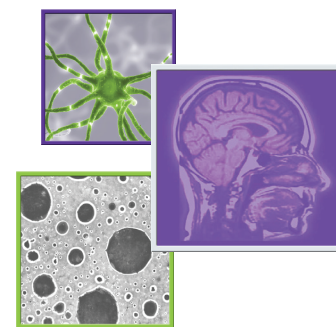# Genetic biomarkers of drug response for small-molecule therapeutics targeting the RTK/Ras/PI3K, p53 or Rb pathway in glioblastoma

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Practice points

- Three major pathways are deregulated in glioblastoma: the RTK/Ras/PI3K signaling, the p53 and the Rb tumor suppressor pathways.

- These pathways are clinically attractive to target due to their frequent hyper- or inactivation.

- Many drugs targeting these pathways appear to respond in only a subset of patients, and therefore the effect of a certain experimental drug will be diluted within an unselected study population.

- Preclinical models that faithfully represent the tumor characteristics as observed in the patient must be applied to identify clinically relevant biomarkers and to filter out compounds that will likely not be effective for the patient.

- Clinical trials need to be stratified according to the molecular predictor for response.

Glioblastoma is the most deadly and frequently occurring primary malignant tumor of the central nervous system. Genomic studies have shown that mutated oncogenes and tumor suppressor genes in glioblastoma mainly occur in three pathways: the RTK/Ras/PI3K signaling, the p53 and the Rb pathways. In this review, we summarize the modulatory effects of genetic aberrations in these three pathways to drugs targeting these specific pathways. We also provide an overview of the preclinical efforts made to identify genetic biomarkers of response and resistance. Knowledge of biomarkers will finally promote patient stratification in clinical trials, a prerequisite for trial design in the era of precision medicine.

The aim of precision medicine is to tailor cancer treatment to the specific tumor characteristics of each individual patient. Glioblastomas carry a heterogeneous set of molecular aberrations driving tumor progression [1–3]. Integrative analyses of mutational and copy number data identified three major deregulated pathways in glioblastoma: the RTK/Ras/PI3K signaling, the p53 and the Rb pathway [1,3]. An average of 80–90% of glioblastomas contains at least one aberration in each pathway, highlighting their significance as possible therapeutic targets [1,3]. In addition to the increased insights into cancer biology, a large array of compounds has been developed that target different signaling pathways. However, many compounds do not pass preclinical testing and even fewer fulfill the required endpoints in clinical trials. An underlying reason may be attributed to the

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Future Medicine part of fsg

large patient-to-patient variability of glioblastoma. Many drugs appear to respond in only a subset of patients, and therefore the effect of a certain experimental drug will be diluted within an unselected study population [4]. As a result it will be difficult to detect a clinically relevant treatment effect that is restricted to a subpopulation with a specific predictive marker (for examples, see [4]). To avoid this problem, clinical trials need to be stratified according to the molecular predictor for response.

Appropriately, based on gene expression data The Cancer Genome Atlas (TCGA) consortium proposed glioblastoma to be divided into the following four subtypes: proneural, neural, classical and mesenchymal [2]. A later study by the TCGA looked at the extent of promoter methylation and identified a hypermethylated group of tumors which correlated with the presence of *IDH1* mutations, which they named the glioma-CpG island methylator phenotype (G-CIMP) [5]. Interestingly, this intertumor heterogeneity can be preserved in the lab for *in vitro* experiments using glioma stem-like cells (GSC) [6]. In respect to the variety of glioma models available, patient-derived glioma cultures and orthotopic xenografts of patient-derived material are gaining more research focus since they appear to mimic the clinical responses more faithfully than established cell lines [7,8]. Since the discovery that serum-free neurobasal medium supplemented with basic FGF and EGF faithfully preserves the molecular aberrations of patient-derived glioblastomas, this culture method is increasingly being implemented in drug development research [7,8].

In this review, we provide an overview of the preclinical studies performed in the last decade on glioblastoma, investigating the value of genetic aberrations for the prediction of response to small-molecule therapeutics specifically targeting the RTK/Ras/PI3K, p53 or Rb pathway.

RTK/Ras/PI3K pathway

Approximately 90% of glioblastomas contain one or more genetic aberrations in the RTK/Ras/PI3K pathway [1,3], making it one of the most intensely studied pathways in drug development. Deregulation of this pathway affects several hallmarks of cancer [9], such as sustaining proliferative signaling, evading growth suppressors, activating invasion and resisting cell death.

EGFR inhibition

RTKs are altered in approximately 70% of glioblastomas, of which *EGFR* is most frequently mutated [3]. Approximately half of all glioblastomas contain amplifications of the *EGFR* gene and half of these contain in-frame deletions [3,10]. *EGFRvIII* is the predominant in-frame deletion, which arises due to the deletion of exons 2–7, leading to a constitutively active mutant receptor [11]. Missense mutations are usually mutually exclusive with the presence of in-frame deletions and occur in more than 20% of glioblastomas. These missense mutations are predominantly located in the extracellular domain of *EGFR* [12,13].

Due to the frequently occurring mutations in *EGFR*, it is a popular drug target. The clinical experience with EGFR inhibitors for glioblastoma was recently reviewed by Reardon and colleagues [14]. First-generation EGFR inhibitors compete with ATP-binding in the catalytic tyrosine kinase domain and bind reversibly to EGFR (such as erlotinib, gefitinib, lapatinib, PKI-166 and vandetanib). On the other side, second-generation EGFR inhibitors bind irreversibly to EGFR (such as NT113, neratinib and dacomitinib).

• *EGFR* aberrations in the context of EGFR inhibition

EGFR amplification has been shown to sensitize glioma stem-like cells (GSC) to erlotinib [15], dacomitinib [16] and lapatinib [12] **(Figure 1)**. However, interestingly, the choice of the tumor model appears to play an important role in determining the eventual drug response. In the study performed by Eimer and colleagues the same clones cultured in serum-supplemented medium (SSM) were more resistant to erlotinib compared with GSCs [15]. There are various possible explanations underlying this observation, such as a failure to retain the increased *EGFR* copy number in SSM [17], the abundance of growth factors facilitating RTK switching [18] or perhaps coactivation of other RTKs [19]. Furthermore, a study performed in a different model, using orthotopic xenografts also did not observe sensitivity to erlotinib in amplified wild-type *EGFR* even in the presence of wild-type phosphatase and tensin homolog (*PTEN*) [20].

Besides *EGFR* amplification, several studies have found *EGFRvIII* to sensitize glioblastoma cell lines *in vitro* to erlotinib [21], gefitinib [22], vandetanib [23] and NT113 [24] compared with

wild-type *EGFR*. This finding also appears to hold in orthotopic xenograft models of glioblastoma cell lines for erlotinib [25]. Especially cell lines with PTEN and EGFRvIII expression are more sensitive to erlotinib [26] and gefitinib [27] compared with wild-type *EGFR*. Recently it has also been reported in a glioblastoma stem-like cell culture carrying an *EGFRvIII* construct that it conveys an increased sensitivity to erlotinib and lapatinib compared with wild-type *EGFR* [28]. In addition, *EGFRvIII* sensitizes orthotopic xenografts of glioblastoma cell lines and patient-derived glioblastoma to vandetanib [23] and NT113 [24]. However other studies find no sensitization by *EGFRvIII* to erlotinib [29,30] or gefitinib [31]. Nevertheless, collectively there is more compelling evidence indicating that *EGFRvIII* sensitizes glioblastoma cells to EGFR inhibitors. A recent report, by Nathanson and colleagues [32] supports this by showing that the presence of more *EGFRvIII* extrachromosomal DNA promotes sensitivity to erlotinib [32].

Other types of *EGFR* in-frame deletions include *EGFRvII* and the recently discovered '*EGFRvII*-extended'. In *EGFRvII*, exons 14–15 are deleted. Whereas in the recently discovered *EGFRvII*-extended, exons 14–16 are deleted [33]. *EGFRvII* is present in around 10% of the *EGFR* mutations in GBM. Recently, Francis and colleagues have shown that *EGFRvII* is oncogenic and sensitive to a panel of EGFR inhibitors **(Figure 1)** [33].

In contrast to the frequently occurring *EGFRvIII*, recurrent in-frame C-terminal deletions are relatively rare [3,34,35]. Therefore it is not surprising that the functional consequences of these mutations have not yet been studied extensively. Cho and colleagues have performed one of the few studies looking into the functional consequence of C-terminal deletions [29]. They demonstrate that the *EGFR* C-terminal deletions CT982NT (exons 25–27 deletion) and CT1054NT (exon 27 deletion) are oncogenic in murine Ba/F3 pro-B lymphocytes and sensitize them to erlotinib [29].
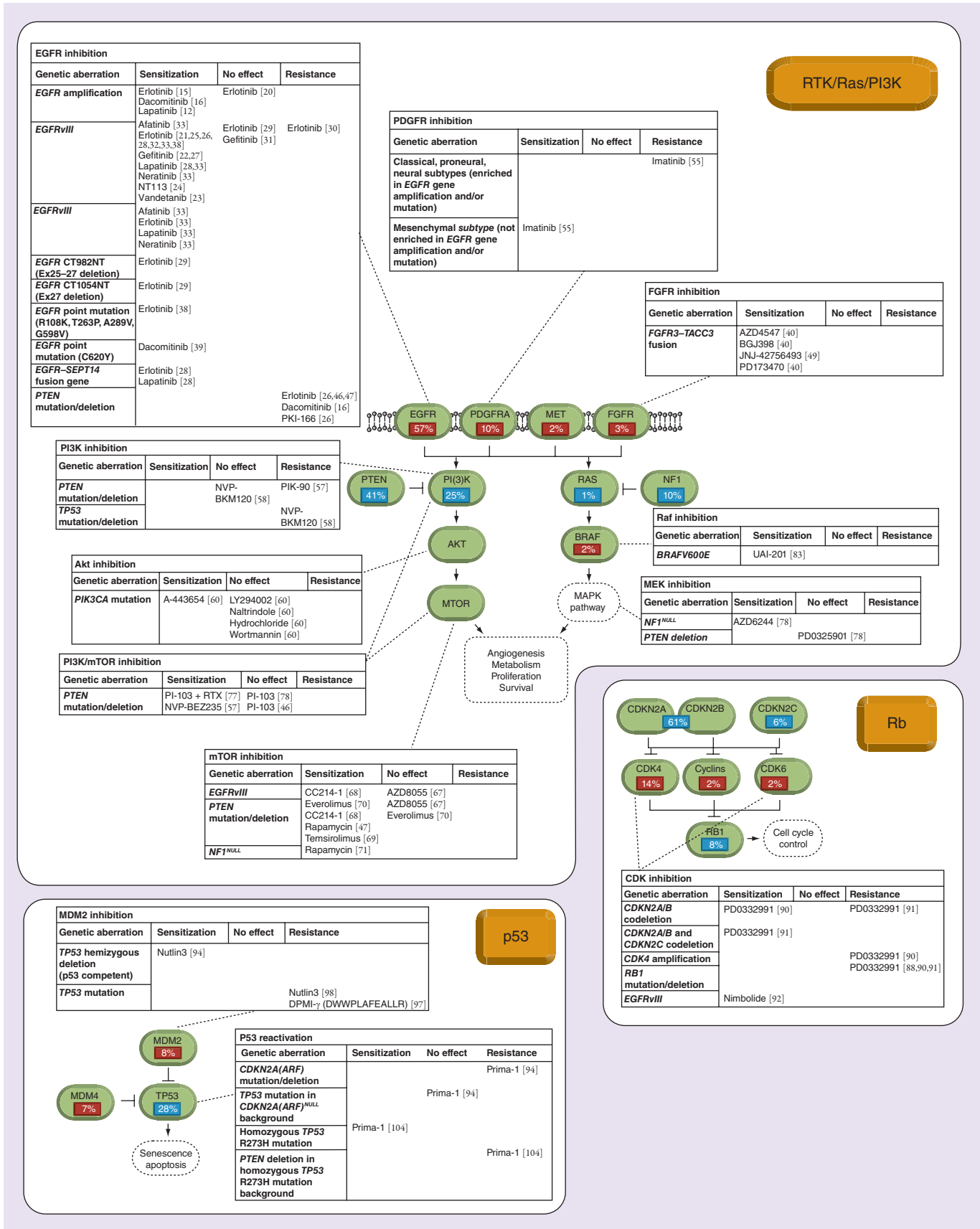
Besides amplification or focal exon deletions of *EGFR*, single base mutations in *EGFR* can have profound effects on the response to EGFR inhibitors. *EGFR* mutations in glioblastoma appear to confer *EGFR* oncogene addiction [36,37]. Using murine Ba/F3 pro-B lymphocytes as a model system to investigate the oncogenic effect of different *EGFR*

mutations, Lee and colleagues demonstrate that the *EGFR* ectodomain mutations (R108K, T263P, A289V, G598V) found in glioblastoma were more sensitive to erlotinib than the *EGFR* wild-type Ba/F3 cells [38]. Afterward Greenall and colleagues investigated the functional effects of additional ectodomain mutants and found that the glioma-specific *EGFR* C620Y mutation confers sensitivity to the pan-ERBB inhibitor, dacomitinib [39]. Interestingly, EGFR dependence cannot be exploited equally effective by all EGFR inhibitors. EGFR inhibitors can either bind to the conformationally active EGFR kinase domain (type I inhibitors, such as erlotinib) [36] or conformationally inactive EGFR kinase domain (type II inhibitors, such as lapatinib) [37]. In glioblastoma, activating point mutations occur predominantly in the extracellular domain of *EGFR* and confer sensitivity to EGFR type II inhibitors in contrast to lung cancer, where kinase domain mutations are predominant which confer sensitivity to EGFR type I inhibitors [12]. It is clear that drug response correlations identified in other types of cancer may not directly be extrapolated to glioblastoma and this should be taken into account when designing trials based on molecular markers independent of tumor histology (i.e., basket trials).

In contrast to the long-known *EGFR* point mutations, *EGFR* fusion genes were only recently found to play a role in glioblastoma, offering unique possibilities to investigate fusion oncogene dependency [3,28,40–45]. *EGFR* has been identified as one of the most frequently rearranged gene, fusing with genes such as *SEPT14* (±4% of glioblastomas) [3,28], *PSPH* (±2%) [28] and *SEC61G* (±2%) [3]. Frattini and colleagues functionally investigated the *EGFR–SEPT14* fusion gene and found that it sensitizes GSCs to erlotinib and lapatinib [28]. This report highlights the potential clinical relevance of fusion oncogenes in glioblastoma.

• *PTEN* mutations in the context of EGFR inhibition

PTEN acts downstream of EGFR and is an important repressor of PI3K–Akt–mTOR pathway activity. Loss of *PTEN* appears to confer resistance of glioblastoma cell lines *in vitro* to EGFR inhibitors such as erlotinib [26,46,47], dacomitinib [16] and PKI-166 [26]. However, resistance due to *PTEN* loss can be overcome by simultaneously inhibiting EGFR and mTOR [47].

RTK/Ras/PI3K

EGFR inhibition

Genetic aberration	Sensitization	No effect	Resistance
EGFR amplification	Erlotinib [15] Dacomitinib [16] Lapatinib [12]	Erlotinib [20]	
EGFRvIII	Afatinib [33] Erlotinib [21,25,26, 28,32,33,38] Gefitinib [22,27] Lapatinib [28,33] Neratinib [33] NT113 [24] Vandetanib [23]	Erlotinib [29] Gefitinib [31]	Erlotinib [30]
EGFRvIII	Afatinib [33] Erlotinib [33] Lapatinib [33] Neratinib [33]		
EGFR CT982NT (Ex25–27 deletion)	Erlotinib [29]		
EGFR CT1054NT (Ex27 deletion)	Erlotinib [29]		
EGFR point mutation (R108K, T263P, A289V, G598V)	Erlotinib [38]		
EGFR point mutation (C620Y)	Dacomitinib [39]		
EGFR–SEPT14 fusion gene	Erlotinib [28] Lapatinib [28]		
PTEN mutation/deletion			Erlotinib [26,46,47] Dacomitinib [16] PKI-166 [26]

PDGFR inhibition

Genetic aberration	Sensitization	No effect	Resistance
Classical, proneural, neural subtypes (enriched in *EGFR* gene amplification and/or mutation)			Imatinib [55]
Mesenchymal *subtype* (not enriched in *EGFR* gene amplification and/or mutation)	Imatinib [55]		

FGFR inhibition

Genetic aberration	Sensitization	No effect	Resistance
FGFR3–TACC3 fusion	AZD4547 [40] BGJ398 [40] JNJ-42756493 [49] PD173470 [40]		

PI3K inhibition

Genetic aberration	Sensitization	No effect	Resistance
PTEN mutation/deletion		NVP- BKM120 [58]	PIK-90 [57]
TP53 mutation/deletion			NVP- BKM120 [58]

Akt inhibition

Genetic aberration	Sensitization	No effect	Resistance
PIK3CA mutation	A-443654 [60]	LY294002 [60] Naltrindole [60] Hydrochloride [60] Wortmannin [60]	

PI3K/mTOR inhibition

Genetic aberration	Sensitization	No effect	Resistance
PTEN mutation/deletion	PI-103 + RTX [77] NVP-BEZ235 [57]	PI-103 [78] PI-103 [46]	

mTOR inhibition

Genetic aberration	Sensitization	No effect	Resistance
EGFRvIII	CC214-1 [68]	AZD8055 [67]	
PTEN mutation/deletion	Everolimus [70] CC214-1 [68] Rapamycin [47] Temsirolimus [69]	AZD8055 [67] Everolimus [70]	
NF1^NULL	Rapamycin [71]		

Raf inhibition

Genetic aberration	Sensitization	No effect	Resistance
BRAFV600E	UAI-201 [83]		

MEK inhibition

Genetic aberration	Sensitization	No effect	Resistance
NF1^NULL	AZD6244 [78]		
PTEN deletion			PD0325901 [78]

EGFR **57%** PDGFRA **10%** MET **2%** FGFR **3%**

PTEN **41%** PI(3)K **25%** RAS **1%** NF1 **10%**

AKT BRAF **2%**

MTOR MAPK pathway

Angiogenesis
Metabolism
Proliferation
Survival

Rb

CDKN2A CDKN2B **61%** CDKN2C **6%**

CDK4 **14%** Cyclins **2%** CDK6 **2%**

RB1 **8%** Cell cycle control

CDK inhibition

Genetic aberration	Sensitization	No effect	Resistance
CDKN2A/B codeletion	PD0332991 [90]		PD0332991 [91]
CDKN2A/B and *CDKN2C* codeletion	PD0332991 [91]		
CDK4 amplification			PD0332991 [90] PD0332991 [88,90,91]
RB1 mutation/deletion			
EGFRvIII	Nimbolide [92]		

MDM2 inhibition

Genetic aberration	Sensitization	No effect	Resistance
TP53 hemizygous deletion (p53 competent)	Nutlin3 [94]		
TP53 mutation			Nutlin3 [98] DPMI-γ (DWWPLAFEALLR) [97]

p53

MDM2 **8%**

MDM4 **7%** TP53 **28%**

Senescence
apoptosis

P53 reactivation

Genetic aberration	Sensitization	No effect	Resistance
CDKN2A(ARF) mutation/deletion			Prima-1 [94]
TP53 mutation in *CDKN2A(ARF)*^NULL background		Prima-1 [94]	
Homozygous *TP53* R273H mutation	Prima-1 [104]		
PTEN deletion in homozygous *TP53* R273H mutation background			Prima-1 [104]

Figure 1. Overview of the genetic aberrations conferring sensitivity or resistance to compounds targeting the RTK/Ras/PI3K, p53 or Rb pathway in glioblastoma (see facing page). The frequency of genetic amplifications (red), mutations and deletions (blue) are indicated in percentages. For each target molecule, a summary of the genetic biomarkers and its status of predicting sensitivity or resistance is given.

FGFR inhibition

FGF signaling has been linked to proliferation, cellular survival and angiogenesis within a wide range of tumors and therefore the development of FGFR inhibitors is actively being pursued [48]. Although only 3% of glioblastomas contain aberrant *FGFR* [3,49], there is a renewed interest in FGFR inhibition in glioma due to the discovery of *FGFR* fusion genes [40,41,50,51]. Inhibition of fusion gene products is attractive due to the remarkable success of imatinib targeting the *BCR–ABL1* fusion gene in chronic myeloid leukemia (CML) [52].

• *FGFR* fusion genes in the context of FGFR inhibition

The *FGFR–TACC* fusion gene was one of the first recurrent fusion genes to be discovered in glioblastoma [40]. Singh and colleagues discovered *FGFR3–TACC1* and *FGFR1–TACC3* (±3%) fusion genes and found that these drive gliomagenesis when introduced into astrocytes [40]. Recently, Di Stefano and colleagues showed that *FGFR* fusion genes occur mutually exclusive with *EGFR* amplification, *EGFRvIII* and *IDH1/2* mutations whereas they appear to co-occur with *CDK4* amplification [49]. Interestingly, GSCs transduced with these fusion genes are highly sensitive to FGFR inhibitors [40,49], suggesting a similar fusion gene dependence as in BCR–ABL-driven CML.

PDGFR inhibition

PDGFR is amplified or mutated in approximately 10% of glioblastomas. Glioblastomas of the proneural subtype are mainly driven by PDGFR signaling and a third of these tumors contain a *PDGFRA* amplification [2,53]. Apart from a sporadic gene fusion between *VEGFRII* and *PDGFRA* [43], *PDGFRA* is not known to be involved in recurrent gene fusions. In contrast, *PDGFRA* does contain a frequent in-frame deletion of 243 bp of exons 8–9, resulting in the constitutively active $PDGFRA^{\Delta 8,\,9}$ [43,54]. Cells carrying this deletion appear to be sensitive to PDGFR inhibitors [43], however it is yet unknown whether $PDGFRA^{\Delta 8,\,9}$ confers more sensitivity to these drugs compared with its wild-type counterpart.

• *EGFR* mutations in the context of PDGFR inhibition

Hägerstrand and colleagues studied 11 GSCs and subdivided them according to the expression of a set of genes [55]. A subset of glioblastomas, termed as 'type A tumors', contained more classical, neural and/or proneural tumors which were enriched for *EGFR* mutations. In contrast, 'type B tumors' were more related to the mesenchymal subtype. They observed that type A cultures were more resistant whereas type B tumors were more sensitive to imatinib. Possibly, type A cultures were more resistant to imatinib due to a decreased dependence on PDGFR signaling in the setting of increased coactivated mutant EGFR.

PI3K inhibition

PI3K is an intensely studied drug target for which inhibitors are being developed and tested within clinical trials for the treatment of glioblastoma (for review, see [56]). PI3Ks are divided into class I–III, of which class I is the most studied in glioblastoma. *PIK3CA*, *PIK3CB* and *PIK3CD* encode the class IA catalytic isoforms: p110α, p110β and p110δ, respectively. These isoforms heterodimerize with any of the regulatory subunits p85α, p85β, p55γ (encoded by *PIK3R1*, *PIK3R2*, *PIK3R3,* respectively). PI3K is mutated in 25% of glioblastomas, with 18% affecting the p110α and/or p85α subunits and 7% affecting other PI3K family genes [3].

• *PTEN* & *TP53* mutations in the context of PI3K inhibition

Since *PTEN* is a known modulator of the PI3K–Akt–mTOR pathway, it is relevant to know whether *PTEN* mutations also modulate the response to PI3K inhibitors. Chen and colleagues found that *PTEN*-mutated glioma cell lines tend to be more resistant to single treatment with PI3K inhibition or radiation [57]. However, PI3K inhibition combined with radiation could decrease cell survival to a level comparable with the response of combinatorial treated *PTEN* wild-type glioma cell lines. So PI3K inhibitors are able to sensitize gliomas to radiation-induced cytotoxicity, only in the absence of PTEN wild-type expression [57].

Koul and colleagues tested NVP-BKM120 (a pan class I PI3K inhibitor) on a panel of 21 established glioma cell lines differing in *PTEN* and *TP53* mutation status [58]. No relation was found between *PTEN* status and drug sensitivity, however there was a clear relation with the *TP53* mutation status. *TP53* wild-type cells were more sensitive and underwent apoptotic cell death, whereas p53 mutant cells underwent a mitotic catastrophe cell death [58].

Akt inhibition

Mutations in RTKs, PI3K and *PTEN* result in downstream hyperphosphorylation of Akt, leading to an increase in cell proliferation and this supports the rationale to inhibit Akt [59]. The development of catalytic and allosteric inhibitors provides the tools to target Akt.

• *PIK3CA* mutation in the context of Akt inhibition

Gallia and colleagues used a *PIK3CA* isogenic cell culture knockout system, in which one cell line contained mutant *PIK3CA* and as a consequence had hyperactivated Akt, whereas the other cell line contained wild-type *PIK3CA* [60]. Several PI3K/Akt inhibitors were screened for selective inhibition of cells with the mutant phenotype and they identified A-443654 as a selective PIK3CA mutant inhibitor. Due to previously reported metabolic toxicity problems in mice [61], Gallia and colleagues delivered A-443654 locally with polymers in a xenograft mouse model [60]. Polymers containing 30% A-443654 were well tolerated, but could only prolong survival for a few more days. Unfortunately, Akt inhibitors have shown limited single-agent efficacy in clinical trials, therefore, future research is focused more on combination therapies.

mTOR inhibition

mTOR is a master regulator of cell growth and is an essential node downstream of PI3K hyperactivation. As a consequence, mTOR has been the focus of numerous drug development programs for the past two decades. mTOR comprises mTORC1 and mTORC2 (for review, see [62,63]). It operates downstream of Akt and regulates functions in growth signaling pathways. First-generation mTOR inhibitors (such as rapamycin, temsirolimus, everolimus and sirolimus) inhibit only mTORC1 and not mTORC2. Whereas second-generation mTOR inhibitors compete for the ATP-binding catalytic site and inhibit both mTORC1 and mTORC2. Dual inhibition of mTORC1 and mTORC2 may increase the efficacy of mTOR inhibitors since compelling evidence points toward involvement of mTORC2 in treatment resistance [64], and gliomagenesis [65,66].

• *EGFRvIII* in the context of mTOR inhibition

EGFRvIII is constitutively activated and consequently leads to constitutive mTOR activation. There are contradictory reports about the predictive role of *EGFRvIII* for dual mTORC1/2 dual inhibitors. Luchman and colleagues studied GSCs differing in *EGFRvIII* status and did not report any difference in cell viability at a concentration of 2 uM of AZD8055 [67]. Whereas Gini and colleagues tested U87-cell lines differing in *EGFRvIII* status and reported that *EGFRvIII* mutant cells were more sensitive to CC214–1 in a dose-dependent manner compared with U87-*EGFRvIII*-negative cells [68].

• *PTEN* mutations in the context of mTOR inhibition

As with *EGFRvIII*, *PTEN* mutations lead to hyperactivated mTOR signaling. The predictive value of *PTEN* mutations for mTOR inhibitors has been debated. Several studies have found *PTEN* mutant glioma cell lines to be more sensitive for rapamycin analogs (such as everolimus and temsirolimus) [47,69,70] and dual mTORC1/2 inhibitors [68] compared with *PTEN* wild-type glioma cell lines. Weiler and colleagues concluded that they did not find any correlation between *PTEN* status and sensitivity to temsirolimus [69]. However a closer evaluation between the *PTEN* status and the provided IC_{50} value actually appears to reveal a strong tendency for *PTEN* mutants to be more sensitive to temsirolimus [69]. Yang and colleagues studied the efficacy of everolimus in glioma cell lines in parallel to a panel of patient-derived orthotopic xenografts [70]. Interestingly, the established *PTEN* mutant glioma cell lines were sensitive to mTOR inhibition, whereas xenografts of patient-derived *PTEN* mutant cells did not respond to everolimus [70]. They hypothesized that this discrepancy could be due to adaptive changes taking place with long-term culturing of tumor cells *in vitro*. A study using only GSCs (which better preserves the parental tumor [7]), supports this hypothesis, since it did not find any predictive value of *PTEN* mutant status for response to a dual mTORC1/2 inhibitor [67].

• *NF1* deletions in the context of mTOR inhibition

Similar to *PTEN*, *NF1* functions as a tumor suppressor gene. *NF1* inactivation is enriched in the more malignant, mesenchymal subtype of glioblastoma [2], and leads to disinhibition of the Ras pathway. McGillicuddy and colleagues reported that in a subset of glioblastomas NF1 protein is degraded as a consequence of PKC hyperactivation, whereas a separate subset of glioblastomas inactivated *NF1* through mutations [71]. Both mechanisms result in loss of NF1 function. Interestingly, they found that *NF1*-allele proficient tumors, in which PKC was hyperactivated, were sensitive to PKC inhibitors. Conversely, $NF1^{-/-}$ tumors were insensitive to PKC inhibitors but were sensitive to mTOR inhibitors [71]. This study highlights that the precise mechanism by which NF1 function is inactivated, determines the selection strategy of the appropriately targeted therapy.

PI3K/mTOR dual inhibition

Active mTORC1 suppresses the PI3K–Akt pathway. Unfortunately, rapamycin analogs abrogate this negative feedback and activate the insulin receptor pathway which may diminish the potential therapeutic benefits of mTORC1 inhibition [72–74]. PI3K/mTOR dual inhibitors can counteract the derepression of the PI3K–Akt pathway and are therefore more effective than mTORC1 inhibitors as a single agent [75,76].

• *PTEN* mutations in the context of PI3K/mTOR dual inhibition

Studies investigating PI3K/mTOR dual inhibitors do not report drug resistance of *PTEN* mutants [46,57,77,78]. There was even a tendency of *PTEN* mutant glioma cell lines to be more sensitive to dual inhibition of PI3K and mTOR [57,77].

Raf inhibition

Specific inhibitors of mutated BRAF have produced impressive responses in patients with melanoma [79,80]. Therefore the treatment of *BRAF*-mutated glioblastomas may elicit similar spectacular responses. In contrast to the high incidence in melanomas, the *BRAF* V600E mutation is only detected in a few percent of glioblastomas [3]. *BRAF* V600E is more prevalent in pediatric patients and lower grade gliomas [81]. In the rare case of a *BRAF* V600E mutation, BRAF inhibition with vemurafenib may be clinically attractive as evidenced by a recent case report [82].

• *BRAF* V600E mutation in the context of BRAF inhibition

Vemurafenib is a BRAF mutant-specific inhibitor that has been effective in the treatment of *BRAF* V600E mutant melanomas [79]. Ahn and colleagues investigated the efficacy of UAI-201, a different BRAF mutant-specific inhibitor [83]. Similar to melanomas, glioma cell lines with the *BRAF* V600E mutation responded better to a BRAF mutant-specific inhibitor compared with BRAF wild-type cells [83].

MEK inhibition

Activated MAPK is an independent prognostic marker for poor overall survival in glioblastoma and since MAPK is activated by MEK, this suggests MEK hyperactivation to be implicated in more aggressive glioblastomas [84]. In support of this study, Brennan and colleagues report that the aggressive mesenchymal glioblastoma subtype, show an increased activation of MEK [3]. Therefore MEK inhibition may impede clinical progression of glioblastoma and may contribute to prolonged survival.

• *NF1* deletions in the context of MEK inhibition

MEK inhibition within *NF1*-deficient cells has proven to be effective within AML [85]. Similarly a subset of *NF1*-deficient glioblastomas is highly sensitive to MEK inhibitors, whereas *PTEN* deficiency does not seem to modulate sensitivity to MEK inhibitors [78].

Rb pathway

Approximately 80% of glioblastomas contain one or more genetic aberrations in the Rb pathway [1,3]. Rb pathway deregulation leads to the sustainment of proliferative signaling, often through deletion or mutation of *RB1* and the cyclin-dependent kinase inhibitors (*CDKN2A*, *CDKN2C*), and amplification of *CDK4* or *CDK6* [1,3].

CDK4/6 inhibition

Numerous studies indicated that the function of CDK4 and CDK6 is redundant in most mammalian cells, whereas glioblastomas appear to be more dependent on CDK4 and CDK6 for cell cycling compared with normal cells [86–88]. As a consequence CDK inhibitors may selectively impair tumor cells, while leaving normal cells intact. However, the first-generation CDK inhibitors were not very selective and inhibited multiple

CDKs, such as CDK1/2/3/7 and CDK9. Second-generation CDK inhibitors are more selective for CDK4 and CDK6 and show more promising clinical responses (for review, see [89]).

• RB1 & CDKN2A/B/C mutation/deletion in the context of CDK4/6 inhibition

PD0332991 is a selective CDK4/6 inhibitor and is the most extensively investigated drug in relation to the Rb pathway. Homozygous deletion/mutation of *RB1* [88,90,91] and *CDK4* [90] amplification contribute to resistance to PD0332991. In contrast, $p16^{INK4A}$ (cyclin-dependent kinase inhibitor 2A, *CDKN2A*), $p15^{INK4B}$ (*CDKN2B*) and $p18^{INK4C}$ (*CDKN2C*) codeletion predicts sensitivity to PD0332991 [91]. The predictive status of *CDKN2A/B* codeletion without *CDKN2C* probably also predicts sensitivity, however reports have been contradictory since it has been linked to both tumor sensitization [90] and tumor resistance [91].

• EGFRvIII in the context of CDK4/6 inhibition

Nimbolide is a cytotoxic component extracted from the leaf of Azadirachta Indica and inhibits multiple proteins, including CDK4 and CDK6. Intriguingly, Karkare and colleagues report that *EGFRvIII* mutant cells are especially sensitive to this natural extract [92]. Nevertheless, the drug is known to also inhibit Akt, MAPK and the JAK-STAT3 pathway, thus the tumor suppressive effect may also be due to the inhibition of proteins other than CDK4/6.

p53 pathway

Approximately 80–90% of glioblastomas contain one or more genetic aberrations in the p53 pathway [1,13]. p53 pathway deregulation leads to resistance of cell death and evasion of growth suppression, often through amplification of *MDM2/4* or inactivation of *TP53* [1,3].

MDM2 inhibition

MDM2 inhibits p53 transcriptional activity and stimulates its nuclear export and degradation. Roughly half of human cancers, contain either deleted or mutated *TP53* [93]. In the remaining cancers p53 protein function is often diminished due to interacting inhibiting proteins, such as MDM2 [93]. Reactivation of p53 by MDM2 interference of the MDM2–p53 interaction is therefore an interesting approach for inducing p53-mediated apoptosis.

• TP53 mutations in the context of MDM2 inhibition

The mode of *TP53* inactivation has a major influence on the response to MDM2 inhibition. First, hemizygous *TP53* deletion (p53 competent tumors) will be discussed. Shchors and colleagues have dissected the different mechanisms involved in the loss of p53 function [94]. They used a genetically engineered mouse model (GEMM) with $Gfap\text{-}HRas^{V12}$ crossed into a $p53ER^{TAM}$ background, resulting in the three mouse strains $Gfap\text{-}HRas^{V12}$; $Tp53^{KI/KI}$, $Gfap\text{-}HRas^{V12}$; $Tp53^{+/KI}$ and $Gfap\text{-}HRas^{V12}$; $Tp53^{+/+}$. The knocked-in $Tp53ER^{TAM}$ is functional only in the presence of 4-hydroxytamoxifen (4-OHT). As a result $Tp53^{KI/KI}$ mice are functionally p53^{null} in the absence of 4-OHT, but change into p53^{wt} upon systemic administration of tamoxifen (TAM), which is metabolized *in vivo* to 4-OHT. $Gfap\text{-}HRas^{V12}$; $Tp53^{+/KI}$ and $Gfap\text{-}HRas^{V12}$; $Tp53^{+/+}$ GEMMs modeled the evolution of gliomas in which Ras pathway activation precedes p53 pathway inactivation. Shchors and colleagues show that functional p53 is present in $Gfap\text{-}HRas^{V12}$; $Tp53^{+/KI}$ tumor cells and could induce p53-mediated apoptosis by irradiation. Since p53 and its downstream apoptosis pathway were intact, the likely explanation for p53 inactivity in $Gfap\text{-}HRas^{V12}$; $TP53^{+/KI}$ and $Gfap\text{-}HRas^{V12}$; $Tp53^{+/+}$ tumors must lie in the upstream signal to activate p53 in response to oncogenic signaling. Normally oncogenic signaling, such as by Myc [95] and Ras [96], leads to activation of p19^{ARF} (p14^{ARF} in humans) and reactivates p53 function by suppression of MDM2. Indeed, MDM2 expression was high in these tumors and exposure to the MDM2 inhibitor (nutlin3) induced significant cell death [94]. This indicates that the upstream Ras oncogene-sensing $p19^{ARF}$/Mdm2 ($p14^{ARF}$/HDM2 in humans) pathway is dysfunctional, eventually resulting in the impaired reactivation of p53. Thus p53 competent tumors (containing at least one $TP53^{wt}$ allele) are likely to inactivate the upstream activating p53 pathway during tumor formation and can therefore benefit from MDM2 inhibition.

Besides *TP53* deletions, *TP53* can be genetically inactivated by point mutations. Studies using small-molecule inhibitors or peptides specifically blocking the MDM2–p53 interaction, reveal that *TP53* mutant glioblastoma cells are resistant to MDM2 inhibition. This is because only p53 wild-type cells are able to undergo p53-dependent apoptosis [97,98].

p53 reactivation

TP53 is mutated (considering missense, truncating and in-frame mutations) in 20% of glioblastomas [3]. Missense mutations can confer loss of the tumor suppressor function and can lead to an oncogenic gain of function (for review, see [99]). For example, patients with germline mutant *TP53* have an earlier onset of cancer than patients with mutations resulting in loss of p53 protein expression [100]. Understandably, restoring wild-type p53 function is an attractive therapeutic approach. Several synthetic peptides and small molecules have been developed to restore p53 wild-type activity from mutant p53 [101–103].

• *TP53* mutation in the context of p53 reactivation

Gfap-HRas^{V12};Tp53^{KI/KI} mice are able to model tumors in which sporadic *Tp53* loss precedes or coincides Ras activation. These mouse models probably behave similarly to human glioblastomas that directly inactivate *TP53* (via homozygous deletion or mutation) while retaining *CDKN2A* [94]. Shchors and colleagues [94] found that p53 reactivation with 4-OHT induced massive cell death in these GEMMs. Similarly, human glioma cell lines containing mutated *TP53* with wild-type p14^{ARF} (i.e., *CDNK2A(ARF)*) benefitted from p53 reactivation with the drug prima-1 [104], whereas the other combinations of mutations of *TP53* and *CDKN2A* did not [94]. Interestingly, the expression of PTEN in the presence of mutant *TP53* induces an even increased sensitivity to p53 reactivation [104].

Conclusion & future perspective

In this review, we provide an overview of the preclinical efforts to identify genetic biomarkers that predict response to small-molecule compounds specifically targeting the RTK/Ras/PI3K, p53 or Rb pathway **(Figure 1)**. The application of targeted therapy with small-molecule kinase inhibitors has yet to lead to clinical breakthroughs in the treatment of glioblastoma (for review, see [105]). Even though the neuro-oncology research community has made great progress, the translation of preclinical findings into clinical trials and to patient benefit is lagging behind. This disconnect is also known as the translational gap [106]. The underlying reasons can be attributed to flawed preclinical models and flawed clinical trial designs.

For several decades, established glioblastoma cell lines were a popular tool to investigate the *in vitro* response to different compounds. However, recent research demonstrates that these models bear little resemblance to the original tumor of patients [6,7]. In contrast, in different types of cancer several reports indicate that response rates of patient-derived primary cultures and xenografts correlate well with that of patients (for review, see [107]). Next to the use of established cell lines, 2D *ex vivo* culture models may not accurately reflect the tumor pathophysiology as observed in the patient. For example, drug sensitivity profiles of 2D glioma cultures do not readily correlate with those of 3D cultured glioma cells [108].

Another factor influencing the lack of correlation between preclinical and clinical efficacy, is that the appropriate concentration of the small-molecule compounds may not reach and sustain therapeutic levels within the glioma tissue or (peri)tumoral area. This may be due to unfavorable pharmacodynamics resulting in inefficient penetration of the blood–brain barrier in the (peri)tumoral area. In addition, drug concentrations that show decrease of tumor cell viability in preclinical models may not be achievable in the patient due to drug toxicity. Even when the drug does effectively reach the tumor, intratumor heterogeneity may impede effective homogeneous tumor cell death.

Intratumor heterogeneity may complicate the translation of *in vitro* drug responses to *in vivo* patient responses, because the presence of biomarkers linked with specific drug responses may vary within the tumor [109–112]. Indeed, recent studies demonstrate that different subclones within the same tumor may have different *in vitro* drug sensitivity profiles [113,114]. To circumvent the problem of intratumor heterogeneity, it may be beneficial to identify targetable driver events that are uniformly expressed in the tumor, such as *IDH1/2* mutations or *FGFR-TACC* fusion genes. This may delay or prevent the outgrowth of subclones without these specific driver events. However, a recent publication showed a case in which an *IDH1* mutation was lost during tumor progression [115]. In this respect it remains to be determined whether biomarkers generally used to stratify patients, may also hold for predicting response in the setting of intratumor heterogeneity. A yet unanswered question is whether the presence of different genetic biomarkers within a specific tumor leads to a

differential *in vivo* sensitivity of the subclones to the drug in question.

Besides intratumor hetereogeneity, also interpatient variability (i.e., intertumor heterogeneity) plays an important role in clinical assessment of drug efficacy. Many clinical trials still test the experimental drug in question in an unselected patient population. Usually only a subset of patients respond to experimental drugs and consequently the effect of the drug will be diluted [4]. Therefore clinical trials should be stratified with validated biomarkers that are predictive for the drug under study. However, predictive biomarkers of drug response identified in other types of cancer may not directly be extrapolated to glioblastoma. For example, inhibition of *BRAF* V600E in melanoma induces dramatic responses, whereas colon cancer patients harboring exactly the same *BRAF* V600E mutation show only a very limited response to this drug [116]. Thus the context in which the mutation occurs has a major influence on the value of predictive biomarkers and cannot immediately be translated to the treatment of other types of cancer.

Besides biomarker-guided patient stratification, the vast array of inhibitors which are already available should be incorporated into 'smart drug schemes'. Many drug dimensions should be considered when designing smart drug schemes, such as: implementing synergistic drug combinations; implementing synergistic sequential drug schemes in order to minimize drug toxicity and maximize drug synergy [117]; implementing drug holidays in order to resensitize tumors to targeted therapy [32].

Finally, it is imperative to incorporate preclinical evidence into transformative clinical trial designs, such as multicenter umbrella trials or basket trials [4,118]. In an umbrella trial patients with a specific type of cancer are matched to a specific treatment arm based on their molecular profile of their cancer. In contrast, in basket trials the effect of a targeted drug is tested in patients whose cancer contains a specific molecular aberration irrespective of their type of cancer. Besides these larger scale trials, Phase 0 trials should also be pursued more. In these studies the experimental drug is administered before tumor resection which allows the investigation of drug-target effects and the assessment of pharmacokinetic–pharmacodynamic relationships in humans. For example, Vivanco and colleagues have applied this approach in order to identify that lapatinib does not reach sufficient intratumor concentrations in glioblastoma patients [12].

In conclusion, targeted therapies still provide hope for the treatment of patients with glioblastoma. Especially drugs targeting the RTK/Ras/PI3K, p53 or Rb pathway are attractive due to the frequent aberrations found in these pathways. However, preclinical models that faithfully represent the tumor characteristics as observed in the patient must be applied to filter out compounds that will likely not be effective in the patient. After compounds have successfully passed the appropriate filters of preclinical models, they should be tested in synergistic drug combination schemes in well-designed, biomarker-guided clinical trials.

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