Department of Radiation Biology

FACULTY OF HEALTH SCIENCES
UNIVERSITY OF COPENHAGEN

PhD thesis

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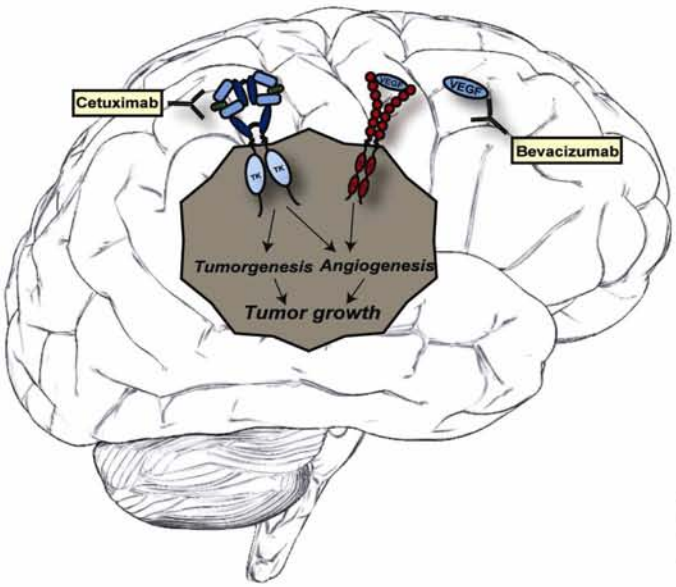
Effects of EGFR and VEGF inhibition in Human Glioblastomas using Cetuximab and Bevacizumab



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Preface

This thesis is submitted to the Faculty of Health Science, University of Copenhagen, Denmark, in order to fulfill the requirements to obtain the PhD-degree in medical sciences.

The study was performed from September 2006 to November 2009 under the supervision of Head supervisor Hans Skovgaard Poulsen, MD, DMSc, Project Supervisor Marie-Thérése Stockhausen, MSc, PhD at the Department of Radiation Biology Copenhagen University Hospital, Denmark and Clinical Supervisor, Ulrik Lassen, MD, PhD, Department of Oncology, Copenhagen University Hospital, Denmark.

The experimental work was conducted at the Department of Radiation Biology, while the immunohistochemical strainings presented in Manuscript IV, were performed at the Department of Neuropathology, Copenhagen University Hospital and Department of Experimental Clinical Oncology, Aarhus University Hospital.

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List of papers

This thesis is based on the following papers, which is refererred in the text by their Roman numerals:

I **Cetuximab insufficiently inhibits glioma cell growth due to persistent EGFR downstream signaling.**
Benedikte Hasselbalch, Ulrik Lassen, Hans Skovgaard Poulsen and Marie-Thérése Stockhausen
In press *Cancer Invest., 2010*

II **Bevacizumab plus irinotecan in the treatment of patients with progressive recurrent malignant brain tumours.**
Hans Skovgaard Poulsen, Kirsten Grunnet, Morten Sorensen,
Preben Olsen, Benedikte Hasselbalch, Knud Nelausen, Michael Kosteljanetz, Ulrik Lassen
Acta Oncol. 2009;48(1):6-8

III **Cetuximab, bevacizumab plus irinotecan for patients with primary glioblastoma and progression after radiation and temozolomide: A phase II trial**
Benedikte Hasselbalch, Ulrik Lassen, Steinbjørn Hansen, Mats Holmberg, Morten Sørensen, Michael Kosteljanetz, Helle Broholm, Marie-Thérése Stockhausen, and Hans Skovgaard Poulsen
NeuroOncol. Feb. 5, 2010

IV **Prospective evaluation of angiogenic, hypoxic and EGFR related biomarkers in recurrent glioblastoma multiforme treated with cetuximab, bevacizumab and irinotecan**
Benedikte Hasselbalch, Jesper Grau Eriksen, Helle Broholm, Ib Jarle Christensen, Michael R. Horsman, Hans Skovgaard Poulsen, Marie-Thérése Stockhausen, and Ulrik Lassen
In press *APMIS, 2010*

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Dansk Resumé

Glioblastoma multiforme (GBM) er en særdeles ondartet kræftsygdom, med en median overlevelse for ny-diagnosticerede GBM patienter på kun 15 måneder. GBM tumorer er yderst vaskulariserede og udtalt tumor vaskularisering er signifikant korreleret med kort overlevelse. Endvidere er GBM tumorer kendt for at være hypoxiske samt nekrotiske. Hypoxi medfører blandt andet stabilisering af hhv. HIF-1α og HIF-2α som efterfølgende initiere transkription af pro-angiogene faktorer som vascular endothelial growth factor (VEGF). Dette inducerer angiogenese i og omkring tumoren. Tumor kar er ofte malformerede og okklusion er hyppig, hvilket medfører tumor ødem og øget intratumoralt tryk som yderligere forværrer tumor hypoxi. GBM er karakteriseret ved hyppig amplifikation/overekspression af den epidermale growth factor receptor (EGFR) samt udtrykket af den muterede EGFR version III (EGFRvIII), hvilket medfører dysregulering af nedstrøms signaleringen igennem, bla. PI3K/Akt vejen. Derudover har 15-40% af GBM inaktiverende mutationer af tumor suppressoren PTEN, medførende øget aktivitet af Akt (pAkt). Signalering igennem PI3K/Akt vejen inducere ekspression af VEGF enten ved at stimulere transkriptionen direkte eller ved opregulering af HIF-1α. Derudover er HIF-1α kendt for at inducere ekspression af EGFR liganden TGF-α. Forbindelsen imellem EGFR, hypoxi og angiogenese kunne være af betydning for patogenesen af GBM og dermed mulige targets i behandlingen af GBM.

Formålet med denne PhD har været at undersøge *in vitro* effekten af EGFR hæmning i gliom celler ved brug af cetuximab samt at undersøge effekten af cetuximab på EGFR relaterede nedstrøms signaleringsveje. Cetuximab viste sig ikke i stand til at hæmme celle overlevelsen trods hæmning af aktiveret EGFR (pEGFR) hverken i gliom celle linier med vild-type eller muteret PTEN.

Derudover var formålet at undersøge, i en klinisk fase II protokol til patienter med recidiverende GBM, om tilføjelsen af cetuximab til det eksisterende behandlings regime brugt ved recidiv af GBM med det anti-angiogenetiske stof bevacizumab kombineret med irinotecan (CBI), kunne inducere forbedret klinisk respons og øget overlevelse. Der viste sig ikke at være nogen behandlingsgevinst ved at tilføje cetuximab til bevacizumab og irinotecan (BI) behandlingens regimet. Med henblik på længere sigt at kunne udpege hvilke patienter som ville have gavn af behandling (targeteret behandling), blev der foretaget, prospektive (CBI) og retrospektive (BI) immunohistokemiske undersøgelser af EGFR og hypoxi/angiogenese relaterede biomarkører på patient tumor væv. Disse resultater blev korreleret med den kliniske respons og overlevelses data på hhv. CBI og BI behandling. Der blev ikke fundet nogen sammenhæng mellem udtrykket af de undersøgte biomarkører og respons eller overlevelse.

English Summary

Glioblastoma multiforme (GBM) is a highly aggressive malignant disease with a median survival for newly diagnosed GBM of only 15 months. GBM are vastly vascularized and pronounced tumor vascularity is significantly correlated with poor survival. Moreover, GBM tumors are hypoxic and also necrotic. Hypoxia leads to, among others, stabilization of the HIF-1α and HIF-2α subunits that initiate transcription of pro-angiogenic factors such as the vascular endothelial growth factor (VEGF). This leads to angiogenesis in and around the tumor. Tumor vessels are often malformed and occlusions are frequent, and as such intratumoral hypoxic areas will remain. Moreover, tumor vessels are leaky, leading to tumor edema and increased intratumoral pressure, which further increases hypoxia. GBM is characterized by frequent amplification/overexpression of the epidermal growth factor receptor (EGFR) and expression of the mutated EGFR version III (EGFRvIII), leading to dysregulated downstream signaling through, among others, the PI3K/Akt pathway. Furthermore, 15-40% of GBM have inactivating mutations of the tumor suppressor PTEN leading to elevated activity of Akt (pAkt). Signaling through the PI3K/Akt pathway induce the expression of VEGF either by stimulating its transcription directly or by upregulation of HIF-1α. Furthermore, HIF-1α induces the expression of the EGFR ligand TGF-α. Thus, there are several links between EGFR, hypoxia and angiogenesis that could be of importance for GBM pathogenesis and thereby possible targets to obtain improved treatment for GBM.

The aim of this PhD thesis has been to study *in vitro* the effect of EGFR inhibition in glioma cell lines using cetuximab and investigate the effect of cetuximab on EGFR related downstream signaling pathways. It was observed that cetuximab did not inhibit cell viability, despite inhibition of activated EGFR (pEGFR) and this was observed in both PTEN wild-type and PTEN mutated glioma cell lines. In addition, the aim was to investigate in a clinical phase II study for recurrent GBM, if the addition of cetuximab to the existing treatment regimen used for patients with recurrent GBM, consisting of the anti-angiogenetic drug bevacizumab in combination with irinotecan (CBI), would induce improved clinical response and survival. It was concluded that the addition of cetuximab did not improve the bevacizumab and irinotecan (BI) regimen. In order to in the future being able to select which patients to benefit from treatment (targeted treatment), prospective (CBI) and retrospective (BI) immunohistochemical analysis of EGFR and angiogenesis/hypoxia related biomarkers were performed on patient tumor material. These results were correlated with the clinical response and survival data to CBI and BI respectively. However, no correlation where found between the expression of the biomarkers investigated and response or survival.

Abbreviations

5FU	5-flourouracil
ADCC	antibody-dependent cellular cytotoxicity
ARNT	aryl hydrocarbon receptor nuclear translocator
BBB	blood-brain barrier
bCSC	brain cancer stem cell
bHLH-PAS	basic helix-loop-helix-PAS
BI	bevacizumab + irinotecan
BT	bevacizumab + temozolomide
CAIN	calcium influx internalization
CA 9/IX	carbon anhydrase 9
CBI	cetuximab + bevacizumab + irinotecan
CDK	cyclin-dependent kinase
CT	computed tomography
Dll	delta-like ligands
EMEA	european medicines agency
EGFR	epidermal growth factor receptor
EGFRvIII	epidermal growth factor receptor variant III
EPO	erythropoietin
FDA	food and drug administration
FDG	fluorodeoxyglucose
FGF	fibroblast growth factor
FLAIR	fluid-attenuated inversion recovery
Flk-1	fetal lever kinase (also known as VEGFR-2)
Flt-1	fms-like tyrosine kinase (also known as VEGFR-1)
Gab1	Grb2-associated protein 1
GBM	glioblastoma multiforme
GLUT1	glucose transporter 1
FIH	factor inhibiting HIF
FGF	fibroblast growth factor
HB-EGF	heparin binding EGF-like growth factor
HGF	hepatocyte growth factor
HGG	high-grade glima
HIF	hypoxia inducible factor
HRE	hypoxia response element
IHC	immunohistochemistry
IGF-1	insulin-like growth factor-1
i.c.	intra cranial
Ig	immunoglobulin
IL	interleukin
IGFR-1	insulin like growth factor receptor-1
KDR	kinase insert domain-containing receptor (also known as VEGFR-2)
LOH	loss of heterozygosity
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MET	mesenchymal epithelial transition factor
MMP	matrix metalloproteinase

MRI	magnetic resonance imaging
MVD	micro vessel density
NICD	notch intracellular domain
Oct-4	octamer-binding transcription factor-4
OS	overall survival
PBT	primary brain tumor
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PET	positron emission tomography
PFS	progression-free survival
PHD	prolyl hydroxylase
PlGF	placenta-like growth factor
PKB	protein kinase B
PLCγ	phospholipase Cγ
PKC	protein kinase C
PTEN	phosphatase and tensin homolog
PI	phosphoinositide
PIP2	phosphatidyl inositol-4,5-biphosphate
PIP3	phosphatidyl inositol-3,4,5-triphosphate
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B (also known as Akt)
RANO	response assessment in neuro-oncology
Rb	retinoblastoma
RR	response rate
RTK	receptor tyrosine kinase
siRNA	small-interference RNA
TGF-α	transforming growth factor-α
TIMP1	tissue inhibitor of metalloproteinases 1
TK	tyrosine kinase
TKI	tyrosine kinase inhibitors
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
sVEGFR-1	soluble vascular endothelial growth factor receptor 1
VHL	von Hippel-Lindau
WHO	world health organization

1. Introduction

1.1 Brain tumors

Brain tumors are a diverse group of neoplasms that can be of primary or metastatic origin. Primary brain tumors (PBT) arise from cells intrinsic to the brain and intracranial cavity, while metastatic brain tumors have their origin outside the brain and arise from a systemic tumor disseminating to the brain parenchyma.

PBT are primarily of neuroepithelial origin and according to WHO classification there are three main types which usally can be distinguished by their histological features; oligodendrogliomas, mixed oligoastrocytomas and astrocytomas (or gliomas).[1] Gliomas are the most common PBT with a yearly incidence of approximately 6/100,000 in western countries.[2] Through analyzing the most malignant region of the tumors, PBT are graded as low-grade tumors (WHO grades I and II), or as high-grade tumors (WHO grades III and IV) dependent on four main features: nuclear atypia, mitoses, microvascular proliferation, and necrosis. By the degree of increasing anaplasia[3], the types of astrocytomas usually include pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III) and the most malignant form, glioblastoma multiforme (GBM/grade IV), which is the main focus of the presented study. Necrosis and/or areas of vascular proliferation in addition to the above mentioned criteria, are mandatory for diagnosing GBM.[1] The pronounced vascularization arises because of increased angiogenesis as described in section 1.4. However, the dense vascularity does not prevent the GBM tumor from being hypoxic, partly because of the dysfunctional nature of the tumor vessels. The molecular consequences of hypoxia will be explained in section 1.3.

GBM represents approximately 70% of astrocytic and oligodendroglial tumors.[2] GBM develops from either pre-existing low-grade astrocytomas into secondary GBM or arise *de novo* as primary GBM.[4] Primary glioblastomas represents the majority of GBMs (95%) and affect mainly the elderly (mean age 62 years), whereas patients with secondary GBM have a mean age of 45 years.[2,5] There is a slight overweight of males affected with primary GBM (female to male ratio 1:1.33) whereas secondary GBM is more frequent among women (female to male ratio 1:0.65).[5] The etiology of gliomas is largely unknown, however some hereditary syndromes such as Neurofibromatosis 1/2, Tuberous sclerosis, Li-Fraumeni and von Hippel-Lindau disease, carry strong predisposition for developing gliomas.[6]

The median survival for newly diagnosed GBM is only 14.6 months.[7] The early invasion of astrocytomas into normal brain prevents surgical cure, even with aggressive resection. Standard treatment for GBM is debulking surgery if possible, followed by concomitant temozolomide (Temodal®); an oral alkylating agent, plus radiotherapy and adjuvant temozolomide, also known as the "Stupp-regime".[7] The introduction of temozolomide has improved the survival of GBM significantly, increasing the 2-year survival from 10 to 27%, compared to previous treatment regimens -however, nearly all patients with GBM will eventually relapse. The prognosis for recurrent GBM is even worse with a median survival of three to nine months when using traditional chemotherapeutic agents.[8,9] GBM is still incurable and accordingly there is a pivotal need for improved treatment strategies for this malignancy.

1.1.1 Genetics of primary and secondary glioblastoma multiforme

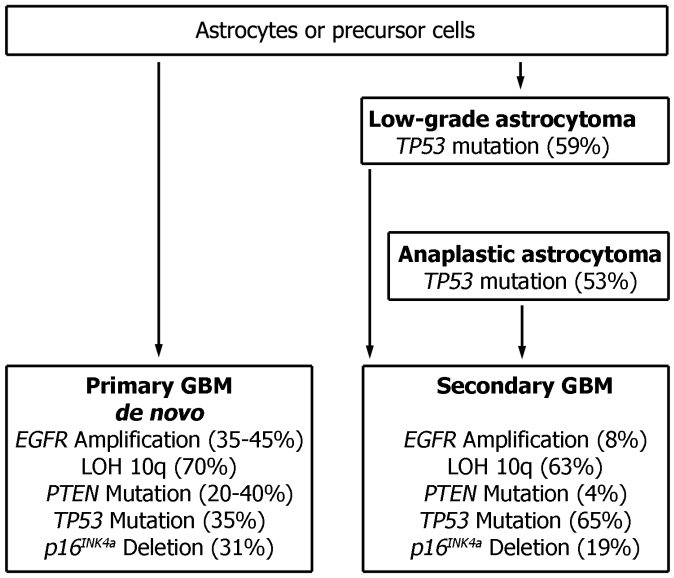

Figure 1: Timing and frequency of genetic alterations during astrocytoma progression. Note that *TP53* mutations are more frequent in low grade and secondary GBM. Moreover, *EGFR* amplification and *PTEN* mutations are more frequent in primary GBM than secondary GBM. Modified from Ohgaki et al.[5]

Gliomas are strikingly heterogeneous tumors in terms of their pathology and gene expression, even within a single tumor. Despite the variability, common alterations in specific cellular signal transduction pathways or cellular functions occur within most malignant gliomas. Primary and secondary GBM are clinically indistinguishable, but, genotypically there are differences, that could be used in the search for improved treatment of these patients (Figure 1).[5,10,11]

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK) that drives cell proliferation and survival. Amplification and overexpression of EGFR is observed in 35-45% of primary GBM and have been correlated with a poor prognosis.[12,13] Accordingly, EGFR has been expected to be of pivotal importance in the pathogenesis of GBM. In addition, EGFR mutations are present in 40-50% of GBM, of which the constitutively activated EGFRvIII is the most common.[14] EGFR and downstream related pathways are one of the main

focuses of the presented study. (Further description of EGFR and EGFRvIII, will follow in section 1.2.1 and section 1.2.5 respectively). Other genetic alterations of relevance for the GBM phenotype are summarized below and in Figure 2.

Loss of heterozygosity (LOH) in chromosome 10 is the most frequent genetic alteration in both primary and secondary GBM and occurs in 70-80% of the cases.[15,16] The majority of GBMs appear to have lost the entire copy of chromosome 10.[15-17] LOH of 10q is equally frequent in primary and secondary GBM, whereas LOH of 10p is mostly present in primary GBM.[16] Several studies have identified at least three commonly deleted loci, of which 10q23-24 harbors the tumor suppressor, phosphatase and tensin homolog (PTEN). PTEN mutations are present in 20-40% of primary GBM but rarely in secondary GBMs.[18,19] (Further description of PTEN, see section 1.2.3).

Mutations of the tumor suppressor p53 is more frequently observed in secondary GBM than in primary GBM (65% versus 35%).[20] p53 induces cell-cycle arrest (at the G1/S and G2/M transition points), DNA repair and apoptosis in response to genotoxic stress (DNA damage, inappropriate oncogene activation, hypoxia, inadequate nucleotide supply and defects in DNA methylation). Accordingly, inactivation of p53 promotes abnormal cell division and facilitates anaplastic transformation through genomic instability.[21] Inactivation of p53 can also occur by amplification of the p53-inhibitors MDM2 or MDM4 or by deletion of the MDM2 inhibitor *p14ARF* (see Figure 2).[22]

Another pathway that seems to be important for both primary and secondary GBM is the 16^{INK4a}/retinoblastoma (Rb), which often harbors genetic alterations. Rb-pathway controls progression through $G_1 \rightarrow$ S phase in the cell cycle. Mitogenic signaling induces cyclin-D1, which associates to cyclin-dependent kinase (CDK) 4. This CDK4/cyclin D1 complex phosphorylates the Rb1 protein, inducing release of the transcription factor E2F that activate genes involved in the $G_1 \rightarrow$ S phase transition. $p16^{INK4a}$ binds to CDK4, inhibits the CDK4/cyclin D1 complex, and thereby inhibiting the $G_1 \rightarrow$ S phase transition.[23] Homozygous deletions are the most common alterations of the $p16^{INK4a}$ gene and are found most frequently in primary GBM, whereas amplification of CDK4, and mutations of $p16^{INK4a}$ are observed in both primary and secondary GBM.[24,25] Homozygous deletion of the *CDKN2A* locus, which encodes both p16 and p14 is one of the most frequent aberrations, accordingly having an impact on both the Rb- and p53-pathways simultaneously.[25]

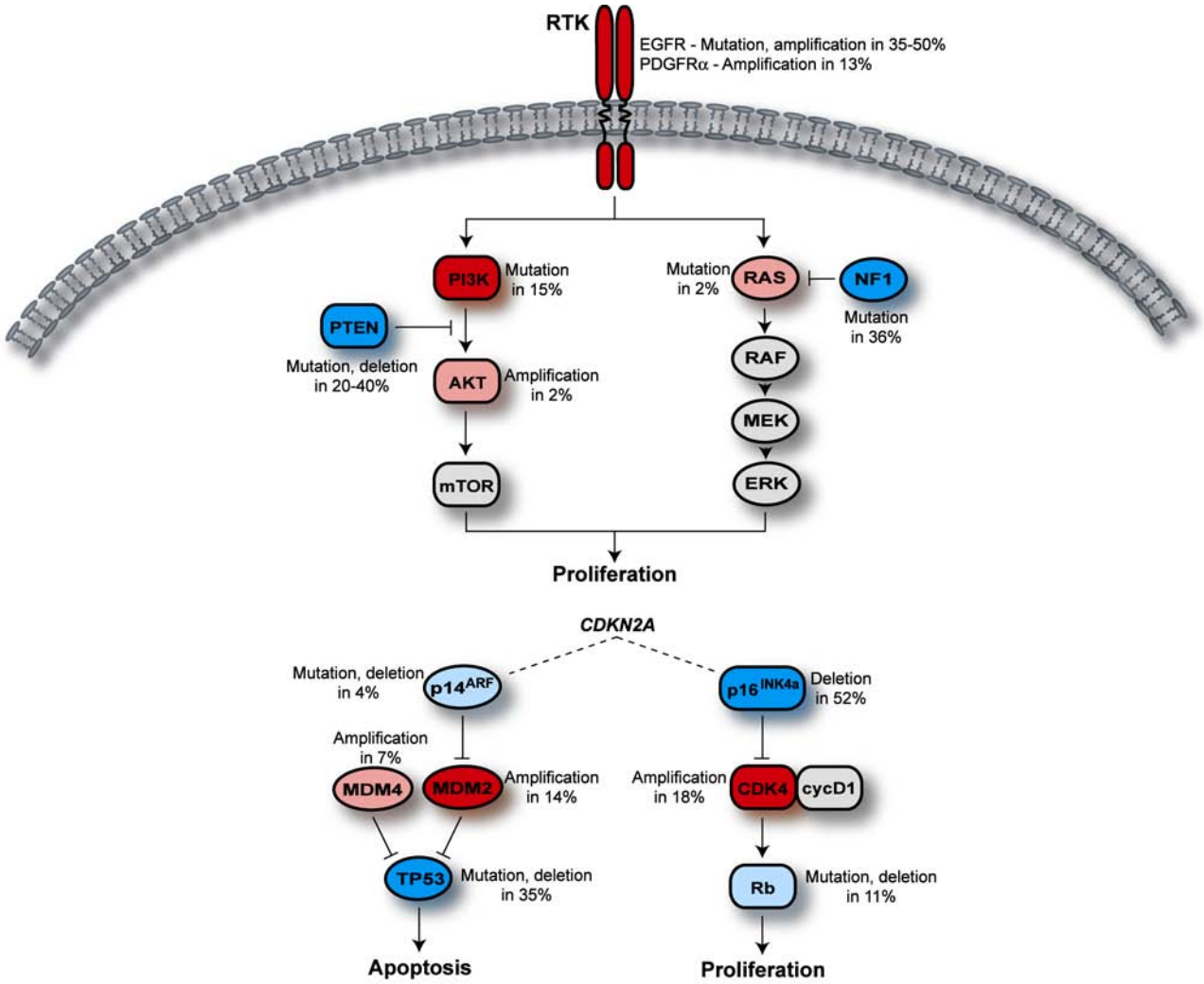

Figure 2: Frequent genetic alterations in RTK- p53-, and Rb- signaling pathways in GBM. Red indicates activating genetic alterations in oncogenes, with frequently altered genes shown in dark red and less frequent in light red. Conversely, blue indicates inactivating alterations in tumor suppressor genes, with dark blue corresponding to a higher frequency of alteration and light blue to less frequent alterations. Mutations or amplifications in the Ras/Raf/Mek/Erk pathway are rare in GBM, but increased activity is often found due to upstream alterations. As both p16 and p14 are transcribed from the *CDKN2A* locus, deletions in this region induce alterations in both the p53 and Rb pathways simultaneously. Figure modified from Chin et al.[27]

The platelet-derived growth factor receptor (PDGFR-α and PDGFR-β) are members of the protein tyrosine kinase family of receptors and are activated by receptor dimerization induced by the PDGF ligand leading to activation of downstream signaling pathways similar to the EGFR. The PDGF ligand consists of disulfide-bonded dimers of A and/or B chains. The isoforms are functionally active when dimerized as either PDGF-AA, PDGF-AB or PDGF-BB. PDGF is an endothelial cell mitogen and both PDGF and PDGFR are expressed in the endothelial cells and by tumor cells

mediating proliferation and angiogenesis.[26] PDGF and PDGFR both are overexpressed in primary and secondary GBM.[3,4,27]

1.2 EGFR

Growth factors and their receptors play a central role in the regulation of a number of cellular processes including cell growth and proliferation. Among the best understood growth factor regulated pathways are those mediated by RTKs which are multifunctional proteins with similar structural features that include an extracellular ligand binding domain and an intracellular kinase domain.[28] The first RTK to be discovered was EGFR, also known as ErbB1/HER1. EGFR belongs to the ErbB/HER family of ligand activated tyrosine kinase receptors, which also include ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER.[29] EGFR has frequently been implicated in various forms of human cancers including those of the breast, lung, brain, prostate and head and neck.[30] The mechanisms by which EGFR becomes oncogenic are several and include autocrine growth factor loops, overexpression of EGFR, and gene mutations giving rise to constitutively active variants of EGFR.[30] The events leading to overexpression can be increased activity of the EGFR promoter, amplification of the EGFR gene or deregulation at the translational and post-translational level.[31,32]

1.2.1 The EGFR gene and structure

EGFR is encoded by the *c-erbB1* proto-oncogene which is located on the human chromosome 7p11.2.[33] EGFR is a highly glycosylated 170 kDa membrane spanning protein, which consists of a single polypeptide chain of 1186 amino acids.[34]

Like all RTKs, EGFR is characterized by a modular structure consisting of three main domains: an extracellular ligand binding domain, a transmembrane domain and a cytoplasmic domain containing the tyrosine kinases.[35] The extracellular domain of EGFR can be further divided into four subdomains designated I, II, III and IV. Crystallographic studies of the EGFR extracellular domain in complex with EGF and transforming growth factor α (TGF-α) have shown that the domains I, II and III form a ligand-binding pocket, as shown in Figure 3.[36,37] The protein kinase domain, which contains the intrinsic tyrosine kinase activity of the receptor, is capable of phosphorylating several cytoplasmic target proteins as well as tyrosine residues on the dimer partner upon receptor dimerization.[38]

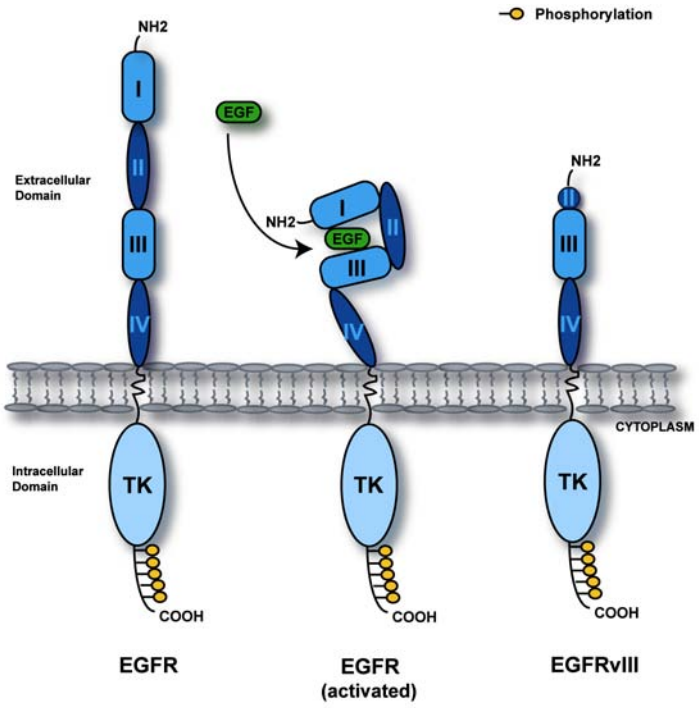

Figure 3. Schematic structures of EGFR and EGFRvIII. The EGFR contains three main domains: a ligand binding domain, a transmembrane domain and an intracellular domain. The extracellular domain is composed of four subdomains designated I, II, III and IV. The intracellular domain contains the tyrosine kinase (TK) domain. The domains I, II and III form a ligand-binding pocket, where a ligand is docked between the domains I and III (activated EGFR). The EGFRvIII lacks the most of domain II and all of domain I resulting in a disrupted binding pocket.

1.2.2 EGFR ligands and activation

Like all known RTKs, EGFR exist as monomers in the cell membrane. Upon ligand binding of EGF or other ligands (i.e. TGF-α, epiregulin, heparin binding EGF-like growth factor (HB-EGF) and amphiregulin) the EGFR pairs, or 'dimerizes'. This dimerization may occur as homodimerization (i.e. EGFR dimerizes with another EGFR) or heterodimerization (i.e. EGFR dimerizes with another ErbB family member) inducing activation of the intrinsic tyrosine kinase activity in the cytosolic domain of the receptor (Figure 4).[39] In the absence of ligand, most RTKs posses a low basal kinase activity, which increases upon ligand induced dimerization.[40]

Receptor dimerization, which results in increased tyrosine kinase activity, is a result of autophosphorylation of tyrosine residues within the kinase domain. When the catalytic activity of the receptors is elevated, additional tyrosine sites on the receptor intracellular domain are autophosphorylated.[34,39] Ligand induced EGFR dimerization leads to autophosphorylation of several key tyrosine residues (Tyr 992, 1045, 1068, 1086, 1148 and 1173) in the cytoplasmic domain of each receptor monomer.[41] Other tyrosine residues including Tyr 845, 891, 920, 954, 974 and 1101 are also present in the cytosolic tail of the receptor but these are phosphorylated by kinases in the cytosol.[34] These phosphorylated tyrosine residues then serve as binding sites for

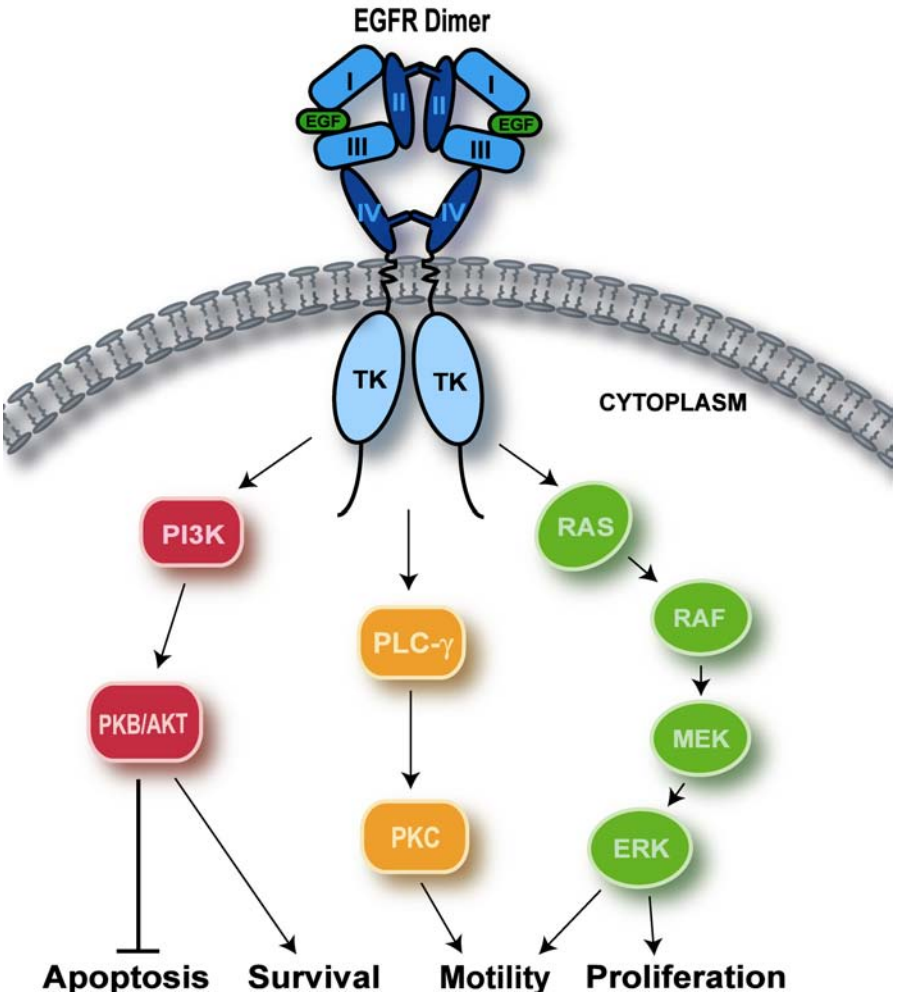

Figure 4: Ligand binding leads to dimerization of the EGFR, and subsequently activation of the intracellular tyrosine kinases. Overview of the signal transduction pathways: PI3K-Akt/PKB, Ras-Raf-Mek-Erk and PLC-PKC, which are activated by the EGFR. (See text for details.)

adapter and signaling molecules leading to the activation of several signaling pathways downstream from the receptor (Figure 4).

1.2.3 EGFR signaling

One of the best characterized EGFR effector pathways are the mitogen activated protein kinase (MAPK) signaling cascade, composed of among others the Ras-Raf-Mek-Erk pathway (Figure 4). The biological effects of the Ras-Raf-Mek-Erk pathway are many, but mainly they lead to cell growth and proliferation.[42] Constitutive active, mutated forms of *Ras* are observed in several epithelial tumors as for example K-Ras in colon cancer and although high Ras-activity often is found in gliomas, Ras mutations are not very common in GBM.[43-45] Accordingly, increased Ras-

signaling observed in gliomas is due to excessive upstream RTK-activation, rather than due to alterations within the Ras-Raf-Mek-Erk pathway itself (Figure 2).[46]

Another pathway downstream of EGFR involves the phosphatidylinositol 3-kinase (PI3K) (Figure 4), which binds to phosphorylated EGFR through the adapter protein Grb2-associated protein 1 (Gab1).[47] The activated PI3K subsequently phosphorylates phosphoinositides (PIs) at their 3-OH position, generating the lipid second messengers phosphatidyl inositol-4,5-biphosphate (PIP2) and phosphatidyl inositol-3,4,5-triphosphate (PIP3). This leads to subsequent recruitment and activation of protein kinase B (PKB, also called Akt).[48] Akt has been shown to promote cell survival by inhibiting apoptosis.[49] The PI3K complex consist of a catalytically active protein (p110α) encoded by *PIK3CA*, and a regulatory protein (p85α), encoded by *PIK3R1* and several studies have shown mutations in the Akt-interacting regions of *PIK3CA* in GBM, which could contribute to increased activation in the PI3K/Akt pathway.[50,51] The PI3K/Akt pathway is regulated by the tumor suppressor PTEN, which antagonizes the action of the PI3K, by dephosphoylation of PIP3 to PIP2 (Figure 6). Inactivation of PTEN due to *PTEN* mutations on top of increased EGFR signalling, contributes the abnormally high activity of the PI3K/Akt pathway, often seen in primary GBM[13,52] and has been correlated to the dismal prognosis of patients with GBM.[5,52,53]

A third signaling pathway, induced by EGFR, is the phospholipase Cγ-protein kinase C (PLCγ-PKC) pathway (Figure 4). PLCγ is activated by binding to activated EGFR and activation of PLCγ has been shown to be necessary for EGF and PDGF-induced cell motility.[54,55]

1.2.4 EGFR downregulation

Due to its role in cell growth and proliferation, the activity of the EGFR is tightly regulated. The most prominent regulator of EGFR signal attenuation is downregulation of the protein, which includes internalization and subsequent degradation of the activated receptor.[56] In the absence of EGF, EGFR is localized to smooth, uncoated, uninvaginated regions of the cell surface.[57] When EGF is added, the receptors are recruited to clathrin coated pits, which then invaginate and pinch off (fission) to become free clathrin coated vesicles, thus internalizing the EGFR localized within these clathrin-coated pits (Figure 5).[56,58] The clathrin coated vesicles then fuse with and deliver their cargo to the endosomes which are characterized by a low pH (pH~6).[58]

In the case of EGFR, ligand-receptor dissociation depends on the ligand bound to the receptor.[59] For example, the affinity of EGF for EGFR is fairly insensitive to endosomal pH, and the majority of the EGF molecules remain bound to the receptor in the endosomes. In contrast, the affinity of TGF-

α for EGFR is very sensitive to the low pH environment in the endosomes, and TGF-α is largely dissociated from the receptor upon reaching the endosomes.[59] In the endosomes the EGFR undergoes sorting and is either recycled back to the plasma membrane or directed to the lysosomes

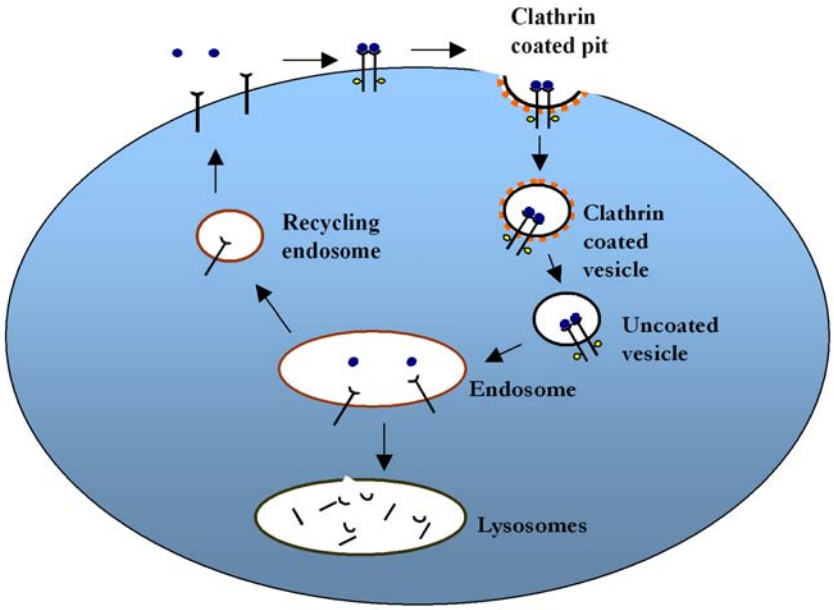

Figure 5. Schematic model of the intracellular trafficking of the EGFR upon ligand binding. Ligand binding results in receptor dimerization and autophosphorylation leading to the formation of clathrin coated pits. The coated pits invaginate to free coated vesicles which then become uncoated before they fuse with the endosomes and deliver their cargo. In the endosomes the EGFR undergoes sorting and is either recycled back to the cell surface or directed to the lysosomes for degradation.

for degradation (Figure 5). Lysosomes have a lower pH (pH~ 5-5.0) than endosomes and they contain many hydrolytic enzymes that are optimally active in low-pH environment and involved in the degradation of proteins.[60] The degradation of EGFR also results in the degradation of EGF in the lysosomes.[61] Lysosomal degradation is the ultimate step in EGFR downregulation.

1.2.5 EGFR mutations

EGFR mutations are present in 40-50% of GBM, with the constitutively activated EGFRvIII being the most common.[14,27] The EGFRvIII arises by an in-frame deletion of 801 base pairs, corresponding to exons 2-7 in the EGFR gene.[62] The deletion of exons 2-7 eliminates 267 amino acids (amino acid 6-273) from the extracellular domain and results in a 145 kDa truncated receptor with a distorted ligand binding area (Figure 3).[62,63] Approximately 40% of astrocytic tumors with EGFR amplification also express EGFRvIII.[64]

EGFRvIII has been found in primary human brain- and ovarian tumors as well as in breast carcinomas and non-small cell lung carcinomas.[63] The frequent expression of this variant in several tumor types suggests a strong selective advantage conferred upon tumor cells *in vivo*.[65,66] Several functional differences between EGFRvIII and EGFR have been characterized. Unlike EGFR,

EGFRvIII lacks ligand binding (EGF and TGF-α), has a constitutively active tyrosine kinase and does not seem to be downregulated from the membrane.[67,68]

Despite the lack of ligand binding, not only does EGFRvIII seem to form homodimers, but the receptor also appears to form heterodimers when co-expressed with EGFR.[69,70] The kinase activity per molecule of dimeric EGFRvIII is similar to that of the EGF stimulated EGFR, but only 10-15 % of the total EGFRvIII proteins are present in the dimeric form and therefore phosphorylated.[71]

The prognostic significance of EGFRvIII when measured by immunohistochemistry in high-grade glioma (HGG) is not clarified, as when with validating and reproducing immunohistochemical results.

1.2.6 EGFR inhibitors

In the recent years, enhanced understanding of molecular abnormalities occurring in malignant gliomas has given rise to the development and use of targeted therapy in the search for an improved treatment, and optimally, a cure for this malignancy.

As detailed above, overexpression and/or amplification of EGFR are frequent in GBM and this has been correlated with a poor prognosis.[12,13] Accordingly, EGFR has been expected to be of pivotal importance in the pathogenesis of GBM. This seems supported by the observations that overactivity of the EGFR pathway results in cell proliferation, increase in tumor invasiveness, motility and angiogenesis.[72,73]

The two major strategies for the inhibition of EGFR signaling are the use of monoclonal antibodies (mAb) targeting the extracellular EGFR domain, and small molecule inhibitors directly targeting the highly conserved tyrosine kinase domain of the EGFR. Mutations of the tumor suppressor gene *PTEN*, which occur in 20-40% of GBM, have been shown by other groups to mediate resistance to anti-EGFR treatment Accordingly, PTEN has been considered of importance for response to EGFR inhibition.[74-77]

Tyrosine kinase inhibitors (TKI) are ATP-competitive inhibitors of the tyrosine kinase located at the intracellular part of the EGFR resulting in inhibition of EGFR autophosphorylation.[78] Gefitinib (Iressa®) and erlotinib (Tarceva®) are reversible TKI inhibitors and are currently the most advanced drugs of the TKI family (Figure 6). Both drugs have been used in phase I and phase II clinical trials for HGG either as monotherapy or in combination with conventional chemotherapy.[79-81] Results from these studies are not uniform although several of them indicate a modest efficacy of TKIs in GBM.[75,82] However, EGFR levels fail to predict the response to EGFR TKIs.[79,83]

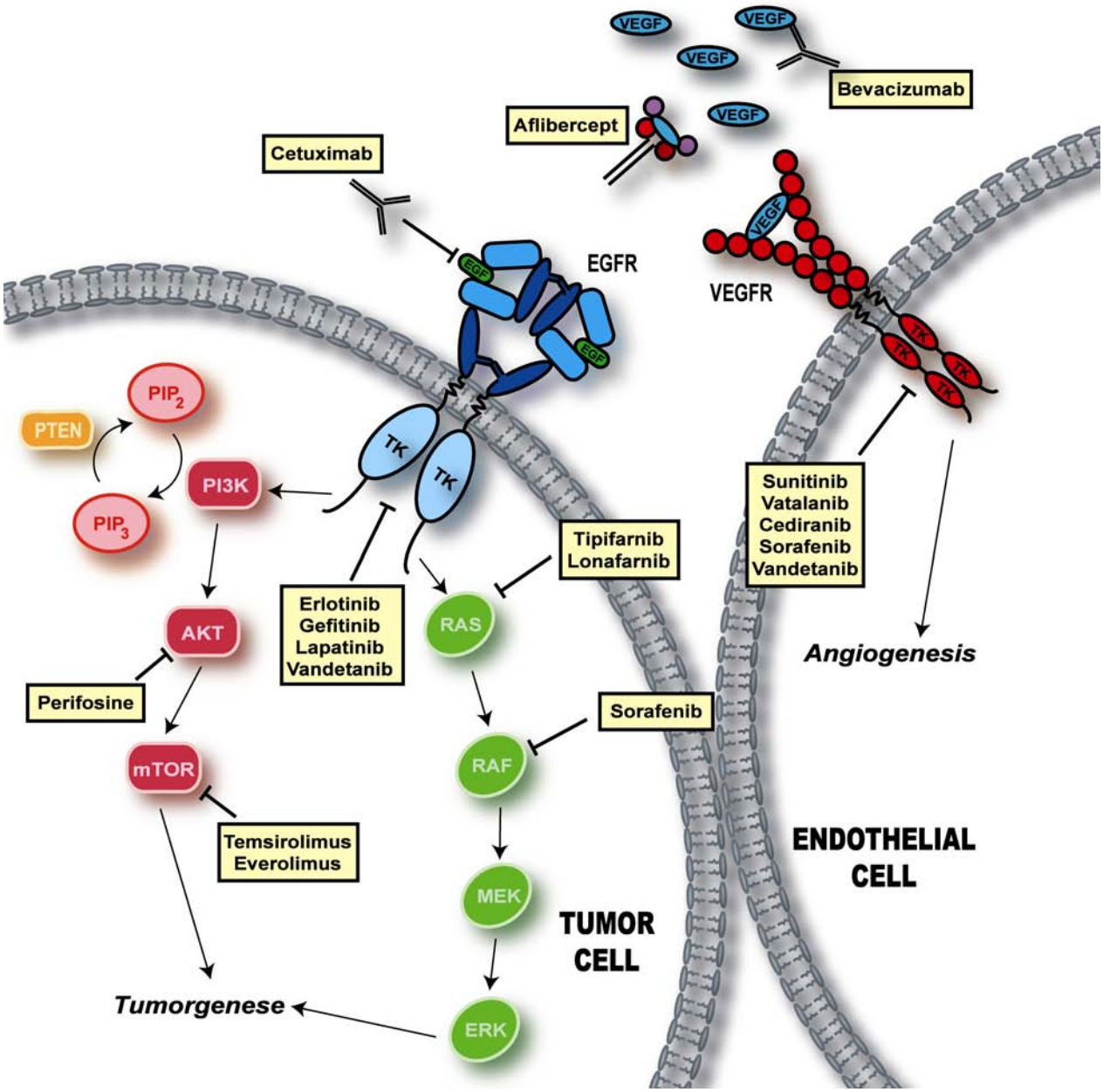

Figure 6: Examples of different targeted therapies investigated in HGG. The different types of therapeutic compounds are for simplification assembled in the yellow boxes according to their respective targets. Tumorigenesis in tumor cells can be inhibited by monoclonal antibodies (mAb) or intracellular tyrosine kinase inhibitors (TKI) targeting EGFR. Moreover, several different compounds can inhibit EGFR downstream signaling. Tumor angiogenesis can be inhibited by mAb against the pro-angiogenic factor VEGF or by TKI targeting VEGFR. See text for further details.

Cetuximab (Erbitux®) is a chimeric mAb of the IgG1 type that binds to the extracellular domain of the EGFR with high affinity, competes for ligand binding and downregulates receptor expression on the cell surface (Figure 6).[84,85] Additionally, cetuximab prevents EGFR extracellular dimerization, hindering the formation of EGFR dimers.[84] Cetuximab also recognizes EGFRvIII and despite the

lack of an extracellular domain, it inhibits autophosphorylation of the mutated receptor and induces internalization of the cetuximab-EGFRvIII complex.[86-88]

Cetuximab is FDA approved for use in colon- and head and neck cancer and is currently used in clinical phase II and III studies, preferentially in combination with conventional chemotherapy in various other cancer types (see [89] for review). *In vitro* and *in vivo* studies with cetuximab using glioma cell lines that overexpress and/or amplify EGFR have shown a reduction in cell viability upon treatment.[90-92] Moreover, it has been demonstrated *in vitro* that cetuximab induces antibody-dependent cellular cytotoxicity (ADCC) activity, a feature also observed with other mAb drugs as for example the HER2/neu mAb trastuzumab (Herceptin®).[93]

The use of cetuximab for HGG patients has been limited. However, Belda-Iniesta and co-workers did show some durable responses when using cetuximab in three patients with recurrent GBM who remained clinically and radiologically stable for 14, 13, and 11 months, respectively.[94]

As the effect of EGFR inhibition in GBM still needs to be clarified, there are ongoing *in vitro* and *in vivo* studies using TKIs or mAb looking at the various effects of EGFR inhibition. The downstream pathway of EGFR is one area to explore and one of the main focuses of the study presented, by the use of an *in vitro* model in glioma cell lines. The other main focus is investigation of the effect of EGFR inhibition, which is investigated in a clinical phase II trial for recurrent GBM.

1.3 Hypoxia

Hypoxia plays a prominent role in tumor development, invasion, angiogenesis, resistance to chemo- and radiotherapy and decreased patient survival in various cancer types, including GBM. The characteristic necrotic regions of GBM are assumed to be regions of hypoxia, although this involvement is not conclusively proven.

When available blood flow cannot fulfill the requirements for maintaining oxygen homeostasis, the partial oxygen pressure of these tumor areas become low, i.e. hypoxic, or close to zero, anoxic. The diffusion limit for oxygen is approximately 100 µm and oxygen transport over further distances requires red blood cells. Tumor hypoxia evolves as a consequence of insufficient oxygen delivery and is a feature of most solid tumors. High rate of cell proliferation and increased distance to existing blood vessels in combination with insufficient neo-vascularization contributes to a tumor microenvironment with low oxygen tension. Moreover, tumor vessels are leaky, leading to tumor edema and increased intratumoral pressure, which further increases hypoxia.[95] Cancer cells undergo numerous changes that enable them to adapt to and survive hypoxia, contributing to a more

aggressive behavior of the tumor. The hypoxia inducible factors (HIF), HIF-1α and HIF-2α, are critical for this adaptive response.[96] HIF-1α upregulates carbon anhydrase 9 (CA9) in response to hypoxia.[97] CA9 (a member of the carbonic anhydrase family, comprising transmembrane enzymes) catalyzes the reversible hydration of carbon dioxide to carbonic acid ($CO_2 + H_20 \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$) and thereby is involved in the pH homeostasis of the cancer cells. CA9 has been shown to be an independent prognostic marker in patients with various cancers including HGG.[98,99] In a recent study by Sathornsumetee *et al*., high CA9 expression predicted poor survival outcome in HGG patients receiving anti-angiogenic treatment.[100]

1.3.1 Cellular response to hypoxia

The HIF-1 transcription factor mediates adaptive responses to changes in tissue oxygenation by regulating numerous genes involved in e.g. angiogenesis, vascular reactivity and remodeling, cell proliferation and survival. HIF-1 is a member of the basic helix-loop-helix-PAS (bHLH-PAS) family, which includes the hypoxia regulated HIF-1α, 2α, 3α and the constitutively expressed HIF-1β (also known as ARNT).[101]

HIF-2α and, especially HIF-1α have been most intensively studied, and the two factors display some differences, which will be mentioned below when relevant (otherwise described as HIF-α). HIF-3α will not be described in further detail. HIF-1α is expressed in an apparently ubiquitous fashion, whereas HIF-2α expression is restricted to particular cell types, including brain and vascular endothelial cells.[102,103] HIF-1α and HIF-2α are both implicated in tumorgenesis and are frequently coexpressed in human tumors.[96,104]

The HIF-α proteins form a transcriptional active heterodimer with HIF-1β during hypoxia, which initiates transcription by binding to hypoxia response elements (HREs) in promoters or enhancers of target genes.[105,106] The HIF-α expression is determined by the rate of protein synthesis regulated via O_2-independent mechanisms whereas protein degradation is regulated via O_2-dependent mechanisms.[107] During normoxia, prolyl hydroxylases 1-4 (PHD 1-4) modify the HIF-α proteins at conserved prolines resulting in interaction with the von Hippel-Lindau (VHL)-E3 ligase protein complex, targeting HIF-α for ubiquitylation which is followed by degradation through the proteasomal machinery.[108,109] Since hydroxylation is oxygen dependent, this ensures a tight regulation of the HIF-α proteins. In addition to being regulated at the protein stability level, HIF-α is also regulated at the level of transcriptional activity by an oxygen-dependent asparagyl

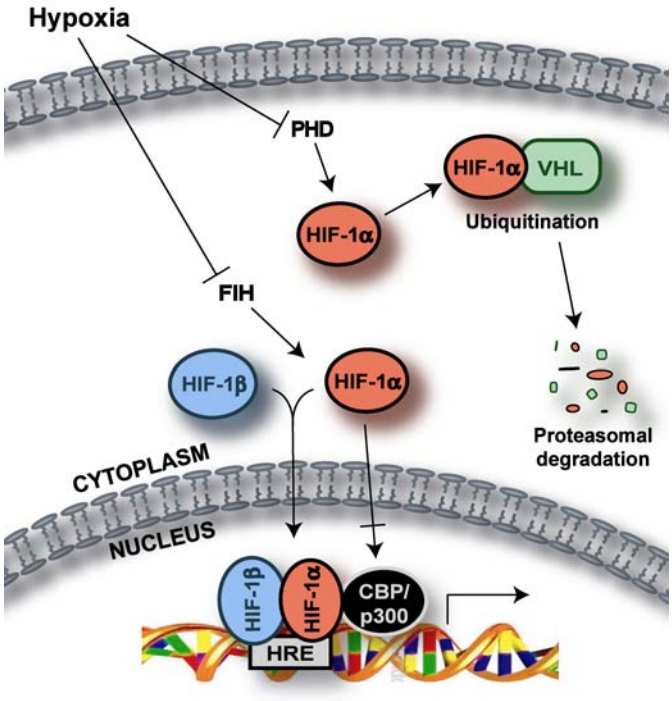

Figure 7: Regulation of HIF-1α. During normoxia, HIF-1α is hydroxylated by PHDs leading to ubiquitination of HIF-1α by interaction with VHL and subsequently degradation by the proteasome. In addition, HIF-1α transcriptional activity is regulated by FIH, which inhibits HIF-1α interaction with CBP/p300. Hypoxia inhibits both PHDs and FIH and leads to heterodimerization between HIF-1α and HIF-1β/ARNT and initiation of transcription

hydroxylase, factor inhibiting HIF (FIH) leading to reduced interaction with the nuclear co-activator CBP/p300 at normoxia.[110] Both these regulatory mechanisms are antagonized under hypoxic (1-2% O_2) conditions. At hypoxia, the HIF-α is no longer hydroxylated by PHDs and VHL does not bind. This results in stabilization of the HIF-α proteins that now can interact with CBP/p300 and initiate transcription from HREs in target gene promoters by forming a complex together with HIF-1β (Figure 7). The stabilization of the HIF-1α and the HIF-2α subunits leads among others to transcription of vascular endothelial growth factor (VEGF) and CA9.[97,106] HIF-1α also controls the expression of angiopoietin-2 (Ang-2) which act as a pro-angiogenic factor like VEGF.[111] Some of the other target genes for HIF-1α are well known and characterized and include those regulating glucose metabolism and proliferation such as glycolytic enzymes, glucose transporters (e.g. GLUT-1), angiogenic factors (e.g. VEGF and erythropoietin (EPO)), and growth factors such as TGF-α.[96] Exclusive target genes for HIF-2α has yet to be identified, however, it has been shown that HIF-2α can regulate cancer stem cell function and/or differentation through the octamer-binding transcription factor (Oct-4) which in turn contributes to HIF-2α activity.[112]

Increase in HIF-1α levels can also be induced by growth factors like EGF, which unlike the hypoxic associated decreased degradation of HIF-1α, stimulates the synthesis of HIF-1α via the PI3K or the Ras/MAPK pathways.[113,114] EGFR inhibition with cetuximab has demonstrated to downregulate of HIF-1α *in vitro*.[115,116] Furthermore, HIF-1α is known to induce the expression of the EGFR ligand TGF-α thus providing an autocrine loop regulating the hypoxic response.[117]

1.3.2 Hypoxia in GBM

The characteristic necrotic regions of GBM are surrounded by a cluster of cells known as pseudopalisading that are suspected to be regions of hypoxia, although this has not been conclusively proven. These necrotic areas do not seem to be related to tumor size, as they have been found in both small and large tumors. Furthermore, it has been demonstrated in animal glioma models that tumors <1 mm in diameter are more hypoxic and poorly perfused with sparse vasculature as compared to larger tumors (1-4 mm in diameter).[118] This suggests that necrosis might not be simply due to inadequate vascular supply but instead a result of intrinsic molecular or genetic changes within the tumor.[119] Hypoxia also seems to induce GBM cell migration and invasion.[118] However, the extent of hypoxia in GBM has still to be elucidated.

HIF-1α overexpression and angiogenesis have been shown to correlate in brain tumors[118,120], and there is a significant association between HIF-1α overexpression and tumor grade.[105] Moreover, in GBM, HIF-1α has been shown to be overexpressed in viable cells surrounding areas of necrosis, suggesting that HIF-1α might mediate cell survival.[120]

1.4 Angiogenesis

High micro vessel density (MVD) is a hallmark for GBM and pronounced tumor vascularity is significantly correlated with poor survival.[121,122]

Vasculogenesis, arteriogenesis and angiogenesis are the three mechanisms of the vascular network development. Vasculogenesis occurs mainly at the embryonic stage by angioblast differentiation into endothelial cells to form blood vessels, a process which among others depends on paracrine stimulation by VEGF.[123]

Arteriogenesis is remodeling and enlargement of the preexisting collateral arteriolar networks to oblige higher metabolic demands, e.g. due to vascular stenosis or occlusion.

Angiogenesis is development of new vessels from preexisting ones by sprouting or by intussusception from their vessels of origin.[124,125] Many molecules are implicated in the positive regulation of angiogenesis, e.g. acidic fibroblast growth factor (aFGF), basic FGF (bFGF), EGF, TGF-α, angiogenin, interleukin 8 (IL 8), angiopoitins and VEGF (Figure 8).[124,126,127] In adults, angiogenesis is essential during the ovarian reproductive cycle and for repair, remodeling and regeneration of tissues, for example during wound heeling.[128] In tumor development, angiogenesis

is essential for tumor growth and being one of the most vasculized tumors, angiogenesis seems fundamental for GBM.

Tumor vasculature is characterized as immature and malformed with abnormal branching resulting in a chaotic structure. Moreover, the leaky nature of tumor vessels induce edema, which further promotes the hypoxic tumor milieu and induction of pro-angiogenic factors like VEGF thus creating a positive paracrine loop, maintaining angiogenesis and conditions necessary for sustained tumor growth.[95] Besides activating tissue endothelial cells, angiogenic factors also activates circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs) from the bone marrow, which enter the circulation and generates new blood vessels in tumor tissue.[129]

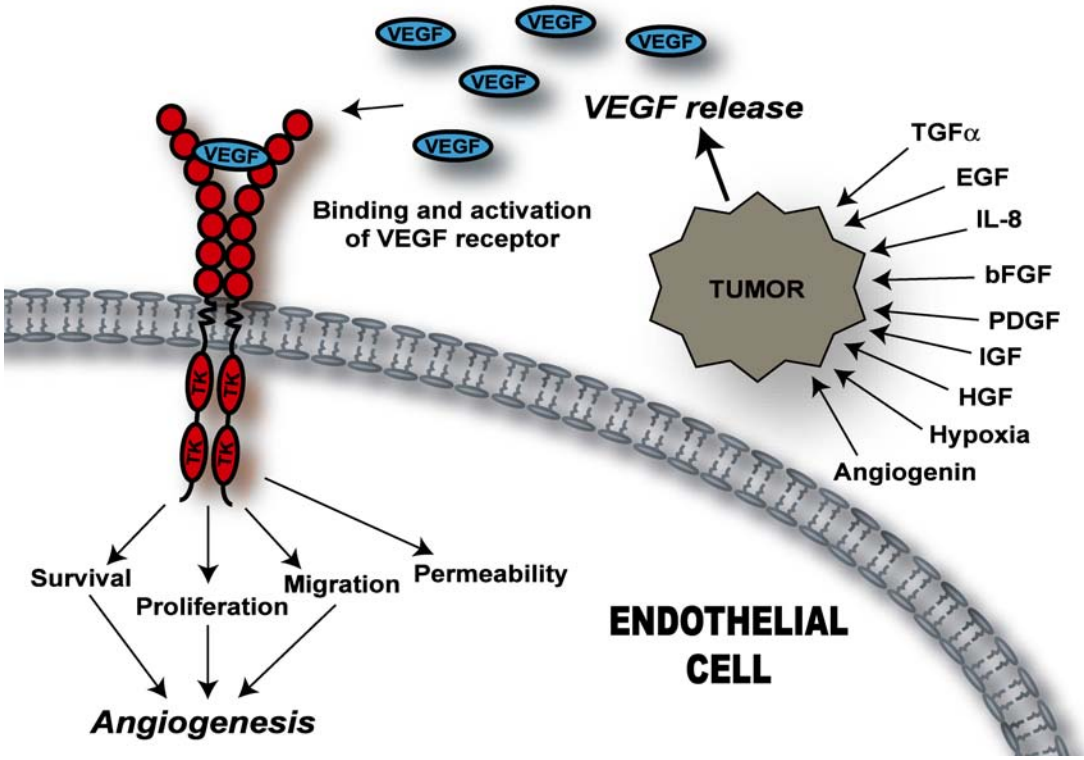

Figure 8. Tumor induced VEGF release gives rise to angiogenesis and increased vessel permeability. Modified from Tabernero et al.[192]

1.4.1 VEGF/VEGFR signaling pathway in pathological conditions

VEGF is the major endothelial mitogen in central nervous system neoplasms[130] and strong VEGF expression has been detected by immunohistochemistry in 65-100% of GBMs.[131-133] Of endogenous angiogenic factors identified, the VEGF family and the angiopoietins are the endothelium-specific angiogenic factors. The VEGF family consists of at least five ligands (VEGF-A, -B, -C, -D, and

placenta-like growth factor (PlGF)) and three tyrosine kinase receptors (VEGFR-1, -2, -3).[134] VEGF-A (also known as VEGF) is a 34-45 kDa dimeric glycosylated protein. Alternative exon splicing of the *VEGF* gene results in at least five isoforms of VEGF ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$), with $VEGF_{165}$ being the predominant form in general and the most common form found in GBM.[134-136] VEGF is a survival factor for endothelial cells of newly formed but not established vessels within the tumor.[95] Moreover, VEGF is a major permeability factor partly responsible for the loss of blood-brain barrier (BBB) during tumor growth.[95]

The elevated expression of VEGF in human cancer is likely induced by numerous mechanisms, of which hypoxia via HIF-1α plays a key role as described above. Tumor cells are the main source of VEGF in GBM, whereas VEGF receptors are predominantly expressed by endothelial cells.[135,137] Moreover, *in vitro* studies have shown that PTEN mutations increases VEGF mRNA levels via the PI3K/Akt pathway.[138] Furthermore, several major growth factors, including EGF, TGF-α and TGF-β, insulin-like growth factor-1 (IGF-1), FGF, IL-8 and PDGF upregulate VEGF mRNA expression in a paracrine or autocrine manner, in cooperation with hypoxia (Figure 8).[126,134] In addition to VEGF, GBM cells are known to produce a variety of pro-angiogenic factors, including bFGF, PDGF, IL 8, HIF-1α and hepatocyte growth factor (HGF) (Figure 8).[139-142] Although tumor cells represent the major source of VEGF, tumor-associated stroma is also an important site of VEGF production.[143] The expression of VEGF is especially prominent in tumor cells around necrotic areas in GBM[130], and increased concentrations of VEGF have been found to correlate with malignancy grade[142] and radiological response to bevacizumab in HGG.[100,144]

VEGF binds to two related RTKs, VEGFR-1 (Flt-1) and VEGFR-2 (KDR or Flk-1) which are both expressed on endothelial cell surface and are found to have increased expression in GBM, when compared to normal brain.[130,144] VEGFR-3 is not a receptor for VEGF, but instead binds VEGF-C and VEGF-D and its expression is largely restricted to lymphatic endothelial cells.[134] Despite being the first VEGF receptor to be identified, the precise role of VEGFR-1 in angiogenesis is to be elucidated. It is known that VEGFR-1 expression is upregulated by hypoxia by a HIF-1α dependent mechanism and that VEGFR-1 interact with VEGF, PlGF and VEGF-B.[134,145] VEGFR-1 binds VEGF with approximately ten times the affinity of VEGFR-2 binding, but its signal-transducing properties are extremely weak.[146] Moreover, an alternatively spliced soluble form of VEGFR-1 (sVEGFR-1) has shown to be an inhibitor of VEGF activity by complexing with the ligand and thus acting as regulator of VEGF bioavailability. In addition, VEGFR-1 forms heterodimers with transmembrane VEGFR-2, preventing autophosphorylation of VEGFR-2.[147,148] Increased

concentration of sVEGFR-1 is found in GBM, when compared with low-grade glioma and normal brain. Despite the elevated level and anti-angiogenic effect of sVEGFR-1, the pro-angiogenic effect of the VEGF ligand is still dominant in GBM.[149]

VEGFR-2 is expressed both on endothelial cells and tumor cells.[144] VEGFR-2 undergoes dimerization and ligand-dependent tyrosine phosphorylation inducing phosphorylation of among others; PKC-γ, PI3K, Ras and the Src kinase familiy.[129,134] VEGFR-2 is the major receptor involved in angiogenesis and VEGF activation leads to endothelial cell survival, proliferation, endothelial cell migration and vascular permability.[127,134] VEGF also interacts with the co-receptors neurophilin-1 and neurophilin-2.[150]

1.4.2 Other angiogenetic mediators

VEGF, EGFR and hypoxia are not the only mediators of angiogenesis, and accordingly a brief presentation introducing some additional pro-angiogenic factors that could be of importance in HGG follow below.

Notch 1, 2, 3 and 4 are transmembrane cell surface receptors expressed in various cell types and are generally involved in cellular processes such as differentiation, proliferation and apoptosis. These receptors interact with the transmembrane ligands (Jagged 1 and -2, and Delta-like ligands (Dll) 1, -2, and -4) on adjacent cells.[151] Ligand binding sensitizes the heterodimeric receptor to cleavage events mediated by members of the ADAM and γ-secretase families of proteases. This leads to release of the Notch intracellular domain (NICD) and stimulation of transcription of multiple target genes.[151] Notch-Dll4 signaling is essential for vascular development in the embryo, as knockout of one *Dll4* allele is lethal to the embryo[152], haploinsufficiency of *VEGF* has a similar effect.[153,154]

The downstream effects of Notch signaling are highly tissue- and time-dependent and Notch has been implicated in both the maintenance of neural progenitors and in the generation of glia during development of the brain.[155] In addition to playing a role in normal development, Notch signaling is also important in tumorigenesis.[156]

Dysregulation of Notch activity is likely to be a part of HGG tumorigenesis.[157] In the work by Purow *et al*., downregulation of Notch1, Dll1 or Jagged1 by RNA interference (siRNA) induced apoptosis and inhibited proliferation in glioma cell lines.[158] These findings were largely reproduced in a recent work by Xu *et al.* who also showed an increasing expression of Notch1 with increased grade of glioma malignancy.[159] Taken together these results indicate that, Notch1 is of importance for glioma cell survival and in the malignant phenotype of HGG. Recent findings indicate that

Notch might play a crucial role in the cellular response to hypoxia[160] and tumor angiogenesis[161], proving yet another target for anti-angiogenic therapy.

Dll4 is upregulated in tumor vasculature[162,163] in part by VEGF[164] and interacts with Notch1 or Notch4, both of which are highly expressed in the vascular endothelial- and tumor cells and which are involved in angiogenesis.[162,164,165] Additionally, VEGF has been shown to induce expression of Notch1 in arterial endothelial cells[166] indicating that pro-angiogenic factors activates Notch signaling, which in turn can promote angiogenesis. Moreover, there is evidence that the Notch pathway is intimately coupled to signaling through EGFR, in the onset and maintenance of cancer[167-169] and angiogenesis.[170] The link between EGFR and angiogenesis has been described in section 1.3.1.

The cross talk between VEGF and Notch, and EGFR and Notch implicates the complexiy of tumor development, but also possible strategies, which could include Notch as an additional target in the treatment of HGG.

Finally, a short remark on the so-called brain cancer stem cells (bCSC) which have been identified in human gliomas.[171] These cells share characteristics of normal neural stem cells as they express markers such as Nestin and CD133 and harbor the capacity of self-renewal and multi-lineage differentiation.[171] Several studies have implicated these cells in treatment resistance and tumor angiogenesis through the production of VEGF.[172] In addition, a recent study found these cells, identified by the cell surface marker CD133, as being of prognostic value in glioma patients correlating with tumor grade and dismal prognosis.[173] bCSC are able to migrate unorganized throughout the brain parenchyma and initiate tumor formation in adjacent brain regions.[174] Accordingly, after debulking surgery, they will remain in their vascular niche and continue to produce migrating progenitors and ultimately cause relapse. It has been suggested that present cytotoxic treatment of HGG fails because it only kills the bulk of the tumor, whereas the tumor initiating bCSC escape and are able to regenerate the tumor and cause relapse.[175] This inadequate effect of cytotoxic treatment is partly ascribed to the fact that it is aimed at fast dividing cells (tumor progenitor cells), while the bCSC are spared as they are normally quiescent[176] or slowly cycling.[177] Recurrent GBM have been reported to have an increased level of CD133 expressing cells as compared to newly diagnosed GBM tumors.[178] As such, it could be speculated that bCSC contribute to factors responsible for tumor recurrence after therapy with increased aggression. As inhibition of Notch signaling in the embryonal brain tumor medulloblastoma almost entirely depleted the CD133

positive cell population[179] one could speculate that Notch plays an important role in the survival of bCSC and thus might be a tempting target also for glioma therapy.

1.5 Anti-angiogenic therapy

Bevacizumab (Avastin®) is a humanized immunoglobulin (Ig) G_1 that binds to and inhibits the activity of all active isoforms of the human VEGF ligand (VEGF-A) (Figure 6).[180] The terminal half-life of bevacizumab in humans is 17–21 days.[181] Bevacizumab was the first inhibitor of angiogenesis to be approved by FDA, based on the survival benefit observed in a randomized phase III trial when used as first line treatment of metastatic colorectal cancer, in combination with conventional chemotherapy (irinotecan, 5-flourouracil (5FU) and leucoverin).[182] Bevacizumab has also been FDA approved as first line treatment of advanced non-small-cell lung cancer (NSCLC) in combination with standard therapy (carboplatin and paclitaxel), for metastatic HER2 negative breast cancer in combination with paclitaxel, and for metastatic renal cancer in combination with interferon alpha.[183-185]

Other anti-VEGF therapies besides bevacizumab in clinical trials are aflibercept (also known as VEGF Trap), a soluble decoy VEGFR that binds both VEGF, VEGF-B and placenta-like growth factor (PlGF) (Figure 6).[186,187]

Another strategy for anti-angiogenic treatment is inhibition of the VEGFR. Several inhibitors of VEGFR are either underway for approval in clinical trials or already approved for cancer therapy.[188] One such drug is cediranib, a multi-targeted TKI which blocks VEGFR-1, VEGFR-2, and VEGFR-3 signaling and shows a response rate of 56% as single-agent therapy in recurrent GBM.[189] Sorafenib (Nexavar®) and sunitinib (Sutent®) are FDA approved small molecule TKIs targeting multiple receptor tyrosine kinases, including VEGFR and PDGFR.[190-193] An overview of EGFR and VEGF/VEGFR inhibitors is presented in Figure 6.

1.5.1 Bevacizumab in GBM

The efficacy of bevacizumab in recurrent HGG, was first described by Stark-Vance[194], which combined bevacizumab and the topoisomerase I inhibitor irinotecan. Irinotecan is able to cross the BBB but demonstrates only limited effect against HGG when used as single-agent therapy, with response rates between 0–15%.[195-198] Subsequently, several studies have shown the efficacy of bevacizumab in recurrent HGG[199-205] and in May 2009, FDA approved the use of bevacizumab as a single agent for patients with recurrent GBM based on two phase II studies showing durable

objective response rates.[205,206] The promising results obtained with bevacizumab are however shown to be only temporary, as recurrence is inevitable and despite prolonged progression-free survival (PFS), overall survival (OS) remains largely unchanged.

Jain *et al.* found that VEGF inhibition transiently "normalizes" the disorganized and dysfunctional tumor vasculature in some experimental models, potentially improves the delivery of oxygen and cytotoxic drugs to tumor cells.[207] Given the transient nature of this phenomenon, it remains unknown whether the proposed "vasculare normalization" model has relevance in the long-term therapeutic effects of bevacizumab-chemotherapy.

The clinical and radiological benefit of bevacizumab and other anti-angiogenic therapies is indisputable. However, only a minority of GBM patients experience this effect and there is a compelling need to select and stratify patients most likely to benefit from the treatment. Consequently, there is an ongoing search for one or more valid biomarkers, which could prove to be predictive for response to treatment.

Treatment with bevacizumab is in general well tolerated with common toxicities related to anti-angiogenic drugs, such as hypertension, proteinuria, fatigue, tromboembolic events and wound-healing complications.

1.5.2 Combination of EGFR and VEGF inhibition

Tumors like GBM are not likely to be dependent on one signaling pathway, and hence monotherapy is insufficient for obtaining tumor control. Furthermore, the heterogeneity of GBM and the ability of nearly all GBM either primarily or over time to bypass signaling pathway blockades, indicate that a multifaceted approach for GBM, involving targeted inhibition of multiple signaling pathways could block potential "escape routes".

Two key elements involved in growth and dissemination of GBM are VEGF/VEGFR and EGFR. The VEGF and EGFR pathways are closely related, sharing common downstream signaling pathways with both indirect and direct effects on the tumor cells and angiogenesis. Besides inducing cell proliferation, motility and survival, the EGFR pathway also induces angiogenesis. Accordingly, targeting EGFR has been shown to inhibit tumor neo-angiogenesis.[208-210] Thus, the effect of VEGF inhibition in GBM might be increased by adding an EGFR inhibitor. Studies with cetuximab used in GEO colon cancer cells growing as xenograft on mice, showed both anti-angiogenic and anti-tumorigenic effect which were amplified with the addition of a VEGF antisense oligonucleotide.[211] Dual inhibition of angiogenesis and EGFR might be achieved with the new drug

vandetanib, a TKI of VEGFR-1, VEGFR-2 and EGFR (Figure 6). *In vitro* results combining vandetanib with a mTOR inhibitor (a downstream mediator of EGFR signaling) induced cell cycle arrest, apoptosis and reduced proliferation.[209] The potential of this multitargeted drug in patients, is still under investigation. A phase I/II study using vandetanib in recurrent GBM has been completed, but the results have to our knowledge not been published yet.*

When the present study was initiated, preliminary data showed promising clinical and radiological effects of bevacizumab combined with irinotecan (BI) in recurrent glioma. Moreover, early reports showing the feasibility of cetuximab in combination with bevacizumab and the topoisomerase I irinotecan (CBI) in metastatic colon cancer were underway.[212] However, the combination of EGFR inhibition and anti-angiogenic treatment in GBM still remained to be investigated in recurrent GBM. Accordingly, a clinical phase II study with CBI in recurrent primary GBM was initiated at Copenhagen University Hospital, Denmark.

* http://www.clinicaltrials.gov

2. Aim of project

Hypothesis

By targeting multiple pathways in primary GBM an additive or synergistic effect leading to inhibition of tumor growth can be achieved.

Aims

Investigate the effect of the EGFR inhibitor cetuximab on growth and EGFR related signaling in glioma cell lines.

Study in a phase II clinical trial, if the addition of cetuximab to the regimen of bevacizumab and irinotecan (CBI) in recurrent primary GBM, would induce increased tumor control and response.

Investigate the biomarkers involved in angiogenesis, hypoxia and the EGFR pathway in tumor material from patients treated with CBI and correlate the expression of these biomarkers to treatment response and survival.

Manuscript I

Cetuximab insufficiently inhibits glioma cell growth due to persistent EGFR downstream signaling.

By

Benedikte Hasselbalch, Ulrik Lassen, Hans Skovgaard Poulsen and Marie-Thérése Stockhausen

In press

Cancer Investigation

2010

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Ulrik Lassen	-Supervision, manuscript revision
Hans Skovgaard Poulsen	-Supervision, manuscript revision
Marie-Thérése Stockhausen	-Supervision, design, manuscript revision

Title: Cetuximab insufficiently inhibits glioma cell growth due to persistent EGFR downstream signaling

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Abstract

Overexpression and/or amplification of the epidermal growth factor receptor (EGFR) is present in 35-45% of primary glioblastoma multiforme tumors and has been correlated with a poor prognosis. In this study, we investigated the effect of cetuximab and intracellular signaling pathways downstream of EGFR important for cell survival and proliferation. We show insufficient EGFR down-regulation and competition with endogenous EGFR ligands upon cetuximab treatment. Dose-response experiments showed inhibition of EGFR phosphorylation without affecting two of the prominent downstream signaling pathways. Our results indicate that amplification and/or overexpression of EGFR is an unsatisfactory predictor for response to cetuximab.

Introduction

Despite recent progress in the treatment of glioblastoma multiforme (GBM), including tumor-reductive surgery, radiotherapy, and concomitant and adjuvant chemotherapy with temozolomide, median survival is only 14.6 months for newly diagnosed GBM[1]. GBM develops from either pre-existing low-grade astrocytomas, secondary GBM, or arises *de novo*, primary GBM, without a previously identified brain tumor[2]. Primary and secondary GBM are clinically indistinguishable but there are genotypic differences, which could be used in the search for improved treatment of these patients[3,4].

Epidermal growth factor receptor (EGFR) is overexpresed and/or amplified in 35-45% of primary GBM tumors and has been correlated with a poor prognosis[5,6]. EGFR is therefore believed to be of pivotal importance in the pathogenesis of GBM. Furthermore, EGFR mutations are present in 40-50% of GBM tumors, of which the constitutively activated EGFRvIII, unable to bind any known ligand, is the most common[7]. Normally, epidermal growth factor (EGF) and other ligands (i.e. transforming growth factor α, epiregulin, heparin-binding EGF-like growth factor, and amphiregulin) bind to EGFR causing receptor dimerization and activation of intrinsic tyrosine kinase activity in the cytoplasmatic domain of the receptor[8]. Upon activation, the receptor is autophosphorylated and initiates downstream signaling by the Ras/MEK/ERK and the phosphatidylinositol 3-kinase (PI3K)/AKT pathways, among others, resulting in increased cell survival, proliferation, migration, invasion, and angiogenesis[9,10,11]. Inhibition of EGFR activity has been shown to be effective in various types of cancer such as lung, colon, and head and neck cancer[12,13,14], but EGFR levels fail to predict the response to EGFR inhibitors[15,16]. The EGFR tyrosine kinase inhibitors (TKI) gefitinib and erlotinib have been used in phase I and II trials for the treatment of recurrent GBM, either in combination with conventional chemotherapy or as single-agent therapy[15,17,18]. Results from these studies are not uniform, although several indicate a modest efficacy for TKIs in GBM[19,20]. Mutations of the tumor suppressor gene, phosphatase and tensin homolog (PTEN), which occur in 20-40% of GBM tumors, have been shown to mediate resistance to anti-EGFR treatment[9,21,22,23]. Accordingly, PTEN is considered of importance for response to EGFR inhibition[20,21,23,24].

Cetuximab is a chimeric monoclonal antibody that binds to EGFR with high affinity, competes for ligand binding and down-regulates receptor expression[25,26]. Additionally, cetuximab prevents EGFR extracellular dimerization, hindering the formation of EGFR dimers[25]. Cetuximab is FDA approved for use in colon and head and neck cancer, and is

currently being investigated in clinical phase II and III studies, usually in combination with conventional chemotherapy, in various other cancer types [for review see [27]]. *In vitro* studies with cetuximab using glioma cell lines that overexpress and/or amplify EGFR have shown a reduction in cell viability[28,29,30]. In the current report we further investigated the effects of cetuximab on glioma cells with respect to central intracellular signaling pathways downstream of EGFR that are important for cell survival and proliferation.

Materials and Methods

Materials

Recombinant EGF was purchased from Calbiochem (San Diego, CA), the PI3 kinase inhibitor LY294002 from Cell Signaling Technology, Inc. (Danvers, MA), and the MEK kinase inhibitor U0126 from Promega (Mannheim, Germany). Cetuximab 2 mg/mL was provided by E. Merck AB, (Stockholm, Sweden).

Cell Culture

The SKMG3 cell line was provided by Joon H. Uhm (Rochester Mayo Clinic, MN)[31], the U87MGvIII and the U87MG-EGFR cell lines by Dr Webster Cavanee (Ludwig Institute for Cancer Research, San Diego, CA), and the head and neck cancer cell line HN5 by Dr Jiri Bartek (Danish Cancer Society, Copenhagen, Denmark), while the U87MG, U118MG, U373MG, and LN229 astrocytoma cell lines and A431 epidermoid carcinoma cell line were all obtained from the American Type Culture Collection. All cells were maintained as adherent cultures in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 50 U/mL penicillin, and 50 µg/mL streptomycin (all from Invitrogen, Taastrup, Denmark).

Western Blotting

Cells were seeded in DMEM with 10% FCS, allowed to grow until 80% confluence, washed once in PBS and serum starved (SS) in DMEM supplemented with 0.5% FCS overnight (ON). SS cells were treated with: A) cetuximab 0-100 µg/mL; B) cetuximab 10 µg/mL with or without LY294002 20 µmol/L; or C) cetuximab 10 µg/mL with or without U0126 10 µmol/L. Cells were incubated with cetuximab/LY294002/U0126 for 1 h, followed by media or EGF 1 or 10 nmol/L for 15 min. Cells were then washed in ice-cold PBS and harvested by centrifugation in the presence of Protease Inhibitor Cocktail Set II (PICS-II) and Phosphatase Inhibitor Cocktail Set III (PICS-III) (Calbiochem). Cell pellets were lysed in RIPA lysis buffer (50 mmol/L Tris-HCL pH 7.4, 1% NP40, 0.25% sodium deoxylate, 150 mmol/L NaCl, and 1 mmol/L EDTA) supplemented with PICS-II and PICS-III for 15 min and subsequently sonicated. Cell debris was removed by centrifugation at 13,000× g for 15 min. Protein concentrations were determined using the BCA protein assay (Pierce, Herlev, Denmark) according to the manufacturers' instructions. Protein lysates (10 µg) were separated on pre-cast 3-8% NuPAGE TA gels and electroblotted onto nitrocellulose membranes (Invitrogen).

After 1 h in blocking buffer (10 mmol/L Tris-HCl, 100 mmol/L NaCl, and 0.1% Tween 20 pH 7.5 with 5% non-fat dry milk powder) at room temperature (RT), the membranes were incubated with mouse monoclonal anti-EGFRvIII (clone DH8.3, Novocastra, Newcastle upon Tyne, UK), mouse monoclonal anti-phospho-p44/42 MAP/Erk1/2 (Thr202/Tyr204) (Cell Signaling Technology, Inc.), mouse monoclonal anti-PTEN (A2B1) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-EGFR pTyr1086 (Biosource, Invitrogen, Carlsbad, CA), rabbit polyclonal anti-EGFR pTyr1148 (Biosource), rabbit polyclonal anti-EGFR pTyr1173 (Biosource), rabbit polyclonal anti-p44/42 MAP/ERK1/2 (Cell Signaling Technology, Inc.), rabbit polyclonal anti-phospho-AKT(Ser473) (Cell Signaling Technology, Inc.), rabbit polyclonal anti-AKT (Cell Signaling Technology, Inc.), or sheep polyclonal anti-EGFR (20-ES04, Fitzgerald Industries International, Concorde, MA) ON at 4ºC. The membranes were then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (swine anti-rabbit, goat anti-mouse, and rabbit anti-sheep (all from Dako, Glostrup, Denmark) for 1 h at RT. The HRP signal was detected using SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL) developed in the Biospectrum Imaging System (UVP, Upland, CA). After blotting with primary antibody, all blots were stripped and re-probed with rabbit monoclonal anti-α-tubulin (11H10) (Cell Signaling Technology, Inc.). Only one Tubulin control is illustrated at each figure.

MTT Assay

For viability assays, cells were seeded in 96-well plates (NUNC A/S, Roskilde, Denmark) [4,000 cells/well for U373MG; 3,000 cells/well for U87MG and U87MGvIII; 5,000 cells/well for LN229 and U87MG-EGFR; and 2,000 cells/well for HN5 and SKMG3, respectively, as found in previous, unpublished experiments to result in sub-confluent control cultures at day of harvest] and incubated ON in 10% FCS or SS medium (if incubated with 10% FCS primarily, SS medium was added the following day and the cells were incubated for one additional night). Cells were then treated with different regimens: A) cetuximab 0-100 µg/mL followed by EGF 1 nmol/L or media; B) cetuximab 10 µg/mL with or without LY294002 20 µmol/L in the presence of EGF 1 or 10 nmol/L or media; or C) cetuximab 10 µg/mL with U0126 10 µmol/L followed by EGF 1 or 10 nmol/L or media. The cells were incubated at 37ºC for 72 h before addition of 20 µL 5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, Broendby, Denmark), followed by 4 h incubation at 37ºC before addition of 100 µL solubilization buffer (10% SDS, 0.03 mol/L

HCL). Plates were read the following day at 570 nm on a Dynatech MR 5000 ELISA reader (Dynatech Laboratories, Chantilly, VA).

Flow Cytometric Analysis

SKMG3 and LN229 cells were seeded in 3 cm plates (NUNC A7S, Roskilde, Denmark) (as duplicates), 180,000 cells/plate and incubated ON in 10% FCS. The following day, 0.5% FCS was added to half of the plates. Subsequently, the plates received 0, 10 or 100 µg/mL cetuximab respectively and the cells were incubated at 37°C for 72 h to mimic the conditions for the MTT assays. For the flow cytometric analysis of cell cycle distribution, both adherent cells and cells in the culture media were harvested, centrifuged, resuspended in 70% ethanol and stored at -20°C. Cells were then washed in cold PBS after which 800 µl Vindelöv solution (3.5 µM Tris (pH 7.6), 10 nM NaCl, 50 µg/mL propidium iodide, 20µg/mL Rnase and 0.1% NP-40) was added to the cells and left incubated for at least 20 min on ice. DNA analyses were performed using a FACSCanto II flowcytometer (Becton Dickinson Biosciences, San Jose, CA) counting 20,000 cells for each sample. The fraction of sub G0/G1 (corresponding to the dead cells), G0/G1 and S/G2/M cells were determined using Becton Dickinson Biosciences, FACSDiva, version 6.1.2 Software (Becton Dickinson Biosciences).

Cycloheximide Experiments

SS cells (80% confluent) were pre-incubated with 100 µg/mL cycloheximide (Sigma-Aldrich, St Louis, MT) for 5 min and then incubated with or without cetuximab 10 µg/mL and/or with or without 10 nmol/L EGF for 0-21 h. Cells were harvested as described above.

Data Analyses

Differences between groups in the MTT viability-assays were analyzed using an unpaired Student's *t* test. Unless indicated, treated cells are compared to unstimulated cells (control), which represent 100% viability. Data are presented as the mean ± SD.

Results

Human Glioma Cell Lines Are Resistant to Cetuximab Treatment *in Vitro*

Previous studies have shown that cetuximab inhibits the viability of glioma cells *in vitro*[28,30]. To verify and extend these results we performed cell viability MTT assays on a panel of human glioma cell lines in the presence of cetuximab. We deliberately did choose to perform all experiments in this paper at SS conditions in order to minimize any uncontrolled effect from EGFR activating ligands like EGF present in serum. However, to exclude that the lack of response to cetuximab in the MTT viability assays was due to poor glioma cell growth in the absence of serum we also performed MMT viability assays in 10% FCS using the SKMG3 and LN229 glioma cell lines. These assays show the same response to cetuximab as observed in SS medium (unpublished results). We could only detect minor effects on glioma cell viability (Fig. 1A). We included the head and neck carcinoma cell line HN5 as a positive control since cetuximab has been shown to be effective in treatment of this type of cancer[32]. The HN5 cell line was extremely sensitive to cetuximab, with an IC_{50} of approximately 0.20 µg/mL (Fig. 1B). Similar results were observed with the A431 cell line (data not shown).

It has been suggested that the response of glioma cell lines to cetuximab is dependent on amplification of the *EGFR* gene, resulting in high EGFR levels[28]. We therefore investigated basal levels of EGFR expression in the cell lines. Previous work has shown that the human epidermoid carcinoma cell A431 and glioma SKMG3 cell lines are amplified for the *EGFR* gene and express 2.5×10^6 and 2.8×10^5 EGF receptors/cell, respectively, whereas the U87MG cell line, not amplified for EGFR, expresses 1×10^4 receptors/cell[31,33]. As shown in Figure 1C, the LN229, U87MG, U373MG, U118MG, and U87MGvIII glioma cell lines express low levels of EGFR, whereas the SKMG3, and A431 cell lines express EGFR at a level comparable with HN5. However, despite EGFR amplification the SKMG3 cell line showed little sensitivity to cetuximab treatment in the cell viability assays (Fig. 1D). GBM cells grown *in vitro* tend to lose their EGFR amplification and SKMG3 is the only established GBM cell line with reported EGFR amplification[34]. We therefore also included the U87MG-EGFR cell line, which is stably transfected with EGFR and express EGFR at similar levels as HN5 (Fig. 1C). However, U87MG-EGFR also failed to respond to cetuximab in MTT assays (Fig. 1D).

To exclude that the results from our viability assays were due to cell cycle arrest, we performed cell cycle analysis by flow cytometry of cells treated with 0, 10 or 100 µg/mL cetuximab in both 0.5% and 10% FCS. As illustrated in Figure 2, these results confirmed that

the lack of response was not due to cell cycle arrest (G0/G1 = P2) or cell death (Sub G0/G1 = P1).

Inhibition of EGFR Activation in Glioma Cell lines

To further investigate the lack of response to cetuximab on cell viability, we analyzed the effect of cetuximab on phosphorylation of EGFR. As shown in Figure 1E, cetuximab inhibited phosphorylation of EGFR (pEGFR: Tyr1086, Tyr1148, and Tyr1173) even at the lowest concentration of 0.1 µg/mL in the SKMG3 glioma cell line. Similar results were observed for the U87MG, U87MGvIII, U87MG-EGFR, and LN229 cell lines (data not shown). On the contrary, cetuximab did not inhibit EGFR phosphorylation in the HN5 cell line, (Fig. 1F). The observed basal level of EGFR phosphorylation most likely originates from spontaneous receptor dimerization and the limited amount of EGF ligand present in the cell environment despite serum-starved conditions.

No Effect of Cetuximab on Downstream Signaling

Despite the inhibition of pEGFR observed in SKMG3 (Fig. 1E), cetuximab failed to inhibit phosphorylation of the downstream mediators AKT (pAKT) or ERK (pERK) (Fig. 3A). Similar results were observed for the U87MG, U87MGvIII, U87MG-EGFR, and LN229 cell lines (data not shown). On the contrary, even though the phosphorylation of the HN5 cell line was not influenced notably be cetuximab, prominent inhibition of phosphorylated ERK was observed in addition to inhibition of phosphorylated AKT (Fig. 3B). To further investigate the downstream PI3K/AKT and Ras/MEK/ERK pathways and reveal any differences between the cell lines used, we performed immunoblotting showing the basal expression levels of total AKT and ERK as well as pAKT and pERK for the glioma cell lines, HN5 and A431. All of the glioma cell lines showed higher expression of total AKT and pAKT as compared to the HN5 and A431 cell lines. Total ERK expression was more pronounced in the glioma cell lines and pERK was especially up-regulated in LN229, U87MG, U87MG-EGFR, and U87MGvIII (Fig. 3C).

PTEN Mutation Is Not a Predictor for Response to Cetuximab

A critical regulator of PI3K/AKT activity is the tumor suppressor PTEN. To examine PTEN status in the cell lines used, we performed immunoblotting on unstimulated glioma cell lines, along with the HN5 and the A431 cell lines. As illustrated in Figure 3D, PTEN expression was below the level of detection in all of the glioma cells examined except for LN229, which

is the only glioma cell line in our study with reported wild-type PTEN[35]. However, as shown in Figure 3C and D the presence of PTEN did neither predict the occurrence of pAKT, since both the PTEN mutated glioma cell lines and the PTEN wild type glioma cell line LN229 expressed almost similar amount of pAKT, nor the response to cetuximab (Fig. 1A and data not shown). This is the opposite to the HN5 and A431 cell lines in which PTEN was readily detected and in which wild-type PTEN was confirmed by sequencing (unpublished observations). Importantly, HN5 and A431 were the only cell lines responding to cetuximab (Figure 1B and data not shown).

Inhibition of Downstream Signaling Leads to Decreased Cell Viability

To further investigate the importance of the PI3K/AKT and Ras/MEK/ERK pathways, we performed MTT viability assays using the PI3K inhibitor LY294002 or the MEK inhibitor U0126 with or without the presence of cetuximab. In advance, IC_{50} for both LY294002 and U0126 was determined for the cell lines used and the respective doses were used for the subsequent experiments (data not shown). As illustrated in Figure 4A and B, both LY294002 and U0126 induced a significant decrease in cell viability in the SKMG3 cell line. When treating the cells with a combination of either LY294002 or U0126 together with cetuximab, no further decrease in cell viability was observed (Fig. 4A,B). Similar results were observed with the LN229 cell line (data not shown). Both LY290042 and U0126 resulted in decreased cell viability in the HN5 cell line, at levels comparable to those observed with SKMG3 (Fig. 4C,D). However, as opposed to SKMG3, cetuximab treatment alone resulted in a significant (P <0.0005) decrease in cell viability in the HN5 cells, which is in line with our earlier results (Fig. 4C,D and Fig. 1B). Furthermore, combining LY290042 or U0126 with cetuximab resulted in a significantly enhanced effect on cell viability as compared to inhibition of the PI3K/AKT and Ras/MEK/ERK pathways alone (Fig. 4C,D). To verify that LY294009 and U0126 inhibited the downstream PI3K/AKT and Ras/MEK/ERK pathways, we performed immunoblotting, which showed that both LY294002 and U0126 completely inhibited pAKT or pERK, respectively, in the SKMG3, LN229, and HN5 cell lines (data not shown).
These results indicate that glioma cell lines are sensitive to inhibition of signaling pathways downstream of EGFR, but that targeting the EGFR itself with cetuximab is insufficient.

Cetuximab Is Ineffective in Inducing EGFR Degradation in Glioma Cells

A small decrease in the EGFR levels can be observed in the HN5 cell line in Figure 3B, indicating degradation of EGFR. Previous work has shown that cetuximab induces

internalization of EGFR to an extent comparable with EGF in the A431 cell line[36]. Additionally, it has been shown by other groups, that cetuximab induces internalization and degradation of mutated EGFR in non-small cell lung cancer (NSCLC) cell lines, whereas receptor down-regulation was not observed in the A431 cell line[37]. To our knowledge, it has not previously been shown whether cetuximab induces internalization with concomitant degradation of EGFR in glioma cell lines. Accordingly, we investigated whether EGFR was degraded upon treatment with cetuximab by performing a degradation assay using the translational inhibitor cycloheximide in the presence of cetuximab. As shown in Figure 5, cetuximab was unable to induce degradation of EGFR in the SKMG3 cell line, as the level of EGFR was unchanged over time. As a positive control, we treated the cells with EGF alone, as it is known to induce EGFR degradation. Indeed, EGF treatment induced EGFR degradation as early as after 3 h, which persisted until the end of the experiment. Notably, the effect of EGF was almost abolished when combined with cetuximab. Despite the observed effect of the EGF ligand, the high EGFR levels present in the SKMG3 cell line could disguise a possible small degrading effect of cetuximab. Accordingly, we repeated the experiment with the U87MG cell line, which confirmed our observations from the SKMG3 cell line, showing no signs of cetuximab-induced EGFR degradation (data not shown). These experiments indicate that cetuximab only induces limited, if any, degradation of EGFR in glioma cells.

EGF Abolishes the Effect of Cetuximab

In vivo, cetuximab would be competing with EGF for binding to EGFR. However, the affinity of cetuximab for EGFR is higher than that of the EGF ligand (average dissociation constant (K_d) for cetuximab ≈0.03 nmol/L versus EGF ≈2.7 nmol/L)[38]. Therefore, we performed the same MTT viability assays as above in the presence of EGF.

As seen from Figure 6A, the effect of cetuximab in the presence of EGF is similar to that observed without EGF in the glioma cell lines. However, the inhibitory effect of cetuximab in the HN5 cell line was almost abolished in the presence of EGF (Fig. 6B).

Cetuximab Induce Inhibition of EGFR Activation in the Presence of EGF

To further investigate the effects on EGFR when cetuximab was combined with EGF, we performed immunoblotting. As illustrated in Figure 6C and D, EGF induces activation of EGFR in the SKMG3 and HN5 cell lines as shown by phosphorylation of several tyrosine residues on the receptor. Similar observations were made for the LN229, U87MGvIII,

U87MG-EGFR and U87MG cell lines (data not shown). This phosphorylation event was completely abolished upon cetuximab addition.

No Inhibition of pAKT or pERK despite Inhibition of pEGFR

In the presence of EGF, pAKT and pERK remained unaffected by cetuximab in the glioma cell line, even though pEGFR was inhibited (Fig. 7A). As opposed to what was shown earlier (Fig. 3B), cetuximab treatment did not inhibit pAKT and pERK in the presence of EGF in the HN5 cell line, even though phosphorylation of EGFR was clearly affected (Fig. 7B).

In addition, we observed that EGF had an inhibitory effect on HN5 cell viability, and this inhibitory effect was diminished by the addition of cetuximab (Fig. 7E, F). On the contrary, EGF showed minimal inhibitory effect on the SKMG3 cell line (Fig. 7C, D), and EGF concentrations as high as 25 nmol/L demonstrated minimal influence (data not shown).

We performed the same experiment as above with the MEK inhibitor (U0126) and the PI3K inhibitor (LY294002) in the presence of EGF. A significant inhibitory effect was observed when treating the SKMG3 cell line with U0126 in the presence of EGF (Fig. 7C). However, addition of cetuximab diminished this inhibition at 10 nmol/L EGF. Similar results were observed with the LN229 cell line (data not shown). Addition of cetuximab to U0126 and EGF counteracted their growth inhibitory effect in the HN5 cell line (Fig. 7E). LY294002 induced a moderate inhibition of viability in the SKMG3 cell line (significant inhibition observed in the presence of EGF 1 nmol/L), which was unaffected by the addition of cetuximab (Fig. 7D). Similar results were observed for the LN229 cell line (data not shown). A significant inhibition of HN5 cell viability was observed when treated with LY294002 in the presence of 10 nmol/L EGF with or without the addition of cetuximab, although addition of cetuximab in the presence of LY294002 and 10 nmol/L EGF significantly diminished the inhibition achieved by LY294002 and EGF alone (Fig. 7F).

Discussion

In this study, we have shown that cetuximab does not exert an inhibitory effect on glioma cell viability, despite amplification and overexpression of EGFR in both PTEN-mutated and PTEN wild-type cell lines. Dose-response experiments showed inhibition of EGFR phosphorylation without affecting activity of the downstream signaling pathways PI3K/AKT and Ras/MEK/ERK. However, inhibition of both PI3K/AKT and Ras/MEK/ERK signaling inhibited glioma cell viability, indicating these pathways to be of importance in survival of these cells. Using the translational inhibitor cycloheximide, we further found that cetuximab fails to induce EGFR degradation in glioma cells and, to some extent, also blocks the receptor degradation induced by EGF.

The head and neck cancer cell line HN5 was used as a positive control in our experiments, as it has been shown to be sensitive to cetuximab[32]. Moreover, the HN5 cell line expresses a high level of EGFR and normal PTEN. We showed that cetuximab inhibits cell viability as well as EGFR activation and downstream signaling in the HN5 cell line and addition of EGF completely abolished the inhibitory effect of cetuximab. In our experiments no growth-promoting effect of EGF could be detected, but rather, EGF seemed to inhibit cell growth at concentrations above 1 nmol/L, an observation also made by others[39]. Importantly, we observed that cetuximab is able to reduce cell viability in the presence of low concentrations of EGF (below 1nmol/L), whereas at high concentrations, cetuximab rescues the cells from EGF-induced growth inhibition (unpublished observations). These observations imply that there is a fine balance between EGF and cetuximab, that in turn affects cellular outcome, and which could possibly be of importance in an *in vivo* situation.

In a previous work by Eller et al.[28], cetuximab was shown to have a substantial effect on EGFR-amplified glioma cell lines, whereas EGFR non-amplified glioma cell lines were only moderately sensitive. Accordingly, they concluded that EGFR amplification was important to obtain an effect with cetuximab treatment[28]. However, we were not able retrieve the same inhibitory effect of cetuximab in our viability assays. Moreover, when performing cell cycle analysis by flow cytometry we did not find an increase in apoptosis, despite increasing concentration of cetuximab, when compared to cells not receiving cetuximab. Accordingly, as opposed to Eller et al.[28], our results indicate that EGFR amplification and/or overexpression is not predictive for response to cetuximab. since the EGFR-amplified glioma cell line SKMG3 and the stably transfected U87MG-EGFR glioma cell line did not respond to cetuximab treatment, despite the observed inhibitory effect on activated EGFR (pEGFR).

Additionally, the inhibitory effect of cetuximab on pEGFR was not reflected by the downstream PI3K/AKT and Ras/MEK/ERK pathways, suggesting that the activity of these pathways is involved in maintaining glioma growth. These results are corroborated in a recently published study by Fukai et al.[40], who observed inhibition of phosphorylated EGFRvIII upon cetuximab treatment without any impact on pAKT in glioma cell lines stably transfected with EGFRvIII. As EGFRvIII is constitutively activated, they proposed that this activity was enough for maintaining the phosphorylation of AKT. However, our results show that cetuximab is unable to inhibit the activity of both the PI3K/AKT and Ras/MEK/ERK pathways downstream of EGFR, even in glioma cells without EGFRvIII and, thus, is more likely to be an effect of other factors influencing these pathways. Indeed, several articles have reported pathways other than EGFR, such as platelet-derived growth factor (PDGF) and insulin-like growth factor I (IGFR-I), to be of importance for glioma cell viability.[41,42] Furthermore, cetuximab has shown to be ineffective when treating colon cancer patients with K-*ras* mutations, however, K-*ras* mutations is not very common in GBM[43,44].

In the work by Martens et al[45] tumor growth inhibition were observed in glioblastoma spheroids implanted into the brains of nude mice, when treated with constant infusion of i.c. cetuximab. However, constant i.c. infusion is not convenient in a clinical setting for patients with high grade glioma. Moreover, in a phase II clinical trial in patients with recurrent primary GBM , cetuximab was used in combination with the vascular endothelial growth factor-binding antibody bevacizumab and the topoisomerase 1 inhibitor irinotecan[46]. Results from this phase II study indicate that cetuximab fails to increase tumor control significantly when compared to bevacizumab and irinotecan alone. Despite promising results published regarding the use of bevacizumab and irinotecan in recurrent high-grade glioma,[47,48] these results could be due to the fact that, in addition to alterations in EGFR expression, the tumors have mutations in PTEN inducing increased activation of the PI3K/AKT pathway[10]. This hypothesis is supported the work by Mellinghoff et al.[20], who showed that expression of wild-type PTEN is associated with response to the EGFR tyrosine kinase inhibitors erlotinib and gefitinib in glioma. However, as illustrated in our MTT viability assays, limited responses to cetuximab were observed in the PTEN-mutated glioma cell lines, whereas the PTEN-expressing cell line HN5 responded significantly. A similar minor response was observed with the LN229 glioma cell line despite expression of wild-type PTEN. This could be due to limited dependence on EGFR-induced signaling in the LN229 cell line. As shown by Fan et al.[49], transfection with EGFR to LN229 resulted in significant reduction in cell viability when treated with TKIs.

Our *in vitro* results indicate that EGFR alone might not be of pivotal importance in the growth of GBM. Accordingly, targeting of EGFR alone would be insufficient for essential inhibition of glioma cell growth, as other pathways most likely are important for the activity of PI3/AKT and cell viability. This was also demonstrated by Stommel et al.[50] who used three different TKIs (targeting EGFR, platelet-derived growth factor-α, and MET) to inhibit viability of the PTEN-mutated and EGFRvIII-containing U87MGvIII glioma cell line. These results illustrate that, by targeting multiple surface receptors involved in the PI3K/AKT and Ras/MEK/ERK pathways, a significant decrease in cell activity can be obtained. Moreover, they obtained complete inhibition of activated AKT (pAKT) when combining these three different TKIs, indicating that the growth-promoting effect of mutated PTEN can be overcome with multi-targeted treatment. Along with these data, our results lend to support their results, since we found, that inhibitors of both PI3K and MEK pathways profoundly influenced the viability of the tumor cells.

EGFR is internalized and efficiently degraded upon interaction and binding to the EGFR ligand EGF. This is opposed to the EGFR ligand transforming growth factor α, which fails to induce proper degradation of EGFR even though it induces receptor internalization, and consequently EGFR is recycled back to the cell surface, again ready for activation.[51] Other groups have shown that cetuximab is able to induce down-regulation of mutated EGFR in NSCLC at levels similar to EGF[37,52] and activated EGFRvIII in the U87MGvIII glioma cell line[53]. Our results show that cetuximab is unable to induce EGFR degradation and even inhibits degradation induced by EGF stimulation.

In conclusion, we suggest several potential resistance mechanisms against cetuximab treatment, including inactivation/mutation of PTEN and involvement of other signaling pathways, both of which would lead to persistent activation of intracellular signaling pathways such as PI3K/AKT and Ras/MEK/ERK, as well as insufficient receptor degradation. These results, in combination with the results from our phase II study, indicate that amplification and/or overexpression of EGFR is an unsatisfactory predictor for response to cetuximab.

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Figure Legends

Figure 1. A, B, D, MTT assays showing the effect of cetuximab on cell viability. Cells were treated with increasing concentrations of cetuximab for 72 h after which the number of viable cells was measured as described in Materials and Methods. **A,** Glioma cell lines U373MG, U87MGvIII, U87MG, LN229, and U118MG. **B,** The EGFR-overexpressing head and neck cancer cell line HN5. $P < 0.0005$ between cetuximab 0 µg/mL and 0.5 µg/mL. **D.** The EGFR-amplified glioma cell line SKMG3 and the U87MG-EGFR glioma cell line stably transfected with EGFR. Data are representative of at least three independently performed experiments in triplicate and presented as mean ± SD. **C.** Western blotting showing basal expression levels of EGFR in the glioma SKMG3, LN229, U87MG, U87MG-EGFR U373MG, U118MG, and U87MGvIII, the head and neck cancer HN5, and the epidermoid cancer A431 cell lines. Western blot results shown are representative for at least three independently performed experiments. Tubulin was used as a control for equal loading. **E.** Western blotting showing the effect of cetuximab on activated EGFR (pEGFR) of the SKMG3 **(E)** and HN5 **(F)** cell lines incubated with increasing concentrations of cetuximab. Western blot results shown are representative for at least three independently performed experiments. Tubulin was used as a control for equal loading.

Figure 2.
Cell cycle distribution as detected by flow cytometric analysis of SKMG3 (Fig 2A-F) and LN229 (Fig 2G-L) grown in 0.5% or 10% FCS and treated with 0, 10 or 100 µg/mL cetuximab for 72 h. Sub G0/G1=p1, G0/G1=P2 and S/G2/M=P3. Experiment was performed in duplicates and representative histograms are shown.

Figure 3. Western blotting showing the effect of cetuximab on downstream signaling pathways (pAKT and pERK) of EGFR in the SKMG3 **(A)** and HN5 **(B)** cell lines incubated with increasing concentrations of cetuximab. **C,** Western blotting showing the basal expression levels of phosphorylated AKT (pAKT) and phosphorylated ERK (pERK) in unstimulated glioma (SKMG3, LN229, U87MG, U87MG-EGFR, U373MG, U118MG, and U87MGvIII), head and neck cancer (HN5), and epidermoid carcinoma (A431) cell lines. **D,** PTEN protein expression in the glioma cell lines. HN5 and A431 were used as a positive control for PTEN expression. Western blot results shown are representative for at least three independently performed experiments. Tubulin was used as a control for equal loading.

Figure 4. MTT assay showing the effect of the PI3K inhibitor LY294002 (**A, C**) and the MEK inhibitor U0126 **(B, D)** on SKMG3 and HN5 cell viability. Data are representative of at least three independently performed experiments in triplicate and presented as mean ± SD. Unstimulated cells are used as control and represent 100% viability. ***, P <0.0005 vs control. *Abbreviations:* 0, unstimulated; C, cetuximab 10 µg/mL; LY, LY294002 20 µmol/L; U0, U0126 10 µmol/L.

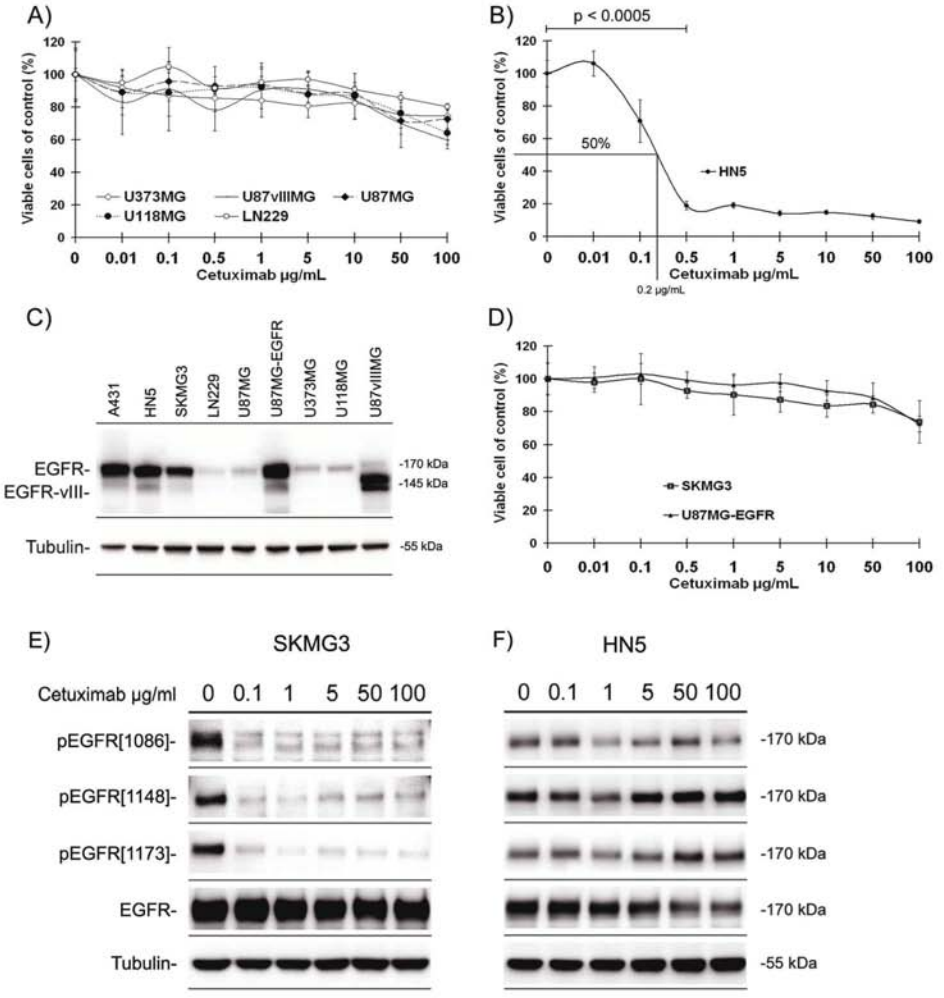
Figure 5. Western blotting showing the effect of cetuximab on EGFR degradation. The SKMG3 cell line was incubated (0-21 h) with the translation inhibitor cycloheximide in the presence of cetuximab and/or EGF 10 nmol/L. After incubation, cells were harvested and EGFR protein levels were subsequently analyzed. As illustrated, only limited degradation is induced by cetuximab. EGF is used as a positive control for EGFR degradation. The combination of cetuximab and EGF almost abolishes the effect of EGF alone. Tubulin was used as a control for equal loading and the experiment was repeated three times.

Figure 6. MTT assays showing cell viability of the **(A)** glioma cell lines SKMG3, LN229, U87MG, U87MGvIII, U373MG, U118M, and U87MG-EGFR with increasing concentration of cetuximab in the presence of EGF 1 nmol/L and **(B)** HN5 cell line treated with cetuximab in the presence of EGF. Data are representative of at least three independently performed experiments in triplicate and presented as mean ± SD. Western blotting showing effects on EGFR in the **(C)** SKMG3 and **(D)** HN5 cell lines incubated with increasing concentrations of cetuximab 0-100 µg/mL in the presence of EGF 1 nmol/L. Western blot results shown are representative for at least three independently performed experiments. Tubulin was used as a control for equal loading.

Figure 7. Western blotting showing effects on downstream signaling pathways (pAKT and pERK) of EGFR in the **(A)** SKMG3 and **(B)** HN5 cell lines incubated with increasing concentrations of cetuximab 0-100 µg/mL in the presence of EGF 1 nmol/L. Western blot results shown are representative for at least three independently performed experiments. Tubulin was used as a control for equal loading.
MTT assays showing the treatment of the **(C,D)** SKMG3 and **(E,F)** HN5 cell lines with the ERK inhibitor U0126 the PI3K inhibitor LY294002 in the presence of 1 or 10 nmol/L EGF with or without the addition of cetuximab 1 µg/mL. Data are representative of at least three

independently performed experiments in triplicate and presented as mean ± SD. **, P <0.01, ***, P <0.0005 vs control or between indicated treatments. *Abbreviations:* 0, EGF 1 or 10 nmol/L as indicated; C, cetuximab 10 µg/mL; U0, U0126 10 µmol/L; LY, LY294002 20 µmol/L; NS, non-significant.

Figure 1


A)
Viable cells of control (%)
U373MG
U87vIIIMG
U87MG
U118MG
LN229
Cetuximab µg/mL
B)
p < 0.0005
50%
HN5
0.2 µg/mL
C)
A431
HN5
SKMG3
LN229
U87MG
U87MG-EGFR
U373MG
U118MG
U87vIIIMG
EGFR-
EGFR-vIII-
-170 kDa
-145 kDa
Tubulin-
-55 kDa
D)
SKMG3
U87MG-EGFR
E)
SKMG3
Cetuximab µg/ml 0 0.1 1 5 50 100
pEGFR[1086]-
pEGFR[1148]-
pEGFR[1173]-
EGFR-
Tubulin-
F)
HN5
0 0.1 1 5 50 100
-170 kDa
-55 kDa

26

Figure 2

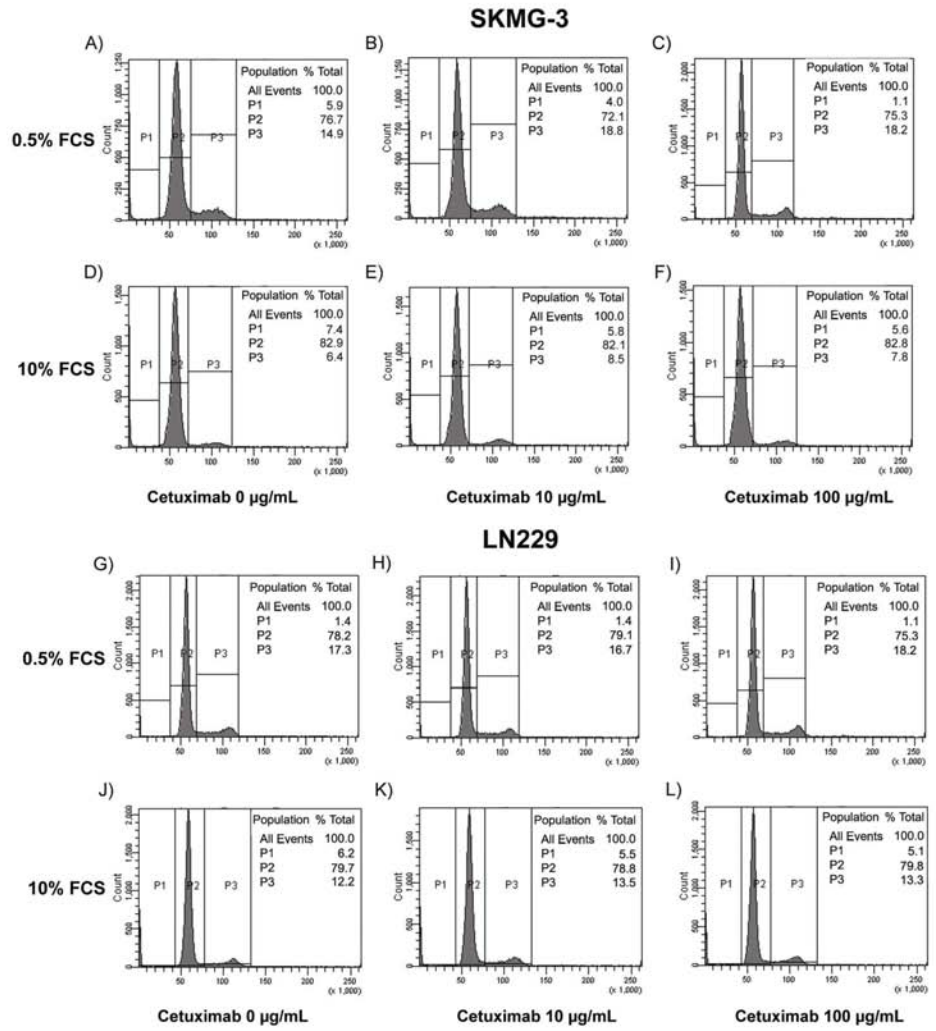
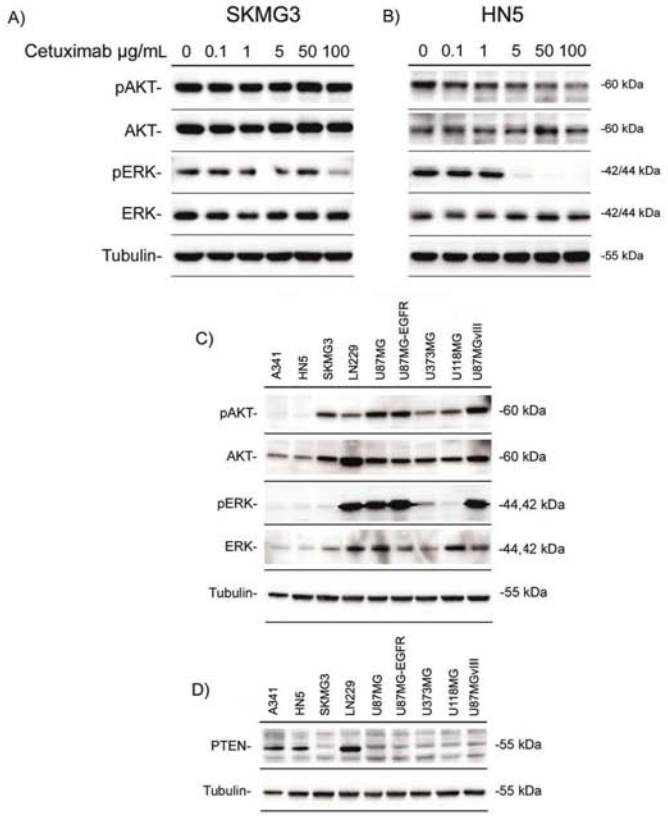
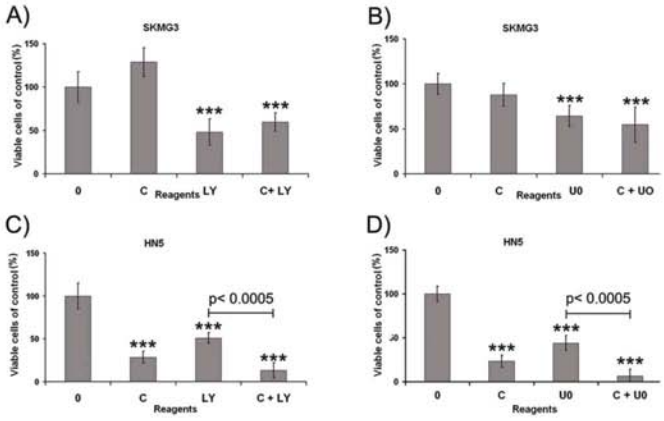

SKMG-3
A)
B)
C)
0.5% FCS
Population % Total
All Events 100.0
P1 5.9
P2 76.7
P3 14.9
Population % Total
All Events 100.0
P1 4.0
P2 72.1
P3 18.8
Population % Total
All Events 100.0
P1 1.1
P2 75.3
P3 18.2
D)
E)
F)
10% FCS
Population % Total
All Events 100.0
P1 7.4
P2 82.9
P3 6.4
Population % Total
All Events 100.0
P1 5.8
P2 82.1
P3 8.5
Population % Total
All Events 100.0
P1 5.6
P2 82.8
P3 7.8
Cetuximab 0 µg/mL
Cetuximab 10 µg/mL
Cetuximab 100 µg/mL
LN229
G)
H)
I)
0.5% FCS
Population % Total
All Events 100.0
P1 1.4
P2 78.2
P3 17.3
Population % Total
All Events 100.0
P1 1.4
P2 79.1
P3 16.7
Population % Total
All Events 100.0
P1 1.1
P2 75.3
P3 18.2
J)
K)
L)
10% FCS
Population % Total
All Events 100.0
P1 6.2
P2 79.7
P3 12.2
Population % Total
All Events 100.0
P1 5.5
P2 78.8
P3 13.5
Population % Total
All Events 100.0
P1 5.1
P2 79.8
P3 13.3
Cetuximab 0 µg/mL
Cetuximab 10 µg/mL
Cetuximab 100 µg/mL

Figure 3


A)
SKMG3
B)
HN5
Cetuximab µg/mL 0 0.1 1 5 50 100
0 0.1 1 5 50 100
pAKT-
AKT-
pERK-
ERK-
Tubulin-
-60 kDa
-60 kDa
-42/44 kDa
-42/44 kDa
-55 kDa
C)
A341
HN5
SKMG3
LN229
U87MG
U87MG-EGFR
U373MG
U118MG
U87MGvIII
pAKT-
AKT-
pERK-
ERK-
Tubulin-
-60 kDa
-60 kDa
-44,42 kDa
-44,42 kDa
-55 kDa
D)
A341
HN5
SKMG3
LN229
U87MG
U87MG-EGFR
U373MG
U118MG
U87MGvIII
PTEN-
Tubulin-
-55 kDa
-55 kDa

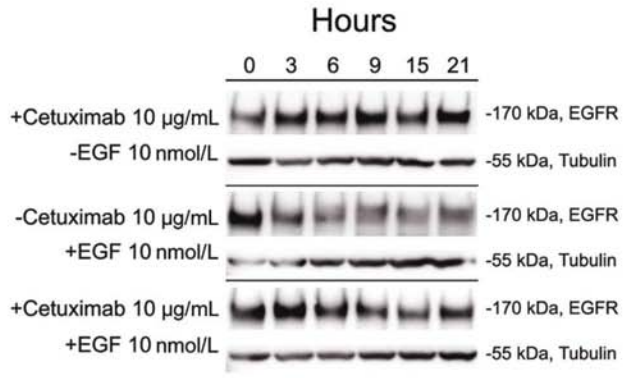
28

Figure 4


A)
SKMG3
Viable cells of control (%)
150
100
50
0
***
***
0
C
LY
C+ LY
Reagents
B)
SKMG3
Viable cells of control (%)
150
100
50
0
***
***
0
C
U0
C + UO
Reagents
C)
HN5
Viable cells of control (%)
150
100
50
0
p< 0.0005
***
***
***
0
C
LY
C + LY
Reagents
D)
HN5
Viable cells of control (%)
150
100
20
0
p< 0.0005
***
***
***
0
C
U0
C + U0
Reagents

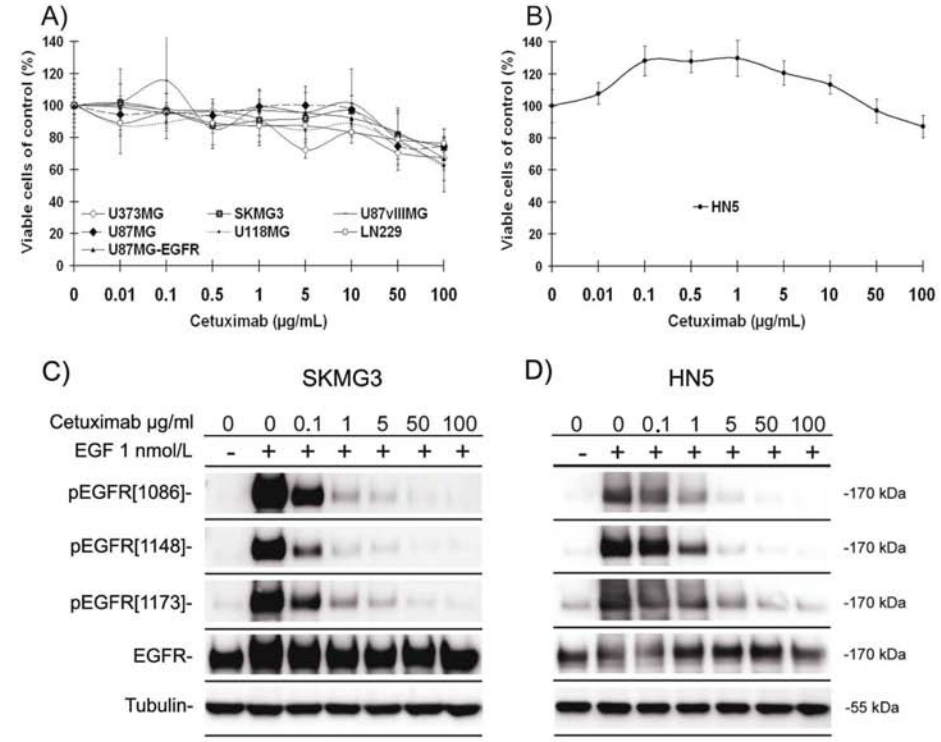
29

Figure 5


Hours
0 3 6 9 15 21
+Cetuximab 10 µg/mL
-EGF 10 nmol/L
-170 kDa, EGFR
-55 kDa, Tubulin
-Cetuximab 10 µg/mL
+EGF 10 nmol/L
-170 kDa, EGFR
-55 kDa, Tubulin
+Cetuximab 10 µg/mL
+EGF 10 nmol/L
-170 kDa, EGFR
-55 kDa, Tubulin

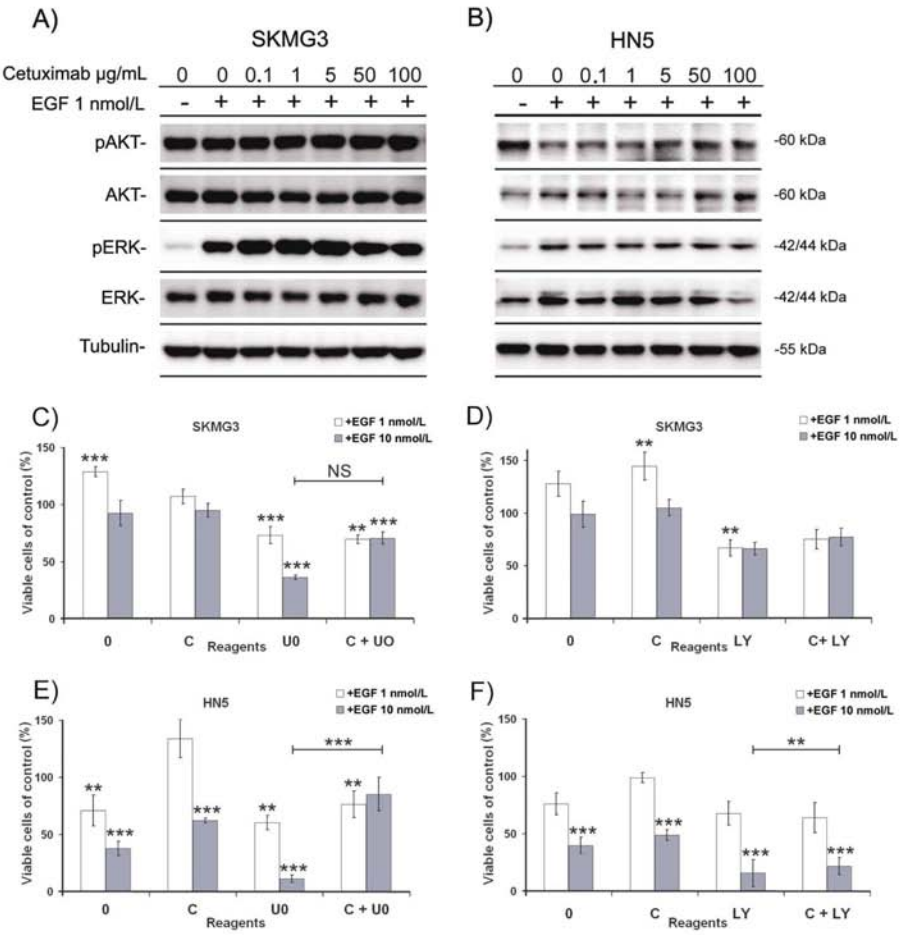
30

Figure 6


A)
Viable cells of control (%)
U373MG
SKMG3
U87vIIIMG
U87MG
U118MG
LN229
U87MG-EGFR
Cetuximab (µg/mL)
B)
HN5
Cetuximab (µg/mL)
C)
SKMG3
Cetuximab µg/ml 0 0 0.1 1 5 50 100
EGF 1 nmol/L - + + + + + +
pEGFR[1086]-
pEGFR[1148]-
pEGFR[1173]-
EGFR-
Tubulin-
D)
HN5
0 0 0.1 1 5 50 100
- + + + + + +
-170 kDa
-170 kDa
-170 kDa
-170 kDa
-55 kDa

31

Figure 7


A)
SKMG3
Cetuximab µg/mL 0 0 0.1 1 5 50 100
EGF 1 nmol/L - + + + + + +
pAKT-
AKT-
pERK-
ERK-
Tubulin-
B)
HN5
0 0 0.1 1 5 50 100
- + + + + + +
-60 kDa
-60 kDa
-42/44 kDa
-42/44 kDa
-55 kDa
C)
SKMG3
+EGF 1 nmol/L
+EGF 10 nmol/L
Viable cells of control (%)
NS
0 C U0 C + UO
Reagents
D)
SKMG3
+EGF 1 nmol/L
+EGF 10 nmol/L
Viable cells of control (%)
0 C LY C+ LY
Reagents
E)
HN5
+EGF 1 nmol/L
+EGF 10 nmol/L
Viable cells of control (%)
0 C U0 C + U0
Reagents
F)
HN5
+EGF 1 nmol/L
+EGF 10 nmol/L
Viable cells of control (%)
0 C LY C + LY
Reagents

32

Summary

EGFR is overexpresed and/or amplified in 35-45% of primary GBM tumors and has been correlated with a poor prognosis. EGFR is therefore thought to be of pivotal importance in the pathogenesis of GBM. Activation of EGFR initiates downstream signaling, resulting in among others, increased cell survival, proliferation, migration, invasion, and angiogenesis. Mutations of the tumor suppressor gene, PTEN, has been shown to mediate resistance to anti-EGFR treatment and accordingly, PTEN is considered of importance for response to EGFR inhibition.

Previous *in vitro* studies using cetuximab in glioma cell lines that overexpress and/or harbor amplified EGFR have shown a reduction in cell viability and clinical phase I and II trials have indicated a modest efficacy of EGFR TKIs in this tumor type.

In the present study further investigation is performed analyzing the effect of the EGFR inhibitor cetuximab on glioma cell lines having different EGFR status with respect to central intracellular signaling pathways downstream of EGFR that are important for cell survival and proliferation.

The study showed that cetuximab did not exert an inhibitory effect on glioma cell viability, despite amplification and overexpression of EGFR in both PTEN-mutated and PTEN wild-type glioma cell lines. Dose-response experiments showed inhibition of EGFR phosphorylation without affecting the activity of the downstream signaling pathways PI3K/Akt and Ras/Mek/Erk. However, inhibition of both PI3K/Akt and Ras/Mek/Erk signaling inhibited glioma cell viability, indicating that these pathways are of importance for survival of the glioma cells. Using the translational inhibitor cycloheximide, we further found that cetuximab failed to induce EGFR degradation in glioma cells and, to some extent, also blocked the EGF induced receptor degradation.

In contrast to the glioma cell lines, the EGFR overexpressing head and neck cancer cell line HN5 and the EGFR amplified ovarian squamous cancer cell line A431 were sensitive for EGFR inhibition by cetuximab. Taken together, these results indicate that EGFR alone might not be of pivotal importance for the growth of GBM. Accordingly, targeting of EGFR alone would be insufficient for inhibition of glioma cell growth as other pathways most likely are essential for the activity of PI3K/Akt- and Ras-Raf-Mek-Erk pathway and also cell viability.

Manuscript II

Bevacizumab plus irinotecan in the treatment of patients with progressive recurrent malignant brain tumours.

By

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ORIGINAL ARTICLE

Bevacizumab plus irinotecan in the treatment patients with progressive recurrent malignant brain tumours

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Abstract

Material and Methods. We retrospectively determined the efficacy and safety of a combination of bevacizumab and irinotecan in a consecutive series of 52 heavily pre-treated patients with recurrent high-grade brain tumours. Patients received bevacizumab (10 mg/kg) and irinotecan [340 mg/m^2 for those receiving enzyme-inducing antiepileptic drugs (EIAEDs) and 125 mg/m^2 for those not receiving EIAEDs] every 2 weeks. Fifty-two patients were included and 47 were evaluable for response. *Results.* Complete or partial response was observed in 25% of all cases (30% response in grade IV glioma and 15% in grade III glioma). Estimated median progression-free survival (PFS) for both grade IV and grade III glioma was 22 weeks. The 6-month PFS was 32% for all patients, 40% for grade IV glioma and 33% for grade III glioma. Estimated median overall survival was 30 weeks for all patients, 28 weeks for grade IV glioma and 32 weeks for grade III glioma. Four patients discontinued treatment because of unmanageable toxicity: cerebral haemorrhage, cardiac arrhythmia, intestinal perforation and diarrhoea, the latter resulting in death. *Discussion.* We conclude that the combination of bevacizumab and irinotecan shows acceptable safety and is a clinically relevant choice of therapy in heavily pre-treated patients with recurrent high-grade brain tumours.

Key Words: *Bevacizumab, Irinotecan, Glioma, recurrent, Treatment, Clinical trial*

Introduction

Treatment of patients with primary brain tumours is a multidisciplinary effort, consisting of maximal cyto-reductive surgery followed by radiotherapy and in some cases chemotherapy [1–3]. Patients with grade IV glioma can be treated with concomitant and adjuvant temozolomide, a regimen that has yielded a significant increase in survival [4]. Nonetheless, median survival remains <15 months and practically all patients eventually die from their disease [4]. The same holds true for grade III glioma patients, for which median survival is approximately 24 months [1]. At first recurrence, prognosis is even poorer with a median survival of 3–9 months, while at second recurrence, life expectancy drops to a few weeks for more than 90% of the patients [5,6]. These facts reflect the relatively poor efficacy of available chemotherapy and the scarcity of objective durable responses. Novel effective treatment modalities are therefore needed.

Malignant gliomas are highly vascular and often express abundant amounts of vascular endothelial growth factor (VEGF) [7]. VEGF stimulates/promotes tumour angiogenesis [8–11] but might also stimulate brain tumour stem cells [12] and decrease bioavailability of chemotherapeutic drugs [8–11]. Consequently, inhibition of VEGF activity may reduce angiogenesis, inhibit stem-cell proliferation, and increase the delivery and effect of cytotoxic chemotherapy [8,11]. Bevacizumab, a humanized monoclonal antibody that binds to and inhibits the activity of VEGF, has demonstrated synergy with

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cytotoxic chemotherapy in the treatment of various solid tumours, e.g., colorectal, lung, breast carcinoma [13–15]. Recently, promising results have been published showing durable responses using a combination of bevacizumab and irinotecan in patients with recurrent high-grade glioma [16–18].

Irinotecan, a topoisomerase I inhibitor, demonstrates excellent CNS penetration but has shown only modest efficacy in patients with recurrent primary brain tumours [3,19]. However, it is the only cytotoxic chemotherapeutic agent that has been administered in combination with bevacizumab to a substantial number of brain tumour patients. The toxicity of this combination has been shown to be manageable [16–18].

We therefore decided to conduct a clinical trial at our Danish centre, administering bevacizumab plus irinotecan to a consecutive series of heavily pretreated brain tumour patients with progression after standard primary and secondary treatment.

Patients and methods

The protocol was approved by The Danish National Board of Health and conducted in accordance with the Declaration of Helsinki. Patients provided signed, informed consent prior to enrolment. Patients had to be >18 years of age and have disease progression after standard treatment of histologically verified primary brain tumour according to WHO classification [20]. Histological diagnosis was based on the most recent surgical biopsy obtained before entering the study. Patients were required to have received at least one non-surgical treatment modality after recurrence. In addition, no other standard treatment was available.

Conditions for eligibility were as follows: measurable progressive disease by contrast-enhanced magnetic resonance imaging (MRI); WHO performance status 0–2; and a minimum of 6 weeks from prior surgery and 4 weeks from the prior chemotherapy. None of the patients received radiotherapy within 3 months of study treatment. Other inclusion criteria included: neutrophils $>3 \times 10^9$/L, haemoglobin >6.2 mmol/L, platelets $>125 \times 10^9$/L, serum ASAT or ALAT $<3 \times$ upper limit of normal (ULN), bilirubin $<1.5 \times$ ULN, and creatinine clearance >45 ml /min. Exclusion criteria included: a history of bleeding diathesis and coagulopathy; significant peripheral vascular disease; cardiac disease including acute myocardial infarction within 6 months; unstable angina pectoris; congestive heart failure; BP >150 /100 mmHg; proteinuria ≥grade 2; immunosuppressive co-medication other than corticosteroids; and any other active malignancy or condition preventing adequate follow-up or data collection.

Treatment

Bevacizumab and irinotecan were administered every 2 weeks and each cycle of treatment was defined as two treatment administrations. Bevacizumab 10 mg/kg was administered by slow IV infusion: over 90, 60 and 30 minutes for the first, second and subsequent doses, respectively. IV irinotecan [340 mg/m^2 for patients receiving enzyme-inducing antiepileptic drugs (EIAEDs) and 125 mg/m^2 for patients not receiving EIAEDs] was administered 60 minutes prior to bevacizumab. Atropine 1 mg SC was given 10 minutes prior to irinotecan to prevent cholinergic syndrome. For patients on corticosteroids, the dose had to be stable for >1 week before the first cycle of treatment. Before starting any treatment, haematological recovery was required as witnessed by ANC $>1.5 \times 10^9$/L and platelets $>100 \times 10^9$/L.

Dose modification was not allowed for bevacizumab. In case of unmanageable, bevacizumab-related side effects (grade 3 or 4 hypertension, venous thrombosis, haemorrhage, arterial thromboembolic event, grade 3 and 4 proteinuria and GI perforation), the patient discontinued study treatment. In case of grade 4 neutropenia or febrile neutropenia, the irinotecan dose was reduced to 80% of the starting dose. In case of grade 4 neutropenia after dose reduction, irinotecan was reduced to 60% of the starting dose. In the case of grade 3 or higher non-haematological toxicity, irinotecan dose was reduced to 80% of the initial dose in the following treatment cycles. Treatment was discontinued in the case of tumour progression, unmanageable grade 4 non-haematological toxicity or at the request of the patient. The physician could terminate study treatment if continuation was deemed unsafe.

Patient evaluation

A full medical history was determined before initiation of study treatment and all patients underwent baseline physical and neurological examination, performance status examination, routine laboratory tests (including blood chemistry and urinalysis) and MRI scans. Contrast and non-contrast MRI was repeated every 8 weeks during treatment, and clinical and laboratory tests every 2 weeks. Toxicity was evaluated according to NCI-CTCAE, version 3.0, criteria [21].

Treatment response evaluation

Response to therapy was evaluated using the MacDonald criteria [22], which comprises measurements of contrast-enhancing tumour size and recording the largest cross-sectional area of the

tumour, neurological status and steroid dose. A complete response (CR) was defined as complete disappearance of measurable disease by MRI, partial response (PR) as >50% reduction of MRI contrast-enhancing tumour, and progressive disease (PD) as a >25% increase in area of contrast enhancement. Patients, by definition, had stable disease (SD) if the criteria for CR, PR or PD were not met and no clinical progression was observed. Furthermore, we sub-defined a minimal response (MR) as a 25 to 49% reduction of MRI contrast enhancement. Patients with CR or PR also had to be taking the same or decreased steroid dose and have stable or improved neurological findings.

Results

Patient characteristics

The baseline demographic and clinical characteristics of the patients enrolled in the study are summarized in Table I. There were 34 males and 18 females. Twenty-seven patients had grade IV glioma, 13 had grade III glioma and five had grade III oligodendrogliomas. In addition, one patient had grade III ependymoma, one had grade III haemangiopericytoma; one had a malignant prolactinoma; three suffered from brain-stem gliomas and one had grade IV medulloblastoma.

All patients had received ≥2 treatment interventions before enrolment and most patients were heavily pre-treated with an average of two surgical interventions (range 1–4), usually at least two chemotherapy regimens (range 1–3) and radiotherapy. All patients had received standard primary treatment including surgery and radiotherapy with or without chemotherapy according to international recommendations [2,3]. At recurrence, most patients had received temozolomide as first-line treatment and some had received additional treatment with PCV (procarbazine, lomustine and vincristine), cisplatin, lomustine plus etoposide, or imatinib plus hydroxycarbamide depending on the local practice of the referring institutions.

Median age at enrolment was 46 (range 26–67) years. Time from primary diagnosis to enrolment ranged from 5 to 183 (median 37) months. This large range is primarily due to two factors: firstly, a number of patients who initially harboured a low-grade glioma presented malignant transformation when they were referred for the present treatment; and secondly, a number of patients had responded for a long time to previous therapy. As seen in Table I, patients with grade IV tumours had a significantly shorter median disease-free interval from primary diagnosis compared with patients who had grade III tumours. Eighteen of the 52 patients used EIAEDs. This group was statistically comparable to those not using EIAEDs and with respect to treatment response (data not shown). The reasons for patient discontinuation from the study and the duration of patients remaining on treatment are summarized in Table II.

Table I. Baseline patient demographic and clinical characteristics (N = 52)

Median age, years (range)	46 (26–67)
Gender	
Male	34
Female	18
WHO performance status	
0	20
1	21
2	11
Histological diagnosis	
Grade IV glioma (glioblastoma multiforme)	27
Grade III anaplastic astrocytoma	13
Grade III anaplastic oligodendroglioma	5
Grade III ependymoma	1
Grade III haemangiopericytoma	1
Malignant prolactinoma	1
Grade brain-stem glioma	3
Grade IV medulloblastoma	1
Concomitant medication	
EIAED	18
Non-EAIAD	15
No AEDs	19
Median time from primary diagnosis to enrolment, months1 (range)	
All patients	37 (5–183)
Grade IV glioma	16 (4–118)
Grade III glioma	47 (9–137)
No. of interventions before enrolment	
2	2
3	17
4	17
5	10
6	5
7	1
No. of responders (CR+PR) to previous chemotherapy	
All patients	8
Grade IV glioma	0
Grade III glioma	6
Others	2

Abbreviations: AED, antiepileptic drug; CR, complete response; EIAED, enzyme-inducing antiepileptic drug; non-EIAED, non-enzyme-inducing antiepileptic drug; PR, partial response.

Response

First response was evaluated after a minimum of 2 cycles of treatment and the best response was noted. Most patients had their best response after 2 to 4 cycles. For the intent-to-treat (ITT) population, response (CR+PR) was found in 13 of 52 patients (25%; 95% CI: 15–40%). Five patients

Table II. Reasons for patient discontinuation from the study and duration of study treatment

Reason for treatment discontinuation	Patients (no.)	Duration on study medication (months)
Disease progression		
Glioblastoma multiforme	12	2–12
Anaplastic Astrocytoma	7	2–14
Anaplastic oligodendroglioma	3	2–10
Other	2	2–6
Adverse events		
Grade 3 CNS haemorrhage	1	3
Grade 5 diarrhoea	1	1
Grade 3 intestinal perforation	1	4.5
Grade 3 cardiac arrhythmia	1	1
Toxicity and consent withdrawal	3	1–3

exhibited CR: four with grade IV glioma and one with grade III anaplastic oligodendroglioma.

For patients with grade IV tumours, response (CR+PR) was observed in 30% (95% CI: 14–57%) of those in the ITT population. Twenty-three of the 27 patients with grade IV tumours could be evaluated: results were 4 CR, 4 PR, 12 SD and 3 PD. Among the SD patients, six experienced MR, with shrinkage of initial contrast-enhancing tumour varying from 30 to 48%. Four grade IV patients could not be evaluated: one patient had clinical PD before evaluation; two patients did not want to continue participation in the study; and one patient died after the first treatment cycle due to unmanageable diarrhoea.

For the patients with grade III tumours, response was seen in three of 20 patients (15%; CI: 6–44%). One patient with grade III astrocytoma was not evaluable for response, because of discontinuation of treatment after 1 cycle of treatment. Among the patients with grade III astrocytoma, there were 2 PR, 9 SD and 1 PD. None of the grade III astrocytoma patients with SD could be sub-classified as MR. There was 1 CR and 4 SD among the five patients with grade III oligodendroglioma. There was 1 PR and 2 SD among the three patients with brain-stem glioma. The patient with prolactinoma experienced a PR. Each patient with grade III ependymoma and grade III haemangiopericytoma showed SD and the patient with grade IV medulloblastoma showed PD.

For the evaluable population, response (CR+PR) was observed in 13 of 47 patients (28%; 95% CI: 16–43%), while 20 of 47 patients had a greater than 25% radiographic response (43%; 95% CI: 29–58%). No correlation could be found between response to study treatment and response to prior radiotherapy or chemotherapy, disease duration before enrolment in the present study, performance status, or the use of steroids or antiepileptic drugs.

Table III summarizes change in steroid dose, WHO performance status and clinical symptoms according to best radiographic response in evaluable patients (N = 47). An improvement in or maintenance of steroid dose, performance status or clinical symptoms was almost invariable (80–100%) in patients with a clinical response (CR+PR), frequent in those with SD (59–96%) and uncommon in those with PD (0–60%).

Survival

Thirty-seven patients were followed for ≥6 months. In this population, progression-free survival at 6 months (6-month PFS) was 32.4% (95% CI: 18–49%). Corresponding 6–month PFS was 40% (95% CI: 16–67%) in 15 patients with grade IV glioma and 33.2% (95% CI: 18–67%) in 17 patients with grade III anaplastic glioma. Kaplan-Meier estimates showed median PFS as 22 weeks (95% CI: 16–28 weeks) in patients with grade IV glioma and 22 weeks (95% CI: 18–25 weeks) in patients grade III anaplastic glioma.

Median overall survival (OS) as estimated by Kaplan-Meier analysis was 30 weeks (95% CI: 24–37 weeks) in the total population. One- and 2-year survival was estimated to be 21% and 18%, respectively. Grade IV glioma patients had a median OS of 28 weeks (95% CI: 13–43 weeks) with 1- and 2 -year survival of 24% and 18%, respectively. Patients who responded (CR+PR) to study therapy had a median OS of 69 weeks (95% CI: 41–99 weeks) compared to 22 weeks (95% CI: 13–32 weeks) in patients with SD or PD. This difference is statistically significant ($p < 0.0001$, log-rank test). Grade III anaplastic glioma patients had an estimated OS of 32 weeks (95% CI: 25–39 weeks) and 1-year survival of 45%. There was no significant difference for OS between responders and non-responders ($p = 0.409$, log-rank test).

Safety

Study treatment was stopped because of toxicity in four patients: one each from grade 5 diarrhoea, grade 3 cerebral haemorrhage, grade 3 cardiac arrhythmia (atrial fibrillation) and grade 3 intestinal perforation. The GI perforation resulted from rupture of an anastomosis originating from a bowel resection performed 20 years prior to study treatment. Other grade 3 adverse events included:

Table III. Change in steroid dose, WHO performance status and clinical symptoms according to best radiological response in evaluable patients (N = 47).

	No. of patients (% improved or unchanged)					
	CR+PR (n = 13)		SD (n = 29)		PD (n = 5)	
Steroid dose						
Decreased	6	} 100%	14	} 96%	1	} 60%
Unchanged	4		8		2	
Increased	0		1		2	
NR*	3		6		0	
WHO performance status						
Improved	2	} 80%	0	} 59%	0	} 33%
Unchanged	6		13		1	
Worsened	2		9		2	
NR*	3		7		2	
Clinical symptoms†						
Improved	8	} 91%	13	} 81%	0	} 0%
Unchanged	2		8		0	
Worsened	1		5		5	
NR*	2		3		0	

*Data not recorded (not included in percent determination).
†Includes neurological symptoms, fatigue and/or mobility.
Abbreviations: CR, complete response; NR, not recorded; PD, progressive disease; PR, partial response; SD, stable disease.

superficial venous thrombosis (n = 1), hypertension (n = 3), neutropenia (n = 1), infection (n = 2) and proteinuria (n = 1). Most patients experienced grade 1 or 2 adverse events, which primarily consisted of neutropenia (21%), infections (14%), nausea and vomiting (33%), diarrhoea (34%), hypertension (11%), fatigue (56%), epistaxis (21%), proteinuria (56%) and increased transaminase values (28%). No difference in adverse events was observed between patients receiving EIAEDs and those not receiving EIAEDs (data not shown).

Discussion

This investigation represents a retrospective analysis of all patients with recurrent malignant brain tumours referred to our department for last-option treatment. We found that combination of bevacizumab and irinotecan induces a significant number of clinically relevant, durable responses (25% response rate). In addition, these responses translated into significant prolongation of survival. The response rate, with some complete responses (n = 5), and the improvement in 6-month PFS and OS compared with historical controls [5,6], was particularly encouraging. In most investigations using chemotherapy alone, response rates in recurrent high-grade glioma were approximately 5 to 20% and in heavily pre-treated patients, such as ours, 6-month PFS could be expected to be <10% [5]. We were particularly encouraged by the high CR rate among grade IV glioma patients (4 of 27; 15%) treated with bevacizumab+irinotecan in our series and the duration of response in these patients, as durable complete responses are extremely rare in this setting with previous treatment modalities. For example, Wong et al. [5] identified only one CR among 375 recurrent glioma patients (225 grade IV and 150 grade III) in pooled data from eight consecutive phase II clinical trials of chemotherapy.

Compared to other populations of patients with recurrent high-grade glioma treated with the combination of bevacizumab and irinotecan, our patient population is similar to that previously described by Stark-Vance [23], who found a response rate of 43% among 21 patients. Our data are comparable to the results for bevacizumab/irinotecan treatment in high-grade glioma patients [18] published by Norden et al. [18] and Guiu et al. [24], who showed response rates of 34% and 36%, respectively, with bevacizumab+irinotecan. However, our results were not comparable with those published by Vredenburgh et al. [16,17], who found response rates of approximately 60% for bevacizumab+irinotecan- our patient population was more heavily pre-treated. However, results of 6-month PFS and OS for grade IV gliomas are comparable to those published by Vredenburgh et al. [16,17]. It is possible that differences in MRI imaging evaluation or patient populations might explain these differences between studies. Our patients with grade III glioma did not show the response rates and the survival benefit reported by Vredenburgh et al. [16]. The reason for this is uncertain but may simply be related to non-comparable patient populations. However, it might also reflect a possible biological impact of

the significantly lower VEGF expression found in grade III as compared to grade IV tumours [25–28], which would make grade III tumours more likely to be less responsive to anti-VEGF therapy.

It has been argued that response rates to bevacizumab treatment using contrast-enhancement MRI scans might be overestimated [10,29]. Tumour blood vessels are leaky and bevacizumab regulates vascular permeability, probably by a transient normalization of tumour blood vessels. Consequently, targeting VEGF directly through bevacizumab may decrease leakage of the vessels resulting in decreased enhancement, although this does not necessarily reflect tumour cell death [8]. However, the responses that we and others have observed [16–18] resulted in clinical improvement and significantly prolonged survival compared with best supportive care [3]. This indicates that decreased enhancement was due to clonogenic tumour cell death, rather than a steroid-like effect. This conclusion is furthermore supported by a recent study by Chen et al. [30], who showed that a reduction in metabolic activity, as measured by ^{18}F-fluorothymidine PET scanning, correlated with response and survival in grade III and IV gliomas treated with bevacizumab plus irinotecan. Furthermore, we found that radiographic response in our patients was correlated with factors related to quality of life such clinical/neurological symptoms, WHO performance status and steroid dose.

Published response rates of irinotecan alone in patients with recurrent high-grade glioma are up to 15% [19] and studies using other anti-angiogenic agents such as thalidomide or vatalanib alone showed response rates of 6 to 9% [31–33]. Furthermore, these studies showed shorter median PFS and OS than in our study. In high-grade glioma patients, treatment with thalidomide plus temozolomide [34–36] or carmustine [37] produced response rates of 7 to 24% at first recurrence. However the combination of thalidomide and carmustine yielded a median PFS of just 14 weeks [37], which is less than we observed. It should be emphasized, however, that all the cited studies are small and other differences between patient populations might possibly explain some of the similarities and differences in efficacy. When responses did occur in these studies [19,31–37], they were almost invariably partial responses, with only the rare, isolated complete response

It appears that the combination of bevacizumab and irinotecan has at least an additive effect. While the reasons for this are still under investigation [9–11], several pathophysiological and non-pathophysiological factors have been proposed. The combination of the two drugs may increase apoptosis and decrease the number of tumour stem cells, decrease interstitial tissue pressure and normalize the tumour vasculature. The latter would increase irinotecan penetration into the tumour and all these mechanisms would facilitate increased tumour cell death [8,9,12].

We found that the safety of the combination of bevacizumab and irinotecan was acceptable. Adverse events were manageable in most cases. There was one treatment-related death (diarrhoea), a well known side effect of irinotecan [38]. There was a suggestion of increased risk of thromboembolic effects including one case each of cerebral haemorrhage, intestinal perforation and superficial venous thrombosis, which may have been related to and have been associated with bevacizumab [39]. With respect to cerebral haemorrhage, however, it should be noted that high-grade gliomas have a particularly high propensity to present with haemorrhage, up to 29% of patients with mixed oligodendroglioma/astrocytoma in a retrospective clinico-pathological review of consecutive brain tumour cases [40].

In conclusion, heavily pre-treated patients with recurrent high-grade gliomas show clinically relevant durable responses, with a substantial number of complete responses. We recommend that bevacizumab and irinotecan be offered to patients with recurrent grade IV tumours and be considered in future protocols for treatment of grade IV gliomas, including the first-line setting.

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Summary

HGG is a devastating disease, and despite multidisciplinary effort with tumor reductive surgery, radiotherapy and chemotherapy, tumor recurrence is almost always inevitable. Consequently, there is an urgent need for an efficient alternative to the putative gold standards of chemotherapy (i.e. temozolomide and nitrosoureas) at recurrence. HGGs are known to be vastly vasculized tumors and pronounced tumor vascularity is significantly correlated with poor survival.[121] Angiogenesis is among other factors induced by VEGF. Moreover, HGG tumors are known to be hypoxic and hypoxia leads to stabilization of the hypoxia inducible factors, HIF-1α and HIF-2α, that subsequently induce transcription of VEGF. Tumor vessels are immature, malformed and leaky resulting in edema and increased intratumoral pressure. This is further promoting the hypoxic tumor milieu and induction of pro-angiogenic factors like VEGF thus creating a positive paracrine loop, maintaining angiogenesis and conditions necessary for sustained tumor growth. Moreover, the increased intratumoral pressure has been suspected to decrease tumor drug delivery. Bevacizumab is a humanized monoclonal antibody that binds to and inhibits the pro-angiogenic activity of VEGF. Irinotecan is a topoisomerase I inhibitor that readily passes the blood-brain-barrier, but only demonstrates limited efficacy against HGG. Promising results using the combination of bevacizumab and irinotecan (BI) in HGG were reported initially from Vredenburgh *et al.*[200] We subsequently used the BI regimen in a group of recurrent HGG patients at the Copenhagen University Hospital, Denmark. Retrospective analysis showed a 25% response rate (complete and partial responses), and an improvement of progression-free and overall survival when compared with historic results. The data were equivalent with the results from most other groups.[201,204] However, our results were not comparable with the results published by Vredenburgh *et al*. who found response rates of approximately 60% although the progression-free survival data were similar with the study presented.[200] It was concluded that the combination of bevacizumab and irinotecan was a feasible regimen, with acceptable side effects inducing a substantial number of clinical and radiological responses and an improved survival in a subset of HGG patients.

Manuscript III

Cetuximab, Bevacizumab plus Irinotecan for Patients With Primary Glioblastoma and progression after Radiation and Temozolomide: A Phase II Trial

By

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Cetuximab, bevacizumab, and irinotecan for patients with primary glioblastoma and progression after radiation therapy and temozolomide: a phase II trial

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The aim of this clinical trial was to investigate safety and efficacy when combining cetuximab with bevacizumab and irinotecan in patients with recurrent primary glioblastoma multiforme (GBM). Patients were included with recurrent primary GBM and progression within 6 months of ending standard treatment (radiotherapy and temozolomide). Bevacizumab and irinotecan were administered IV every 2 weeks. The first 10 patients received bevacizumab 5 mg/kg, but this was increased to 10 mg/kg after interim safety analysis. Irinotecan dose was based on whether patients were taking enzyme-inducing antiepileptic drugs or not: 340 and 125 mg/m^2, respectively. Cetuximab 400 mg/m^2 as loading dose followed by 250 mg/m^2 weekly was administered IV. Forty-three patients were enrolled in the trial, of which 32 were available for response. Radiographic responses were noted in 34%, of which 2 patients had complete responses and 9 patients had partial responses. The 6-month progression-free survival probability was 30% and median overall survival was 29 weeks (95% CI: 23–37 weeks). One patient had lacunar infarction, 1 patient had multiple pulmonary embolisms, and 3 patients had grade 3 skin toxicity, for which 1 patient needed plastic surgery. One patient was excluded due to suspicion of interstitial lung disease. Three patients had deep-vein thrombosis; all continued on study after adequate treatment. Cetuximab in combination with bevacizumab and irinotecan in recurrent GBM is well tolerated except for skin toxicity, with an encouraging response rate. However, the efficacy data do not seem to be superior compared with results with bevacizumab and irinotecan alone.

Keywords: bevacizumab, cetuximab, EGFR, glioblastoma multiforme, irinotecan

Glioblastoma multiforme (GBM) continues to be a devastating disease with a median survival for newly diagnosed GBM of only 15 months.[1] The prognosis for recurrent GBM is even worse with a median survival of 3–9 months when using traditional chemotherapeutic agents.[2,3] However, several recent publications have demonstrated a significant improvement in the treatment of recurrent GBM using the angiogenesis inhibitor bevacizumab plus the topoisomerase 1 inhibitor irinotecan.[4–6]

Primary GBM arises de novo, whereas secondary GBM develops from pre-existing low-grade astrocytomas.[7] Primary and secondary GBM are clinically indistinguishable. However, genotypically, there are differences, which could be used in the search for improved treatment.[8,9] One target could be the epidermal growth factor receptor (EGFR), which is known to

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be overexpressed and/or amplified in 35%–45% of primary GBM tumors and has been shown to correlate with poor prognosis.[9–12] The EGFR tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib, have been used in phase I and II trials for treatment of recurrent GBM, either alone or in combination with conventional chemotherapy.[13–15] Results from these studies are not uniform, although several indicate modest efficacy for TKIs in GBM.[16,17] Cetuximab is a chimeric monoclonal antibody that binds to EGFR with high affinity, competes for ligand binding, and down-regulates cell-surface receptor expression.[18,19] In vitro and in vivo studies using glioma cells that overexpress and/or amplify EGFR have shown reduction in cell viability with cetuximab.[20–22]

GBM is one of the most highly vascularized tumors with extremely elevated levels of proangiogenic factors, including vascular endothelial growth factor (VEGF) that induces tumor angiogenesis.[23] VEGF promotes endothelial cell proliferation and migration in human gliomas and has been associated with poor prognosis in high-grade glioma.[24] Bevacizumab is a humanized immunoglobulin G_1 that binds to and inhibits the activity of the human VEGF ligand (VEGF-A) and has been used in combination with cytotoxic chemotherapy in colorectal, lung, and breast cancers.[25] At the initiation of the study, there were several reports of promising effect when combining bevacizumab with irinotecan in high-grade glioma, and the results from these clinical trials have subsequently been published, confirming these observations.[4,5,26] Irinotecan is able to cross the blood-brain barrier (BBB) but demonstrates only limited effect against high-grade glioma as a single-agent therapy, with response rates between 0% and 15%.[27–30]

Phase I studies have shown that erlotinib and gefitinib cannot be combined with irinotecan[31,32] and, at the time of initiation of this study, the BOND-2 data showed that the combination of cetuximab, bevacizumab, and irinotecan is feasible,[33] and clinical activity of cetuximab in GBM has been reported.[34] We therefore combined bevacizumab and irinotecan with cetuximab in patients with high-grade glioma. With this combination, the aim was to target both angiogenesis through VEGF inhibition and tumors likely to overexpress EGFR, which, accordingly, were expected to benefit the most from EGFR inhibition. In addition, in vitro and in vivo results have shown that EGFR inhibition leads to reduced angiogenesis, which indicates a possible synergistic effect of cetuximab and bevacizumab on angiogenesis.[35–37]

Patients and Methods

Patient Selection

Adult patients (age ≥18 years) with histologically proven primary GBM (WHO classification)[38] and MRI-verified recurrent or progressive disease (PD) were eligible for inclusion. Moreover, patients had to have progression within 6 months of finishing standard treatment with concomitant radiotherapy and temozolomide followed by adjuvant temozolomide.[1] Reintroduction of temozolomide was not allowed. Debulking surgery was performed, if possible, before entering the study but no other tumor reductive treatments were accepted. Basic clinical and laboratory evaluations were performed within 2 weeks and MRI scan within 4 weeks of starting study treatment. Eligibility criteria were: WHO performance status 0–2; ≥4 weeks from prior surgery and/or chemotherapy; life expectancy >3 months; neutrophils ≥1500/mm^3; platelets ≥125 000/mm^3; hemoglobin ≥6.2 mmol/L; ASAT and/or ALT <3× upper limit of normal (ULN); bilirubin ≤1.5× ULN; cholesterol ≤7 mmol/L; normal creatinine clearance; and activated partial thromboplastin time (APTT) ≤35 seconds and/or international normalized ratio (INR) from 0.8 to 1.2. Fertile women had to use contraception. Exclusion criteria were: prior EGFR- or VEGFR-based therapy; any medical, social, or physiological condition which could prevent adequate follow-up; any other active malignancy or previous malignancies within the previous 5 years, except adequately treated basal or squamous cell carcinoma of the skin or carcinoma in situ; any significant cardiac disease (New York Heart Association Class II or greater), arrhythmia, congestive heart failure, acute myocardial infarction within 6 months, or unstable angina pectoris; any serious on-going infection, illness, or medical condition; requirement of therapeutic anticoagulation, aspirin, nonsteroidal anti-inflammatory drugs, or clopidogrel; BP >150/100 mm Hg; proteinuria WHO grade 2 or greater; and pregnant or breast-feeding women.

The study was financed by the Danish National Board of Health and conducted in accordance with the Declaration of Helsinki and ICH Guideline for Good Clinical Practice.[39] Approval was obtained from the Ethics Committee and Danish Medicines Agency. Each patient signed written informed consent prior to enrollment.

Treatment

Bevacizumab and irinotecan were administered every 2 weeks (days 1 and 15) and each cycle of treatment was defined as 2 treatment administrations (4 weeks). The first 10 patients included in the study received bevacizumab 5 mg/kg without significant side effects and with no dose-limiting toxicities observed defined as grade 4 hematological toxicity or grade 3 nonhematological toxicity (according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0 [CTCAE 3.0]) except for headache, fatigue, nausea, vomiting, and alopecia if not sufficiently medically palliated. Consequently, after a planned safety analysis when these patients had received at least 2 cycles, bevacizumab was increased to 10 mg/kg in these and subsequent patients. Bevacizumab was administered by slow IV infusion over 90, 60, and 30

minutes for the first, second, and subsequent doses, respectively. IV irinotecan 340 mg/m^2 for patients receiving enzyme-inducing antiepileptic drugs (EIAEDs) and 125 mg/m^2 for patients not receiving EIAEDs was administered 60 minutes prior to bevacizumab. Atropine 1 mg SC was given 10 minutes prior to irinotecan to prevent cholinergic syndrome. Cetuximab was administered by slow IV infusion on days 1, 8, 15, and 22, with 400 mg/m^2 as the loading dose on day 1 followed by 250 mg/m^2 weekly. Appropriate antiemetics and/or antidiarrheal agents were permitted. Patients on corticosteroids were required to have a stable dose for at least 7 days before baseline MRI scan.

Cetuximab could be reduced once to 200 mg/m^2 for grade 3 or 4 skin toxicity and was discontinued for grade 3 or 4 hypersensitivity reactions. These patients were allowed to continue on-study without cetuximab. Reduction in bevacizumab dose was not permitted. For unmanageable bevacizumab-related side effects (grade 3 or 4 hypertension, pulmonary embolism, severe hemorrhage, arterial thromboembolic event, grade 3 or 4 proteinuria, and GI perforation), study treatment was discontinued. Irinotecan was reduced to 80% of starting dose for grade 4 neutropenia or febrile neutropenia or ≥ grade 3 toxicity (except alopecia) in the following cycles. For grade 4 neutropenia or febrile neutropenia after dose reduction, irinotecan was reduced to 60%. No further dose reduction was allowed. Treatment was discontinued in the case of tumor progression, unmanageable grade 4 toxicity, or at the request of the patient. The physician could terminate study treatment if continuation was deemed unsafe. Patients went off-study if treatment had to be postponed for more than 2 weeks.

Patient Evaluation

Evaluation was performed within 14 days of initiating therapy and included full medical history, physical and neurological examinations, performance status examination, complete blood count with differential and platelet counts, APTT or INR, serum chemistry profile, creatinine clearance, and urinary protein dipstick analysis. T1 and T2 contrast and noncontrast MRIs were repeated every 8 weeks during treatment, and clinical and laboratory tests were repeated every 2 weeks. Toxicities were evaluated during each cycle and graded according to CTCAE 3.0.

Treatment Response Evaluation

Response to therapy was evaluated after at least 2 cycles of study treatment using the MacDonald criteria.[40] These criteria use the largest cross-sectional area of the postcontrast images, neurological status, and corticosteroid dose. Complete response (CR) was defined as complete disappearance of measurable disease by MRI, partial response (PR) as >50% decrease in the area of enhancement, and PD as >25% increase in the area of enhancement, appearance of a new lesion, or deterioration in clinical status, likely secondary to tumor progression. Patients with CR or PR had to be on the same or decreased steroid dose and have stable or improved neurological findings. Stable disease (SD) is defined for patients not fulfilling CR, PR, or PD criteria.

Immunohistochemistry

Surgical specimens were routinely formalin-fixed and paraffin-embedded. Histological sections (4 μm) were stained with TissuGnost monoclonal mouse EGFR antibody (E 30; 1:200 dilution, Merck KgaA, Darmstadt, Germany). Briefly, tissue sections were deparaffinized and rehydrated followed by pretreatment in a microwave oven for 15 minutes at 95°C in *tris*-ethylene glycol tetraacetic acid buffer pH 9. Subsequently, staining was performed using a DAKO Autostainer (DAKO, Copenhagen, Denmark), allowing primary antibody to be incubated for 30 minutes at room temperature (RT). After washing with phosphate-buffered saline (PBS), sections were incubated with DAKO antimouse Envision+ System labeled with HRP (K4001, DAKO) for 30 minutes at RT and washed with PBS. DAKO Liquid DAB+ Substrate Chromogen System (K3468, DAKO) was applied for 10 minutes and sections washed with PBS. Sections were counterstained with hematoxylin.

Evaluation of the slides was performed independently and under blind conditions by H.B. (neuropathologist) and B.H. (MD, PhD researcher). EGFR labeling of tumor cells was scored semiquantitatively on a scale from 0 to 3 (0 = 0%; 1 = 1%–10%; 2 = 11%–50%; 3 = >50% cells stained positive).

Statistical Considerations

The primary endpoint of this study was 6-month progression-free survival (PFS). Yung et al.[3] reported a median PFS of 3 months with a 6-month PFS of 21% (95% CI: 13%–29%) among patients with first-relapse GBM who were treated with temozolomide. These data were used as the historical basis for the design of our phase II study. With 43 included patients and an assumed median PFS of historical controls of 3 months, there will be an approximate 80% power to detect an improvement of 2 months, and an approximate 60% power to detect an improvement of 1.4 months. If the median PFS from our trial is 3 months, the lower 95% CI will be approximately 2 months. We estimated 6-month PFS, time-to-progression (TTP), overall survival (OS), and associated 95% CIs using SPSS software, version 15.0 (Chicago, Illinois) and Kaplan–Meier methodology. The log-rank test was performed to compare survival in responders vs nonresponders. Pearson χ^2 and Fischer's exact tests were used for correlation between the EGFR level and response. Probability values (P values) <.05 were considered statistically significant. The Kaplan–Meier methodology was used for correlation between EGFR and survival.

Results

Patient Characteristics

Forty-three patients were enrolled from August 2006 to February 2008. Baseline characteristics of the patients are shown in Table 1. All patients had histologically verified primary GBM and had received standard treatment,[1] after which they showed progressive or recurrent disease within 6 months. Median study treatment duration was 14 weeks (range: 2–84 weeks).

Response Rate

The response rate of all patients based on intention-to-treat (ITT) (CR + PR) was 26% (95% CI: 14%–41%; Table 2). Eleven of the patients included ($n = 43$) went off study prior to MRI evaluation due to early deterioration or severe adverse events leading to early discontinuation of the treatment. Among evaluable patients ($n = 32$), best response was recorded after 2–4 treatment cycles. Both patients with CR had minor tumor load at the initiation of study treatment. Figure 1 shows serial MRI for a patient with PR.

Table 1. Patient characteristics of the ITT population

Characteristic	Cetuximab/bevacizumab/irinotecan ($n = 43$)	
	Number	Percentage
Gender		
Male	25	58
Female	18	42
Age (y)		
Median	54	
Range	23–70	
WHO performance status		
0	9	21
1	26	60
2	8	19
Reoperation before study treatment		
Yes	12	28
No	31	72
Site of treatment		
Copenhagen	32	74
Aalberg	9	21
Odense	3	7
Concomitant medications		
EIAED	6	14
Non-EIAED	37	86
Corticosteroids	35	81
Time from diagnosis until starting study treatment (d)		
Median	266	
Range	164–937	
Time from first recurrence until starting study treatment (d)		
Median	59	
Range	15–162	

Abbreviations: EIAED, enzyme-inducing antiepileptic drug; non-EIAED, non-enzyme-inducing antiepileptic drug; ITT, intention-to-treat.

Table 2. Response in patients intended to treat

Characteristic	Cetuximab/bevacizumab/irinotecan ($n = 43$)	
	Number of patients	Percentage
ORR: CR + PR	11	26 (95% CI: 14%–41%)
CR	2	5
PR	9	21
SD	17	40
PD	4	9
Not evaluable	11	26

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ORR, objective response rate.

Progression-Free Survival

Median follow-up time was 15 months (range: 7–25 months) and median PFS was 16 weeks (95% CI: 13–20 weeks). The 6-month PFS was 33% (95% CI: 19%–48%). Of the 2 patients with CR, 1 had 24 weeks to tumor progression and the other had not progressed at the time of study evaluation, 90 weeks after initiating study treatment. Figure 2A shows the Kaplan–Meier PFS plot, illustrating TTP for those with CR + PR vs SD + PD, which showed a significant difference between these groups ($P < .004$).

Overall Survival

Median OS as estimated by the Kaplan–Meier analysis (Fig. 2B) was 30 weeks (95% CI: 23–37 weeks). One patient with CR, 4 patients with PR, and 2 patients with SD were still alive at the time of study evaluation.

EGFR Expression

EGFR expression was determined for 39 of the 43 patients included, of which 2 were missing in the evaluable group of patients ($n = 32$). Of the 11 patients with CR and PR, 8 had <10% and 3 had >50% EGFR expression. Of the 19 patients with SD or PR, 13 had <10%, 4 had 11%–50%, and 2 had >50% EGFR expression. Kaplan–Meier methodology showed no correlation between EGFR expression and survival, and no significant correlation was found between EGFR expression and response using the Pearson χ^2 and Fischer's exact tests (data not shown). Figure 3 shows examples of EGFR staining.

Tolerability

Adverse events are summarized in Table 3. Six patients discontinued study treatment: one each for multiple pulmonary embolisms, lacunar infarction, severe skin

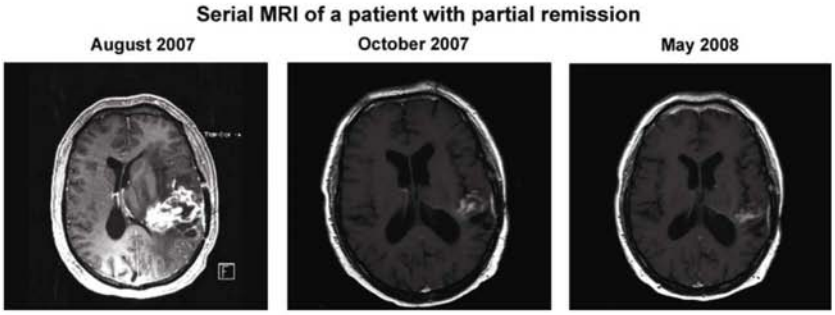

Fig. 1. MRI scan of a 64-year-old man with a PR and a TTP of 342 days. The patient initiated treatment within 4 weeks of the MRI scan originating from August 2007.

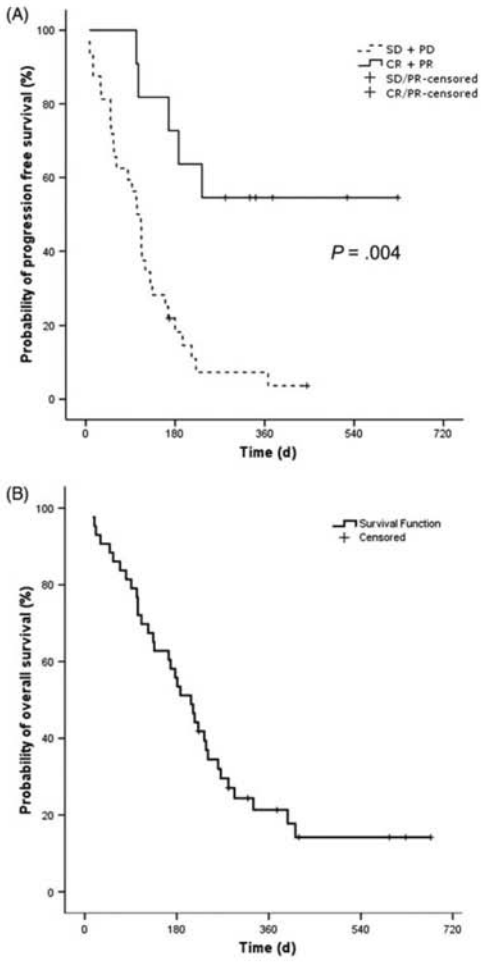

Fig. 2. Kaplan–Meier estimates showing TTP for evaluable patients ($n = 32$) (A) and OS for the ITT population ($n = 43$) (B).

toxicity which needed plastic surgery, pneumonia resulting in >2 weeks treatment suspension, suspicion of interstitial lung disease which normalized after discontinuation of treatment, and infection in a scalp scar originating from a reoperation procedure causing intracerebral air embolism and eventual death. Furthermore, 1 patient had cardiac arrest on day 24 and died the following day: autopsy showed acute pulmonary edema and no sign of intracerebral, cardiac or pulmonary bleeding, or thrombosis. Possible cause of death was epileptic seizure resulting in cerebral-triggered cardiac arrest, not related to study therapy. Three patients developed deep-vein thrombosis, all continued study treatment after initiation of a low-molecular–weight heparin, although one of these experienced grade 3 GI bleeding of unknown origin but continued study treatment after recovery.

Three patients experienced grade 3 or 4 allergic reactions during the first cetuximab administration despite premedication and all continued on study without cetuximab according to study protocol. Cetuximab is known for its skin toxicity: 12 patients had grade 1, 14 had grade 2, and 3 had grade 3 skin toxicity. Of the latter, one discontinued study treatment and the other two continued on study treatment without cetuximab. No patient developed grade 4 hematologic toxicity or grade 4 nonhematological toxicity except as noted above. Three patients developed arterial hypertension during study treatment and all were treated with appropriate antihypertensive medication and continued study treatment.

Discussion

We report the first phase II trial of irinotecan and bevacizumab in combination with cetuximab for the treatment of recurrent primary GBM. This study demonstrates that cetuximab, bevacizumab plus irinotecan, has an acceptable safety profile and induces a considerable number of clinically relevant, durable responses. For the ITT population, 6-month PFS was 33% (95% CI: 19%–48%), being 73% and 25% for

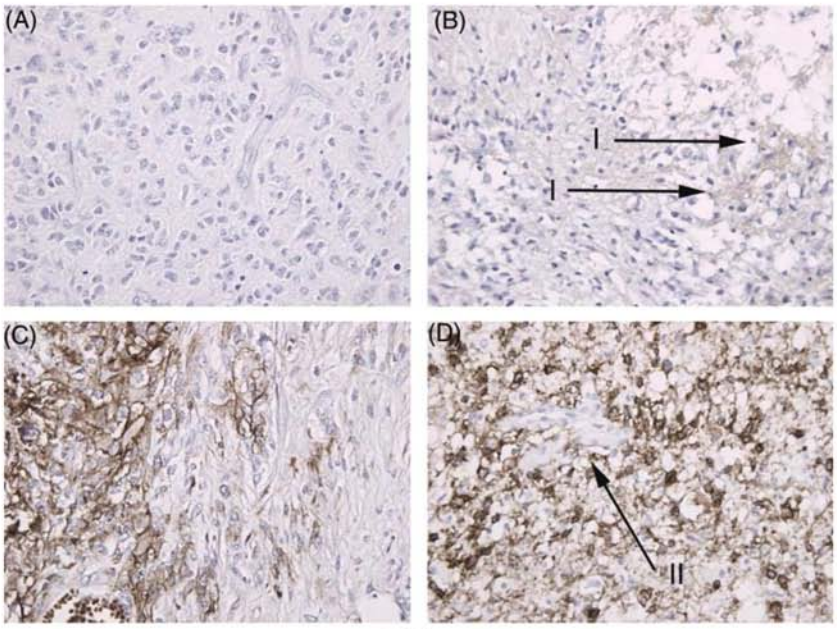

Fig. 3. Examples of EGFR expression by immunohistochemistry scored semiquantitatively on a scale from 0 to 3. (A) 0 = 0%; (B) 1 = 1%–10%; (C) 2 = 11%–50%; (D) 3 = >50% cells stained positive. Arrowheads I showing positive EGFR staining. Arrowhead II showing a vessel, not staining for EGFR.

Table 3. Adverse events in the ITT population

Adverse event	Cetuximab/bevacizumab/irinotecan (n = 43)	
	Grade 1/2 (No. [%])	Grade 3/4 (No. [%])
Nausea	13 (30)	0
Vomiting	5 (12)	1 (2)
Diarrhea	14 (9)	3 (7)
Stomatitis	12 (28)	0
Constipation	16 (37)	1 (2)
Loss of appetite	6 (14)	1 (2)
Fatigue	22 (51)	0
Neutropenia	5 (12)	2 (5)
Fever	5 (12)	0
Infection	9 (21)	6 (14)
Thrombosis	0	4 (9)
CNS hemorrhage	1 (2)	0
Skin reaction	26 (60)	3 (7)
Bleeding	6 (14)	2 (5)
Interstitial lung disease	0	1 (2)

Abbreviation: ITT, intention-to-treat.

those with CR/PR and SD/PD, respectively, and median PFS was 16 weeks (95% CI: 13–20 weeks). The response rate was 26% (95% CI: 14%–41%). The initial sample size of 43 patients was reduced to 32 evaluable patients; patients with recurrent GBM are particularly vulnerable and since the first MRI evaluation was performed after 8 weeks, 11 patients discontinued within this time due to early deterioration or severe adverse events. However, these patients were included in all ITT analyses.

The efficacy of bevacizumab plus irinotecan for recurrent high-grade glioma was first shown by Stark-Vance,[6] who found a response rate of 43% among 21 patients. In our study, we only included patients with primary GBM, which may explain our lower response rate. Our results are comparable with those obtained with the combination of bevacizumab and chemotherapy in high-grade glioma patients by Norden et al.,[26] Guiu et al.,[41] and Poulsen et al.,[5] who showed response rates of 34%, 36%, and 25%, respectively. Bevacizumab was combined with irinotecan in the latter 2 studies. Our results are not comparable with those of Vredenburgh et al.,[4] who showed a response rate of 63% and median OS of 40 weeks with bevacizumab plus irinotecan in patients with recurrent malignant glioma. However, 6-month PFS was 32% in their study, which is comparable with our result. In our study, 9 of the 17 patients with SD (53%) had tumor reduction between 25% and 48%, which clearly indicates a clinical benefit.

EGFR is known to be amplified and/or overexpressed in 35%–45% of primary GBM tumors,[9–12] and 40% of

GBM tumors with EGFR amplification express the mutant EGFRvIII receptor which induces ligand-independent constitutive activation.[12,42] Accordingly, targeting EGFR and EGFRvIII and their down-stream pathways has been of considerable interest in the search for new treatments of high-grade glioma. Moreover, EGFR activation can increase VEGF production in glioma cell lines,[43] and EGFR inhibition by cetuximab reduces the VEGF production of both in vitro and in vivo in various cancer cell lines.[35–37] In addition, it has been shown that cetuximab reduces the level of hypoxia-inducible factor-1 alpha (HIF-1α), which is a transcriptional regulator of VEGF expression.[44] Necrosis and hypoxia are mandatory in GBM and hypoxia leads, among other factors, to stabilization of HIF-1α and HIF-2α subunits that initiate VEGF transcription.[45] Thus, combining inhibition of EGFR and VEGFR (by inhibiting binding of the ligand VEGF) might be expected to have a beneficial effect in primary GBM. In anticipation of EGFR being an essential target in the treatment of high-grade glioma, gefitinib and erlotinib have been studied as single-agents or in combination with chemotherapy, radiotherapy, and other targeted therapies, but with only modest effect.[13–17] Cetuximab in combination with radiotherapy has been shown to reduce the viability of EGFR-amplified glioma cell lines both in vitro and in vivo and has been shown to bind EGFRvIII and induce internalization of the receptor.[20,21] In addition, cetuximab has been shown to induce 40%–50% inhibition of cell proliferation in vitro.[46] The use of cetuximab for high-grade glioma patients has been limited. However, Belda-Iniesta et al.[34] showed some durable responses when using cetuximab in 3 patients with recurrent GBM who remained clinically and radiologically stable for 14, 13, and 11 months, respectively. These 3 patients all had positive EGFR staining. In our study, EGFR were overexpressed in 11 (37%) of evaluable patients and the expression was not correlated with response or survival.

Cetuximab has shown to be ineffective when treating colon cancer patients with K-*ras* mutations;[47] however, K-*ras* mutations are not very common in GBM.[48] The lack of an improved response rate when combining cetuximab with bevacizumab and irinotecan might be caused by mutations in the tumor suppressor gene, phosphatase, and tensin homolog (PTEN). Importantly, PTEN mutations occur in 20%–40% of GBM tumors and have been shown by other groups to mediate resistance to anti-EGFR treatment.[17,49] Thus, it would appear that EGFR is not of such pivotal importance for maintenance of glioma tumor growth as had been expected previously, despite the fact that EGFR is often found to be overexpressed and/or amplified in primary GBM.

At the time of initiation of our trial, there were no data showing the effect of bevacizumab alone vs the combination of bevacizumab and irinotecan in patients with GBM. Subsequently, Cloughesy et al.[50] have shown that 6-month PFS (50% vs 35%) and response rate (33% vs 20%) are not significantly higher in patients treated with bevacizumab and irinotecan when compared with those receiving bevacizumab alone, respectively. Moreover, a recent study by Kreisl et al.[51] showed that single-agent bevacizumab resulted in significant activity in heavily pretreated patients with GBM with a 6-month PFS of 29% and a response rate of 35%, without benefit from the addition of irinotecan at progression. These results are comparable with the 6-month PFS and response rate found in our study (33% and 26%, respectively). On the basis of the results of the studies by Cloughesy et al.[50] and Kreisl et al.,[51] bevacizumab has now been approved by the US Food and Drug Administration as single-agent treatment for patients with progressive GBM following prior therapy.

In contrast to small-molecule TKIs, cetuximab is a large molecule, which will possibly not cross the intact BBB. However, this should also be the case with bevacizumab, and the significant clinical benefit of this agent may be related to the fact that BBB is not intact in areas of active tumor cells.[52] We found it appropriate to add cetuximab to a backbone of bevacizumab and irinotecan, since it has been shown in 2 phase I studies that combination of erlotinib or gefitinib with irinotecan induced dose-limiting diarrhea.[31,32] Other new interesting agents also interfere with vascularization. Cilengitide is an integrin inhibitor with clinical activity in recurrent GBM.[53] AZD2171 (cediranib) is a multitargeted TKI that blocks VEGFR-1, VEGFR-2, and VEGFR-3 signaling that showed a response rate of 56% as single-agent therapy in recurrent GBM.[54] Both these agents are now being tested in phase III trials for GBM.

In our study, 11 patients were not evaluable for response. This indicates that patient selection is very important when evaluating new regimens in GBM, because these patients are vulnerable due to immobilization, and minor changes in the primary tumor may result in global alteration and severe deterioration.

In conclusion, cetuximab in combination with bevacizumab and irinotecan in patients with recurrent GBM was found to be a well-tolerated regimen, except for skin toxicity, with an encouraging response rate, including 2 patients with CR. However, the response rate does not appear to be superior with the addition of cetuximab to that which can be obtained with single-agent bevacizumab or the combination of bevacizumab plus irinotecan. Consequently, there would appear to be no rationale for adding cetuximab to the bevacizumab-based regimens in recurrent high-grade glioma in the future.

Conflict of interest statement. U.L. has previously received a research grant from Roche A/S and Merck and has received speaking fees from the same companies.

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Summary

GBM is a dreadful disease with a median survival of only 15 months. The prognosis for recurrent GBM is even worse with a median survival of only 3-9 months when using traditional chemotherapeutic agents. Several recent publications, including one from our own institution (Manuscript II), have demonstrated significant improvement of response in the treatment of a subset of recurrent GBM patients when using bevacizumab in combination with irinotecan. The heterogeneity of GBM and the ability of nearly all GBM to either primarily or over time, bypass signaling pathway blockade, could indicate that a multifaceted approach involving targeted inhibition of multiple signaling pathways could block potential "escape routes". Primary GBM are known to have overexpressed and/or amplified epidermal EGFR and this has been shown to correlate with poor prognosis. Results from previous studies using EGFR TKIs are not uniform, but several indicate a modest efficacy for TKIs in GBM. The intension of this phase II study was to investigate if the addition of the EGFR inhibitor cetuximab would induce increased tumor control, survival and the number of patients benefiting from the treatment, as compared with the effect of BI in recurrent GBM.

Forty-three patients were included on this phase II study. It was demonstrated that CBI is a feasible combination, although some patients did experience skin toxicity. CBI induced a considerable number of clinically relevant, durable responses, including two complete responses. However, the response rate and survival data obtained did not appear to be superior the regimen of BI and consequently the CBI regimen is not recommended in recurrent GBM.

Manuscript IV

Prospective evaluation of angiogenic, hypoxic and EGFR related biomarkers in recurrent glioblastoma multiforme treated with cetuximab, bevacizumab and irinotecan

By

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Title: Prospective evaluation of angiogenic, hypoxic and EGFR related biomarkers in recurrent glioblastoma multiforme treated with cetuximab, bevacizumab and irinotecan

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Running head: Immunohistochemical evaluation in recurrent GBM

Abstract

Aim

Several recent studies have demonstrated a beneficial effect of anti-angiogenic treatment with the vascular endothelial growth factor (VEGF) –neutralizing antibody, bevacizumab in recurrent high-grade glioma. In the current study, immunohistochemical evaluation of biomarkers involved in angiogenesis, hypoxia and mediators of the epidermal growth factor receptor (EGFR) pathway were investigated.

Experimental design

Tumor tissue was obtained from a previous phase II study, treating recurrent primary glioblastoma multiforme patients with the EGFR inhibitor cetuximab in combination with bevacizumab and irinotecan. Of the 37 patients with available tumor tissue, twenty-nine were evaluable for response. We concurrently performed immunohistochemical stainings on tumor tissue from 21 glioblastoma multiforme patients treated with bevacizumab and irinotecan.

Results

We found a tendency of correlation between the hypoxia related markers, indicating that they share the same regulatory mechanisms. None of the EGFR related biomarkers showed any significant correlations to each other. None of the biomarkers tested alone or in combination could identify a patient population likely to benefit from bevacizumab and irinotecan, with or without the addition of cetuximab.

Conclusion

There is still an urgent need for one or more reliable and reproducible biomarkers able to predict the efficacy of anti-angiogenic therapy.

Key words: Angiogenesis, hypoxia, glioblastoma multiforme, bevacizumab, cetuximab

Introduction

Despite recent improvement in the treatment of glioblastoma multiforme (GBM), it continues to be a devastating disease with a median survival for newly diagnosed GBM of only 15 months (1). GBMs are known to be vastly vascularized, and pronounced tumor vascularity significantly correlates with poor survival (2). Several molecular mechanisms contribute to the continued growth of this tumor and inhibition of angiogenesis with the vascular endothelial growth factor (VEGF) inhibitor bevacizumab (Avastin®) has recently been shown to contribute to prolonged progression-free survival (PFS) in a not yet characterized group of GBM patients (3,4,5,6,7,8). Similar results have been obtained from a phase II study using the EGFR inhibitor, cetuximab (Erbitux®) in combination with bevacizumab and irinotecan (CBI) in recurrent primary GBM (9).

Hypoxia is mandatory in GBM and the hypoxia inducible factor 1 (HIF-1) transcription factor mediates adaptive responses to changes in tissue oxygenation by regulating numerous genes involved in, for instance, angiogenesis, vascular reactivity and remodeling. HIF-1 is a member of the basic helix-loop-helix-PAS (bHLH-PAS) family, which include the hypoxia regulated HIF-1α and HIF-2α (10). HIF-1α is expressed in an apparently ubiquitous fashion, whereas HIF-2α expression is restricted to particular cell types, including vascular endothelial cells and brain (11,12). Hypoxia induces stabilization of the HIF-1α and the HIF-2α subunits which leads to transcription of VEGF (13,14). HIF-1α furthermore upregulates carbonic anhydrase 9 (CA9), a transmembrane enzyme that catalyzes the reversible hydration of carbon dioxide to carbonic acid and thereby is involved in the pH homeostasis of the cancer cells in response to hypoxia (15). Moreover, hypoxia induces enhanced transcription of the glucose transporter *GLUT-1* (16).

Increased activity of the EGFR pathway results in cell proliferation and an increase in tumor invasiveness and motility (17). Amplification and overexpression of the EGFR is observed in 35-45% of primary GBM and have been correlated with a poor prognosis (18,19). Accordingly, EGFR has as such been expected to be of pivotal importance in the pathogenesis of primary GBM. Moreover, EGFR mutations are present in 40-50% of GBM, of which the constitutively activated EGFRvIII is the most common (20). In

addition, EGFR induces angiogenesis by stimulating the synthesis of HIF-1α via the PI3K/AKT or the Ras/MAPK pathways (21,22).

The tumor suppressor phosphatase and tensin homolog (PTEN) regulates the PI3K/AKT kinase pathway and thereby the activation/phosporylation of AKT (pAKT) (23). Inactivation of PTEN due to *PTEN* mutations contributes to an abnormally high activity of the PI3K/AKT pathway which is often seen in primary GBM (19,24) and has been correlated to a dismal prognosis and resistance to anti-EGFR therapy (25,26).

Despite encouraging results using anti-angiogenic therapy in malignant gliomas, only a subset of the patients receiving bevacizumab experience radiographic response or prolonged survival.

Immunohistochemical (IHC) analysis of tumor tissue has been used in two previous studies to reveal biomarkers predicting response to the EGFR inhibitors erlotinib and gefitinib (27,28) and in a recent study by Sathornsumetee et al.(29), high CA9 expression was found to predict poor survival outcome whereas high VEGF expression was associated with radiographic response, in HGG patients receiving bevacizumab. In the current study, we used semiquantitative IHC analysis of biological markers involved in angiogenesis, hypoxia and EGFR signaling on tumor tissue from patients treated with CBI. In this prospective study, the aim was to identify markers, which could be used as predictive biomarkers of response and prolongation of progression-free survival. Moreover, we retrospectively analyzed the same biomarkers and used the same statistical methods in a group of recurrent GBM patients, which had previously been receiving bevacizumab and irinotecan (BI) at our institution (7). The observations obtained from CBI and BI are presented separately. As the treatment regimens as well as the response and survival data are very similar in CBI and BI, we also merged the data from these two groups, thereby achieving a larger material for our statistical analysis.

Patients and Methods

Patient selection and Tissue Acquisition

From August 2006 to February 2008, forty-three (43) patients with recurrent primary GBM were included in a prospective phase II trial and treated with CBI (9). All patients had received standard treatment (1) after which they showed progressive or recurrent disease within 6 months. We prospectively collected paraffin-embedded tumor material for IHC analysis from initial surgical specimens. Of the 37 patients with available tumor tissue, eight patients went off study prior to MRI evaluation due to early deterioration or severe adverse events leading to early discontinuation of the treatment. Survival data were available from all patients included in the study. Thus response to therapy was available on 29 patients with corresponding tumor tissue material, and the patients were evaluated after at least two cycles of study treatment using the MacDonald criteria (30). Each patient signed written informed consent prior to enrollment. The study was approved by the Ethics Committee and Danish Medicines Agency. In addition, we retrospectively collected tumor tissue from the initial surgical procedure and performed IHC on GBM tumor tissue from 24 patients, treated for recurrent GBM with BI. The clinical results from these patients have previously been published by Poulsen et al (7). Briefly, patients in this protocol had recurrent primary or secondary GBM, with one (n = 18), two (n = 5) or four (n = 1) prior chemotherapy regimens, none of which contained bevacizumab or irinotecan. Survival data were available on all patients from this study. Response data were available on 21 evaluable patients as described above. The study was conducted in accordance with the Declaration of Helsinki. One tumor block from each patient was evaluated in both CBI and BI. Independent confirmation of the initial diagnosis and the presence of tumor in each specimen were endorsed by a neuropathologist (H.B) at Copenhagen University Hospital. Histological material was assigned by anonymous numbers to the investigators (B.H., H.B. and J.G.E.).

Immunohistochemistry

Surgical specimens were routinely formalin-fixed and paraffin-embedded. Histological sections (4 µm) underwent IHC staining for ten protein markers with the following

antibodies: TissuGnost EGFR (E 30) (1:200 dilution, Merck KgaA, Darmstadt, Germany); EGFRvIII (Ua30) (1:100 dilution, InRo BioMedTek, Umeå, Sweden) (31), HIF-1α (clone 54) (1:250 dilution, BD Biosciences, San Jose, NJ) (32), HIF-2α (ep190b) (1:300 dilution, Novus Biological, Littleton, CO) (33), VEGF (C-1) (1:400 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA) (34), CD34 (NCL-L-END) (1:200 dilution, Novo Castra, Newcastle Upon Tyne, UK) (34), CA9 (M75) (1:200 dilution, (from S. Pastorekova, Bratislava, Slovakia) (32) and GLUT-1 (SPM498) (1:200 dilution, Abcam, Cambridge, MA) (35), pAKT (736E11) (1:40 dilution, Cell Signaling Technology Inc., Danvers, MA); PTEN (138G6) (1:50 dilution, Cell Signaling Technology Inc.). All were mouse monoclonal antibodies (mAb) except from pAKT and PTEN, which were rabbit mAb.

VEGF, CA9, CD34, GLUT-1, HIF-1α, HIF-2α stainings were performed on LabVision 480 autostainer (Thermo Fisher Scientific Inc., Fremont, CA), EGFRvIII where performed manually, whereas the EGFR, pAKT, PTEN stainings were performed on DAKO Cytomation Plus autostainer (Dako Glostrup, Denmark). Briefly; formalin-fixed and paraffin-embedded slides were melted for one hour at 60º C, followed by deparaffination in petroleum and ethanol. Endogenous peroxidase was blocked in hydrogen peroxide 0.5% (diluted in ethanol 99%) for 10-20 min. Afterwards the slides were boiled in tris-ehtylen-diamin-tetra-acid (EDTA) glycerol buffer or with regards to EGFR, EGFRvIII, PTEN and pAKT in tris-ethylene-glycol-tetraacetic-acid (EGTA) at pH 9.0 for 20 min (GLUT-1 only 10 min) and cooled at room temperature (RT) before embedment in serum-free protein block (DAKO X0909 (Dako) or (with regards to CA9, GLUT-1 and CD34) Ultra V Block TA-125-UB (Thermo Fisher Scientific Inc.)) or nothing (EGFR, EGFRvIII, PTEN and pAKT). Incubation with primary antibody was performed either over night (ON) at 4ºC (VEGF, EGFRvIII and HIF-1α) or for 30 min at RT for the remaining antibodies. Subsequently, Envision^{+} (DAKO K4001 or DAKO K4003 (used for EGFRvIII, PTEN and pAKT), Dako) or Ultravision (AH diagnostics, Aarhus, Denmark) (used for GLUT-1, CA9 and CD34) were applied for 30 min. DAKO Liquid DAB+ Substrate Chromogen System (K3468, DAKO) was applied for 10 min (CA9 only 5 min). Enhancement with 0.5% $CuSO_4$ was used for VEGF, CA9, HIF-2α, EGFR, EGFRvIII, PTEN and pAKT, and NovaRed (SK4800) (Vector Laboratories Inc., Burlingame, CA) was used for HIF-1α. Sections were counterstained with

Mayer's haematoxylin. Negative controls were performed by replacing primary antibodies with antibody diluents. Staining of normal brain tissue was performed with each of the protein markers. IHC performed on cytospin from a verified PTEN wild type positive head and neck cancer cell line and a glioma cell line with verified deleted PTEN (36) was used for positive and negative control respectively (unpublished results). To verify the specificity of the pAKT antibody, the PTEN deleted glioma cell line was treated with the PI3 kinase inhibitor LY294002 (Cell Signaling Technology, Inc.) resulting in inhibition and lack of expression of pAKT (unpublished results). Moreover the EGFRvIII antibody was tested on EGFRvIII positive and negative glioma cell lines, which demonstrated high specificity of the antibody in a western blotting setting (unpublished results).

Semiquantitative IHC Analysis

All optical fields on each sample were evaluated. Necrotic areas and normal brain tissue were excluded. Positive staining was determined semiquantitatively by two independent investigators. B.H. and J.G.E. scored VEGF, CA9, GLUT-1, HIF-1α, HIF-2α and CD34 whereas B.H. and H.B. scored EGFR, EGFRvIII, pAKT and PTEN respectively. Conflicting results (<10%) were revised and consensus was reached. EGFR and EGFRvIII were scored semiquantitatively on a scale from 0 to 3 (0 = 0%; 1 = 1–10%; 2 = 11–50%; 3 = >50% cells stained positive), CD34 positive vessels were counted in 3 hotspots in x400 magnification, and the mean value was used in the analysis (37). The remaining markers were scored semiquantitatively on a scale from 0 – 100% of positive staining cells in non-necrotic tumor tissue. Positive representative areas were selected for imaging (x200 and x400 magnification).

Statistical Analysis

Survival was determined from the time of treatment initiation until the time of death or last follow-up. Response was scored as complete response (CR) or partial response (PR) versus stable disease (SD) or progressive disease (PD) according to the MacDonald criteria (30). Response rate (RR) was defined as CR + PR. The actual values of the markers were used to score each marker, except CD34, which was analysed on the log scale (base2). Measures of association between levels of the

biomarkers were calculated using Spearman's rank correlation. The effect of each biomarker for response was screened using a logistic regression model. Estimates of survival probabilities were done using the Kaplan-Meier method and survival rates were compared using logrank statistics. The relationship between the biomarkers and progression free survival (PFS) as well as overall survival (OS) was analysed using the Cox proportional hazards model. The effects are presented by the hazard ratios (HR) with 95% confidence limits (CI). $P < 5\%$ were considered significant. Statistical analysis was performed using SAS program (SAS institute, Cary, NC).

Results

Patients

All of the 60 patients included with available tumor tissue had WHO grade IV astrocytoma/GBM. Twenty-nine of the 37 patients treated with CBI were evaluable for response, whereas 21 of the 24 patients treated with BI were evaluable for response. The patient subset in the CBI group is similar to the BI group (Table 1). All patients included in the CBI study had to have progression within 6 months of finishing standard treatment with concomitant radiotherapy and temozolomide followed by adjuvant temozolomide (1). Debulking surgery was performed, if possible, before entering the study but no other tumor reductive treatments were accepted. The majority (75%) of the patients in the BI group had received one prior chemotherapy regimen containing temozolomide. Median PFS in CBI ($n = 37$) and BI ($n = 21$) corresponded to 17 weeks (range 7 – 125 weeks) and 29 weeks (range 6-206 weeks) and median OS was 38.4 weeks (range 2-141 weeks) and 44.9 weeks (range 1-225 weeks) in CBI ($n = 37$) and BI ($n = 24$), respectively. Univariate logistic regression analysis showed no difference in Response ($P = 0.99$, OR = 1.01 (95%CI, 0.32 – 3.20)), OS ($P = 0.45$, HR = 1.23 (95%CI, 0.72 – 2.10)), or PFS ($P = 0.17$, HR = 1.46 (95%CI, 0.85 – 2.53)) between the CBI and BI treatment. The median time from termination of treatment with temozolomide and start of either CBI or BI treatment, were 64 (range 32 – 371) and 108 (range 45 - 2039) days respectively. One patient from the BI group had previously confirmed grade 2 astrocytomas whereas the primary diagnosis was GBM in the remaining patients. Of the 29 evaluable patients

receiving CBI treatment, two had CR defined as complete disappearance of measurable disease by magnetic resonance (MRI) scan, and nine patients had PR defined as >50% decrease in the area of enhancement. Patients with CR or PR had to be on the same or decreased steroid dose and have stable or improved neurological findings. Four patients had CR and four patients had PR in the BI group (*n* = 21). Nine (of which 5 had CR or PR) of the 37 patients in the CBI group and six (all having CR or PR) of the 24 patients in the BI group were alive one year after initiation of treatment. Median follow-up were 30 weeks for the CBI group and 37 weeks for the BI group. Two patients from the BI group were alive at the time of the analysis (September the 1st, 2009), 26 and 51 months after initiation of treatment.

Biomarkers

Representative images of each biomarker in tumor tissue are illustrated in Figure 1-3. The endothelial marker CD34 was evaluated by counting the number of vessels in three hot spots in tumor tissue, using the median value for the analysis. The remaining biomarkers were quantified by semiqualitative technique (the distribution of biomarkers evaluated is presented in supplementary Figure 1). It could be considered, whether the EGFR- and the EGFRvIII antibodies would detect the same epitope, however there was no convergence in the staining and intensity of EGFR and EGFRvIII which where found to be diverse in 55.7% (*n*=34/61) of the slides. Accordingly, detection of the same epitope seemed unlikely. PTEN expression was not detected in 47% of the slides (*n*=28/61). Expression of pAKT was not found in 29% of the tumors (*n*=18/61). The hypoxia markers HIF-1α and HIF-2α not were found in 37.8% (*n*= 23/61) and 43.3% (*n*=26/61) of the slides respectively.

Correlation between biomarkers

Spearman rank correlation was used to assess the association of uncategorized reactivity between markers. Correlation coefficients above 0.5 found in the analyzes are illustrated in Table 2. GLUT-1 and CA9, GLUT-1 and HIF-1α plus HIF-1α and CA9 showed a tendency of being correlated in both CBI and BI. This could indicate a hypoxic molecular profile of these tumors. Moreover, these findings suggest that GLUT-1, HIF-1α and CA9 share regulatory mechanisms.

No positive biomarker for response

The effect of each biomarker was screened for response (CR + PR) using univariate logistic regression. The only biomarker, found to be nearly significant (P = 0.07, OR = 1.04 (95%CI, 0.99 – 1.08)) was CD34, however, this was only found in the BI group and could not be found in neither CBI nor when pooling data from the two groups.

Biomarkers fail to predict survival outcome

The relationship between the expression of each biomarker and survival is shown in supplementary Table 1-3. We tested the CBI and BI groups individually. Moreover, in order to expand the size of the material and thereby increase the possibility of finding a significant interaction between one or more biomarkers and survival, logistic regression analysis were performed on the merged material of CBI and BI. The only biomarker showing a tendency toward predicting survival and response was the endothelial marker CD34. PFS showing P = 0.07 and HR was found to be 1.04 (95% CI: 0.99-1.08). In addition, OS showed P = 0.08 and a HR of 0.99 (95% CI: 0.98 – 1.00). However, it must be emphasized that this trend was only found in the BI group, and although the tendency showed in both response and progression-free survival it could be due to variance in values of the biomarkers.

Discussion

In the recent development of cancer therapy, a broad selection of "molecular targeted" anticancer drugs are in clinical evaluation or have been approved for specific cancer diseases. The intention is to accomplish a prolonged tumor control and survival for the individual patient. As most of these drugs are not without side effects, there is a compelling need to select and stratify the patients most likely to benefit from the treatment. In order to achieve this, there is an ongoing search for one or more valid biomarkers, which could prove to be predictive of response to treatment. One such method is IHC analysis of patient tumor material. In brain tumors there are obvious limitations to acquisition of tumor material, as neurosurgical complications precludes repeated tumor sampling. Consequently, GBM tumor tissue used for biomarker analysis often originates from the primary diagnosis, as it is infrequent that the patient is undergoing surgery or biopsy at recurrence. Available tumor tissue after treatment for recurrent disease is even more rare and consequently, any changes or differences in the expression of the biomarkers after receiving treatment are not systematically revealed.

The aim of the phase II CBI study (9) was to explore if the addition of the EGFR inhibitor cetuximab to the regimen of bevacizumab and irinotecan, would indicate an increase in the response rate and survival in primary GBM, which could be expected to have overexpressed and/or amplified EGFR. However, the response and survival data obtained were not different from what have been reported from other studies using bevacizumab and irinotecan or bevacizumab as monotherapy, and the CBI combination is thus not further explored (3,4,5,7,8). In addition, the secondary aim was to investigate if there were any tumor tissue biomarkers that could predict a group of patients, which would benefit from the treatment. The value of biomarkers in HGG has only been tested in few earlier studies (27,28,29), and none of these results have to our knowledge been confirmed in a different and/or larger material. In this immunohistochemical study of patients receiving CBI or BI, we were not able to confirm the association of VEGF and CA9 with radiographic response or survival respectively, as found in the study by Sathornsumetee et al (29). Hypoxia is suspected to play a prominent role in tumor development, angiogenesis and

decreased patient survival in various cancer types, including GBM. The expression of VEGF is increased with hypoxia, due partly to hypoxia-induced stabilization of the HIF-1α and HIF-2α subunits, that initiate VEGF transcription (13). Hypoxia contributes to a more aggressive behavior of the tumor and HIF-1α and HIF-2α are critical for this adaptive response (38), however neither HIF-1α nor HIF-2α showed to be predictive for response to treatment or survival in our material.

The micro vessel density (MVD) in tumors as measured by the hot spot method has been shown to be a valuable prognostic indicator for a wide range of tumors (39,40,41,42). When using an anti-angiogenic agent for the treatment of a highly vascularized tumor like GBM, it is intriguing to assume that MVD would be of importance for response. MVD estimated in our material, using the endothelial marker CD34 showed a tendency towards correlation with response, OS and PFS, but only in the BI material. Accordingly, a cautious approach should be taken to this observation. This is in accordance with the review by Hlatky et al., who emphasized that MVD was not equivalent to the degree of tumor angiogenetic activity and that measurement of MVD was not predictive of tumor response under anti-angiogenic treatment (43). Consequently the level of MVD in the tumor should not be used to decide which patients would benefit from anti-angiogenic treatment.

The EGFR tyrosine kinase inhibitors erlotinib and gefitinib have been used in previous studies for the treatment of HGG, and EGFR as well as coexpression of EGFRvIII and PTEN have been showed to be predictive for treatment response (27,28). When using the EGFR inhibitor cetuximab in combination with bevacizumab and irinotecan in our phase II trial, our hypothesis was that the expression of EGFR, EGFRvIII or mediators downstream from these (pAKT or PTEN), might predict to response to treatment or survival. As demonstrated, none of the above-mentioned biomarkers could identify a patient population likely to benefit from the CBI treatment. The same result was not surprisingly obtained from the BI group.

We also investigated, whether there would be a molecular profile between the angiogenic and hypoxic biomarkers and/or amongst the EGFR related biomarkers. We did find a tendency of correlation between the hypoxic related markers GLUT-1 and CA9, GLUT-1 and HIF-1α plus HIF-2α and CA9 respectively in CBI and BI and also when pooling the CBI and BI data. This could suggest that GLUT-1, HIF-1α and

CA9 share regulatory mechanisms. None of the EGFR related biomarkers showed any significant correlations.

A limitation of this study was the relatively small sample size and the well known heterogeneity of the GBM tumor material. Accordingly, these observations should be verified in a larger material.

The clinical and radiological benefit of anti-angiogenic therapy in a minority of GBM patients is indisputable. However, it remains to be found one or more reproducible biomarkers such as e.g. 1p19q deletion (44) in anaplastic oligodendrogliomas or O^6-methylguanine-DNA methyltransferease (MGMT) methylation in GBM (45) could be used to predict the response in an anti-angiogenic treatment regimen. Contrary to what has been found in other studies, none of the biomarkers tested alone or in combination in our material, could identify a patient population likely to benefit from either CBI or BI treatment. This could be due to difference in treatment regimens (CBI and BI) or difference in patient selection and sample size. It might also be due to different IHC techniques and antibodies, which may well demonstrate the difficulty of using IHC as a reproducible method in the search for biomarkers in anti-angiogenic therapy. Hence, there is still an urgent need for one or more reliable and reproducible biomarkers able to predict the efficacy of anti-angiogenic therapy. Since tumor tissue is difficult to obtain repeatedly from brain tumors, other non-invasive approaches like imaging techniques would be attractive, however this area is still under development.

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Figure and Table Legends

Fig. 1. Representative immunostaining detection of angiogenic/hypixic markers. (A and B) Vascular endothelial growth factor (VEGF) staining of tumor cell cytoplasm. Notice that positive cells are located especially in the pseudopallisading cells around necrotic areas (arrows). (C and D) Hypoxia-inducible factor-1α (HIF-1α) in tumor cell nuclei. Note that the positive cells are located in the pseudopallisading areas around necrosis (arrows). (E and F) HIF-2α diffuse staining of nuclei and cytoplasm of tumor cells (arrows). (G and F) Carbonic anhydrase 9 (CA-9) staining of tumor cell membrane and cytoplasm. Note that positivity is seen in the pseudopallisading tumor cells around necrotic areas(arrows). (I and J) GLUT-1 staining of tumor cell membrane and cytoplasm. Like VEGF, HIF-1α and CA9, the positivity are found in the pseudopallisading tumor cells around necrotic areas (arrows). The images are shown in x200 magnification (top row) and x400 magnification (bottom row).

Fig. 2. The epidermal growth factor receptor (EGFR) and downstream mediators detected by representative immunostaining. (A and B) EGFR positivity in tumor cell cytoplasm, diffusely distributed in a tumor with more than 50% positive staining tumor cells. No staining of endothelial cells (arrow heads). (C and D) Mutated EGFR (EGFRvIII) with positive staining of the cytoplasm (arrow head). (E and F) The tumor suppressor, phosphatase and tensin homolog (PTEN) showing diffuse staining of tumor cell cytoplasm (arrows). (G and H) pAKT staining of the tumor cell cytoplasm (arrows). Notice no staining of endothelial cells (arrow heads). The images are shown in x200 magnification (top row) and x400 magnification (bottom row).

Fig. 3. Immunostaining of the endothelial marker CD34 showing low (A) versus high (B) micro vessel density (MVD) in tumor tissue.

Table 2. Evaluated tumor specimens obtained from the initial surgery are grouped according to treatment at recurrence (CBI and BI respectively). Spearman rank correlation is used to assess the association between the biomarkers studied. Correlation coefficients above 0.5 and the corresponding *P*-values are shown.

Table 1

Characteristics	Treatment (*N*=61) CBI	BI
Treated	37	24
Evaluable	29	21
Gender		
Male	21	17
Female	16	7
Age		
Median	57.9	50.5
Range	23.8-70.3	29.1-67.9
WHO performance status		
0	8	7
1	24	13
2	5	3
Prior chemotherapy		
1	37	18
2	-	5
4	-	1
Radiographic response rate, %	37.9	38.1
Overall survival (OS), weeks		
Median	38.4	44.9
Range	2-141	1-225
Progression free survival (PFS), weeks		
Median	17	29[#]
Range	7-125	6-206
PFS at 6 months, %	44.8	52.4

Abbreviations: CBI, cetuximab, bevacizumab and irinotecan; BI, bevacizumab and irinotecan

Note[#]: Registration of response or progression were missing in three of the 24 patients.

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Table 2

Treatment	Number of samples	Biomarkers	Correlation Coefficient	*P*
CBI		HIF-1α – CA9	0.56	0.0003
CBI	N = 37	GLUT-1 – CA9	0.84	<0.0001
CBI		GLUT-1 – HIF-1α	0.63	<0.0001
BI		HIF-1α – CA9	0.58	0.0031
BI		GLUT-1 – CA9	0.52	0.0091
BI	N = 24	GLUT-1 – HIF-1α	0.54	0.0062
BI		PTEN - EGFRvIII	0.52	0.0098
BI		VEGF – CA9	0.66	0.0005
CBI + BI		HIF-1α – CA9	0.52	<0.0001
CBI + BI	N = 61	GLUT-1 – CA9	0.73	<0.0001
CBI + BI		GLUT-1 – HIF-1α	0.58	<0.0001

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Fig 1

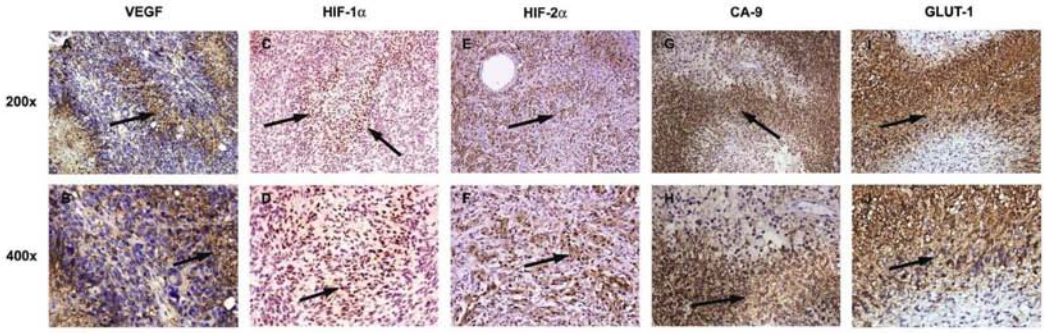

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Fig 2

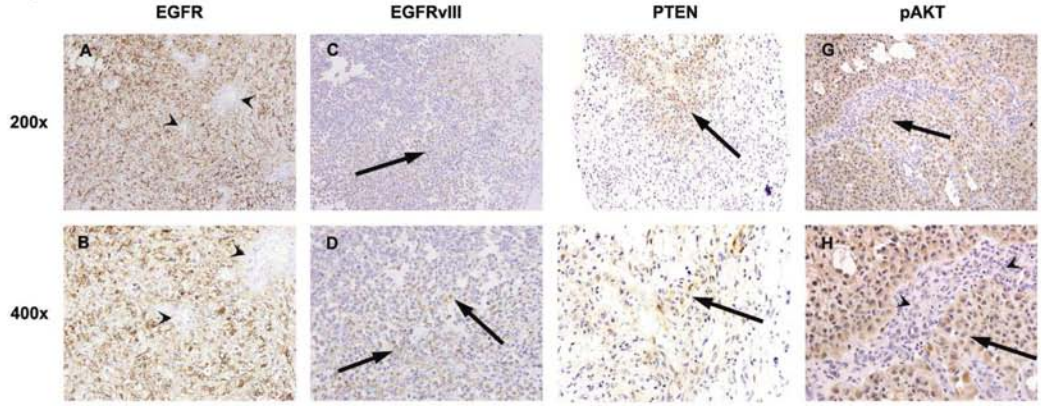

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Fig 3

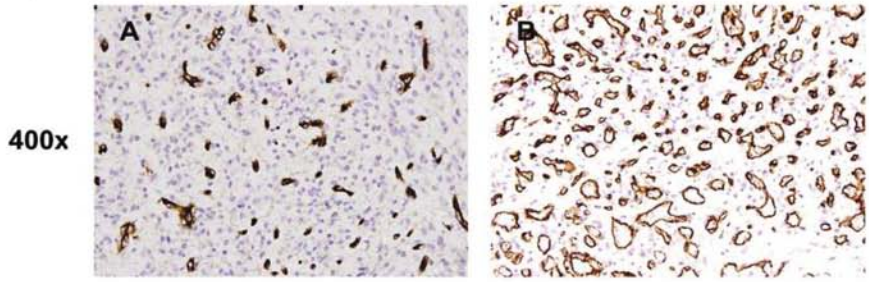

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Figure Legends, Supplementary

Fig. 1. Histograms showing the distribution of the staining values found for each biomarker evaluated.

Supplementary

Figure 1

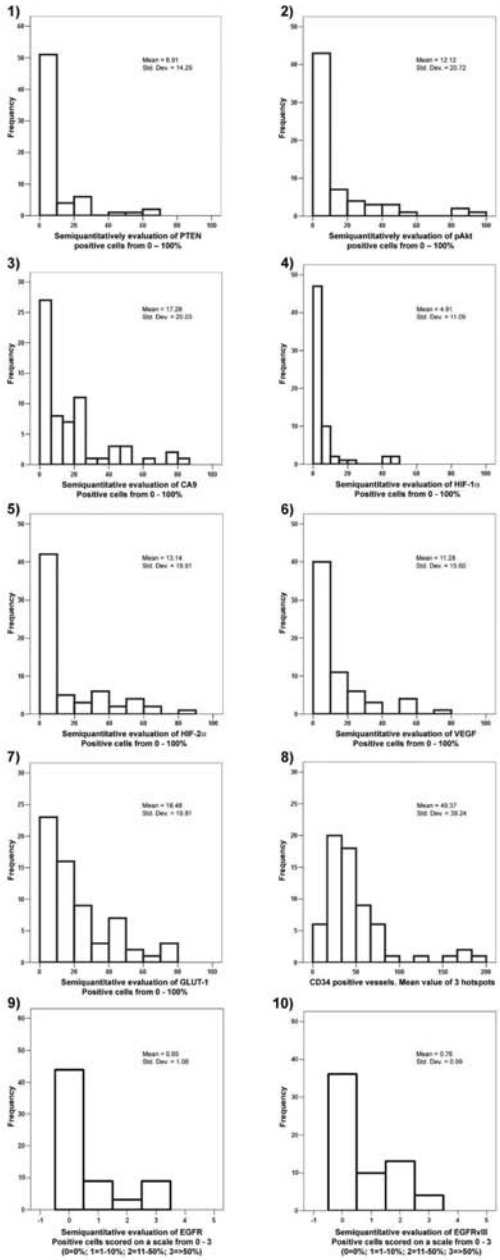

Table 1

	Regression analysis (CBI)		
	Response (n=29)	**PFS (n = 37)**	**OS (n=37)**
EGFR	OR: 0.98 95%CI, 0.54 – 1.78 *P* = 0.94	HR: 0.98 95%CI, 0.75 – 1.28 *P* = 0.89	HR: 1.02 95%CI, 0.77 – 1.34 *P* = 0.92
EGFRvIII	OR: 0.88 95%CI, 0.41 – 1.89 *P* = 0.74	HR: 0.92 95%CI, 0.64 – 1.33 *P* = 0.67	HR: 0.84 95%CI, 0.57 – 1.24 *P* = 0.38
PTEN	OR: 1.03 95%CI, 0.98 – 1.08 *P* = 0.22	HR: 1.00 95%CI, 0.99 – 1.02 *P* = 0.97	HR: 0.99 95%CI, 0.98 – 1.01 *P* = 0.72
pAKT	OR: 1.00 95%CI, 0.96 – 1.04 *P* = 0.97	HR: 1.00 95%CI, 0.99 – 1.02 *P* = 0.87	HR: 1.00 95%CI, 0.99 – 1.02 *P* = 0.35
HIF-1α	OR: 1.00 95%CI, 0.96 – 1.05 *P* = 0.87	HR: 0.99 95%CI, 0.97 – 1.02 *P* = 0.77	HR: 0.99 95%CI, 0.96 – 1.01 *P* = 0.29
HIF-2α	OR: 0.99 95%CI, 0.94 – 1.04 *P* = 0.61	HR: 1.01 95%CI, 0.99 – 1.03 *P* = 0.39	HR: 1.00 95%CI, 0.98 – 1.02 *P* = 0.78
VEGF	OR: 1.03 95%CI, 0.96 – 1.09 *P* = 0.40	HR: 1.01 95%CI, 0.98 – 1.03 *P* = 0.50	HR: 1.01 95%CI, 0.99 – 1.04 *P* = 0.37
CA9	OR: 1.00 95%CI, 0.97 – 1.04 *P* = 0.80	HR: 1.00 95%CI, 0.99 – 1.02 *P* = 0.96	HR: 0.99 95%CI, 0.98 – 1.01 *P* = 0.56
CD34	OR: 0.98 95%CI, 0.96 – 1.01 *P* = 0.29	HR: 1.00 95%CI, 0.99 – 1.01 *P* = 0.23	HR: 1.00 95%CI, 0.99 – 1.01 *P* = 0.98

Table 2

	Regression analysis (BI)		
	Response (n=21)	**PFS (n=21)**	**OS (n=24)**
EGFR	OR: 0.88 95%CI, 0.34 – 2-28 *P* = 0.79	HR: 1.17 95%CI, 0.75 – 1.82 *P* = 0.50	HR: 1.28 95%CI, 0.84 – 1.95 *P* = 0.26
EGFRvIII	HR: 2.09 95%CI, 0.85 – 5.17 *P* = 0.11	HR: 0.44 95%CI, 0.44 – 1.06 *P* = 0.09	HR: 0.73 95%CI, 0.48 – 1.09 *P* = 0.13
PTEN	OR: 1.05 95%CI, 0.96 – 1.16 *P* = 0.25	HR: 0.99 95%CI, 0.95 – 1.03 *P* = 0.53	HR: 0.99 95%CI, 0.95 – 1.03 *P* = 0.49
pAKT	OR: 1.02 95%CI, 0.98 – 1.05 *P* = 0.38	HR: 0.99 95%CI, 0.97 – 1.01 *P* = 0.38	HR: 0.99 95%CI, 0.97 – 1.01 *P* = 0.46
HIF-1α	OR: 0.87 95%CI, 0.59 – 1.28 *P* = 0.47	HR: 1.06 95%CI, 0.85 – 1.32 *P* = 0.63	HR: 1.01 95%CI, 0.82 – 1.24 *P* = 0.95
HIF-2α	OR: 0.95 95%CI, 0.89 – 1.02 *P* = 0.13	HR: 1.01 95%CI, 0.98 – 1.04 *P* = 0.71	HR: 1.00 95%CI, 0.97 – 1.03 *P* = 0.92
VEGF	OR: 1.01 95%CI, 0.97 – 1.06 *P* = 0.54	HR: 0.99 95%CI, 0.97 – 1.01 *P* = 0.39	HR: 0.99 95%CI, 0.96 – 1.01 *P* = 0.31
CA9	OR: 0.99 95%CI, 0.95 – 1.04 *P* = 0.81	HR: 0.99 95%CI, 0.97 – 1.03 *P* = 0.83	HR: 1.01 95%CI, 0.98 – 1.04 *P* = 0.73
CD34	OR: 1.04 95%CI, 0.99 – 1.08 *P* = 0.07	HR: 0.99 95%CI, 0.98 – 1.00 *P* = 0.09	HR: 0.99 95%CI, 0.98 – 1.00 *P* = 0.08

Table 3

	Regression analysis (CBI+BI)		
	Response (*n* = 50)	**PFS (*n* = 58)**	**OS (*n* = 61)**
EGFR	OR: 1.05 95%CI, 0.64–1.74 *P* = 0.84	HR: 1.07 95%CI, 0.86–1.34 *P* = 0.53	HR: 1.10 95%CI, 0.88–1.39 *P* = 0.39
EGFRvIII	OR: 0.78 95%CI, 0.45–1.36 *P* = 0.38	HR: 0.81 95%CI, 0.62–1-05 *P* = 0.11	HR: 0.79 95%CI, 0.61–1.05 *P* = 0.11
PTEN	OR: 0.97 95%CI, 0.93–1.01 *P* = 0.11	HR: 0.99 95%CI, 0.98–1.01 *P* = 0.82	HR: 0.99 95%CI, 0.98–1.01 *P* = 0.57
pAKT	OR: 0.99 95%CI, 0.97–1.02 *P* = 0.0.46	HR: 0.99 95%CI, 0.98–1.01 *P* = 0.53	HR: 1.00 95%CI, 0.99–1.01 *P* = 0.0.87
HIF-1α	OR: 0.99 95%CI, 0.95–1.05 *P* = 0.95	HR: 0.94 95%CI, 0.98–1.023 *P* = 0.94	HR: 0.99 95%CI, 0.97–1.02 *P* = 0.48
HIF-2α	OR: 1.03 95%CI, 0.99–1.07 *P* = 0.16	HR: 1.00 95%CI, 0.99–1.02 *P* = 0.74	HR: 1.00 95%CI, 0.99–1.02 *P* = 0.91
VEGF	OR: 0.92 95%CI, 0.95–1.02 *P* = 0.33	HR: 0.99 95%CI, 0.98–1.01 *P* = 0.58	HR: 0.99 95%CI, 0.98–1.01 *P* = 0.74
CA9	OR: 0.99 95%CI, 0.97–1.03 *P* = 0.95	HR: 0.99 95%CI, 0.99–1.01 *P* = 0.87	HR: 0.99 95%CI, 0.99–1.01 *P* = 0.83
CD34	OR: 0.99 95%CI, 0.98–1.01 *P* = 0.39	HR: 0.99 95%CI, 0.99–1.00 *P* = 0.34	HR: 0.99 95%CI, 0.99–1.00 *P* = 0.14

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Summary

Despite encouraging results using anti-angiogenic therapy in malignant glioma, only a subset of the patients receiving the anti-VEGF antibody bevacizumab, experience radiographic response or prolongation of survival.

In the current study, semiquantitative IHC analysis of biological markers involved in angiogenesis and hypoxia in addition to mediators of the EGFR pathway was performed. The aim was to identify biomarkers, which could be used as predictors of response and prolonged PFS upon treatment with CBI and BI. Tumor tissue was obtained from patients included in a previous phase II study (Manuscript III), treating recurrent primary GBM with the EGFR inhibitor cetuximab in combination with bevacizumab and irinotecan (CBI). Of the 37 patients with available tumor tissue, 29 were evaluable for response. We concurrently performed immunohistochemical stainings on tumor tissue from 24 GBM patients treated with bevacizumab and irinotecan (BI) (Manuscript II). Survival data were available from all patients included in the study.

As opposed to what has been found in other studies, none of the angiogenic-, hypoxia- or EGFR related biomarkers tested alone or in combination, could identify a patient population likely to benefit from either CBI or BI treatment in this material. It was not possible to confirm the association of VEGF and CA9 with radiographic response or survival respectively, found in the study by Sathornsumetee *et al.*[100] It was also investigated, if there could be created molecular profiles amongst the different the different markers, within or between the angiogenic-, hypoxic- and/or the EGFR related biomarkers. We found a tendency of correlation between the hypoxic related markers GLUT-1 and CA9, GLUT-1 and HIF-1α plus HIF-1α and CA9 respectively in both CBI and BI. This could suggest that GLUT-1, HIF-1α and CA9 share regulatory mechanisms. However, this hypoxic profile failed to predict response or PFS. None of the EGFR related biomarkers showed any significant correlations to each other.

Hence, there is still an urgent need for one or more reliable and reproducible biomarkers able to predict the efficacy of anti-angiogenic therapy.

4. Summarizing discussion and perspectivation

The development of targeted therapies designed to inhibit or block key cellular pathways in tumor growth have brought with it an increased awareness of the heterogeneity of the tumors and the ability of most tumors to bypass signaling pathway blockade. Accordingly, some tumors may be primarily resistant or could become resistant to therapies targeting a specific pathway. A multifaceted approach involving targeted inhibition of multiple signaling pathways may be more effective than inhibition of a single target and may help to overcome tumor resistance by blocking potential "escape routes". EGFR and VEGF could potentially be two key elements in the growth and dissemination of GBM tumors and accordingly an additive or synergistic tumor inhibiting effect might be achieved by targeting both concurrently.

4.1 Inhibition of EGFR is insufficient for reducing glioma cell growth *in vitro*

The frequent overexpression and/or amplification of EGFR and its correlation with a poor prognosis in GBM[12,13] in addition with the expression of the constitutive active EGFRvIII[14] have led to the assumption that EGFR is of pivotal importance in the pathogenesis of GBM. Furthermore, activated EGFR has shown to increase the expression of VEGF, thereby increasing neo-angiogenesis.[113,213]

Previous *in vitro* and *in vivo* studies with cetuximab using primary glioma cell lines that overexpress and/or are amplified for EGFR have shown reduction in cell viability.[90-92] At he initiation of the present study, there were conflicting reports of the clinical effect of EGFR inhibition in HGG[79-81], although a few indicated an effect of the TKIs erlotinib and gefitinib.[75,82] The importance of EGFR for maintenance of tumor growth, and the effect obtained when inhibiting EGFR needed further exploration. Therefore, the effects of cetuximab on glioma cell lines *in vitro* with respect to central intracellular signaling pathways downstream of EGFR important for cell survival and proliferation were further investigated in this study.

As described in Manuscript I, cetuximab did not induce an inhibitory effect on glioma cell viability, despite amplification and overexpression of EGFR in both PTEN-mutated and PTEN wild-type cell lines. The SKMG3 cell line used in Manuscript I is the only glioma cell line described in the literature with amplified EGFR.[214] In addition, we also tested the U87MG-EGFR glioma cell line (with stable transfected EGFR), however, neither SKMG3 nor U87MG-EGFR (which both are PTEN-mutated) responded to cetuximab. It must be emphasized that the results presented are

achieved from *in vitro* experiments which do not recapitulate the *in vivo* brain tumor environment or the cellular diversity within the tumor of origin. Tumor cell lines cultured for decades in the laboratory achieve alterations in gene expression, acquisition of additional mutations and are under the pressure of differential selection resulting in clonal expansion of a certain cell population during culture time.[215] Malignant gliomas cultured in normal cell culture conditions lose both cellular expression of EGFR and mitogenic dependence of EGFR, whereas gliomas maintained *in vivo* as xenografts maintain EGFR expression and dependency.[216] This could possibly explain the previously reported growth inhibition of cetuximab *in vitro* and *in vivo* when using primary glioma cell lines.[90-92] This might also explain the discrepancy with the study by Martens *et al*. who obtained tumor growth inhibition of glioblastoma spheroids implanted into the brains of nude mice, when treated with constant infusion of i.c. cetuximab.[217] The limited dependence of EGFR for maintenance of cell survival and proliferation was further strengthen by the observation that the glioma cell lines used in Manuscript I did not respond to the addition of the EGF ligand (unpublished results) as opposed to the cetuximab responsive head and neck cancer cell line (HN5), which demonstrated growth inhibition upon EGF stimulation at concentration above 1 nmol/l, an observation also made by others.[218]

Mutation of PTEN had been shown to mediate resistance to anti-EGFR treatment[74,77] and accordingly, PTEN was considered to be of importance for response to EGFR inhibition.[74-77]

Consequently, we also investigated effect of cetuximab in cell viability assays on the LN229 glioma cell line that is harboring wild-type PTEN and which is not amplified for EGFR. However, LN229 demonstrated the same lack of responsiveness to cetuximab as the PTEN mutated glioma cell lines investigated (Manuscript I). In addition, we tested an EGFR transfected LN229 glioma cell line (LN229-EGFR) previously used by Fan *et al.* who demonstrated erlotinib-induced inhibition of cell proliferation *in vitro*.[219] However, despite expressing wild-type PTEN and being amplified for EGFR, no inhibition of cell viability was observed for LN229-EGFR when treated with cetuximab (unpublished results). But these observations must be interpreted with caution as LN229-EGFR subsequently was tested positive for mycoplasma virus and accordingly not used for further experiments.

In Manuscript I both PTEN mutated and PTEN wild-type glioma cell lines were used, but the effect of cetuximab on cell viability and the activity of Akt (pAkt) was identical. This was opposed to a pronounced inhibition observed in the PTEN wild-type HN5 cell line used as a positive control in the experiments. There is no commercially available glioma cell line, with amplified EGFR and

wild-type PTEN. Since it has previously been shown that cetuximab inhibit cell viability in EGFR amplified glioma cell lines it was therefore attempted to stable express the PTEN gene in the EGFR amplified glioma cell line SKMG3. A plasmid containing CMV-PTEN and the selection marker G418 (neomycin) was transfected into SKMG3. However, only transient expression of wild-type PTEN lasting a few hours was obtained. Furthermore, after continuing exposure to the selection media containing G418, none of the CMV-PTEN transfected cells survived. The negative outcome of the experiment could be due to either incompatible conditions of having both amplified EGFR and wild-type PTEN in a glioma cell line *in vitro* or due to the lack of CMV-PTEN integration in the SKMG3 genome. However, the experiment remains inconclusive since no further investigation or attempt of stable transfection were performed.

Dose-response experiments showed inhibition of EGFR phosphorylation without affecting the activity of the downstream signaling pathways PI3K/Akt and Ras/Mek/Erk. However, direct inhibition of both PI3K/Akt and Ras/Mek/Erk signaling inhibited glioma cell viability, indicating both of these pathways to be of importance for survival of these cells. Accordingly, inhibition of cell survival through targeting signaling pathways downstream of EGFR is not obtained by inhibition of the EGFR alone. As demonstrated by Stommel *et al.* the use of three different TKIs (targeting EGFR, PDGFR-α, and mesenchymal epithelial transition factor (MET)) induced a considerably inhibition of viability in the U87MGvIII glioma cell line (which was PTEN-mutated and expressed EGFRvIII).[220] Moreover, they obtained complete inhibition of activated Akt (pAkt) when combining these three different TKIs, indicating that the growth-promoting effect of mutated PTEN can be overcome with multilateral treatment. This further supports the assumption that a multitargeted approach is essential to obtain tumor control.

In addition, as shown in Manuscript I, cetuximab failed to induce EGFR degradation in glioma cells and, to some extent, also blocked the receptor degradation induced by EGF. In conclusion it was shown that cetuximab failed to inhibit cell viability, inhibit downstream signaling pathways of EGFR and degradation of the EGFR *in vitro*.

- **Future perspective:** *In vivo* experiments investigating the activity of EGFR and downstream signaling pathways, using cetuximab and/or the TKIs erlotinib and gefitinib with and without the addition of other growth factor inhibitors (e.g. TKIs targeting PDGFR-α, insulin like growth factor 1 receptor (IGFR-1) or MET) for the treatment of primary human glioma xenografts on mouse. Subsequently investigate the expression of activated

EGFR (pEGFR) and downstream mediators (i.e. pAkt, pErk and PTEN), by e.g. immunohistochemistry (IHC) and western blotting.

4.2 Addition of the EGFR inhibitor cetuximab to bevacizumab and irinotecan did not improve response rate or progression-free survival

At the initiation of this study, standard treatment for GBM was debulking surgery if possible plus concomitant radiotherapy and temozolomide followed by adjuvant temozolomide.[7] Despite the improved survival obtained for GBM after introduction of this regimen, the median survival was still only 14.3 months.[7] At recurrence, the prognosis was even worse with a median survival of three to nine months when using traditional chemotherapeutic agents.[8,9] However, promising results started to emerge from reports describing the use of bevacizumab in combination with irinotecan (BI) for recurrent HGG. At Copenhagen University Hospital, Denmark, the first patients were treated with BI at the beginning of 2006 (Manuscript II).

As described above, a multitargeted approach is most likely necessary to obtain tumor control and prolonged survival. EGFR TKIs had been used in some clinical studies, and although the conclusion failed to be uniform, a few studies indicated an effect of erlotinib and gefitinib.[75,82] Moreover, knowing the frequent overexpression and/or amplification of EGFR, it was still generally accepted, that EGFR might be of importance in tumor growth and accordingly a promising target for GBM therapy. To improve the BI regimen and conceivably achieve a potential benefit of multitargeted therapy, a phase II trial was initiated for primary GBM with the first recurrence within six months of finishing standard therapy (Manuscript III). With the addition of cetuximab to the regimen of BI (CBI) the intention was to inhibit EGFR and thus proliferation, migration and survival of the tumor. Moreover, the aim was to achieve dual inhibition of VEGF and thereby angiogenesis, by inhibiting the EGFR induced transcription of VEGF using cetuximab and the VEGF interaction and activation of VEGFR using bevacizumab.

The regimen of CBI was feasible and induced two complete responses (5%) and nine partial responses (21%), which is comparable with most other studies using the BI regime.[200,202,203,206] However, due to a significant number of reported skin toxicities, a well known complication of cetuximab[221], and the fact that the results from this phase II trial was not superior to previous studies using BI, the conclusion was not to continue the use of cetuximab in combination with BI in e.g. a phase III study. Moreover, when taking into consideration the limited effect of EGFR inhibition observed in previous clinical studies with EGFR TKIs in HGG, and our results obtained

in vitro (Manuscript I), the importance of EGFR in maintaining of GBM tumor growth, might be questioned. However, as illustrated in Manuscript I and by other groups[219,220], the signaling pathways downstream of EGFR seem important for GBM tumor growth and could be potential targets in cancer treatment. Examples of different therapeutic compounds, targeting mediators downstream of EGFR, are illustrated in Figure 6.

- **Future perspective:** Addition of compounds targeting downstream mediators of EGFR (i.e. Akt, mTOR, Ras or Raf) with or without the addition of other growth factor inhibitors (inhibiting e.g. PDGFR or IGFR-1) used in combination with anti-angiogenic therapy in the clinic.

4.3 The use of anti-angiogenic therapy in the clinic

To date, the importance of irinotecan in the regime of BI still needs to be elucidated. The FDA approval of bevacizumab as monotherapy in recurrent GBM was based on two phase II studies from Kreisl *et al*.[205] and Friedman *et al*.[206] The first study demonstrated that the effect of bevacizumab as monotherapy in recurrent GBM was feasible and responses were comparable with previous reports using BI in recurrent GBM. In the second study, patients with recurrent GBM were randomized in a noncomparative phase II trial to bevacizumab alone or in combination with irinotecan.[206] The primary objective of this trial[206] was evaluation of safety and efficacy, and there was no intension of comparing the outcome of the two treatment groups, although it was observed that data did not indicate a treatment benefit of the addition of irinotecan. Subsequently, bevacizumab was FDA approved as monotherapy in recurrent GBM. However, a randomized phase III study (randomizing between bevacizumab versus bevacizumab and irinotecan) is still necessary to with certainty determine the effect of irinotecan in the regimen of bevacizumab in recurrent GBM. Even better would be a randomized trial also including irinotecan monotherapy as control, because phase III trials showing a survival benefit of bevacizumab in recurrent GBM still remains to be performed. For this reason, European Medicines Agency (EMEA) recently did not approve bevacizumab for this indication[#], in contrast to the FDA approval.

Several ongoing phase II trials are investigating the effect of bevacizumab in the primary treatment of GBM. One such study is currently recruiting patients in Denmark at Copenhagen University Hospital in cooperation with Odense University Hospital and Aarhus University Hospital. In this

[#] http://www.emea.europa.eu

phase II trial, patients are randomized to first line treatment with either neoadjuvant BI followed by concomitant radiotherapy and BI and subsequently adjuvant BI, or the same schedule but instead with bevacizumab and temozolomide (BT). The primary objective is response rate and feasibility, and secondary objective is PFS. The intension of this study is to investigate if the combination of BI could potentially be better than the combination of bevacizumab and temozolomide in first line treatment of GBM.

Moreover, there are two ongoing phase III trials, randomizing between the standard regime (temozolomide and radiotherapy[7]) or standard regime plus bevacizumab as first line treatment for GBM.[*] If the above mentioned phase II trial (BI versus BT) indicates a potential benefit of irinotecan, it should be carefully considered, if a future phase III trial should include the BI regime.

4.4 The difficulty in evaluating clinical response when using anti-angiogenic therapy

The effects of bevacizumab on tumor vasculature have given rise to challenges in response evaluation. Disruption of the BBB by the tumor results in increased accumulation of fluid and plasma proteins peritumorally and in the surrounding brain.[222] Because of the lack of lymphatic vasculature in the brain, and the fact that it is located in a confined space, the fluid leakage leads to increased interstitial pressure within the tumor and accumulation of fluid outside the tumor, resulting in vasogenic brain edema. Corticosteroids have been used for decades as temporary control of vasogenic brain edema, with moderate efficacy but also numerous side effects. The vascular normalization induced by anti-angiogenic agents like bevacizumab has shown to alleviate brain edema.[200,223,224] This “steroid effect” might also improve drug delivery.[225-227] However, the steroid effect from anti-angiogenic therapy gives rise to additional challenges when evaluating tumor load and response by MRI scan. The MacDonald criterias are still used for evaluation and definition of response to treatment.[228] These criterias are based on the WHO criteria using the contrast-enhanced largest cross-sectional area of tumor on CT or MRI scan in combination with corticosteroid use and changes in neurological function. However, enhancement is nonspecific and primarily reflects a disrupted BBB. Besides bevacizumab, enhancement can be influenced by changes in corticosteroid dose and radiologic technique.[229] Thus, when treating HGG with anti-angiogenic therapy like bevacizumab, the response to treatment observed may result at least partially from the bevacizumab induced normalization of abnormally permeable blood vessels and

[*] http://www.clinicaltrials.gov

not from anti-tumor activity.[223] Furthermore, anti-angiogenic treatment might control the contrast enhancing tumor more effectively than non-enhancing tumor, causing problems in interpretation of CT or MRI scan, as to if the reduction in contrast enhancement reflects a true anti-tumor effect.[230] This is also reflected in the overall survival data from patients treated with anti-angiogenic therapy, which fail to show prolonged OS in recurrent HGG despite a promising response rate and PFS.[231] Accordingly, other response measurements are needed in evaluation of tumor response in GBM taking into account both enhancing and non-enhancing tumor, the latter being best visualized on T2 weighted and fluid-attenuated inversion recovery (FLAIR) MRI sequences. In order to improve endpoints in clinical trials and response criteria, an international working party [Response Assessment in Neuro-Oncology](RANO)] has been established. However, recommendations are still to come.

- **Future perspective:** Establishing an adequate criterion of response, which provides more reliable indicators of outcome. This requires improved radiographic imaging, e.g. FLAIR, fluorodeoxyglucose (FDG)-positron emission tomography (PET), and/or MRI, and most likely a combination of the different imaging techniques.

4.5 Biomarkers as surrogate markers for clinical response

As a consequence of the development and use of targeted therapies, there is ongoing investigation for one or more biomarkers predictive for response and survival. In breast cancer, detection of overexpression or amplification of *HER2/neu* has proven to be predictive for response to the monoclonal HER2 antibody trastuzumab or the EGFR-HER2 TKI lapatinib.[232,233] One or more biomarkers predicting response and survival in GBM when using bevacizumab still needs to be discovered.

In Manuscript IV, prospective and retrospective IHC evaluations were made of biomarkers involved in angiogenesis and hypoxia in addition to EGFR/EGFRvIII and downstream related pathways. Some of these biomarkers have previously been investigated in a a small number of clinical studies.[75,82,100] Tumor tissue from patients included in the phase II study, treated with CBI (Manuscript III) was used for the analysis. In addition, retrospectively collected tumor tissue from most of the GBM patients described Manuscript II was analyzed as nearly all the patients had primary GBM, and most had progressed from standard treatment with radiotherapy and temozolomide. Besides the obvious difference in treatment (CBI versus BI), there were few

differences in the patient material. The CBI patients were all primary GBM, with tumor recurrence within six months of finishing standard therapy and no other tumor reductive interventions were allowed, except for tumor reductive surgery. Moreover, one of the inclusion criterias for the CBI protocol was available tumor tissue from either primary diagnosis and/or at recurrence. The intervening period from finishing first line treatment until unset of BI/CBI was slightly longer in the BI group. By choosing several mediators of angiogenesis, in combination with different markers of hypoxia the expectation and aim was to discover one or more biomarkers that could indicate treatment response and/or survival outcome.

VEGF and CA9 have been found to be associated with radiologic response and survival outcome in HGG patients treated with BI in one previous study.[100] These observations were however not confirmed in our patient material (Manuscript IV). The hypoxia markers HIF-1α and HIF-2α also failed to predict patient response or survival outcome. However, as illustrated in Table 1, the analysis did demonstrate correlations between some of the biomarkers, of which the three combinations of GLUT-1 and CA-9, GLUT-1 and HIF-1α and CA9 and HIF-1α were found to be consistent in CBI and BI. Although these observations need to be read with caution, they are indicative of a hypoxic molecular profile within the tumors analyzed and could indicate that hypoxia is unable to predict response to treatment in these regimens.

Treatment	Number of samples	Biomarkers	Correlation Coefficient	*P*
CBI	N = 37	HIF-1α – CA9	0.56	0.0003
CBI		GLUT-1 – CA9	0.84	<0.0001
CBI		GLUT-1 – HIF-1α	0.63	<0.0001
BI	N = 24	HIF-1α – CA9	0.58	0.0031
BI		GLUT-1 – CA9	0.52	0.0091
BI		GLUT-1 – HIF-1α	0.54	0.0062
BI		PTEN - EGFRvIII	0.52	0.0098
BI		VEGF – CA9	0.66	0.0005
CBI + BI	N = 61	HIF-1α – CA9	0.52	<0.0001
CBI + BI		GLUT-1 – CA9	0.73	<0.0001
CBI + BI		GLUT-1 – HIF-1α	0.58	<0.0001

Table 1: Significant or nearly significant correlations found between the biomarkers tested. From patients treated with cetuximab, bevacizumab and irinotecan (CBI) or bevacizumab

Because of heterogeneity and insufficiency of tumor vasculature in GBM tumors, hypoxia within the tumor can be chronic or acute (fluctuating) although the importance of this is not known with respect to survival and/or response to anti-angiogenic treatment in GBM. This could also influenze the potential information obtained from the hypoxic biomarkers used in this study, as their precise role in GBM when treated with anti-angiogenic compounds, needs to be revealed before they can be used as predictive biomarkers. The microvessel density (MVD) in tumors has been shown to be a valuable prognostic indicator for a wide range of tumors.[234-237] The endothelial marker CD34 used for counting MVD in hot spots was the only biomarker showing tendency towards predicting survival outcome or response, although not significantly. However, it most be emphasized that this

observation was only done in the BI group and accordingly could be due to a statistical Type 1 error. When using an anti-angiogenic agent for the treatment of a highly vascularized tumor as GBM, it is intriguing to assume that MVD would be of importance for response and hence could be used as a predictive biomarker. However, as emphasized in the review by Hlatky *et al*., MVD is not equivalent to the degree of tumor angiogenic activity and measurement of MVD is not predictive of tumor response under anti-angiogenic treatment.[238] Consequently the level of MVD in the tumor should not be used to decide which patients would benefit from anti-angiogenic treatment.

Since we used the EGFR inhibitor cetuximab in the CBI regimen, EGFR, EGFRvIII and mediators downstream from these (pAkt and PTEN), were also examined (Manuscript IV). The EGFR tyrosine kinase inhibitors erlotinib and gefitinib have been used in previous studies for the treatment of HGG, and EGFR as well as co-expression of EGFRvIII and PTEN have been shown to be predictive of response to treatment.[75,239] However, as demonstrated in Manuscript IV, none of the above-mentioned biomarkers could identify a patient population likely to benefit from the CBI treatment. The same result was not surprisingly obtained from the BI group.

The lack of positive predictive biomarkers could be due to the limited number of patients included in the study, resulting in insufficient statistic power. Moreover, the biomarkers investigated might not be representative for the underlying biological mechanisms inducing response (or no response) to anti-angiogenic therapy in GBM. This area still remains to be thoroughly investigated. In addition, it must be emphasized that most new drugs including the drugs (bevacizumab and cetuximab) used in Manuscript II-IV are often tested in recurrent disease, from which tissue is not available. Recurrent tumor may be different from the primary tumor in terms of genetic expression and relevance of specific targets. Thus correlative studies may not capture meaningful associations of e.g. biomarkers and response and/or survival data investigated in Manuscript IV.

In addition, the immunohistochemical methods used in this study and by others could be inadequate or imprecise for detection of the target requested. GBMs are known to be heterogeneous tumors and accordingly, the small tumor sample investigated might not be representative for the gross mass. The tumor tissue used in this study, was collected at four different institutions, and although similar techniques are used for tissue handling and formalin fixation, small differences in procedures could influence antigen preservation and thereby staining. Moreover, the protocols and antibodies used in this study are not equivalent to protocols used by other groups. This also emphasizes the difficulty of reproducing IHC observations in-between research groups. The inter- and intra-observer

variations were however not significant in our study, thus interpretation of the stainings seemed reproducible (Manuscript IV).

In the search of biomarkers that predict response and resistance to anti-angiogenic therapy, additional modalities are under investigation. This include systemic, circulating, tissue and imaging biomarkers (reviewed in[240]). However, as described above, the use of anti-angiogenic treatment has given rise to difficulty in establishing adequate criterias of response. Accordingly, this issue must be solved before any information regarding response can be used in combination with potential predictive biomarkers.

- **Future perspective:** Validation of the hypoxia and angiogenic related biomarkers in a larger material from HGG/GBM patients receiving anti-angiogenic therapy.

  Concurrently, confirmation of the staining in a randomized selection of tumor material previously investigated (CBI and/or BI) which would strength the validation of antibodies used, IHC technique and interpretation of staining

4.6 Resistance to VEGF pathway inhibitors is inevitable

VEGF pathway inhibitors induce only temporary tumor stasis or shrinkage but fail to produce enduring clinical responses in GBM.[241] Despite the frequent benefit of bevacizumab and other anti-angiogenic drugs used for treatment of HGG, tumor progression is inevitable.

The traditional concept of drug resistance involves mutational alterations of the target gene or alteration in the drug uptake and/or efflux resulting in treatment failure. This appears to be different when using angiogenic inhibitors, as the tumor functionally evade the therapeutic blockade of angiogenesis even though the specific therapeutic target remains inhibited. Instead, alternative pathways are activated resulting in angiogenesis and sustained tumor growth.[242]

In order to improve the effect of anti-angiogenic treatment and achieve improved OS, we need to understand this mechanism of resistance.[243] In a recent study by Lucio-Eterovic *et al*., it was demonstrated *in vitro* that bevacizumab was able to sequester the majority of secreted VEGF in glioblastomas.[230] In addition, it was observed that bevacizumab induced upregulation of several pro-angiogenic molecules *in vivo* (bFGF) and *in vitro* (i.e. bFGF, IL-1α, angiogenin and TGFα), which supports the idea that one of the reasons for lack of sustained effect from anti-angiogenic treatment is caused by upregulation of additional pro-angiogenic molecules. Another concern is that inhibition of angiogenesis leads to an infiltrative tumor growth pattern with co-option of existing

cerebral blood vessels thereby achieving vascular sufficiency.[244-249] Although not pathologically confirmed, this observation is supported in several clinical studies, which suggest an increased invasive growth on MRI scans from bevacizumab treated HGG patients.[201,250-252] In addition, it has been demonstrated in an *in vitro* model, that addition of bevacizumab induced an increased migration/invasion of glioma cells in a concentration-dependent manner. As glioma cells releases VEGF, this is suggesting that autocrine VEGF signaling blockade plays an important role in glioma cell invasion.[230] This could also be of importance in the resistance pattern observed upon anti-angiogenic therapy, thus reduced levels of VEGF gives rice to increased invasion. In addition, these findings also indicate that VEGF can influence glioma cells directly which are known to express both VEGFR-1 and VEGFR2[230,253] and that the effects are not restricted to the influence on endothelial cells. Moreover, Lucio-Eterovic *et al.* demonstrated both *in vitro* and *in vivo*, that bevacizumab treatment induced upregulation of invasion-related proteins (e.g. matrix metalloproteinase (MMP) -2, -9 and -12 and tissue inhibitor of metalloproteinases 1 (TIMP1)) which further supports the idea that GBM cells can escape from anti-angiogenic treatment by upregulating molecules that allow them to invade into surrounding brain areas.

The mechanism of resistance to anti-angiogenic therapy has been profoundly reviewed by Bergers and Hanahan[242] who suggest four adaptive mechanisms that induce resistance to anti-angiogenic therapy. The first two overrule the necessity of VEGF by (1) activation and/or upregulation of alternative pro-angiogenic pathways as mentioned above or (2) recruitment of bone marrow-derived pro-angiogenic cells.[244] Next, (3) the increased pericyte coverage of the tumor vasculature, which is known to occur[254,255], is serving to support its integrity, attenuating the necessity for VEGF-mediated survival signaling. Finally, and discussed above (4) they also state that inhibition of angiogenesis leads to an infiltrative tumor growth. This could originate from the activation and increased invasion of tumor cells into normal tissue, by co-option of normal blood vessels thereby achieving vascular sufficiency[241,244-249,256], and could explain the frequently observed decrease in neurological status, despite the relative stability of contrast-enhancing tumor on MRI scans.[257]

Furthermore, tumor recurrence could also originate from bCSC that are not known to be influenced by anti-angiogenic treatment. The self-renewing, multipotent and tumorigenic capacities of bCSC are yet another option for inducing tumor recurrence. In addition, bCSC are able to migrate throughout the brain parenchyma, which along with the above mentioned infiltrative growth induced by anti-angiogenic treatment might explain the diffuse recurrence pattern observed by MRI scan.

Thus, the absence of response to anti-angiogenic therapy could be due to intrinsic (pre-existing) resistance or reflect a rapid adaptation to the above-mentioned evasive resistance mechanisms.

4.7 Other inhibitors of angiogenesis

The effect of anti-angiogenic therapy, although transient, is indisputable. However, it seems that a more comprehensive approach targeting several different mediators of angiogenesis is needed in order to achieve tumor control. As mentioned in section 1.4.2, there are other pro-angiogenic mediators than VEGF, as e.g. Notch1, Dll-4 and bCSC. Since Notch signaling and especially Dll-4 are involved in tumor angiogenesis, it is likely that this pathway is involved in anti-VEGF resistance. As shown by Li *et al*., although initially responsive to bevacizumab, Dll-4-expressing U87MG glioma cells continued to grow at the same rate as control-treated tumors after terminating treatment.[163] Blocking Notch signaling by using a soluble form of Dll-4 reduced tumor burden and prolonged survival of the Dll-4 expressing tumors. Most importantly, soluble Dll-4 inhibited growth of both bevacizumab-sensitive and -insensitive tumors indicating that targeting Notch in addition to VEGF would result in improved treatment outcome. The effect of Dll-4 inhibition in cancer therapy is still premature, however, one anti-Dll-4 drug (REGN421) is tested in a phase I clinical trial for solid tumors.[*] Inhibition of Notch activation with the γ-secretase inhibitor MK0752 is also a new approach in the clinic currently used for young patients (3 to 21 years) with recurrent or refractory CNS malignacies in a phase I clinical trial.[*]

Detection, evaluation and the prognostic/predictive significance of Notch1, Dll-4, Nestin, Oct-4 and CD133 positive bCSC in the material used in Manuscript IV is currently underway (unpublished results). However, this area was not within the scope of the presented study and additional work and analysis remain to be performed in future studies.

- **Future perspective:** Evaluation Notch, Dll-4, Nestin, Oct-4 and CD133 positive cells in GBM tissue and concurrently investigation of prognostic and predictive significance of these biomarkers.

[*]http::// www.clinicaltrials.gov

5. Conclusion

Cetuximab did not induce any inhibitory effect on cell viability *in vitro* regardless of the EGFR expression level and in spite of the expression of wild-type PTEN in the glioma cell lines tested.

The observed lack of growth inhibition could be due ineffective inhibition of downstream signaling pathways from EGFR, despite the observed inhibition of activated EGFR (pEGFR). When inhibiting either the PI3K/Akt or Ras/Mek/Erk signaling pathways a significant growth inhibition was observed. Thus we concluded that these pathways are of importance for glioma cell viability although clearly their activity is not solely dependent on EGFR signaling. Consequently, a multitargeted approach targeting several different growth factors and/or downstream mediators is necessary in order to achieve a therapeutic effect.

The use of bevacizumab and irinotecan (BI) in recurrent GBM induced a substantial number of responses and prolonged PFS.

The addition cetuximab to the bevacizumab and irinotecan regime (CBI) did not improve the number of responses or the survival data obtained when comparing with BI. Accordingly, the CBI regime will not be used in the future for the treatment of recurrent GBM.

Immunohistochemical evaluation of hypoxia, angiogenesis and EGFR related biomarkers in tumor tissue from patients treated with either CBI or BI did not show any correlation with response or survival data. This could be a result of an insufficient technique, lack of statistic power or because the biomarkers investigated were not representative for the underlying biological mechanisms inducing response (or no response) to anti-angiogenic therapy in GBM.

The expression of EGFR should not be used for stratifying GBM patients most likely to benefit from anti-EGFR or anti-angiogenic treatment.

Despite frequent overexpression and/or amplification of EGFR, the importance of EGFR for tumor maintenance could be questioned, which is contrary to previous assumptions.

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6. Declaration of co-authorship

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Declaration of co-authorship

This declaration concerns the article: *Cetuximab insufficiently inhibits glioma cell growth due to persistent EGFR downstream signaling*

A part of the thesis: *Targeting the EGFR and VEGF signaling pathways in human glioblastomas, using cetuximab and bevacizumab*

submitted for the defense/obtainment of the PhD degree at the University of Copenhagen.

Name of the PhD student: Benedikte Hasselbalch

The proportion of the PhD students contribution to the article in question is evaluated from the following scale:

A. Has contributed to the co-operation (0-33%)
B. Has contributed considerably to the co-operation (34-66%)
C. Has predominantly executed the work independently (67-100%)

Declaration regarding specific elements (1.-4.)	Extent (A,B,C)
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable via experiments.	C
2. Planning of the experiments and methodology design, including selection of methods and method development.	C
3. Involvement in the experimental work.	C
4. Presentation, interpretation and discussion in a journal article format of the obtained data.	C

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Declaration of co-authorship

This declaration concerns the article: *Bevacizumab plus irinotecan in the treatment patients with progressive recurrent malignant brain tumours.*

A part of the thesis: *Targeting the EGFR and VEGF signaling pathways in human glioblastomas, using cetuximab and bevacizumab*

submitted for the defense/obtainment of the PhD degree at the University of Copenhagen.

Name of the PhD student: Benedikte Hasselbalch

The proportion of the PhD students contribution to the article in question is evaluated from the following scale:

A. Has contributed to the co-operation (0-33%)
B. Has contributed considerably to the co-operation (34-66%)
C. Has predominantly executed the work independently (67-100%)

Declaration regarding specific elements (1.-4.)	Extent (A,B,C)
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable via experiments.	A
2. Planning of the experiments and methodology design, including selection of methods and method development.	A
3. Involvement in the experimental work.	A
4. Presentation, interpretation and discussion in a journal article format of the obtained data.	B

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3. Involvement in the experimental work.	B
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This declaration concerns the article: *Prospective evaluation of angiogenic, hypoxic and EGFR related biomarkers in recurrent glioblastoma multiforme treated with cetuximab, bevacizumab and irinotecan*

A part of the thesis: *Targeting the EGFR and VEGF signaling pathways in human glioblastomas, using cetuximab and bevacizumab*
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