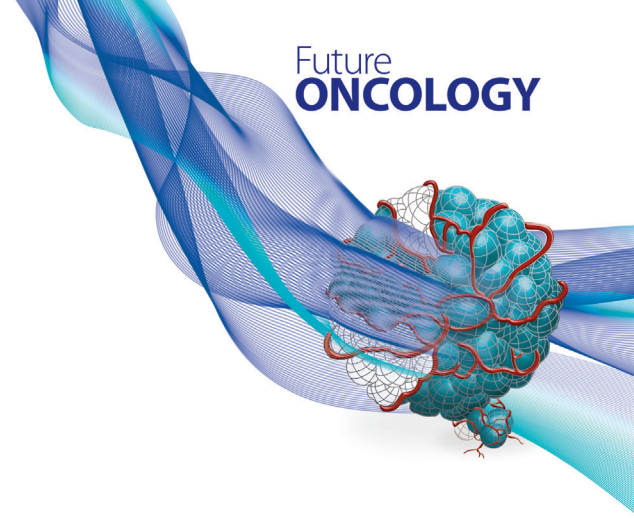
REVIEW

For reprint orders, please contact: reprints@futuremedicine.com



Molecular targets in glioblastoma

Maira Zorzan[1], Enrico Giordan[1], Marco Redaelli[1,2], Antonio Caretta[2,3] & Carla Mucignat-Caretta*[,1,2]

ABSTRACT Glioblastoma is the most lethal brain tumor. The poor prognosis results from lack of defined tumor margins, critical location of the tumor mass and presence of chemo- and radio-resistant tumor stem cells. The current treatment for glioblastoma consists of neurosurgery, followed by radiotherapy and temozolomide chemotherapy. A better understanding of the role of molecular and genetic heterogeneity in glioblastoma pathogenesis allowed the design of novel targeted therapies. New targets include different key-role signaling molecules and specifically altered pathways. The new approaches include interference through small molecules or monoclonal antibodies and RNA-based strategies mediated by siRNA, antisense oligonucleotides and ribozymes. Most of these treatments are still being tested yet they stay as solid promises for a clinically relevant success.

KEYWORDS
• diagnosis • glioblastoma
• intracellular pathways
• signaling • therapy

The WHO classification of the CNS tumors relies on histomorphological criteria to differentiate 15 tumor categories [1]. Gliomas are graded as low (I/II) and high grade (III/IV). The latter comprise 85% of all gliomas and are still incurable. The WHO classification includes a combination of criteria for tumor grading, which drives the choice on the use of adjuvant radiation therapy and specific chemotherapic protocols [1]. Besides histological appearance, additional criteria are: patient's clinical condition, performance status, tumor localization, radiological characteristics, extent of surgical resection, proliferation index and genetic alterations. Noteworthy, most low-grade gliomas eventually progress to a higher grade [2], which leads them to a malignant phenotype, characterized by clonal evolution of transformed cells, after abrogation of cell cycle control and activation of cellular proliferation signals. Supported by increased angiogenesis, tumor cells invade the surrounding tissue [3].

Gliomas showing necrosis and malignant cytology, including mitotically active behavior (grade IV, glioblastoma [GB]) result in a poor clinical prognosis. GB is the most frequent and encompasses 51% of all gliomas [4]. Its incidence is three to five cases per 100,000 persons every year, with a peak between the V and VI decade. Due to location in the brain, aggressiveness and low survival time, GB is considered one of the most lethal forms of cancer [5]. The overall median survival time for GB patients is 14.6 months: only about 3% of patients survive longer than 5 years [5]. The surgical outcome of GB resection is uncertain due to the lack of a defined tumor margin and to the location in close proximity to vital anatomical structures in the brain. A better outcome in eradication can be achieved with subsequent radiotherapy (RT) and adjuvant chemotherapy. However, the presence of chemo-resistant and radio-resistant glioma stem cells (GSCs), which may play a role in initiating relapse [6], should be considered during the evaluation of prospective therapeutic targets. Malignant tumors possibly derive from a population of cells

[1]Department of Molecular Medicine, University of Padova, Padova, Italy
[2]National Institute of Biostructures & Biosystems, Rome, Italy
[3]Pharmaceutical Department, University of Parma, Parma, Italy
*Author for correspondence: Tel.: +39 049 8275304; Fax: +39 049 8272328; carla.mucignat@unipd.it

Future Medicine part of fsg

that share some biologic properties with normal adult stem cells [7]. Cells with stem-like feature in human brain tumors were first described in surgery specimens of human GB [8]. GSCs may be involved in controlling the molecular tumor phenotype and in promoting the recruitment of vascular and stromal cells to sustain tumor growth; they may contribute to resistance and hamper the efficacy of drugs [9]. It is thus necessary to re-evaluate current strategies and find alternative approaches to eradicate malignant gliomas, and revisit the fundamental biology to explore the potential cancer resistance mechanisms in GB.

Treatment & protocols

Current standard treatment for GB patients is neurosurgery, when feasible, followed by fractionated external beam RT and chemotherapy with systemic temozolomide (TMZ) administration [5]. TMZ is a prodrug converted into its own active form, monomethyl–triazeno–imidazole–carboxamide, in all cells at physiological pH. The cytotoxicity of monomethyl–triazeno–imidazole–carboxamide results from various events, including methylation of adenine at N3, which accounts for 9% of compounds, and most importantly of guanine, mainly at N7, accounting for 70% of compounds, and at O6, for a minor extent [10]. However, TMZ preferentially targets guanine triplet sequences in their middle guanine residue, to create O6-methyl-guanine (O6MeG): this is indeed the most potent killing agent [11]. TMZ can cross the blood–brain barrier, resulting in almost complete bioavailability [12]. A study by the European Organization for the Research and Treatment of Cancer and the National Cancer Institute of Canada Clinical Trials Group (EORTC/NCIC) revealed a significant increase in the overall median survival of patients, from 12.1 months in the controls to 14.6 months in the TMZ-treated group, which results in an increase in the 2-year survival, from 10% to 27% [13,14]. Hence, the methylation status of *MGMT* is used as a GB prognostic factor, since it is the most relevant biomarker for response to TMZ treatment. MGMT is a DNA repair enzyme that restores the TMZ-induced O6MeG damage [12]. It irreversibly binds to O6MeG adducts leading to their degradation. In this way, it may counteract the cytotoxicity of TMZ or alkylating drugs. In patients with recurrent GB, O6-benzyl-guanine (O6-BG) may restore TMZ sensitivity [15], an approach that may be further ameliorated by gene therapy (trial NCT00669669) [16]. Moreover, MGMT activity correlates with resistance to methylating chemotherapeutic drugs [17]. Most importantly, a better response to TMZ may be observed in patients that show methylation of *MGMT* promoter, since their median survival increases up to 21.7 months [18]. However, till now, despite all the treatment options, the average lifespan after diagnosis for GB patients remains limited by a high rate of recurrences [19].

Molecular diagnosis of malignant gliomas

Classification of malignant gliomas is switching from morphology-based guidelines to molecular criteria, with the definition of a glioma genomic landscape and a better understanding of its relationship with tumor development [20]. Mechanisms of tumorigenesis, growth and resistance to treatment are critical for development and efficacy of new-targeted therapies. Various alterations acting on gene expression and protein functions have been identified in GB. These span from activation of oncogenes to silencing of tumor-suppressor genes **(Figure 1)**. Based on gene expression analyses and DNA sequencing, The Cancer Genome Atlas (TCGA) research network confirmed that three signaling pathways are frequently altered in GB. They are related to receptor tyrosine kinase (RTK)/Ras/PI3K, p53 and retinoblastoma (Rb) signaling. TGCA ranks GBs into mesenchymal, proneural, neural and classical subtypes [20]. The mesenchymal subtype presents an overexpression of YKL-40 (CHI3L1), MET, CD44 and MERTK, but is mostly characterized by deletions of emizygotic 17q11.2, which comprises *NF1* gene. The proneural type displays amplification of *PDGFRA*, mutations of IDH1 and of p53, while genes such as *PDGFRA, NKX2–2* and *OLIG2*, which are related to oligodendrocytic lineage, are upregulated. The neural subtype expresses tumor markers such as NEFL, GABRA1, SYT1 and SLC12A5. The classical subtype overexpresses neural stem cell markers such as nestin, as well as components of Notch and Sonic Hedgehog (SHH) signaling pathways, in addition to upregulation of p16, INK4A and p14ARF. It also shows an amplification of chromosome 7, which affects EGFR expression.

In adult gliomas, p53 is mutated in 87% of GB, Rb in 78% and RTK/Ras/PI3K pathway

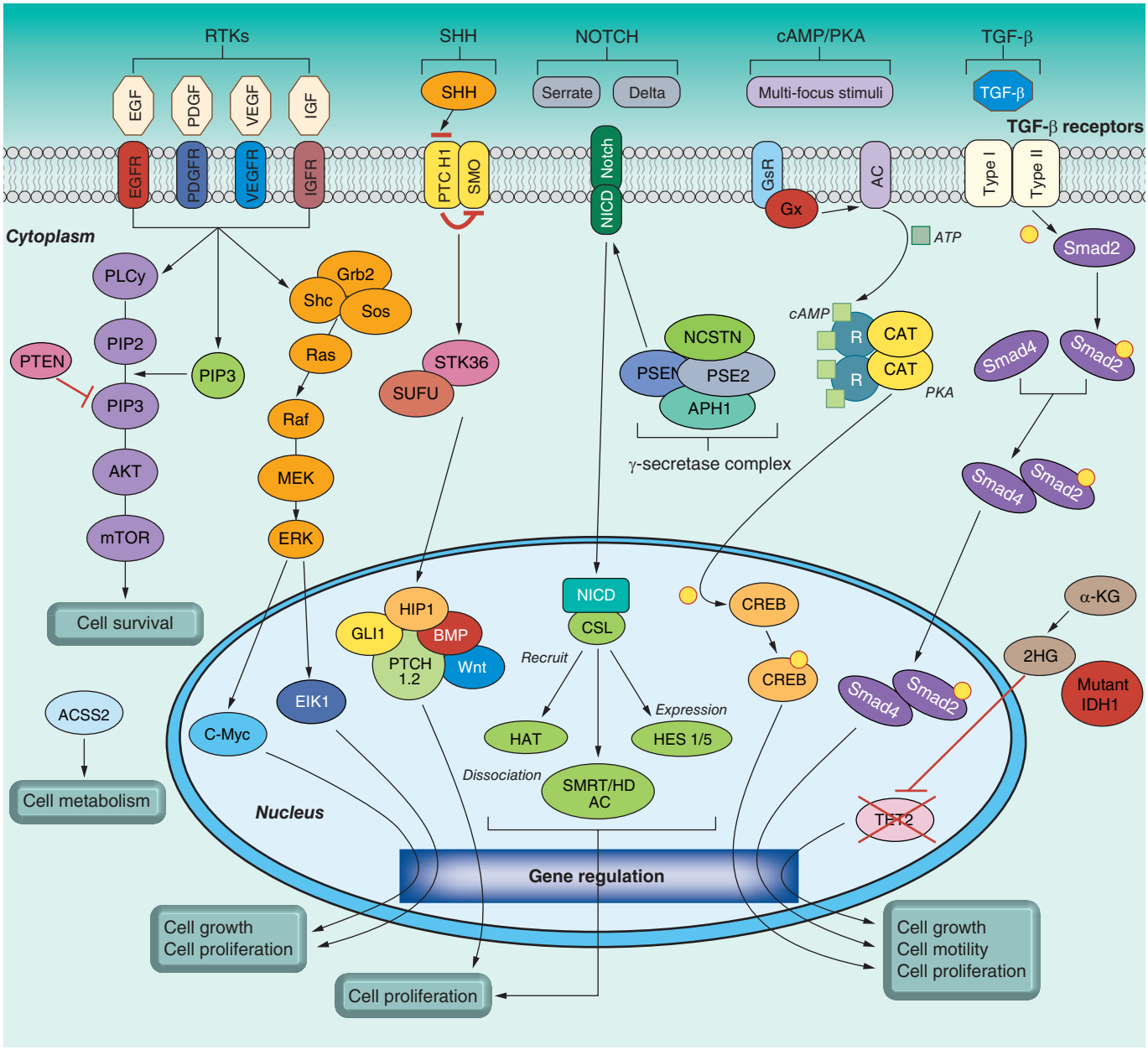


Figure 1. Signaling pathways altered in malignant gliomas.

2-HG: 2-Hydroxyglutarate; AC: Adenylate cyclase; BMP: Bone morphogenetic protein; cAMP: Cyclic AMP; CAT: PKA catalytic subunit; EGFR: EGF receptor; GsR: G-proteins receptor; Gx: G-protein generic; HAT: Histone acetyltransferase; HDAC: Histone deacetylase; IGFR: IGF receptor; NCSTN: Nicastrin; NICD: Notch intracellular domain; PDGFR: PDGF receptor; PKA: Protein kinase A; PSEN: Presenilin; R: PKA regulatory subunit; RTK: Receptor tyrosine kinase; Sos: Son of Sevenless; VEGFR: VEGF receptor.

in 88% of malignant gliomas. Among these, the RTK/Ras/PI3K pathway is now considered one of the most suitable pathways for pharmacological intervention. Mutations such as amplification of *EGFR* can be found in 45% of GBs, gain of function of PI3K in 15% and loss of *PTEN* in 36% [21]. This activates the lipid kinase PI3K and its target, Akt, that has over 40 downstream targets, including GSK-3, PRAS40, FOXO, BAD, mTOR and the TSC1/2 proteins. Alterations in these pathways are essential for the development of GB, but it is still possible that other pathways will be revealed through different types of biomolecular analysis [22]. The study of the molecular profile of GB aims at establishing a personalized therapy for each tumor subtype [20].

These observations point to a better understanding of the molecular and genetic

Table 1. Main agents acting on intracellular pathways† that have been tested in glioblastoma studies.

Pathway	Agents
IDH	AGI-5198, IDH1 inhibitor
	AG-120, inhibitor of the mutated form of the IDH1 enzyme
	AG-221, inhibitor of the mutated IDH2 protein
PI3K/Akt/mTOR	LY294002, inhibitor of PI3Ks
	Wortmannin, inhibitor of PI3Ks
	GDC-0941 (thienopyrimidine), PI3K inhibitor
	Perifosine, Akt inhibitor
	Temsirolimus, mTOR inhibitor
	Rapamycin
	XL765, inhibitor of PI3K and mTOR
	BKM 120, inhibitor of the pan-class I PI3K family
	INC280, MET inhibitor
	XL147, inhibitor of PI3K
EGFR	Gefitinib,first generation of EGFR inhibitors
	Erlotinib, first generation of EGFR inhibitors
	Afatinib, first generation of EGFR inhibitors
	Canertinib, new class of EGFR inhibitors
	Pelitinib, new class of EGFR inhibitors
	Bay846, new class of EGFR inhibitors
	Lapatinib, new class of EGFR inhibitors
	Cetuximab, monoclonal antibody against EGFRvIII
	Panitumumab, monoclonal antibody against EGFR
	Nimotuzumab, monoclonal antibody against EGFR
	Rindopepimut, EGFRvIII-targeted peptide vaccine
NOTCH	MK0752, γ-secretase inhibitor
	RO4929097, γ-secretase inhibitor
VEGF	Bevacizumab, humanized monoclonal antibody against VEGF
	Cabozantinib (XL-184, Exelixis), inhibitor of MET/VEGFR2
	Dasatinib, SFK inhibitor
	Cediranib, inhibitor of VEGFR tyrosine kinases
PDGF	Imatinib (Gleevec), PDGFR inhibitor
	Sunitinib (Sutent), PDGFR inhibitor
	Sorafenib (Nexavar), PDGFR inhibitor
	Vandetanib (Caprelsa), PDGFR inhibitor
	Tandutinib, inhibitor of type III receptor tyrosine kinases
SHH	Vismodegib, Smo inhibitor
	Itraconazole
	Arsenic trioxide
	LDE225, Smo antagonist
TGF-β	SD-208, novel TGF-βR1 kinase inhibitor
	LY364947, inhibitor of TGF-β type1 receptor
	Trabedersen,TGF-β-specific antisense oligonucleotide
	LY2157299, TGF-βR1 and TGF-βR2 kinase inhibitor
	GC1008, human anti-TGF-β monoclonal antibody
	Cilengitide, integrin inhibitor

†Several agents act indirectly on PTEN, see text. Drugs acting on Protein kinase A have not yet been tested on glioblastoma patients.
EGFR: EGF receptor.

anomalies in GB to improve therapy, by using single agents or combination protocols, to effectively target these pathways in patients (Table 1).

Novel targeted therapies for malignant glioma

• IDH

Somatic mutations in the metabolic enzyme isocitrate dehydrogenase (IDH) have been identified in different human cancers, including gliomas [23,24]. The mechanism by which mutant IDH1 contributes to the pathogenesis of human glioma is still not completely clear. Mutations of IDH1 are found in 50–80% of human low-grade gliomas, in 50% of anaplastic gliomas and in approximately 5% of GBs [25]. Further studies revealed that *IDH1* mutation is an independent prognostic marker of favorable prognosis [26]. The aberrant function of mutated IDH1 is the conversion of alphaketoglutarate to 2-hydroxyglutarate [27]. However, its role may extend beyond epigenetic effects [28]. Mutant IDH1 (mIDH1) action was investigated in fully transformed cells with endogenous *IDH1* mutations by using a selective IDH1 inhibitor (AGI-5198) that impedes the formation of R-2-hydroxyglutarate (R-2HG) by the mutant enzyme, resulting in histone H3K9me3 demethylation and in subsequent action on genes related to glial differentiation. This effect is not present in non-mutated glioma cells, while it is sufficient to block the growth of IDH1-mutant cells. At present, at least two clinical trials are targeting mutated IDH in various tumors, including gliomas (e.g., NCT02073994 and NCT02273739, as listed in www.clinicaltrials.gov).

• PTEN

PTEN suppresses Akt phosphorylation through reversion of PI3K-induced phosphorylation, with the consequent inhibition of PIP3 signaling and the suppression of cell proliferation. Even though the status of PTEN in GSCs has not been elucidated yet, it is considered one of the most important targets involved in GSC activity. *PTEN* mutations are common in primary GBs, but are rare in secondary GBs and are considered a potential prognostic marker. Low PTEN transcript levels are associated with a significantly shorter survival, compared with patients with high levels of PTEN mRNA. Also, PTEN may sensitize glioma cells to chemotherapy and RT, and also to CD95L-induced apoptosis [29]. Recent studies suggest the importance of PTEN in defining the response to EGFR tyrosine kinase inhibitors (TKIs). The expression of mutant EGFR and wild-type PTEN enhances the tumor's response to erlotinib and gefitinib. In contrast, loss-of function mutations in *PTEN* and phosphorylation at tyrosine 240 are associated with resistance to these drugs [30]. The response of *PTEN*-deficient tumors to TKIs can be increased by simultaneous inhibition of EGFR and downstream signaling molecules of PI3K/Akt pathway [31]. However, the role of PTEN in determining sensitivity/resistance to EGFR-TKI therapy is unclear [32]. Several studies showed that various miRNAs, which act as gene regulators, may be involved in glioma development, since they appear deregulated in GB specimens and cell lines [33,34]. These miRNAs act directly or indirectly on the modulation of the EGFR/PTEN/Akt pathway [35–37]. For example, the oncogenic miR-26a is upregulated in some high-grade gliomas, where it co-occurs with mono-allelic *PTEN* loss and Akt activation. This correlation was confirmed also in a murine model, in which miR-26a downregulated PTEN and facilitated glioma formation [38,39]. PTEN is also a target for other miRNAs, including miR-21, which however may modulate the EGFR/Akt pathway in a PTEN-independent way. The complex interplay of miRNAs in gliomas is still under scrutiny [39,40], as is the response of gliomas to PTEN modulators [41].

• PI3K/Akt/mTOR

Cell growth and proliferation require the activation of the PI3K/Akt/mTOR pathway. Several indications suggest that PI3K, Akt and mTOR may represent potential therapeutic targets for malignant glioma treatment [31]. Preclinical studies demonstrated that LY294002 and wortmannin can inhibit PI3K, while the thienopyrimidine drug GDC-0941 was active as an anticancer drug [42]. Akt has also been deeply investigated as a molecular target for drugs. The phospholipid perifosine may possibly interfere with the association of the Akt PH domain with PIP3, and is currently in Phase II clinical trials for different tumors [43]. Another possible target, the mTOR kinase, is also strictly related to PI3K/Akt pathway and hence may be involved in the regulation of various aspects of cell survival, from protein synthesis to cell growth [44]. Actually, mTOR inhibitors such as temsirolimus have been already tested in clinical trials for glioma treatment. Despite the fact that this molecule alone could not increase survival, it could ameliorate it when given in combined regimens [45]. Recent studies showed that multiple mechanisms may exist related to mTOR

inhibitor resistance, some of which might be exploitable [46,47], like the promyelocytic leukemia (*PML*) gene. GBs may be very resistant to mTOR-targeted therapy, an effect apparently mediated by PML [48], which is variously related to PI3K/Akt/mTOR pathway. PML may prevent mTOR and EGFR inhibitor-dependent cell death. It may oppose the function of nuclear Akt [49] and act as repressor of mTOR during hypoxia [50] and as repressor of transcriptional activity from the *EGFR* gene promoter [51]. It is possible that PML by acting via the RTK/PI3K/Akt/mTOR pathway may influence the GB cell cycle and ultimately results in resistance to various agents, including rapamycin, ATP-competitive mTOR kinase inhibitors, and EGFR tyrosine kinase inhibitors. Inhibition of PML expression reverses the resistance to mTOR kinase inhibitors *in vivo* and results in tumor growth inhibition and cell death. PML is degraded by arsenic trioxide [52]. Therefore, PML acts as a major player in the resistance to mTOR and EGFR inhibitor drugs, urging for the inclusion of PML as an additional target in the therapeutic schedule [48]. At present, various agents are being evaluated in clinical trials (e.g., NCT00704080, NCT01576666, NCT01349660, NCT01870726, NCT01339052 and NCT01240460) [53].

• EGFR pathway

EGFR gene amplification and high EGFR protein expression levels are reported in 40–60% of GB cases [54]. EGFR activation may affect the PI3K/Akt pathway. Development of EGFR-targeting molecular approaches to control the growth and recurrence of GB resulted in major progress in the last few years and revealed many factors that may significantly affect *in vivo* treatment.

The first generations of EGFR inhibitors such as gefitinib, erlotinib and afatinib have been studied in clinical trials [55], but gave no satisfactory outcomes [56]. Gefitinib is an effective therapeutic option for a subset of patients carrying an activating *EGFR* mutation [57], while *in vitro* Afatinib, an irreversible erbB family blocker, is active in tumor cells which are resistant to reversible EGFR TKI [58]. Irreversible TKIs that covalently bind to cysteines in the ATP cleft of the EGFR-TK domain represent the newest class of EGFR inhibitors [59]. This class of EGFR inhibitors includes canertinib and pelitinib, which are still in clinical studies [60], while lapatinib showed no significant activity in GB patients [61]. Also Bay846 is a recently developed irreversible small molecule inhibitor, which is more potent than lapatinib [62]. Altogether, despite the increasing *in vitro* potency of this group of drugs, TKIs are not demonstrated to have an *in vivo* effect in GB as in other cancers [59,63]. Monoclonal antibodies against EGFRvIII are being explored as therapeutic agents for GB (see e.g., clinical trial NCT00643097) and in some cases may increase the survival time [64,65]. Preclinical studies have shown an effect of cetuximab against GB, studies on the potential adjuvant effect in combination with RT and TMZ are ongoing. In a single case report, combination therapy including cetuximab and bevacizumab resulted in 20 months of progression-free survival in a patient with recurrent GB [66]. Additional monoclonal antibodies against EGFR, such as panitumumab and nimotuzumab, have shown similar efficacy as cetuximab [67,68]. However, first-line use of bevacizumab did not improve overall survival in GB patients: progression-free survival was prolonged but not enough to reach the target [69].

Several strategies may focus on the translation of selected molecules at the RNA level, these include antisense oligonucleotides, RNAi and ribozymes. All these three RNA-based strategies have been used in experimental systems to induce GB cell death [70]. Injection of vectors containing antisense RNAs that target EGFRvIII into a GB xenograft induces significant inhibition of tumor growth [71].

siRNA targeting the TK domain of EGFR can prolong survival in glioma cell lines and in an intracranial xenograft model of GB [72]. Cyclodextrin-modified dendritic polyamine complexes (DexAms) have been applied as vehicles to translocate siRNAs and deliver EGFRvIII-specific siRNAs selectively to GB: these lead to decrease systemic toxicity and mortality associated with the intervention [73]. Anti-EGFRvIII hairpin ribozymes can also significantly reduce the expression of EGFRvIII and inhibit glial tumor proliferation in cell culture [74]. Both monoclonal and vaccine approaches are influenced by the immunogenicity of the target, and intrinsic and extrinsic factors that control the host's immune response. The success of RNA-based therapies, besides experimental studies, is still dependent on a large number of factors that we need to consider [75], so a clinical translation is far.

Notch pathway

Notch signaling affects the survival of non-neoplastic neural precursors by acting on proliferation and differentiation signals. It is aberrantly activated in embryonic brain tumors [76]. This pathway activates the PI3K/Akt pathway and the prosurvival protein Mcl-1, and thus is involved in the response to DNA damage [77]. The inhibition of Notch pathway via γ-secretase inhibitors (GSIs; MK0752) affects cell growth and survival, reduces tumor formation and sensitizes GSCs to radiation [78]. Several studies demonstrated that MSI1, a RNA-binding protein, acts as a translational repressor for Numb protein mRNA [79], which is a negative regulator of the Notch pathway [80]. MSI1 expression is increased in glioma [81], astrocytoma [82] and other solid tumors [83]. In human gliomas, a correlation was demonstrated between the expression of MSI1 and the grade of malignancy, proliferative activity and cell differentiation [81]. At present, the link between MSI1 and GB is still obscure. A recent study examined the role of MSI1 in glioma cells growth [84]. MSI1 knock-down repressed Notch signaling and led to the accumulation of Numb. Since MSI1 represses Numb translation, it increases Notch signaling [85]. In many tumors MSI1 acts as an upregulating agent of Notch signaling activity [84]. Increased proliferation and inhibition of apoptosis are both hallmarks of tumorigenesis and are increased by the nuclear translocation of Notch1, which activates its downstream pathway [86]. Notch1 is upregulated while Notch2 appears downregulated in most glioma specimens and in GB cell lines [87]. Knock down of Notch1 by siRNAs in GB cells leads to inhibition of cell growth and invasion, and to induction of apoptosis. In addition upregulation of Notch2 suppressed cell growth and invasion and caused apoptosis. These data reveal that Notch1 and Notch2 play different roles in the regulation of GB growth [88].

• VEGF signaling

Anti-angiogenic agents have emerged as important therapeutic options in glioma treatment [89–93]. The humanized monoclonal antibody against VEGF, Bevacizumab, was approved in 2009 by FDA for treating recurrent GB. A large randomized Phase III trial is currently evaluating its combination with the standard-of-care therapy in patients with newly diagnosed GB [94]. However, several preclinical and clinical studies suggest that anti-angiogenic GB therapy increases tumor invasiveness [95–98]. This appears to be a consequence of the Src family kinases (SFKs) activation, associated with induced hypoxia [99,100] or with the activation of c-Met signaling [101]. Combined inhibition of angiogenesis and tumor cell invasion is now being investigated as a potential more effective therapeutic approach [102]. Treatment of mice carrying highly aggressive orthotopic glioma xenografts with the inhibitor of MET/VEGFR2 cabozantinib (XL-184, Exelixis), resulted in a significant increase in overall survival, not observed with other previously used angiogenesis inhibitors [102]. Bevacizumab-induced invasion and infiltration of orthotopically xenografted GB cells were effectively blocked by treatment with the SFK inhibitor dasatinib [103], a molecule otherwise ineffective in patients with recurrent bevacizumab-resistant GB [104]. In 2013 the results of a randomized Phase III study comparing cediranib, a potent inhibitor of VEGFR tyrosine kinases and lomustine (CCNU) in patients with recurrent GB proved no significant increase in progression-free survival or overall survival, despite some secondary beneficial effects [105]. Other clinical trials are now evaluating the efficacy of cediranib either as monotherapy or in combination with other agents [29], or γ-secretase inhibitor blocking the activation of Notch receptors (clinical trials NCT01122901, NCT01269411, NCT01119599 and NCT01189240) [53].

• PDGF signaling

PDGF and its receptor PDGFR sustain gliomagenesis [106]. Alterations in PDGF signaling are commonly observed in high-grade gliomas [20,107] and many PDGFR inhibitors have been introduced in clinical trials, among which imatinib (Gleevec) [108], sunitinib (Sutent) [109], sorafenib (Nexavar) [110] and vandetanib (Caprelsa) [111]. Imatinib inhibits the BCR, ABL, KIT tyrosine kinase proteins and PDGFR by blocking their ATP binding site [106]. A randomized Phase III study of patients with progressive, TMZ-refractory GB indicates that there is no clinical benefit of combined imatinib and hydroxyurea therapy [112]. Single-agent imatinib showed limited activity with moderate toxicity in recurrent oligodendroglioma and mixed oligoastrocytoma patients [113]. Sunitinib, an oral small molecule inhibitor of multiple RTKs including PDGFR-α and -β, was tested for treating recurrent GB and anaplastic astrocytoma in a Phase II trial, but it

demonstrated no significant activity [114,115]. A Phase I/II trial is evaluating tandutinib for treatment of recurrent or progressive GB. Sorafenib and vandetanib had only limited or no significant activity for recurrent glioma in Phase II trials [111,116], but other studies are underway to further evaluate their efficacy.

• SHH signaling

The Hedgehog (Hh) pathway modulates cell differentiation and self-renewal during embryo development but is usually silenced in adult tissues [117]. Sonic Hedgehog (SHH) activates a signal transduction cascade that comprises the membrane proteins PTCH1 and SMO, leading to the action of GLI transcription factors [118]. Aberrant activation of this signaling pathway has been described previously in basal skin carcinoma and in medulloblastoma [119] and mutations in Hh pathway have been connected to the pathogenesis of up to 30% of sporadic medulloblastomas [120]. SHH-GLI signaling is implicated not only in glioma growth and survival but also for GSC survival and proliferation [121]. Based on current research on SHH pathway, different Smo inhibitors are currently under clinical evaluation for the treatment of different cancers [122]. A Phase I clinical trial demonstrates that the Smo inhibitor vismodegib has a good tolerability and an acceptable safety profile in refractory locally advanced metastatic solid tumors such as basal cell carcinoma and medulloblastoma [123]. Phase II trials are now ongoing to study vismodegib in patients with recurrent or refractory medulloblastoma and patients with recurrent GB. Itraconazole and arsenic trioxide are two agents inhibiting Hh signaling by mechanisms distinct from that of current Smo antagonists: treatment with these molecules has recently been proved to inhibit the growth of medulloblastoma with acquired resistance to Smo inhibitors [124].

Protein kinase A

The cAMP/protein kinase A (PKA) pathway is deeply involved in the regulation of cell growth, differentiation and apoptosis of both normal and cancer cells. Abnormalities in PKA activity or expression have been reported in many different cancers [125–129]. SHH-driven proliferation of cerebellar granule cell progenitors is inhibited by pituitary adenylate cyclase activating polypeptide through a mechanism that involves activation of protein kinase A, a major inhibitor of SHH signaling. Despite this, elevated total PKA activity can coexist with moderately high levels of SHH signaling. To explain this apparent paradox it has been proposed that SHH regulates a compartmentalized pool of PKA, whereas a second PKA pool responds to stimulation by G-protein-coupled receptors (GPCRs) distributed throughout the plasma membrane. The interplay between PKA pools results in fine-tuning of the SHH-induced transcriptional activity and plays a role in cerebellar healthy development or disease [130].

Many studies indicate that activation of cAMP/PKA pathway in glioma cells induces cell cycle arrest, differentiation and apoptosis [131–134]. In a previous study we showed a distinctinve presence of PKA RIIα regulatory subunit in the Golgi complex of glioma cells, not detectable in the healthy tissue and in other types of central nervous system cancers [125,135]. PKA-dependent phosphorylation of Dock180 mediates EGFRvIII stimulation of GB tumorigenesis and invasion, suggesting that EGFRvIII-PKA-Dock180-Rac1 axis may represent a novel target to develop therapeutic tools for malignant gliomas [136].

• TGF-β

TGF-β is involved in different cellular processes. Cell growth, differentiation and survival, but also migration and immune cell activation are differentially affected by TGF-β according to cell type and extracellular environment [137,138]. High serum levels of TGF-β were observed in malignant gliomas: they directly correlate with tumor grade and outcome [139–142]. TGF-β acts on multiple targets by promoting the malignant phenotype of gliomas, which includes invasiveness, stemness, angiogenesis, immunosuppression, chemo- and radio-resistance [143].

In preclinical models, various molecules targeting TGF-β have been exploited and were demonstrated to possess antitumor activity [143]. In orthotopic glioma murine models, a novel TGF-βR1 kinase inhibitor (SD-208) promoted tumor infiltration by natural killer cells, CD8T cells and macrophages and increased the median survival [144]. The treatment of cultured murine and human glioma cell lines with an inhibitor of TGF-β type1 receptor (LY364947) increased the sensitivity to radiation. Murine and human glioma models treated with the TGF-β interfering agents following irradiation and standard chemotherapy showed a decrease in tumor growth [145,146].

A number of TGF-β targeting molecules are currently under evaluation in early clinical studies showing good tolerability and safety for glioma patients [143]. In three Phase I/II studies evaluating the TGF-β-specific antisense oligonucleotide Trabedersen, a prolonged survival compared with literature data was observed in patients with recurrent or refractory high-grade glioma [147]. Moreover, a randomized controlled dose-finding Phase IIb study showed significant effects compared with standard chemotherapy in patients with anaplastic astrocytoma receiving 10 μM Trabedersen [148]. Other clinical trials are ongoing with the TGF-βR1 and TGF-βR2 kinase inhibitor LY2157299 and the human anti-TGF-β monoclonal antibody GC1008.

EXECUTIVE SUMMARY

High-grade gliomas

- Malignant gliomas are the most aggressive tumors affecting the CNS. Despite advances in treatments and therapeutics, the prognosis of these tumors is still poor. The mean survival of patients with glioblastoma, the most malignant glioma subtype, is 14.6 months, with only 3% of patients surviving longer than 5 years.

Current approach

- The treatment approved by the US FDA for glioblastoma is total or subtotal resection followed by radiotherapy and concomitant systemic chemotherapy with temozolomide, a prodrug converting to an alkylating agent. This treatment results in 2-year survival in 27% of patients.

Failure of the current approach

- As an outcome of decades of studies, the improvement due to temozolomide treatment is still unsatisfactory. Many factors account for this failure: the lack of defined tumor margins, the critical localization of the tumor and the presence of different subpopulations of cells such as glioma stem cells.

Molecular signature

- Molecular biology is strongly conditioning the clinical practice due to its accurate and reliable results. For this reason a new molecular signature of malignant gliomas could improve not only the quality of diagnosis and prognosis, but also the effectiveness of new therapeutic tools.

Molecular targeting

- In recent years, the basis for different fates of glioblastoma patients has been unraveled by the discovery of molecular and genetic heterogeneity of this tumor. Different alterations have been demonstrated to lead to different outcomes. In particular key-role signaling molecules have been identified in many pathways specifically altered in glioblastoma: isocitrate dehydrogenase, PTEN, PI3K/Akt/mTOR, EGF, Notch, VEGF, PDGF, Sonic Hedgehog, Protien Kinase A and TGF-β. Targeting these molecules with specific chemical and/or biological agents can be the necessary step toward a new generation of therapeutics.

Main advantage

- The molecular signature of glioblastoma patients points to the advent of new more effective personalized therapies. This, hopefully, should increase the quality of the outcome and as a consequence the survival rate.

Main disadvantage

- The advantage of the personalized molecular therapy is also to be seen as a disadvantage. That's why, even if a personalized therapy should be more effective, we have to consider the economical impact of such a strong change in the clinical practice, at least in the first period when new tools may be costly. The diagnosis will be more expensive and also the direct and indirect costs of the treatment will increase.

Conclusion & future perspective

- A large number of the approaches presented in this review are still under preclinical and clinical evaluation, but none of these looks like the piece that will solve the puzzle. It is possible that single-shot approaches will not work, while multitargeted strategies appear more likely to succeed. The focus today should be oriented also toward a better integration of all these pieces of information to reach a unified model clarifying the large number of still obscure points. This will allow, hopefully in 10 years, to reach the reliable and affordable personalized molecular therapy.

Downregulation of the TGF-β pathway in GB has been explored by interfering with the expression or function of specific integrins through neutralizing antibodies, gene silencing through RNA interference or pharmacological inhibition with cilengitide. This molecule proved satisfactory until Phase II trial, but failed Phase III [149,150].

Despite these results, the inhibition of integrin remains a promising therapeutic strategy to block TGF-β-dependent features of malignancy in human GB [151].

Metabolic targets

The last frontier to fight GB appears to involve GB cell metabolism, which is related to an increased oxidation of acetate in the citric acid cycle, to support biosynthetic pathways and histone modification [152]. The enzyme ACSS2 is the main actor of this pathway; interestingly its expression is correlated with GB survival, therefore it may represent an exploitable target.

Conclusion

The complexity of malignant gliomas opens a large number of questions and controversies to discussion and investigation. Our knowledge on malignant gliomas is continuously increasing thanks to the application of new techniques and to the integration of new findings within a multidisciplinary framework. We are now able to design therapies targeting specific altered pathways and to hypothesize new connections between them. Despite these new evidences, at present the approaches targeting various pathways have been proved unsatisfactory. There may be different reasons for such failure, including poor study design and suboptimal administration schedule/formulation. A better outcome may result from combining multistep approaches, targeted at different mechanisms. Today we are still looking to turn these new advances into clinical protocols, to translate all this knowledge from the lab bench to the patient bed.

Future perspective

The current understanding of malignant gliomas is providing a wide range of opportunities to improve the accuracy of diagnostic tools and the efficacy of therapies. Many data are still needed to elucidate what is the role of each single molecular mechanism in tumor development and progression. The major goal will be the integration of all these results and new findings into a complex multifocal model clarifying the glioma ethiopathogenesis. This will justify all the efforts of the last decades providing a real outstanding progress.

Financial & competing interests disclosure

Supported by the University of Padova. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 Louis DN, Ohgaki H, Wiestler OD *et al.* The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* 114, 97–109 (2007).

• **Shows the state of the art concerning the glioma morphological classification according to WHO criteria.**

2 Jiang Y, Uhrbom L. On the origin of glioma. *Ups. J. Med. Sci.* 117, 113–121 (2012).

3 Louis DN. Molecular pathology of malignant gliomas. *Annu. Rev. Pathol.* 1, 97–117 (2006).

4 Kleihues P, Burger PC, Scheithauer BW. The new WHO classification of brain tumours. *Brain Pathol.* 3, 255–268 (1993).

5 Preusser M, de Ribaupierre S, Wohrer A *et al.* Current concepts and management of glioblastoma. *Ann. Neurol.* 70, 9–21 (2011).

6 Stupp R, Hegi ME, Neyns B *et al.* Phase I//II a study of cilengitide and temozolomide with concomitant radiotherapy followed by cilengitide and temozolomide maintenance therapy in patients with newly diagnosed glioblastoma. *J. Clin. Oncol.* 28, 2712–2718 (2010).

7 Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat. Rev. Cancer* 8, 755–768 (2008).

8 Ignatova TN, Kukekov VG, Laywell ED, Suslov ON, Vrionis FD, Steindler DA. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers *in vitro*. *Glia* 39, 193–206 (2002).

9 Persano L, Rampazzo E, Basso G, Viola G. Glioblastoma cancer stem cells: role of the microenvironment and therapeutic targeting. *Biochem. Pharmacol.* 85, 612–622 (2013).

10 Donson AM, Addo-Yobo SO, Handler MH, Gore L, Foreman NK. MGMT promoter methylation correlates with survival benefit and sensitivity to temozolomide in pediatric glioblastoma. *Pediatr. Blood Cancer* 48, 403–407 (2007).

11 Roos WP, Batista LF, Naumann SC *et al.* Apoptosis in malignant glioma cells triggered by the temozolomide-induced DNA lesion O6-methylguanine. *Oncogene* 26, 186–197 (2007).

12 Nishikawa R. Standard therapy for glioblastoma a review of where we are. *Neurol. Med. Chir.* 50, 713–719 (2010).

13 Stupp R, Hegi ME, Mason WP *et al.* Effects of radiotherapy with concomitant and

adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised Phase III study: 5 year analysis of the EORTC-NCIC trial. *Lancet Oncol.* 10, 459–466 (2009).

- **Presents the recent advances in the clinical application of the FDA approved TMZ/RT treatment for glioblastoma ("Stupp protocol").**

14 Tanaka S, Louis DN, Curry WT, Batchelor TT, Dietrich J. Diagnostic and therapeutic avenues for glioblastoma: no longer a dead end? *Clin. Oncol.* 10, 14–26 (2013).

15 Quinn JA, Jiang SX, Reardon DA *et al.* Phase II trial of temozolomide plus o6-benzylguanine in adults with recurrent, temozolomide-resistant malignant glioma. *J. Clin. Oncol.* 27, 1262–1267 (2009).

16 Adair JE, Johnston SK, Mrugala MM *et al.* Gene therapy enhances chemotherapy tolerance and efficacy in glioblastoma patients. *J. Clin. Invest.* 124, 4082–4092 (2014).

17 Happold C, Roth P, Wick W *et al.* Distinct molecular mechanisms of acquired resistance to temozolomide in glioblastoma cells. *J. Neurochem.* 122(2), 444–455 (2012).

18 Hegi ME, Diserens AC, Gorlia T *et al.* *MGMT* gene silencing and benefit from temozolomide in glioblastoma. *N. Engl. J. Med.* 352, 997–1003 (2005).

19 Bao S, Wu Q, McLendon RE. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 444, 756–760 (2006).

20 Verhaak RG, Hoadley KA, Purdom E *et al.* Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 17, 98–110 (2010).

21 Walker C, du Plessis DG, Joyce KA *et al.* Molecular pathology and clinical characteristics of oligodendroglial neoplasms. *Ann. Neurol.* 57, 855–865 (2005).

22 Manning B.D., Cantley L.C. AKT/PKB signaling: navigating downstream. *Cell* 129, 1261–1274 (2009).

23 Yen KE, Bittinger MA, Su SM, Fantin VR. Cancer-associated IDH mutations: biomarker and therapeutic opportunities. *Oncogene* 29, 6409 (2010).

24 Dang L, White DW, Gross S *et al.* Cancer-associated *IDH1* mutations produce 2-hydroxyglutarate. *Nature* 462, 739 (2009).

25 Ward PS, Patel J, Wise DR *et al.* The common feature of leukemia-associated *IDH1* and *IDH2* mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell* 17, 225 (2010).

26 Cahoy JD, Emery B, Kaushal A *et al.* A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J. Neurosci.* 28, 264 (2008).

27 Sasaki M, Knobbe CB, Itsumi M *et al.* D-2-hydroxyglutarate produced by mutant *IDH1* perturbs collagen maturation and basement membrane function. *Genes Dev.* 26, 2038 (2012).

28 Rohle D, Popovici-Muller J, Palaskas N *et al.* An inhibitor of mutant *IDH1* delays growth and promotes differentiation. *Science* 340, 626–630 (2013).

29 Ohka F, Natsume A, Wakabayashi T. Current trends in targeted therapies for glioblastoma multiforme. *Neurol. Res. Int.* 2012 878425 (2012).

30 Fenton TR, Nathanson D, Ponte de Albuquerque C *et al.* Resistance to EGF receptor inhibitors in glioblastoma mediated by phosphorylation of the PTEN tumor suppressor at tyrosine 240. *Proc. Natl Acad. Sci. USA* 109, 14164–14169 (2012).

31 Fan QW, Weiss WA. Targeting the RTK-PI3K-mTOR axis in malignant glioma: overcoming resistance. *Curr. Top. Microbiol. Immunol.* 347, 279–296 (2010).

32 Mellinghoff IK, Wang MY, Vivanco I *et al.* Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N. Engl. J. Med.* 353, 2012–2024 (2005).

33 Ciafre' SA, Galardi S, Mangiola A *et al.* Extensive modulation of a set of microRNAs in primary glioblastoma. *Biochem. Biophys. Res. Commun.* 334, 1351–1358 (2005).

34 Guessous F, Zhang Y, Kofman A *et al.* MicroRNA-34a is tumor suppressive in brain tumors and glioma stem cells. *Cell Cycle* 9, 1031–1036 (2010).

35 Esquela-Kerscher A, Slack FJ. Oncomirs-microRNAs with a role in cancer. *Nat. Rev. Cancer* 6, 259–269 (2006).

36 Garzon R, Fabbri M, Cimmino A, Calin GA, Croce CM. MicroRNA expression and function in cancer. *Trends Mol. Med.* 12, 580–587 (2006).

37 Garzon R, Calin GA, Croce CM. MicroRNAs in cancer. *Annu. Rev. Med.* 60, 167–179 (2009).

38 Huse JT, Brennan C, Hambardzumyan D *et al.* The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis *in vivo*. *Genes Dev.* 23, 1327–1337 (2009).

39 Wang Y, Wang X, Zhang J *et al.* MicroRNAs involved in the EGFR/PTEN/AKT pathway in gliomas. *J. Neurooncol.* 106, 217–224 (2012).

40 Zhou X, Ren Y, Moore L *et al.* Downregulation of miR-21 inhibits EGFR pathway and suppresses the growth of human glioblastoma cells independent of PTEN status. *Lab. Invest.* 90, 144–155 (2010).

41 Boosani CS, Agrawal DK. PTEN modulators: a patent review. *Expert Opin. Ther. Pat.* 23, 569–580 (2013).

42 Workman P, Clarke PA, Raynaud FI, Van Montfort RLM. Drugging the PI3 kinome: from chemical tools to drugs in the clinic. *Cancer Res.* 70, 2146–2157 (2010).

43 Calvo E, Bolós V, Grande E. Multiple roles and therapeutic implications of Akt signaling in cancer. *Onco Targets Ther.* 2, 135–150 (2009).

44 Azim H, Azim HA, Escudier B. Targeting mTOR in cancer: renal cell is just a beginning. *Target Oncol.* 5, 269–280 (2010).

45 De Witt Hamer PC. Small molecule kinase inhibitors in glioblastoma: a systematic review of clinical studies. *Neurooncology* 12, 304–316 (2010).

- **Analyzes the efficacy of small-molecule kinase inhibitors directed against six main targets: EGFR, mTOR, KDR, FLT1, PKC-β and PDGFR.**

46 Schwanbeck R, Martini S, Bernoth K, Just U. The Notch signaling pathway: molecular basis of cell context dependency. *Eur. J. Cell Biol.* 90, 572–581 (2011).

47 Cloughesy TF, Yoshimoto K, Nghiemphu P *et al.* Antitumor activity of rapamycin in a Phase I trial for patients with recurrent PTEN-deficient glioblastoma. *PLoS Med.* 22, e8 (2008).

48 Iwanami A, Gini B, Zanca C *et al.* PML mediates glioblastoma resistance to mammalian target of rapamycin (mTOR)-targeted therapies. *Proc. Natl Acad. Sci. USA* 110, 4339–4344 (2013).

49 Trotman LC, Alimonti A, Scaglioni PP, Koutcher JA, Cordon-Cardo C, Pandolfi PP. Identification of a tumour suppressor network opposing nuclear Akt function. *Nature* 441, 523–527 (2006).

50 Bernardi R, Scaglioni PP, Bergmann S, Horn HF, Vousden KH, Pandolfi PP. PML regulates p53 stability by sequestering Mdm2 to the nucleolus. *Nat. Cell Biol.* 6, 665–672 (2004).

51 Vallian S, Gäken JA, Trayner ID *et al.* Transcriptional repression by the promyelocytic leukemia protein, PML. *Exp. Cell Res.* 237, 371–382 (1997).

52 Lallemand-Breitenbach V, Jeanne M, Benhenda S *et al.* Arsenic degrades PML or PML-RARalpha through a SUMO-triggered RNF4/ubiquitin-mediated pathway. *Nat. Cell Biol.* 10, 547–555 (2008).

53 ClinicalTrials.gov. www.clinicaltrials.gov

54 Ohgaki H, Dessen P, Jourde B *et al.* Genetic pathways to glioblastoma: a population-based study. *Cancer Res.* 19, 6892–6899 (2004).

• **Is focused on the importance of genetic analysis of glioblastoma, trying to determine the effect of most important genetic alterations on patient survival.**

55 Strumberg D, Schultheis B, Scheulen ME *et al.* Phase II study of nimotuzumab, a humanized monoclonal antiepidermal growth factor receptor (EGFR) antibody, in patients with locally advanced or metastatic pancreatic cancer. *Invest. New Drugs* 30, 1138–1143 (2012).

56 Reardon DA, Wen PY, Mellinghoff IK. Targeted molecular therapies against epidermal growth factor receptor: past experiences and challenges. *Neuro-oncology*16(Suppl. 8), viii7–viii13 (2014).

57 Uhm JH, Ballman KV, Wu W *et al.* Phase II evaluation of gefitinib in patients with newly diagnosed grade 4 astrocytoma: Mayo/North Central Cancer Treatment Group Study N0074. *Int. J. Radiat. Oncol. Biol. Phys.* 80, 347–353 (2010).

58 Eisenstat DD. A Phase II study of daily afatinib (BIBW 2992) with or without temozolomide (21/28 days) in the treatment of patients with recurrent glioblastoma. *J. Clin. Oncol.* 29, 2010 (2011).

59 Ou SH. Second-generation irreversible epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs): a better mousetrap? A review of the clinical evidence. *Crit. Rev. Oncol. Hematol.* 83, 407–421 (2012).

60 Vivanco I, Robins HI, Rohle D *et al.* Differential sensitivity of glioma- versus lung cancer-specific *EGFR* mutations to EGFR kinase inhibitors. *Cancer Discov.* 2, 458–471 (2012).

61 Thiessen B, Stewart C, Tsao M. A Phase I/II trial of GW572016 (lapatinib) in recurrent glioblastoma multiforme: clinical outcomes, pharmacokinetics and molecular correlation. *Cancer Chemother. Pharmacol.* 65, 353–361 (2010).

62 Longo SL, Padalino DJ, McGillis S *et al.* Bay846, a new irreversible small molecule inhibitor of EGFR and Her2, is highly effective against malignant brain tumor models. *Invest. New Drugs.* 30, 2161–2172 (2012).

63 Agarwal S, Sane R, Oberoi R, Ohlfest JR, Elmquist WF. Delivery of molecularly targeted therapy to malignant glioma, a disease of the whole brain. *Expert Rev. Mol. Med.* 13, 17 (2011).

64 Sampson JH, Aldape KD, Archer GE *et al.* Greater chemotherapy-induced lymphopenia enhances tumor-specific immune responses that eliminate EGFRvIII-expressing tumor cells in patients with glioblastoma. *Neuro-oncology* 13, 324–333 (2011).

65 Babu R, Adamson DC. Rindopepimut: an evidence-based review of its therapeutic potential in the treatment of EGFRvIII-positive glioblastoma. *Core Evid.* 7, 93–103 (2012).

66 Blesa JM, Mollá SB, Esparcia MF *et al.* Durable complete remission of a brainstem glioma treated with a combination of bevacizumab and cetuximab. *Case Rep. Oncol.* 5, 676–681 (2012).

67 Taylor DD, Gercel-Taylor C. Exosomes/microvesicles: mediators of cancer-associated immunosuppressive microenvironments. *Semin. Immunopathol.* 33, 441–454 (2011).

68 Hegi, ME, Rajakannu P, Weller M. Epidermal growth factor receptor: a re-emerging target in glioblastoma. *Curr. Opin. Neurol.* 25, 774–779 (2012).

69 Gilbert MR, Dignam JJ, Armstrong TS *et al.* A randomized trial of bevacizumab for newly diagnosed glioblastoma. *N. Engl. J. Med.* 370, 699–708 (2014).

70 Bora RS, Gupta D, Mukkur TK, Saini KS. RNA interference therapeutics for cancer: challenges and opportunities. *Mol. Med. Rep.* 6, 9–15 (2012).

• **Presents major advances in development of RNA interference therapeutics and current challenges for their clinical application.**

71 Shir A, Levitzki A. Inhibition of glioma growth by tumor-specific activation of double-stranded RNA-dependent protein kinase PKR. *Nat. Biotechnol.* 20, 895–900 (2002).

72 Kang CS, Zhang ZY, Jia ZF *et al.* Suppression of EGFR expression by antisense or small interference RNA inhibits U251 glioma cell growth *in vitro* and *in vivo*. *Cancer Gene Ther.* 13, 530–538 (2006).

73 Kim C, Shah BP, Subramaniam P, Lee KB. Synergistic induction of apoptosis in brain cancer cells by targeted codelivery of siRNA and anticancer drugs. *Mol. Pharm.* 8, 1955–1961 (2011).

74 Halatsch ME, Löw S, Mursch K *et al.* Candidate genes for sensitivity and resistance of human glioblastoma multiforme cell lines to erlotinib. Laboratory Investigation. *J. Neurosurg.* 111, 211–218 (2009).

75 Kalman B, Szep E, Garzuly F, Post DE. Epidermal growth factor receptor as a therapeutic target in glioblastoma. *Neuromol. Med.* 15, 420–434 (2013).

76 Bray SJ. Notch signalling: a simple pathway becomes complex. *Nat. Rev. Mol. Cell. Biol.* 7, 678–689 (2006).

77 Bleau AM, Hambardzumyan D, Ozawa T *et al.* PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells. *Cell Stem Cell* 4, 226–235 (2009).

78 Pannuti A, Foreman K, Rizzo P *et al.* Targeting Notch to target cancer stem cells. *Clin. Cancer Res.* 16, 3141–3152 (2010).

79 Masuda H, Maruyama T, Hiratsu E *et al.* Noninvasive and real-time assessment of reconstructed functional human endometrium in NOD/SCID/gamma c(null) immunodeficient mice. *Proc. Natl Acad. Sci. USA* 104, 1925–1930 (2007).

80 Shen Q, Zhong W, Jan YN, Temple S. Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts. *Development* 129, 4843–4853 (2002).

81 Toda M, Iizuka Y, Yu W *et al.* Expression of the neural RNA-binding protein Musashi1 in human gliomas. *Glia* 34, 1–7 (2001).

82 Nakano A, Kanemura Y, Mori K *et al.* Expression of the Neural RNA-binding protein Musashi1 in pediatric brain tumors. *Pediatr. Neurosurg.* 43, 279–284 (2007).

83 Schulenburg A, Cech P, Herbacek I *et al.* CD44-positive colorectal adenoma cells express the potential stem cell markers musashi antigen (msi1) and ephrin B2 receptor (EphB2). *J. Pathol.* 213, 152–160 (2007).

84 Sureban SM, May R, George RJ *et al.* Knockdown of RNA binding protein musashi-1 leads to tumor regression *in vivo*. *Gastroenterology* 134, 1448–1458 (2008).

85 Imai T, Tokunaga A, Yoshida T *et al.* The neural RNA-binding protein Musashi1 translationally regulates mammalian numb gene expression by interacting with its mRNA. *Mol. Cell Biol.* 21, 3888–3900 (2001).

86 Schwanbeck R, Martini S, Bernoth K, Just U. The Notch signaling pathway: molecular

basis of cell context dependency. *Eur. J. Cell Biol.* 90, 572–581 (2011).

87 Purow BW, Haque RM, Noel MW *et al.* Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. *Cancer Res.* 65, 2353–2363 (2005).

88 Xu P, Zhang A, Jiang R *et al.* The different role of Notch1 and Notch2 in astrocytic gliomas. *PLoS ONE* 8, e53654 (2013).

89 Rubenstein JL, Kim J, Ozawa T *et al.* Anti-VEGF antibody treatment of glioblastoma prolongs survival but results in increased vascular cooption. *Neoplasia* 2, 306–314 (2000).

90 Laird AD, Vajkoczy P, Shawver LK *et al.* SU6668 is a potent antiangiogenic and antitumor agent that induces regression of established tumors. *Cancer Res.* 60, 4152–4160 (2000).

91 Kunkel P, Ulbricht U, Bohlen P *et al.* Inhibition of glioma angiogenesis and growth *in vivo* by systemic treatment with a monoclonal antibody against vascular endothelial growth factor receptor-2. *Cancer Res.* 61, 6624–6628 (2001).

92 Goldbrunner RH, Bendszus M, Wood J, Kiderlen M, Sasaki M, Tonn JC. PTK787/ZK222584, an inhibitor of vascular endothelial growth factor receptor tyrosine kinases, decreases glioma growth and vascularization. *Neurosurgery* 55, 426–432 (2004).

93 Fong TA, Shawver LK, Sun L *et al.* SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. *Cancer Res.* 59, 99–106 (1999).

94 Chinot OL, de La Motte Rouge T, Moore N *et al.* AVAglio: Phase 3 trial of bevacizumab plus temozolomide and radiotherapy in newly diagnosed glioblastoma multiforme. *Adv. Ther.* 28, 334–340 (2011).

95 Norden AD, Young GS, Setayesh K *et al.* Bevacizumab for recurrent malignant gliomas: efficacy, toxicity, and patterns of recurrence. *Neurology* 70, 779–787 (2008).

96 Plate KH, Scholz A, Dumont DJ. Tumor angiogenesis and anti-angiogenic therapy in malignant gliomas revisited. *Acta Neuropathol.* 124, 763–775 (2012).

97 de Groot JF, Fuller G, Kumar AJ *et al.* Tumor invasion after treatment of glioblastoma with bevacizumab: radiographic and pathologic correlation in humans and mice. *Neuro-oncology* 12, 233–242 (2010).

98 Narayana A, Gruber D, Kunnakkat S *et al.* A clinical trial of bevacizumab, temozolomide, and radiation for newly diagnosed glioblastoma. *J. Neurosurg.* 116, 341–345 (2012).

99 Zagzag D, Friedlander DR, Margolis B *et al.* Molecular events implicated in brain tumor angiogenesis and invasion. *Pediatr. Neurosurg.* 33, 49–55 (2000).

100 Keunen O, Johansson M, Oudin A *et al.* Anti-VEGF treatment reduces blood supply and increases tumor cell invasion in glioblastoma. *Proc. Natl Acad. Sci. USA* 108, 3749–3754 (2011).

101 Lu KV, Chang JP, Parachoniak CA *et al.* VEGF inhibits tumor cell invasion and mesenchymal transition through a MET/VEGFR2 complex. *Cancer Cell* 22, 21–35 (2012).

102 Navis AC, Bourgonje A, Wesseling P *et al.* Effects of dual targeting of tumor cells and stroma in human glioblastoma xenografts with a tyrosine kinase inhibitor against c-MET and VEGFR2. *PLoS ONE* 8, e58262 (2013).

103 Huveldt D, Lewis-Tuffin LJ, Carlson BL *et al.* Targeting Src family kinases inhibits bevacizumab-induced glioma cell invasion. *PLoS ONE* 8, e56505 (2013).

104 Lu-Emerson C, Norden AD, Drappatz J *et al.* Retrospective study of dasatinib for recurrent glioblastoma after bevacizumab failure. *J. Neurooncol.* 104, 287–291 (2011).

105 Batchelor TT, Mulholland P, Neyns B *et al.* Phase III randomized trial comparing the efficacy of Cediranib as monotherapy, and in combination with Lomustine, versus Lomustine alone in patients with recurrent glioblastoma. *J. Clin. Oncol.* 31, 3212–3218 (2013).

106 Razis E, Selviaridis P, Labropoulos S *et al.* Phase II study of neoadjuvant imatinib in glioblastoma: evaluation of clinical and molecular effects of the treatment. *Clin. Cancer Res.* 15, 6258–6266 (2009).

107 Brennan C, Momota H, Hambardzumyan D *et al.* Glioblastoma subclasses can be defined by activity among signal transduction pathways and associated genomic alterations. *PLoS ONE* 4, e7752 (2009).

- **Suggests the importance of a new classification strategy based on genetic mutations of major signalling pathway components.**

108 Reardon DA, Desjardins A, Vredenburgh JJ *et al.* Phase II study of Gleevec plus hydroxyurea in adults with progressive or recurrent low-grade glioma. *Cancer* 118, 4759–4767 (2012).

109 Czabanka M, Bruenner J, Parmaksiz G *et al.* Combined temozolomide and sunitinib treatment leads to better tumour control but increased vascular resistance in O6-methylguanine methyltransferase-methylated gliomas. *Eur. J. Cancer* 8049, 149–154 (2013).

110 Den RB, Kamrava M, Sheng Z *et al.* A Phase I study of the combination of sorafenib with temozolomide and radiation therapy for the treatment of primary and recurrent high-grade gliomas. *Int. J. Radiat. Oncol. Biol. Phys.* 5, 321–328 (2013).

111 Kreisl TN, McNeill KA, Sul J, Iwamoto FM, Shih J, Fine HA. A Phase I/II trial of vandetanib for patients with recurrent malignant glioma. *Neuro-oncology* 14, 1519–1526 (2012).

112 Dresemann G, Weller M, Rosenthal MA *et al.* Imatinib in combination with hydroxyurea versus hydroxyurea alone as oral therapy in patients with progressive pretreated glioblastoma resistant to standard dose temozolomide. *J. Neurooncol.* 96, 393–402 (2010).

113 Jaeckle KA, Anderson SK, Kosel M *et al.* NCCTG N0272: Phase II trial of imatinib mesylate; (Gleevec; STI571) in treatment of recurrent oligodendroglioma and mixed oligoastrocytoma. A North Central Cancer Treatment Group study. *Neuro-oncology* 13, 85–91 (2011).

114 Pan E, Yu D, Yue B *et al.* A prospective Phase II single-institution trial of sunitinib for recurrent malignant glioma. *J. Neurooncol.* 110, 111–118 (2012).

115 Kreisl TN, Smith P, Sul J *et al.* Continuous daily sunitinib for recurrent glioblastoma. *J. Neurooncol.* 111, 41–48 (2013).

116 Reardon DA, Vredenburgh JJ, Desjardins A *et al.* Effect of CYP3A-inducing anti-epileptics on sorafenib exposure: results of a Phase II study of sorafenib plus daily temozolomide in adults with recurrent glioblastoma. *J. Neurooncol.* 101, 57–66 (2011).

117 Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* 15, 3059–3087 (2001).

118 Li SH, Fu J, Watkins DN, Srivastava RK, Shankar S. Sulforaphane regulates self-renewal of pancreatic cancer stem cells through the modulation of Sonic hedgehog-GLI pathway. *Mol. Cell Biochem.* 373, 217–227 (2013).

119 Kasper M, Toftgård R. Smoothing out drug resistance. *Cancer Cell* 23, 3–5 (2013).

120 Thompson MC, Fuller C, Hogg TL *et al.* Genomics identifies medulloblastoma subgroups that are enriched for specific genetic alterations. *J. Clin. Oncol.* 24, 1924–1931 (2006).

121 Clement V, Sanchez P, de Tribolet N, Radovanovic I, Ruiz i Altaba A. HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity. *Curr. Biol.* 17, 165–172 (2007).

122 Lin TL, Matsui W. Hedgehog pathway as a drug target: smoothened inhibitors in development. *Onco Targets Ther.* 5, 47–58 (2012).

123 LoRusso PM, Rudin CM, Reddy JC *et al.* Phase I trial of hedgehog pathway inhibitor vismodegib (GDC-0449) in patients with refractory, locally advanced or metastatic solid tumors. *Clin. Cancer Res.* 17, 2502–2511 (2011).

124 Kim J, Aftab BT, Tang JY *et al.* Itraconazole and arsenic trioxide inhibit Hedgehog pathway activation and tumor growth associated with acquired resistance to smoothened antagonists. *Cancer Cell* 23, 23–34 (2013).

125 Mucignat-Caretta C, Cavaggioni A, Redaelli M, Malatesta M, Zancanaro C, Caretta A. Selective distribution of protein kinase A regulatory subunit RII{alpha} in rodent gliomas. *Neuro-oncology* 10, 958–967 (2008).

126 Nesterova M, Yokozaki H, McDuffie E, Cho-Chung YS. Overexpression of RII beta regulatory subunit of protein kinase A in human colon carcinoma cell induces growth arrest and phenotypic changes that are abolished by site-directed mutation of RII beta. *Eur. J. Biochem.* 235, 486–494 (1996).

127 Carlson CC, Smithers SL, Yeh KA, Burnham LL, Dransfield DT. Protein kinase A regulatory subunits in colon cancer. *Neoplasia* 1, 373–378 (1999).

128 Gu L, Lau SK, Loera S, Somlo G, Kane SE. Protein kinase A activation confers resistance to trastuzumab in human breast cancer cell lines. *Clin. Cancer Res.* 15, 7196–7206 (2009).

129 Loilome W, Juntana S, Namwat N *et al.* PRKAR1A is overexpressed and represents a possible therapeutic target in human cholangiocarcinoma. *Int. J. Cancer* 129, 34–44 (2011).

130 Niewiadomski P, Zhujiang A, Youssef M, Waschek JA. Interaction of PACAP with Sonic hedgehog reveals complex regulation of the Hedgehog pathway by PKA. *Cell Signal.* 25, 2222–2230 (2013).

131 Chen TC, Hinton DR, Zidovetzki R, Hofman FM. Up-regulation of the cAMP/PKA pathway inhibits proliferation, induces differentiation, and leads to apoptosis in malignant gliomas. *Lab. Invest.* 78, 165–174 (1998).

132 Li Y, Yin W, Wang X, Zhu W, Huang Y, Yan G. Cholera toxin induces malignant glioma cell differentiation via the PKA/CREB pathway. *Proc. Natl Acad. Sci. USA* 104, 13438–13443 (2007).

133 He S, Zhu W, Zhou Y *et al.* Transcriptional and post-transcriptional down-regulation of cyclin D1 contributes to C6 glioma cell differentiation induced by forskolin. *J. Cell Biochem.* 112, 2241–2239 (2011).

134 Ku BM, Lee YK, Jeong JY *et al.* Caffeine inhibits cell proliferation and regulates PKA/GSK3β pathways in U87MG human glioma cells. *Mol. Cell* 31, 275–279 (2011).

135 Mucignat-Caretta C, Denaro L, Redaelli M, D'Avella D, Caretta A. Protein kinase A regulatory subunit distribution in medulloblastoma. *BMC Cancer* 10, 141 (2010).

136 Feng H, Hu B, Vuori K *et al.* EGFRvIII stimulates glioma growth and invasion through PKA-dependent serine phosphorylation of Dock180. *Oncogene* 33, 2504–2512 (2013).

137 Seoane J. Escaping from the TGFbeta anti-proliferative control. *Carcinogenesis* 27, 2148–2156 (2006).

138 Massagué J. TGFbeta in Cancer. *Cell* 134, 215–230 (2008).

139 Rich JN. The role of transforming growth factor-beta in primary brain tumors. *Front. Biosci.* 8, 245–260 (2003).

140 Sasaki A, Naganuma H, Satoh E *et al.* Secretion of transforming growth factor-beta 1 and -beta 2 by malignant glioma cells. *Neurol. Med. Chir.* 35, 423–430 (1995).

141 Gold LI. The role for transforming growth factor-beta (TGF-beta) in human cancer. *Crit. Rev. Oncog.* 10, 303–360 (1999).

142 Platten M, Wick W, Weller M. Malignant glioma biology: role for TGF-beta in growth, motility, angiogenesis, and immune escape. *Microsc. Res. Tech.* 52, 401–410 (2001).

143 Joseph JV, Balasubramaniyan V, Walenkamp A, Kruyt FA. TGF-β as a therapeutic target in high grade gliomas - promises and challenges. *Biochem. Pharmacol.* 85, 478–485 (2013).

144 Uhl M, Aulwurm S, Wischhusen J *et al.* SD-208, a novel transforming growth factor beta receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells *in vitro* and *in vivo*. *Cancer Res.* 64, 7954–7961 (2004).

145 Hardee ME, Marciscano AE, Medina-Ramirez CM *et al.* Resistance of glioblastoma-initiating cells to radiation mediated by the tumor microenvironment can be abolished by inhibiting transforming growth factor-β. *Cancer Res.* 72, 4119–4129 (2012).

146 Zhang M, Herion TW, Timke C *et al.* Trimodal glioblastoma treatment consisting of concurrent radiotherapy, temozolomide, and the novel TGF-β receptor I kinase inhibitor LY2109761. *Neoplasia* 13, 537–549 (2011).

147 Hau P, Jachimczak P, Schlingensiepen R *et al.* Inhibition of TGF-beta2 with AP 1 2009 in recurrent malignant gliomas: from preclinical to Phase I/II studies. *Oligonucleotides* 17, 201–212 (2007).

148 Bogdahn U, Hau P, Stockhammer G *et al.* Targeted therapy for high-grade glioma with the TGF-β2 inhibitor trabedersen: results of a randomized and controlled Phase IIb study. *Neuro-oncology* 13, 132–142 (2011).

149 Nabors LB, Mikkelsen T, Hegi ME *et al.* A safety run-in and randomized Phase II study of Cilengitide combined with chemoradiation for newly diagnosed glioblastoma (NABTT 0306). *Cancer* 118, 5601–5607 (2012).

150 Stupp R, Hegi ME, Gorlia T *et al.* Cilengitide combined with standard treatment for patients with newly diagnosed glioblastoma with methylated MGMT promoter (CENTRIC EORTC 6071–22072 study): a multicentre, randomised, open-label, Phase 3 trial. *Lancet Oncol.* 15, 1100–1108 (2014).

151 Roth P, Silginer M, Goodman SL *et al.* Integrin control of the transforming growth factor-β pathway in glioblastoma. *Brain* 136, 564–576 (2013).

152 Mashimo T, Pichumani K, Vemireddy V *et al.* Acetate is a bioenergetic substrate for human glioblastoma and brain metastases. *Cell* 159, 1603–1614 (2014).