Specific biomarkers of receptors, pathways of inhibition and targeted therapies: pre-clinical developments

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ABSTRACT. A deeper understanding of the role of specific genes, proteins, pathways and networks in health and disease, coupled with the development of technologies to assay these molecules and pathways in patients, promises to revolutionise the practice of clinical medicine. Especially the discovery and development of novel drugs targeted to disease-specific alterations could benefit significantly from non-invasive imaging techniques assessing the dynamics of specific disease-related parameters. Here we review the application of imaging biomarkers in the management of patients with brain tumours, especially malignant glioma. In our other review we focused on imaging biomarkers of general biochemical and physiological processes related with tumour growth such as energy, protein, DNA and membrane metabolism, vascular function, hypoxia and cell death. In this part of the review, we will discuss the use of imaging biomarkers of specific disease-related molecular genetic alterations such as apoptosis, angiogenesis, cell membrane receptors and signalling pathways and their application in targeted therapies.

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Glioma-specific molecular genetic alterations

From a molecular perspective, malignant gliomas are extremely heterogeneous. Despite this variability, common alterations in several key pathways controlling cell growth, proliferation, invasion and resistance to cell death have been identified in gliomas. These highly complex signal transduction cascades, which are differentially activated and silenced, involve signalling between multiple parallel and inter-related pathways. Growth factors and their receptors, such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), platelet-derived growth factor receptor (PDGFR) and transforming growth factor- β , primarily acting through receptor tyrosine kinases, have been implicated in the initiation and progression of gliomas [1, 2]. Stimulation of these receptors activates several shared downstream targets and effector molecules (Figure 1). Other genetic alterations include a loss, mutation or hypermethylation of tumour suppressor genes (such as TP53) and other genes involved in the regulation of the cell cycle [such as cyclin-dependent kinase N2A/p16, p14ARF and phosphatase and tensin homologue (PTEN)] as well as activation or amplification of oncogenes, such as MDM2, cyclin-dependent kinase 4, cyclin D1 and D3, inactivation of the p16 cyclin-dependent kinase (CDK)-retinoblastoma (Rb) tumour suppressor pathway, loss of heterozygosity (LOH) on chromosomes 9p, 17p,

22q, 13q, 19q or 10q and O6-methylguanine-DNA-methyltransferase (MGMT) promoter methylation status [2, 3].

For a number of these pathways and molecules, specific inhibiting agents are available. Most research focused on the development of molecules targeting growth factors and/or their receptors (Table 1); in clinical trials, EGF and VEGF signalling pathways were addressed in particular [4, 5]. However, heterogeneity at the cellular and molecular level and redundant or overlapping signalling between these pathways may explain, in part, the therapeutic resistance of tumours seen in clinical trials. Therefore, it seems unlikely that a drug directed at a single molecular target will be curative. There is significant interest both in agents designed to inhibit several targets simultaneously (e.g. multitargeted tyrosine kinase inhibitors [6]) and in combinations of complementary targeting agents, with the potential to inhibit several critical pathways of tumour growth (e.g. anti-EGFR antibody combined with tyrosine kinase inhibitor) [7, 8]. Some of these pathways can be targeted directly with imaging agents, and it is expected that non-invasive imaging will significantly improve the selection of patients and the time window in which such targeted therapies could be successful, and the evaluation of therapeutic efficacy.

Apoptosis

Apoptosis is an essential component of normal human growth and development, immunoregulation, and tissue homeostasis. Apoptotic cell death can be initiated

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Figure 1. Relevant glioma signalling pathways and potential targets for molecular therapeutic agents. Akt, protein kinase B; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; HIF-1, hypoxia-inducible factor-1; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; mTOR, mammalian target of rapamycin; NF- κ B, nuclear factor kappa B; PDGF, platelet-derived growth factor, and their respective receptors EGFR, VEGFR and PDGFR; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PLC, phospholipase C; PTEN, phosphatase and tensin homology deleted on chromosome 10; VEGF, vascular endothelial growth factor.

Table 1. Molecular targeted agents. Selected molecular targeted agents and their specific targets with potential efficacy against gliomas

Agent class	Agent name
Anti-VEGF antibodies	Bevacizumab, VEGF Trap
Anti-VEGFR-2 antibodies	CT332
Anti-EGFR antibodies	Cetuximab, panitumumab
EGFR inhibitor	HKI272
EGFR TK inhibitors	Erlotinib, gefitinib, lapatanib, EKB569
VEGFR TK inhibitors	Vatalanib, SU5146, XL820, XL880, XL999
PDGFR TK inhibitors	Imatinib, dasatinib
EGFR TK and VEGFR TK inhibitors	AEE78, vandetanib, ZD6474
PDGFR TK, VEGFR TK and cKit inhibitors	Cediranib, pazopanib, AMG706, sunitinib
PDGFR TK, VEGFR TK and Raf inhibitors	Sorafenib
Farnesyltransferase inhibitors	Tipifarnib, lonofarnib
Raf inhibitors	Sorafenib (also PDGFR and VEGFR inhibitor)
PKC inhibitors	Enzastaurin
mTOR inhibitors	Sirolimus, temsirolimus, everolimus, AP23573
Protease inhibitors	Bortezomib

EGF, epidermal growth factor; PDGF, platelet-derived growth factor, and their respective receptors EGFR, VEGFR and PDGFR; TK, tyrosine kinase; VEGF, vascular endothelial growth factor.

through an extrinsic pathway involving activation of cell surface death receptors or by an intrinsic pathway via the mitochondria. Both pathways lead to activation of initiator (e.g. caspase-1, -8, -10) and effector caspases (e.g. caspase-3, -6, -7) [9, 10] that trigger a proteolytic cascade resulting in fragmentation of intracellular components. The final enzyme activated within the cascade is caspase-3, and once the apoptotic pathway is activated, caspase-mediated proteolysis is irreversible and ultimately leads to typical cellular changes, such as cell shrinkage, plasma membrane blebbing, nuclear chromatin condensation and aggregation, and nuclear fragmentation. One of the earliest effects of caspase activation is the disruption of the translocase system that normally maintains phosphatidylserine (PS) on the interior of the cell membrane. This results in the redistribution of PS to the outer membrane leaflet, where it serves as a signal to phagocytic cells to engulf and digest the membrane-enclosed apoptotic cells. The most recent developments in the biochemical characterisation of the many intracellular pathways involved in this complex process and how multimodality imaging can be used to visualise these processes *in vivo* have been described by Blankenberg [11].

Modulating the apoptotic pathway represents special opportunities for targeted therapeutic interventions and direct imaging of caspase activity or PS expression can be used for non-invasive monitoring of early drug responses.

Caspase

Caspase peptide substrates containing either a nuclear (a ^{18}F -labelled caspase-inhibiting analogue [12]), or a bioluminescence label [13] or a far-red [14] or near-infrared optical fluorochrome [15] have been developed to detect apoptosis. Using firefly luciferase-based bioluminescence imaging in nude mice, Laxman et al [13] developed a caspase-cleavable reporter probe able to detect tumour apoptosis following chemotherapy. Although not relevant clinically, optical imaging techniques may become particularly useful in the screening of targeted drugs in preclinical studies.

Annexin V

PS exposure has been the most pursued target for the detection of cell death using molecular imaging methods [16]. The 40 kDa vesicle-associated protein annexin V (AnxV) has been the most widely used PS-targeting moiety. AnxV binds PS in a calcium-dependent manner and with high (nanomolar) affinity. Initially, AnxV was coupled to fluorescent dye molecules and used as an apoptosis detection reagent for fluorescence microscopy and flow cytometry [17]. Subsequently AnxV was coupled to a radionuclide ($^{99\text{m}}\text{Tc}$) and used to detect apoptosis non-invasively in animals [18] and in the clinic [19, 20] using radionuclide imaging techniques. Recent studies in oncology suggest a single scan 24–48 h after the start of treatment can identify patients with a response after one course of chemotherapy [19, 21]. Other radionuclide derivatives of AnxV have been developed, including AnxV labelled for single photon emission computed tomography (SPECT) with ^{123}I [22] or for positron emission tomography (PET) labelled with

^{18}F [23, 24] or ^{124}I [25] and ^{64}Cu -labelled streptavidin for PET imaging after pre-targeting of PS with biotinylated annexin V [26]. However, there are currently no clinical trials using radionuclide-labelled AnxV to further evaluate its potential to assess the outcome of cancer therapy.

Photonic imaging methods have also been used to image AnxV labelled with fluorochromes [27] or near-infrared (NIR) fluorochromes [28], and Schellenberger et al [29] were the first to label AnxV with superparamagnetic iron oxide (SPIO) nanoparticles for MR detection. Later also Gd-containing AnxV-coated liposomes for positive or bimodal MR contrast were developed [30, 31] as well as multimodality contrast agents for combined MRI and fluorescent imaging [32].

Synaptotagmin-I

More recent studies have focused on the use of a smaller PS targeting agent, based on the C2A domain (14.2 kDa) of another vesicle-associated protein, synaptotagmin-I, which also binds PS with nanomolar affinity in a calcium-dependent manner. This protein has been labelled with SPIO nanoparticles and Gd $^{3+}$ chelates for MRI-based detection of PS exposure *in vivo* after chemotherapy [33].

Angiogenic switch and transcriptional regulation

During the last three decades, intensive research has been performed to characterise the tumour-associated angiogenic processes that result from the imbalance between pro- and anti-angiogenic factors, and a comprehensive review has been recently published by Jouanneau [34]. Both hypoxia and genetic anomalies can trigger this imbalance between pro- and anti-angiogenic factors.

A variety of genetic anomalies have been identified that trigger angiogenesis independent of hypoxia, such as VEGF expression; expression of the oncogenes EGF, PDGF and their receptors EGFR and PDGFR; tumour suppressor gene mutations (p16 $^{\text{INK4a}}$, p14 $^{\text{ARF}}$, PTEN, RB and p53); and the loss of heterozygosity of chromosomes 1p, 10p, 10q, 19q, and 22q [2]. Most of these genetic anomalies that accumulate in glioma cells result in chronic activation of the hypoxia-inducible transcription factor-1 (HIF-1) via the intracellular phosphatidylinositol 3-kinase (PI3K) or mitogen-activated protein kinase (MAPK) pathways [35].

Although these anomalies are more or less frequent in malignant gliomas, the main trigger of the angiogenic switch is tumour hypoxia that occurs in growing tumours when tumour cells are distant from vessels (1–2 mm) [36]. Tumour hypoxia upregulates pro-angiogenic factors and downregulates anti-angiogenic factors. Key endogenous angiogenesis agonists include VEGF, basic fibroblast growth factor (bFGF), angiopoietins, matrix metalloproteases (MMPs), integrins and catherins; key endogenous angiogenesis inhibitors are thrombospondins, brain angiogenesis inhibitor-1, endostatin, angiotatin, troponin, metallospindins, interleukins and tissue inhibitors of MMPs, among others [34, 37]. Hypoxia is the primary stimulus for VEGF and VEGFR expression via HIF-1 and the hypoxia-signalling pathway and

VEGF-A is the most potent angiogenic factor for brain tumour angiogenesis. Thus, HIF-1 and the hypoxia-signalling pathway is the common pathway for both angiogenesis triggers (genetic or hypoxic) [35]. HIF-1 is a heterodimeric protein composed of two subunits: a constitutively expressed HIF-1 β and the α -subunit. During normoxia, HIF-1 α is rapidly degraded by ubiquitination, whereas exposure to hypoxic conditions prevents its degradation. Under hypoxic conditions, the HIF-1 α level is increased and it forms a complex with HIF-1 β and the CBP/p300 coactivator and translocates into the nucleus, where it binds to the core DNA sequence 5'-RCGTG-3' [38]. Many oncogenic signalling pathways overlap with the HIF-1 signalling pathway and cause upregulation of many HIF-1-inducible genes (e.g. VEGF, erythropoietin and glucose transporters) and lytic enzymes even under normoxic conditions [39].

VEGF binds primarily to three transmembrane receptors with tyrosine kinase activity (VEGFR-1/3) and activates several intracellular signalling pathways by induction of many kinases of the Ras/Raf/MEK/MAPK, PI3K/AKT/PKB and protein kinase C- β pathways. These signals finally lead to activation, proliferation and migration of endothelial cells, new vessel formation and organisation. These newly formed tumour vessels are immature and highly fenestrated and present an increased permeability [40].

Both VEGF and VEGFR are overexpressed in glioma tissue and its vasculature, and present valuable targets for novel, mechanism-based therapies. Therapeutic strategies for targeting the VEGF/VEGFR signalling pathway have focused on VEGFR tyrosine kinase inhibitors, anti-VEGF-A and VEGFR-2 monoclonal antibodies [41] and protein kinase C- β inhibitors, with promising initial clinical results [42]. Most VEGFR tyrosine kinase inhibitor drugs are not specific and act on other tyrosine kinase receptors.

Although conventional imaging techniques like MRI and PET focus on the measurement of physiological parameters, such as blood flow, blood volume, vascular perfusion, permeability and/or structure, and represent the radiographic tools in current clinical trials of anti-angiogenic therapy [6], the more specific approach to image anti-angiogenesis is to target with an imaging probe the same molecular events that are targeted with the therapeutic drug, enabling early assessment of response to therapy, before physiological changes become apparent.

VEGF/VEGFR

During the last few years, intense research focused on VEGF/VEGFR-targeted molecular imaging and a wide variety of targeting molecules (peptides, proteins, antibodies and nanoparticles) have been labelled with various imaging labels (such as radioisotopes, fluorescent dyes and microbubbles) for PET, SPECT, optical imaging and contrast-enhanced ultrasound imaging of tumour angiogenesis, and many excellent reviews exist [43, 44].

Various radiolabelled (^{123}I , $^{99\text{m}}\text{Tc}$, ^{111}In , ^{64}Cu) VEGF-A isoforms have been developed and tested for tumour detection [45–48] and imaging of tumour vasculature before and after chemotherapy [48] with SPECT or PET.

Also humanised anti-VEGF antibodies have been labelled with ^{111}In [49] for SPECT imaging or with ^{89}Zr [49] or ^{124}I [50] for PET imaging.

Human VEGF conjugated to the fluorescent dye Cy5.5, and microbubbles conjugated to anti-VEGFR-2 monoclonal antibodies can also be used for non-invasive imaging of tumour angiogenesis, as well as for the *in vivo* monitoring of the vascular effects after therapy [51, 52].

Hsu et al [53] used multimodality [bioluminescence imaging (BLI), MRI and PET] molecular imaging to determine the antiangiogenic and antitumour efficacies of a vasculature-targeting fusion toxin [VEGF(121)/rGel] composed of the VEGF-A isoform VEGF(121) linked with a G(4)S tether to recombinant plant toxin gelonin (rGel) in an orthotopic glioblastoma mouse model. In this study, the level of target expression was monitored before therapy by [^{64}Cu]-1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA)-VEGF(121)/rGel PET, whereas [^{18}F]-FLT scans were obtained before and after treatment to evaluate VEGF(121)/rGel therapeutic efficacy. In VEGF(121)/rGel-treated mice, a significant decrease in [^{18}F]-FLT uptake and peak BLI tumour signal intensities could be observed compared with non-treated mice; these results were validated by histological analysis.

Integrins

Expression of cell adhesion molecules is significantly upregulated during tumour growth and angiogenesis. Integrins are a family of cell adhesion molecules consisting of two non-covalently bound transmembrane subunits (α and β) that pair to create heterodimers with distinct adhesive capabilities [54]. Natural integrin ligands include laminin, fibronectin and vitronectin, but they also include fibrinogen and fibrin, thrombospondin, MMP-2 and fibroblast growth factor 2 [55]. Integrins bind ligands by recognising short amino acid stretches on exposed loops, particularly the arginine-glycine-aspartic acid (RGD) sequence. On ligation, integrins mediate complex signalling events, alone or in combination with growth factor receptors, regulating cell adhesion, proliferation, survival and migration by activating canonical pathways, such as integrin-linked kinase (ILK), protein kinase B (PKB/Akt), MAPK, Rac or nuclear factor kappa B (NF- κ B). In resting vessels, integrins interact with the basal membrane, thereby maintaining vascular quiescence. During tumour angiogenesis and metastasis, they are essential for endothelial cell migration, proliferation and survival [56]. Integrin α v β 3, the most extensively studied integrin to date, is significantly upregulated on tumour vasculature but not on quiescent endothelium [56], and integrin α v β 3 expression correlates well with tumour aggressiveness [57]. Many monoclonal antibodies (e.g. vitaxin), cyclic RGD peptide antagonists (e.g. cilengitide) and peptidomimetic agents, which are orally bioavailable non-peptidic molecules mimicking the RGD sequence (e.g. S247), against integrin α v β 3 have been used for anti-angiogenic cancer therapy [46, 55].

Most studies on imaging of integrin α v β 3 have used RGD peptides as the targeting ligands, but also nanoparticle probes have been developed for labelling with nuclear [58–61], paramagnetic [62–64] and fluorescent

[65] molecules, and those methods can be used to selectively target suicide gene therapy [66] or drug delivery [67, 68]. Recently, an $\alpha v\beta 3$ -targeted bimodal liposome tagged with a paramagnetic and fluorescent label has been developed [69] and tested for its ability to quantitate tumour angiogenesis and evaluate the therapeutic efficacy of angiogenesis inhibitors [70]. Jin et al [71] developed an RGD-targeted and self-quenched Cy5.5-labelled probe for $\alpha v\beta 3$ imaging. Many excellent reviews on imaging of integrin $\alpha v\beta 3$ expression are available [44, 72, 73].

Also, the expression of other cell adhesion molecules has been monitored non-invasively. Several antibodies against intercellular adhesion molecule (ICAM)-1 and the E-selectin molecule, coupled with paramagnetic liposomes or nanoparticles, have made angiogenesis studies possible on subcutaneous or intracranial animal models [74, 75].

Ongoing tumour angiogenesis and neovascularisation can also be non-invasively imaged by magnetically labelled endothelial precursor cells [76], which are known to home to sites of angiogenesis and are being investigated as angiogenesis-selective gene-targeting vectors [77].

Transcriptional regulation

HIF-1 Serganova et al [38] developed a dual-reporter gene cassette to monitor non-invasively the dynamics and spatial heterogeneity of HIF-1-specific transcriptional activity in tumours and showed that HIF-1-mediated activation of TKGFP (thymidine kinase green fluorescent protein) reporter gene expression in hypoxic tumour tissue can be non-invasively and repeatedly visualised in living mice using PET imaging with [^{18}F]-2'-fluoro-2'-deoxy-1 β -D-arabionofuranosyl-5-ethyl-uracil (FEAU).

p53 In a similar approach, the same group showed that p53-dependent gene expression can be imaged *in vivo* with PET and by *in situ* fluorescence using a retroviral vector containing a Cis-p53/TKGFP dual-reporter gene under control of a p53-specific response element [78]. The PET images corresponded with upregulation of genes in the p53 signal transduction pathway (including p21) in response to DNA damage induced by BCNU [1, 3-bis(2-chloroethyl)-1-nitrosourea] chemotherapy. p53 is a transcription factor encoded by the TP53 (tumour protein 53) gene, one of the major tumour suppressor genes. p53 is central to many of the cell's anticancer mechanisms [79]. It can induce growth arrest, apoptosis and cell senescence. In normal cells, p53 is usually inactive, bound to the protein MDM2, which prevents its action and promotes its degradation by acting as ubiquitin ligase. Active p53 is induced after the effects of various cancer-causing agents such as UV radiation, oncogenes and some DNA-damaging drugs. Mutation in the TP53 gene occurs in about 50% of low-grade gliomas, resulting in a loss of its tumour suppressor function [80].

E2F-1 Our group recently constructed a Cis-E2F/LucIRES TKGFP reporter system to non-invasively assess E2F-1-dependent transcriptional regulation in culture and *in vivo*

[81]. We can show this reporter system is sensitive to non-invasively monitor various changes in cellular E2F-1 levels following DNA damage-induced up-regulation of E2F-1 activity (Figure 2). Activation of the transcription factor E2F-1 via alteration of the p16-cyclinD-Rb pathway is one of the key molecular events in the development of gliomas and represents a promising anticancer target.

PET imaging using such reporter systems could be used to assess the effects of new drugs or other novel therapeutic paradigms that are mediated through hypoxic, p53- or E2F-dependent pathways.

Receptor tyrosine kinases

The human epidermal growth factor receptor (HER) family of receptor tyrosine kinases controls critical pathways involved in epithelial cell differentiation, growth, division and motility [82]. The HER family consists of four closely related members: EGFR (HER1 or ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). Together, the HER family controls a complex network of ligand-receptor interactions and cellular responses known as the HER-kinase axis.

Ligands that bind to the HER-kinases include over 10 independent proteins such as EGF, transforming growth factor (TGF)- α , β -cellulin and neuregulins/hereregulins, many of which exhibit relatively similar receptor-binding characteristics. Each HER-kinase contains a large extracellular (ligand-binding) domain, a membrane-spanning region and a cytoplasmic [protein tyrosine kinase (TK)] domain and phosphorylation sites. Upon ligand binding, EGFR dimerises either with itself to form a homodimer or with other HER family members to form heterodimers (e.g. EGFR:HER2 or EGFR:HER3). Ligand-induced dimerisation causes a conformational change in the receptors that promotes the activation of the TK domain. Subsequent phosphorylation of the HER-kinase itself and/or other proteins, which then pass on to various signalling cascades [e.g. phosphoinositide 3-kinase (PI3K)/Akt and MAPK pathways] can lead to different cellular events such as growth, migration and division. From this axis the EGF/EGFR ligand-receptor pair was the first to be discovered and is the best studied.

EGFR is a 170 kDa cell surface protein overexpressed in many epithelial cancers [83]. Dysregulation of EGFR is associated with several key features of cancer, such as autonomous cell growth, inhibition of apoptosis, angiogenic potential, invasion and metastases (Figure 1). EGFR amplification and overexpression, present in up to 60% of glioblastomas, are associated with a poor prognosis, especially when occurring in younger patients [84]. EGFR mutations are frequent in glioblastomas and may be present in up to 50–70% of EGFR-overexpressing tumours. Two main categories of EGFR mutations exist: deletion or point mutations in the extracellular domain and somatic mutations in the TK domain. The Type III EGFR mutant (EGFRvIII), which lacks the amino acid residues 6–273 in the extracellular domain, is the most common [85] and is observed in 60–70% of EGFR-overexpressing glioblastomas [42]. Since EGFRvIII lacks a large portion of the ligand-binding domain and hence is unable to bind either EGF or other EGFR ligands, it is constitutively active and can initiate downstream

Figure 2. Regulated E2F-1 activity as determined *in vivo*. (a) E2F-regulated cells and negative and positive control cells were implanted as a set of four tumours in the back of different groups of experimental mice. Mice were followed over time by bioluminescence imaging until tumours could be clearly visualised. Mice were then subjected to BCNU treatment (50%) or control treatment (50%), and repeat imaging was performed 24 h later. An increased luciferase signal was observed only in mice bearing E2F-1-regulated cells and not in mice bearing negative and positive control cells. Colour scale, luminescent signal intensity; blue, least intense signal; red, most intense signal. (b) Mean of the total bioluminescent signals emitted from E2F-1-regulated tumours and negative and positive control tumours in response to BCNU administration. Columns, mean of three independent experiments with $n=6$ animals per group; bars, SD. Significant differences are indicated by $*p<0.05$ and $**p<0.05$ (modified with permission from [81]).

signalling because the deletion results in a conformational change that mimics the one induced by ligand binding in wild-type EGFR [86]. These EGFRvIII-positive tumours are reported to be associated with a worse prognosis and shorter life expectancy [84]. The other category of EGFR mutations involves changes in the TK domain that can influence the binding of TK inhibitors (TKIs) [87]. These mutants exhibit increased activation compared with wild-type EGFR, but at the same time are much more sensitive to inhibition by EGFR-TKIs. However, to date, mutations in the TK domain have not been shown in malignant glioma [88]. Monoclonal antibodies against EGFR and small molecule inhibitors targeting TKs that bind to the intracellular part of the receptor and thereby block receptor phosphorylation have been introduced in clinical practice for patients

with malignant gliomas. However, findings reported in studies evaluating EGFR inhibitors are surprisingly contradictory. A prospective Phase I/II trial with a monoclonal EGFR antibody on patients with recurrent malignant gliomas failed to show these antibodies were of therapeutic efficacy [89] and also the first generation of studies with EGFR TKIs have found confounding results. Four prospective trials also using *in vitro* biomarkers reported negative findings [90–93], whereas two other retrospective trials suggested these inhibitors had some sort of activity in a subset of patients with particular molecular characteristics (high EGFR expression and low phospho-Akt levels [94] or EGFRvIII and PTEN co-expression [95]). These results indicate the need for the development and validation of non-invasive imaging techniques targeted to EGFR expression to

eventually be able to predict which patients will likely respond to anti-EGFR therapy and monitor their response to such personalised cancer management.

EGFR

Molecular therapies targeting the TK domain of EGFR for therapy and non-invasive molecular imaging *in vivo* with PET have been the focus of several research groups for more than a decade. Gelovani [96] recently published a comprehensive review on PET imaging of EGFR expression activity and Cai et al [82] on multimodality imaging of the HER-kinase axis in cancer.

A number of ^{11}C , ^{18}F and radioiodine-labelled EGFR kinase inhibitors that bind reversibly or irreversibly to the EGFR kinase ATP-binding pocket have been developed. The radiolabelled irreversible EGFR inhibitors demonstrate the greatest potential for derivatisation into effective EGFR kinase imaging agents compared with those developed on the basis of reversible inhibitors. In particular, PET imaging with (E)-but-2-enedioic acid [4-(3-124I-iodoanilino)-quinazolin-6-yl]-amide-(3-morpholin-4-yl-propyl)-amide (morpholino- ^{124}I -IPQA) seems to be very promising as this tracer binds covalently to the ATP-binding site in activated EGFR but not in inactive EGFR [97]. *In vitro* studies demonstrated rapid accumulation and progressive retention post washout of morpholino- ^{131}I -IPQA in A431 cells and in U87MG human glioblastoma cells genetically modified to express EGFRvIII, but not in the wild-type U87MG cells under serum-starved conditions. Thus, it was suggested that PET imaging with morpholino- ^{124}I -IPQA should allow for identification of tumours with high EGFR signalling activity, such as brain tumours expressing the EGFRvIII mutant and non-small cell lung cancer expressing gain-of-function EGFR mutants expected to have an increased sensitivity to EGFR TKIs [82].

As the natural ligand of EGFR, EGF has been labelled with ^{76}Br [98] or ^{68}Ga [99] for direct targeted PET imaging or with $^{99\text{m}}\text{Tc}$ [100] or ^{131}I [101] for tumour detection and evaluation of therapy response with SPECT imaging. Monoclonal antibodies (mAbs) against EGFR, used for anti-EGFR therapy such as cetuximab, have been labelled with positron emitters, e.g. ^{64}Cu [102] or gamma-emitters, e.g. $^{99\text{m}}\text{Tc}$ and ^{111}In [82], with good tumour contrast. However, MRI with a ferromagnetic anti-EGFR mAb in athymic rats bearing EGFR-positive tumours revealed the targeted contrast agent only gave modest EGFR-specific MR contrast *in vivo* [103]. Optical imaging studies on EGFR expression have been carried out with fluorescently labelled EGF or anti-EGFR mAbs [104].

Non-invasive imaging of EGFR status in patients pre-treatment might be of great clinical importance to identify and select patients who are likely to respond to anti-EGFR therapy.

Akt

Recent advances in pre-clinical research have led to the development of several targeted reporters to interrogate the activity of other receptors or elements in intracellular signalling cascades related to cell growth and proliferation. Zhang et al [105] constructed a bioluminescence Akt reporter based on split-luciferase technology. By using this reporter, the authors could monitor quantitatively

and dynamically Akt activity (Akt phosphorylation) in cultured cells and tumour xenografts (human glioma cell line) in response to upstream signalling pathway modulators such as EGF or PI3K inhibitors. On the other hand, mTOR inhibitors acting downstream of Akt did not result in reporter bioluminescence alteration. A similar reporter, based on split-luciferase technology, has been developed for non-invasive quantification of EGFR activity *in vivo* [106]. With this reporter a rapid and sustained EGFR signal activation after radiotherapy and signal suppression after EGFR inhibitor treatment could be observed in the same tumour xenografts (non-small cell lung adenocarcinoma cell line) over a period of weeks.

TRAIL

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potent promoter of programmed cell death in diverse tumour types and binds to a family of receptors, including death receptors 4 and 5 [TRAIL receptor-1 and -2 (TRAIL-R1 and TRAILR2)] [107]. Ligand-receptor interaction leads to apoptosis via a conserved cytoplasmic death-signalling module. Recently, clinical trials have been initiated with recombinant TRAIL and with TRAIL-R1 and TRAIL-R2 specific agonist monoclonal antibodies that mimic the activity of native TRAIL [108]. Such trials could be optimised by pre-treatment based on non-invasively obtained information on the receptor expression status. Gong et al [109] used $^{99\text{m}}\text{Tc}$ -labelled TRAIL-R1 and TRAIL-R2 agonist antibodies (HGS-ETR1 and HGS-ETR2) to monitor receptor expression prior to therapy. The authors showed the antitumour activity of HGS-ETR1 and HGS-ETR2 against human colorectal cancer xenografts could be remarkably enhanced by prior administration of the antimicrotubule paclitaxel. Pre-treatment with tolerable doses of paclitaxel resulted in a striking, time-dependent upregulation of TRAIL-R1 and TRAIL-R2, as shown by *in vivo* tumour SPECT imaging using radiolabelled HGS-ETR1 and HGS-ETR2 and by analysis of steady-state protein levels from the colorectal tumour xenografts. This receptor upregulation was responsible for the enhanced antitumour activity of the agonist antibodies.

Conclusion

Over the past years, various fields in clinical medicine and in particular oncology are undergoing a rapid evolution from relatively broad histopathology-based diagnoses and non-specific therapies to a disease-specific molecular targeted approach of disease diagnoses and treatment. Nevertheless, the first molecular targeted therapy Phase II clinical trials in malignant glioma have not translated into current clinical practice, yet [42]. Several signalling pathways that are differentially activated or silenced with both parallel and converging complex interactions drive the oncogenic process in malignant glioma and, therefore, the inhibition of a specific pathway may result in subsequent activation of a compensatory pathway and account for the resistance to a single molecular targeted therapy. As a consequence, most current clinical trials are exploring the possibility of addressing multiple targets through the use of (i)

multitargeting single agents, (ii) combinations of single-targeting agents or (iii) combination with cytotoxic chemo- and/or radiotherapy.

Molecular imaging can significantly aid in the development of such molecular targeted novel therapies through early lesion detection, patient stratification and treatment monitoring in the setting of personalised cancer management. Clinically applicable methods and imaging agents for the non-invasive and repetitive four-dimensional molecular imaging will facilitate quantitative assessments of levels and heterogeneity of drug target expression and activity in tumours of individual patients. Non-invasive molecular imaging can serve as a new paradigm for assessing the efficacy of molecular targeted cancer therapy, improving cancer management, and elucidating the role and modulation of cancer-related signalling pathways during cancer development and intervention.

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