EGFRvIII : molecular insights and therapeutic potential

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EGFR overexpression and mutations in the EGFR have significant impact on tumor growth, treatment response, and patient survival, as outlined in **chapter 1**. Currently, EGFR deregulated tumors are treated with TKIs or monoclonal antibodies like Cetuximab. Cetuximab is designed to block ligand binding of the EGFR and binds domain 1 and 3 of this receptor. EGFRvIII, a commonly found mutation variant of EGFR, lacks the majority of domain 1 and 2. Despite this truncation we hypothesized that Cetuximab can also bind to EGFRvIII and can thereby be used to treat EGFRvIII expressing tumors. **Chapter 2** describes the binding of Cetuximab to EGFRvIII and its biologicals consequences in vitro.
Currently, EGFR expression levels, mutations, and its downstream effectors like k-RAS and PTEN are routinely evaluated for diagnosis and clinical treatment of patients. However, EGFRvIII is not routinely determent despite its clinical relevance[1,2] and its adverse effect on anti-EGFR therapy.[3] We therefore attempted to develop an EGFRvIII specific llama derived antibody suitable for in vitro and in vivo usage (**chapter 3**). Llama nanobodies (single domain heavy chain only antibody fragments) are in general suitable for both PET-imaging[4] and detection on cryosections.[5] The selected antibody had a high affinity for EGFRvIII but unfortunately also for wtEGFR.
In **chapter 4 en 5** the consequences of EGFR and EGFRvIII expression in vitro and in vivo were investigated to elucidate the biological relevance of their expression. Interestingly, EGFRvIII expressing cells were more resistant to stresses like serum starvation and hypoxia in vitro and EGFRvIII expressing tumors grew faster. We hypothesized that EGFRvIII expressing cells and tumors require autophagy for their enhanced growth and survival. Indeed, EGFRvIII expressing cells growth and survival advantage could be abrogated by autophagy inhibition. Additionally, radio resistant EGFRvIII expressing tumors could be radio sensitized through autophagy inhibition. Moreover, in a cohort of glioblastoma patients, conventional therapy supplemented with chloroquine improved the overall survival of these patients. Interestingly, this effect was even more pronounced in patients with an EGFRvIII positive tumor.
In **chapter 6** we assessed the relation between EGFR expression and autophagy. A positive correlation between EGFR and LC3b protein and mRNA

was observed in a panel of primary Head and Neck Squamous cell Carcinoma xenografts (HNSCC). Additionally, EGFR overexpressing glioma xenografts showed a reduction in growth when treated with chloroquine. Although in most tumors LC3b is predominantly expressed in hypoxic regions, LC3b expression in EGFR expressing tumors was predominantly located within the non-hypoxic regions. In contrast to non-EGFR expressing tumors, chloroquine administration lead to a large reduction in tumor growth suggesting that not only hypoxic cells, but also other cells that depend on autophagy, e.g. nutrient deprived cells are targeted. Taken together, inhibition of autophagy could therefore be explored as a novel treatment opportunity for EGFR and EGFRvIII overexpressing tumors.

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Chapter 1

General introduction

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EGFR signaling and autophagy dependence for growth, survival, and therapy resistance
B.Jutten and K.M.A.Rouschop

Abstract

The epidermal growth factor receptor (EGFR) is amplified or mutated in various human epithelial tumors. Its expression and activation leads to cell proliferation, differentiation, and survival. Consistently, EGFR amplification or expression of EGFR variant 3 (EGFRvIII) is associated with resistance to conventional cancer therapy through activation of pro-survival signaling and DNA-repair mechanisms. EGFR targeting has successfully been exploited as strategy to increase treatment efficacy. Nevertheless, these targeting strategies have only been proven effective in a limited percentage of human tumors.

Recent knowledge indicates that EGFR deregulated tumors display differences in autophagy and dependence on autophagy for growth and survival and the use of autophagy to increase resistance to EGFR-targeting drugs. In this review the dependency on autophagy and its role in mediating resistance to EGFR-targeting agents will be discussed. Considering the current knowledge, auto-phagy inhibition could provide a novel strategy to enhance therapy efficacy in treatment of EGFR deregulated tumors.

Introduction

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase within the ErbB family consisting of 4 members; EGFR (ErbB1, HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). ErbBs are typical receptor tyrosine kinases that were implicated in cancer in the early 1980s, when the avian erythroblastosis tumor virus was found to encode an aberrant form of the human epidermal growth factor receptor.[1] In many different cancer cell types, the ErbB pathway becomes hyperactivated by a range of mechanisms, including overproduction of ligands, overproduction of receptors, or constitutive activation of receptors.[2] In general, EGFR signaling is triggered by ligand binding to the extracellular ligand binding domain. This initiates receptor homo-/hetero-dimerization and autophosphorylation through the intracellular kinase domain, resulting in receptor activation. Following activation, cytoplasmic substrates are phosphorylated and initiate a signaling cascade that drives multiple cellular responses, including changes in gene expression, cytoskeletal rearrangement, apoptosis inhibition, and increased cell proliferation.[3,4] In cancer, EGFR signaling is often deregulated, leading to treatment resistance of the tumor and poor survival of patients. This deregulation is often mediated by overexpression (e.g., through gene amplification) and numerous mutations that lead to uncontrolled and sustained EGFR-signaling. Several EGFR targeting therapies have been developed (e.g., tyrosine kinase inhibitors (TKI) that inhibit EGFR signaling and antibodies that prevent EGFR expression and dimerization). Unfortunately, these therapies have only been proven effective in a limited percentage of cancer patients despite the presence of EGFR in many of the targeted tumors.[5] Novel strategies that, potentially combined with earlier EGFR-targeting agents, lead to enhanced cell killing are therefore still desired.

Current research has indicated that EGFR-deregulated cells and tumors display alterations in their autophagic response, a pro-survival mechanism that allows cells to recycle nutrients for energy- and macromolecule production.[6] Importantly: (1) EGFRderegulated cells seem to be more dependent on autophagy for growth and survival; and (2) resistance to EGFR-targeting agents can be reduced through autophagy inhibition, providing a potential novel

modality to target these tumors. In this review we highlight current knowledge that may provide insights as to why EGFR-deregulated cells display differences in autophagic responses and dependency on autophagy for survival and provide rationale for combining autophagy inhibition with conventional cancer therapy.

Gene Amplification and Overexpression

One of the most investigated alterations in the EGFR function is activation of signaling through increased gene copy number arising from amplification or polysomy.[7-9] Elevated EGFR expression is a strong prognostic indicator in head and neck, ovarian, cervical, bladder, and esophageal cancer. In gastric, breast endometrial, and colorectal cancers (CRC) EGFR expression is a modest predictor. This in contrast to non-small cell lung carcinoma (NSCLC), where increased EGFR expression rarely has a prognostic value.[10]

EGFR mutations often determine the responsiveness of tumors to EGFR inhibitors; this is often related to the dependency of cancer on continued oncogenic signaling (oncogene addiction). For a number of different oncogenes, data supporting addiction in tumors have been gathered.[11,12] For EGFR in particular, positive results in clinical trials with different antagonists have been considered as clinical evidence of oncogene addiction, even though the clinical benefits from the use of either monoclonal antibodies (mAbs) or TKIs have been limited.[5] Only a small portion (9-20%) of tumors with hyperactive EGFR signaling is exquisitely sensitive to such specific inhibitors.[13-15] This percentage is much higher (88-94.1%) when sensitizing mutations (e.g., L858R) in the EGFR gene are present.[16,17] In NSCLC and CRC, increased EGFR gene copy number has been associated with increased clinical efficacy of EGFR antagonists erlotinib and cetuximab.[18] Both drugs have shown clinical promise, and the anti-EGFR antibody cetuximab is used in treatment of head and neck squamous cell cancer (HNSCC) and CRC. Despite clinical gain, both intrinsic resistance and the development of acquired resistance have been observed.[19]

The Tyrosine Kinase Domain

Both mutations associated with drug resistan,ce and sensitivity have been described within the tyrosine kinase (TK) domain of EGFR in subsets of NSCLC, rare cases in HNSCC, CRC, small cell lung carcinomas (SCLC), ovarian, esophageal, and pancreatic cancers.[20] Distribution of mutations is not random and may be related to cancer etiology. For instance, in NSCLC the incidence of EGFR mutations among clinical responders to gefitinib or erlotinib is 77%, compared with only 7% in NSCLC cases that are refractory to tyrosine kinase inhibitor (TKI) treatment.[20] Multiple studies have shown differences in treatment outcome associated with EGFR mutations. For example, mutations in exon 18 (nucleotide-binding loop), accounting for 5% of the mutations, are usually amino acid substitutions that contribute to drug sensitivity. Mutations in exon 19 are characterized by small in-frame deletions and account for 45% of EGFR mutations, making it the most prominent EGFR kinase domain mutation in NSCLC. These tumors are, in general, sensitive to TKIs like gefitinib and erlotinib.[20] The L858R substitution in exon 21, within the activation loop of EGFR, comprises approximately 40-45% of EGFR mutations. Tumors harboring the L858R mutation are, in general, sensitive to TKIs, although some clinical studies have shown that these tumors are not as responsive in comparison to tumors with deletion mutations in exon 19.[20]

EGFR exon 20 mutations, typically located after the C-helix of the tyrosine kinase domain, may account for up to 4% of all EGFR mutations, with the T790M substitution as the most prominent one (up to 50% of all mutations in exon 20). This T790M mutation is considered an acquired mutation and converts TKIsensitive tumors into (reversible) TKI-resistant tumors.[21] Like the T790M mutation, other exon 20 mutated proteins are resistant to clinically achievable doses of reversible (gefitinib, erlotinib) and irreversible (neratinib, afatinib, PF00299804) TKIs in preclinical models.[22] Growing clinical experience with tumors harboring EGFR exon 20 insertions correspond with the preclinical data; only few patients have shown responsiveness to EGFR TKIs.[22]

EGFRvIII

In a significant proportion of tumors, amplification of the EGFR gene is accompanied by rearrangements, although amplification is not mandatory for gene rearrangement.[23] The most abundant rearrangement is a deletion variant that lacks exon 2–7 of the extracellular domain, yielding a constitutively active receptor, EGFRvIII or Δ2–7.[24-26] This mutation is most prevalent in malignant gliomas (20–30% in unselected patients with a glioblastoma multiforme [GBM] and 50–60% in patients whose tumors show amplification of wild-type EGFR).[27] Recent studies identified EGFRvIII in head and neck squamous cell carcinomas (~21%),[28] squamous cell carcinomas of the lung (~5%),[29,30] and breast (~5%),[31] suggesting broader implications to human cancer.[32] EGFRvIII is known to contribute to radio resistance of tumor cells[33] at least in part through enhanced repair of DNA doublestrand breaks.[34] Additionally, EGFRvIII expression is associated with resistance to gefitinib and leads to sustained EGFR signaling and AKT activity.[35] Furthermore, the tumor microenvironment, and in particular tumor hypoxia, significantly contributes to therapy resistance.[36-38] Expression of EGFRvIII provides cells with a survival advantage when exposed to stresses such as hypoxia and nutrient starvation.[39] Although EGFRvIII expression is frequently observed in GBM, culturing GBM cells in vitro will lead to a rapid loss of EGFRvIII expression,[40,41] and thus complicates assessment of EGFRvIII-targeting strategies in GBM. Researchers therefore often use cell lines that artificially express EGFRvIII. Although informative, these cell lines have their limitations as, unlike in primary GBM (a range of 1–100% EGFRvIII positive cells in GBM is observed),[23] all cells will express EGFRvIII. Furthermore, the heterogeneous expression levels observed in GBM are difficult to mimic due to the use of artificial promoters; in addition, the cells were established without EGFRvIII and are thus not dependent on EGFRvIII for growth and survival. To maintain EGFRvIII expression in vitro, cells could be cultured under stem cell culture conditions.[42] Alternatively, EGFRvIII expression is also maintained when primary tumors are xenografted subcutaneously on mice[42] and should be considered in validating results obtained in transgenic models.

For GBM patients, EGFR overexpression is a significant prognostic value for predicting survival, and the expression of EGFRvIII with EGFR amplification

plays an important role in enhanced tumorigenicity. EGFRvIII overexpression in the presence of EGFR amplification is the strongest indicator for poor survival prognosis in 2 large cohorts of patients. Shinojima and colleagues found in a cohort of 87 patients that EGFRvIII expression, assessed by immunohistochemistry (IHC), was not a predictor for overall survival (OS). However, in patients with EGFR amplification, multivariate analysis revealed that EGFRvIII expression was an independent, significant, poor prognostic factor for OS (P = 0.0044, HR = 2.71).[23] These findings were endorsed by Pelloski et al.,[43] who observed that the median survival of a patient group with EGFRvIII expression (n = 36, assessed by IHC) was reduced from 85 to 47 wk compared with EGFRvIII-negative patient group (n = 81). In contrast, Montano et al.[44] showed, in a cohort of 73 patients, that EGFRvIII (assessed by reverse transcription-PCR) is a molecular predictor of improved overall survival (P = 0.0023, HR = 2.59) in GBM patients treated with surgery followed by adjuvant radiotherapy and temozolomide (TMZ). This discrepancy could potentially be explained by the EGFRvIII detection method. Montano used the more sensitive RT-PCR, whereas Pelloski and Shinojima used IHC and may have missed very low levels of EGFRvIII expression. Another possible explanation for the differences could be the uniformness of the patient group. Montano used patients that all underwent surgery, radiotherapy, and TMZ treatment, whereas the other cohorts were treated more heterogeneously. Furthermore, all patients in Pelloski's study were wild-type for YKL-40 (a Ras activator), were Montano does not discriminate between Ras activator status, and the Karnofsky performance status (KPS score) of the patients in Pelloski's and Shinojima's cohort was much higher.[23,43,44] Taken together, more and lager cohorts with uniform treatment are required to gain additional insight in the clinical relevance of EGFRvIII.

Cancer Stem Cells

Recent data showed that EGFR and EGFRvIII signaling are involved in maintaining a cancer stem cell (CSC) phenotype. In glioblastoma, both the EGFRpos and EGFRneg tumor-initiating cells (TICs) derived from primary GBM can give rise to experimental tumors. However, the EGFRpos TICs displayed

enhanced tumorigenic potential and highly invasive behavior.[45] Conversely, EGFRneg TICs formed tumors with low efficiency and needed to re-upregulate their EGFR expression to become tumorigenic. These “potential” CSCs might be kept in a dormancy-like state by EGFR downregulation and be reactivated when exposed to stimuli of the in vivo tumor microenvironment.[45] Indeed, GBMs that were EGFRneg in origin expressed EGFR on recurrence.[46] The idea of EGFRpos and EGFRneg CSC is further supported by the finding that CSC propagation is possible in the absence of exogenous growth factors (like EGF), suggesting that EGF signaling is not critical for GBM CSC maintenance.[47] In contrast, EGFR signaling is required for GMB CSC proliferation,[48,49] and gefitinib treatment decreases CSC number in nasopharyngeal carcinoma models.[50] In this study, cisplatin-treated tumor cells regrew rapidly upon re-implantation, whereas regrowth of gefitinib-treated tumor cells was severely diminished.[50] Furthermore, Clark et al.[51] showed that GBM CSC lines displayed tumor-initiating capacity after EGF withdrawal or cetuximab treatment by compensatory activation of ErbB2 and ErbB3, suggesting a resistance mechanism for EGFR-targeted therapy. Lapatinib, a dual EGFR/ErbB2 inhibitor, treatment inhibited CSCs proliferation, indicating that a simultaneous blockade of multiple ErbB family members could be needed for more efficient GBM treatment.

In relation to EGFRvIII in CSC, a population of the cells derived from pediatric diffuse intrinsic pontine gliomas (DIPG) neurospheres displayed co-expression of the CSC marker CD133 and EGFRvIII.[52] In another study, EGFRvIII expression on invasive breast cancer carcinomas resulted in increased expression of genes related to self-renewal and epithelial–mesenchymal transition, along with a higher percentage of CSC-like cells.[31] Furthermore, Liu et al.[53] showed that the CD133+ fraction of GBM exclusively expressed EGFRvIII, whereas wild-type EGFR was not detected. These data indicate a role for EGFRvIII in the propagation of CSC that could explain the relative therapy resistance of EGFRvIII tumors.

EGFR Signaling Pathways Implicated in Autophagy

After ligand binding by EGFR or constitutive signaling by EGFRvIII the

activation of several parallel pathways has been described. These include: (1) activation of the PI3K-AKTmTOR pathway; (2) increased Ras and (3) STAT3 signaling; and (4) Beclin1 (Fig. 1).[54] All pathways involved in autophagy regulation.

Autophagy is a catabolic process that allows cells to recycle cellular components through degradation by the lysosomal machinery.[55,56] Autophagy is an evolutionarily conserved process that results in the targeting of cellular proteins and organelles to lysosomes for degradation. Autophagy serves to regulate normal organelle turnover and the removal of those with compromised function to maintain cellular homeostasis. Additionally, autophagy is a survival mechanism during periods of metabolic stress, where self-digestion provides an alternative energy source and facilitates the disposal of unfolded proteins.[57-60]

Previously, we and others showed that cells with deregulated EGFR signaling display differences in autophagic response.[61-63] Interestingly, EGFR expression represses autophagy activity. For example, EGFR reduction by siRNA treatment leads to an induction of autophagy activity in prostate cancer cells.[63] Furthermore, induction in autophagy was observed after targeting with TKIs or cetuximab.[64] Recently, in a panel of HNSSC xenografts, we observed a correlation between EGFR and expression of the autophagy marker Lc3b,

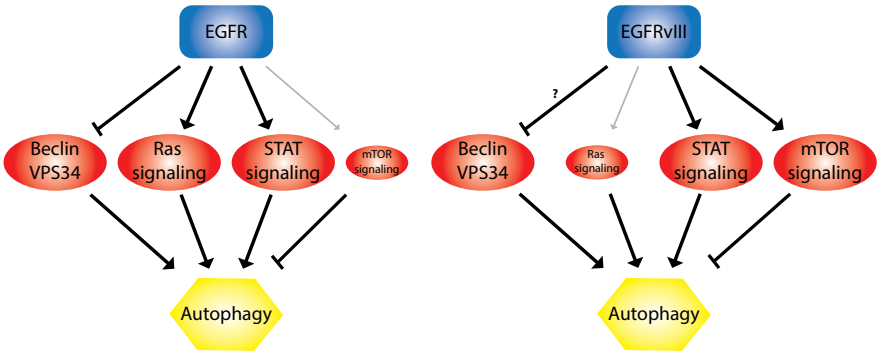

Figure 1. EGFR- and EGFRvIII-signaling pathways associated with auophagy regulation. Both receptors signal through all 4 pathways; nevertheless, EGFR preferentially signals via the RAS pathway, whereas EGFRvIII predominantly uses mTOR signaling.

suggesting a close interplay between EGFR signaling and autophagy. This correlation is most likely mediated through controlling Lc3b protein production, as this correlation was also observed on the mRNA level.[61] This was further confirmed in a panel of cell lines, where EGFR expression negatively correlated with autophagic flux, as determined by Lc3b-turnover. Interestingly, the suppressive activity of EGFR in these cells can be independent of its kinase activity[65] and mediated through maintaining high glucose levels through association with sodium/glucose cotransporter 1 (SGLT1).[63] In addition, EGFR can suppress autophagy dependent on its kinase domain through maintaining high activation of the PI3K/Akt/mTOR pathway.[66] Furthermore, EGFR activity results in inhibition of autophagy through inhibition of beclin1,[62] a potent inducer of autophagy. Together these data indicate that the expression of EGFR is closely related to expression of autophagic markers and autophagic activity of cells.

Although the effect of EGFR seems to be mostly autophagysuppressive, in constitutive EGFR-signaling cells the effect on autophagy activity is less pronounced during normal conditions and seems to be stimulatory during metabolic stresses. For example, in stably transduced glioblastoma cell lines and prostate cancer cells that express EGFRvIII, a faster and more pronounced autophagic response during starvation or severe hypoxia is observed (unpublished data). The enhanced autophagic response provides these cells with survival and growth advantage.

The suppressive action of EGFR on autophagy activity and the opposing action of EGFRvIII during stressful conditions could result from signaling via different signal-transduction pathways. For example, Wolf-Yadlin et al.[67] showed that EGFR predominantly signals via Erk1, Erk2, and STAT3, whereas EGFRvIII favors signaling via the PI3K and STAT3 pathway.[68,69] This difference in signaling preference of these pathways associated with autophagy activity is likely to result in differences between EGFR and EGFRvIII.

EGFR–PI3K–AKT–mTOR Pathway

Activated EGFR binds GRB2-associated binding protein 1 (GAB1) together with growth factor receptorbound protein 2 (GRB2) to recruit phosphoinositide-3-

kinase (PI3K). PI3K phosphorylates PI(4,5)P2 (phosphatidylinositol) into PI(3,4,5)P3. This process is negatively regulated by phosphatase and tensin homolog (PTEN). 3-phosphoinositide dependent protein kinase-1 (PDK1) brings v-akt murine thymoma viral oncogene homolog 1 (AKT) to the plasma membrane, where PIP3 is located, to phosphorylate and activate AKT. AKT subsequently activates mTOR (mammalian target of Rapamycin).[54] mTOR, a central growth regulator downstream of oxygen, energy, nutrient, and growth factor signaling, inhibits autophagy. Hence, insufficiency in either results in mTOR inhibition and rapid induction of autophagy in most systems. In conditions of nutrient sufficiency, high mTOR activity prevents Unc-51-like kinase (ULK1) activation by phosphorylating ULK1 Ser757 and disrupting the interaction between ULK1 and 5′ AMP-activated protein kinase (AMPK), thereby preventing ULK1 to initiate an autophagy activating complex with FIP200 and ATG13.[70,71] During periods of starvation, mTOR dissociates from the ULK1 complex, leading to less ULK1 phosphorylation, and increases ULK1 kinase activity.[72,73] Recently, a role for ULK1 activation for survival of hypoxic cells was identified.[74,75]

EGFR–RAS Signaling Pathway

The RAS oncogene is a member of small GTPase family involved in the regulation of cell survival and growth and is frequently activated in cancer.[76] Next to frequently detected activating mutations in RAS, growth factor signaling, e.g., through EGFR, can lead to uncontrolled RAS signaling. After auto-phosphorylation, the adaptor protein growth factor receptor-bound protein 2 (GRB2) binds EGFR at the phosphorylated sites and activates Son of sevenless (SOS), a GTP-exchange factor for RAS. SOS then converts RAS-GDP into active RAS-GTP. Several studies have implicated RAS activity in the induction of autophagy, as displayed by a high autophagic flux after oncogenic RAS transformation.[77] Increased autophagy in these cells is required to sustain a high metabolic rate, to prevent accumulation of damaged mitochondria, reduce oxygen consumption, and to prevent metabolic substrate depletion.[77-79] In relation, autophagy inhibition in RAS transformed cells leads to enhanced cell killing during nutrient deprivation.[77] Furthermore, it has been shown that

RAS plays a role in regulating the redox state of the cell, and that constitutive production of ROS correlates with RAS-induced cell transformation[80,81] and mediates autophagy induction through activation of protein kinase 8 (JNK) and subsequent upregulation of ATG5 and ATG7.[80]

EGFR–STAT3 Signaling Pathway

The third main signaling mediator downstream of activated EGFR is the signal transducer and activator of transcription (STAT3) protein. STAT3 belongs to a family of at least 7 transcription factors that share conservation in coiled-coil, SRC homology (SH2), and DNA-binding domains.[82] STAT3 is a latent transcription factor present in the cytoplasm of cells. Phosphorylation at Y705, is mediated through activation of several transmembrane receptors, such as EGFR,[83] and is required for transcriptional activity or transactivation of members of the Janus kinase (JAK) protein family.[84] Phosphorylation leads to dimerization, nuclear translocation, DNA binding, and gene activation.[85] Recently, STAT3 has been recognized as a new autophagy regulator through suppression of PKR.[86] Shen et al.[86] proposed that in normal conditions, latent cytoplasmic STAT3 binds to protein kinase R (PKR), inhibiting its activity, and reduces autophagy levels through eIF2α inhibition, a signaling cascade involved in both transcriptional and translational regulation of Lc3b and ATG5 production.[60] Hence, STAT3 phosphorylation leads to homodimerization and enables the free PKR to phosphorylate eIF2α via direct interaction between STAT3 and PKR.[87] Furthermore, STAT3 controls the expression of several autophagy-associated proteins, including BCL-2, Bcl-XL, and MCL-1,[88,89] which inhibit autophagy through sequestration of Beclin 1.[60]

EGFR-Beclin 1

Beclin 1 is a coiled-coil protein involved in the regulation of autophagy in mammalian cells and is a component of the class III phosphatidylinositol-3-kinase (PI3K) complex.[90] Beclin 1 promotes autophagy, and cells with reduced Beclin 1 expression exhibit reduced autophagic activity.[91] Beclin 1 is an essential gene for early embryonic development and is a haploinsufficient tumor

suppressor.[92] Intriguingly, Beclin 1 is tumor suppressive in breast cancer cells; mice that have only one functional allele of Beclin 1 display higher incidence of spontaneous tumors, and mono-allelical deletions of Beclin 1 have been described for 40–75% of human ovarian, breast, and prostate cancers.[91,93-95] Beclin 1 may also promote survival as an interacting partner of an anti-apoptotic protein Bcl-2.[96] Binding of Bcl-2 to Beclin 1 inhibits Beclin 1-dependent autophagy and Beclin 1-dependent autophagic cell death.[91,97] Recently, it was shown that EGFR phosphorylates Beclin 1 at 3 different tyrosine residues, Y229, Y233, and Y352, after activation by EGF. This tyrosine phosphorylation favors the formation of Beclin 1 dimers, which are incapable of VPS34 binding, and results in reduced autophagy activation (Fig. 1).[62]

EGFRvIII Tumors Require Increased Metabolism

Why EGFRvIII-expressing tumors require higher activation of autophagy during metabolic stress remains unclear, but could be related to the higher proliferation rate and associated nutritional demand. For example, Guo et al.[98] showed that EGFRvIII expression induces major shifts in GBM cell metabolism. Uptake of 18FDG in EGFRvIII-expressing U87 xenografts was doubled compared with volume matched control xenografts. In relation, gene expression arrays showed upregulation of genes involved in regulation of the cell metabolism, e.g., glucose transporter 1 (GLUT1) and GLUT3, Hexokinase2 (HK2), and pyruvate dehydrogenase kinase (PDK1).[99] In general, EGFRvIII-expressing tumors require upregulation of cell metabolism proteins and require increased glucose uptake to maintain their elevated growth rate. This might explain why these tumors may display increased dependence on autophagy for their energy supply in a tumor microenvironment that is low in glucose or deprived of oxygen.

EGFR Mediates Mitochondrial Homeostasis

In relation to the involvement of EGFR in cell metabolism, Rasbach et al. showed the involvement of EGFR in mitochondrial biogenesis after oxidant injury via EGFR-dependent p38 MAPK activation of the mitochondrial

biogenesis regulator PPAR-γ cofactor-1α (PGC-1),[100] allowing the cells to maintain high metabolism and their increased proliferation rate.

Additionally, EGFR is involved in stabilizing mitochondria and preventing apoptosis. Synergistic interaction between EGFR and c-Src via phosphorylation of EGFR at Y845 causes translocation to the mitochondria. There, it interacts with cytochrome c oxidase subunit II (COX II) and prevents apoptosis. This seems independent of EGFR kinase activity but is enhanced by EGF treatment.[101,102] Although cells did not undergo apoptosis, ATP production was drastically reduced by binding of EGFR to COX II.[102] Similar mechanisms and translocation to the mitochondria to antagonize apoptosis have been observed for EGFRvIII.[103,104] COX II inhibition by EGFR leaves the cell with reduced ATP production after insults such as chemo- and radiotherapy or starvation and must revert to other sources for their ATP production. Autophagy may provide an alternative source for energy production, and could be exploited therapeutically in stressed cells with EGFR overexpression. This could also explain why EGFR-expressing cells are more dependent on autophagy for their survival.[61]

EGFR, Treatment Resistance, and Therapeutic Potential of Autophagy Inhibition

EGFR expression or mutations contribute to tumor treatment resistance. For instance, acquired mutations in the kinase domain of EGFR (like the T790M) can abrogate the susceptibility to TKIs like gefitinib or erlotinib.[21] Furthermore, EGFR contributes to radiotherapy resistance either through activation of the pro-survival pathway PLCγ-PKC-RAF[105] or through activation of DNA repair via DNA-PK.[106] We have also shown that expression of EGFRvIII contributes to stress resistance typical for the tumor microenvironment, including nutrient deprivation and hypoxia.[39] Hypoxia is a common feature of tumors and an important contributor to malignancy and treatment resistance,[36,37,107] and in HNSCC, the degree of hypoxia is the most significant factor explaining variability in survival.[37] Targeting hypoxia in pre-clinical models has been shown to sensitize tumors to therapy through various modalities.[60,108,109] Importantly, a metaanalysis in HNSCC demonstrated therapeutic benefit of hypoxia modification.[110] Tumor cells adapt to hypoxia through multiple mechanisms,

including activation of autophagy.[6,60,111-115] Genetic and pharmacological inhibition of autophagy sensitizes human tumor cells to hypoxia, reduces the fraction of viable hypoxic tumor cells, and sensitizes human tumors xenografts to irradiation (Fig. 2A).[60] In relation to EGFR expression, although we showed reduced autophagic flux in cells expressing EGFR, these cells were already under normal conditions dependent on autophagy for proliferation and survival.[61] In general, EGFR-expressing tumors are considered highly radioresistant;[116] also in our setting, a large dose irradiation had only a minor effect on tumor delay. Interestingly, chloroquine administration to inhibit autophagy led to a large delay in tumor growth that exceeded the effect of irradiation and, in addition, sensitized tumors to irradiation.[61]

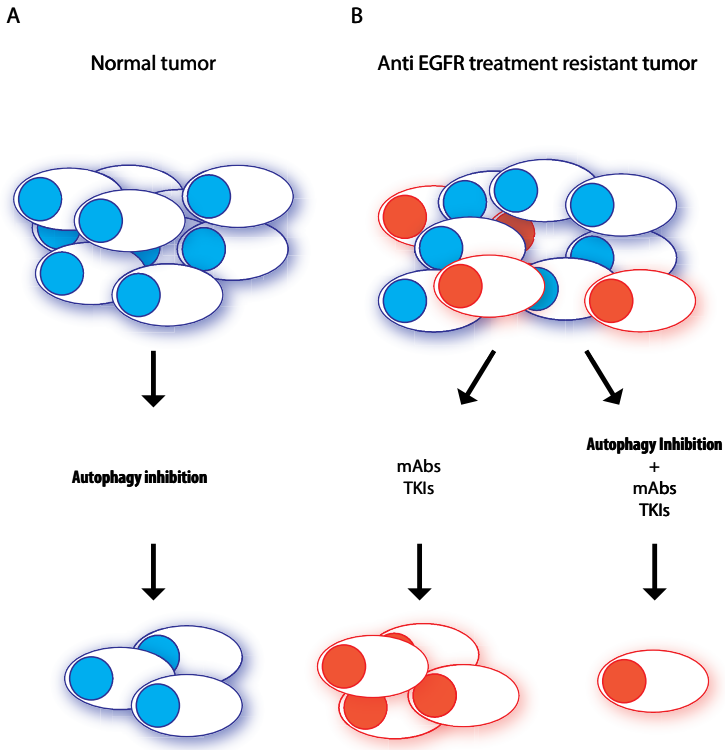

Figure 2. (A) In EGFR-deregulated tumors, inhibition of autophagy leads to increased cell killing of metabolic stressed cells. (B) Resistance of tumor cells with active EGFR signaling to monoclonal antibodies (mAbs) or tyrosine kinase inhibitors (TKIs) can be reduced by autophagy inhibition.

Recent data showed that EGFR and EGFRvIII signaling is involved in maintaining a CSC phenotype, and recently it was shown that autophagy is important for CSC self-renewal and tumorigenic potential in breast cancer stem cells,[117] and for regulation of energy metabolism and migration and invasion of GBM-derived stem cells.[118] Taken together, these data suggest that autophagy and EGFR or EGFRvIII signaling are very important in CSC and could therefore be considered for dual targeted therapy for treatment of CSCs in patients. Why EGFRand EGFRvIII-expressing cells and tumors are more sensitive to chloroquine treatment remains matter of investigation.

Clinical efficacy of anti EGFR drugs to date has been limited by both acquired and intrinsic resistance. Cancer cells adapt rapidly to EGFR inhibition treatment, resulting in only a small success rate for EGFR inhibition as mono therapy in cancer treatment[119,120] (Fig. 2B). Nevertheless, inhibition of EGFR signaling in combination with autophagy inhibition looks promising in vitro. In NSCLC cell lines with activating EGFR mutation (exon 19 deletion) erlotinib induces both apoptosis and autophagy. Inhibition of autophagy can further enhance sensitivity to erlotinib in these NSCLC cells, suggesting that autophagy serves as a protective mechanism.[121] Moreover, wild-type EGFR-expressing NSCLC cells' resistance to erlotinib can be abrogated through autophagy inhibition.[122] Furthermore, ZD6474, a small molecule inhibitor of VEGFR, EGFR, and RET induces apoptosis in 2 glioblastoma cell lines, which can be enhanced by the inhibition of autophagy.[123] These findings suggest a therapeutic opportunity for the inhibition of autophagy in combination with conventional cancer therapies.

Conclusion

Over the last decades EGFR has evolved as highly investigated target in the field of anti-cancer treatment. This has led to the development of EGFR-targeting antibodies like cetuximab or panitumumab and TKIs like gefitinib, erlotinib, and lapatinib. More recently, the potential of autophagy inhibition as therapy in cancer is being evaluated. Several reports indicate that cells and tumors with amplified or overactivated EGFR are particularly sensitive to autophagy inhibition for growth, survival, and resistance to conventional therapies. Additionally, resistance to EGFR-targeting therapies can also be

reduced by autophagy inhibition. Inhibition of autophagy may therefore provide a novel treatment opportunity for EGFR-overexpressing tumors and should be pursued clinically.

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Chapter 2

Binding of cetuximab to the EGFRvIII deletion mutant and its biological consequences in malignant glioma cells

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Abstract

Background and purpose: Despite the clinical use of cetuximab, a chimeric antibody against EGFR, little is known regarding its interaction with EGFRvIII, a frequently expressed deletion mutant of EGFR. Therefore, we investigated the interaction and the functional consequences of cetuximab treatment on glioma cells stably expressing EGFRvIII.

Materials and methods: The human glioma cell line U373 genetically modified to express EGFRvIII was used to measure the binding of cetuximab and its internalization using flow cytometry and confocal microscopy. Proliferation and cell survival were analyzed by cell growth and clonogenic survival assays.

Results: Cetuximab is able to bind to EGFRvIII and causes an internalization of the receptor and decreases its expression levels. Furthermore, in contrast to EGF, cetuximab was able to activate EGFRvIII which was evidenced by multiple phosphorylation sites and its downstream signaling targets. Despite this activation, the growth rate and the radiosensitivity of the EGFRvIII-expressing glioma cells were not modulated.

Conclusions: Cetuximab binds to EGFRvIII and leads to the initial activation, internalization and subsequent downregulation of EGFRvIII, but it does not seem to modulate the proliferation or radiosensitivity of EGFRvIII-expressing glioma cells. Thus, approaches to treat EGFRvIII-expressing glioma cells should be evaluated more carefully.

Introduction

EGFR-targeted strategies, such as EGFR-specific tyrosine kinase inhibitors (TKIs) and monoclonal antibodies are actively being investigated, and these are showing promising results.[1-3] However, recent evidence has demonstrated that certain cancer-associated mutations, including k-Ras and PTEN can result in persistent growth pathways activation despite the blockade of EGFR[4,5], thereby counteracting the efficiency of these anti-EGFR-targeted treatments. Furthermore, it has also become apparent that the different strategies to inhibit EGFR (e.g. antibody versus TKI) may act differently in the same tumour[6-8.] Compensatory responses by other receptors, including mutant EGFR species might be one likely reason for such failures.[8,9] Furthermore, the presence of naturally occurring mutants of EGFR may in part also account for the limited clinical response to some EGFR-targeted therapies. The most common variant of the EGFR, an exon 2-7 deletion mutant named EGFRvIII, has not been detected in normal tissue, but it is found in many malignancies, such as glioblastoma, non-small lung cell carcinoma, breast cancer, prostate cancer and head and neck cancer.[10-12] Based on these observations, EGFRvIII should also be considered in EGFR-targeted approaches.

Next to small synthetic TKIs, monoclonal antibodies have been developed to target EGFR.[13] Cetuximab, a human-mouse chimeric monoclonal antibody, is developed to target EGFR specifically.[13,14] It has been designed to inhibit endogenous ligand binding to EGFR and thereby prevent the dimerization and activation of the tyrosine kinase domain.[15] Cetuximab binding leads to an internalization of the receptor resulting in the downregulation of the EGFR expression on the cell.[16] However, despite its frequent clinical use, little is known regarding its interaction or activity against EGFRvIII. Therefore, the present study was undertaken to investigate the interaction and the functional consequences of cetuximab treatment on malignant glioma cells stably expressing EGFRvIII.

Materials and methods

Reagents and cell lines

Unless specified otherwise, all reagents were obtained from Sigma Chemical Co. (Sigma–Aldrich) and all electrophoresis reagentswere from BioRad (Bio-Rad). MEM-α and Geneticin were obtained from Invitrogen (Invitrogen) and fetal bovine serum (FBS) was from Hyclone.
The anti-EGFR monoclonal antibody (Sc-03 mAb) was purchased from Santa Cruz (Santa Cruz Biotechnology). The anti-Tyr1173, anti-Tyr-1086, anti-Tyr-1068, anti-Tyr-992 and anti-Tyr-845 EGFR phosphorylation polyclonal antibodies (pAbs), and the anti-Akt mAb, anti-ser473-phosphorylated Akt mAb and anti-phosphorylated ERK mAb were all purchased from Cell Signaling Technology (Bioke). Anti-actin mAb was purchased from MP Biomedicals (MP Biomedicals). Secondary Abs, anti-mouse HRP-conjugated and anti-rabbit HRP-conjugated, were purchased from Cell signaling Technology (Bioke). The phbAc.EGFRvIII expression plasmid was kindly provided by D. Bigner (Dept. of Pathology, Duke University, Durham, NC, USA). The U373 glioma cell line, the Chinese Hamster Ovary (CHO) cell line and the A431 squamous carcinoma cell line were obtained from the American Type Tissue Collection (ATTC; Rockville, MD, USA). U373 cells were grown and routinely maintained in MEM-α, supplemented with 10% FBS; the stable U373 cells expressing EGFRvIII were incubated in the same medium but also with 300 μg/ml Geneticin. The CHO and A431 cell lines were maintained in RPMI with 5% FBS (10% for A431). Cells were incubated at 37°C with 5% CO_2 and 95% air.

Flow cytometric analysis

Flow cytometric analysis was performed as described previously.[17]

Immunofluorescence and confocal microscopy

Cells were grown on plastic 10-cm (immunofluorescence) or 6-cm (confocal microscopy) dishes until 80% confluency and were incubated with cetuximab-

OG (60 nM, 24 or 3 h, respectively, 37°C). Cells were washed thrice using phosphate-buffered saline (PBS), and fixed in 3 ml methanol on ice (10 min). Propidium iodide (1 µg/ml) and Hoechst, containing 1 µl/ml RNase (15 min, RT), were used as nuclear markers. Cells were washed with PBS, pH 8, and cyto-spinned (5' 500 g), mounted with Fluorescent Mounting Medium (DAKO) and analyzed using a Leica DM5000B fluorescence microscope (Leica Microsystems) or Leica TCS SPE confocal microscope.

Immunoblotting

Experiments were performed as described previously.[18] Briefly, 10 µg (20 µg for phosphor-specific antibodies) of each sample was resolved on a 4-12% Criterion SDS–PAGE gel, blotted onto a Protran nitrocellulose membrane (Schleicher & Schuell) by electrotransfer and probed overnight with antibodies as described above. Immobilized proteins were detected using SuperSignal West Pico chemiluminescent substrate (Perbio) and by exposing the blot to X-ray film.

Cell growth assay

To determine the growth rate, $1.4*10^5$ cells were seeded in triplicate on 6-cm dishes. The plates were incubated under normal culture conditions in a 5% CO_2 incubator for 1-6 days. After attachment, medium was supplemented with 60 nM cetuximab. Plates were harvested by washing thrice with PBS followed by trypsinization. Total cell numbers were enumerated using a Coulter Z Series particle counter (Beckman Coulter). Doubling times were calculated from the slope of the best-fit line during the exponential phase of growth.

Radiation of cells and clonogenic survival assay

Cells were seeded in 10-cm dishes and cultured for 72 h in the medium with and without 60 nM cetuximab. During irradiation, dishes were placed in a Plexiglas jig filled with water. Cells were irradiated using an MCN 225 industrial X-ray tube (Philips) operated at 225 kV and 10 mA to deliver a dose of 4 Gy at

a rate of 0.85 Gy/min. Cells treated with cetuximab were seeded continuously in the medium supplemented with 60 nM cetuximab. The clonogenic survival assay was performed as described previously.[18]

Statistics

Statistical analysis was carried out using GraphPad Prism version 5.01 for Windows (GraphPad Software, 2007, California, USA). Unpaired Student's t-test and non-parametric Mann–WhitneyU test for small groups were used to determine the statistical significance of differences between two independent groups of variables. For all tests a $p < 0.05$ was considered to be significant.

Results

Cetuximab binds to EGFRvIII and internalizes the cetuximab-EGFRvIII complex

Despite the lack of the ligand binding domain in EGFRvIII, we demonstrate that cetuximab is able to bind to EGFRvIII[17] (Fig. 1). We further investigated this binding using different doses of cetuximab in a range from 5.2 fM to 5.2 μM (Fig. 1). We found that cetuximab significantly binds to EGFRvIII reaching saturation at 5.2 nM, while the parental U373 cells do not show any significant binding (Fig. 1A). The cetuximab binding was further confirmed using immunofluorescence (Fig. 1B). A membranous staining can be observed for the U373-vIII(+) cells, while no staining is detectable for the parental U373 cells (data not shown). The A431 cells, known to overexpress the EGF receptor, served as a positive control. These observations clearly demonstrate that cetuximab binds to both EGFR and EGFRvIII.

We further analyzed the localization of the cetuximab-EGFRvIII complex using confocal microscopy. Besides a membranous staining, we observed internalization of the cetuximab-receptor complex in A431 cells for EGFR and in U373-vIII(+) cells for EGFRvIII after 3 h of cetuximab treatment (Fig. 1C). This suggests that cetuximab not only binds to EGFRvIII and EGFR but also internalizes both these receptors.

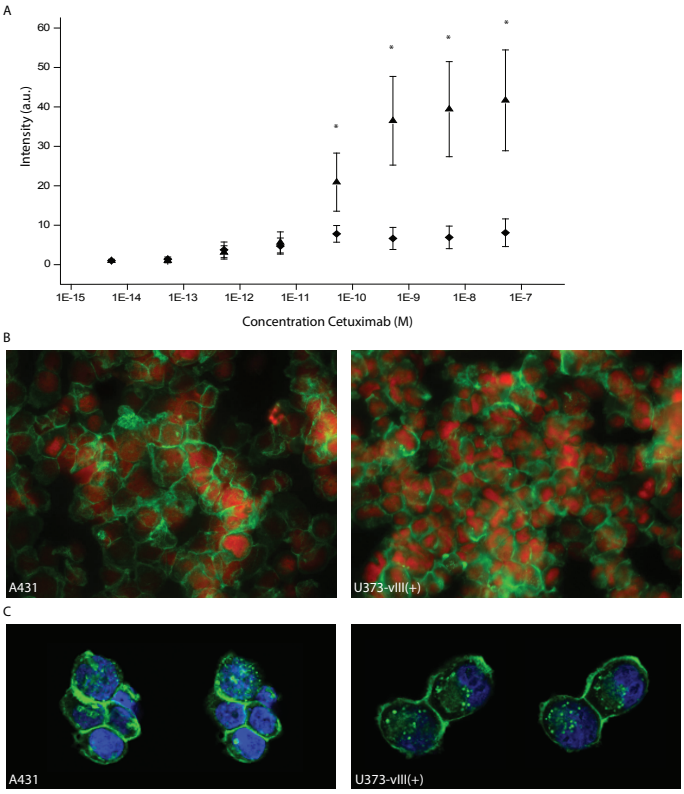

Figure 1. Cetuximab binds to and internalizes EGFRvIII. (A) Flow cytometry analysis of the binding of cetuximab to two U373 cell lines (▲) U373-vIII(+) and (●) U373. Error bars indicate standard deviation of triplicate measurement, (*) indicates significance $p < 0.05$. (B) Immunofluorescence analysis of U373-vIII(+) and A431 cells incubated for 24 h with cetuximab-OG. Propidium iodine was used for nuclear staining. (C) Confocal microscopy of U373-vIII(+) and A431 cells incubated for 3 h with cetuximab-OG. Hoechst was used for nuclear staining. Two representative slices at different depths are presented.

Cetuximab induces an initial activation of EGFRvIII

To study the biological consequences of the cetuximab binding on EGFRvIII expression, we assessed the protein expression levels of EGFRvIII under cetuximab treatment. Incubation of U373-vIII(+) cells with cetuximab in a time course revealed a slight, although not significant initial upregulation of EGFRvIII expression with a maximum at 4 h, followed by a downregulation after 2 days

(Fig. 2A). Interestingly, activation of EGFRvIII, as assessed by the phosphorylation at Tyr1173, strongly and significantly increased during the first hours of treatment reaching a maximum in between 1 and 4 h before downregulation to baseline levels at 72 h.

In order to confirm the unresponsiveness of EGFRvIII upon ligand treatment with EGF, we incubated U373-vIII(+) and A431 cells with 3 ng/ml of EGF.

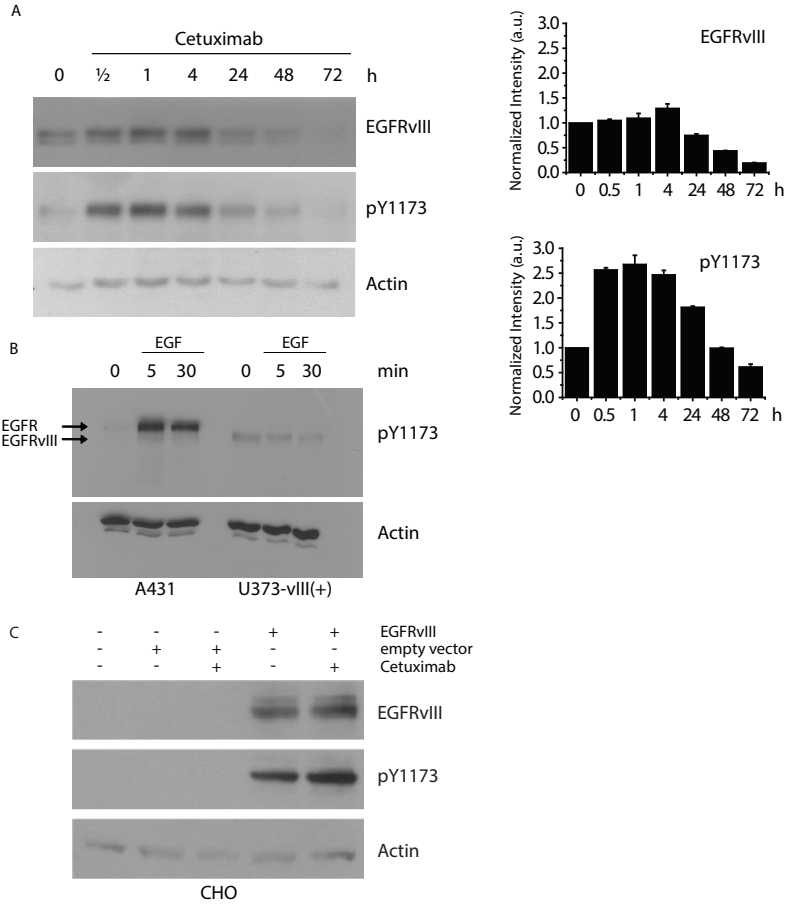

Figure 2. Cetuximab but not EGF activates EGFRvIII. (A) Western blot analysis of (phospho-) EGFRvIII expression of U373-vIII(+) cells incubated with 60 nM cetuximab for the indicated time (h). Densitometric quantification of the band intensities is shown on the right panel normalized for actin. (B) Western blot analysis of phosphorylated EGFR in a time course after 3 ng/ml EGF treatment of A431 and U373-vIII(+) cells. (C) Western blot analysis of CHO cells transiently expressing EGFRvIII or an empty vector treated with cetuximab for 2.5 h. Actin was used as a loading control.

Western blot analysis revealed that EGFRvIII cannot be activated by EGF in U373-vIII(+) cells (Fig. 2B). Taken together, cetuximab but not EGF can initially activate EGFRvIII.

To further investigate the possible influence of other receptors expressed in U373 cells on the activation of EGFRvIII upon cetuximab treatment,we transiently overexpressed EGFRvIII in CHO cells, which are known to lack receptors of this family. In contrast to U373-vIII(+) cells, cetuximab did not induce an increased phosphorylation in these CHO cells at pY1173, indicating that the cetuximabinduced activation of EGFRvIII is cell line dependent (Fig. 2C).

These data prompted us to further evaluate the initial activation of EGFRvIII and its downstream consequences in more detail. We investigated all known EGFRvIII phosphorylation sites and the important downstream effector kinases Erk and Akt at 30-min intervals up to 6 h of cetuximab treatment. Western blot

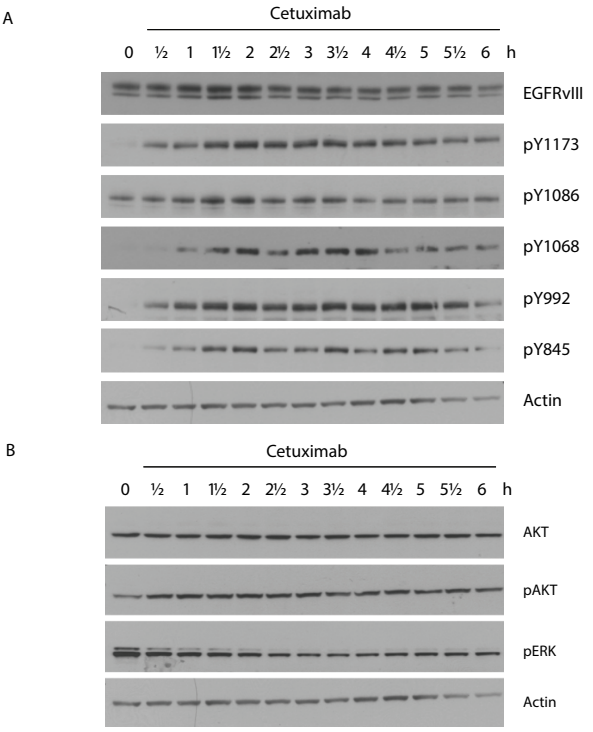

Figure 3. Phosphorylation of EGFRvIII and downstream effectors after cetuximab treatment. Western blot analysis of different EGFR phosphorylation sites (A) and Akt and Erk phosphorylation (B) for every 0.5 h for 6 h after 60 nM cetuximab treatment. Actin was used as a loading control.

analysis revealed that the activation of EGFRvIII resulted in phosphorylation of all tested sites (Y1173, Y1086, Y1068, Y992 and Y845). All phosphorylation sites showed a similar activation pattern with an increase up to 2.5 h followed by a subsequent decrease over time (Fig. 3A).

Importantly, the downstream effectors of EGFRvIII, Akt and Erk are differentially affected by the cetuximab-induced activation of EGFRvIII. While the phosphorylation of Erk constantly decreased over time, the phosphorylation of Akt slightly increased after 30 min, indicating a potential initial activation of Akt (Fig. 3B). These data suggest that cetuximab treatment activates EGFRvIII upon binding, and that it also initially stimulates Akt, which is known to be involved in cellular proliferation, apoptosis and DNA repair.[9,19,20]

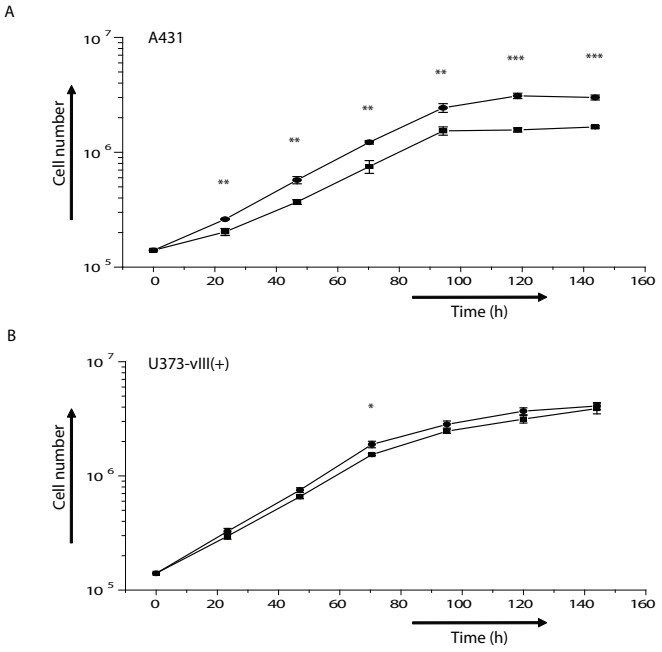

Figure 4. Cell growth upon cetuximab treatment. The influence of cetuximab on growth was evaluated by counting total numbers of A431 (A) and U373-vIII(+) (B) cells. (●) Cells incubated with 60 nM control IgG, (■) cells incubated with 60 nM cetuximab. Error bars indicate standard deviation of triplicate measurement. Asterisk indicates significance (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

Growth of EGFRvIII-expressing glioma cells upon cetuximab treatment

Since cetuximab has been shown to reduce the proliferation of EGFR-expressing cells, we further investigated the effect of cetuximab on the proliferation of EGFRvIII-expressing cells in vitro. We compared the growth of A431 and U373-vIII(+) cells upon incubation with cetuximab. Whereas the doubling time of A431 cells increased significantly from approximately 23 to 30 h upon longterm cetuximab treatment (Fig. 4A), the doubling time of U373-vIII(+) cells remained unchanged at approximately 20 h (Fig. 4B).

Radiosensitivity of EGFRvIII-expressing glioma cells upon cetuximab treatment

Finally, to evaluate the consequences of cetuximab treatment on the radioresistant phenotype of glioma cells expressing EGFRvIII, we performed a clonogenic survival assay with the parental U373 and the U373-vIII(+) cell lines. Both cell lines were pre-treated with cetuximab for 72 h, before the cells were irradiated with a clinically relevant single dose of 4 Gy.
In line with earlier reports[18,21], the EGFRvIII-expressing cell line U373-vIII(+) demonstrated a higher survival rate compared to the parental U373 cell line. Cetuximab treatment alone only slightly decreased the survival rate of the two

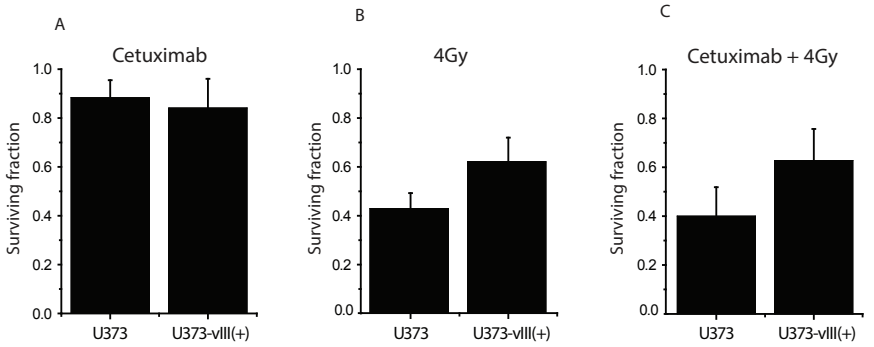

Figure 5. Clonogenic survival upon cetuximab treatment. Clonogenic survival assays were performed to determine the survival of the cell lines U373 and U373-vIII(+) 72 h after 60 nM cetuximab treatment (A), 24 h after IR at 4 Gy (B) and the combination of cetuximab treatment and IR at 4 Gy (C). The mean values of three independent experiments are shown. Error bars indicate standard deviation.

tested cell lines.Importantly, the combination of cetuximab and irradiation did not alter the survival rate in both cell lines as compared with irradiation alone (Fig. 5). Thus, despite the binding and internalization of EGFRvIII upon cetuximab treatment, cetuximab did not alter the radiosensitivity of EGFRvIII-expressing U373 cells in vitro.

Discussion

In the present report we describe the interaction of cetuximab with EGFRvIII in malignant glioma cells genetically modified to stably express EGFRvIII. We prove that in contrast to recent literature and despite the absence of the ligand binding domain, cetuximab indeed binds to EGFRvIII and initially activates and internalizes EGFRvIII before subsequent downregulation.[3]

To our knowledge, this is the first evidence that cetuximab not only binds to EGFRvIII but also activates it. Although binding of cetuximab to either EGFR or EGFRvIII should not lead to the activation of the receptors, this has been observed previously for the wild-type receptor.[16,28] Interestingly, this activation was not observed in CHO cells transiently expressing EGFRvIII. This might indicate an activation mechanism related to trans-receptor activation following heterodimerization with other receptors present in U373 cells. CHO cells are characterized by a lack in ErbB receptor expression[21] whereas U373 cells also express ErbB2 and IGF-1R, both known to heterodimerize with EGFR in a variety of epithelial cancers.[26,27] However, other mechanisms both upstream (integrins, integrin-growth receptor linker proteins, etc.) and downstream (e.g. caveolae related) might be involved and this might also influence the specific type of internalization of the cetuximab–EGFRvIII complex we have observed.[29,30] As such, our observations can serve as a starting point to elucidate these mechanisms in more detail. Also, in contrast to EGFR, no studies have yet determined possible translocation pathways for EGFRvIII and whether they can be inhibited.[31]

Importantly, despite the interaction with the receptor and its eventual downregulation, cetuximab failed to reduce the growth, the survival and the displayed increased radioresistance of the U373-vIII(+) cells.[8,18,21] Thus, in our model under in vitro conditions, EGFRvIII imparts a growth and survival

advantage, which does not seem to be affected by cetuximab treatment in our performed assays.[8,18,21] This might indicate that the current clinical approaches using cetuximab to radiosensitize malignant glioma cells, which express EGFRvIII, should be reconsidered. Indeed, other strategies targeting the phenotypical consequences of the EGFRvIII expression rather than the protein itself should be taken into consideration. These strategies could include, e.g. the inhibition of the EGFRvIII-mediated sustained downstream signaling pathways, which have also been shown to significantly affect the DNA repair machinery in cellular responses to ionizing radiation.[4,22]

Nevertheless, cetuximab can bind to EGFRvIII, which makes it a potential candidate for approaches to image EGFR and EGFRvIII. In this context, studies have been initiated recently.[17,23] The fact that the cetuximab–EGFRvIII complex subsequently internalizes also implies that cetuximab might be potentially used in EGFR and EGFRvIII-expressing tumours as a carrier for intracellular drug delivery, e.g. approaches covalently linking monoclonal antibody fragments of cetuximab to stabilized liposomes containing chemo-therapeutic drugs or probes .[24,25]

Obviously, the in vitro transfection model that was used has some limitations. Indeed, such stable in vitro overexpression might probably not resemble the native in vivo situation. Not only is the current understanding of the true role of EGFRvIII in real solid tumours limited, but the homogeneous cellular overexpression levels also largely differ from the endogenous expression levels and the distribution pattern seen in mature tumours.[32] Despite this, the model used can definitely provide valuable information on the EGFRvIII-related biological mechanisms, because it enables the measurement of biological responses which would otherwise probably not have been detected at the endogenous in vivo levels due to the current detection limits.

In summary, we have implicated cetuximab as an antibody capable of binding to EGFRvIII in glioma cells with even an initial activation of the receptor. However, despite the binding and the subsequent decrease in expression, cetuximab did not modulate the cellular growth rate or the radiosensitivity of the tested cells. Our findings highlight the value of cetuximab as an imaging probe for EGFRvIII, but they also suggest that targeted approaches using cetuximab to treat malignant glioma cells expressing EGFRvIII should be

evaluated more carefully also considering other strategies targeting the phenotypical consequences of the EGFRvIII expression rather than the protein itself. The mechanisms underlying the vIII-dependent biological changes warrant further investigation.

Acknowledgements

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Chapter 3

Discriminative in vivo imaging of wtEGFR and EGFRvIII using nanobody technology

In preparation
Jutten B, Khoulati, R. el, Lammering G, Lambin, P, Van Bergen en Henegouwen PM, and Roovers RC

Abstract

The epidermal growth factor receptor (EGFR) has been shown to be a valid cancer target for antibody-based therapy. At present, several anti-EGFR monoclonal antibodies (mAbs) have been clinically used, but with varying degrees of success. The constitutive active variant vIII (lacking exon 2-7) of the EGFR is an oncogenic mutant of the receptor that is frequently expressed in (e.g. brain-) tumours. The expression of this truncation mutant of the receptor is an important characteristic of tumours relatively resistant to anti-EGFR therapy. Clinically used antibodies (e.g. cetuximab), do not discriminate between EGFR and EGFRvIII. As most EGFR-targeting antibodies target ligand binding and the vIII mutant does not require ligand binding for activation, vIII is resistant to antibody mediated targeting inhibition. Furthermore, EGFRvIII expressing tumours are less sensitive to the tyrosine kinase inhibitor gefitinib and require different treatment. The ability to assess the 'EGFR status' in EGFR positive tumours is therefore clinically very important. The 7D12 nanobody, which we have previously shown to be a suitable tool for imaging of EGFR-expressing tumours, also recognises both variants of the receptor and cannot discriminate wtEGFR from EGFRvIII. To be able to discriminate wtEGFR from EGFRvIII expression in vivo by means of imaging, single-domain antibody fragments were developed that specifically recognise wtEGFR or EGFRvIII. These nanobodies recognised their cognate antigen in an excess of the other form of the receptor, thereby demonstrating exquisite specificity for one of the two forms of the EGFR. Single domain antibody fragments that were developed recognised either wtEGFR (but not vIII) or demonstrated preferential binding to EGFRvIII. The latter fragments did not exclusively recognise EGFRvIII, but also bound wtEGFR when present in abundance.

Introduction

The epidermal growth factor receptor (EGFR) is a member of a family of four related receptor tyrosine kinases (RTK), named Her or cErbB1, -2, -3 and -4.[1,2] Its extracellular domain consists of four subdomains, two of which are involved in ligand binding and one of which is involved in homodimerisation and heterodimerisation.[3-5] EGFR propagates extracellular signals from its variety of ligands to yield diverse intracellular responses. Once activated, EGFR drives proliferation, differentiation and survival.

Mutations in the EGFR are common in various types of cancer. Over-expression of the EGF receptor is seen in over 70% of all metastatic colorectal cancers[6], whereas mutations in the tyrosine kinase domain are often observed in non-small cell lung cell (NSCLC) tumours.[7] The truncated and constitutively active form of the EGFR, EGFRvIII or Δ2-7, is noted in approximately 50% of EGFR over-expressing brain tumours[8] and approximately 21% of all head and neck squamous cell carcinomas.[9] Expression of this mutant EGFRvIII form is correlated with poor prognosis and survival.[10, 11] Currently, expression of EGFRvIII is not routinely tested in clinical settings. Therefore we attempted to make antibodies suitable for both in vivo and ex vivo detection of wtEGFR or EGFRvIII expression. Detection of overexpression of wtEGFR and/or EGFRvIII is crucial for the selection of the correct treatment. E.g., wtEGFR overexpressing tumours can be treated with TKIs like gefitinib, while EGFRvIII expressing tumours require higher doses, repeated dosing, and longer exposure.[12]

Llama single domain heavy chain only antibody fragments, or nanobodies, are suitable for both PET-imaging[13] and diagnosis on cryosections.[14] Nanobodies are very stable, easy to engineer, have specificity and affinities comparable to conventional antibodies and efficiently expressed on bacteriophage. Since nanobodies are relatively small, 12-15kD, they are ideally suited for imaging. This is because of their high tumour penetrating capacity and rapid clearance, giving a high tumour to blood ratio.

Cetuximab or other commercially available antibodies directed against EGFR do not discriminate between wtEGFR and EGFRvIII. Therefore we set out to select an EGFRvIII specific nanobody from a phage-library expressing

nanobodies derived from a llama immunized with EGFRvIII to complement the previous selected wtEGFR specific nanobody (Egb5) from Roovers et.al. (Unpublished data).
The selected wtEGFR antibody was selected to bind to domain I since domain I is for the greater part missing in EGFRvIII. For EGFRvIII detection we sought for nanobodies that specifically recognised the exon 1 and 8 fusion containing the EGFRvIII unique glycine.

Materials and Methods

E.coli strain and cell lines

The bacterial strain used was TG1: supE thi-1 Δ(lac-proAB)Δ(mcrB-hsdSM)5 (rK– mK–) [F'traD36 proAB lacIqZΔM15]. Her14 cells are derived from NIH 3T3 fibroblasts and stably express roughly 105 copies of the human EGFR on their cell surface.[15,16] The human head and neck squamous cell carcinoma (HNSCC) cell line UM-SCC-14C was developed by- and a kind gift of Dr. T.E. Carey, Ann Arbor, MI, USA (abbreviated as 14C). The glioblastoma cell line U373-MG (cat. nr. HTB-17) and the epidermoid squamous carcinoma cell line A431 (cat. nr. CRL-1555) were bought from the ATCC. All cells were cultured in Dulbecco's Modification of Eagle Medium (DMEM: Gibco, Invitrogen, Paisley, United Kingdom) containing 7.5% (v/v) foetal calf serum and 2 mM L-glutamine in a humidified atmosphere without antibiotics at 37°C under 5% CO_2. Cells were regularly tested by PCR for the presence of mycoplasma and consistently found to be mycoplasma-free.

Plasmids and constructs

The cDNA encoding the truncated (de2-7, or vIII) variant of the EGFR[17] was a kind gift from Dr. Shibuya (Graduate School of Medicine, the University of Tokyo, Tokyo, Japan). The cDNA was first re-cloned in the multiple cloning site of pCDNA3.1 Zeo (+) as Kpn1 fragment. Subsequently, the cDNA was liberated using HindIII and Not1 digestion. The insert was ligated at the 3' end to EcoR1-Not1 digested PMX-IRES-GFP (Roovers et. al., unpublished), sticky ends were

blunted by Klenow DNA polymerase and the 5' end of the cDNA was subsequently ligated to the vector. To synthesise an immuno-fusion of the extracellular domain (ECD) of EGFRvIII, the ECD was amplified using primers vIII-sense (5'-caagtcgtttaaacgccaccatgcgaccctccgggacggccggggc-3') and vIII anti-sense (5'-ccgtatgagatcttcaggcccattcgttggacagccttc-3'). The resulting PCR product was then digested with Pme1 and BglII and ligated in a Pme1-BamH1 digested, in-house developed Fc fusion vector. This construct (comprising a.a. 1-374 of EGFRvIII fused to human hinge-IgG1 Fc) was then expressed and purified as has been described for wtEGFR ECD-Fc.[18] Purified, recombinant EGFR domain I (a.a. 1-183) and domain I and II (a.a. 1-308) were a kind gift from Dr. K.M. Ferguson (Department of Physiology, University of Pennsylvania school of Medicine, Philadelphia, USA). The domain I protein was biotinylated using the reactive ester (+)-Biotin N-hydroxy succinimide ester (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's recommendations. Excess nonreacted ester was removed by dialysis through a dialysis membrane with a cut-off of 3.5kDa. A peptide comprising the first 16 a.a. of EGFRvIII, biotinylated at the C-terminal residue (N term-LEEKKGNYVVTDHGSK-Biotin) was synthesised and purified by Isogen (de Meern, the Netherlands).
The pUR8100-myc-his vector was used for the construction of the phage library and the pUR8100-flag-his vector was used for the production of soluble nanobodies.

Immunisation of Llama Glama and library construction

Llama Glama (2 animals) were immunized with cell lines (alternating HCT116-vIII and U373-vIII) stably expressing EGFRvIII, as has been described for cells expressing wtEGFR.[19,20] After immunization, sera from immunized animals were tested for the successful induction of a humoral immune response towards the cell line by whole cell ELISA and nanobody phage libraries were constructed as has been described.[19,20]

Nanobody phage selections on EGFR domain I and EGFRvIII

For selection of nanobodies recognising EGFRvIII, the purified EGFRvIII ECD-

Fc fusion was used. An antiserum to human Fc (Dako, Glostrup, Denmark) was coated (1 in 2000 in PBS) to the wells of a Maxisorp (NUNC, Rochester, USA) plate (o/n at 4°C) and the next day, wells were washed and blocked with 4% Marvel skimmed milk powder in PBS (MPBS) before 1µg of antigen was captured (in 2% MPBS) for the first selection round. Phage (roughly 10^{10} cfu) were blocked with 2% MPBS and added to the captured antigen. After incubation for 1.5 hours with shaking, the Maxisorb plate was extensively washed with PBS containing 0.1% (v/v) tween-20 and antigen-bound phage were retrieved by competitive elution[21] with 5µM vIII-peptide (30min, rt). For the second round of selection, rescued phage were blocked with 2% MPBS and added to a near confluent dish of U373vIII cells blocked with selection medium (comprising DMEM, 10% FCS, 25mM HEPES and 2% Marvel). For negative (counter-) selection, a 10-fold excess of with selection medium preblocked HER14 cells (overexpressing wtEGFR[15]) were added in suspension during phage selection. After incubation of 1.5 hours with shaking at 4°C, the dish was extensively washed with PBS and antigen bound phages were retrieved by competitive elution with 5µM vIII-peptide (30min, 4°C).

ELISA

A rabbit polyclonal serum to human IgG (Dako, Glostrup, Denmark) was coated (at a 1:2000 dilution in PBS) to the wells of a Maxisorp plate overnight at 4 degrees. Next day, plates were washed three times with PBS, blocked with 2% MPBS and ECD-Fc fusion protein (of either wtEGFR or EGFRvIII) was captured (25ng per well) for an hour at rt with shaking. Plates were washed as before and either phage (roughly 10^{8} colony forming units: CFU) or nanobody (200nM) were added and incubated for an hour at rt. Bound phage was detected with 1:10000 diluted peroxidase-coupled monoclonal anti-fd phage antibody (Amersham/GE Healthcare, Buckinghamshire, England); staining was performed using OPD (orthophenylene diamine) as substrate. Bound VHH was detected using a1:1000 diluted murine antibody against the C-terminal cMyc-derived epitope tag (clone 9E10), 1:2500 diluted peroxidase-coupled donkey anti-mouse antibodies (Jackson Immuno-research Laboratories) and staining using OPD. Optical density was measured at 490nm.

Immuno-fluorescence staining

Cells were seeded on glass slides and transfected with the respective plasmids using Fugene HD transfection reagent (Promega, Madison, USA) according to the manufacturer's recommendations. Two days later, cells were washed with PBS, fixed with 4% (w/v) formaldehyde in PBS for 30min. at room temperature (rt) and stained with the respective nanobodies. Briefly, fixed cells were blocked with glycine (100mM in PBS) and 2% MPBS for 10 min each and then incubated with 200nM of the respective nanobodies or cetuximab in 2% MPBS. Bound nanobody was detected with an in-house developed rabbit serum to nanobodies and alexa-555 coupled goat anti-rabbit antibodies (Jackson Immuno-research Laboratories, Suffolk, UK). Bound cetuximab was detected with a rabbit anti-human IgG serum (Dako, Glostrup, Denmark) and alexa-555 coupled goat anti-rabbit antibodies. Nuclei were stained with DAPI and cells were embedded in Mowiol, containing PPD. Images were taken using an Olympus AX70 microscope equipped with a Nikon CCD camera (DXM1200) microscope.

Immuno-fluorescence staining of xenografts

Nanobodies were labelled with fluoProbes594 labelling kit according manufacturers protocol (Antibodies-online, Aachen, Germany). 4µm cryosections of A431 and U373vIII xenografts were air dried for 15' and blocked with 2% BSA in PBS for 30'. Slides were stained for 2 hours with fluoProbes594 labelled nanobodies. After 3 times 5' washing with PBS, nuclei were stained with Hoechst H 33342. After 1 additional wash, slides were mounted with immunomount (Shandon, Thermo Fisher, Breda, the Netherlands). Slides were analysed using a Leica TCS SPE confocal microscope (Leica, Rijswijk, the Netherlands)

Flow cytometry

34-E5 nanobody was added in a serial dilution (1.2µM to 12pM) to $3*10^5$ cells (A431,U373vIII, U373wt (U373 cells with wtEGFR overexpression) and U373ev

cells) in DMEM supplemented with 10% FCS and incubated for 1 hour on ice in a 96 well V-shape plate. Bound nanobodies were detected using anti FLAG antibody (Sigma-Aldrich, St. Louis, USA) in a 1:2000 dilution followed by an anti-mouse alexa 488 antibody (Invitrogen, Paisley, United Kingdom) for 1 hour each. Cetuximab coupled to Oregon green[22,23] was used in a 60nM concentration as a positive control. After a short fixation in 2% formalin, cells were analysed using on a Canto II (BD, Breda, the Netherlands).

Surface Plasma Resonance

All measurements were performed on a BIACORE T100 instrument (Biacore Life sciences, Uppsala, Sweden) at 25°C. A 1:1 mixture of 1-ethyl-3-(3-dimethylaminopropyl carbodiimide) hydrochloride and N-hydroxysuccinimide was injected at a flow rate of 10µL/min for 7 minutes to activate the surface of a CM5 sensor chip flow cell. wtEGFR and EGFRvIII Fc-fusion constructs diluted in 10mM acetate pH 4.0 were coupled to the activated CM5 sensor chip up to 200 RU at a flow rate of 10µl/min. The flow cell was then deactivated by means of injection of 1 mol/L ethanolamine-HCl (pH 8.5; 10 µL/min) for 7 minutes. The reference cell was activated and blocked for background subtraction. IMAC purified nanobodies were diluted in HBS-EP (0.01 mol/L HEPES, 0.15 mol/L NaCl, 3 mmol/L EDTA, and 0.005% vol/vol surfactant P20, pH 7.4) and injected at 5 µL/min

Results

Induction of a humoral anti-EGFRvIII response in Llama Glama

Llama Glama were immunised with human glioma cells (U373-MG) or human colorectal cancer cells (HCT-116) genetically modified to constitutively express EGFRvIII. These cell lines were genetically modified since natural expression of EGFRvIII on tumour cells is rapidly lost when these cells are cultured under normal cell culture conditions.
The induction of a humoral immune response was followed by testing sera of the animals before and after immunisation by (whole cell-) ELISA. Both animal

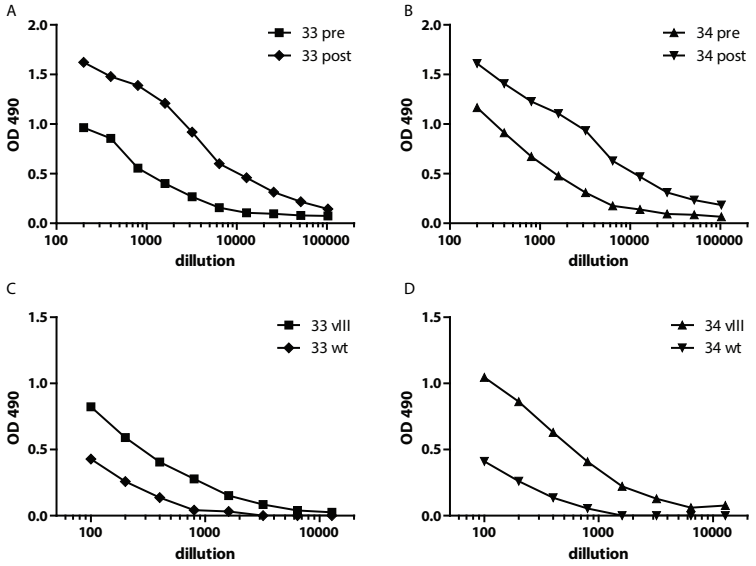

Figure 1. (A+B) whole cell ELISA of pre- and post-immunization sera showing immune response towards EGFRvIII. (C+D) ELISA showing reactivity of the immune sera against the extracellular domain of the EGFRvIII receptor and in a lesser extent towards the extracellular domain of wtEGFR.

33 and 34 showed an induced antibody response directed towards EGFRvIII expressing U373 cells (Fig. 1A+B). In addition, immune sera of both animals were also reactive with purified extracellular domain of the EGFRvIII but to a lesser extent with the wt extracellular domain (Fig. 1C+D). These data clearly demonstrate the successful induction of a humoral immune response towards the mutant EGF receptor.

Selection and characterisation of nanobodies

Phage nanobody repertoires were then synthesised by RT-PCR from two different lymphoid sources obtained from immunised animals 33 and 34: peripheral blood lymphocytes (PBLs) and a biopsy from a lymph node draining the site of immunisation. This resulted in libraries of approximately 6 to $8*10^7$ transformants each. Direct sequencing of selected clones revealed two distinct families of nanobodies (table 1). Selected clones were retested for binding towards EGFRvIII in an ELISA using soluble nanobodies (data not shown). One

Table 1

	FW 1	CDR 1	FW 2	CDR2	FW 3	CDR3	FW4
33-A5	EVQLVESGGGLVQAGGSLRLSCAASGRTFS	SYAMG	WFRQAPGKEREFVA	AISWSSGSTYYADSVKG	RFTISRDNAKNTVYLQMNSLKPEDTAGYYCAA	GYNFYPDYSVSAYYSSRELYDY	WGQGTQVTVSS
33-A10	EVQLVESGGGLVQAGGSLRLSCAASGRTFS	SYAMG	WFRQAPGKEREFVA	AISWSSGSTYYADSVKG	RFTISRDNAKNTVYLQMNSLKPEDTAGYYCAA	GYNFYPDYSVSAYYSSRELYDY	WGQGTQVTVSS
33-B5	EVQLVESGGGLVQAGGSLRLSCAASGRTFS	SYAMG	WFRQAPGKEREFVA	AISWSSGSTYYADSVKG	RFTISRDNAKNTVYLQMNSLKPEDTAGYYCAA	GYNFYPDYSVSAYYSSRELYDY	WGQGTQVTVSS
33-D1	EVQLVESGGGLVQAGGSLRLSCAASGRTFS	SYAMG	WFRQAPGKEREFVA	AISWSSGSTYYADSVKG	RFTISRDNAKNTVYLQMNSLKPEDTAGYYCAA	GYNFYPDYSVSAYYSSRELYDY	WGQGTQVTVSS
34-B1	EVQLVESGGGLVQPGGSLRLSCTASGMIFS	FNVMG	WYRQAPGNQRELAA	TITTDGSTNYADSVKG	RFTISRDKNTVYLQMNSLKPEDTAVYYCHL	KNIRDYRNYSNY	WGQGTQVTVSS
34-B2	EVQLVESGGGLVQPGGSLRLSCTASGMIFS	FNVMG	WYRQAPGNQRELAA	TITTDGSTNYADSVKG	RFTISRDKNTVYLQMNSLKPEDTAVYYCHL	KNIRDYRNYSNY	WGQGTQVTVSS
34-B7	EVQLVESGGGLVQPGGSLRLSCTASGSIFN	INVMG	WYRQAPGNQRELVA	TITTDGSTYYADSVKG	RFTIRRDKNTVYLQMNSLKPEDTAVYYCHL	KNIRDYRNYYNY	WSQGTQVTVSS
34-B8	EVQLVESGGGLVQPGGSLRLSCTASGMIFS	FNVMG	WYRQAPGNQRELAA	TITTDGSTNYADSVKG	RFTISRDKNTVYLQMNSLKPEDTAVYYCHL	KNIRDYRNYSNY	WGQGTQVTVSS
34-C1	EVQLVESGGGLVQPGGSLRLSCTASGMIFS	FNVMG	WYRQAPGNQRELAA	TITTDGSTNYADSVKG	RFTISRDKNTVYLQMNSLKPEDTAVYYCHL	KNIRDYRNYSNY	WGQGTQVTVSS
34-C11	EVQLVESGGGLVQPGGSLRLSCTASGSIFS	INVMG	WYRQAPGNQRELVA	TITTDGSTYYADSVKG	RFTISKDKSTVYLQMSSLKPEDTAVYYCHL	KNIRDYRNYWNY	WGQGTQVTVSS
34-C12	EVQLVESGGGLVQPGGSLRLSCTASGMIFS	FNVMG	WYRQAPGNQRELAA	TITTDGSTNYADSVKG	RFTISRDKNTVYLQMNSLKPEDTAVYYCHL	KNIRDYRNYSNY	WGQGTQVTVSS
34-D9	EVQLVESGGGLVQPGGSLRLSCTASGMIFS	FNVMG	WYRQAPGNQRELAA	TITTDGSTNYADSVKG	RFTISRDKNTVYLQMNSLKPEDTAVYYCHL	KNIRDYRNYSNY	WGQGTQVTVSS
34-E5	EVQLVESGGGLVQPGGSLRLSCTASGMIFS	FNVMG	WYRQAPGNQRELAA	TITTDGSTNYADSVKG	RFTISRDKNTVYLQMNSLKPEDTAVYYCHL	KNIRDYRNYSNY	WGQGTQVTVSS
34-E6	EVQLVESGGGLVQPGGSLRLSCTASGMIFS	FNVMG	WYRQAPGNQRELAA	TITTDGSTNYADSVKG	RFTISRDKNTVYLQMNSLKPEDTAVYYCHL	KNIRDYRNYSNY	WGQGTQVTVSS
34-F4	EVQLVESGGGLVQPGGSLRLSCTASGMIFS	FNVMG	WYRQAPGNQRELAA	TITTDGSTNYADSVKG	RFTISRDKNTVYLQMNSLKPEDTAVYYCHL	KNIRDYRNYSNY	WGQGTQVTVSS
34-F5	EVQLVESGGGLVQPGGSLRLSCTASGMIFS	FNVMG	WYRLAPGNQRELAA	TITTDGSTNYADSVKG	RFTISRDNDTVYLQMNSLKPEDTAVYYCHL	KNIRDYRNYSNY	WGQGTQVTVSS
34-G2	EVQLVESGGGLVQPGGSLRLSCTASGMIFS	FNVMG	WYRQAPGNQRELAA	TITTDGSTNYADSVKG	RFTISRDKNTVYLQMNSLKPEDTAVYYCHL	KNIRDYRNYSNY	WGQGTQVTVSS
34-G12	EVQLVESGGGLVQPGGSLRLSCTASGSIFN	INVMG	WYRQAPGNQRELVA	TITTDSSTYYADSVKG	RFTIRRDKNTVYLQMASLKPEDTAVYYCHL	KNIRDYRNYYNY	WSQGTQVTVSS

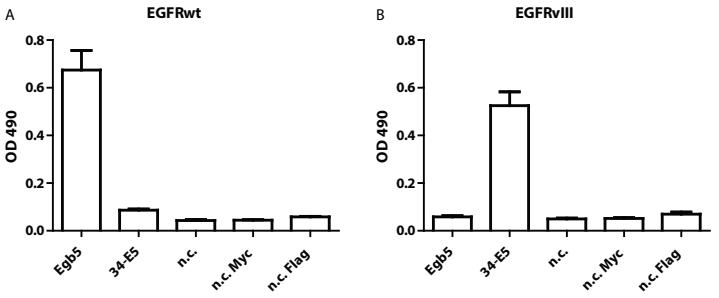

Figure 2. (A+B) ELISA showing reactivity of Egb5 (wtEGFR binder) for the extracellular domain of wtEGFR and not EGFRvIII, and 34-E5 (EGFRvIII binder) showing reactivity for the extracellular domain of EGFRvIII and not wtEGFR.

nanobody, 34-E5, was retested and found strongly reactive towards EGFRvIII with negligible reactivity towards wtEGFR (Fig. 2A+B). These results suggest the successful isolation of a specific anti-EGFRvIII nanobody.

Specificity of the 34-E5 nanobody for EGFRvIII on the surface of cells was tested by mixing HER14 cells with EGFRvIII expressing U373 cells. This mixed culture was stained with rhodamine coupled egf and 34-E5 nanobody. Bound nanobody was detected using 9E10 and alexa 488 coupled anti mouse antibody. Figure 3A shows HER14 cells, with wtEGFR expression, with clear red staining and internalized egf. No binding of 34-E5 nanobody to HER14 cells is observed. EGFRvIII expressing U373-MG cells, on the other hand, show clear binding of 34-E5 nanobody with minimal binding of egf (due to low expression of wtEGFR).

To test whether the 34-E5 nanobody was capable of specifically detecting EGFRvIII in an excess of wtEGFR, HER 14 cells were transfected with a plasmid encoding EGFRvIII and GFP. Cells that express EGFRvIII (Fig. 3B, left panel) clearly show binding of 34-E5 nanobody (Fig. 3B, middle panel). This result indicates that the 34-E5 nanobody specifically binds to EGFRvIII even in the presence of wtEGFR (Fig. 3B, bottom panel).

Tumour xenografts from U373vIII and A431 cells were used to prove specificity on actual tumour sections. 34-E5 nanobody was directly labelled with fluoProbes-594. Confocal images of U373vIII xenografts showed areas with intense 34-E5 nanobody binding (Fig. 4B). In the same section unstained cell were observed, possibly stromal or other mouse tissue cells. In the A431

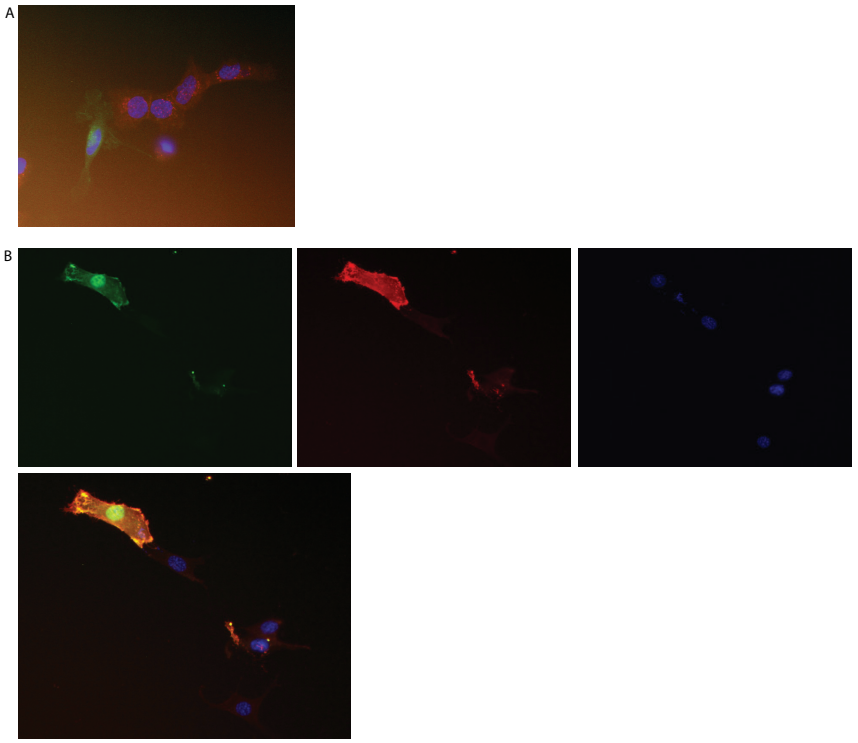

Figure 3. (A) Fluorescent immunostaining of a mix of U373vIII and HER14 cells. 34-E5 (green) binds specifically to EGFRvIII cells, whereas rhodamine coupled egf (red) binds to the HER14 cells. Nuclei in blue. (B) Fluorescent immunostaining of HER14 trasnfected with EGFRvIII and GFP. Only cells expressing EGFRvIII (green cells) are recognized by 34-E5 (in red), nuclei in blue. Overlay in bottom panel.

xenografts only marginal staining was observed in areas with cells abnormal nuclei, probably necrotic cells (Fig. 4A, left and middle panel). This was confirmed by an image of a clear necrotic area showing very intense staining due to aspecific binding which was not observed in the sections of the U373vIII xenograft. Aspecific binding to necrotic areas was confirmed using the R2 nanobody directed against the hapten ate-dye Reactive Red (RR6)[24,25] showing the same staining in stromal and necrotic areas. These data suggest that the 34-E5 nanobody specifically binds to EGFRvIII.

To assess the exact affinity of the 34-E5 nanobody for EGFRvIII and wtEGFR, Surface Plasma Resonance (SPR) kinetic analysis was performed. 34-E5 showed a 0.74nM affinity for the Fc-EGFRvIII fusionconstruct coated on a CM5

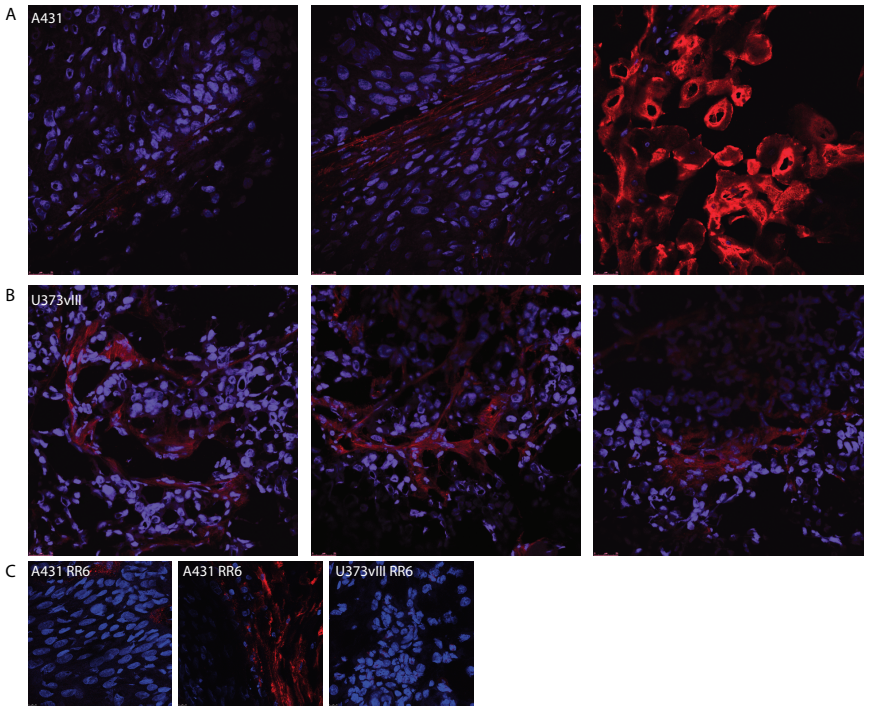

Figure 4. (A) Fluorescent immunostaining of A431 xenograft with direct labelled 34-E5. No staining was observed in the viable part of the tumour (left), a slight staining in regions with abnormal nuclei (middle, probably necrotic cells) and strong staining in clear necrotic areas (right). (B) Fluorescent immunostaining of U373vIII xenograft showing clear staining throughout the tumor. (C) Fluorescent immunostaining with RR6 for control of aspecific nanobody binding.

sensor chip (Ka $4.370*10^{5}$, Kd $3.242*10^{-4}$). Unfortunately, the 34-E5 nanobody also bound to the Fc-wtEGFR fusion construct with an affinity of 1.77nM (Ka $3.579*10^{5}$, Kd $6.335*10^{-4}$). The lower affinity for wtEGFR is mostly due to its almost 2 fold higher dissociation rate constant.

To explain the observed binding of the 34-E5 nanobody to wtEGFR in the SPR and in the staining of the A431 xenografts (Fig. 4A, middle panel) an ELISA with a substantial larger amount of antigen was performed. 1µg of fusion construct was coated per well and binding of the nanobodies was tested. The control nanobody Egb5, specific for wtEGFR, only showed binding in the wells coated with wtEGFR fusion construct. The 34-E5 showed clear binding in the wells coated with EGFRvIII fusion constructs, but unfortunately also in the wells

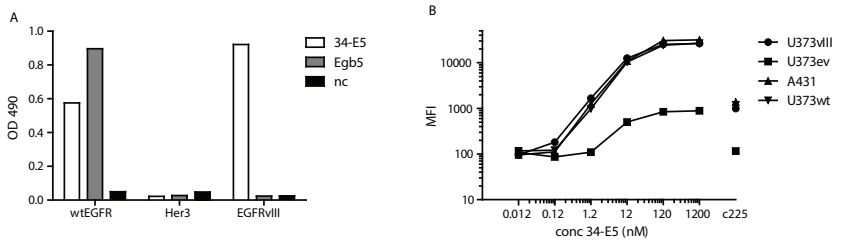

Figure 5. (A) ELISA showing specific binding of 34-E5 to EGFRvIII, but also binding to wtEGFR (B) Flow cytometry showing increased binding of 34-E5 with increased concentration of 34-E5. (U373ev is control cell line with low amounts of wtEGFR, U373wt is U373 with overexpression of wtEGFR). Cetuximab (c225) was used as a control.

coated with wtEGFR fusion construct, albeit less. Neither of the nanobodies tested showed binding to Her3, indicating that the observed binding is limited to wtEGFR/EGFRvIII (Fig. 5A).

To address the binding of 34-E5 to cells with more copies of the EGF receptor a flow cytometry analysis was performed on A431, U373vIII, U373wt (U373 cells with wtEGFR overexpression) and U373ev cells (Fig. 5B). Increasing amounts of 34-E5 nanobody showed increased binding to U373vIII cells. Unfortunately, the same was observed for A431 and U373wt cells. When concentrations of the nanobody were high enough, even binding to U373ev cells was observed. These results regrettably indicate that the selected 34-E5 nanobody is not specific for EGFRvIII when copy numbers of wtEGFR are high.

Discussion

This study set out to develop a nanobody based probe to identify EGFRvIII expression in in vitro and in vivo. Ideally this nanobody, together with the previously developed wtEGFR specific nanobody (Egb5, Roovers et al. unpublished results), could be used to discriminate between wtEGFR and EGFRvIII expressing tumours in vivo. Nanobodies were previously generated that specifically recognised domain I of the EGFR, thereby not binding to EGFRvIII. In addition, nanobodies were attempted to be selected that recognised EGFRvIII and that did not bind to wtEGFR. Although selection of the wtEGFR specific nanobody was very successful, the nanobody selected

against EGFRvIII was only specific when low amounts of wtEGFR were present. When bigger amounts of wtEGFR, e.g. A431 cells, were presented, the EGFRvIII specific nanobody turned out not to be fully specific for EGFRvIII. This was quite unexpected since selection and screening were planned out carefully. Selections were done with depletion of wtEGFR binders by means of an access amount of HER14 cells and by selective elution with EGFRvIII peptide. During screening specificity for wtEGFR was also elaborately tested in ELISA, FACS and fluorescent-IHC. Throughout the whole screening process no indications for wtEGFR specificity were found. Unexpectedly, in the SPR experiment affinity of the EGFRvIII specific antibody to wtEGFR was detected. Indeed, when the EGFRvIII specific antibody was tested for binding to A431 cells in FACS, or higher amounts of wtEGFR were presented in ELISA, the nanobody showed binding to wtEGFR. A potential explanation could be the sensitivity of the secondary antibodies used to test nanobody binding. The used anti-FLAG antibody has a much lower detection limit then the anti-MYC 9E10. Thereby low amount of 34-E5 bound to wtEGFR could have been missed, while they were detected by the anti-FLAG antibody.

Unfortunately no real EGFRvIII specific antibody was acquired during the selection most probably due to the strong presence of the 34-E5 nanobody family. This assumption is heightened by the fact that a selection with specific elution with L8A4, a proven EGFRvIII specific mouse monoclonal[26], solely resulted in 34-E5 family members. Nevertheless, we still believe a specific EGFRvIII nanobody is present in the library because of the difference in ELISA signal when post immunisation sera were tested for wtEGFR and EGFRvIII binding (Fig. 1).

Camelid derived nanobodies are able to bind to hidden antigens that are not accessible to whole antibodies, for example to the active sites of enzymes or the ligand binding domain of receptors. This property has been shown to result from their extended CDR3 loop, which is able to penetrate such sites.[27,28] While the EGFRvIII peptide used in many selection and immunization[26,29-31] has a type II′ β-hairpin conformation, resulting in a structural conformation suitable for nanobody binding, the full length ECD of EGFRvIII on the cell surface is considered to be quite amorphous. Perhaps this makes it very difficult to find nanobody with affinity for EGFRvIII. While highly specific full length rabbit,

mouse and human IgGs directed against EGFRvIII have been made[26,32,33], the selection of antibody fragments like scFvs or nanobodies remain difficult. Affinity of these antibody fragments has been proven for EGFRvIII peptide or EGFRvIII on a cell surface but the required proof of absence of affinity for wtEGFR is still lacking.[29,30,34]

For future selections we would like to recommend more extensive depletion of wtEGFR binders. Perhaps even blocking of the epitope recognized by the 34-E5 family with excessive amounts of soluble 34-E5 nanobody could be suggested, avoiding selection of the 34-E5 family.[35]

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Chapter 4

The deletion mutant EGFRvIII significantly contributes to stress resistance typical for the tumour microenvironment

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Abstract

Background and purpose: The epidermal growth factor receptor (EGFR) is overexpressed or mutated in many tumour types. The truncated, constitutively active EGFRvIII variant has not been detected in normal tissues but is found in many malignancies. In the current study, we have investigated the hypothesis that EGFRvIII contributes to a growth and survival advantage under tumour microenvironment-related stress conditions.

Materials and methods: U373MG doxycycline-regulated isogenic cells expressing EGFRwt or EGFRvIII were created and validated using Western blot, FACS and qRT-PCR. In vitro proliferation was evaluated with standard growth assays. Cell survival was assayed using clonogenic survival. Animal experiments were performed using NMRI-nu-xenografted mice.

Results: Inducible isogenic cell lines were created and showed high induction of EGFRwt and EGFRvIII upon doxycycline addition. Overexpression of EGFRvIII but not of EGFRwt in this model resulted in a growth and survival advantage upon different tumour microenvironment-related stress conditions in vitro. Induction of EGFRvIII increased tumour growth in vivo, which was reversible upon loss of expression.

Conclusions: Under conditions where nutrients are limited and stress is apparent, as in the tumour microenvironment, expression of EGFRvIII leads to a growth and survival advantage. These data indicate a potential selection of EGFRvIII-expressing tumour cells under such stress conditions.

Introduction

The epidermal growth factor receptor (EGFR) is overexpressed, amplified or altered in various human epithelial tumours.[1] Upon ligand binding and receptor activation, tyrosine residues in their cytoplasmatic domain are phosphorylated, resulting in the activation of downstream substrates that control cell proliferation, differentiation and survival. It is therefore not surprising that overexpression of EGFR has been shown to be associated with tumour aggressiveness and treatment resistance[2,3] and that targeting of EGFR is a potentially promising treatment strategy.[4-8]

Several reports have documented rearrangements within the EGFR gene, especially in primary glioblastomas.[9] The most common variant is EGFRvIII, which harbours an in-frame deletion resulting in a truncated receptor that lacks 267 amino acids in the extracellular binding domain. This truncation leads to important functional changes: the receptor cannot bind ligand, but is constitutively active, resulting in uncontrolled pro-oncogenic effects, thereby promoting neoplastic transformation and tumourigenicity. The EGFRvIII variant is found in many malignancies, including breast, head and neck, prostate, lung and brain tumours. In patients with glioblastoma, the EGFRvIII mutation has an overall prevalence of 50-60% in patients whose tumours show amplification of EGFR.[10] Strikingly, EGFRvIII has not been detected in normal tissues. Moreover, expression in tumour tissues in vivo is rapidly lost when cultured in vitro.[11] This observation suggests that the tumour microenvironment, which is characterized by heterogeneities in nutrient supply and oxygenation, may provide suitable conditions for growth and survival of EGFRvIII-expressing tumour cells. The establishment of such a microenvironment, unlike that of any normal tissue, is a consequence of alterations in the metabolic and proliferative status of tumour cells together with a highly irregular vascular supply.[12] This process is believed to occur early in the development of a tumour, and has been implicated in promoting metastasis, angiogenesis and increased malignancy. Cellular adaptive responses to this environment are critical for the continued growth of tumours. The role of EGFRvIII expression in the context of the tumour microenvironment has only been minimally defined. The present study was performed to investigate the contribution of EGFRvIII expression on

tumour growth, as well as its importance in determining the response to microenvironmental stress.

Materials and methods

Plasmids and generation of cell lines

The glioblastoma cell line U373MG (ATCC, Manassas, VA, USA) was cultured in MEMa medium, supplemented with 10% FBS. Isogenic cell lines that can be induced to express either EGFR wildtype (wt) or EGFRvIII were generated using the Flp-In T-Rex Core Kit from Invitrogen (Breda, NL), according to the manufacturer's recommendations. These cell lines were designated U373FlpIn/EGFRvIII and U373FlpIn/EGFRwt, respectively. To induce gene expression, doxycycline (1 µg/ml) was added to the medium.

Antibodies and Western blotting

Western blotting was done according to standard protocols as described.[13] Antibodies used were Sc-03 (Santa Cruz Biotechnology, Huissen, NL) for EGFRwt, L8A4 (a gift from D. Bigner, Duke University, Durham, NC, USA) for EGFRvIII and HRP-linked antirabbit (EGFRwt) or anti-mouse (EGFRvIII) secondary antibodies. Immobilized proteins were detected using ECL-plus reagent (Kirkegaard & Perry Laboratories, NL) and by exposing blots to X-ray film.

Quantitative real-time PCR

RNA extraction was performed using the SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA). cDNA was prepared using the iScript cDNA Synthesis kit (BioRad Laboratories, CA, USA). Reactions were carried out in a 25 µl volume using SYBR Green Master Mix (Applied Biosystems, CA) with the ABI Prism 7700 Sequence Detection System. Values for each gene were normalized to expression levels of 18S RNA. The primer sequences used were:

EGFRvIII forward: 5'AAGAAAGGTAATTATGTGGTGACA3';
EGFRvIII reverse: 5'CCGTCTTCCTCCATCTCATAGC3';
EGFRwt forward: 5'ACCTGCGTGAAGAAGTGTCC3'; and
EGFRwt reverse: 5'CCGTCTTCCTCCATCTCATAGC3'

Flow cytometric analysis

FACS analysis with Oregon Green-labeled cetuximab was performed as described.[14]

In vitro growth and survival assays

For growth under control aerobic conditions, $2.5*10^5$ cells were seeded and incubated in 5% CO_2 for 1–7 days. To monitor growth under stress conditions, cells were seeded and allowed to attach during overnight incubation in 5% CO_2. The following day (day 0), dishes were placed in the hypoxic chamber or medium was replaced according to the appropriate conditions. Total cell numbers were counted after trypsinization using a Coulter Z series particle counter (Beckman, Mijdrecht, NL) and cell numbers normalized to the amount of cells present at day 0. Clonogenic assays were performed as previously described.[13]

Tumour xenograft model

In vivo experiments were performed using adult NMRI-nu (nu/nu) female mice. Animal facilities and experiments were in accordance with local institutional guidelines. U373 FlpIn/EGFRvIII cells were mixed with matrigel and $3*10^6$ cells were subcutaneously injected in the lateral flank. Induction of gene expression was done by giving animals doxycycline (2 g/l) in their drinking water. Tumours were measured with calipers in 3 orthogonal diameters and volumes calculated with the formula A x B x C x π/6. The tumour doubling time (T_d) was calculated using the equation $Td = (T-T_0) \times \log 2/[\log(V) - \log(V_0)]$ where $T - T_0$ indicates the length of time between two measurements and V_0 and V denote the tumour volume at two points of measurement.

Statistics

Statistical analysis was carried out using the program GraphPad Prism version 5.01 for Windows (GraphPad Software, 2007, CA, USA). A Mann–Whitney U test was used to assess differences in tumour doubling times. Non-linear regression analysis was performed to compare differences between in vivo growth curves. Student's t-test was applied to determine differences in growth and survival assays.

Results

To investigate the potential influence of EGFRvIII on tumour phenotype, and to compare the effect of EGFRvIII overexpressio with that of EGFRwt, we created doxycycline-inducible U373MG glioma isogenic cell lines expressing EGFRvIII or EGFRwt. The unmodified parental U373MG cells express moderate levels of endogenous EGFRwt and have been widely recognized as a valuable in vitro and xenograft model. Previously, we constructed a Flp-In T-Rex host acceptor cell line, designated U373FlpIn. Following transfection and selection, we identified clones for both EGFRvIII and EGFRwt. Quantitative PCR revealed induction of mRNA levels of 7.15 ± 0.6 for EGFRvIII and 7.55 ± 0.6 for the EGFRwt in response to doxycycline (Fig. 1A). As shown in Fig. 1B, protein levels of EGFRvIII were strongly induced after exposure to doxycycline, with virtually undetectable background levels. EGFRwt is also induced in the U373FlpIn/EGFRwt cells, but to a lesser extent than EGFRvIII in the U373FlpIn/EGFRvIII cells, presumably due to the already expressed endogenous EGFRwt levels in U373FlpIn. To show that the vIII mutant and the wt receptor were properly processed and located at the cell membrane after doxycycline administration, we performed a flow cytometry experiment using Oregon Greenlabeled cetuximab (C225-OG) (Fig. 1C). Without doxycycline induction, both cell lines showed similar levels of C225-OG binding, indicating binding of C225-OG to endogenous EGFRwt receptor. Increased C225-OG binding was observed when doxycycline was added, reaching a 5- to 6-fold increase at 72 h. Taken together,these data demonstrate that the use of this inducible system is feasible and that it results in an increase of correctly

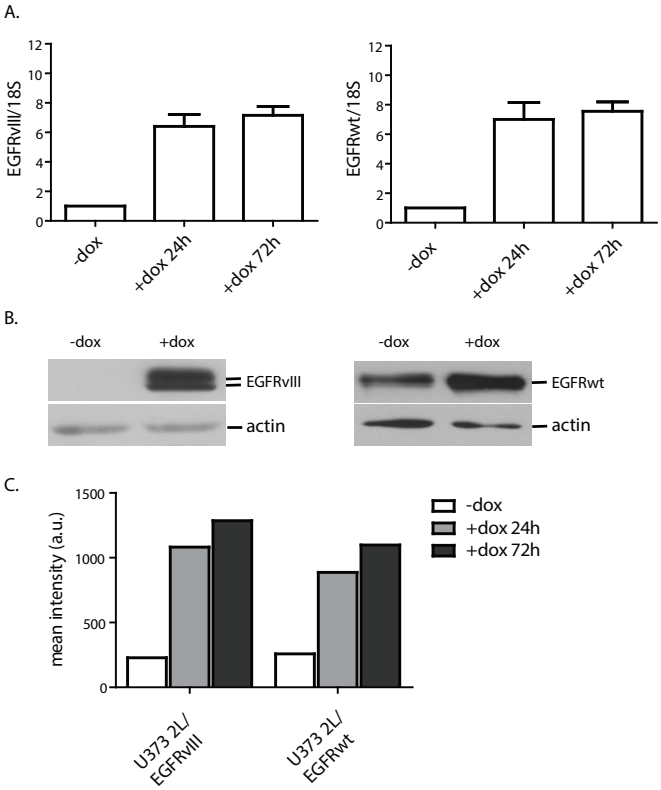

Figure 1. Validation of U373FlpIn/EGFRvIII and U373FlpIn/EGFRwt doxycycline-inducible isogenic cell line. (A) EGFRvIII and EGFRwt transcript levels in U373FlpIn transfected cells following addition of doxycycline for 24-72 h, reported as mean ± SEM of triplicate measurements. (B) Representative Western blots showing EGFRvIII and EGFRwt protein levels after doxycycline induction for 48 h. (C) FACS analysis of C225-OG binding to U373FlpIn/EGFRvIII and U373FlpIn/EGFRwt cells after 24–72 h induction with doxycycline.

processed receptors at the membrane of induced U373FlpIn cells. To assess the biological relevance of EGFRvIII expression in relation to stress typically found in the tumour microenvironment, we evaluated the growth of U373FlpIn/EGFRvIII and U373FlpIn/ EGFRwt inducible isogenic cell lines under conditions of serum starvation, low pH, glucose deprivation, hypoxia (0.2%) and anoxia. As shown in Fig. 2, no effect on growth after induction of EGFRvIII or EGFRwt was observed in medium containing 10% FBS. However, in conditions of serum starvation, addition of doxycycline to overexpress EGFRvIII

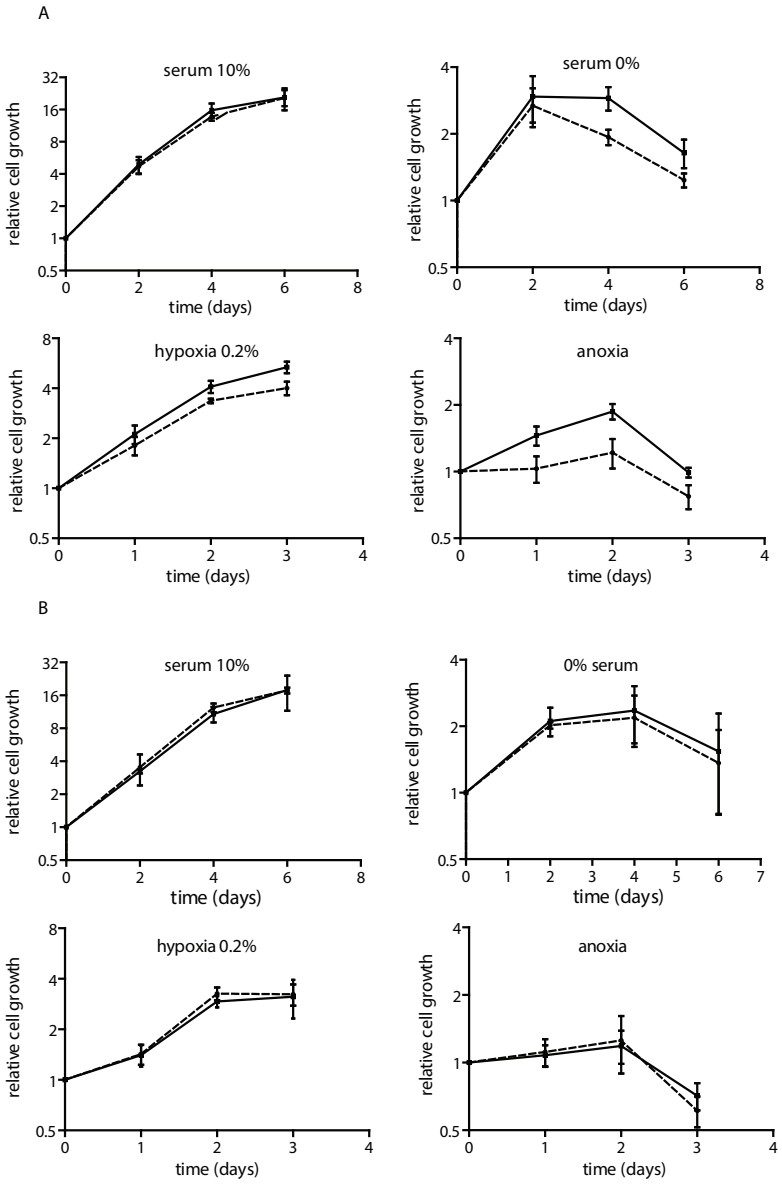

Figure 2. EGFRvIII expression results in a growth advantage in tumour microenvironment-related stress conditions. Growth was evaluated by counting cells at the indicated time points and normalized towards the number of cells present at day 0. Experiments were done in triplicate. Data points represent the mean ± SEM. U373FlpIn/EGFRvIII cells (A) and U373FlpIn/EGFRwt cells (B) were grown in the absence (dashed line) or presence (full line) of doxycycline at 10% or 0% serum, 0.2% hypoxia or in anoxia.

resulted in a consistent growth advantage over non-induced cells (Fig. 2A). In contrast, no proliferation effect was detected upon induction of the EGFRwt in U373FlpIn/EGFRwt (Fig. 2B). Similarly, overexpression of EGFRvIII resulted in significant induction of proliferation in conditions of low oxygen (0.2%) (Fig. 2A, $p < 0.05$) and complete anoxia (Fig. 2A, $p < 0.05$ at 48 h). Overexpression of EGFRwt did not lead to increased proliferation in either hypoxic or anoxic conditions (Fig. 2B), and remained at the same levels as the non-induced cells. No EGFRvIII-mediated growth promoting effect could be detected when cells were exposed to low glucose concentrations or when grown at pH 6.2 (data not shown).

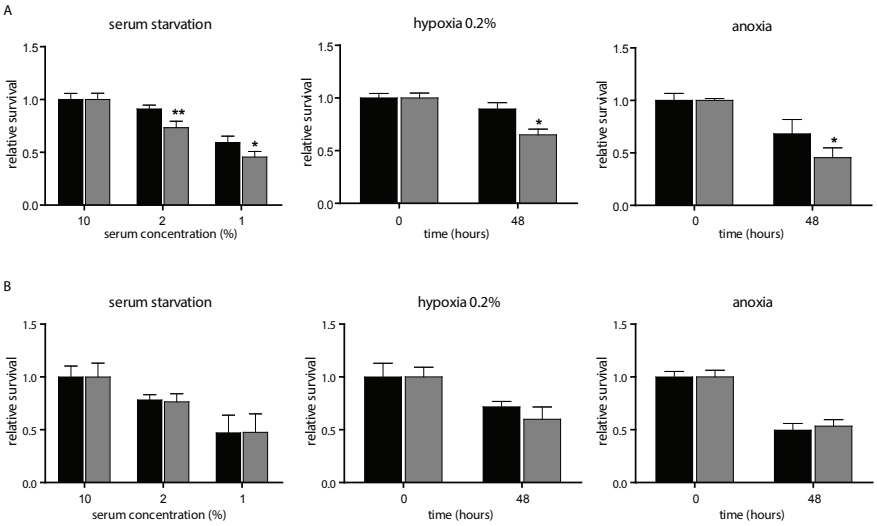

Figure 3. EGFRvIII expression influences cell survival in tumour microenvironment-related stress conditions. Clonogenic survival assay of U373FlpIn/EGFRvIII (A) and U373FlpIn/EGFRwt (B) after exposure to different concentrations of serum, to 0.2% hypoxia or to anoxia for 48 h with (black bars) or without (grey bars) induction with doxycycline. The mean values of three independent experiments are shown. Error bars represent SEM. (*$p < 0.05$; **$p < 0.01$).

To further address the biological significance of EGFRvIII overexpression, we performed cell survival assays. In conditions with low serum concentration, EGFRvIII-expressing cells survived significantly better as cells without EGFRvIII ($p < 0.01$ at 2% and $p < 0.05$ at 1%, Fig. 3A). We have previously demonstrated that EGFRvIII expression promotes hypoxia tolerance.[13] However, those

experiments were performed using cells that stably expressed very high levels of EGFRvIII. We therefore also compared the hypoxia tolerance of the U373FlpIn/EGFRvIII and U373FlpIn/ EGFRwt cell lines following exposure to 48 h of hypoxia or anoxia. As shown in Fig. 3A, cells expressing EGFRvIII tolerated hypoxia ($p < 0.05$) and anoxia ($p < 0.05$) better than the cells that did not express the mutant receptor. Similar to our observations in proliferation experiments, overexpression of EGFRwt did not increase hypoxia or anoxia tolerance (Fig. 3B).

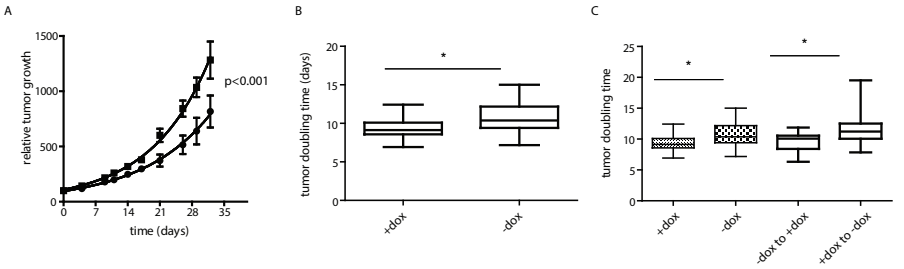

Figure 4. Induction of EGFRvIII increases tumour growth in vivo. (A) U373FlpIn/EGFRvIII xenografts were established in nude mice (n = 28). Mice were separated in two groups of which one group (n = 14) received doxycycline (j) and the other group (n = 14) did not (d). Mean tumour volumes ± SEM are shown. Volumes were normalized to the start of the measurements (day 0). Data were fitted using an exponential growth equation. Non-linear regression analysis was subsequently performed to compare whether both these fits are significantly different (F-test). (B) Doubling times for tumours growing in animals that received doxycycline (left panel) or did not receive doxycycline (right panel) (*$p < 0.05$). (C) Tumour doubling times in animals after switching of animals of both groups (n = 6) to the other treatment group by withdrawing or administering doxycycline (*$p < 0.05$).

To investigate the influence of EGFRvIII expression on tumour growth in vivo, and to evaluate whether the observed in vitro observations could be translated to an effect in vivo, we injected U373FlpIn/EGFRvIII cells in NMRI-mice to form tumours. Mice were divided in two equal groups (n = 14) of which one received doxycycline. Cells in both groups formed tumours with similar efficiency and palpable tumours became visible at 12 days postinjection. As illustrated in Fig. 4A, the tumours in animals that were given doxycycline grew significantly ($p < 0.001$) faster as those in animals without doxycycline administration, as

evidenced by the shorter doubling time of doxycycline-induced tumours versus the non-induced xenografts (9.3 days versus 10.7 days, $p < 0.05$) (Fig. 4B). When tumours reached volumes of 250-400 mm^3, animals from both groups (n = 6) were randomly switched to the other treatment group by withdrawing or administering doxycycline. Intriguingly, the tumour growth rate in animals that were switched from no doxycycline to receiving doxycycline increased and their doubling time, calculated from the switch onwards, decreased (mean T_d = 9.5 days) to levels similar to that of tumours in which EGFRvIII was already expressed from the start of the experiment. Conversely, tumour growth rate in animals that were taken off doxycycline decreased, and doubling times (mean T_d = 11.7 days) became similar to those of tumours that had never been induced with doxycycline (Fig. 4C). These data indicate that EGFRvIII promotes the growth of established tumours.

Discussion

In this study, we have shown that overexpression of EGFRvIII, but not of EGFRwt, results in a consistent proliferative and survival advantage when cells were grown under low serum, hypoxia or anoxia. Interestingly, the observed in vitro effects were also translated in increased rates of tumour growth in vivo. Previous reports have demonstrated a prominent role for EGFRvIII driving the progression of neoplastic events.[15,16] However, these studies have been mainly carried out using tumour models that massively and stably overexpress the mutant receptor. To rule out clonal effects of stable transfectants and to specifically investigate the tumourigenic role of EGFRvIII, we therefore developed an isogenic inducible system. Because several different transgenes can be inserted in an identical way into the same parental cell line, it is possible to compare their contribution and effectiveness in exactly the same genetic background, overcoming a general disadvantage of models used until now. Moreover, because EGFRvIII is constitutively active, an inducible model is particularly useful for studying such a mutant. Strikingly, whereas expression of the EGFRvIII-mutant confers proliferation and survival advantages in conditions of low serum and oxygen availability, overexpression of the wt receptor did not. This might be explained by the endogenous levels of wt

receptor that are already present on these cells (Fig. 1C), masking the effects of a further increase of receptor expression. However, other mechanisms may explain the differential phenotypic effect we observed. EGFRvIII has been shown to be increasingly associated with the adapter proteins Shc and Grb2, and increased levels of the active form of Ras.[17] A recent large-scale analysis of phosphorylated proteins induced by EGFRvIII expression pointed towards PI3K signaling as the dominant pathway in glioblastoma cells.[18] The significance of this pathway has also been confirmed in other studies.[19,20] In a differential gene expression analysis, Ramnarain et al. revealed that expression of EGFRvIII resulted in specific upregulation of a small group of genes that are not induced by the wt receptor. These genes influence signaling pathways known to play a key role in oncogenesis and function in interconnected networks. Importantly, EGFRvIII but not EGFRwt plays an important role in generating an autocrine loop in glioma cells by increasing expression of ligands for the wt receptor[21]. The signaling potency of EGFRvIII might also be increased by its ability to signal for a prolonged time as a result of impaired endocytosis, inefficient ubiquitination and rapid recycling.[22,23] EGFRwt has been shown to be translationally upregulated under hypoxia.[24] In the stress conditions we have tested, however, this did not translate into a measurable phenotypic effect. Interestingly, we also observed an increase in EGFRvIII expression under hypoxia (unpublished observations). Although the underlying mechanism for the increased EGFRvIII levels is not yet clear, our data presented here suggest that this induction may contribute to the observed proliferative and survival advantage. In addition, this might lead to a selection of EGFRvIII-expressing cells in vivo, thereby contributing to the aggressive tumour phenotype. Interestingly, with the inducible model we have developed, tumours can be established with a parental genotype and subsequently induced to express a gene of interest to selectively examine its effect. The fact that tumours start to grow faster as soon as they are induced to express EGFRvIII (Fig. 4C), underscores the important role of EGFRvIII in the tumourigenesis process. Conversely, we also demonstrate that the increased growth rate of EGFRvIII-expressing tumours can be reversed when the mutant receptor is no longer expressed. These data indicate that targeting EGFRvIII by silencing its expression and/or blocking its downstream effects[25], even when EGFRwt

receptor levels are relatively high as in our model, might be a promising strategy for treating cancers that contain this mutated receptor.

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Chapter 5

Autophagy inhibition abrogates the survival advantages of EGFRvIII^{+} cells, sensitizes tumors to irradiation and improves prognosis of GBM patients

In preparation
Jutten B, Schaaf MBE, Keulers TGH, Clarijs R, Theys J, Colaris RME, Dubois L, Savelkouls KGM, Vooijs MA, Lambin P, Bussink J, Sotelo J, Lammering GMA and Rouschop KMA

Abstract

Expression of Epidermal growth factor receptor variant 3 (EGFRvIII), a constitutively active deletion variant of EGFR, is frequently observed in glioblastoma multiforme (GBM). We and others have shown that EGFRvIII expression is advantageous for cellular proliferation rate in vitro and in vivo. In addition, these cells display enhanced survival following radio- and chemotherapy and after exposure to metabolic stresses typically found in the tumor microenvironment such as hypoxia and starvation. Recent studies indicate that metabolically challenged cells activate autophagy to enhance their survival. In this study, we evaluated the consequence of enhanced autophagy activation in EGFRvIII expressing following exposure to starvation and hypoxia. Furthermore we assessed the therapeutic potential of targeting autophagy in EGFRvIII expressing GBM tumors.

EGFRvIII expressing cells displayed a faster and more pronounced autophagic response compared to non-EGFRvIII expressing cells during exposure to hypoxia or serum starvation. Autophagy inhibition through chloroquine (CQ) addition abrogated the enhanced proliferation and survival of EGFRvIII positive cells during stress conditions in vitro. As expected, EGFRvIII positive tumors are more radio resistant than control tumors, as displayed by decreased tumor growth delay. Interestingly, the increased radio resistance of EGFRvIII expressing tumors was abrogated by CQ treatment prior to irradiation. Although effective in both GBM patient cohorts, retrospective analysis of GBM patients confirmed the enhanced effect on EGFRvIII positive GBM. (Increase in median survival for EGFRvIII 12 months versus 5 months for other).

In conclusion, EGFRvIII overexpressing cells are more dependent on autophagy for survival during metabolic stresses. Inhibition of autophagy diminished the proliferation and survival advantage of EGFRvIII overexpressing cells and sensitizes tumors to treatment. In line, although CQ treatment improves survival of GBM patients, patients with EGFRvIII expressing GBM benefited most. Therefore, CQ treatment could be considered as additional treatment strategy for GBM patients, and can reverse the poorer prognosis of patients with EGFRvIII positive GBM.

Introduction

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein and one of four members of the erbB family of tyrosine kinase receptors. Binding of one of its natural ligands results in homo- or heterodimerization of the receptor and subsequent activation of signal transduction pathways. These pathways are involved in regulating cellular proliferation, differentiation, and survival.[1,2] Although present in normal cells, EGFR is overexpressed in a variety of tumors and has been associated with poor prognosis and decreased survival.[3] EGFR activation also plays a role in resistance to chemotherapy and radiation treatment in tumor cells.[4,5]

EGFR is often mutated, leading to enhanced or sustained receptor signaling. One of the most common variant of the EGFR is an exon 2-7 deletion mutant, EGFRvIII.[6-9] This deletion results in a truncated receptor that lacks 267 amino acids in the extracellular binding domain. This deletion leads to important functional changes: the receptor cannot bind its ligands, but is constitutively active, resulting in uncontrolled pro-oncogenic effects, thereby promoting neoplastic transformation and tumorigenicity.[10-13] The EGFRvIII variant is found in many malignancies, including glioblastoma, non-small lung cell carcinoma, breast cancer, prostate cancer and head and neck cancer.[6,8,9] More recently, EGFRvIII signaling has been implied in tumor stem cell maintenance.[14-16] In GBM patients with amplification of the EGFR gene, the overall prevalence of EGFRvIII is 50-60%.[17] Moreover, expression in tumor tissues in vivo is rapidly lost when tumor cells are subsequently cultured in vitro.[18] This observation suggests that the tumor microenvironment, which is characterized by heterogeneities in nutrient supply and oxygenation, may provide suitable conditions for growth and survival of EGFRvIII-expressing tumor cells. Cellular adaptive responses to this environment are critical for the continued growth of tumors.

To survive in such environment cells will frequently upregulate autophagy. Autophagy (Greek for “self-eating”) is a mechanism that protects cancer cells from low nutrient supply or therapeutic insults.[19,20] It enables a cell to recycle constituents and provide itself with the necessary nutrients. Besides starvation, autophagy is upregulated during exposure to other physiological stress stimuli

(e.g., hypoxia, energy depletion, endoplasmic reticulum stress, high temperature, and high density conditions).[21,22]
In initial experiments we observed that EGFRvIII expressing cells displayed increased proliferation and survival in association with increased autophagy activation when metabolically challenged. We therefore assessed the dependence of EGFRvIII expressing cells and tumors on autophagy for their increased proliferation, survival and resistance to therapy.

Materials and Methods

Reagents and cell lines

Unless specified otherwise, all reagents were obtained from Sigma Chemical Co. (the Netherlands) and all electrophoresis reagents were from Bio-Rad (the Netherlands). MEM-α medium was obtained from Invitrogen (Life Technologies, the Netherlands), glutamax-I (35050-038) was obtained from Life Technologies (the Netherlands), DMEM and fetal bovine serum (FBS) was from GE-Healthcare (the Netherlands). The U373-MG, U-87 MG and T-47D cell lines were obtained from ATCC (USA). Cells were maintained as described by ATCC. The L8A4 antibody was kindly provided by D. Bigner (Dept. of Pathology, Duke University, Durham, NC, USA).

Western blotting

Cells were lysed and processed as described previously[7] and proteins were separated on mini-PROTEAN precast gels (4-20%, Bio-Rad). After transfer, proteins were probed with antibodies against EGFR (Santa-Cruz, USA, sc-03), MAP1LC3b (Cell Signaling, Bioke, the Netherlands, 2775S), phosphorylated ERK (Cell signaling, 4370) and Actin (MP Biomedicals, the Netherlands, 8961001). Bound antibodies were visualized using HRP-linked anti-rabbit (Cell Signaling, 7074S) or anti-mouse (Cell Signaling, 7076S) antibodies.

Immunofluorescence and confocal microscopy

Cells were grown on 14mm round coverslips (VWR, the Netherlands) in a 24-well culture plate (Greiner Bio One, the Netherlands) until 70% confluency. The medium was replaced with either medium without FBS, chloroquine was added in a concentration of 5µg/ml and cells were incubated for 6 hours. Cells were washed 3 times with PBS and fixed in 4% PBS-buffered paraformaldehyde (pfa) and permeabilized using 0.5% Tween-20 in PBS. Cells were probed with MAP1LC3b (Cell Signaling, 2775S) followed by anti-rabbit Alexa Fluor 488 (Invitrogen, A11006), and analyzed using a Leica TCS SPE confocal microscope.

Proliferation curves

Cells were seeded in a 24-well culture plate (Greiner Bio One) and incubated under either normal, serum starved or hypoxic conditions. Chloroquine was used at a 5µg/ml. Every 24 hours a plate was washed and fixed with 4% pfa. Plates were stained with 0.5ml 0.1% cristalviolet for 15' at RT, after extensive washing with double distilled water cells were lysed with 0.5ml 10% acetic acid for 15' at RT. Absorbance was measured at 590 nm.

Clonogenic Survival assay

Cells were counted using a Coulter Z Series particle counter (Beckman Coulter, the Netherlands) and seeded in triplicate 6 cm dishes. For starvation experiments, cells were seeded for clonogenic survival prior to the starvation. After serum starvation for up to 4 days, the medium was replaced with normal medium and cells were incubated under standard culture conditions until colonies formed (10 days). For hypoxia experiments, cells were incubated at hypoxic conditions (O_2< 0.02%) for up to 3 days. Cells, including detached cells, were harvested, seeded and incubated under standard culture conditions until colonies formed (10 days). Colonies were fixed and stained with 0.4% bromophenol blue in 70% ethanol. Plating efficiency was determined by counting colonies consisting of ≥50 cells and corrected for the number of cells

seeded.

In vivo experiments

Animal facilities and experiments were in accordance with local institutional guidelines and approved by the local animal welfare committee. Experiments were performed as described previously.[22] Briefly, tumors were grown in NMRI-nu (nu/nu) female mice. U373-EGFRvIII and U373 control cells in matrigel were injected subcutaneously ($1.5x10^6$ cells). Tumor size was assessed by caliper measurement in 3 orthogonal diameters. Mice treated with CQ received 60 mg/kg CQ for 7 consecutive days via intraperitoneal injection. Tumors were positioned in the irradiation field using a custom-built jig and irradiated with a single dose of 10 Gy (15 MeV e-) using a linear accelerator (Varian).[23]

Immunohistochemistry and image processing

Frozen, acetone-fixed sections were stained by using anti-pimonidazole (Chemicon, Merck Millipore, Germany), anti LC3b (Abgent, USA, AP1806a) and anti EGFR (Santa Cruz, sc-03). For quantitative analysis, the slides were scanned by a computerized digital image processing system by using a high-resolution intensified solid-state camera on a fluorescence microscope (Axioskop; Zeiss, the Netherlands) with a computer-controlled motorized stepping stage. Tumor necrosis was evaluated, relative to the total tumor area, morphologically by using H&E staining. Tumor hypoxic fraction and vascular density (structures per square millimeter) were determined relative to the viable tumor tissue (necrosis excluded).

Results

EGFRvIII cells depend more on autophagy during starvation in vitro

EGFR is often overexpressed or mutated in glioblastoma. One of the most commonly found mutation is the constitutive active deletion variant EGFRvIII.

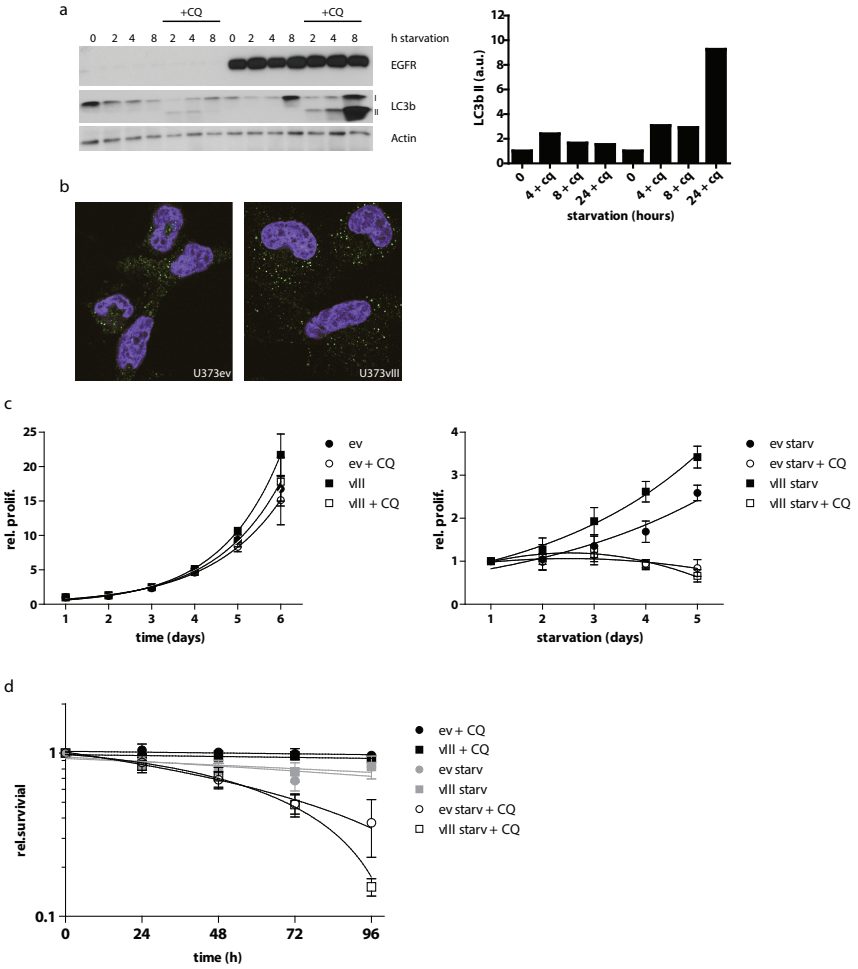

Figure 1. (A) EGFRvIII expressing glioma cells display increased autophagy under starvation. (B) EGFRvIII expressing cells display more autophagosomes under starvation (nuclei in blue, LC3b in green). (C) Proliferation assay of EGFRvIII and control cells under normal growth conditions, CQ was used at a non-toxic concentration (5µg/ml) (left panel). EGFRvIII cells proliferate faster under starvation but this can be abrogated by inhibition of autophagy (right panel). (D) EGFRvIII survive equally well under starvation as control cells but are more sensitive for autophagy inhibition under this condition.

The tumor microenvironment is characterized by the presence of regions that are low in oxygen (hypoxia) and nutrient supply. Previously we and others have shown that cells deprived of nutrients or oxygen rapidly activate autophagy.[22]

Considering the high proliferation rate and nutritional demand of EGFRvIII cells[24], we explored autophagic flux in EGFRvIII cells. To determine autophagy activity, cells were exposed to nutrient deprivation in the absence and presence of chloroquine (CQ). Assessment of LC3b-II accumulation allows determination of autophagic flux.[25,26] Indeed, when malignant glioma U373 cells were deprived from serum we observed a faster and more pronounced induction of autophagy in the EGFRvIII expressing cells (Fig. 1A left panel, right panel is a quantification of LC3b-II). We observed similar effects in another glioma line U-87 MG (fig S1) and in a prostate cancer line T-47D (data not shown). In accordance, more and larger autophagosomes were observed in the EGFRvIII expressing cells compared to the control cells (Fig. 1B). To determine if the higher autophagic flux contributed to survival advantages of EGFRvIII expressing cells, we assessed proliferation during normal and starvation culture conditions. As described previously[12], during normal culture conditions, EGFRvIII expressing cells proliferate faster. Addition of chloroquine had no significant effect on proliferation of either control or EGFRvIII expressing cells (Fig. 1C), indicating that the used concentration is non-toxic at this dose. As expected, we observed that EGFRvIII expressing cells proliferate faster during serum starvation conditions compared to control cells (Fig. 1C) and this could be abrogated by the addition of chloroquine (CQ) to the medium. To assess whether targeting autophagy decreases survival of EGFRvIII expressing cells during metabolic stress, survival was assessed by clonogenic survival. During normal culture conditions, the chosen CQ dose proved nontoxic, and also serum starvation did not significantly alter clonogenic survival (Fig. 1D). However, when combining starvation with addition of CQ more EGFRvIII cells were killed. Taken together, these data suggest a more pronounced autophagic response and a higher dependency of autophagy in EGFRvIII expressing cells in vitro.

EGFRvIII cells depend more on autophagy during starvation in vitro

To assess if the enhanced autophagic response was restricted to nutrient deprivation, we assessed the autophagic response and dependency during hypoxia exposure, another stress typically found in the tumor microenvironment. Again, EGFRvIII cells displayed an enhanced autophagic

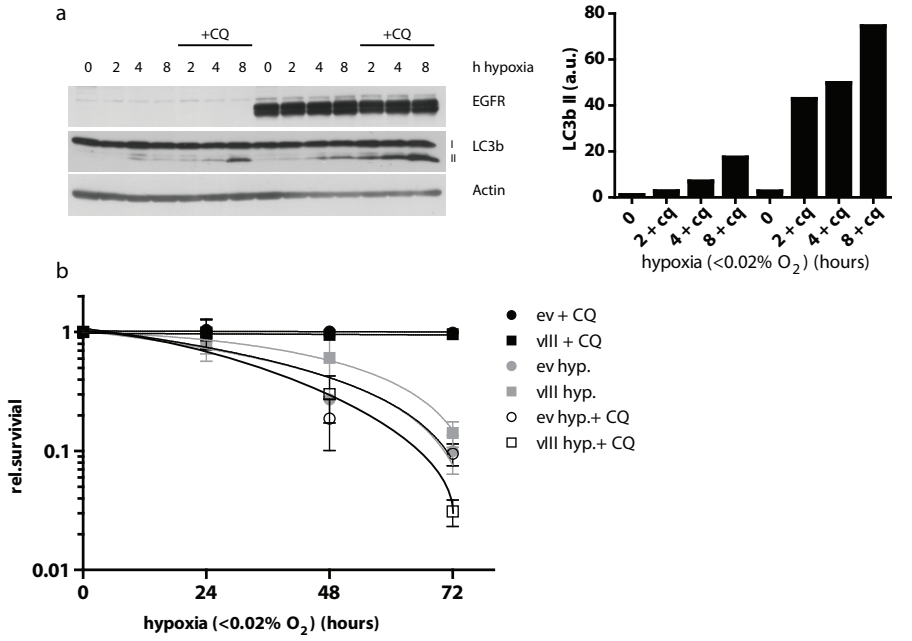

Figure 2. (A) EGFRvIII cells display increased autophagic flux under severe hypoxia (left panel, right panel is quantification of LC3b II). (B) EGFRvIII cells survive slightly better under severe anoxia compared to control cells but depend much more on their autophagy for survival.

response compared to the isogenic control cells as indicated by faster LC3b-II accumulation (Fig. 2A). To assess dependency on autophagy of EGFRvIII expressing cells during hypoxia, clonogenic survival after hypoxia exposure was determined. As expected, reduced survival during severe hypoxia was observed in both cell lines. However, EGFRvIII cells displayed a survival advantage over control cells (Fig 2B, grey lines). Addition of CQ during these conditions had no effect on survival of the control cells. Strikingly, EGFRvIII cells were greatly sensitized to severe hypoxia (Fig 2B, open symbols). These data indicate that EGFRvIII cells are more dependent on autophagy hypoxic conditions.

EGFRvIII xenografts depend on autophagy for accelerated growth?

As shown previously[12], EGFRvIII tumors grow faster than control tumors (Fig.

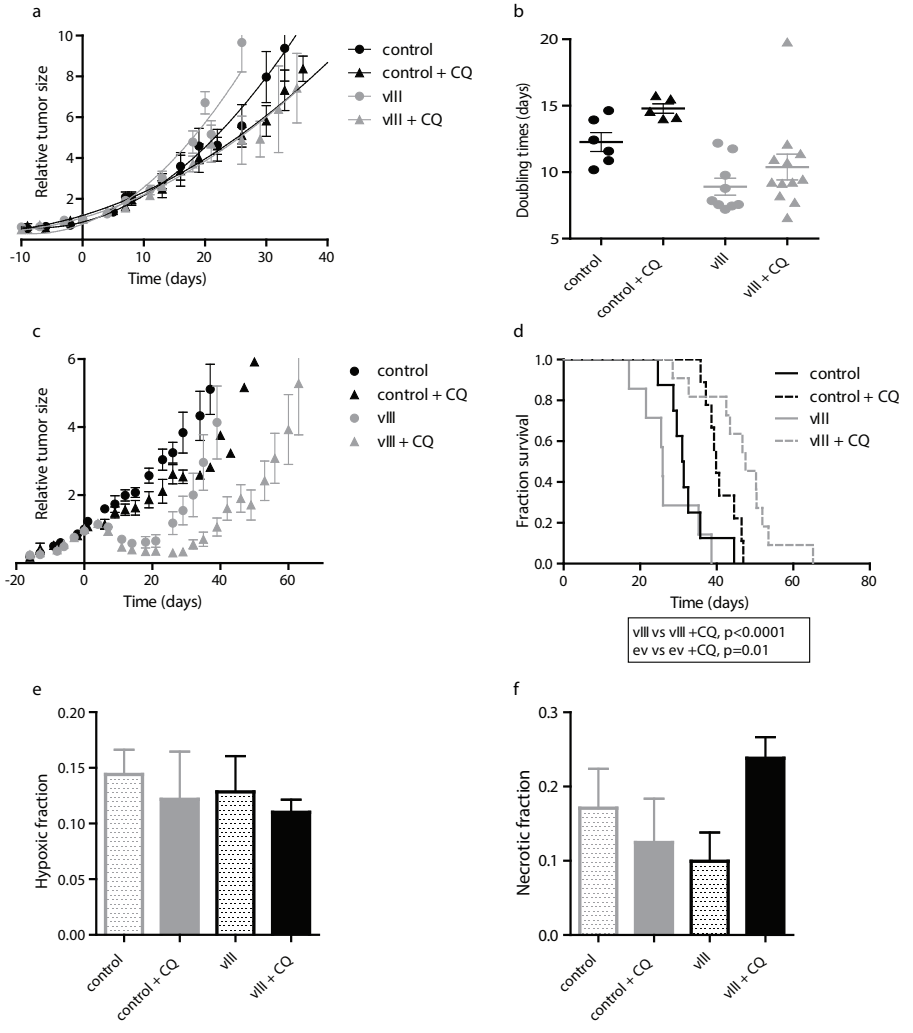

Figure 3. (A) EGFRvIII tumors grow faster than control but this can be abrogated by CQ treatment by which EGFRvIII tumors' growth rate is reduced to control tumors' growth rate. (B) EGFRvIII tumors display a faster doubling time compared to control tumors, but tumor doubling times are reduced when treated with CQ. However, control tumors are more affected in their growth than EGFRvIII tumors suggesting that the targeted cells, most likely hypoxic cells, are not the main contributor to tumor growth. (C) EGFRvIII tumors regrow faster after radiotherapy but this can be overcome but CQ treatment (D) Time to regrow to eight times the irradiated volume after irradiation with 10Gy. (E-F) Hypoxic and necrotic fraction of EGFRvIII and control tumors. (control n=6, control + CQ n=6, EGFRvIII n=5, EGFRvIII + CQ n=5)

3A. To investigate if our in vitro findings were also valid in vivo, CQ was administered to tumor bearing animals. Interestingly, treatment with CQ for seven days resulted in a small decrease in growth as also indicated by an increase in tumor doubling time in all tumors (Fig. 3B). Unexpectedly, the tumor volume doubling times of control tumors was relatively more increased than EGFRvIII tumors (20% for control tumors vs 16.8% for EGFRvIII tumors)(Fig. 3b). Nevertheless these changes are moderately suggesting that the targeted cells, most likely hypoxic cells, are not the main contributor to tumor growth. These findings are in line with earlier data.[22,27]

In contrast to the effects on growth, we previously showed that CQ treatment sensitizes tumors to irradiation by decreasing the hypoxic fraction.[22] Our in vitro data suggests that EGFRvIII expressing cells are highly dependent on autophagy for their survival during hypoxic exposure (Fig. 1B).

As hypoxic cells contribute to radiotherapy resistance, we determined if EGFRvIII expressing tumors could be sensitized to irradiation. Therefore we treated EGFRvIII and control tumor bearing mice with CQ for 7 days prior to irradiation and monitored tumor growth. We observed that control tumors hardly showed a growth delay after a single dose of 10Gy but could be radio sensitized by CQ treatment (Fig. 3C, black symbols). EGFRvIII tumors were much more

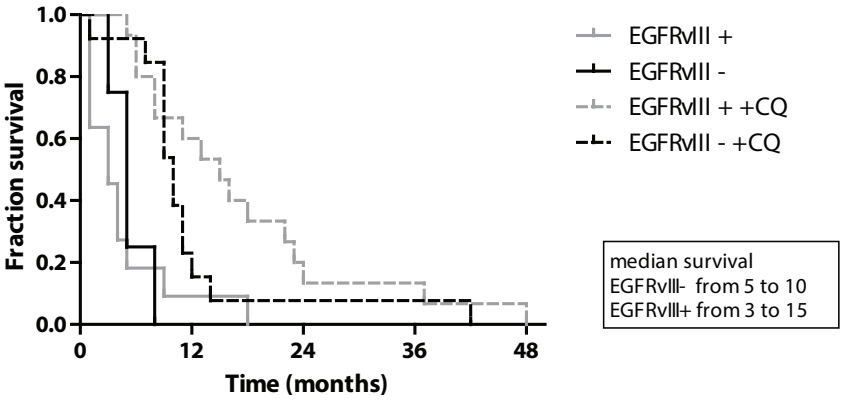

Figure 4. Survival plot of 43 GBM patients categorized for EGFRvIII status and CQ treatment. Patients with an EGFRvIII positive tumor benefit substantially from CQ treatment.

affected by radiotherapy than control tumors but regrew drastically faster. CQ treatment of EGFRvIII tumors postponed the regrowth even longer (Fig. 3C, grey symbols). When the time to reach 4x the irradiated was plotted we observed no difference between EGFRvIII tumors and control tumors (Fig. 3D). CQ treatment prior to radiotherapy postponed the regrowth of control tumors significantly. However, EGFRvIII tumors were even more delayed in their regrowth, suggesting that EGFRvIII tumors can be radio sensitized by CQ treatment. This effect is even more pronounced after a single dose of 15Gy (Fig. S2). To determine whether the enhanced radio sensitizing effect in EGFRvIII-tumors was related to a more pronounced decrease in hypoxic fraction compared to the control tumors, tumor sections were stained for tumor hypoxia (pimonidazole). No differences were observed between groups (Fig. 3E). The BrdU labeling index showed more BrdU incorporation in EGFRvIII tumors compared to control tumors (Fig. S3A), but CQ treatment did not affect the proliferation of these tumor cells. The vessel density in control tumors is increased probably due to vessel normalization as a consequence of CQ treatment (Fig. S3B). Contradictory, EGFRvIII tumors showed a high vessel density when untreated. CQ treatment reduced the vessel density to the level of control tumors treated with CQ. We could not detect a difference in perfused fraction of these tumors (Fig. S3C), this in contrast to the necrotic fraction (Fig. 3F). The necrotic fraction of CQ treated tumors was drastically increased compared to untreated tumors suggesting that CQ treatment indeed contributes to the killing of tumors cells. Taken together, CQ treatment is a promising treatment of rapidly growing EGFRvIII tumors.

Patients with an EGFRvIII positive GBM benefit from CQ treatment

To assess the clinical relevance of our findings we retrospectively analyzed 43 GBM patients, of which 28 patients received CQ in combination with conventional treatment. EGFRvIII expression was assessed immunohistochemically and observed in 22 of 43 patients. In line with previous reports[28,29], EGFRvIII expression was associated with poor survival (Fig. 4). CQ treatment improved median survival from 5 to 10 months in patients with EGFRvIII negative GBM. Strikingly median survival of patients with EGFRvIII

positive GBM increased from 3 to 15 months, confirming the therapeutic potential CQ in treatment of EGFRvIII expressing tumors.

	no CQ treatment		CQ treatment	
EGFRvIII status	vIII -	vIII +	vIII -	vIII +
male	5/8	3/7	10/13 (77%)	6/15 (40%)
female	3/8	4/7	3/13 (23%)	9/15 (60%
Karnofsky preformace (pre-surgery)				
< 70	0 (0%)	1 (14%)	0 (0%)	1 (7%)
70 - 80	7 (87%)	6 (86%)	7 (54%)	7 (47%)
90 -100	1 (13%)	0 (0%)	6 (46%)	7 (47%)
Karnofsky preformace (post-surgery)				
< 70	1 (13%)	2 (29%)	0 (0%)	0 (0%)
70 - 80	1 (13%)	1 (14%)	1 (8%)	3 (20%)
90 -100	6 (74%)	4 (57%)	12 (92%)	12 (80%)
2nd surgery	4/8 (50%)	2/7 (29%)	2/13 (15%)	2/15 (13%)
Radiotherapy	3/8 (38%)	2/7 (29%)	8/13 (62%)	6/15 (40%)

Table 1. Characteristics of GBM patients

Discussion

In this manuscript we showed that EGFRvIII expressing cells displayed increased autophagic flux during metabolic stresses like severe hypoxia and starvation. We also showed that EGFRvIII expressing cells proliferate faster and have increased survival under these stresses and that these advantages can be abrogated through inhibition of autophagy with CQ. In vivo, EGFRvIII tumors showed increased tumor growth and increased tumor regrowth after radiotherapy. However, CQ treatment augmented the increased growth and regrowth of EGFRvIII tumors. Unexpectedly, CQ treatment did not change the hypoxic fraction of treated tumors, this in contrast with previous observations[22], nor did CQ treatment affect proliferation as expected from in vitro results. However, the necrotic fraction was drastically increased due to CQ treatment of EGFRvIII tumors, suggesting that CQ treatment is effective in EGFRvIII tumors.

The underlying mechanism for the increased autophagic flux in EGFRvIII expressing cells remains to be elucidated. One would expect reduced autophagic flux in EGFRvIII cells due to constitutive signaling of EGFRvIII through mTOR, a known autophagy inhibitor. However, preliminary results

suggest that the RAS-RAF pathway[30-32], one of the EGFR signaling pathways, plays a more important role in the increased flux in EGFRvIII cells (Fig. S4). Here we observed a block in autophagic flux when cell were treated with Vemurafenib (plx4032, a V600E BRAF inhibitor used at wtBRAF inhibiting concentration).[33] Another possible explanation is the recently proposed hypothesis by Shen et.al.[34]; under normal conditions, latent cytoplasmic STAT3 binds to Protein Kinase R (PKR) inhibiting its activity and reduces autophagy levels through eIF2α inhibition. In contrast, phosphorylated STAT3 forms dimers would leave PKR free to phosphorylate eIF2α.[35] More recently, a new autophagy regulating mechanism has been described for EGFR; activated EGFR phosphorylates Beclin 1. Beclin 1 phosphorylation favors the formation of Beclin 1 dimers that are unable to bind VPS34 and promote autophagy.[36] However, elucidating this mechanism warrants further investigation.

Most importantly, the effect of CQ on the outcome of patients with EGFRvIII expressing tumors was investigated. Analysis of the EGFRvIII staining revealed a drastically improvement in survival for patients with EGFRvIII positive GBM after concurrent CQ treatment (Fig. 4). Although these initial clinical results are promising, larger cohorts of patients are required to fully understand the therapeutic potential of CQ administration in treatment of GBM.

In conclusion, CQ treatment abrogates the growth and survival advantages of EGFRvIII expressing cells under metabolic stress and radio sensitizes EGFRvIII tumors for radiotherapy. Further, preliminary findings suggest that EGFRvIII positive cells regulate autophagy, at least in part, via the Ras-pathway, and patients with an EGFRvIII expressing tumor benefit from CQ treatment.

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Supplementary figures

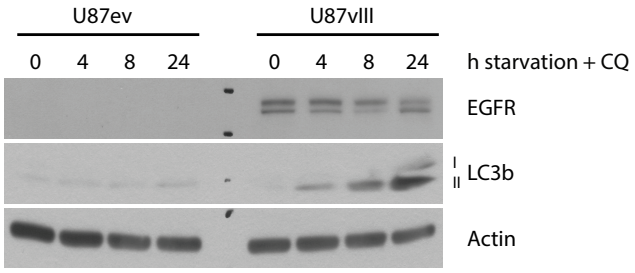

Figure S1. EGFRvIII expressing cells display increased autophagy under starvation; U-87 MG glioma cells

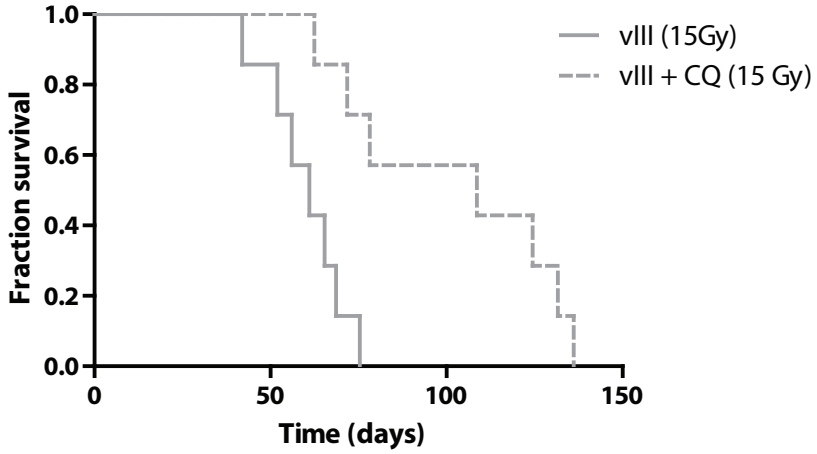

Figure S2. Time to regrow to eight times the irradiated volume after irradiation with 15Gy.

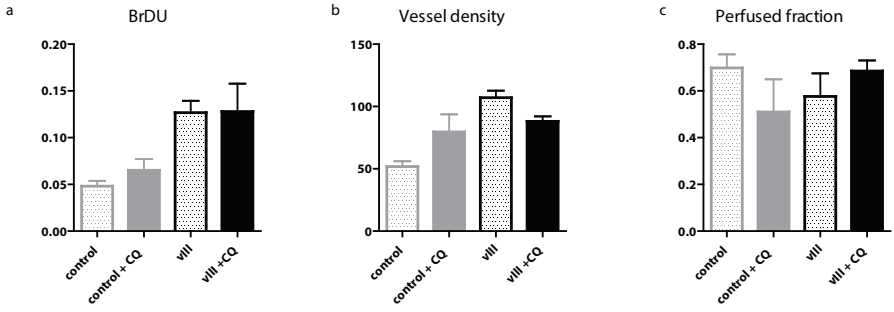

Figure S3. (A-C) Proliferation (BrDU)(n=5,6,6,5), vessel density (n=5,6,6,5) and perfused fraction (n=5,4,3,2) of EGFRvIII and control tumors. (n=control, control + CQ, vIII, vIII + CQ)

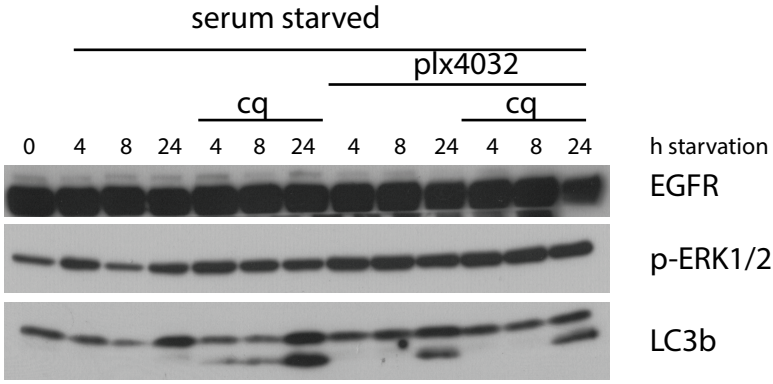

Figure S4. PLX4032 (Ras inhibitor) treatment blocks autophagic flux suggesting that Ras signaling is important for the autophagy process.

Chapter 6

EGFR overexpressing cells and tumors are dependent on autophagy for growth and survival

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Jutten B, Keulers TG, Schaaf MB, Savelkouls K, Theys J, Span PN, Vooijs MA, Bussink J, and Rouschop KM

Abstract

Background and purpose: The epidermal growth factor receptor (EGFR) is overexpressed, amplified or mutated in various human epithelial tumors, and is associated with tumor aggressiveness and therapy resistance. Autophagy activation provides a survival advantage for cells in the tumor microenvironment. In the current study, we assessed the potential of autophagy inhibition (using chloroquine (CQ)) in treatment of EGFR expressing tumors.
Material and methods: Quantitative PCR, immunohistochemistry, clonogenic survival, proliferation assays and in vivo tumor growth were used to assess this potential.
Results: We show that EGFR overexpressing xenografts are sensitive to CQ treatment and are sensitized to irradiation by autophagy inhibition. In HNSSC xenografts, a correlation between EGFR and expression of the autophagy marker LC3b is observed, suggesting a role for autophagy in EGFR expressing tumors. This observation was substantiated in cell lines, showing high EGFR expressing cells to be more sensitive to CQ addition as reflected by decreased proliferation and survival. Surprisingly high EGFR expressing cells display a lower autophagic flux.
Conclusions: The EGFR high expressing cells and tumors investigated in this study are highly dependent on autophagy for growth and survival. Inhibition of autophagy may therefore provide a novel treatment opportunity for EGFR overexpressing tumors.

Introduction

The epidermal growth factor receptor (EGFR) is overexpressed, amplified or mutated in various human epithelial tumors.[1] Its expression and activation leads to activation of several downstream effectors that control cell proliferation, differentiation, survival and acquisition of an EMT-like phenotype.[2,3] Consistently, EGFR amplification is associated with increased radioresistance through activation of pro-survival signaling and DNA-repair mechanisms.[4-7] EGFR targeting is therefore regarded as a potential treatment strategy to sensitize tumors to therapy.[8-10]

Besides the intrinsic radiosensitivity of tumor cell, the tumor microenvironment has also influence on the tumor's responsiveness to irradiation. This microenvironment is characterized by extreme heterogeneity in oxygenation ranging from normal values close to vessels to complete anoxia in the peri-necrotic regions. Poorly oxygenated (hypoxic) cells are highly resistant to chemoand radiotherapy. Therefore, targeting the hypoxic cells is likely to improve treatment efficacy.[11-13] Tumor cells respond to hypoxia by activating a variety of different mechanisms, one of the most important ones being autophagy. Autophagy (''to eat oneself'') is a lysosomal degradation mechanism that allows recycling of proteins and nutrients and is upregulated in response to various stresses, including hypoxia.[13,14] During the initiation of autophagy phosphatidylethanol-amine (PE) is conjugated to MAP1LC3B (LC3b), allowing autophagic membrane association. Fusion with a lysosome allows degradation of the autophagosomal content but simultaneously degrades part of the LC3b associated with the inner membrane of the autophagosome.[15,16] Consistent with this, degradation of LC3b is considered the golden standard for autophagy.[15] CQ, a lysosomotropic compound, accumulates in the lysosomes and raises intralysosomal pH, thereby preventing fusion autophagosome lysosome fusion. Previously we observed sensitization of tumors to irradiation
after CQ administration through reduction of the hypoxic fraction.[14] As EGFR-expressing tumors are radioresistant and require novel additions to increase tumor responsiveness, we explored the use of CQ in treatment of EGFR overexpressing tumors. Interestingly, we observed not only a radiosensitizing effect as observed previously, but revealed dependency of EGFR expressing

tumors on autophagy to maintain accelerated growth and survival. Autophagy inhibition may thus provide a novel treatment opportunity to target EGFR overexpressing tumors.

Materials and methods

Reagents and cell lines

Unless specified otherwise, all reagents were obtained from Sigma Chemical Co. (Sigma-Aldrich) and all electrophoresis reagents were from BioRad. MEMα medium was obtained from Invitrogen, glutamax-I (35050-038) was obtained from Life Technologies, DMEM and fetal bovine serum (FBS) was from PAA. The U373-MG, T47D, HT29 and A431 cell lines were obtained from ATCC and the E2 glioma cell line was kindly provided by A. Chalmers (Beatson Institute for Cancer Research & Beatson West of Scotland Cancer Centre, University of Glasgow). Cells were maintained as described by ATCC, E2 cells were maintained in MEMa supplemented with 2 mM glutamax-I.

Tumor xenograft model

Animal facilities and experiments were in accordance with local institutional guidelines and approved by the local animal welfare committee. Experiments were performed as described previously.[17] Briefly, tumors were grown in NMRI-nu (nu/nu) female mice. U373-EGFRwt and U373 control cells in matrigel were injected subcutaneously ($1.5*10^6$ cells). Tumor size was assessed by caliper measurement in 3 orthogonal diameters. Mice treated with CQ received 60 mg/kg CQ for 7 consecutive days administered IP. Tumors were positioned in the irradiation field using a custom-built jig and irradiated with a single dose of 10 Gy (15 MeV e) using a linear accelerator (Varian).

Immunohistochemistry and image processing

Frozen, acetone-fixed sections were stained by using antipimonidazole (Chemicon), anti LC3b (Abgent, AP1806a) and anti EGFR (Santa Cruz, sc-03).

For quantitative analysis, the slides were scanned by a computerized digital image processing system by using a high-resolution intensified solid-state camera on a fluorescence microscope (Axioskop; Zeiss) with a computer-controlled motorized stepping stage. Tumor necrosis was evaluated, relative to the total tumor area, morphologically by using H&E staining. Tumor hypoxic fraction and vascular density (structures per square millimeter) were determined relative to the viable tumor tissue (necrosis excluded).

Quantitative real-time PCR

RNA extraction was performed using the NucleoSpin RNA II kit (Bioke). cDNA was prepared using the iScript cDNA Synthesis kit (BioRad). Reactions were carried out in a 25 Il volume using sensiMix SYBR low-ROX kit (GC Biotech) with the ABI Prism 7500 Sequence Detection System. Values for each gene were normalized to expression levels of RPL13a RNA. The primer sequences used were:
EGFR for: ACCTGCGTGAAGAAGTGTCC
EGFR rev: CGTTACACACTTTGCGGCAAGG
LC3b for: AACGGGCTGTGTGAGAAAAC
LC3b rev: AGTGAGGACTTTGGGTGTGG
RPL13a for: CCGGGTTGGCTGGAAGTACC
RPL13a rev: CTTCTCGGCCTGTTTCCGTAG

Clonogenic assay

After seeding and allowing cells to attach, indicated concentrations of CQ were added to the medium and incubated for 96 h. After standard incubation formed colonies were fixed (0.4% methylene blue in 70% ethanol) and colonies consisting of >50 cells were counted manually.

Cellular proliferation

Cells were plated in a 24 well cell culture plate (Greiner) and allowed to attach to the plate. CQ was added to the medium in the indicated concentration and

cell densities were measured for 5 days using an IncuCyte™ Live-Cell Imaging System (SelectScience).

Western blotting

Cells were lysed and processed as described previously[18] and proteins were separated on mini-PROTEAN precast gels (4–20%, BIORAD). After transfer, proteins were probed with antibodies against EGFR (Santa-Cruz, sc-03), MAP1LC3b (Cell Signaling, 2775S), and Actin (MP Biomedicals, 8961001). Bound antibodies were visualized using HRP-linked anti-rabbit (Cell Signaling, 7074S) or anti-mouse (Cell Signaling, 7076S) antibodies.

Results

EGFR is often overexpressed in Glioblastoma Multiforme, which contributes to their radioresistant phenotype.[4-6] Previously, we have shown that treatment of tumors with chloroquine (CQ) decreases the hypoxic fraction and sensitizes them to radiation.[14] To assess if similar results could be obtained in EGFR overexpressing tumors, U373-MG with constitutive EGFR overexpression were implanted in mice. As described before, these EGFR overexpressing tumors were highly resistant to irradiation as reflected by rapid regrowth after irradiation (single dose, 10 Gy) (Fig. 1A and B). As observed previously, CQ administration sensitized tumors to irradiation and increased radiation-induced growth delay. Yet the effect of CQ treatment alone was far more pronounced (Fig. 1A and B). In agreement with the tumor growth delay, the individual doubling time of EGFR-expressing tumors increased dramatically after CQ administration, whereas the control tumors (U373-MG transfected with control vector) remained largely unaffected in proliferation (data not shown). These data indicate that EGFR overexpressing tumors are dependent on autophagy for growth. Although we previously observed a reduction of the hypoxic fraction in tumors after CQ addition that explained the radiosensitizing effect of CQ addition[14], the large effect on growth of EGFR overexpressing tumors was neither due to decreased hypoxic fraction (Fig. 1D)[14] nor due to differences in necrosis, apoptosis or proliferating cells (Fig. 1E–G).

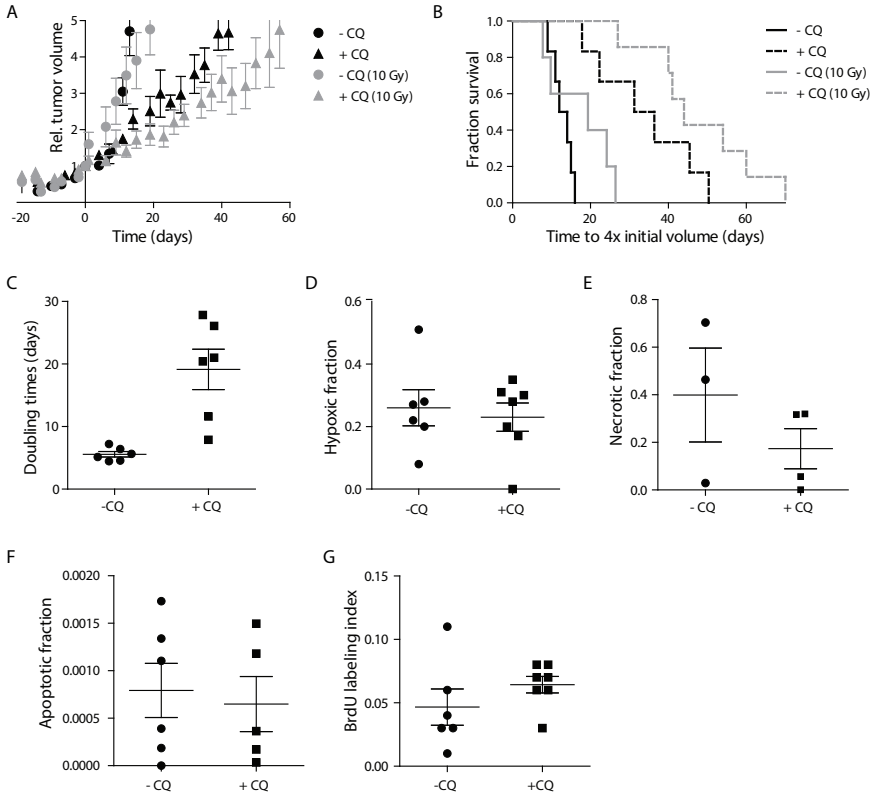

Figure 1. EGFR overexpressing U373-MG xenografts are sensitive to CQ treatment. (A) Growth of control (CQ, n = 6), irradiated (CQ (10 Gy), n = 5), CQ treated (+CQ, n = 6) and irradiated in combination with CQ treated (+CQ (10 Gy), n = 7) U373-MG xenografts with EGFR overexpression. (B) Time to reach 4 initial volume was plotted for the individual xenografts. Control (CQ) vs. CQ treated (+CQ), $p < 0.05$. Irradiated (CQ (10 Gy)) vs. irradiated in combination with CQ (+CQ (10 Gy)), $p < 0.05$. (C) Tumor volume doubling times, (D) Hypoxic fraction, (E) Necrotic fraction (F) Apoptotic fraction, (G) Brdu labeling index of the individual xenografts.

To determine whether a relation between EGFR expression and autophagy dependency also existed in other tumor types we performed immunohistochemical analysis of EGFR and LC3b in a panel of human head and neck squamous cell carcinoma (HNSCC) primary xenografts. Interestingly, we observed a correlation between EGFR and LC3b protein expression (Fig. 2A). As LC3b has been reported to be primarily expressed in the hypoxic tumor regions, we assessed the correlation between EGFR and LC3b in the hypoxic (pimonidazole positive) area of the tumor (Fig. S1a) and between EGFR

expression and the hypoxic fraction (Fig. S1b). However, no correlations were observed. We did find a correlation in mRNA abundance between EGFR and LC3b, while this was absent for EGFR and CA IX, a known hypoxia regulated gene (Fig. S1c). These findings suggest that EGFR is associated with expression and transcriptional regulation of LC3b in a non-hypoxia dependent manner. In agreement, immunohistochemical analysis of primary HNSCC xenografts revealed high LC3b expression in hypoxic regions in lowest EGFR expressing tumors, whereas in highest EGFR expressing tumors most LC3b was localized within the non-hypoxic regions (Fig. 2C).

To further address the high dependency of EGFR expressing cells on autophagy we explored the effect of CQ addition on cellular survival and proliferation. In agreement with our in vivo findings, U373-EGFR cells were more dependent on autophagy for survival and proliferation than U373 control

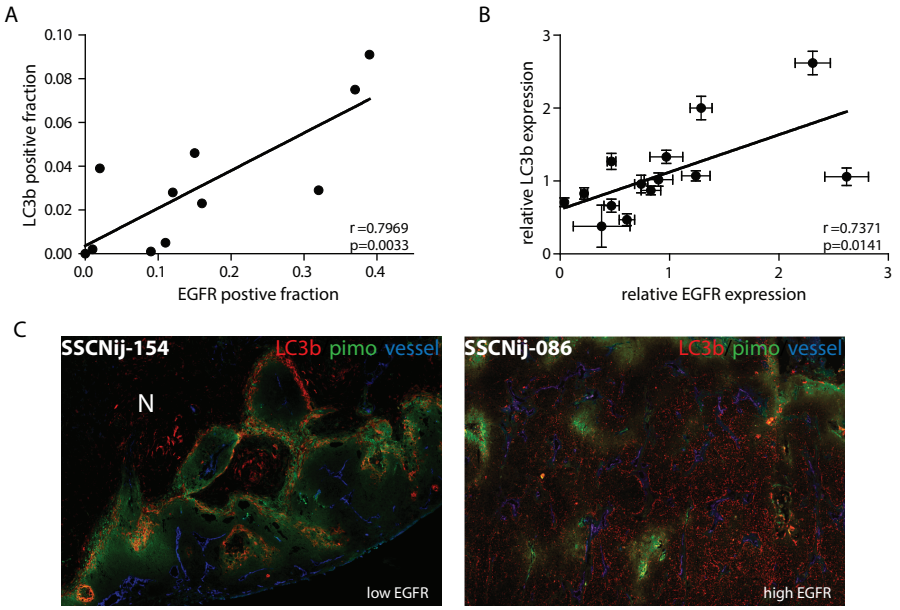

Figure 2. EGFR expression correlates with LC3b expression. (A) Correlation between LC3b positive fraction and EGFR positive fraction determined by IHC in a panel of primary HNSCC xenografts. (B) Correlation between EGFR and LC3b mRNA in a panel of primary HNSCC xenografts. (C) IHC of HNSSC with low EGFR (left panel) and high EGFR (right panel) expression, green: hypoxic fraction, blue: vessels and red: LC3b. N indicates necrotic area.

cells as assessed by clonogenic survival (Fig. 3A) and cellular proliferation (Fig. 3B).

We observed that low CQ concentrations reduced proliferation without reducing survival, suggesting that high EGFR expressing cells are highly dependent on cell cycle progression e.g. through maintaining high energy and nutrient availability. To investigate the high autophagy dependency further, we used a panel of high and low EGFR expressing cell lines (Fig. 3E) and tested their sensitivity to CQ by clonogenic survival (Fig. 3C). We observed that high EGFR expressing cells (HT29 and A431) were more sensitive to CQ compared to low EGFR expressing cell lines (E2 and T47D). This was confirmed by proliferation assessment (Fig. 3D) where CQ addition to EGFR high expressing cells led to a larger reduction in proliferation than in EGFR low expressing cells.

To determine if the high autophagy dependence of EGFR expressing cells was reflected by a high autophagic activation, autophagic flux was determined under normal growth conditions (flux is represented by differences in the amount of LC3-II between samples in the presence and absence of lysosomotrophic compounds[16], like CQ). Unexpectedly we observed higher flux in the low EGFR expressing cells. These results indicate that although a lower autophagic flux in high EGFR expressing cells is observed, their dependency on autophagy for proliferation and survival is higher. The underlying reason for this observation remains to be elucidated.

Discussion

Our data presented here show that CQ drastically reducesis growth of EGFR overexpressing U373-MG xenografts and that this is not due to the previously described reduction of the hypoxic fraction.[14] A possible explanation for this discrepancy could be the overexpression of EGFR which was not taken into account in our previous study.[14] Neither the necrotic nor the apoptotic areas of xenografts are affected by CQ treatment, suggesting that not the oxygen deprived cells are targeted but other cells that depend on autophagy, e.g. nutrient deprived cells. Additionally, in primary HNSCC xenografts we observed a positive correlation between LC3b and EGFR protein expression. In the same HNSCC xenografts we observed a correlation between LC3b and EGFR mRNA

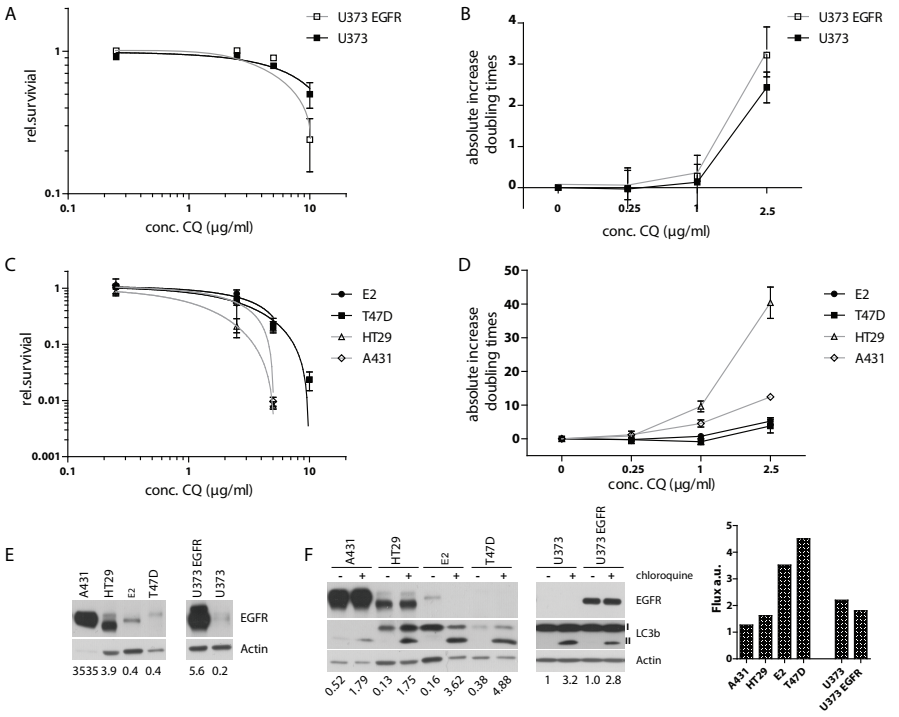

Figure 3. EGFR high expressing cells are more sensitive to CQ treatment than EGFR low expressing cells (A) Dose-dependent clonogenic survival of EGFR overexpressing and control U373-MGs after CQ addition. (B) Doubling times of EGFR high and low U373-MGs after exposure to CQ. (C) Clonogenic survival of a panel of EGFR high and low cells after exposure to CQ. (D) Doubling times of panel of EGFR high and low cells after exposure to CQ. (E) WB showing total amount of EGFR normalized to actin expression. (F) Representative WB assessing autophagic flux under normal conditions. (Quantification of LC3b-II levels normalized to actin expression). (A–C and D) n = 4 and SEM is plotted.

expression, suggesting a role for EGFR in the production of LC3b. Although in most tumors LC3b is predominantly expressed in hypoxic regions, LC3b expression in EGFR expressing tumors displayed no correlation with tumor hypoxia. In agreement, most LC3b in high EGFR expressing HNSCC xenografts was located within the non-hypoxic regions. In vitro experimentation in a panel of high and low EGFR expression cell lines revealed a relation between EGFR expression and CQ sensitivity under normal cell growth conditions. Based on our in vivo findings, the in vitro sensitization of U373 with

EGFR overexpression is not as high as expected. This may be due to the artificial introduction of EGFR, survival and proliferation is thus not dependent on EGFR overexpression in vitro, but provides a growth advantage in vivo. All other cell lines with endogenous EGFR expression levels behaved in line with our findings; high EGFR expression, high sensitivity for CQ. Strikingly, EGFR high expressing cells showed remarkable low flux compared to EGFR low expressing cells under normal growth conditions, indicating that flux does not necessarily reflect the cells' dependency on autophagy for its survival. In line with our findings, knocking down EGFR rapidly leads to activation of autophagy as a result of reduced glucose uptake[19-21], providing a potential explanation for the dramatic effect observed after CQ administration. Nevertheless other effects of CQ, besides inhibition of autophagy, should be considered.

Another potential explanation for the high sensitivity to CQ and autophagy inhibition of EGFR high expressing cells and tumors could be the EGFR signaling to RAS. It has been described that cells depend on autophagy during Ras-mediated oncogenic transformation.[22,23] Further, it has been described that cells with oncogenic Ras signaling require autophagy to maintain their oxidative metabolism and that down-regulation of essential autophagy proteins impaired cell growth. As cancers with Ras mutations have a poor prognosis, this ''autophagy addiction'' suggests that targeting autophagy is a valuable new approach to treat these aggressive cancers.[24]

Next to Ras-signaling, mTOR signaling also influences autophagy.[25,26] In the presences of sufficient nutrients mTOR drives proliferation but inhibits autophagy. This could explain the low autophagic flux in EGFR overexpressing cells. Additional inhibition of flux by CQ could be lethal for these cells. However, the potential benefits from CQ treatment of EGFR high expressing tumors and the underlying mechanisms require further investigation.

In conclusion, EGFR high expressing cells and tumors investigated in this study are highly dependent on autophagy for growth and survival. Inhibition of autophagy may therefore provide a novel treatment opportunity for EGFR overexpressing tumors.

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Chapter 7

General discussion and summary

General Discussion and Summary

This thesis describes how the Epidermal Growth Factor Receptor (EGFR) and one of its most commonly observed mutated form, variant III (EGFRvIII), contribute to a more malignant tumor phenotype and describes possible new treatment opportunities for patients with deregulated EGFR signaling.

The EGFR is a receptor tyrosine kinase of the ErbB family, consisting of four members; EGFR (ErbB1, HER1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4). EGFR is activated by ligand binding to the extracellular ligand binding domain. This initiates a conformational change of the receptor and exposure of the dimerization domain, allowing homo-dimerization or hetero-dimerization with other family members and autophosphorylation by the intracellular kinase domain. EGFR activates a number of signaling cascade that drive many cellular responses, including changes in gene expression, cytoskeletal rearrangement, apoptosis inhibition, increased cell proliferation and acquisition of an EMT-like phenotype.[1-3] Not long after their discovery in the early 1980s the ErbB family members were implicated in cancer research. In many different cancer cell types, the ErbB pathway becomes hyper activated by a range of mechanisms, including overproduction of ligands, overproduction of receptors, or constitutive activation of receptors.[4]

Consistently, amplification of the EGFR gene is associated with increased radio resistance through activation of pro-survival signaling and DNA-repair mechanisms.[5-7] Targeting EGFR is therefore regarded a potential treatment strategy to sensitize tumors to therapy.[8-10] Despite many efforts that have been made to develop EGFR targeting therapies like inhibition of EGFRsignaling through tyrosine kinase inhibitors (TKI) and preventing EGFR expression and dimerization using antibodies , these therapies have only been proven effective in a limited percentage of patients.[11]

Recent data have emerged demonstrating that certain cancer-associated mutations, including k-Ras and PTEN can result in persistent growth pathways activation despite the blockade of EGFR[12,13] and abrogate the efficacy anti-EGFR-targeted treatments. Furthermore, it has also become apparent that different treatment strategies to inhibit EGFR (e.g. antibody versus TKI) yield different effects in the same tumor.[9,14] Compensatory responses by other

receptors, including mutant EGFR species, are likely reasons for such failures. The most common variant of the EGFR is an exon 2–7 deletion mutant EGFRvIII.[15-17] EGFRvIII is known to contribute to the radio resistance of tumor cells[18] at least in part through enhanced repair of DNA double strand breaks.[19] Furthermore, EGFRvIII expression contributes to resistance to the TKI gefitinib and leads to sustained EGFR signaling and AKT activity.[20]

Additionally, the tumor microenvironment, and in particular the hypoxic tumor regions, significantly contribute to therapy resistance.[21-23] Expression of EGFRvIII provides cells with a survival advantage when exposed to stresses as hypoxia and nutrient starvation.[24] We therefore investigated whether EGFRvIII expressing tumor cells can be target with Cetuximab, a monoclonal antibody directed against EGFR. Cetuximab prevents binding of ligands to EGFR and as EGFRvIII lacks the majority of domain I and II (domain I and III are responsible for ligand binding) we questioned whether Cetuximab binds to EGFRvIII and whether Cetuximab binding to EGFRvIII would have functional therapeutic effects. Indeed, in **chapter 2** we showed that Cetuximab binds to EGFRvIII resulting in a reduction of EGFRvIII expression on the cell membrane. Unexpectedly, this reduction had no effect on the proliferation rate of these EGFRvIII expressing glioma cells. Potentially, EGFRvIII expression is not beneficial for proliferation under normal cell growth conditions or the down regulation was insufficient to evoke phenotypic changes. In line, Cetuximab treatment also failed to radio sensitize these EGFRvIII expressing cells, again suggesting that, the residual EGFRvIII receptors left on the membrane are sufficient to maintain the radio resistant phenotype of these cells. Nevertheless, ERK signaling is reduced in these EGFRvIII expressing cells suggesting that Cetuximab reduces EGFRvIII signaling and could mediate therapeutic effects against EGFRvIII in cells with less extensive EGFRvIII expression.

Currently, EGFR expression and mutations in its tyrosine kinase domain are assessed for diagnosis and clinical treatment of patients. Also downstream effectors of EGFR like k-RAS and PTEN are routinely evaluated as k-RAS mutations can compromise Cetuximab efficacy and loss of PTEN ablates effectiveness of TKIs directed against EGFR. The expression of these proteins is therefore tested for optimal treatment. However, EGFRvIII is not routinely determined despite its clinical relevance.[25,26] Additionally, EGFRvIII expression

adversely effects anti-EGFR therapy.[20] Cells expressing EGFRvIII are relatively resistant to gefitinib and require higher doses, repeated dosing, and longer exposure to achieve decreased receptor phosphorylation. However, this decrease does not effectively inhibit the biologically relevant processes of DNA synthesis, cellular growth, and invasion in cells expressing EGFRvIII.[20] A potential explanation for not routinely testing EGFRvIII expression is the lack of commercially availability of an EGFRvIII specific antibody. We therefore attempted to develop an EGFRvIII specific llama derived antibody which could be used for in vitro and in vivo assessment (**chapter 3**) as llama single domain heavy chain only antibody fragments, or nanobodies, are in general suitable for both PET-imaging[27] and detection on cryosections.[28] This EGFRvIII nanobody could be used in combination with a previously selected EGFR specific nanobody (Roovers et al. unpublished results) to determine EGFR and EGFRvIII expression in vitro or in vivo and thereby helping to make the best suited treatment. Unfortunately, the selected nanobody against EGFRvIII showed also affinity for EGFR making this nanobody unsuitable to discriminate between EGFR and EGFRvIII expression as EGFRvIII expression is almost always accompanied with EGFR overexpression.

In **chapter 4** we investigated the effect of EGFR and EGFRvIII expression on the stress resistance typical for the tumor microenvironment. We observed that EGFRvIII but not EGFR expressing cells maintained a higher proliferation potential during stress conditions as serum starvation or hypoxia. Similar, in the same model we also observed that EGFRvIII but not EGFR overexpressing cells displayed improved survival after exposure to nutrient starvation and hypoxia. Interestingly, the observed in vitro effects were also translated in increased tumor growth rates in vivo. We noted that the increased growth rate of EGFRvIII-expressing tumors could be reversed when the mutant receptor was no longer present. These data indicate that targeting EGFRvIII by silencing its expression and/or blocking its downstream effects might be a promising strategy for treating cancers that express this mutated receptor.

In **chapter 5** we demonstrated that EGFRvIII expressing cells and tumors depend on autophagy to maintain their proliferative state and for their survival during metabolic stresses like starvation or severe hypoxia. We also showed that these advantages can be abrogated by inhibiting autophagy. As observed

previously, EGFRvIII tumors displayed increased tumor growth and accelerated tumor regrowth after radiotherapy. Interestingly, chloroquine (CQ) treatment abrogated the growth and regrowth advantage of EGFRvIII tumors. The underlying mechanism for the increased autophagic flux in EGFRvIII expressing cells remains to be elucidated. Intuitively, one would expect reduced autophagic flux in EGFRvIII cells due to constitutive signaling of EGFRvIII through mTOR[29,30], a known negative regulator of autophagy. However, preliminary results suggest that the RAS-RAF pathway[31-33], one of the EGFR signaling pathways, plays a more important role in the increased flux in EGFRvIII cells.

Although autophagy is neither regulated via transcription nor translation, yet sufficient transcription and translation of autophagy related proteins is needed to sustain autophagic flux.[34] Signaling through the Signal Transducer and Activator of Transcription 3 (STAT3) pathway could be responsible for sustaining the increased autophagic flux under hypoxic conditions of EGFRvIII expressing cells through Protein Kinase R (PKR) and eIF2α.[35,36] More recently, a fourth autophagy regulating mechanism has been described for EGFR.[37] EGF activated EGFR phosphorylates Beclin 1 at three tyrosine residues favoring Beclin 1 to form dimers and thereby preventing binding of Beclin 1 to the Vps34 complex and initiation of autophagy.[38,39] This may also explain why EGFR overexpressing cells display low basal autophagic flux as described in chapter 6. Nevertheless, to elucidate the exact mechanism at play more research is needed.

Furthermore, the effect of CQ on the outcome of patients with EGFRvIII expressing Glioblastoma Multiforme (GBM) was investigated. Importantly, in cohort of 52 GBM patients (18 conventional treatment and 34 treated with additional CQ) large improvements in overall survival were observed. Patients harboring EGFRvIII negative tumors displayed an increase in median survival from 5 to 10 months, where patients with EGFRvIII positive tumors displayed an increase in median survival from 3 to 15 months. However, these results should be treated with caution since this group of patients is relative small and was treated heterogeneously. Nevertheless, these promising results should be further pursued. Taken together, inhibition of autophagy may provide a novel treatment opportunity for EGFRvIII expressing tumors.

To assess whether there is also a relation between non-mutated EGFR and

autophagy we engrafted EGFR overexpression glioblastoma cells in a xenograft model and assessed CQ treatment efficacy, described in **chapter 6**. We observed a reduction in growth of EGFR expressing xenografts when treated with CQ. As expected EGFR expressing tumors were highly radio resistant. Interestingly, within these radio resistant tumors were we also observed a radio sensitization effect after CQ treatment, as also observed in EGFRvIII expressing xenografts (chapter 5). Interestingly, this was not due to the previously described reduction of the hypoxic fraction.[34] Neither the necrotic nor the apoptotic areas of xenografts appear affected by CQ treatment, suggesting that not only oxygen deprived cells are targeted but other cells, illustrating the high dependence of EGFR expressing cells on autophagy for survival. Additionally, in primary Head and Neck Squamous Cell Carcinoma (HNSCC) xenografts we observed a positive correlation between LC3b and EGFR protein expression. In the same HNSCC xenografts we observed a correlation between LC3b and EGFR mRNA expression, suggesting a role for EGFR in the production of LC3b. Although in most tumors LC3b is predominantly expressed in hypoxic regions, LC3b expression in EGFR expressing tumors displayed no correlation with tumor hypoxia, suggesting once more that other cells that depend on autophagy, e.g. nutrient deprived cells are targeted but not the oxygen deprived cells. In agreement, most LC3b in high EGFR expressing HNSCC xenografts was located within the non-hypoxic regions. Taken together, EGFR high expressing tumors investigated in this study are highly dependent on autophagy for growth, survival and radio resistance. Inhibition of autophagy may therefore provide a novel treatment opportunity for EGFR overexpressing tumors.

Concluding remarks and future perspectives

EGFR has become a highly investigated molecule since its first implication in cancer in the early 1980s. In many different cancer types, the EGFR pathway becomes hyper activated by a range of mechanisms, including overproduction of ligands, overproduction of receptors, or constitutive activation of receptors.[4] As a consequence, EGFR and related signaling molecules are routinely tested for diagnostically purposes and treatment selection. Surprisingly, EGFRvIII

expression is not routinely tested despite the increasing amount of research and clinical data that suggest that EGFRvIII expression adversely effects the therapy success and thereby the outcome for the patient and should be additionally tested to optimize patient treatment.

Many anti-EGFR therapies, like Tyrosine Kinase Inhibitors (TKI) or monoclonal antibodies (mAbs) have been proven effective in experimental settings; however show only little clinical efficacy.[11] Tumors could potentially become resistant to anti-EGFR treatment by induction of compensatory mechanisms or expression of EGFRvIII. To overcome this resistance novel strategies are desired.

In this thesis we described a possible new treatment possibility for the treatment of EGFR and EGFRvIII overexpressing tumors. We have shown that both EGFR and EGFRvIII expressing tumors respond very well to autophagy inhibition through CQ administration. Additionally, within a cohort of patients with CQ in combination with conventional therapy, patients with EGFRvIII expressing GBM benefited most. Nevertheless, larger and more clinical trials with uniformly treated patients are required to evaluate effectiveness of CQ addition in treatment of (EGFRvIII positive) GBM.

Unraveling the exact signaling mechanism how EGFR and EGFRvIII regulate autophagy, could create new insight on how these tumors could be treated more efficiently. Many inhibitors of downstream effectors of EGFR e.g. PI3K, mTOR, RAS or RAF are currently available for researchers or even already tested in clinical trial. These inhibitors in combination with anti-EGFR treatment could provide new treatment possibilities. Moreover, it has become clear that single therapies are not effective in most cancer treatments and combinations of existing therapies should be tested. In that perspective, GBM patients could be treated with Avastin (Bevacizumab, a mAb directed against Vascular Endothelial growth factor (VEGF) that inhibits angiogenesis) in combination with autophagy inhibition and radiotherapy (RT). Bevacizumab treatment effects are mainly mediated trough induction of tumor hypoxia. Combining Bevacizumab with CQ is likely to increase cell killing and the effectiveness of Bevacizumab as single agent treatment but also to prevent or delay recurrence after irradiation.[40]

Another approach in targeting EGFRvIII expressing tumors is the use of cancer

vaccination.[41] EGFRvIII is the ideal cancer vaccination candidate as it is solely expressed on tumors and not on normal tissue. Moreover, vaccination of patients with an EGFRvIII positive GBM with an EGFRvIII peptide led to a 5-fold improved overall survival.[42] Unfortunately, Natural Killer (NK) cells are less effective in a hypoxic tumor environment[43] and autophagy protects tumor cells from cytotoxic T-lymphocyte (CTL) mediated lysis.[44] Combination of RT, CQ treatment and vaccination could therefore eradicate all normoxic and hypoxic tumors cells. This approach not only targets all 'normal' tumor cells but also the very radio resistant glioblastoma cancer stem cell (CSC) which express EGFRvIII.[45,46]

Taken together, despite many decennia of research, new anti-EGFR drugs and clinical trials, EGFR and EGFRvIII expressing tumors remain difficult to treat and more research is required to elucidate the molecular mechanisms at play and to identify the treatment with highest efficacy.

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Nederlandse samenvatting

Samenvatting

Deze thesis beschrijft hoe de Epidermale Groei Factor Receptor (EGFR) en een van de meest voorkomende afwijkende (mutant) vorm, variant III (EGFRvIII), bijdragen aan de agressiviteit van een tumor. Tevens worden nieuwe behandeling mogelijkheden besproken voor patiënten met een tumor met ontregelde EGFR signalering.

EGFR is een lid van de ErbB familie van receptoren welke bestaat uit 4 leden; EGFR (Erb1, HER1), Erb2 (HER2), Erb3 (HER3) en Erb4 (HER4). EGFR wordt geactiveerd door binding van een ligand, hierdoor veranderd de receptor van conformatie. Door deze vormverandering kunnen twee EGFR moleculen een interactie aangaan, dimeriseren (homo-dimeren) of dimeriseren met een ander lid uit de ErbB-familie (hetero-dimeren). Dimerisatie leidt tot phosphorylatie aan de binnenkant van de cel waardoor signalering geactiveerd wordt. EGFR werkt via verschillende signaleringroutes die de cel aanzetten tot gen expressie, cytoskelet veranderingen, remming van geprogrammeerde celdood (apoptose), toename van celgroei en deling en zorgt ervoor dat de tumorcel een fenotype krijgt dat lijkt op dat van 'tumorstamcellen'. Niet lang na de ontdekking van de ErbB receptor familie, in de vroege jaren '80, werden deze al geassocieerd met kanker. In veel kankersoorten zijn de ErbB signalering routes hyper-geactiveerd door o.a. overproductie van liganden, overproductie van receptoren (amplificatie) of door continue activatie van de receptoren.

Hierdoor wordt amplificatie van het EGFR gen geassocieerd met resistentie tegen radiotherapie door de activatie van overlevingssignalering en DNA-herstel mechanismen door EGFR. Onderbreking van EGFR signalering kan daarom beschouwd worden als een mogelijke strategie om tumoren gevoeliger te maken voor behandeling. Ondanks het feit dat er al veel onderzoek is gedaan naar het remmen van EGFR signalering met behulp van medicijnen die de phosphorylatie tegen gaan (TKIs) en met antilichamen die de dimerisatie voorkomen, blijken deze in de praktijk slechts effectief te zijn in een beperkte groep patiënten.

Recent onderzoek heeft uitgewezen dat kanker gerelateerde mutaties in bijvoorbeeld het k-RAS- of PTEN gen kan leiden tot ongecontroleerde celgroei en deling ondanks de blokkade van EGFR signalering. Verder kunnen

verschillende strategieën om EGFR signalering te blokkeren (TKIs versus antilichamen) verschillende resultaten kunnen opleveren in dezelfde tumor. Compensatie via andere receptoren, inclusief mutaties in EGFR, zijn waarschijnlijk de reden hiervoor.

De meest voorkomende mutatie van EGFR is de zogenoemde variant 3 of EGFRvIII. EGFRvIII staat bekend voor zijn bedrage aan de radioresistentie van tumorcellen, o.a. door zijn bedrage aan DNA herstel na bestraling. Verder leidt EGFRvIII expressie tot resistentie tegen Gefitinib (een TKI) en tot constante EGFR signalering. We hebben daarom onderzocht of tumorcellen met EGFRvIII expressie behandeld kunnen worden met Cetuximab, een antilichaam tegen EGFR. Cetuximab voorkomt de binding van een ligand aan domein I en III van EGFR. EGFRvIII mist een groot deel van domein I en II. Daarom stelde wij ons de vraag of Cetuximab zou binden aan EGFRvIII en of deze binding dan een functionele therapeutische waarde zou hebben? In hoofdstuk 2 laten wij zien dat Cetuximab inderdaad kan binden aan EGFRvIII. Deze binding leidde tot een verminderde expressie van EGFRvIII op de cel. Dit had echter geen effect op de groeisnelheid van deze cellen. Mogelijk geeft de expressie van EGFRvIII onder normale omstandigheden geen groeivoordeel of de vermindering in EGFRvIII expressie was niet voldoen om een effect te hebben. Verder leidde de behandeling met Cetuximab ook niet tot radiosensitisatie, suggererend dat voldoende EGFRvIII nog aanwezig is om radioresistentie te handhaven. Toch was de ERK signalering verminderd in EGFRvIII cellen wanneer deze behandeld werden met Cetuximab. Dit suggereert dat Cetuximab EGFRvIII signalering vermindert en zou mogelijk gebruikt kunnen worden voor de behandeling van tumoren met lage EGFRvIII expressie.

In de dagelijkse klinische routine worden de hoeveelheid van en de mutaties in EGFR bepaald om een optimale behandeling van de patiënt te bereiken. Tevens worden mutaties in signaleringmoleculen van EGFR bepaald omdat mutaties hierin kunnen leiden tot ongevoeligheid van de tumor tegen anti-EGFR behandelingen. De expressie van EGFRvIII wordt echter niet meegenomen in deze analyse ondanks bewezen klinische relevantie en de verlaagde effectiviteit van de anti-EGFR therapie door EGFRvIII. Tumorcellen die EGFRvIII tot expressie brengen zijn resistenter tegen Gefitinib en hebben dus

meer en hogere dosissen nodig. Deze hogere dosissen zijn echter niet voldoende om alle processen die leiden tot therapieresistentie van EGFRvIII stil te leggen. Mogelijk is het ontbreken van een goed commercieel verkrijgbaar antilichaam specifiek voor EGFRvIII een reden voor het niet testen op EGFRvIII expressie. Wij hebben daarom getracht een EGFRvIII specifiek antilichaam te genereren afgeleid van een lama antilichaam (hoofdstuk 3). Dit EGFRvIII specifiek mini-antilichaam of nanobody zou gebruikt kunnen worden in combinatie met een eerder gevonden nanobody tegen normaal EGFR, om de expressie niveaus van EGFR en EGFRvIII te bepalen. Met deze nanobodies kan dit zowel in de patiënt als in verwijderd tumormateriaal. Het ontwikkelde antilichaam had hoge affiniteit voor EGFRvIII, maar bleek ook te binden aan normaal EGFR wanneer dit in grote hoeveelheden aanwezig was. Dit maakt het nanobody tegen EGFRvIII ongeschikt voor het stellen van een goede diagnose omdat EGFRvIII expressie vaak gepaard gaat met een hoge expressie van normaal EGFR.

In hoofdstuk 4 hebben we onderzocht hoe tumorcellen met EGFR en EGFRvIII expressie verschillend omgaan met de stress die hoort bij het tumor-micro milieu, zoals lage zuurstof concentratie of weinig voedingsstoffen. Wij zagen dat cellen met EGFRvIII sneller bleven groeien wanneer ze weinig zuurstof of voedingsstoffen kregen. Dit bleek specifiek voor EGFRvIII aangezien cellen met EGFR dit niet vertoonden. Tevens observeerden we dat de cellen met EGFRvIII beter overleefden dan controle cellen of cellen met EGFR wanneer ze weinig zuurstof of voedingsstoffen kregen. Ook tumoren die voortkwamen uit de cellen met EGFRvIII groeiden sneller. Deze verhoogde groeisnelheid kon worden teruggebracht wanneer EGFRvIII productie in de cel werd uitgezet. Deze bevindingen suggereren dat het remmen van EGFRvIII expressie en/of een blokkade in de signaleringsroute een veelbelovende strategie kunnen zijn in de behandeling van tumoren met EGFRvIII expressie.

Cellen met EGFRvIII groeien sneller en overleven beter wanneer ze blootgesteld worden aan weinig zuurstof (hypoxie) of voedingsstoffen (starvatie) vergeleken met cellen zonder EGFRvIII. In hoofdstuk 5 hebben we aangetoond dat remming van autofagie (een proces dat de cel gebruikt om beschadigde onderdelen af te breken en hergebruiken) in cellen met EGFRvIII leidt tot een verlaagde groei en overleving wanneer ze blootgesteld worden aan hypoxie of

starvatie. Cellen met EGFRvIII zijn zelfs gevoeliger voor remming van autofagie wanneer ze blootgesteld worden aan hypoxie of starvatie dan cellen zonder EGFRvIII. Vergelijkbaar met eerdere experimenten, zagen we dat tumoren met EGFRvIII expressie sneller groeiden en ook sneller terug groeiden na radiotherapie in vergelijk met tumoren zonder EGFRvIII expressie. Opvallend was dat chloroquine (CQ, een medicijn dat autofagie remt) behandeling deze versnelde groei en hergroei teniet kon doen. De onderliggende mechanismen hierbij moeten nog worden uitgezocht. Intuïtief zou men verlaagde autofagie verwachten in cellen met EGFRvIII omdat een van de belangrijkste signaleringsroutes van EGFR, de zo gehete mTOR signalering, juist lijdt tot verlaagde autofagie. Voorlopige data wijzen erop dat een ander signaleringsroute, de RAS-RAF signalering, een meer belangrijke rol in de verhoogde autofagie van cellen met EGFRvIII speelt.

Autofagie wordt noch transcriptioneel noch translationeel gereguleerd. Desondanks is voldoende transcriptie en translatie nodig om voldoende autofagie flux te behouden. Signal Transducer and Activator of Transcription 3 (STAT3) signalering zou verantwoordelijk kunnen zijn voor het behoud van de verhoogde autofagie flux in EGFRvIII cellen tijdens zuurstof tekort via Protein Kinase R (PKR) en eIF2α. Recent is een vierde autofagie regulerende signalering van EGFR ontdekt. EGF geactiveerd EGFR phosphoryleerd Beclin 1 op 3 tyrosines waardoor Beclin 1 dimeren vormt en niet meer aan het Vps34 complex kan binden en daardoor autofagie niet kan activeren. Dit kan mogelijk verklaren waarom cellen met EGFR overexpressie lage base autofagie flux hebben zoals beschreven in hoofdstuk 6. Echter, de exacte mechanismen die hierbij een rol spelen moeten nog verder worden uitgezocht.

Analyse van een cohort van 52 glioblastoma patiënten (18 conventioneel en 34 met additioneel CQ behandeld) werden grote toenames in overleving gezien. Patiënten met een EGFRvIII negatieve tumor hadden een toename in overleving van 5 naar 10 maanden na diagnose, waar patiënten met een EGFRvIII positieve tumor een toename van 3 naar 15 maanden hadden. Deze resultaten zijn in lijn met de verkregen preklinische data. Enige voorzichtigheid is echter geboden omdat het een relatief kleine groep is. Echter, deze veel belovende resultaten dienen verder onderzocht te worden in gerandomiseerde studies met patiënten stratificatie gebaseerd op EGFRvIII expressie.

Samengevat, de inhibitie van autofagie door middel van CQ is een veelbelovende toevoeging voor de therapie van EGFRvIII positieve tumoren.
Om de relatie tussen niet-gemuteerd EGFR en autofagie te bepalen werden glioblastoma cellen met EGFR overexpressie in een xenograft model gebruikt en de effectiviteit van CQ behandeling getest, zoals beschreven in hoofdstuk 6. CQ behandeling leidde tot een afname in de groei van tumoren met EGFR overexpressie. Zoals verwacht waren tumoren met EGFR overexpressie zeer resistent tegen radiotherapie. Deze resistente tumoren konden gevoelig gemaakt worden voor radiotherapie door CQ behandeling, zoals gezien met de EGFRvIII tumoren (hoofdstuk5). CQ behandeling op zich zelf vertraagde de groei van tumoren met EGFR overexpressie zelfs meer dan de radiotherapie (enkele dosis van 10Gy).
Opvallend was dat dit niet kwam door de afname van de hypoxische fractie, zoals we in het verleden gezien hebben. Noch de necrotische of de apoptotische fractie leken beïnvloed te worden door CQ behandeling. Dit suggereert dat niet alleen de cellen met weinig zuurstof maar ook andere cellen getarget worden en geeft aan dat cellen met EGFR overexpressie sterk afhankelijk zijn van autofagie voor hun overleving. Tevens werd in xenografts van primaire hoofd-hals tumoren een positieve relatie gevonden tussen LC3b en EGFR RNA en eiwit expressie. Dit suggereert dat EGFR betrokken is in de productie van LC3b. Ondanks dat LC3b voornamelijk gemaakt wordt in hypoxische gebieden van een tumor, werd deze correlatie niet gevonden in tumoren met EGFR overexpressie. Dit suggereert dat andere cellen dan die met te weinig zuurstof (bij-voorbeeld die cellen die te weinig voedingsstoffen krijgen) getarget worden. Dit vermoeden werd versterkt doordat de meeste LC3b expressie in hoofd-hals tumoren met EGFR overexpressie gevonden werd in de niet-hypoxische gebieden. Samengevat, de tumoren met hoge EGFR expressie gebruikt in deze studie zijn erg afhankelijk van autofagie voor hun groei, overleving en resistentie tegen radiotherapie. Het blokkeren van autofagie kan daarom een goede additionele behandeling zijn voor tumoren met EGFR overexpressie.

Tot slot

Sinds de ontdekking in begin jaren 80 is er veel onderzoek gedaan naar EGFR.

In veel verschillende kankersoorten is de EGFR signaleringsroute over actief om verschillende redenen zoals overproductie van de liganden, overproductie van de receptor of constante activatie van de receptor door mutaties. Daarom worden tumoren in klinische setting getest op EGFR en gerelateerde signalering moleculen om de juiste diagnose en behandeling te bepalen. Opvallend hierbij is dat EGFRvIII hierin niet wordt meegenomen ondanks een toenemende hoeveelheid aan onderzoeks- en klinische data die suggereren dat EGFRvIII expressie een negatieve invloed heeft op de behandeling en de overleving van de patiënt. Daarom zou in een klinische setting het routinematig testen van tumoren op EGFRvIII overwogen moeten worden.

Veel therapieën gericht tegen EGFR zoals TKIs en antilichamen die zeer effectief zijn in het laboratorium blijken maar minimaal succesvol in de klinische praktijk. Tumoren kunnen resistent worden tegen de behandeling door compensatie mechanismen te activeren of de expressie van EGFRvIII.

In dit proefschrift beschrijven we een potentieel nieuwe toevoeging aan de therapie om tumoren met EGFR en EGFRvIII overexpressie te behandelen. We hebben laten zien dat tumoren met zowel EGFR als EGFRvIII overexpressie zeer sterk reageren op inhibitie van autofagie door middel van CQ. Verder blijkt dat CQ in combinatie met conventionele behandeling goed werkt en zelfs beter in patiënten met een tumor met EGFRvIII. Meer en grotere klinische trials zijn echter nodig om de effectiviteit van CQ in de behandeling van (EGFRvIII positieve) hersentumoren vast te stellen.

Het ontrafelen van de exacte autofagie signalering routes van EGFR en EGFRvIII in tumoren zou nieuwe inzichten kunnen opleveren over hoe deze meer efficiënt behandeld kunnen worden. Veel inhibitoren van signalering moleculen van EGFR zoals PI3K, mTOR, RAS of RAF zijn momenteel beschikbaar voor onderzoek of worden zelfs al toegepast in de kliniek. De combinatie van deze inhibitoren en anti-EGFR therapie zou nieuwe behandeling strategieën kunnen opleveren.

Men beseft meer en meer dat enkelvoudige therapieën niet effectief genoeg zijn tegen de meeste kanker soorten en dat nieuwe behandelingsstrategieën gezocht moeten worden in combinaties van bestaande of nieuwe therapieën. In dat licht zouden patiënten met een glioblastoma behandeld kunnen worden met Avastin (Bevacizumab, een antilichaam tegen Vascular Endothelial growth

factor (VEGF) welk de aanmaak van nieuwe bloedvaten tegen gaat) in combinatie met inhibitie van autofagie en radiotherapie. Behandeling met Bevacizumab leidt tot een toename van de hypoxische fractie van een tumor. Wanneer dit dan gecombineerd wordt met CQ is een toename in tumorceldood te verwachten en wordt de effectiviteit van Bevacizumab als enkelvoudige therapie verhoogd. Hiermee kan de hergroei van de tumor na bestraling uitgesteld of zelfs voorkomen worden.

Een andere mogelijkheid om tumoren met EGFRvIII expressie te bestrijden is door middel van vaccinatie. EGFRvIII is ideaal als vaccinatie kandidaat omdat het alleen voorkomt op tumorcellen en niet op normaal weefsel. Vaccinatie van patiënten met een EGFRvIII positieve tumor met een EGFRvIII peptide leverde een 5-voudige toename in overleving. Echter, Natural Killer (NK) cellen zijn minder effectief in een hypoxische omgeving en tumor cellen gebruiken autofagie als bescherming tegen cytotoxische T lymfocyten (CTLs). De combinatie van radiotherapie, CQ en vaccinatie zou daarom alle normoxische en hypoxische cellen moeten vernietigen. Deze benadering richt zich niet alleen op ‘normale’ tumor cellen maar ook op de zeer radioresistente glioblastoma kanker stem cellen (CSC) welke EGFRvIII tot expressie brengen.

Samengevat, ondanks enkele decennia aan onderzoek, nieuwe anti-EGFR medicijnen en klinische trials, blijven tumoren met EGFR en EGFRvIII expressie zeer moeilijk te behandelen. Daarom is meer onderzoek nodig om alle mechanismen die hierbij een rol spelen te onderzoeken een zo best mogelijke behandeling te bereiken.

Dankwoord

Dankwoord

Bedankt !!

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Curriculum Vitae

Curriculum Vitae

Barry Jutten was born on September 21st in Stein, the Netherlands. After high school at the Groenewald in Stein, he studied chemistry at the University of Utrecht for two years before he switched to the University of Applied Sciences (Hogeschool Limburg) to study biochemistry, from which he graduated in 1997. In the period from 1997 to 2006 Barry worked as a research technician at the department of Pathology at Maastricht University, and from 2006 to 2008 he worked as a research technician at the department of Radiotherapy (MAASTRO Lab) at Maastricht University. In 2008 Barry started his Master in Molecular Life Sciences at Maastricht University and started as a PhD student within MAASTRO Lab simultaneously. He studied how EGFR and EGFRvIII contribute to a more aggressive tumour phenotype and how the inhibition of autophagy could contribute to the treatment of tumours with deregulated EGFR signalling under supervision of Prof. dr. Ph. Lambin. He finished his PhD thesis in 2014 and continued this research as a post-doc at MAASTRO.

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List of publications

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