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The anti-EGFR antibody cetuximab sensitizes human head and neck squamous cell carcinoma cells to radiation in part through inhibiting radiation-induced upregulation of HIF-1 α

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Abstract

In this study, we investigated the mechanisms underlying cetuximab-mediated radiosensitization of HNSCC. Irradiation of HNSCC cells upregulated hypoxia-inducible factor-1 α (HIF-1 α) via a mechanism involving *de novo* synthesis of HIF-1 α protein. Radiation-induced upregulation of HIF-1 α was completely abolished by concurrent treatment of HNSCC cells with cetuximab. Experimental elevation of constitutively expressed HIF-1 α abolished cetuximab-mediated radiosensitization in HNSCC cells, whereas downregulation of HIF-1 α by siRNA or a small molecule inhibitor enhanced responses of cetuximab-resistant HNSCC cells to cetuximab plus radiation. Our data suggest that cetuximab sensitizes cancer cells to ionizing radiation in part through inhibition of radiation-induced upregulation of HIF-1 α .

1. Introduction

Radiotherapy is an important therapy for patients with locally advanced and inoperable head and neck squamous cell carcinoma (HNSCC); unfortunately, however, about 50% of patients treated with definitive radiotherapy with or without chemotherapy go on to experience local recurrence or even remote metastasis [1,2]. More than 90% of HNSCCs express a high level of epidermal growth factor receptor (EGFR) [3,4]. Overexpression of EGFR makes HNSCC resistant to radiation [5–10]. The combination of radiotherapy and cetuximab, an EGFR-blocking antibody, improved the radiosensitivity of HNSCC in preclinical models [5,11]. In a pivotal phase III trial and further follow-up studies, the combination of radiotherapy and cetuximab resulted in prolonged survival [12,13]. The combination therapy has thus been approved by the US Food and Drug Administration for treatment of HNSCC. However, the mechanisms underlying cetuximab-mediated radiosensitization of HNSCC remain to be fully elucidated, exploration of which may help to improve response of HNSCC to the combination therapy.

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Disclosure statement

None declared.

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For over half a century, tumor hypoxia has been known to contribute to tumor radioresistance and poor clinical outcomes [14,15]. The presence of oxygen during radiotherapy is necessary to generate free oxygen radicals for tumor killing due to radiation-induced DNA damage [16,17]. Hypoxia-inducible factor-1 (HIF-1), a master regulator of tumor hypoxia, has recently been implicated in radiation resistance in several preclinical and clinical studies [18–22]. HIF-1 is a heterodimer consisting of an oxygen-sensitive alpha subunit (HIF-1 α) and a constitutively expressed beta subunit (HIF-1 β) [23–27]. Overexpression of HIF-1 α in biopsied tissues was associated with an increased risk of failure to achieve complete remission after radiotherapy in patients with oropharyngeal cancer [28]. Ectopic overexpression of HIF-1 α in cancer cells conferred radiation resistance [29]. Conversely, HIF-1 α -null mouse embryo fibroblasts manifested increased radiation sensitivity [30]. Also, inhibition of HIF-1 α by small molecule inhibitors or siRNA sensitized cancer cells to radiation [31–37].

Studies in the literature also showed that radiation can upregulate HIF-1 activity [31,36,38]. Radiation can dismantle so-called stress granules, which are protein-mRNA complexes that are formed during hypoxic stress to prevent HIF-1-regulated mRNAs from being translated into protein during hypoxia and that are disaggregated upon radiation-induced reoxygenation, leading to a burst of HIF-1-regulated proteins [31]. HIF-1 activity can also be upregulated by tumor-reactive free oxygen radicals and free nitrogen radicals induced by radiation through both a phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR)-dependent increase in HIF-1 α expression and a heat shock protein 90-mediated stabilization of HIF-1 α protein [31,36,38]. The impacts of radiation-induced HIF-1 activation on cancer cell response to radiation, however, are complex: on the one hand, activation of HIF-1 leads to upregulation of vascular endothelial growth factor and other proangiogenic and prosurvival factors to protect the tumor cells and tumor microvasculature from the cytotoxic effects of radiation; on the other hand, upregulation of HIF-1 can promote p53-mediated apoptosis and thus decrease clonogenic survival of p53 wild-type tumor cells, sensitizing tumors to radiotherapy [39–41].

HIF-1 transcriptionally activates over 100 genes involved in regulating cell metabolism, tumor angiogenesis, cancer cell survival, proliferation, invasion, and resistance to various treatments [42,43]. We previously reported that cetuximab downregulates HIF-1 α by inhibiting new HIF-1 α protein synthesis, an effect that is mediated through inhibition of both the PI3K/Akt/mTOR and MEK/Erk pathways [44]. We also showed that response of cancer cells to cetuximab correlates with downregulation of HIF-1 α by cetuximab through inhibition of EGFR-mediated activation of the PI3K/Akt/mTOR and MEK/Erk pathways [44–46]. Our previously reported data indicate that downregulation of HIF-1 α by cetuximab is required, although may not be sufficient, to mediate cetuximab-induced antitumor activity [44,46]. Silencing of HIF-1 α by RNA interference or small molecule inhibitors substantially restored sensitivity to cetuximab in cancer cells expressing an oncogenic Ras mutant [44,47,48].

In this study, we expanded our previous study by examining the expression of HIF-1 α and the transcriptional activity of HIF-1 in HNSCC cell lines after treatment with ionizing radiation with and without concurrent cetuximab treatment. We hypothesized that cetuximab sensitizes HNSCC cells to radiation in part through inhibiting radiation-induced upregulation of HIF-1 α . We tested this hypothesis by exploring the impacts of experimental elevation of HIF-1 α by overexpression and experimental downregulation of HIF-1 α by RNA interference and by a small molecule inhibitor on clonogenic survival of HNSCC cell lines after treatment with ionizing radiation. Our findings provide novel insights into the mechanisms underlying cetuximab-mediated radiosensitization that may be critically

important for developing novel strategies to improve the clinical response of HNSCC to radiotherapy.

2. Materials and methods

2.1. Cell lines and culture

HNSCC cell lines FaDu, HN5, UMSCC1, and OSC19 were maintained in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin under conditions of 5% CO₂ at 37°C in an incubator.

2.2. Reagents, antibodies, and plasmids

Cetuximab was provided by ImClone System Inc. (New York, NY), and 1-methyl-1, 9 pyrazoloanthrone (1-methyl 1, 9 PA) was purchased from CalBiochem/EMD Chemicals, Inc. (Gibbstown, NJ). Antibodies against HIF-1α and Ras were purchased from BD Transduction Laboratories (San Diego, CA) and Cell Signaling Technology Inc. (Beverly, MA), respectively. HIF-1α siRNA duplexes (Target DNA sequence: AACTGATGACCAGCAACTTGA, dTdT overhang) was purchased from Qiagen (Valencia, CA). Constructs of pBI-GL-V6L, HIF-1α-ΔODD, and H-RasG12V were described previously [44,45,47].

2.3. Western blot analysis

Cultured cells were lysed in a lysis buffer containing 50 mM TrisHCl (pH 7.4), 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml aprotinin, and 25 µg/ml leupeptin and kept on ice for 15 min. The lysates were cleared by centrifugation, and the supernatants were collected. Equal amounts of protein lysate, as determined by the Pierce Coomassie Plus colorimetric protein assay (Thermo Fisher Scientific), were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with the intended primary antibodies. The signals were visualized using the enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ).

2.4. Luciferase activity assay

Luciferase activity was measured with a luciferase activity assay kit purchased from Promega (Madison, WI). Briefly, cultured cells were lysed in lysis buffer provided in the kit. Cell lysates were cleared by centrifugation, and the supernatants were collected. Protein concentration was determined by Lowry protein assay. Protein samples (20 µg each in 20 µl of lysis buffer) were added into wells of an opaque, clear-bottom, 96-well microplate and then mixed with 80 µl in each well of the luciferase assay reagent. Luciferase activity was read immediately with a FLUOstar Omega luminescence microplate reader (BMG Labtech).

2.5. MTT proliferation assay

Cells were cultured in 24-well plates with 0.5 ml of medium per well at 37°C in a CO₂ incubator. At the end of the desired treatment in cell culture, cells were incubated for an additional 2 h after addition of 50 µl/well of 10 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cells were then lysed with a lysis buffer (500 µl/well) containing 20% SDS in dimethyl formamide/H₂O (1:1, v/v; pH 4.7) at 37°C for at least 6 h. We determined the relative number of surviving cells in each group by measuring the optical density (OD) of the cell lysates at an absorbance wavelength of 570 nm. The OD value in each treatment group was then expressed as a percentage of the OD value of the untreated control cells, and OD values were plotted against the treatments.

2.6. Clonogenic survival assay

Cells grown on 6-cm dishes were irradiated at room temperature with γ -rays generated from a high-dose-rate ^{137}Cs unit (4.5 Gy/min). The irradiated cells were then seeded in triplicate into 10-cm dishes, with densities varying from 500 to 3000 cells/dish according to the dose of γ -rays the cells received (to yield 50 to 300 colonies per dish). The cells were cultured in a 37°C, 5% CO_2 incubator for 8–14 days depending on the growth rate of the cells. Individual colonies (>50 cells/colony) were fixed and stained with a solution containing 0.2% crystal violet in 10% ethanol for 30 minutes and counted. The surviving fraction, expressed as a function of irradiation, was calculated as follows: surviving fraction = colonies counted/(cell numbers seeded $\times$ plating efficiency), where plating efficiency is the percentage of cells seeded that grow into colonies under a specific culture condition of a given cell line

3. Results

3.1. Treatment with ionizing radiation upregulates HIF-1 α through de novo protein synthesis in HNSCC cell lines

We first examined whether treatment with ionizing radiation can upregulate HIF-1 α in HNSCC cell lines. We chose two HNSCC cell lines that express intermediate (FaDu) and high (HN5) levels of EGFR and that display inhibited proliferation in response to cetuximab treatment. We found that both the level of HIF-1 α and the transcriptional activity of HIF-1 were upregulated in HN5 and FaDu cells after treatment with ionizing radiation in normoxic culture. Figure 1A shows the upregulation of HIF-1 α protein after ionizing radiation in the two cell lines measured by Western blot analysis. Figure 1B confirms that the radiation-induced HIF-1 α was functional, as measured by a hypoxia response element (HRE) luciferase reporter assay after transient transfection of the cells with the luciferase reporter gene construct pBI-GL-V6L, which contains six tandem repeats of the HRE from the human vascular endothelial growth factor gene [45]. We found that addition of cycloheximide right after irradiation of the cells effectively inhibited radiation-induced regulation of HIF-1 α expression (Figure 1C), which supported a conclusion that the radiation-induced upregulation of HIF-1 α involves *de novo* synthesis of new proteins.

3.2. Overexpression of HIF-1 α renders HNSCC cells radioresistant, whereas silencing of HIF-1 α sensitizes HNSCC cells to radiation

To determine the role of radiation-induced upregulation of HIF-1 α in cell response to radiation, we investigated how experimentally induced changes in the level of HIF-1 α affect cell responses to radiation in HN5 and FaDu cells. Because of the instability of wild-type HIF-1 α in normoxia, we transfected HN5 and FaDu cells with a HIF-1 α mutant with deletion of the oxygen-dependent degradation (ODD) domain (HIF-1 α - Δ ODD). Deletion of the ODD domain enables HIF-1 α - Δ ODD to be stable in normoxia following overexpression and retain the majority of transcriptional activity of HIF-1 α . We found that experimental elevation of HIF-1 α - Δ ODD conferred marked radioresistance to both HN5 and FaDu cells, as measured by clonogenic cell survival assays (Figure 2A and 2B). In contrast, knockdown of HIF-1 α expression by HIF-1 α siRNA for 48 hours before treatment with ionizing radiation sensitized the cells to radiation (Figure 2C and 2D). The X-ray film of the Western blot in Figure 2C had to be exposed longer than the one in Figure 2A in order to reveal the silencing effect of siRNA on the basal level of HIF-1 α in the cells. Together, these findings indicated that the level of HIF-1 α can affect cellular sensitivity to radiation.

3.3. Cetuximab inhibits radiation-induced upregulation of HIF-1 α in HNSCC cells, leading to radiosensitization

We then tested our hypothesis that cetuximab sensitizes cancer cells to radiation through inhibition of radiation-induced upregulation of HIF-1 α . Figure 3A shows that addition of cetuximab after treatment with ionizing radiation substantially enhanced radiation-induced inhibition of clonogenic survival of HN5 and FaDu cells, compared to the effect after radiation treatment alone. We previously reported that cetuximab can reduce the basal level of HIF-1 α in normoxia and, to a lesser extent, reduce the elevation of HIF-1 α in hypoxia [45]. In this study, addition of cetuximab after irradiation also abolished radiation-induced upregulation of HIF-1 α (Figure 3B). A similar pattern of changes was found when the same cell lysates as in Figure 3B were assayed for HIF-1 transcriptional activity with the HRE luciferase assay (Figure 3C).

It is noteworthy that the radiation-induced upregulation of HIF-1 α was less prominent than the upregulation of HIF-1 α under hypoxia. Also, the inhibitory effect of cetuximab was much stronger for radiation-induced upregulation of HIF-1 α than for hypoxia-induced upregulation of HIF-1 α : cetuximab nearly completely inhibited the radiation-induced upregulation of HIF-1 α (Figure 3C). While the hypoxia-induced increase in HIF-1 α was mainly due to HIF-1 α stabilization, which makes the increase only partially sensitive to cetuximab treatment when the flow of HIF-1 α synthesis is inhibited by cetuximab (note: inhibition of hypoxia-induced regulation of HIF-1 α was observed in a less-exposed film in Figure 3B, data not shown), the fact that the radiation-induced upregulation of HIF-1 α could be completely abolished by cetuximab suggests that this upregulation was caused mainly by new HIF-1 α synthesis.

To determine whether downregulation of HIF-1 α is required for cetuximab-mediated radiosensitization, we established pooled HN5 and FaDu cells stably expressing HIF-1 α - Δ ODD. Overexpression of HIF-1 α - Δ ODD conferred nearly complete resistance of HN5 cells to cetuximab and slightly less but still substantial resistance of FaDu cells to cetuximab (Figure 3D). Figure 3E shows that while overexpression of HIF-1 α - Δ ODD conferred only slightly increased clonogenic survival of these cells after treatment with radiation alone as compared with the survival of the parental cells (see Figure 3A), it strongly abolished the cetuximab-mediated radiosensitization that was observed in the parental cells (see Figure 3A)—there were no differences in the clonogenic survivals of HN5-HIF-1 α - Δ ODD and FaDu-HIF-1 α - Δ ODD cells with and without the addition of cetuximab after irradiation. These findings indicated that cetuximab-mediated inhibition of HIF-1 α plays a critically important role in mediating cetuximab-mediated radiosensitization of HNSCC cells.

3.4. Silencing of HIF-1 α improves radiosensitization by cetuximab and overcomes oncogenic H-Ras-mediated radiation resistance

We next explored whether silencing HIF-1 α may sensitize HNSCC cells to radiation. Compared with HN5 and FaDu cells, in which HIF-1 α was strongly downregulated by cetuximab, UMSCC1 and OSC19 HNSCC cells, which contain EGFR levels similar to FaDu cells, were resistant to HIF-1 α downregulation by cetuximab (Figure 4A). Similar resistance to HIF-1 α downregulation by cetuximab was observed in HN5 and FaDu cells transfected with a constitutively active H-Ras mutant (H-Ras G12V) (Figure 4A). Figure 4B shows that while cetuximab only minimally inhibited proliferation of control siRNA-treated UMSCC1 cells (20–25% inhibition) and OSC19 cells (~10% inhibition), cetuximab more strongly inhibited proliferation of HIF-1 α siRNA-treated UMSCC1 cells (~50% inhibition) and OSC19 cells (40–50% inhibition). Consistent with the responses of the cells to cetuximab, cetuximab did not sensitize the control siRNA-transfected UMSCC1 and OSC19 cells to radiation as measured by clonogenic survival assay; however, cetuximab

substantially sensitized HIF-1 α -silenced UMSCC1 and OSC19 cells to radiation (Figure 4C).

We conducted similar experiments in the HN5 and FaDu cells that were transfected for stable expression of constitutively active H-Ras (G12V). Consistent with these cells' resistance to cetuximab-induced downregulation of HIF-1 α (Figure 4A), experimental expression of H-Ras G12V conferred considerable resistance to cetuximab-induced growth inhibition in control siRNA-treated HN5-RasG12V cells (<10% inhibition) and FaDu-RasG12V cells (only 10–20% inhibition) but not in HIF-1 α -silenced HN5-RasG12V cells (50–60% inhibition) and FaDu-RasG12V cells (40–50% inhibition) (Figure 4D). Whereas cetuximab sensitized parental HN5 and FaDu cells to radiation (Figure 3A), cetuximab failed to sensitize HN5-RasG12V or FaDu-RasG12V cells to radiation (Figure 4E). However, cetuximab substantially sensitized HIF-1 α -silenced HN5-RasG12V and FaDu-RasG12V cells to radiation (Figure 4E). These results indicated a causal relationship between cetuximab-induced downregulation of HIF-1 α and the response of cells to cetuximab-induced proliferation inhibition and radiosensitization.

3.5. The combination of cetuximab with 1-methyl 1, 9 PA sensitizes HNSCC cells to radiation

We recently reported that 1-methyl 1, 9 PA, a small molecule compound that is a derivative of anthrapyrazolone, downregulates HIF-1 α through promoting HIF-1 α degradation and sensitizes cancer cells to cetuximab treatment [47]. We thus evaluated whether the combination of cetuximab with 1-methyl 1, 9 PA can sensitize UMSCC1 and OSC19 cells to radiation treatment. 1-methyl 1, 9 PA alone at a dose of 10 μ M can strongly downregulate HIF-1 α in various types of cancer cells [47]. Figure 5A shows that combination of 10 μ M 1-methyl 1, 9 PA with 20 nM cetuximab, which alone minimally inhibited the growth of UMSCC1 and OSC19 cells (Figure 4B) but strongly inhibited the growth of HN5 and FaDu cells (Figure 3A), significantly enhanced growth inhibition in both UMSCC1 and OSC19 cells ($p < 0.01$). Figure 5B shows that while neither cetuximab nor 1-methyl 1, 9 PA alone enhanced the response of UMSCC1 or OSC19 cells to radiation, the combination of these two agents increased the sensitivity of cells to radiation. These results of this pilot study provide proof-of-principle evidence supporting the novel concept that inhibition of HIF-1 α with a small molecule compound may improve response of cetuximab-resistant HNSCC cells to treatment with the combination of radiation and cetuximab.

4. Discussion

In this study, we report two major findings: (1) inhibition of radiation-induced HIF-1 α upregulation by cetuximab in HNSCC cells and (2) the role of inhibition of radiation-induced HIF-1 α upregulation in cetuximab-mediated radiosensitization. Consistent with the findings reported in the literature for other types of cancers [31,36,38], our data show that ionizing radiation can upregulate HIF-1 α in HNSCC cells irrespective to hypoxia. Our findings support a novel strategy of adding approaches targeting HIF-1 α to enhance the response of HNSCC cells to treatment with the combination of radiation and cetuximab.

This study is of potential clinical significance because radiation is a major treatment modality for patients with cancers in the head and neck region (e.g., oral cavity, tongue, pharynx, and larynx). Cetuximab is approved for enhancing the response of HNSCC to radiation. Despite the benefits observed in clinical studies, there exist multiple mechanisms in HNSCC that can render cancer cells resistant to treatment with the combination of radiation and cetuximab. Common resistance mechanisms include constitutive activation of important signaling molecules downstream of EGFR, such as oncogenic activation of H-Ras mutant (in contrast to K-Ras mutation in colorectal cancers), mutational inactivation of

tumor suppressors, such as PTEN, or cross-activation of EGFR downstream signaling pathways by other growth factor receptors in the same family (e.g., HER2, HER3) or different families (e.g., IGF-1R) or through tumor-stromal interactions [49–51]. We recently reported that HIF-1 α , a component of HIF-1 transcription factor, is one of the most important effector molecules downstream to EGFR pathways and appears to be a promising target for developing novel therapies to overcome cetuximab resistance caused by these mechanisms [44–48]. The current work expanded our serial studies reported in recent years demonstrating the role of downregulating HIF-1 α in mediating cetuximab-induced antitumor activities and supporting the novel approach of co-targeting HIF-1 α to overcome resistance of cancer cells to cetuximab treatment.

An important mechanism reported in the literature by which HIF-1 α is upregulated upon irradiation is radiation-induced tumor reoxygenation [31]; however, in our study, we found that HIF-1 α can be upregulated in the absence of tumor reoxygenation, as the cells were cultured *in vitro* in normoxia. Our observation that the induction of HIF-1 α by radiation involves *de novo* synthesis of new proteins in head and neck cancer cell models is consistent with a recent study with lung cancer cell models showing that radiation induced HIF-1 α protein expression mainly through two distinct pathways, including an increase in *de novo* protein synthesis via activation of PI3K/Akt/mTOR and stabilization of HIF-1 α protein via augmenting the interaction between heat shock protein 90 and HIF-1 α protein [36]. This finding from the recent study indicates that there are multiple mechanisms by which HIF-1 α is upregulated after radiation.

Although increased levels of HIF-1 α caused by tumor hypoxia or aberrant signaling in cancer cells have been associated with a poor response to radiation [28–37], the impact of radiation-induced HIF-1 α upregulation on tumor response to radiation has been less clear. The impact of radiation-induced upregulation of HIF-1 α on tumor radiosensitivity is pleiotropic: by promoting p53 activation, HIF-1 has a radiosensitizing effect on tumors; however, through stimulating endothelial cell survival, HIF-1 promotes tumor radioresistance [41]. Our current findings, which are derived from clonogenic survival assays performed in cell culture, support a conclusion that upregulation of HIF-1 α conferred radioresistance whereas silencing of HIF-1 α sensitized cancer cells to radiation. Our data further show that downregulation of HIF-1 α by cetuximab plays an important role in cetuximab-mediated radiosensitization. However, future studies with *in vivo* models are needed to determine the exact role of inhibition of radiation-induced HIF-1 α upregulation in cetuximab-mediated radiosensitization. Findings from such studies may provide important guidance for designing novel therapeutic strategies in which cetuximab is combined with approaches targeting HIF-1 α to enhance the response of HNSCC to radiation.

In summary, our results suggest that downregulation of HIF-1 α contributes to cetuximab-mediated radiosensitization in HNSCC cells and suggest that the combination of cetuximab with approaches targeting HIF-1 α is a promising strategy for sensitizing cetuximab-resistant HNSCC cells to radiation.

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Fig. 1. Ionizing radiation upregulates HIF-1α through *de novo* protein synthesis in HNSCC cells. (A) HN5 and FaDu cells were irradiated with 3 Gy of ionizing radiation (IR) or not and then cultured for the indicated time periods. At each time point, the irradiated cells and the accompanying unirradiated cells were collected for detection of the level of HIF-1α protein by Western blot analysis. The level of β-actin was used as an internal control of equal protein loading in each lane. (B) HN5 and FaDu cells were transiently transfected with pBI-GL-V6L construct for 24 hours prior to irradiation. The cells were then irradiated and collected as described in A for measurement of the transcriptional activity of HIF-1 on HRE-luciferase reporter by a luciferase activity assay as described in Materials and methods. The results shown are the means of triplicate samples plus and minus standard deviations. (C) HN5 and FaDu cells were irradiated with 3 Gy of ionizing radiation, and 10 μM cycloheximide (CHX) or vehicle (DMSO) was added immediately after irradiation. Cells were harvested at each indicated time point for measurement of the level of HIF-1α protein by Western blot analysis.

Fig. 2.

Role of HIF-1 α in mediating HNSCC cell response to radiation-induced clonogenic survival inhibition. (A) and (B) HN5 and FaDu cells were transiently transfected with HIF-1 α - Δ ODD construct or control vector (pcDNA3.1) for 48 hours. In (A), the cells were harvested for detection of HIF-1 α - Δ ODD expression by Western blot analysis. A nonspecific band below HIF-1 α serves as a reference of equal protein loading in each lane. *NS: nonspecific band. In (B), the cells were irradiated with the indicated doses of radiation and subjected to a clonogenic survival assay as described in Materials and methods. (C) and (D) HN5 and FaDu cells were transiently transfected with HIF-1 α -specific siRNA or control siRNA for 48 hours. In (C), the cells were harvested for detection of the basal level of HIF-1 α by Western blot analysis. A nonspecific band below HIF-1 α serves as a reference of equal protein loading in each lane. In (D), the cells were irradiated with the indicated doses of radiation and subjected to a clonogenic survival assay as described in Materials and methods.

Fig. 3.

Cetuximab sensitizes HNSCC cells to radiation through downregulating HIF-1 α . (A) HN5 and FaDu cells were irradiated with the indicated doses of ionizing radiation and then subjected to clonogenic survival assays in the presence or absence of 2 nM cetuximab (HN5) or 5 nM cetuximab (FaDu) as described in Materials and methods. (B) and (C) HN5 and FaDu cells were transfected with pBI-GL-V6L vector 24 hours prior to irradiation. After exposure to 3 Gy, the cells were cultured in the presence or absence of 10 nM cetuximab for 8 hours. Unirradiated HN5 and FaDu cells cultured in hypoxia with and without 10 nM cetuximab for the same time period were used to compare HIF-1 α upregulation. The cells were harvested for detection of the level of HIF-1 α by Western blot analysis (B) and for detection of HIF-1 transcriptional activity on HRE-luciferase reporter by a luciferase assay as described in Materials and methods (C). (D) and (E) HN5 and FaDu cells were transiently transfected with HIF-1 α - Δ ODD construct or control vector (pcDNA3.1) for 48 hours. The cells were then assayed for responses to cetuximab treatment for 5 days by MTT assays (D) and for responses to treatment with ionizing radiation in the presence and absence of cetuximab (2 nM for HN5 cells and 5 nM for FaDu cells) in the postradiation period for 14 days by clonogenic survival assays (E).

Fig. 4. Silencing HIF-1 α enhances responses of cetuximab-resistant HNSCC cells to cetuximab-induced growth inhibition and radiosensitization. (A) The indicated HNSCC cell lines (FaDu, HN5, OSC19, UMSCC1, and FaDu and HN5 cells transfected with H-Ras G12V) were cultured in the presence or absence of 20 nM cetuximab for 16 hours, and then the cells were collected for detection of the levels of HIF-1 α and EGFR protein by Western blot analysis. The level of β -actin was used as an internal control of equal protein loading in each lane. (B) and (C) UMSCC1 and OSC19 cells were transiently transfected with HIF-1 α -specific siRNA or control siRNA for 48 hours. The cells were then assayed for responses to cetuximab treatment for 5 days by MTT assays (B) and for responses to treatment with ionizing radiation in the presence and absence of 10 nM cetuximab for 14 days by clonogenic survival assays (C). (D) and (E) HN5-RasG12V and FaDu-RasG12V cells were similarly transfected with HIF-1 α siRNA or control siRNA for 48 hours and subjected to

MTT assays (D) and clonogenic survival assays (E) for response to treatment with cetuximab and ionizing radiation as described in (B) and (C). Cetu, cetuximab. siHIF-1 α , HIF-1 α siRNA.

Fig. 5.

The small molecule compound 1-methyl-1, 9 PA sensitizes HNSCC cells to treatment with cetuximab and ionizing radiation.

(A) UMSCC1 and OSC19 cells were treated with 20 nM cetuximab, 10 μ M 1-methyl-1, 9 PA or the combination of these two agents for 5 days, and then MTT assays were performed for determination of the responses of the cells to the treatment. Results of statistical analysis are shown. (B) UMSCC1 and OSC19 cells were irradiated with the indicated doses of ionizing radiation and subjected to clonogenic survival assays in the presence or absence of 5 nM cetuximab, 2.5 μ M 1-methyl-1, 9 PA, or the combination of these two agents in the postradiation period for 14 days. Cetu, cetuximab; 1-Me 1,9 PA, 1-Methyl 1,9 PA.