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Interactions between PTEN and receptor tyrosine kinase pathways and their implications for glioma therapy

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Abstract

Gliomas are the most common and deadly form of malignant primary brain tumors. Loss of the tumor-suppressor PTEN and activation of the receptor tyrosine kinases (RTKs) EGF receptor, c-Met, PDGF receptor and VEGF receptor are among the most common molecular dysfunctions associated with glioma malignancy. PTEN interacts with RTK-dependent signaling at multiple levels. These include the ability of PTEN to counteract PI3K activation by RTKs, as well as possible effects of PTEN on RTK activation of the MAPK pathway and RTK-dependent gene-expression regulation. Consequently, PTEN expression affects RTK-induced malignancy. Importantly, the PTEN status was recently found to be critical for the outcome of RTK-targeted clinical therapies that have been developed recently. Combining RTK-targeted therapies with therapies aimed at counteracting the effects of PTEN loss, such as mTOR inhibition, might also have therapeutic advantage. This article reviews the known molecular and functional interactions between PTEN and RTK pathways and their implications for glioma therapy.

Keywords

c-Met; EGF receptor; glioma; mTOR; PDGF receptor; PTEN

Gliomas are extremely aggressive brain tumors that account for the majority of deaths due to primary brain neoplasms. Each year, more than 14,000 new cases of glioma are diagnosed in the USA [1]. Despite the most advanced treatment, with combinations of surgery, radiotherapy and chemotherapy, as well as novel molecularly targeted therapies, glioma mortality and morbidity remain very high. Glioblastoma multiforme, the most malignant glioma, is associated with an average life expectancy of only 12 months [1]. Factors responsible for glioma malignancy and poor prognosis include rapid glioma cell proliferation, resistance against apoptosis, distant invasion of the surrounding brain and high levels of angiogenesis. Human and experimental gliomas express a number of receptor tyrosine kinases (RTKs) and corresponding ligands that contribute to their malignancy by enhancing glioma cell mitogenicity and motogenicity, inhibiting apoptosis and stimulating angiogenesis. These RTKs include the EGF receptor (EGFR), the HGF receptor c-Met, the PDGF receptor (PDGFR) and the VEGF receptor (VEGFR). Loss of tumor-suppressor function in high-grade gliomas also significantly contributes to their malignancy. The most common cytogenetic abnormalities in human glioblastomas are deletions in the long arm of chromosome 10, where the *PTEN* tumor-suppressor gene is located. PTEN expression in glioma cells suppresses tumorigenicity and

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malignant progression by inhibiting mitogenicity, cell migration, spreading and focal adhesions, and by stimulating apoptosis [2,3].

Based on their critical involvement in cancer and glioma malignancy, a number of RTK pathway inhibitors have been developed. Some of these inhibitors have been or are currently being tested in a clinical setting, and initial results indicate modest responsiveness in glioma patients. For a more efficient use of these inhibitors, it is important to determine the molecular factors that determine sensitivity to these drugs. Since PTEN and RTKs are frequently and simultaneously deregulated in gliomas, and because PTEN can interact with RTK-dependent signaling, the PTEN status has emerged as one such critical determinant of sensitivity to RTK-targeted therapies in human cancer and gliomas. As a consequence, strategies that aim at restoring PTEN function or counteracting the effects of PTEN loss might synergize with RTK-targeted therapies. In this article, we review the functional and molecular interactions that exist between PTEN and RTK pathways and their implications for RTK-targeted and combination therapies.

RTKs in gliomas

Receptor tyrosine kinases are a large family of cell surface receptors that are endowed with intrinsic protein tyrosine kinase activity. They are activated by a wide variety of ligands and play an important role in the control of most fundamental cellular processes, including the cell cycle, cell migration, metabolism and survival, as well as cell proliferation and differentiation. Their functions are mediated by a complex network of cell signaling cascades, the most well known of which are Ras/MAPK and PI3K/Akt. Deregulations of several RTKs, including c-Met, EGFR, PDGFR and VEGFR, have been associated with glioma malignancy. This article discusses the interactions between PTEN and c-Met, EGFR and PDGFR. While VEGFR is an important player in glioma malignancy and angiogenesis, the effects of PTEN on VEGFR-targeted therapies have, to our knowledge, not been well investigated to date. In addition, since RTKs share many similarities in function and signaling, these will be discussed in most detail for the c-Met receptor.

c-Met

The RTK c-Met, and its multifunctional growth factor, HGF, also known as scatter factor, have emerged as key determinants of brain tumor growth and angiogenesis [4]. c-Met and HGF are frequently deregulated in gliomas via transcriptional overexpression, autocrine loop formation and gene amplification. HGF and c-Met are expressed in brain tumors and their expression levels frequently correlate with tumor grade and poor prognosis [5-8]. Activation of c-Met in gliomas enhances their tumorigenicity, tumor growth and tumor-associated angiogenesis [8-10]. Conversely, inhibition of c-Met and/or HGF in experimental tumor xenografts leads to inhibition of tumor growth and tumor angiogenesis [11-13]. HGF is expressed and secreted mainly by tumor cells and acts on c-Met receptors that are expressed in tumor cells and vascular endothelial cells. Activation of c-Met leads to the induction of tumor cell proliferation, migration and invasion, and to the inhibition of apoptosis, as well as to resistance to death induced by chemotherapy and radiation [4,6,8,10,14-17]. The malignant effects of HGF/c-Met are mediated by a network of signal transduction pathways and transcriptional events. Activation of c-Met results in the recruitment of scaffolding proteins, such as Gab1 and Grb2, which lead to the activation of Ras and ERK/MAPK. This causes changes in gene expression of cell cycle regulators, such as *pRb*, *cdk6* and *p27*, as well as extracellular matrix proteinases, such as matrix metalloproteinases and urokinase-type plasminogen activator, leading to alterations of cytoskeletal and cell adhesive functions that control cell proliferation, migration and invasion [18-20]. Active c-Met also binds directly to PI3K at the receptor's phosphotyrosine residues 1349 (Y-1349VHV) and 1356 (Y-1356VNV) [21,22]. This leads to the activation of Akt, a well-characterized mediator of cell survival [23,24]. The malignant

effects of PI3K/Akt activation are mediated by various signal-transduction molecules, among which mTOR appears to play a critical role [25-27].

EGF receptor

Activation of EGFR is observed in up to 60% of glioblastomas [28]. EGFR deregulation is mostly found in primary glioblastomas. Mutations of the *EGFR* gene are frequent in glioblastomas and may be present in up to 50–70% of EGFR-overexpressing tumors. Most mutations affect the extracellular domain and involve a large deletion in exons 2–7. The resulting variant receptor, designated EGFRvIII, has ligand-independent kinase activity and is found in 60–70% of EGFR-overexpressing glioblastomas [29]. Several studies have linked EGFR expression patterns and differential prognosis in glioblastomas, but conflicting results have also been described. This could be explained by use of varying methodologies, including different assessment techniques of EGFR expression, heterogeneous patient characteristics and often small sample sizes. Some studies have suggested that EGFRvIII is associated with a less favorable prognosis [30,31], whereas such association is less clear in wild-type EGFR-overexpressing tumors [32,33].

PDGF receptor

PDGF and its receptor are frequently overexpressed in glial tumor cell lines and tumor surgical samples, and increased expression correlates with higher tumor grade [34,35]. The overexpression of ligand and receptor is mostly due to deregulated expression, although genetic abnormalities have been identified [36]. In a few cases of glioblastomas and anaplastic oligodendrogliomas, the *PDGFRα* gene was found to be amplified, and one of the most common chromosomal abnormalities in astrocytomas is aneuploidy of chromosome 7, where the *PDGFRα* gene is located [37]. One study examined 103 low-grade gliomas (WHO grade II) and found that PDGFR was expressed in 50% of the samples and correlated with a poorer prognosis [38]. PDGFRα and PDGF were found to be expressed in tumor cells, whereas PDGFRβ was found in glioma-associated endothelial cells [39,40]. The coexpression of both ligand and receptor in the tumor cells indicates autocrine and paracrine forms of stimulation, while the expression of receptor in associated blood vessels suggests that paracrine effects are possible [36]. Disruption of autocrine PDGFR signaling with small-molecule inhibitors leads to inhibition of glioblastoma cell colony-forming activity and cell proliferation [41]. Inhibition of PDGFR kinase activity is associated with a decrease in phospho-ERK and phospho-AKT levels, probably a result of decreased signaling through the MAPK and PI3K pathways, respectively. PDGF receptor and ligand over-expression are usually associated with p53 tumor-suppressor loss and is characteristic of secondary glioblastomas [37].

VEGF receptor

The VEGF family and its receptors are essential regulators of angiogenesis and vascular permeability. The VEGF family consists of VEGF-A to VEGF-E, PlGF and snake venom VEGF [42]. VEGF-A binds to and activates two RTKs, VEGFR-1 and VEGFR-2. VEGFR-2 mediates most of the endothelial growth and survival signals, but VEGFR-1-mediated signaling plays important roles in pathological conditions, such as cancer [42]. VEGF mRNA and protein are highly expressed in glioma cells, with high-grade glioblastoma producing more VEGF than lower-grade astrocytomas [40,43]. Inhibition of VEGFR kinase leads to inhibition of glioma xenograft growth [44].

RTK inhibitors

Based on their widespread and profound involvement in human cancer, RTKs and their ligands have emerged as attractive targets for cancer therapy in general, and for gliomas in particular. Different approaches to inhibiting RTKs have been developed. These include receptor and

ligand gene-expression inhibition with ribozymes, antisense oligonucleotides and shRNA, receptor antagonism with ligand fragments, competitive ligand binding with soluble receptors, neutralizing antibodies to ligands and receptors, and small-molecule kinase inhibitors (Figure 1). Based on their clinical applicability, small-molecule kinase inhibitors and neutralizing monoclonal antibodies are, perhaps, currently the most promising approaches for inhibiting RTK activation. Clinically applicable kinase inhibitors of EGFR include gefitinib, erlotinib and others. Clinically applicable kinase inhibitors of c-Met include PF-2341066, SGX523, JNJ38877605 and others. PDGFR kinase inhibitors include imatinib mesylate (Gleevec®), sorafenib, XL999 and others. Monoclonal antibodies that target EGFR include cetuximab and panitumumab and antibodies that target the c-Met ligand, HGF, include AMG102 and L2G7. Most of these inhibitors are currently in clinical trials. An update on all worldwide active and completed clinical trials using the aforementioned and other agents can be found on the NIH website [101].

PTEN in gliomas

The *PTEN* gene encodes a 403-amino acid polypeptide that possesses phosphatase activity toward both lipids and proteins. It is located on chromosome 10q, which is deleted in the vast majority of human glioblastomas [45,46]. Somatic mutations of *PTEN* are detected in over 40% of glioblastomas and PTEN protein expression is very low or absent in two-thirds of these tumors [47-49]. The frequency of *PTEN* mutations and loss differs from study to study, and also depends on ethnicity [50-52]. Since *PTEN* mutations in low-grade gliomas and glioneural tumors are rare, it has been suggested that PTEN loss plays a role in the progression of lower-grade astrocytomas to glioblastoma multiforme [53].

Expression of PTEN in *PTEN*-null glioma cell lines causes growth suppression [54]. *PTEN* gene transfer to mutated glioblastoma cells inhibits anchorage-independent growth and *in vivo* tumor growth when the cells are implanted subcutaneously into nude mice [2]. Restoration of PTEN to mutant glioblastoma cells also inhibits cell migration, invasion, growth and focal adhesions [3,55]. Growth inhibition is partly caused by suppression of proliferation due to G₁ cell cycle arrest and corresponding increases in the levels of p27 and decreased levels of retinoblastoma protein phosphorylation [54]. PTEN tumor-suppressive functions are also mediated by induction of tumor cell apoptosis. Reconstitution of PTEN induces apoptosis in glioblastoma by reducing the levels of phosphatidylinositol-3,4,5-triphosphate (PIP3) in the cells [56,57].

The function of PTEN is mediated by its action at various levels of cell signaling [58]. PTEN dephosphorylates the lipid secondary messenger PIP3. PIP3 accumulation at the membrane allows recruitment and phosphorylation of the proto-oncogene serine/threonine kinase *Akt*, a well-established survival factor with antiapoptotic activity [59]. PTEN effects on Akt lead to inhibition of mTOR and glioma malignancy resulting from PTEN deficiency is, in part, due to mTOR activation [60]. PTEN knockdown was also shown to lead to Jun-*N*-terminal kinase (JNK) pathway activation in an AKT-independent manner [61]. PTEN also dephosphorylates the phosphoprotein focal adhesion kinase (FAK) [3,62] and the shc tyrosine kinase that links tyrosine kinases to Ras signaling [63]. Recently, PTEN has also been shown to enhance the levels and tumor-suppressive activity of wild-type p53, as well as the tumor promoting properties of gain-of-function mutant p53 [64,65].

PTEN has been localized to both cytoplasm and nucleus [66-68]. Nuclear PTEN expression appears to be important for the tumor-suppressive effects of PTEN, as decrease or loss of nuclear PTEN has been observed in a number of neoplasms [69,70]. Several studies have shown that cytoplasmic and nuclear PTEN have different functions. The lipid phosphatase activity of PTEN that leads to Akt dephosphorylation and p27 induction occurs primarily in the cytoplasm,

while dephosphorylation of MAPK by PTEN, and the resulting cyclin D1 downregulation, regulation of chromosomal integrity, p53 acetylation and the DNA-damage response through the regulation of RAD51 appear to be nuclear events [71-75]. Nucleocytoplasmic shuttling of PTEN is not very well understood and involves simple diffusion, a Ran-dependent mechanism, transport by the major vault protein and monoubiquitination of PTEN protein [76-78].

Interactions between PTEN & RTKs in gliomas

PTEN interacts with RTK-dependent signaling at multiple levels. The most investigated and well-known interaction is the antagonistic effect of PTEN on PI3K, which plays an essential role in mediating RTK-dependent cell signaling and malignancy. RTKs activate PI3K either directly or via intermediary molecules. PI3K catalyzes phosphorylation of the D3 position on phosphoinositides to generate the biologically active moieties PIP3 and phosphatidylinositol-3,4-bisphosphate (PIP2). Upon generation, PIP3 binds to the pleckstrin homology domains of 3-phosphoinositide-dependent kinase 1 and the serine/threonine kinase Akt, causing both proteins to be translocated to the cell membrane, where Akt is subsequently activated. The tumor-suppressor PTEN antagonizes PI3K by dephosphorylating PIP3 and PIP2, thereby preventing activation of Akt (Figure 2) [79].

Another possible interaction between RTKs and PTEN consists of PTEN's ability to bind and dephosphorylate the p52 isoform of Shc, thus inhibiting the recruitment of the Grb2 adaptor and the subsequent activation of the MAPK cascade, which is another key mediator of c-Met and other RTK malignant effects [18,23]. Shc also directly interacts with Gab1, which is a substrate of c-Met and is involved in c-Met-specific branching morphogenesis. It associates directly with c-Met via the c-Met-binding domain, which is not related to known phosphotyrosine-binding domains [80]. Expression of PTEN was shown to selectively inhibit activation of the ERK/ MAPK pathway. PTEN expression in glioblastoma cells lacking the protein resulted in the inhibition of integrin-mediated MAPK activation. EGF-induced and PDGF-induced MAPK activation were also blocked. Shc phosphorylation and Ras activity were inhibited by expression of PTEN, whereas EGFR autophosphorylation was unaffected. The ability of cells to spread at normal rates was partially rescued by coexpression of constitutively activated MEK1, a downstream component of the pathway. In addition, focal contact formation was enhanced, as indicated by paxillin staining. The phosphatase domain of PTEN was essential for all of these functions because PTEN with an inactive phosphatase domain did not suppress MAPK or Ras activity [62,63]. PTEN was also shown to inhibit integrin- and growth factor-stimulated FAK phosphorylation and migration in glioma cells [62,63]. PTEN was shown to localize to the nucleus coincident with the G₀/G₁ phases of the cell cycle, and compartmentalization-regulated cell cycle progression, dependent upon the downregulation of cyclin D1. It was demonstrated that nuclear PTEN downregulates cyclin D1 transcription and this event is mediated by the downregulation of MAPK specifically by nuclear localized PTEN (Figure 2) [72].

PTEN and RTKs were also found to coregulate gene expression in glioma cells. One study used expression microarrays to investigate the effects of PTEN on gene-expression changes caused by activating c-Met in human glioblastoma cells. c-Met activation by HGF altered the expression of 27-fold more genes in *PTEN*-null glioblastoma cells than in *PTEN* homozygous primary normal human astrocytes (523 vs 19 genes). Restoring wild-type PTEN to glioblastoma cells dramatically altered patterns of c-Met-regulated gene expression. This effect varied depending on the specific gene in question. PTEN reduced the number of c-Met-regulated transcripts from 931 to 502, decreased the relative number of genes upregulated by c-Met from 46 to 25% and increased the relative number of downregulated genes from 54 to 75%. PTEN and c-Met coregulated many genes involved in cell growth regulation, such as oncogenes, growth factors, transcription factors and constituents of the ubiquitin pathway. c-

Met activation in *PTEN*-null (but not *PTEN* reconstituted) cells led to upregulation of the EGFR agonist TGF- α and, subsequently, to EGFR activation. Using *PTEN* mutants, the study found that *PTEN*'s transcriptional effects were either lipid-phosphatase dependent, protein-phosphatase dependent or phosphatase-independent. These results showed that *PTEN* has critical and mechanistically complex effects on RTK-regulated gene transcription [81].

Effects of *PTEN* on RTK-targeted therapies

Based on the profound and multilevel interactions between *PTEN* and RTKs, it is not surprising that *PTEN* was found to affect RTK-targeted therapies. The *PTEN* status of human tumors in general, and gliomas in particular, has recently emerged as a critical predictor of the success of therapies that target RTKs. Since the involvement of EGFR in glioma malignancy has been uncovered earlier than that of other RTKs, EGFR kinase inhibitors were developed and used in clinical trials earlier than inhibitors of other RTKs. Therefore, based on history and drug availability, most research on the effects of *PTEN* on RTK-targeted therapies in gliomas focused on EGFR kinase inhibitors.

A first seminal study investigated the molecular determinants of the response of glioblastomas to EGFR kinase inhibitors [82]. The study analyzed the effects of *PTEN* and EGFR statuses on the responsiveness to EGFR inhibitors of 49 patients with recurrent malignant gliomas. The authors sequenced kinase domains in the *EGFR* and human *EGFR* type 2 (*Her2/neu*) genes and analyzed the expression of EGFR, EGFRvIII and the tumor-suppressor protein *PTEN* in recurrent malignant gliomas from patients who had received EGFR kinase inhibitors. The molecular correlates of clinical response were determined and validated in an independent data set. The results showed that coexpression of EGFRvIII and *PTEN* was significantly associated with a clinical response. These findings were validated in 33 patients who received similar treatment for glioblastoma at a different institution. The study also found that, *in vitro*, coexpression of EGFRvIII and *PTEN* sensitized glioblastoma cells to the EGFR kinase inhibitor erlotinib.

A contemporary study to that mentioned previously investigated the association between the expression of EGFR and downstream signaling components and the response of malignant gliomas to erlotinib in a Phase I trial of erlotinib administered either alone or with the alkylating agent temozolomide [83]. Expression of EGFR and ligand-independent EGFRvIII mutant proteins, and of phosphorylated Akt, *EGFR* gene amplification and mutations in *PTEN* and *EGFR* were evaluated in glioma tissue specimens. Response to therapy was evaluated by sequential MRI every 2 months. Of 41 glioma patients, eight responded to treatment. Response to erlotinib was associated with EGFR expression and EGFR amplification. These associations were stronger and statistically significant among the 29 patients initially diagnosed with glioblastoma. Among responders with sufficient tumor tissue, none had *EGFRvIII* mutations. None of the 22 tumors with high levels of phosphorylated Akt responded to erlotinib treatment, whereas eight of the 18 tumors with low levels of phosphorylated Akt did respond to erlotinib treatment. The level of phosphorylated Akt was also associated with time to progression. Paradoxically, most tumors with elevated levels of phosphorylated Akt did not have *PTEN* mutations, indicating that *PTEN* expression was not a positive predictor of responsiveness to the EGFR inhibitor. The authors concluded that, among glioma patients, those with glioblastoma tumors who have high levels of EGFR expression and low levels of phosphorylated Akt had a better response to erlotinib treatment than those with low levels of EGFR expression and high levels of phosphorylated Akt [83].

The conclusions of the two aforementioned studies differed with respect to the involvement of *EGFRvIII* mutations and *PTEN* expression, which were associated with responsiveness to erlotinib in the first study but not in the second study. This stresses the need for additional

investigations with a larger number of patients to draw more definitive conclusions on this subject.

A subsequent animal xenograft study sought to identify associations between glioblastoma molecular characteristics and tumor sensitivity to the EGFR kinase inhibitor erlotinib [84]. The authors examined a panel of serially passaged glioblastoma xenografts in the context of an intracranial tumor therapy response model. From an initial evaluation of 11 distinct glioblastoma xenografts, two erlotinib-sensitive tumors were identified, each having amplified EGFR and expressing wild-type PTEN. One of these tumors expressed truncated EGFRvIII, whereas the other expressed full-length EGFR. Subsequent cDNA sequence analysis revealed the latter tumor to be expressing an EGFR sequence variant with arginine, rather than leucine, at amino acid position 62; this was the only EGFR sequence variant identified among the 11 xenografts, other than the aforementioned vIII sequence variant. EGFR cDNAs were then examined from 12 more xenografts to determine whether additional missense sequence alterations were evident, and this analysis revealed one such case: expressing threonine rather than alanine at amino acid position 289 of the extracellular domain. This glioblastoma was also amplified for EGFR, but did not display significant erlotinib sensitivity, presumably due to its lacking PTEN expression. This study, therefore, identified two erlotinib-sensitive glioblastoma xenografts, with the common molecular characteristics shared by each being the expression of wild-type PTEN in combination with the expression of amplified and aberrant EGFR [84].

In summary, the few studies that have investigated the effects of PTEN expression on anti-RTK therapies seem to indicate that PTEN is a major predictor of the RTK success of therapy in gliomas, but additional research with larger cohorts of patients is required to confirm these findings and extend them to RTKs other than EGFR.

Combining manipulation of PTEN expression & function with RTK inhibition

As described earlier, RTK inhibitors seem to achieve greater antiglioma effects in tumors with wild-type PTEN expression. Consequently, it is projected that PTEN functional restoration to PTEN-mutated tumors could synergize with RTK inhibitors in inhibiting glioma growth. PTEN-expression restoration would provide proof of principle to this projected synergy but can currently only be achieved in a laboratory setting owing to the delivery limitations associated with gene therapy. Clinically applicable approaches to counteracting the effects of PTEN loss include PI3K, Akt and mTOR inhibitions. PI3K, Akt and mTOR inhibitors are currently in clinical trials. Since combinations of RTK and mTOR inhibitors are currently being tested in glioma patients and because the existence of a feedback loop between mTOR and RTKs provides one more rationale for combining RTK and mTOR inhibitors, we will focus on this latter combination.

PTEN-expression restoration sensitizes gliomas to RTK inhibitors

A few studies have shown that restoring PTEN expression to *PTEN*-null glioma cells using derived xenografts sensitizes them to RTK inhibition. An *in vitro* study found that wild-type PTEN transfection inhibited the growth and transforming ability of U87MG glioblastoma cells by 69.3 and 73.5%, respectively. Antisense-EGFR transfection inhibited the growth and transforming phenotype of these cells by 50.3 and 46.8%, respectively. Cotransfection of U87MG cells with wild-type PTEN and antisense EGFR constructs inhibited the cellular growth by 91.7%. The transforming phenotype of these cells was completely inhibited. In addition, the cotransfected cells showed a differentiated form and expressed much lower telomerase activity than cells transfected with wild-type PTEN or antisense-EGFR alone [85].

Our group recently studied the effects of PTEN on c-Met-induced malignancy and associated molecular events and assessed the potential therapeutic value of combining PTEN restoration approaches with HGF/c-Met inhibition [86]. We studied the effects of c-Met activation on cell proliferation, cell cycle progression, cell migration, cell invasion and associated molecular events in the settings of restored or inhibited PTEN expression in glioblastoma cells. We also assessed the experimental therapeutic effects of combining anti-HGF/c-Met approaches with PTEN restoration. PTEN significantly inhibited HGF-induced proliferation, cell cycle progression, migration and invasion of glioblastoma cells. PTEN attenuated HGF-induced changes of signal transduction proteins AKT, GSK-3, JNK and mTOR, as well as the cell cycle regulatory proteins p27, cyclin E and E2F-1. Combining PTEN restoration to *PTEN*-null glioblastoma cells with c-Met and HGF inhibition additively inhibited tumor cell proliferation and cell cycle progression. Systemic *in vivo* delivery of a humanized monoclonal anti-HGF antibody (L2G7) and PTEN restoration additively inhibited intracranial glioma xenograft growth.

Restoration of the *PTEN* gene is currently not a clinically feasible approach. Instead, counteracting some effects of PTEN loss by inhibiting mTOR in combination with RTK inhibition represents one promising approach with clinical applicability in gliomas as discussed later.

Combining RTK inhibitors with mTOR inhibitors in PTEN-null cells

The mTOR is a protein kinase that is centrally involved in the control of cancer cell metabolism, growth and proliferation. mTOR is a downstream target of Akt, which is activated by RTK activation and PTEN loss. Activation of mTOR mediates some of the malignant effects of PTEN loss [87]. Therefore, inhibition of mTOR with available clinically applicable drugs, such as rapamycin, could counteract some of the malignant effects of PTEN loss [60,88,89]. Interestingly, a recent study also showed that constitutive activation of the Akt/mTOR pathway in cancer cells induces upstream feedback inhibition of signaling via RTKs [90]. This study found that inhibition of mTOR causes the release of this feedback inhibition, paradoxically leading to Akt activation and reduction of the anti-tumor effects of mTOR inhibitors. Another recent study also found that inhibition of mTOR complex 1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer [91]. In addition, mTOR inhibitors are already in clinical trials in glioma patients [92]. Therefore, there is a multi-pronged rationale for combining RTK inhibitors with mTOR inhibitors, and recent studies that have tested this combination are discussed later (Figure 3).

One study tested the combined anti-tumor effects of the EGFR/VEGFR inhibitor AEE788 and the mTOR inhibitor RAD001 (everolimus) [93]. AEE788 is an orally active tyrosine kinase inhibitor that decreases the kinase activity associated with EGFR and, at higher concentrations, the VEGFR-2 (kinase domain region). RAD001 is an orally available mTOR inhibitor, structurally related to rapamycin. The authors hypothesized that combined inhibition of upstream EGFR and kinase domain-region receptors with AEE788 and inhibition of the downstream mTOR pathway with RAD001 would result in increased efficacy against gliomas compared with single-agent therapy. *In vitro* experiments demonstrated that the combination of AEE788 and RAD001 resulted in a greater increase in rates of cell cycle arrest and apoptosis, and greater reduction in proliferation than either agent alone. Combined AEE788 and RAD001, administered orally to athymic mice bearing established human malignant glioma tumor xenografts, resulted in greater tumor growth inhibition and greater increases in median survival than monotherapy. These studies suggested that simultaneous inhibition of RTK and mTOR pathways offer increased benefit in glioma therapy [93].

A separate study examined the cellular and molecular effects of a combined kinase inhibition of mTOR (rapamycin) and EGFR (EKI-785) in glioblastoma cells [94]. Simultaneous

treatment with rapamycin and EKI-785 resulted in synergistic antiproliferative, as well as proapoptotic, effects. At a molecular level, rapamycin alone significantly decreased S6 phosphorylation, whereas EKI-785 alone promoted substantially reduced signal transducer and activator of transcription 3 phosphorylation. Treatment with rapamycin alone also increased Akt phosphorylation on serine-473, but this effect was blocked by a simultaneous administration of EKI-785. Individually, EKI-785 diminished, while rapamycin promoted, the binding of the translation inhibitor eukaryotic initiation factor 4E-binding protein (4EBP1) to the eukaryotic translation initiation factor 4E (eIF4E). In spite of these opposing effects, the highest level of 4EBP1–eIF4E binding occurred with the combination of the two inhibitors. These results indicated that the inhibition of EGFR and mTOR has distinct, as well as common, signaling consequences and provided a molecular rationale for the synergistic anti-tumor effects of EGFR and mTOR inhibitions [94].

Subsequently, another study showed that the mTOR inhibitor rapamycin enhances the sensitivity of PTEN-deficient tumor cells to the EGFR kinase inhibitor erlotinib. In two isogenic model systems (U87MG glioblastoma cells expressing EGFR, EGFRvIII and PTEN in relevant combinations, and SF295 glioblastoma cells in which PTEN protein expression has been stably restored), the study showed that combined EGFR/mTOR kinase inhibition inhibits tumor cell growth and has an additive effect on inhibiting downstream PI3K pathway signaling. The study also showed that combination therapy provides added benefit in promoting cell death in PTEN-deficient tumor cells [95].

Based on laboratory evidence, a research group reported a Phase I trial of the EGFR inhibitor gefitinib plus the mTOR inhibitor sirolimus in adults with recurrent malignant glioma [96]. The trial aimed at determining the maximum tolerated dose and dose-limiting toxicity of gefitinib plus sirolimus among patients with recurrent malignant glioma. In total, 34 patients, with progressive disease after prior radiation therapy and chemotherapy, were enrolled, including 29 (85%) with glioblastoma multiforme and five (15%) with anaplastic glioma. The maximum tolerated dose was gefitinib 500 mg plus sirolimus 5 mg for patients not on enzyme-inducing antiepileptic drugs (EIAEDs) and gefitinib 1000 mg plus sirolimus 10 mg for patients on EIAEDs. Dose-limiting toxicity included mucositis, diarrhea, rash, thrombocytopenia and hypertriglyceridemia. Gefitinib exposure was not affected by sirolimus administration but was lowered significantly by concurrent EIAED use. Two patients (6%) achieved a partial radiographic response and 13 patients (38%) achieved stable disease. They, therefore, showed that gefitinib plus sirolimus can be safely coadministered on a continuous, daily-dosing schedule, and established the recommended dose level of these agents in combination for future Phase II clinical trials [96].

A similar study tested the combination of EGFR and mTOR inhibitors in recurrent malignant gliomas [97]. A total of 28 heavily pretreated patients with recurrent malignant gliomas were administered EGFR inhibitors (gefitinib or erlotinib) in combination with the mTOR inhibitor sirolimus. The regimens were reasonably well tolerated. In total, 19% of patients experienced a partial response and 50% had stable disease. The 6-month progression-free survival for glioblastoma patients was 25% [97].

Recently, our group combined a clinically applicable humanized monoclonal anti-HGF antibody (L2G7) with the mTOR inhibitor rapamycin in glioma *in vitro* and *in vivo* studies [86]. We found that L2G7 and rapamycin had additive inhibitory effects on glioblastoma cell proliferation. Systemic *in vivo* deliveries of L2G7 and rapamycin additively inhibited intracranial glioma xenograft growth. These preclinical studies showed, for the first time, that combining anti-HGF/c-Met approaches with mTOR inhibition is worth testing in a clinical setting.

Altogether, the aforementioned studies demonstrated that combining RTK inhibition with mTOR inhibition in *PTEN*-null tumors could have a therapeutic advantage against gliomas.

Conclusion

In conclusion, *PTEN* and RTK deregulations are among the most common molecular dysfunctions in gliomas. Since *PTEN* interacts with RTK-dependent signaling at multiple levels, *PTEN* expression affects RTK-dependent malignant functions and influences RTK-targeted therapies. As a consequence, the *PTEN* status should be considered when newly developed RTK inhibitors are used in the clinic. In addition, strategies to counteract the effects of *PTEN* loss, such as mTOR inhibition, represent a promising approach to improving the efficacy of RTK inhibition in cancer and gliomas.

Expert commentary

Gliomas are extremely heterogeneous, with numerous histopathologically distinct entities. Due to this complexity, it is most likely that only a small subset of patients will benefit from RTK-pathway inhibition as single therapies. To achieve greater therapeutic efficiency from RTK targeting, it will be important to better understand the molecular determinants of RTK-pathway dependency. This will require that the clinical testing of pathway inhibitors include the rigorous analysis of RTK activation levels, mutations, *PTEN* status and the coexpression and coactivation of all RTKs commonly active in malignant brain tumors. Knowledge of the factors that determine sensitivity and dependence to RTKs, and the development of tests for determining such factors will, hopefully, identify patient subsets most likely to respond to pathway inhibitors. It is also likely RTK targeting will be most useful in combination with other existing and emerging therapies. Basic and translational research has provided a rationale for combining anti-RTK therapies with traditional cytotoxic therapies, such as radiotherapy and chemotherapy. New therapeutically relevant inter-relationships between different RTKs, such as crossactivation and pathway switching, have been discovered in glioma models and systemic human cancers, raising the prospect for considerable therapeutic benefit by simultaneously targeting these pathways. Based on the studies described in the present review, targeting RTKs in combination with approaches that counteract the effects of *PTEN* loss are likely to also be of value for the success of molecularly targeted therapies of gliomas.

Five-year view

The clinical success of RTK-targeted therapies in gliomas has been modest. These therapies are likely to depend on the *PTEN* status of tumors, which might be taken into consideration in future clinical applications. In *PTEN*-mutated tumors, combinations of anti-RTK therapies with therapies that counteract the effects of *PTEN* loss are likely to improve the efficacy of RTK inhibitors. As our knowledge of *PTEN*–RTK interactions improves, similar therapies will be developed and tested in a clinical setting. These will include combinations of anti-RTK agents with PI3K, Akt and mTOR, as well as with other as yet unknown approaches for counteracting the effects of *PTEN* loss.

Key issues

- Loss of the tumor-suppressor *PTEN* and activation of the receptor tyrosine kinases (RTKs) EGF receptor, c-Met, PDGF receptor and VEGF receptor are among the most common molecular dysfunction in gliomas.
- *PTEN* interacts with RTK-dependent signaling at multiple levels, including the ability of *PTEN* to counteract PI3K activation by RTKs, as well as possible effects

of PTEN on RTK activation of the MAPK pathway and RTK-dependent gene-expression regulation.

- PTEN expression affects RTK-induced malignancy.
- Clinically applicable RTK inhibitors have been developed but their success in gliomas has been modest.
- The PTEN status affects the success of RTK-targeted experimental and clinical therapies.
- PTEN restoration sensitizes experimental tumors to RTK inhibitors.
- Combining RTK inhibitors with approaches that counteract the effects of PTEN loss, such as mTOR inhibitors, could be of value for future therapies for glioma patients.

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Website

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Figure 1. Strategies used to inhibit RTK activation
 LIG: Ligand; RTK: Receptor tyrosine kinase.

Figure 2. Known molecular interactions between PTEN and RTK-dependent signaling
 FAK: Focal adhesion kinase; LIG: Ligand; PIP2: Phosphatidylinositol-3,4,-bisphosphate;
 PIP3: Phosphatidylinositol-3,4,5-triphosphate; RTK: Receptor tyrosine kinase.

Figure 3. Rationale for combining RTK-targeted and mTOR-targeted therapies

Arrow thickness is proportional to signal strength. Font weight of 'malignancy' is proportional to malignant effects.

LIG: Ligand; PIP2: Phosphatidylinositol-3,4,-bisphosphate; PIP3: Phosphatidylinositol-3,4,5-triphosphate; RTK: Receptor tyrosine kinase.