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Epidermal growth factor receptor

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

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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.

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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 chemo- and 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 phosphatidylethanolamine (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.

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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. MEM-α 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 MEMα 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 anti-pimonidazole (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 µl volume using sensiMix SYBR low-ROX kit (GC Biotech) with the ABI Prism

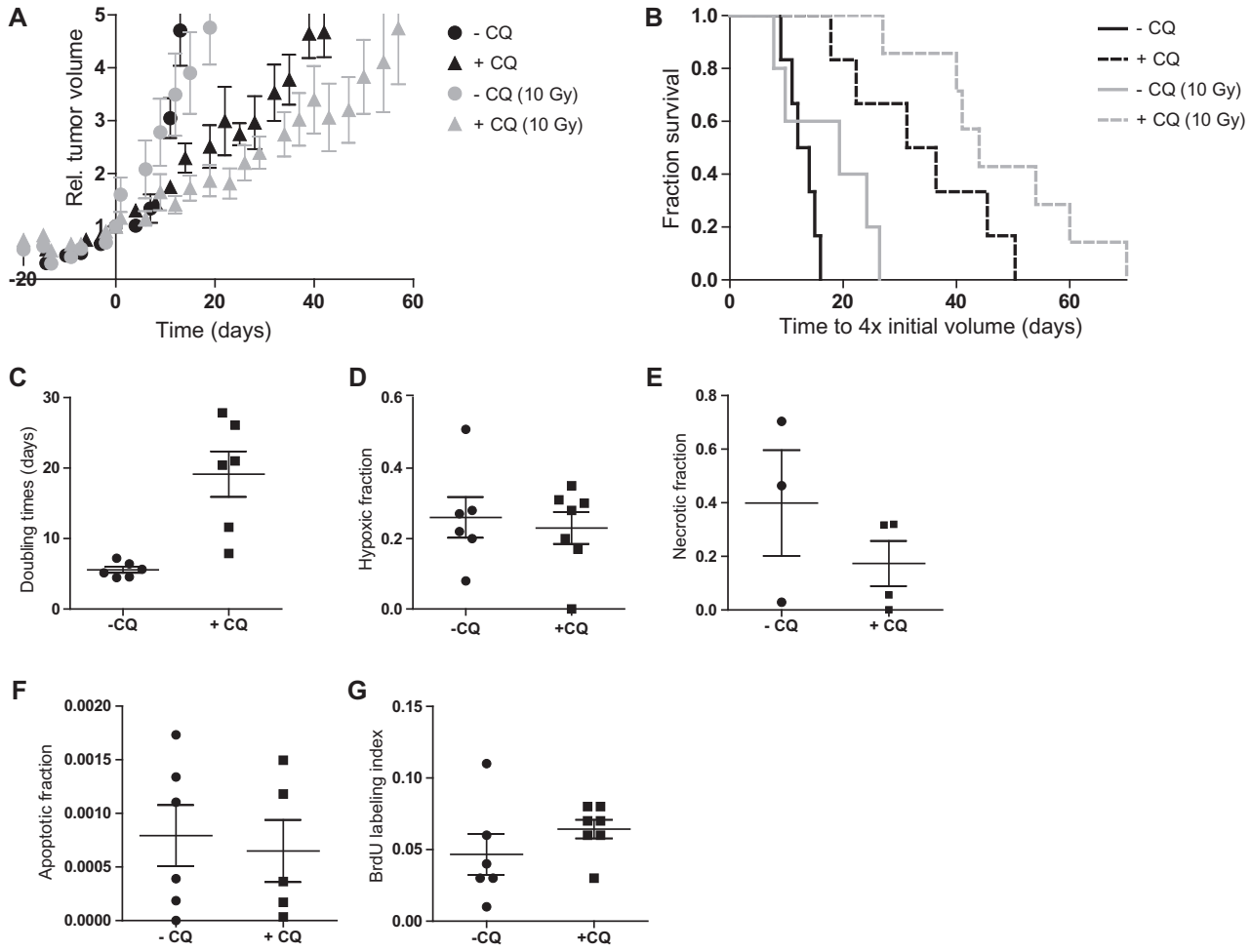

Fig. 1. EGFR overexpressing U373-MG xenografts are sensitive to CQ treatment. (A) Growth of control (−CQ, $n = 6$), irradiated (−CQ (10 Gy), $n = 5$), CQ treated (+CQ, $n = 6$) and irradiated in combination with CQ treated (+CQ (10 Gy), $n = 7$) U373-MG xenografts with EGFR overexpression. (B) Time to 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.

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).

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

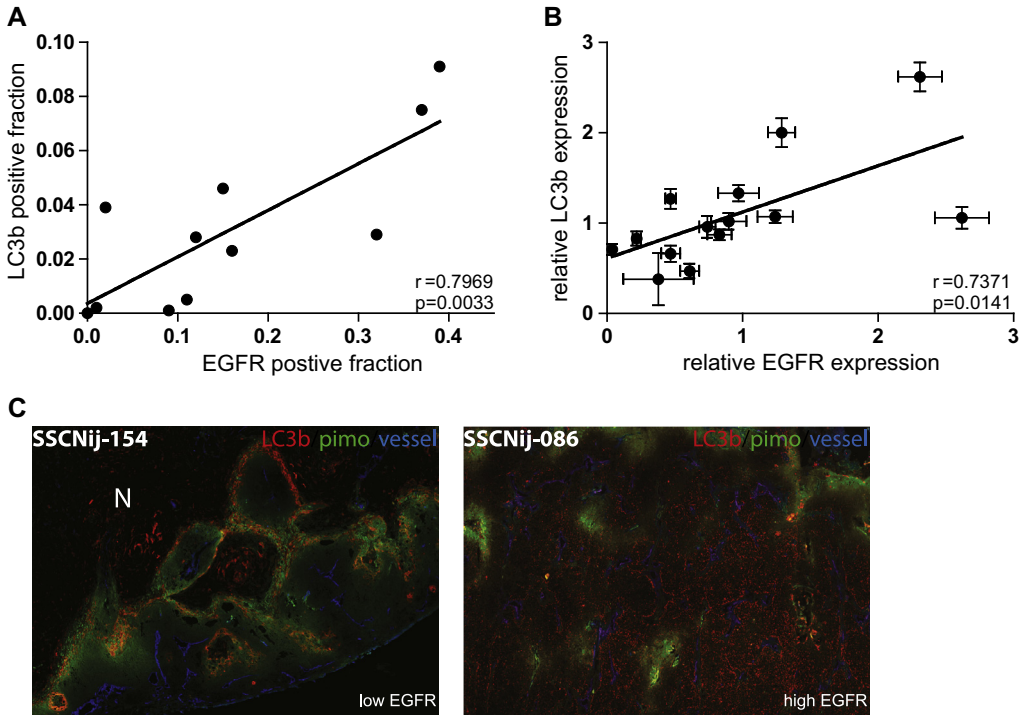

Fig. 2. EGFR expression correlates with LC3b expression. (A) Correlation between LC3b positive fraction and EGFR positive fraction determined by IHC in a panel of primary HNSCC xenografts. (B) Correlation between EGFR and LC3b mRNA in a panel of primary HNSCC xenografts. (C) IHC of HNSSC with low EGFR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

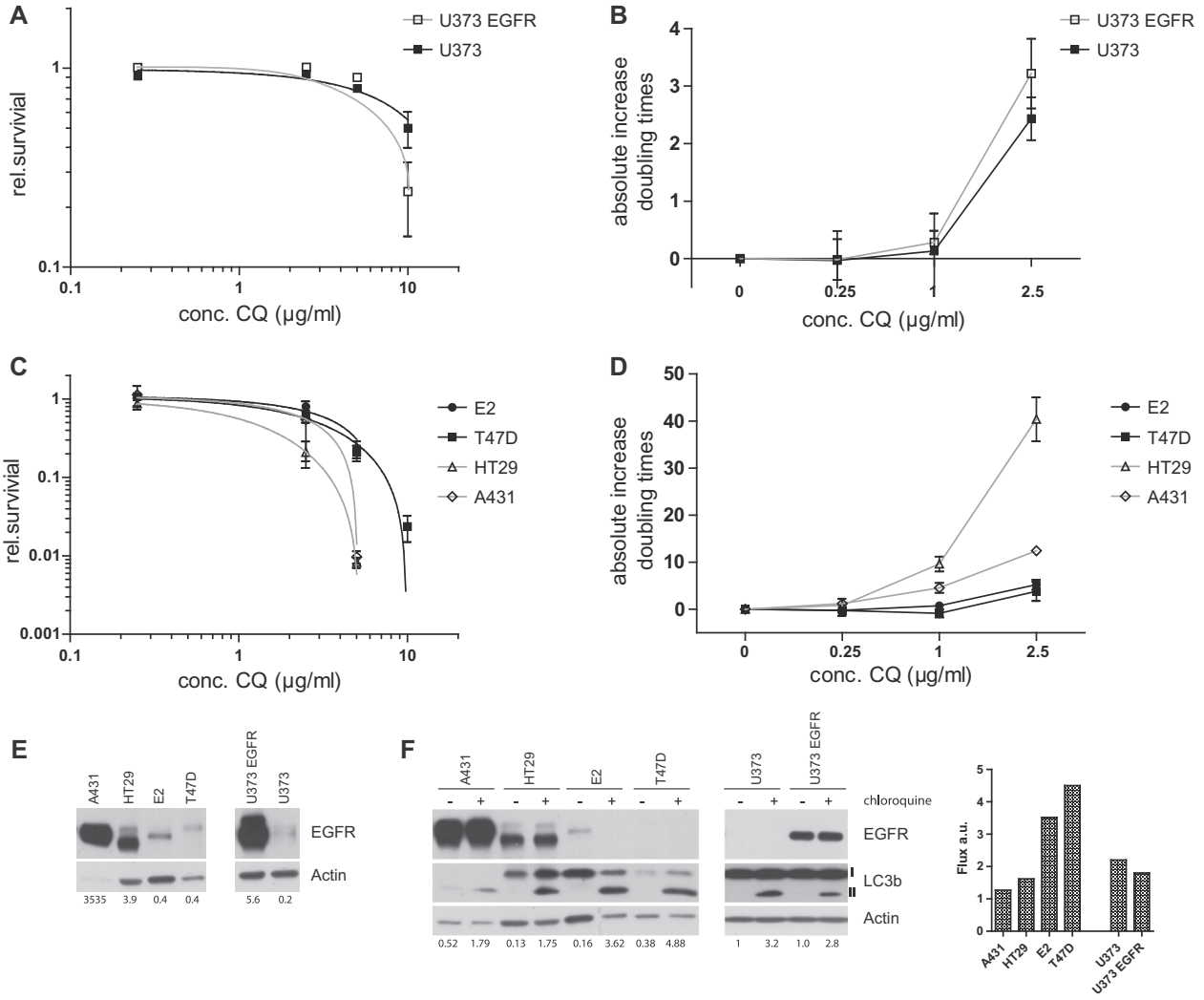

Fig. 3. EGFR high expressing cells are more sensitive to CQ treatment than EGFR low expressing cells (A) Dose-dependent clonogenic survival of EGFR overexpressing and control U373-MGs after CQ addition. (B) Doubling times of EGFR high and low U373-MGs after exposure to CQ. (C) Clonogenic survival of 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.

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 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 reduces 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 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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2013.06.033.

References

[1] Herbst RS. Review of epidermal growth factor receptor biology. Int J Radiat Oncol Biol Phys 2004;59:21–6.
[2] Holz C, Niehr F, Boyko M, et al. Epithelial–mesenchymal-transition induced by EGFR activation interferes with cell migration and response to irradiation and cetuximab in head and neck cancer cells. Radiother Oncol 2011;101:158–64.
[3] Theys J, Jutten B, Habets R, et al. E-Cadherin loss associated with EMT promotes radioresistance in human tumor cells. Radiother Oncol 2011;99:392–7.
[4] Dittmann K, Mayer C, Fehrenbacher B, Schaller M, Kehlbach R, Rodemann HP. Nuclear EGFR shuttling induced by ionizing radiation is regulated by phosphorylation at residue Thr654. FEBS Lett 2010;584:3878–84.
[5] Huang W-C, Chen Y-J, Hung M-C. Implication of nuclear EGFR in the development of resistance to anticancer therapies. BioMedicine 2011;1:2–10.
[6] Schmidt-Ullrich RK, Valerie K, Fogleman PB, Walters J. Radiation-induced autophosphorylation of epidermal growth factor receptor in human malignant mammary and squamous epithelial cells. Radiat Res 1996;145:81–5.
[7] Dittmann K, Mayer C, Fehrenbacher B, Schaller M, Kehlbach R, Rodemann HP. Nuclear epidermal growth factor receptor modulates cellular radio-sensitivity by regulation of chromatin access. Radiother Oncol 2011;99:317–22.
[8] Astsaturov I, Cohen RB, Harari PM. Clinical application of EGFR inhibitors in head and neck squamous cell cancer. Cancer Treat Res 2008;139:135–52.
[9] Krause M, Baumann M. Clinical biomarkers of kinase activity: examples from EGFR inhibition trials. Cancer Metastasis Rev 2008;27:387–402.
[10] Raju U, Riesterer O, Wang ZQ, et al. Dasatinib, a multi-kinase inhibitor increased radiation sensitivity by interfering with nuclear localization of epidermal growth factor receptor and by blocking DNA repair pathways. Radiother Oncol 2012;105:241–9.
[11] Stegeman H, Kaanders JH, van der Kogel AJ, et al. Predictive value of hypoxia, proliferation and tyrosine kinase receptors for EGFR-inhibition and radiotherapy sensitivity in head and neck cancer models. Radiother Oncol 2013;106:383–9.
[12] Overgaard J. Hypoxic modification of radiotherapy in squamous cell carcinoma of the head and neck – a systematic review and meta-analysis. Radiother Oncol 2011;100:22–32.
[13] Starmans MH, Chu KC, Haider S, et al. The prognostic value of temporal in vitro and in vivo derived hypoxia gene-expression signatures in breast cancer. Radiother Oncol 2012;102:436–43.
[14] Rouschop KM, van den Beucken T, Dubois L, et al. The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. J Clin Invest 2010;120:127–41.
[15] Kabeya Y, Mizushima N, Ueno T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 2000;19:5720–8.
[16] Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. Autophagy 2007;3:542–5.
[17] Theys J, Jutten B, Dubois L, et al. The deletion mutant EGFRvIII significantly contributes to stress resistance typical for the tumour microenvironment. Radiother Oncol 2009;92:399–404.
[18] Jutten B, Dubois L, Li Y, et al. Binding of cetuximab to the EGFRvIII deletion mutant and its biological consequences in malignant glioma cells. Radiother Oncol 2009;92:393–8.
[19] Weihua Z, Tsan R, Huang WC, et al. Survival of cancer cells is maintained by EGFR independent of its kinase activity. Cancer Cell 2008;13:385–93.
[20] Dittmann K, Mayer C, Rodemann HP, Huber SM. EGFR cooperates with glucose transporter SGLT1 to enable chromatin remodeling in response to ionizing radiation. Radiother Oncol 2013;107:247–51.
[21] Huber SM, Misovic M, Mayer C, Rodemann HP, Dittmann K. EGFR-mediated stimulation of sodium/glucose cotransport promotes survival of irradiated human A549 lung adenocarcinoma cells. Radiother Oncol 2012;103:373–9.
[22] Lock R, Roy S, Kenific CM, et al. Autophagy facilitates glycolysis during Ras-mediated oncogenic transformation. Mol Biol Cell 2011;22:165–78.
[23] Kim MJ, Woo SJ, Yoon CH, et al. Involvement of autophagy in oncogenic K-Ras-induced malignant cell transformation. J Biol Chem 2011;286:12924–32.
[24] Guo JY, Chen HY, Mathew R, et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. Genes Dev 2011;25:460–70.
[25] Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol 2011;13:132–41.
[26] Nazio F, Strappazzon F, Antonioli M, et al. MTOR inhibits autophagy by controlling ULK1 ubiquitylation, self-association and function through AMBRA1 and TRAF6. Nat Cell Biol 2013;15:406–16.