Pathology: Commonly Monitored Glioblastoma Markers: EFGR, EGFRvIII, PTEN, and MGMT

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KEYWORDS

• EGFR • EGFRvIII • PTEN • MGMT • Glioblastoma

In 1926, Bailey and Cushing established the first widely accepted classification scheme of astrocytic neoplasms.[1] Since then, neuropathologists have worked to improve this classification system to guide clinicians with diagnostic and prognostic information. Recent advances and discoveries in immunocytochemistry markers, radiographic imaging modalities, and genetic/molecular markers have helped further characterize these tumors. Many of these advances have not yet changed the management of astrocytic neoplasms. Nevertheless, they have provided a wealth of information and continue to challenge current understanding of tumor biology and patient management while providing insight into possible novel therapeutic strategies.

The most common astrocytoma, glioblastoma (GB), is also the most malignant primary brain tumor in adults.[2] There are 2 types of GBs distinguished by their origin and molecular phenotype: primary (de novo) and secondary tumors. De novo cases represent the majority (>90%) of GB patients and develop rapidly over the course of weeks, presenting as a grade IV tumor. Secondary GBs present as lower-grade gliomas (grade II or III) and eventually progress to grade IV. Regardless of its classification, once a diagnosis of GB has been made, the overall median survival time for patients treated with surgery and concomitant radiation plus temozolomide, followed by adjuvant temozolomide, is approximately 15 months.[3]

It is still unclear which molecular and cellular alterations transform a normal cell into tumorigenic GB cells. Previous research has begun to identify and elucidate the molecular pathways that are often perturbed in GBs. These signaling pathways can be therapeutically beneficial because they not only help identify and classify glioma tumors but also may provide novel targets for therapy. This article highlights and reviews 4 important GB molecular markers: epidermal growth factor receptor (EGFR), EGFR variant III (EGFRvIII), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), and O^6-methylguanine-DNA methyltransferase (MGMT).

EGFR

Background on EGFR

EGFR is a cell surface transmembrane tyrosine kinase (TK) receptor that belongs to a family of 4 related receptors: ErbB1/EGFR, ErbB2/Neu/Her2, ErbB3/Her3, and ErbB4/Her4.[4] All members of this family contain 3 basic components: an extracellular ligand-binding domain, a transmembrane portion, and an intracellular TK domain.[5] Upon the binding of a ligand, the receptor transforms from an inactive monomer into a catalytically active homodimer that autophosphorylates its own C-terminal tyrosines.[6] This dimerization stabilizes the active receptor confirmation and generates a docking site for proteins to be phosphorylated

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by the now-active TK domain. Activation of EGFR leads to phosphorylation of downstream proteins, including phosphatidylinositol 3-kinase (PI3K), AKT, RAS, RAF, and mitogen-activated protein kinases. These downstream proteins have been associated with cell division, migration, adhesion, differentiation, and apoptosis, making EGFR an important player in tumorigenicity.[7]

EGFR signaling has been implicated in the pathogenesis of many human cancers, including head and neck, ovarian, cervical, bladder, esophageal, gastric, breast, endometrial, colorectal, and GB.[8,9] Often, EGFR expression or activity is enhanced in tumors through gene amplification, aberrant activity through autocrine overproduction of the receptor ligands, or mutations to the *EGFR* gene. In GBs, EGFR is overexpressed in approximately 40% to 50% of cases, and clinical and research studies show that tumors with overexpressed or amplified *EGFR* exhibit worse prognosis, increased tumor aggressiveness, and resistance to therapeutic treatments.[4] Furthermore, in vitro studies have shown increased resistance to radiation therapy in immortalized GB cell lines stably transduced with EGFR.[10,11] *EGFR* gene amplification is 5-fold higher in primary GBs compared with secondary tumors, and EGFR overexpression occurs in approximately 60% of primary cases, although this is found only in 10% of secondary GBs.[12]

Because aberrant EGFR activity plays an important role in malignant transformation, new therapeutic strategies have been developed targeting this gene. For instance, small-molecule TK inhibitors and monoclonal antibodies (mAbs) have been studied in clinical trials. Recently, newer treatments, including RNA-based therapies, ligand-toxin conjugates, and radioimmunoconjugates, have had promising preclinical results.[13–19]

EGFR Inhibitors Targeting the ATP-Binding Pocket

One of the earliest and most common methods of inhibiting EGFR is the use of small molecules that bind to the ATP-binding pocket of the TK domain, thereby preventing autophosphorylation and subsequent activation of the signal mechanism. Imatinib and lapatinib are two examples of molecular inhibitors of tyrosine kinases (others include erlotinib and gefitinib) that were originally designed to target similar TKs (ABL and HER2, respectively) but were found to also inhibit EGFR. Phase I and II trials investigating these inhibitors in GBs have demonstrated only modest clinical effects.[20,21] Even with small molecules that specifically inhibit EGFR, such as erlotinib and gefitinib, the clinical results are modest. In a phase II trial, 38 recurrent GB patients were treated with erlotinib monotherapy after radiotherapy. Patients were found to have a median progression-free survival (PFS) of 8 weeks, with only 3% of patients meeting a target goal of PFS at 6 months.[22] A separate, randomized phase II trial of 110 patients with progressive GB after prior radiotherapy showed that only 11.4% of erlotinib-treated patients with recurrent GB had PFS after 6 months compared with 24% of patients in the control arm treated with temozolomide or carmustine (BCNU).[23] No significant difference in overall survival was observed between the 2 treatment arms. When gefinitib was used as the main treatment in a phase II trial, 6-month PFS occurred in 13% of the GB patients with no significant increase in median overall survival compared with historical controls.[24,25] In another phase II trial involving 98 newly diagnosed GB patients treated with adjuvant gefitinib postradiation, the overall survival at 1 year was 54.2% and PFS at 1 year was 16.7%, results that are not significantly different compared with historical controls.[26] In order to enhance the efficacy of gefitinib, a combinational study with an inhibitor to a mammalian target of rapamycin (mTOR) was also conducted. mTOR is located downstream of 2 well-known EGFR substrates, AKT and PI3K, and by combining the 2 inhibitors the goal was to inhibit the PI3K/AKT signaling pathway in concert with EGFR antagonism. In a phase I trial, patients with recurrent, high-grade gliomas treated with gefitinib and sirolimus (an mTOR inhibitor) showed that 44% of the patients achieved either partial response or stable disease, with PFS similar to that in a separate phase II study involving gefitinib treatment alone.[25,27] More recently, the phase II trial of erlotinib plus sirolimus in adults with recurrent GB showed negligible activity.[28] Another study using gefitinib and everolimus treatment in 22 patients with recurrent GB showed 36% of patients with stable disease and 14% with a partial response but only one patient with PFS at 6 months.[29]

Monoclonal Antibodies Blocking EGFR Ligand Binding

Another method of inhibiting EGFR activity is by blocking the ligands that bind to the EGFR (epidermal growth factor, transforming growth factor, heparin-binding epidermal growth factor–like growth factor, amphiregulin, betacellulin, epiregulin, and epigen). mAbs, such as cetuximab and nimotuzumab, were devised to compete with EGF binding and were shown in vitro to decrease the downstream signaling cascade of EGFR.

Cetuximab in preclinical studies was shown to inhibit growth and increase apoptosis in GB cell lines.[30,31] There are conflicting data, however, regarding whether EGFR amplification imparts cetuximab sensitivity.[30,32] In a phase I/II trial (GERT), 17 patients with confirmed GB pathology underwent standard postoperative treatment of radiation and temozolomide, followed by weekly infusions of cetuximab. The median follow-up was 13 months in this group. At 6 months, 81% of patients had PFS, and at 12 months 87% of patients were still alive. In another study, cetuximab was used to treat patients with recurrent high-grade glioma after surgery, radiotherapy, and chemotherapy. Patients were stratified into 2 treatment arms according to EGFR amplification status. A total of 55 patients underwent treatment with cetuximab (28 with and 27 without an increased EGFR copy number). The median duration of PFS was 1.9 months, and the median overall survival was 5 months. The rates of 6-month PFS and overall survival were 10% and 40%, respectively.[33] Lastly, cetuximab has also been tried in a phase II trial in combination with bevacizumab (an mAb that inhibits vascular endothelial growth factor A) and irinotecan (an inhibitor topoisomerase) for patients with primary GBs after tumor progression after radiation therapy and temozolomide treatment. The mean duration of overall survival observed was 29 weeks, with a mean time to tumor progression of 24 weeks. Thirty percent of patients were free of tumor progression at 6 months.[34] Overall, use of cetuximab has shown limited activity in the GB patient population as seen through the results of various phase I/II trials.

Studies using nimotuzumab, which targets the extracellular domain of EGFR, found it to have both antiangiogenic and proapoptotic effects. A phase I trial using nimotuzumab and whole-brain radiation in 28 individuals with newly diagnosed high-grade gliomas after tumor resection (16 with GB) found an objective response rate of 7.9% (defined as either a complete or partial response).[35] Furthermore, the median duration of overall survival observed was 22 months with a median follow-up of 29 months. The results are promising but require further studies, including a randomized control trial to assess efficacy.

In order to generate a better GB therapy, EGFR mAbs were attached to cytotoxic agents and evaluated in clinical trials. mAb-425 (a murine mAb raised against human carcinoma cells that express high levels of EGFR) was conjugated with sodium iodide I 125 and was used to treat high-grade gliomas in a phase II study of 180 patients with either GB or astrocytomas with anaplastic foci.[36] After radiolabeled mAb-425 was administered after surgery and radiation therapy, the median duration of overall survival rates of patients with GB or astrocytomas with anaplastic foci was 13.4 and 50.9 months, respectively.

EGFRvIII

Background on EGFRvIII

There are at least 10 classes of EGFR mutation variants described in gliomas. EGFRvIII is the most common variant of EGFR and is present in approximately 24% to 67% of GBs.[37,38] EGFRvIII results from an in-frame deletion corresponding to exons 2–7 of the *EGFR* gene, resulting in the deletion of a large portion of the extracellular domain.[39] Despite the loss of the ligand-binding region, EGFRvIII can homodimerize and autophosphorylate, rendering it constitutively active. In vitro studies have demonstrated that EGFRvIII expression can enhance cellular growth and tumorigenicity, indicating that this mutant receptor is oncogenic.[40] The molecular mechanism by which EGFRvIII can transform normal cells into malignant brain tumors is not completely understood, but expression of EGFRvIII was found to correlate with increased activity of PI3K.[41,42]

Clinical studies of EGFRvIII revealed that this mutant EGFR expression is expressed in a variety of tumors, including medulloblastomas, non–small cell lung carcinoma, breast cancer, and GBs.[43] In addition, many in vitro and in vivo studies show that EGFRvIII is specific to tumors and not present in normal cells, making it an intriguing biomarker for cancer. Yet, although its expression is cancer specific, there is currently a debate over the prognostic relevance of EGFRvIII in patients with GB. In some studies, EGFRvIII has not been found an independent prognostic indicator of survival.[44–46] In other studies, however, results have been inconclusive or EGFRvIII found an unfavorable predictor of survival.[47–49] For instance, a recent study showed no significant change in short-term GB survivors in patients with EGFRvIII expression, 0.96 to 1.07 years. In long-term GB survivors, there was a negative correlation in which patients who were EGFRvIII positive had a survival of 1.21 years, whereas EGFRvIII-negative patients survived on average 2.03 years.[50,51] Despite this controversy, EGFRvIII is an attractive target for malignant gliomas and a variety of therapeutics have been developed toward this gene.

Monoclonal Antibodies to EGFRvIII

Because EGFRvIII is expressed on GB and not in normal brain, mAbs have been developed to target and destroy EGFRvIII-expressing tumor cells. Two

such antibodies, mAb 806 and mAb Y10, have shown promising preclinical results as evident by a significant reduction in tumor volume in vivo and prolonged length of survival in mice.[52–55] Consistently, EGFRvIII mAbs conjugated to cytotoxic agents decrease glial tumor growth in mice,[56] and radiolabeling of EGFRvIII mAbs increased glial cell death.[57] Although these reports indicate that EGFRvIII mAbs may be an attractive GB therapeutic agent, no clinical trials have been conducted on their efficacy in humans.

EGFRvIII Vaccine Treatment

Another active area of research has been the development of an EGFRvIII vaccine for GB patients. The preclinical studies had promising results that culminated in series of recent clinical trials evaluating active immunization with an EGFRvIII peptide.[58–61] During accrual for the ACTIVATE study, the Stupp and colleagues[62] study was published and the addition of postoperative temozolomide became standard of care in treatment of GB patients. In response, the ACT II trial was started. Twenty-one patients with newly diagnosed GB who underwent gross total resection followed by concurrent radiotherapy and temozolomide were recruited for treatment with the EGFRvIII peptide vaccine, now called rindopepimut (CDX-110). The median PFS of all patients was 15.2 months, and the overall survival from time of diagnosis was 23.6 months. After adjustment for age and Karnofsky performance status, the risk of death of the vaccinated patients was significantly lower than that observed in the temozolomide-treated historical control group. This trial also evaluated if lymphopenia from temozolomide inhibits the immune response from the peptide vaccination.[63] Patients received either a standard 5-day temozolomide schedule or a daily dose-intensified regimen. Although the dose-intensified group exhibited more profound lymphopenia, those patients mounted a stronger immune response. Histologic samples were available for EGFRvIII expression by immunohistochemical analysis from 12 of 17 recurrent tumors. Eleven of these samples had lost expression of EGFRvIII.

As a result of these promising trials, ACT III was started as a multicenter single-arm trial evaluating the peptide vaccine in 65 patients with newly diagnosed EGFRvIII-positive GBs enrolled after gross total resection and standard chemoradiation. The preliminary results demonstrate a median overall survival of 24.3 months from the initial diagnosis. Furthermore, more than 30% of patients have survived more than 36 months from diagnosis. ACT IV, a multicenter international, randomized, placebo-controlled trial was verified in January of this year to begin enrolling patients.

PTEN

Background on PTEN

One of the most frequent genes that is either mutated or deleted in tumors is PTEN. PTEN was originally identified in 1997 as a tumor suppressor that was mutated in a variety of cancers, including prostate, breast, and GB.[64] PTEN is a phosphatase that controls the activity of an upstream regulator of PI3K, phosphatidylinositol (3,4,5)-trisphosphate. PTEN plays an important role in regulating cell growth by regulating kinases, such as PI3K, and subsequently AKT.

PTEN Role in GB Cell Lines

Much of what is known about PTEN in GB has been done in cell lines and mouse models. PTEN deletions in astrocytic cell lines were found to have increased proliferation,[65,66] whereas reintroduction of PTEN to GB cell lines deficient in PTEN suppressed proliferation.[66,67] PTEN has also been implicated in migration and invasion, traits particularly notorious in GBs. For instance, *PTEN* gene deletions in early neural precursors resulted in profound neuronal migration defects.[68,69] Additionally, glioma cells deficient in PTEN demonstrated reduced cell invasiveness when they were treated with PI3K inhibitors or through the overexpression of PTEN. Together these findings demonstrate that PTEN is involved in a variety of cellular processes in GBs, including cell growth, survival, and tumor invasiveness.

Clinical Significance of PTEN in GBs

Clinical studies monitoring PTEN expression in GBs indicated that there could be a correlation between PTEN loss and poorer patient prognosis. Prior studies have shown that pediatric patients with PTEN mutations found in pediatric malignant astrocytomas have a poorer prognosis.[70,71] In adults, loss of chromosome 10q has been found to negatively affect survival for both high-grade gliomas and GB independently.[72–74] Unfortunately, the conclusions drawn from these studies are limited due to small sample size, and further studies must be conducted to conclude whether PTEN loss is a harbinger of poorer outcome. Loss of chromosome 10q occurs more frequently than genetic mutations (70% vs 25%). A study by Zhou and colleagues[75] found that 28% of GBs, 7% of anaplastic astrocytomas, and 0% of low-grade gliomas had PTEN mutations. The

lack of PTEN mutations in low-grade gliomas was validated in several other studies and suggests that PTEN loss most likely does not promote a growth advantage early in GB development but could be linked to increased invasiveness.

Although the clinical relevance of PTEN is still unclear, a recent report studying drug efficacy in GB patients indicated that PTEN might be a useful biologic marker. In this study, a correlation between coexpression of EGFRvIII and intact PTEN in recurrent malignant glioma patients was found to predict sensitivity to EGFR inhibitor monotherapy.[76] Because PTEN expression decreases PI3K activity, then mTOR inhibitors that inhibit the PI3K pathway are expected to function in a similar fashion if used in combination with EGFR inhibitors—both lower PI3K levels. As discussed previously, however, in phase I and II trials, EGFR inhibition (ie, gefitinib) in combination with mTOR inhibitor therapy has not proved effective in GBs.[27,28] It is possible, however, that positive outcomes could have been missed because coexpression of EGFRvIII and intact PTEN was not specifically selected for in the 2 clinical phase trials.

MGMT

Background on MGMT

MGMT is a highly conserved protein involved in DNA repair. The enzyme protects cells against DNA damage by reversing alkylation at the O^6 position of guanine.[77] Specifically, the transfer of an alkyl group to the active site of the enzyme results in the removal of DNA base pairs incorrectly bound to thymidine. If this correction is not made, the cell undergoes apoptosis or cellular senescence.[78] Consequently, the presence of MGMT enhances cell survival after DNA damage.

Clinical Significance of MGMT in GBs

MGMT became clinically relevant in GBs after reports showed some predictive value attributed to the absence of MGMT in patients undergoing chemotherapy with alkylating agents. It was observed that patients with low levels of MGMT derived considerable benefit from BCNU compared with patients with higher levels of MGMT expression.[79,80] In addition, low levels of MGMT protein could predict prolonged PFS in patients with glioma who were treated with temozolomide.[81]

Because there was mounting evidence that MGMT expression predicted GB patient outcome, researchers began studying how this gene is regulated in GBs. Epigenetic methylation at the MGMT promoter region was discovered to decrease transcription of MGMT, thereby making the cells vulnerable to DNA damage and cell death. The process that promotes DNA damage could also make mismatch repair-deficient cells more vulnerable to alkylating agents. Applying this logic, researchers subsequently investigated whether gliomas with increased levels of methylation at the MGMT promoter site were more susceptible to alkylating agents. Silencing the MGMT promoter has been observed in up to 93% of low-grade gliomas[82] and 45% of GBs.[83] These findings suggest that a large percentage of GB patients could be more responsive to chemotherapy if MGMT expression is decreased through methylation.

MGMT Promoter Methylation as a Prognostic Marker for GBs

The strongest support linking MGMT promoter methylation status to prognosis of patients with gliomas came from 2 large randomized clinical trials, the European Organisation for Research and Treatment of Cancer (EORTC) 26981/22981 and the National Cancer Institute of Canada (NCIC) CE.3 trial.[62,83] These trials demonstrated that MGMT promoter methylation was able to predict prolonged PFS in patients treated with temozolomide and radiotherapy. The 5-year follow-up data from the EORTC study further confirmed that MGMT methylation was predictive of prognosis in patients with GB.[84] In this study, the median overall survival for MGMT promoter methylated patients treated with radiotherapy and temozolomide was 23.4 months versus 15.3 months for those receiving radiotherapy alone. The median overall survival in patients with unmethylated MGMT promoters was 12.6 months in the radiotherapy plus temozolomide group and 11.8 months for the radiotherapy-alone group.

The heterogeneity of malignant GBs across patients, specifically the heterogeneity of MGMT promoter methylation, requires more attention and research. When this unresolved issue becomes clarified, it will undoubtedly stratify patients into further classes of treatment responders and lead to more distinct prognostic groups. Currently, methylation-specific polymerase chain reaction (MSP) remains the only test that has repeatedly shown predictive and prognostic value in identifying methylated areas in clinical trials.[85] Methylation occurs at CpG islands by epigenetic forces. Tests like MSP can detect the fraction of CpG islands that are methylated at the MGMT promoter site in patients with GBs. More recently, Shah and colleagues,[86] using quantitative bisulfate sequencing, determined the methylation status of all 97 CpG sites at the MGMT promoter site in tumor

samples from 70 newly diagnosed GB patients who subsequently underwent resection and radiation therapy with concurrent temozolomide adjuvant therapy. Of the 70 patients, 39 had 1-year PFS data based on which Shah and colleagues[86] were able to propose a new classification scheme using the methylation patterns observed.

Studies have also begun to show that variability of methylation can lead to affects in survival. For instance, a study by Krex and colleagues[87] showed that 75% of 5-year GB survivors demonstrated MGMT promoter methylation. In another study, patients with more than 29% MGMT methylation (over 12 CpG islands) had significantly better outcomes than those with less methylation.[88] These preliminary studies provide important insights not only into the variability between GB patients that has been known for years but also in regards to the variability between MGMT methylation in patients with respect to treatment responsiveness. Despite this new evidence, the picture remains incomplete and still complex. For instance, there are subsets of GB patients who survive well beyond 5 years who do not possess MGMT methylation, indicating other complex biologic processes and interactions in GB. Nevertheless, methylation of the MGMT promoter region seems an important factor influencing treatment responsiveness.

MGMT promoter methylation has emerged as one of the most important biomarkers of GB with respect to predictive and prognostic value, following the results of EORTC-NCIC clinical trials. Several questions, however, remain: (1) How static is the methylation status throughout temozolomide and radiation treatment? (2) How do variations in the degree of methylation affect outcomes? (3) What ultimately becomes the most sensitive test to analyze methylation status? (4) What other biomarkers along with MGMT further stratify prognosis in patients? and (5) How will this information be managed in clinical practice? (ie, Who will receive therapy and who will be encouraged not to seek treatment despite having a GB?) In the coming years, further insights into the role of MGMT promoter methylation in GB patients will provide answers to these questions, as improving treatment of the devastating disease, GB, continues to make strides.

MGMT Resistance to Temozolomide

Many GB cells develop chemoresistance to temozolomide, and MGMT is thought to play an important factor in GB resistance to temozolomide therapy. GBs that possess high expression of MGMT were found to have higher resistance to temozolomide.[89,90] To combat this resistance, recent studies have begun to use therapeutic agents to suppress, overcome, or sensitize MGMT activity. A recent phase II trial study used the psuedosubstrate O^6-benzylguanine concurrently with temozolomide in recurrent, temozolomide-resistant malignant glioma to overcome the MGMT activity. The investigators were unable to produce significant restoration of temozolomide sensitivity in GBs, however. Other investigators have attempted to overcome MGMT activity by using dose-intensified temozolomide regimens, but thus far, this method has not improved overall survival in these patients.

SUMMARY

To date, EGFR, EGFRvIII, PTEN, and MGMT are the most clinically relevant molecular markers in GB. This article reviews the biology and clinical studies for these markers. There is a great need to find more effective treatments for GB patients afflicted with this highly aggressive and biologically complex disease. As technology improves, more methods will be established to identify new and better diagnostic and prognostic markers. Advances in genomics, proteomics, and molecular understanding of GBs will enable novel therapeutic targets to be identified and tested.

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