4A \), also functions as a tumor suppressor, but with a different mechanism and specificity than \( ARF \). The \( p16INK4A \) locus indirectly regulates RB by blocking the action of cyclin-dependent kinases, preventing phosphorylation of RB, and maintaining RB-mediated inhibition of cell cycle progression. Thus, each of the genes encoded by the \( INK4A \) locus regulate tumor suppressors, and loss of each has been shown to cause transformation of cells to a malignant phenotype. Therefore, both are considered tumor suppressors and have been implicated in gliomagenesis. Recent studies in mice indicate that \( ARF \) may play a more prominent role in gliomagenesis than \( p16 \), although there may be differences in the function of these proteins in mice compared with humans.

Separate studies have demonstrated variability of gene expression in different sets of GBMs. In particular, tumors expressing the EGFR, which is associated with a particularly poor prognosis, can clearly be identified by microarray expression analysis. In other studies, investigators have been able to identify subtypes of GBM based on their molecular signature. Some of these researchers have further demonstrated that overexpression of specific genes correlates with a more favorable prognosis, although the mechanism by which they were overexpressed was not evaluated. In one study, expression analysis was correlated with copy number changes determined using comparative genomic hybridization, and global gene expression changes were observed in genes not associated with chromosomal regions implicated in gene amplification or deletion. Specifically, copy number changes in genes located on chromosome 10 were associated with global gene expression changes. Interestingly, loss of heterozygosity at the chromosomal location 10q23 is a common feature in GBM.

Chromosomal deletions, mutations, and amplifications all play roles in the development of malignancies, including GBMs. Many GBMs may have common features attributable to these genetic changes. Nevertheless, the circuitous path each individual tumor has followed to reach malignant transformation is likely unique to that tumor and patient, and thus may require therapeutic strategies tailored specifically for each patient and GBM.
Aberrant Signal Transduction

In addition to loss of tumor suppressor function, gain of function changes also occur in GBMs and, as in other tumors, often affect signal transduction pathways (Fig. 1). The EGFR is an RTK that is commonly overexpressed in GBMs, and this overexpression typically occurs as a result of gene amplification.\textsuperscript{4,14,52} The EGFR-mediated signal transduction results in activation of a number of downstream pathways including PI3K-AKT and RAS–MAPK, inhibiting apoptosis and driving proliferation.\textsuperscript{14} Alternatively, deletion within the \textit{EGFR} coding sequence can result in the expression of a truncated, mutant EGFR protein that signals constitutively.

Other mutations common in GBM affect RTKs other than EGFR. Expression of PDGFR\textbeta \textsuperscript{31} as well as its ligand is frequently found in GBMs.\textsuperscript{31} The PDGFR\textbeta \textsuperscript{31} activates RAS–MAPK and PI3K-AKT as well as JAK-STAT signaling, which is involved in cell survival, proliferation, and differentiation.\textsuperscript{31} Specific dysregulation of RAS-MAPK caused by loss of the \textit{NF1} gene in neurofibromatosis Type 1 syndrome has been associated with an increased risk of malignant astrocytomas in affected individuals.\textsuperscript{53} Therefore, the RAS-MAPK signaling cascade has been presumed to be important for gliomagenesis. Recent evidence indicates, however, that mutant EGFR and overexpressed wild-type EGFR signal preferentially down divergent pathways in GBM: down RAS-MAPK and PI3K-AKT pathways, respectively.\textsuperscript{52}

Inducible models of tumorigenesis in mice have yielded findings that particular tumors become dependent on specific mutations, and that repression of the initiating oncogenic stimulus can result in widespread apoptosis and tumor regression.\textsuperscript{8,10,18,30} Targeting of specific molecular defects on which tumor cells rely is the goal of many novel therapeutic strategies (Table 1). Many of these strategies, however, depend on comprehensive mutation or expression analysis. Because RTKs and signaling pathways represent attractive targets for molecular therapeutics, distinguishing the nature of pathway activation in individual tumors will be important for the selection of the appropriate therapeutic modality. Again, the pathway to complete malignant transformation is likely unique in every patient and, although similar pathways are implicated in gliomagenesis by changes in various upstream signaling molecules, the overall mechanisms of pathway activation in

Fig. 1. Schematic depicting signaling with the growth factor receptors through the JAK-STAT and RAS pathways. (Signaling occurs through a number of pathways, including JAK-STAT and RAS-MAPK.) Known interactions between specific effectors are indicated by \textit{bold arrows}, and \textit{arrowheads} indicate downstream signaling. Similar schematics could be drawn depicting RTK signaling, but there would be no dependence on JAK activation, because many RTKs can activate STATs directly. Evidence indicates that SHP-2–dependent signaling activates PI3-K or RAS-MAPK differentially from wild-type EGFR or EGFRvIII. GDP = guanosine 5’-diphosphate; GTP = guanosine 5’-triphosphate.
each tumor may play an important role in the ultimate malignant phenotype. Analysis of the pathways involved in individual tumors should therefore be performed to assist in the selection of therapies that address the unique biology of the specific tumor.

For instance, molecular inhibitors of MDM2 have been recently developed for clinical use. These compounds, called “nutlins,” interfere with the interaction between MDM2 and p53, releasing p53 from negative regulation and allowing transcription of cell cycle inhibitory molecules.20 Because MDM2 is the second most commonly amplified gene in GBM, use of nutlins in this disease seems a rational therapeutic strategy. Nevertheless, this approach is likely to be most effective only in patients with MDM2 overexpression or low p14ARF expression. Clinical trials using nutlins as a component of therapy should therefore be designed to include expression and mutation analysis of these two genes as a criterion for patient selection.

Expression Analysis for Rational and Patient-Tailored Therapy

An increasing body of evidence demonstrates the utility of expression profiling in stratification of patients with GBM in terms of tumor classification and survival. Unfortunately, a marker of survival in GBM is a relative term that belies the fact that less than 1% of patients survive more than 5 years.2122 Although identification of markers relevant for patient stratification will undoubtedly contribute to improvements in treatment modality, it is important to expand these studies to include the identification of rational therapeutic targets, which seems a more compelling goal in evaluation of GBM at the transcriptional level.

In addition to intertumoral heterogeneity, studies evaluating differential gene expression within the same tumor have demonstrated intratumoral heterogeneity between core GBM tissue and invasive, proliferating peripheral tumor tissue.18 Expression differences in one study correlated with differences in the intensity of contrast enhancement on magnetic resonance imaging.15 This intratumoral heterogeneity again highlights the need for multifaceted modalities addressing all aspects of the biology of GBMs. These studies demonstrate the utility of expression profiling in the stratification of patients as well as the selection of targeted molecular and gene therapies used for successful treatment of this disease.

The identification of BTSCs as candidates for the true tumor-initiating cells poses an interesting challenge for conducting routine, high-throughput gene expression analysis of GBMs to tailor therapy to rational targets. It is vital to kill the BTSCs as well as the more differentiated tumor cells to achieve the maximum antitumor effect. Accordingly, it may be critical to purify CD133-positive BTSCs for expression analysis to determine which class of drugs is most likely to kill these cells. In addition, expression analysis could be conducted in the CD133-negative tumor cells to identify a second drug tailored for their elimination.

**CONCLUSIONS**

In-depth expression analysis with respect to mutation status has not been performed with GBMs to the same extent as with other cancers.2324 With the development of molecular inhibitors targeting multiple pathways implicated in gliomagenesis, the combination of expression analysis and mutation status may be the next step in enabling the development of therapeutic modalities better tailored for individual disease characteristics. It is clear that the appropriate drugs for GBM will not be the same for all patients, and that choosing the best drug may require routine expression analysis. Therefore, expression analysis may be most useful as a standard clinical test performed before drug therapy commences to choose rationally patient-tailored drugs that target the appropriate molecular pathways. For instance, expression analysis could be conducted immediately after resection in multiple biopsy samples of core and invasive regions of the tumor, perhaps identified before resection on magnetic resonance imaging.

Implementing expression analysis as a standard clinical test, however, will require significant changes in the way clinicians approach GBM treatment, as well as the development of substantial infrastructure for rapid, high-throughput expression and mutation analysis of tumor tissue. The challenge of interpreting large data sets generated from routine, high-throughput array analysis from individual tumors will require an expansion of expertise from academic, research-oriented studies to wider clinical practice. Despite these obstacles, it is vital to take into account the genetic mutations present in the patient’s individual tumor before the use of drugs that target molecular pathways can ever be expected to achieve maximal clinical efficacy. Furthermore, it is important to consider that GBMs are composed of a group of related but genetically distinct tumor cells, containing both BTSCs and differentiated tumor cells, and capitalize on this knowledge by using therapies that target all populations present in the tumor.

**Table 1**

<table>
<thead>
<tr>
<th>Authors &amp; Year</th>
<th>Phenotypic or Genetic Change</th>
<th>Targeted Therapy</th>
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<tbody>
<tr>
<td>Carroll, et al., 1997</td>
<td>PDGFR/PDGFR overexpression</td>
<td>Imatinib mesylate (Gleevec)</td>
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<td>Debinski, et al., 1999</td>
<td>IL13R overexpression</td>
<td>Immunotoxins</td>
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<tr>
<td>Hussain, et al., 2001</td>
<td>IL13R overexpression</td>
<td>Immunotoxins</td>
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<td>EGFR amplification, EGFRvIII</td>
<td>OSI-774, PK1166, C11033, EKB569</td>
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<tr>
<td>Shawver, et al., 2002</td>
<td>EGFR amplification, EGFRvIII</td>
<td>OSI-774, PK1166, C11033, EKB569</td>
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<tr>
<td>Ferrara, et al., 2004</td>
<td>VEGF overexpression</td>
<td>Antiangiogenesis</td>
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<td>ARF loss</td>
<td>Nutlins</td>
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<td>PTEN loss</td>
<td>CCI-779, RAD001</td>
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<td>CCI-779, RAD001</td>
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<tr>
<td>Ohlfest, et al., 2005</td>
<td>VEGF overexpression</td>
<td>Antiangiogenesis</td>
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* VEGF = vascular endothelial growth factor.

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