Gliomas are the most frequent types of tumor occurring in the central nervous system and include astrocytoma (World Health Organization grades I–IV), oligodendroglioma (World Health Organization grades II–III), and ependymoma (World Health Organization grades I–III). The prognosis of the most common glioma, glioblastoma (GBM, World Health Organization grade IV), remains poor with 2-year survival rates at less than 20% despite significant advances in therapeutic options available to patients. With the advent of drugs targeting the epidermal growth factor receptor (EGFR) and its second messenger signaling pathways, it is important to both gain some understanding of the functional significance of these pathways and to understand the role the pathologist might play in characterizing the activation status of certain downstream messenger proteins that are targeted in these brain tumor therapies. We have reviewed the literature regarding histologic assays that have been incorporated into trials of these new drugs and report on the methods used to study these proteins and the conclusions of these studies.

**Data Sources.**—Literature review and primary material from Duke University (Durham, NC) Department of Pathology archives.

**Conclusions.**—To date, drug trial reports indicate that identification of the presence of the EGFR variant, EGFRvIII, and measurement of the activated downstream targets, phospho-Akt, phospho-S6, and phospho-MAPK, may be useful in predicting sensitivity to some of the EGFR kinase inhibitors. No studies to date have identified prognostic significance related to immunoreactivity status among any of these markers that is independent of histologic grade.
Figure 1. Second messenger pathways commonly exploited by epidermal growth factor receptor ligand binding.

In these systemic cancers, overexpression of EGFR has been associated with early relapse, aggressive growth, late-stage disease, chemotherapy resistance, hormone-therapy resistance or poor response, and poor outcome in general. A variety of mechanisms appear to control EGFR expression; however, the most important mechanism in gliomas appears to be polysomy of the \textit{EGFR} locus or amplification of the locus (as defined by more than 5 copies of the locus per nucleus).2,3

Epidermal growth factor receptor and various mutants are not the only growth factor receptors driving activation of the PI3K-Akt pathway. Growth factor receptors such as the platelet-derived growth factor receptors are also often overexpressed in malignant gliomas. Both EGFR and platelet-derived growth factor receptor are also implicated in downstream activation of the Ras-Raf-MAPK mitogenic protein kinase pathway, also known as the MAPK or extracellular signal-regulated kinase (ERK1/2) pathway (Figure 1). Both the PI3K and Ras pathways have been linked to tumor progression of gliomas.4,5

There are now many anti-EGFR therapies under development for the treatment of gliomas. However, as noted previously, targeting the receptor alone may not be sufficient as other parallel receptors are driving oncogenic pathways. In clinical trials, \textit{EGFR} expression, amplification, or mutation is poorly predictive of patient response to EGFR inhibitors, resulting in some \textit{EGFR}-expressing gliomas exhibiting resistance to anti-EGFR therapy. Therefore, investigators have taken to exploiting sensitivities of downstream targets in these pathways to downregulate the neoplastic messages being transmitted. For example, both Choe and colleagues6 and Goudar and colleagues7 have demonstrated that immunohistochemistry against phosphorylated, or activated, downstream messenger proteins can be used to measure the levels of activated downstream measures as well as to measure the effect of drugs' inhibitory actions. Further Goudar and colleagues7 found, in an experimental mouse model, that combination therapy that resulted in the inhibition of both EGFR and a downstream messenger target (mammalian target of rapamycin [mTOR]) offered greater benefit than single therapy alone.

Mellinghoff and colleagues8 and Hass-Kogan and colleagues9 have used histologic methods (immunohistochemistry and fluorescent in situ hybridization) profiling of \textit{EGFRvIII} and activated Akt to identify those patients who would respond to EGFR second messenger inhibitory drugs. The intention is that truly personalized cancer che-
motherapy can be tailored by using tissue biopsy material and histologic methods presently available.

**EPIDERMAL GROWTH FACTOR RECEPTOR**

In 1986, Stanley Cohen was awarded a Nobel Prize for the isolation of a protein from the submaxillary gland of mice that promoted accelerated eruption of incisors and eyelid opening in newborn animals; a protein that became known as epidermal growth factor. It is clear that most gliomas express both the ligands of EGFR, epidermal growth factor and transforming growth factor α, and the receptor, EGFR, forming an autocrine loop of growth stimulation. However, by far the most significant event in high-grade gliomas is the increased number and functional changes rendered to the receptor.

The genetic locus encoding epidermal growth factor receptor, EGFR, located on chromosome 7p12.3-p12.1, frequently demonstrates polysomy in all grades of astrocytoma and is amplified in some GBMs. Epidermal growth factor receptor mediates its effect on the cell predominantly through 2 second messenger pathways, the Ras-Raf-MAPK pathway and the PI3K-Akt pathway (Figure 1). In the latter pathway, EGFR acts to phosphorylate phosphatidylinositol-2-phosphate (PIP2) to PIP3, an action that results in the downstream activation of the Akt pathway. PTEN’s action is to dephosphorylate PI3K and effectively inhibit the growth factor signal. As described in more detail later, loss of this antagonism results in inhibited signaling of the apoptotic pathway, up-regulation of vascular endothelial growth factor production, and increased cell-cycle progression via loss of a G1 arrest signal. Expression of EGFR is frequent in low-grade gliomas. Polysomy of EGFR locus by fluorescence in situ hybridization (FISH) often is seen in aggressive low-grade gliomas and in anaplastic astrocytomas. Some suggest that percent positive cells exhibiting polysomy of EGFR may be useful in distinguishing among the grades of astrocytoma. It is clear that in the higher grade of astrocytoma, there is a higher percentage of cells exhibiting polysomy. This finding is distinctive from, but probably influenced by, mechanisms mediating true amplification that is found only in GBMs and very aggressive anaplastic astrocytomas.

In some GBMs, an EGFR variant characterized by an absence of the extracellular receptor domain and called EGFRvIII is expressed. Studies have shown that this protein preferentially activates the PI3K-Akt pathway but also signals a second messenger pathway(s) not available to wild-type EGFR. These studies indicate that EGFRvIII activation has numerous additional effects including the up-regulation of genes implicated in glioma migration and the down-regulation of the CDK inhibitor CDKN1b (p27). Indeed EGFRvIII is found in less than one third of tumors with loss of PTEN but is expressed in almost 90% of tumors with intact PTEN. However, recent studies have also identified other novel EGFR mutations that activate the extracellular kinase domains and relieve the autoinhibitory constraints on kinase activity thus identifying a novel mechanism for oncogenic EGFR activation.

**PTEN**

PTEN is one of the most commonly altered tumor suppressor genes in solid cancers. PTEN gene alterations are associated with aggressive tumor phenotypes. Among brain tumors, mutations and allelic deletions affecting PTEN have been linked to progression of astrocytoma to GBM. PTEN is a tumor suppressor gene located on the long arm of chromosome 10 at 10q23 that encodes a dual specificity phosphatase. PTEN antagonizes the activities of PI3K on the phosphatidylinositol (PIP2 and PIP3) in the PI3K-AKT pathway that affects control of the cell cycle and cell survival. Unrestricted PIP3 generation caused by loss of PTEN results in unopposed activation of Akt (protein kinase B), a crucial enzyme that is involved in controlling multiple downstream effectors including opposing the apoptotic pathway, increasing vascular endothelial growth factor production, and increased transit through the G1 cell-cycle phase, among others.

A number of studies have implicated losses of PTEN genetic dosage with a significantly worse survival in both adult and childhood high-grade astrocytoma patients. Although loss of heterozygosity of 10q has been found in up to 75% of GBMs, deactivating mutations of the remaining PTEN allele are found in only 20% to 40% of GBMs. However, it is clear that the prognostic effect of PTEN loss in a tumor is increased when the loss of PTEN is found in a patient whose tumor also exhibits an abnormality in the RB1 pathway. PTEN loss is mediated not only by a decrease in genetic dosage but also by a negative regulation of gene transcription by methylated CpG islands in the PTEN promoter region. Immunohistochemistry for the PTEN protein is being used both alone and in combination with FISH to evaluate PTEN status in individual tumors.

Thus, a hyperexpression of EGFR via polysomy, the truncating mutation of EGFRvIII, and PTEN hypoexpression via loss of heterozygosity of PTEN all result in overcoming the attenuating effects of PTEN control over the PI3K-Akt second messenger system.

**PI3K-AKT**

**AKT,** also known as protein kinase B, is an oncogene that not only plays a central role in cell survival but also regulates proliferation, motility, and expression of angiogenic factors. Akt is a kinase that phosphorylates numerous intracellular targets. Elevated levels of PIP3 results in the location of Akt to lipid signaling rafts in the cell membrane, which allow the activation of Akt. Later the enzyme translocates by unknown mechanisms to the cytoplasm and nucleus where its various substrates related to different pathways are located. The activation of Akt results in up-regulation of cell proliferation through the cyclin-dependent kinase inhibitors, p21Waf/Cip1 and p27Kip2, and protein synthesis through targets such as the mTOR and the S6 kinase (p70S6K). Activation of the tuberous sclerosis gene product TSC1 by phosphorylation further amplifies the downstream signaling of the PI3K-Akt-mTOR pathway.

Through inhibition of the glycogen synthase kinase 3, Akt prevents the phosphorylation of β-catenin, which impedes its degradation, allowing the β-catenin to accumulate in the nucleus to stimulate transcription of different transcription factors that increase the expression of genes such as cyclin D1 involved in the down-regulation of the cell-cycle inhibitor RB1.

Activated Akt promotes cell survival through inhibition of proapoptotic pathways, thus improving cell survival in the toxic environment related to the cancer’s own environment of oxidative and ischemic stress as well as inflicted DNA damage via chemotherapy and radiation therapy. It
Figure 2. A, High-grade astrocytoma used in subsequent immunohistochemical assays (hematoxylin-eosin, original magnification ×400). B, Anti–epidermal growth factor receptor (anti-EGFR) wild-type immunohistochemistry showing strong diffuse cytoplasmic immunoreactivity, a pattern that is frequently associated with EGFR genetic amplification (original magnification ×400). C, Anti-EGFRvIII immunohistochemical reactivity exhibiting strong cytoplasmic localization. EGFRvIII immunoreactivity is most commonly encountered in tumors also exhibiting amplification of the EGFR locus (original magnification ×400). D, Anti-phosphatase and tensin homolog (anti-PTEN) immunoreactivity demonstrating 80% of tumor cells with cytoplasmic reactivity, a pattern associated with an intact PTEN status in the tumor (original magnification ×400). E, Anti–phospho-S6 immunohistochemistry revealing approximately 20% of tumor cells labeling, indicating this messenger is activated in this tumor (original magnification ×400). F, Anti–phospho-Akt immunohistochemistry demonstrating approximately 80% of tumor cells labeling, indicating this messenger is activated in this tumor (original magnification ×400).
mediates this action through a variety of targets including the well-known tumor suppressor, p53.

Downstream of Akt, P70 S6 kinase 1 phosphorylates the 40S ribosomal protein S6 and is also an important modulator of the PI3K pathway. S6 suppresses apoptosis and promotes cellular survival. The expression of phosphorylated S6 is associated with adverse patient outcome and reduced time to death. It has been shown that the inhibition of S6 with the immunosuppressant drug rapamycin blocks cell-cycle progression through G1 and can inhibit cell growth. Because kinase inhibitors can be an effective treatment for some types of gliomas, glioma tissues with high levels of phospho-S6 would be excellent candidates for treatment with kinase inhibitors.

MAPK PATHWAY

Members of the MAPK pathway have been associated with a variety of roles in tumorigenesis. The MAPK pathway regulates cell proliferation, differentiation, survival, angiogenesis, and metastasis in various tumor types. More specifically, the MAPK pathway members play important roles in mediating glioma cell proliferation and differentiation. MAPK expression correlates with MIB-1 expression, suggesting a role for MAPK in mediating tumor cell proliferation. This contributes to the reason that the increased number of tumor cell nuclei with elevated expression of MAPK is associated with a poorer survival in GBM patients. However, the MAPK pathway is not necessarily tied to malignant progression because expression of MAPK is seen in low-grade gliomas as well.

METHODS

Uncontrolled activation of the PI3K-Akt pathway through amplification of \textit{EGFR} or \textit{EGFrVIII} or through mutation or loss of \textit{PTEN} in gliomas leads to the highly malignant phenotype of GBM. A variety of methods are available to test permutations of this pathway (Figure 2, A through F). Fluorescence in situ hybridization for \textit{EGFR} allows the identification of polysomy or amplification of the gene locus but does not allow identification of the variant \textit{EGFrVIII}. Although antibodies to \textit{EGFrVIII} have been made (Figure 2, C), at present the entire immunohistochemical identification of this protein expression is locked up by patents. Alternative methods include polymerase chain reaction for the DNA in-frame deletion or reverse transcriptase polymerase chain reaction for the RNA transcript. Immunohistochemical identification of \textit{EGFR} wild-type protein is readily performed (Figure 2, B), and a US Food and Drug Administration–approved method is available on the market and the interpretation is based on that methodology. Our laboratory performs both immunohistochemistry for the wild-type protein and FISH for gene dosage levels. The Vysis (Chicago, Ill.) \textit{EGFR}/cep 7 dual-color probe mixture binds to the entire \textit{EGFR} gene (300 kb region) and to the alpha satellite DNA located at the centromere of chromosome 7. Enumeration of both signals provides a mechanism for determining \textit{EGFR} copy number relative to total chromosome 7 number. It is designed for use in multiple types of samples including formalin-fixed, paraffin-embedded tissues. \textit{PTEN} measurements can also be performed by either FISH for the 10q23 locus or loss of heterozygosity analysis to identify loss of the locus or allele. Sequencing will identify the 20% of cases in which there is a \textit{PTEN} mutation, but the 75% of GBMs with \textit{PTEN} allelic loss will be missed. Immunohistochemistry is also available (Figure 2, D) and has been shown to correlate with drug sensitivity to an \textit{EGFR} kinase inhibitor in 1 trial. In that trial, moderate to strong expression of \textit{PTEN} in greater than 75% of cells indicated the gene was intact and correlated with drug response. Although FISH was not used in that study, we accept monosomy in greater than 25% of cells to indicate genetic loss of the locus.

Immunoreactivity for Akt (Figure 2, F) has been shown to be dependent on fixation with the periphery of a biopsy frequently demonstrating the most intense staining with little or no reactivity of either tumor cells or indigenous endothelial cells found centrally. In such cases, the immunoreactivity of 2+ is based on comparison with cytoplasmic reactivity of endothelial cells, and a positive reaction is considered to be greater than 20% of tumor cells with 2+ cytoplasmic immunoreactivity. ERK1 and ERK2 (also known as p44 and p42) are 2 members of the MAPK family of protein kinases that have been extensively studied. Both are regulated through phosphorylation. Antibodies are available for use in studying the activation states of ERK1 and ERK2 immunohistochemically. It has been shown that the immunohistochemical staining of ERK1/2 is heterogeneous, with ERK1/2 being seen mostly in the nucleus because of its transcriptional role. It has also been observed in the cytoplasm (because of nontranscriptional roles such as cell motility and cytoarchitecture) but not as often. In addition, elevated expression has been observed around areas of tumor neovasculature and tumor necrosis. The reported cut-offs for positivity are somewhat complex, and the reader is referred to the original publication by Mizoguchi and colleagues. In brief, a positive reaction is considered any 3+ nuclear intensity or 2+ nuclear intensity in greater than 20% of tumor cells as defined by 2+ intensity equal to the immunoreactive nuclei of indigenous endothelial cells.

To date, measurement of the phosphorylated downstream targets has shown that phospho-Akt, phospho-S6 (Figure 2, E), and phospho-MAPK may be useful in identifying downstream targets of \textit{EGFR} kinase inhibitors. Antibodies to phospho-mTOR are also available, but to date there have been no clinical studies to indicate levels of this enzyme predict response to any of the currently available drugs. Furthermore, no studies to date have identified prognostic significance related to immunoreactivity status among any of these markers that is independent of histologic grade.

We gratefully acknowledge the technical assistance of Ms Cynthia Welsh, Mr Alan Austin, and Ms Debra Young in preparing the immunohistochemical preparations illustrated. We also are indebted to Ms Bonnie Lynch who offered sincere criticism and significant editorial assistance.

References

5. Jiang R, Mircean C, Shimulevich I, et al. Pathway alterations during glioma...


