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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.
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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 reagents

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were 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 phβAc.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 cytospinned (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.

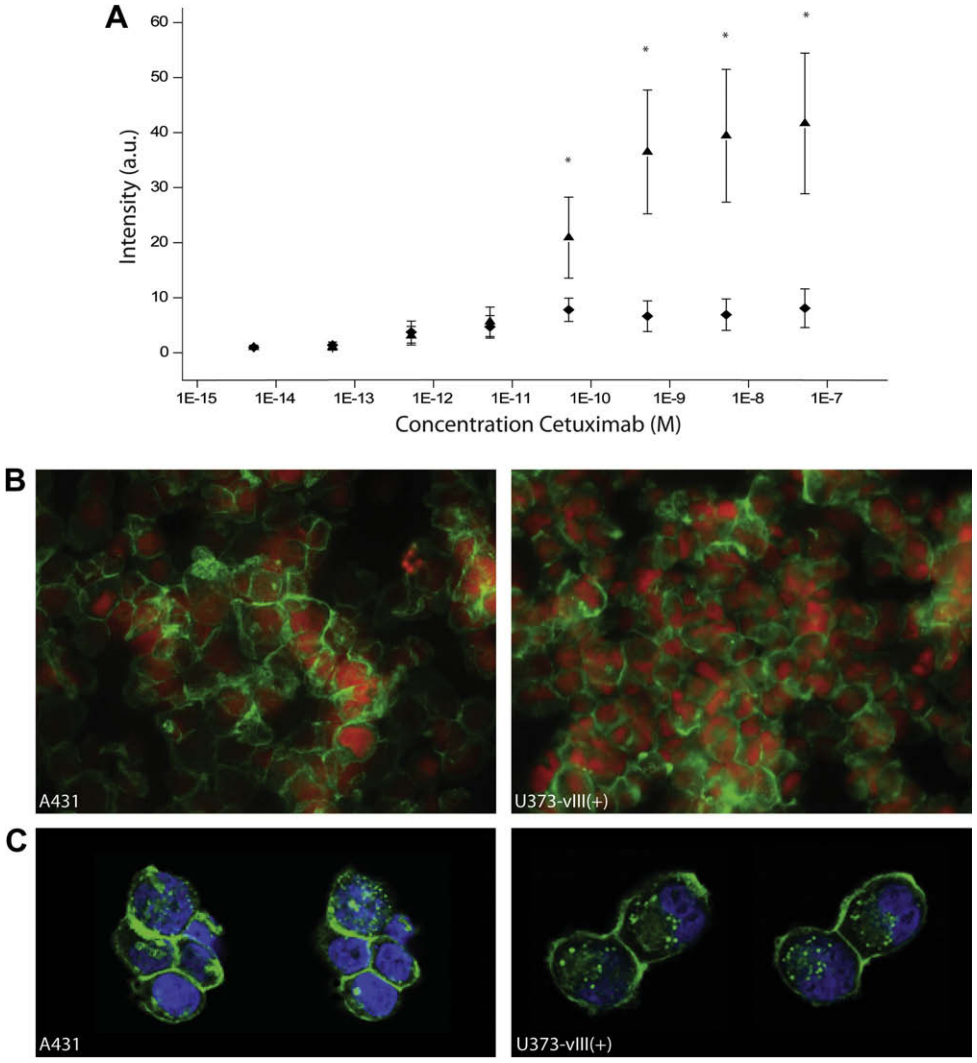

Fig. 1. Cetuximab binds to and internalizes EGFRvIII. (A) Flow cytometry analysis of the binding of cetuximab to two U373 cell lines (▲) U373-vIII(+) and (♦) U373. Error bars indicate standard deviation of triplicate measurement, (∗) indicates significance $p < 0.05$. (B) Immunofluorescence analysis 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.

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–Whitney

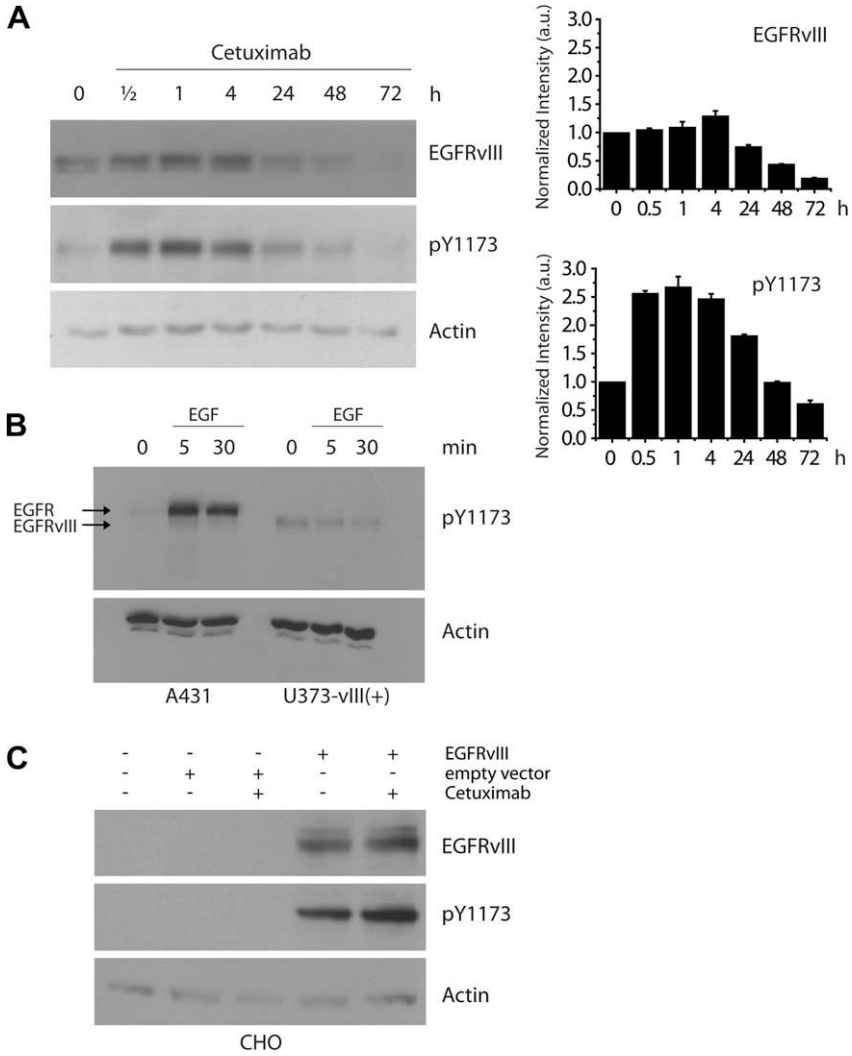

Fig. 2. Cetuximab but not EGF activates EGFRvIII. (A) Western blot analysis of (phospho-) EGFRvIII expression of U373-vIII(+) cells incubated with 60 nM cetuximab for the indicated time (h). Densitometric quantification of the band intensities is shown on the right panel normalized for actin. (B) Western 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.

U 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.

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. 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 cetuximab-induced 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 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].

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 long-term 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-trea-

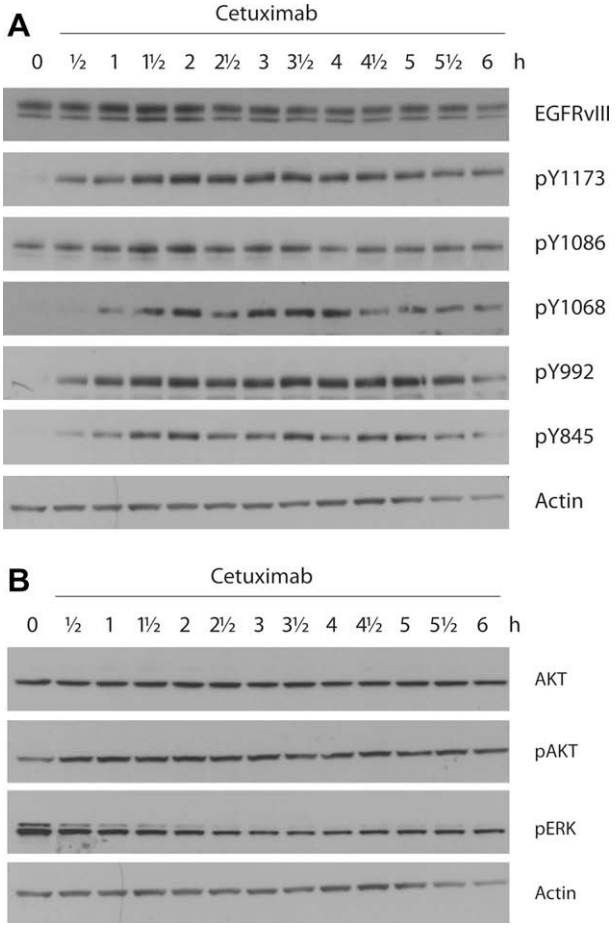

Fig. 3. Phosphorylation of EGFRvIII and downstream effectors after cetuximab treatment. Western blot analysis of different EGFR phosphorylation sites (A) and Akt and Erk phosphorylation (B) for every 0.5 h for 6 h after 60 nM cetuximab treatment. Actin was used as a loading control.

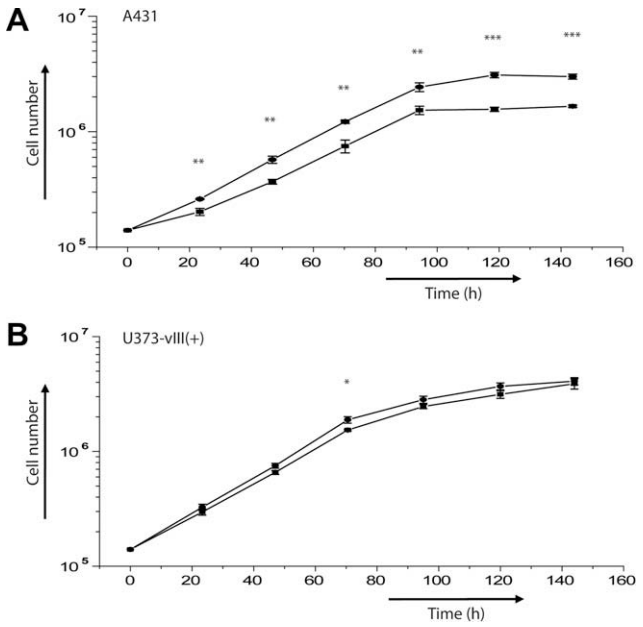

Fig. 4. Cell growth upon cetuximab treatment. The influence of cetuximab on growth was evaluated by counting total numbers of A431 (A) and U373-vIII(+) (B) cells. (●) Cells incubated with 60 nM control IgG, (■) cells incubated with 60 nM cetuximab. Error bars indicate standard deviation of triplicate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

ted 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 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

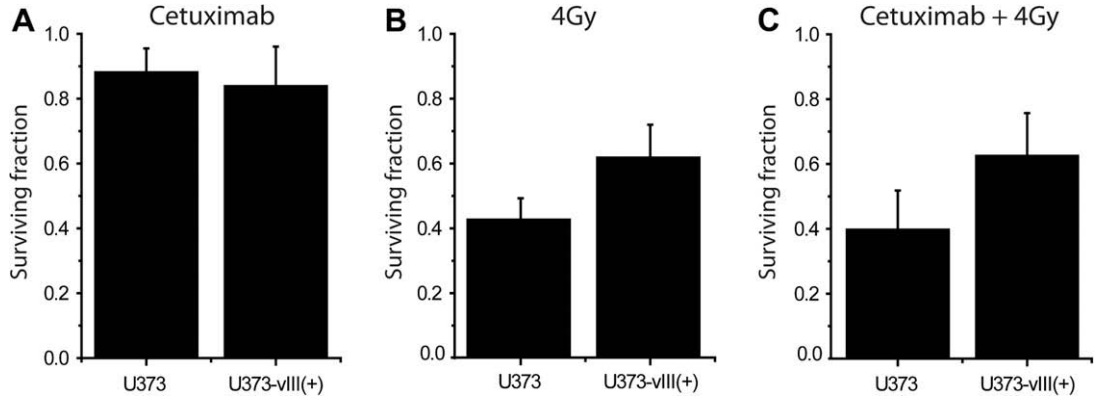

Fig. 5. Clonogenic survival upon cetuximab treatment. Clonogenic survival assays were performed to determine the survival of the cell lines U373 and U373-vIII(+) 72 h after 60 nM cetuximab treatment (A), 24 h after IR at 4 Gy (B) and the combination of cetuximab treatment and IR at 4 Gy (C). The mean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

delivery, e.g. approaches covalently linking monoclonal antibody fragments of cetuximab to stabilized liposomes containing chemotherapeutic 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.

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