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Dasatinib blocks cetuximab- and radiation-induced nuclear translocation of the epidermal growth factor receptor in head and neck squamous cell carcinoma

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Abstract

Background and Purpose—The aberrant expression of epidermal growth factor receptor (EGFR) has been linked to the etiology of head and neck squamous cell carcinoma (HNSCC). The first major phase III trial combining cetuximab with radiation confirmed a strong survival advantage. However, both cetuximab and radiation can promote EGFR translocation to the nucleus where it enhances resistance to both of these modalities. In this report we sought to determine how to block cetuximab and radiation-induced translocation of EGFR to the nucleus in HNSCC cell lines.

Material and Methods—We utilized three established HNSCC cell lines, SCC1, SCC6 and SCC1483 and measured nuclear translocation of EGFR after treatment with cetuximab or radiation. We then utilized dasatinib (BMS-354825), a potent, orally bioavailable inhibitor of several tyrosine kinases, including the Src Family Kinases, to determine if SFKs blockade could abrogate cetuximab and radiation-induced nuclear EGFR translocation.

Results—Cetuximab and radiation treatment of all three HNSCC lines lead to translocation of the EGFR to the nucleus. Blockade of SFKs abrogated cetuximab and radiation-induced EGFR translocation to the nucleus.

Conclusions—The data presented in this report suggests that both cetuximab and radiation can promote EGFR translocation to the nucleus and dasatinib can inhibit this process. Collectively these findings may suggest that dasatinib can limit EGFR translocation to the nucleus and may enhance radiotherapy plus cetuximab in HNSCC.

Keywords

EGFR; cetuximab; radiation; Src family kinases; dasatinib; head and neck cancer

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Conflict of Interest DLW has held a sponsored research agreement with BMS.

INTRODUCTION

A major class of the RTK super family is comprised of the HER or epidermal growth factor (EGF) receptors and consists of the EGFR (ErbB1/HER1), HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). The EGFR is a 170kD transmembrane receptor that contains an extracellular ligand-binding domain, a single membrane-spanning region, a nuclear localization signal and a cytoplasmic tyrosine kinase domain (TKD). Ligand binding allows for receptor homo- or hetero-dimerization at the plasma membrane. This interaction activates the receptor tyrosine kinase and, thereby, causes autophosphorylation of the cytoplasmic tails of each dimer pair. The phosphorylated cytoplasmic tail serves as docking sites for numerous proteins and stimulates two primary pathways 1) RAS/RAF/MEK/ERK and 2) phosphatidylinositol 3-kinase (PI3K)-Akt axes. In addition, SRC tyrosine kinases, PLC γ , PKC and signal transducer and activator of transcription (STATs) activation have also been documented as downstream of EGFR signaling. Tumor cell proliferation, survival, invasion and angiogenesis ultimately can be promoted through these pathways [1].

Besides classic cytoplasmic signaling, the EGFR has been consistently detected in the nuclei of cancer cells, primary tumor specimens and highly proliferative tissues [2–6]. Increased nuclear EGFR localization correlates with poor clinical outcome in patients with breast cancer [7], oropharyngeal SCC [8] and ovarian cancer [9]. Recent reports have characterized a novel nuclear localization sequence in the EGFR and its family members [10–12]. Furthermore, mechanisms of transport of the EGFR to the nucleus have been reported [13]. These mechanisms involve binding of ligand, dimerization, activation and internalization. Endosomal sorting to the ER allows for the EGFR to associate with the Sec61 translocon leading to retrograde translocation from the ER to the cytoplasm [14]. Here the EGFR binds importin β , which facilitates its movement into the nucleus [13]. To date nuclear EGFR has been shown to regulate the promoters of several target genes including, Cyclin D1 [4], iNOS [10], B-myb [15], Aurora Kinase A [16] and COX2 [12]. Mechanisms of EGFR-mediated gene regulation involve direct interaction with the EGFR and STAT3 to regulate the iNOS [10] and COX2 [12] promoters, STAT5 for regulation of the Aurora Kinase A promoter [16], and E2F1 transcription factors for the regulation of the B-Myb promoter [15]. In addition, nuclear EGFR has recently been shown to function as a tyrosine kinase in the nucleus, phosphorylating and stabilizing PCNA and thus enhancing proliferative potential of cancer cells [17]. In addition to ligand induced translocation of the EGFR to the nucleus, radiation has been shown to induce EGFR transport to the nucleus mediated by the Src family kinases (SFKs) [18]. Further, cetuximab, a monoclonal antibody targeting the EGFR, has also been shown to lead to EGFR translocation to the nucleus [19]. Collectively these findings suggest that EGF ligand, radiation and cetuximab enhance nuclear accumulation of the EGFR.

Targeting the EGFR with molecular inhibitors has been intensely pursued in the last decade as a cancer treatment strategy. Two primary strategies have been developed to target the EGFR, including anti-EGFR monoclonal antibodies (mAbs) and small molecule tyrosine kinase inhibitors (TKI). Dating back to the early 1980s, Mendelsohn and colleagues purified a series of mAbs to the EGFR to test these agents as inhibitors of tumor growth. Born out of these efforts, cetuximab (IMC-C225, Erbitux) was developed to target the extracellular ligand-binding domain of the EGFR and thereby block natural ligand binding. Cetuximab prevents receptor activation and dimerization and ultimately induces receptor internalization and downregulation. Cetuximab exhibits promising antitumor activity as monotherapy or in combination with chemotherapy and/or radiation in HNSCC. In a phase III clinical trial using cetuximab in locally advanced HNSCC, Bonner et al. compared the efficacy of radiotherapy alone to radiotherapy plus cetuximab [20]. The results of this trial showed an improvement in overall survival and progression-free survival by 20 and 5 months, respectively, with the addition of cetuximab. Locoregional control was also improved by a

median of 9.5 months with the addition of cetuximab to radiotherapy. The results of this phase III trial demonstrating significant improvement in locoregional control, overall survival, and progression-free survival by the addition of cetuximab to radiotherapy led to the Food and Drug Administration (FDA) approval of cetuximab for use in HNSCC in combination with radiation in 2006. Several other trials, at various phases, further investigating the role of cetuximab have also been reported.

Since the time of this report, basic research has indicated that both cetuximab [19] and radiation [18] can drive EGFR to the nucleus and that nuclear EGFR can result in resistance to both of these modalities [18,21]. In the present study we aimed to determine if cetuximab and radiation-induced EGFR translocation to the nucleus shared a common molecular pathway. The data presented herein indicates that dasatinib (BMS-354825), a potent, orally bioavailable inhibitor of several kinases, including BCR-ABL, SRC family kinases (SFKs) c-KIT and PDGFR- β , blocks both radiation and cetuximab-induced EGFR translocation to the nucleus. These findings suggest that administering dasatinib concomitantly during therapeutic regimens containing cetuximab and/or radiation may enhance the efficacy of these therapies by preventing membrane to nucleus translocation, and therefore nuclear function, of the EGFR.

RESULTS

Cetuximab induces EGFR translocation to the nucleus in NSCLC, CRC and HNSCC tumor cell lines

It has been reported that EGF and cetuximab can induce translocation of the EGFR from the extracellular membrane to the nucleus in breast cancer tumor cell lines [11,19]. To further expand upon these findings and determine the universality of cetuximab induction of EGFR to the nucleus, we treated a series of NSCLC (H226, A549, Calu-3), CRC (HT29, SW480, SK-CO-1) and HNSCC (SCC1, SCC6, SCC1483) cell lines with cetuximab and evaluated nuclear translocation of the EGFR (Figure 1A). The results of these experiments indicated that cetuximab could induce EGFR translocation to the nucleus within 24 hours treatment.

Cetuximab has been approved for use as a single agent in platinum-refractory, metastatic HNSCC and in combination with radiation therapy for initial treatment of locally advanced HNSCC. Therefore we focused our investigation of cetuximab and radiation-induced nuclear translocation of the EGFR in the HNSCC setting. Cetuximab dose responses indicated that as little as two nanomolar could lead to translocation of EGFR to the nucleus in 24 hours (Figure 1B). Time course analysis indicated that cetuximab treatment led to induction of nuclear EGFR by 1 hour, which was maintained up to 96 hours (Figure 1C and 1D) in SCC1, SCC6 and SCC1483 tumor cell lines.

Radiation induces EGFR translocation to the nucleus in HNSCC tumor cell lines

It has been previously shown that radiation can induce translocation of the EGFR to the nucleus in A431 and FaDu cell lines [18]. Furthermore, this translocation has been linked to hyperactivity of DNA-PK and thus enhancing DNA repair and manifesting in resistance to radiation therapy [18]. We sought to determine 1) if radiation could induce EGFR translocation to the nucleus in SCC1, SCC6 and SCC1483 cells and 2) if radiation-induced EGFR translocation was temporally related to cetuximab-induced EGFR translocation to the nucleus (Figure 1C and D). Cells were irradiated (4 Gy) and collected at 0.5, 1 and 4 hours after treatment and fractionated for nuclear protein (Figure 2A). We found that radiation treatment resulted in EGFR nuclear translocation and this translocation returned to baseline levels within four hours after irradiation. To compare the temporal relationship between EGF, cetuximab and radiation-induced nuclear translocation of the EGFR, cells were treated

with EGF, cetuximab or radiation for the indicated times (Figure 2B). Nuclear fraction were obtained, fractionated by SDS-PAGE and quantitated. Relative nuclear EGFR level for each group was normalized to untreated controls and plotted as relative nuclear EGFR. The results of this experiment showed that EGF leads to a robust translocation of the EGFR within 1 hour whereas cetuximab induction continues to accumulate for greater than 4 hours (Figure 1C and D). Radiation treatment led to a brisk low-level translocation of the EGFR to the nucleus with return to baseline within four hours (Figure 2A and B).

Cetuximab treatment induces the phosphorylation of the EGFR in HNSCC tumor cells

To analyze the phosphorylation status of the EGFR after EGF or cetuximab treatment we treated SCC1, SCC6 and SCC1483 cells for 30 minutes and 24 hours, respectively. The EGFR was immunoprecipitated from whole cell lysate, followed by analysis of total phosphorylation using a phosphotyrosine antibody (Figure 3A–B). Both EGF and cetuximab treatment resulted in increased total phosphorylation of the EGFR as measured by a pan-phosphotyrosine antibody. To confirm the presence of EGFR in the nuclear fraction after cetuximab treatment and to determine its phosphorylation status, we next subjected cytoplasmic and nuclear extracts from SCC1, SCC6 and SCC1483 cells to immunoprecipitation with EGFR antibody followed by immunoblotting with a phosphotyrosine antibody (Figure 3C–D). The results indicated that nuclear EGFR levels increased after treatment with cetuximab. Further, the EGFR that accumulated in the nucleus was tyrosine-phosphorylated.

Src family kinases mediate cetuximab-induced EGFR translocation to the nucleus

It has been reported that Src family kinases (SFKs) play a role in both ligand and radiation-induced translocation of the EGFR [18,21]. We have previously reported that SFKs are critical for ligand-induced EGFR translocation to the nucleus [21]. Therefore, we tested whether or not the SFK inhibitor, dasatinib, could block cetuximab-induced EGFR translocation to the nucleus. SCC1, SCC6 and SCC1483 cells were plated and pre-treated with dasatinib or DMSO for 24 hours followed by 24 hours stimulation with cetuximab (Figure 4). The cells were then collected and nuclear fractions prepared. The results suggested that cetuximab-induced nuclear translocation of the EGFR and was accompanied by a robust phosphorylation of tyrosine 845 (Y845) of the EGFR, a site solely phosphorylated by SFKs [22]. Pre-treatment of cells with dasatinib, followed by cetuximab treatment, was able to abrogate cetuximab-induced phosphorylation and translocation of the EGFR to the nucleus. Phosphorylation of tyrosine 419 of SFK in cytoplasmic fractions was measured as a control for dasatinib efficacy. These results suggest, in part, that SFK phosphorylation of EGFR^{Y845} may be necessary for cetuximab-induced EGFR translocation to the nucleus.

Src family kinases mediate radiation-induced EGFR translocation to the nucleus

To determine if dasatinib could block radiation-induced EGFR translocation to the nucleus SCC1, SCC6 and SCC1483 cells were plated and pre-treated with dasatinib or DMSO for 24 hours and collected 30 minutes after radiation treatment (Figure 5). Nuclear and cytoplasmic fractions were prepared and determined for nuclear levels of EGFR and phosphorylation of EGFR at Y845. The results of these experiments indicated that dasatinib could block radiation-induced EGFR translocation to the nucleus. In addition, analysis of EGFR^{Y845} indicated increased phosphorylation after radiation treatment and this was blocked with dasatinib. Phosphorylation of tyrosine 419 of SFK in cytoplasmic fractions was measured as a control for dasatinib efficacy. These results suggest, in part, that phosphorylation of EGFR^{Y845} may be necessary for radiation-induced EGFR translocation to the nucleus.

Dasatinib blocks cetuximab and radiation-induced EGFR translocation to the nucleus

Dittmann *et al.* showed that radiation-induced nuclear import of the EGFR could be blocked by the addition of cetuximab [23]. Data presented in Figures 1 and 2 indicated that both cetuximab and radiation can induce EGFR translocation to the nucleus in HNSCC tumor lines, albeit with different temporal relationship. To determine nuclear translocation of the EGFR after treatment with cetuximab and radiation concomitantly, we treated cells with cetuximab for 1 hour prior to irradiation followed by collection of protein 24 hours post irradiation (Figure 6A). These results were consistent with data presented in figures 1 and 2 indicating that, at the 24-hour time point, radiation-induced EGFR translocation to the nucleus had returned to baseline whereas cetuximab treatment led to continued nuclear EGFR accumulation. It is important to note that in the cetuximab plus radiation combination group we did not observe additive effects of the two modalities (Figure 6A). This is most likely due to the fact that samples were collected after 24 hours and radiation induced nuclear EGFR returns to baseline within 4 hours (Figure 2A). Collectively these results suggest that cetuximab leads to a sustained nuclear EGFR accumulation whereas XRT leads to rapid EGFR translocation to the nucleus, followed by a rapid return to baseline. These results also suggest that cetuximab-induced, rather than radiation-induced nuclear translocation of EGFR may be more important in long-term cetuximab/radiation based therapies.

To determine if dasatinib could block the cetuximab/radiation-induced translocation of EGFR to the nucleus, we pre-treated SCC1, SCC6 and SCC1483 cells with dasatinib for 24 hours, then treated with cetuximab for 24 hours and collected protein 30 minutes after XRT treatment (Figure 6B). Phosphorylation of tyrosine 419 of Src was measured as a control for dasatinib efficacy. In all cases dasatinib could block cetuximab/radiation-induced nuclear translocation of EGFR and EGFR^{Y845} phosphorylation.

DISCUSSION

Modalities such as surgery, radiation, chemotherapy and combinations thereof have led to small improvements in overall survival of HNSCC patients. The most significant advance in the treatment of HNSCC came with the combination of radiation and the anti-EGFR antibody cetuximab. Although there was an improvement in progression free survival and overall survival the results of this phase III study were not curative. Both cetuximab (Figure 1 and [19]) and radiation (Figure 2 and [18]) have been shown to induce the translocation of the EGFR to the nucleus. Nuclear EGFR has been clearly associated with resistance to both radiation and cetuximab treatment [18,21,24]. Here we show that SFKs play a role in both cetuximab and radiation-induced EGFR translocation to the nucleus.

In Figures 1 and 2 we investigated the temporal relationship between cetuximab and radiation-induced nuclear translocation of the EGFR. Our results showed a marked temporal difference in each modalities ability to lead nuclear EGFR accumulation. Cetuximab treatment of HNSCC lines could promote EGFR nuclear translocation within in 1 hour and nuclear expression was maintained greater than 96 hours (Figure 1C and 1D). These results are similar to those reported by Liao *et al.* where they showed cetuximab treatment led to nuclear translocation within 30 minutes [19]. However, their time course only extended to 6 hours. In contrast to cetuximab stimulation, radiation treatment of HNSCC cells resulted in the movement of EGFR to the nucleus within 30-minutes followed by a return to baseline levels between 1 and 4 hours (Figure 2). These results are consistent with Dittmann *et al.* where they showed between 10–40 minutes after radiation EGFR had translocated to the nucleus [18]. However, data presented herein extends on this initial finding showing that EGFR returned to baseline between 1 and 4 hours after XRT. Collectively these data suggest

that cetuximab-induced and radiation-induced translocation of the EGFR to the nucleus differ temporally.

It has been shown that cetuximab results in the paradoxical phosphorylation of the EGFR at tyrosine 1173 [25]. We extended on these findings first by determining if the EGFR had increased total phosphorylation levels after cetuximab treatment (Figure 3). SCC1, SCC6 and SCC1483 cells were stimulated with cetuximab or EGF as a positive control. After immunoprecipitation with EGFR antibody from whole cell lysate, both of these treatments had a robust EGFR phosphorylation. We then immunoprecipitated with EGFR antibody from the cytoplasmic and nuclear fractions and found that the cytoplasmic fraction had phosphorylated EGFR in both the untreated and cetuximab treatments, albeit, the cetuximab treated samples exhibited a marked (3.3–4.9 fold) increased in phosphorylation although total EGFR levels were unchanged (Figure 3C). Likewise the nuclear EGFR was present in both untreated and cetuximab-treated cells. However, cetuximab treated cells exhibited a 2.9–4.6 fold increase in nuclear EGFR levels (Figure 3D). Further analysis of the EGFR in the nuclear fraction indicated that the cetuximab treated cells were highly phosphorylated (6.7–8.8 fold increase) compared to untreated cells. These results suggest that cetuximab treatment may result in altered phosphorylation of the EGFR leading to increased translocation to the nucleus.

It has been reported that the EGFR^{Y845}, which is phosphorylated solely by SFKs, may play a critical role for the translocation to the nucleus when treated with EGFR ligands and/or radiation [18,21]. This site has also been attributed to the subcellular distribution of the EGFR movement to the mitochondria [26]. Our results are consistent with these findings in that SCC1, SCC6 and SCC1483 cells exhibit phosphorylation of EGFR^{Y845} after cetuximab or XRT treatment and the use of dasatinib, led to decreased phosphorylation of EGFR^{Y845} followed by subsequent inhibition of nuclear translocation (Figures 4 and 5). As shown for autophosphorylation of EGFR^{Y1173} [25], we demonstrated that combined treatment with cetuximab and radiation treatment also increases phosphorylation of EGFR^{Y845} in both nuclear and cytoplasmic fractions of three cell lines (Figure 6B). Furthermore, dasatinib could block cetuximab and radiation-induced nuclear translocation of the EGFR and this was correlated with decreased phosphorylation of EGFR^{Y845}. Collectively these data suggest that both cetuximab and radiation can induce phosphorylation of EGFR^{Y845}, which may enhance nuclear translocation of the EGFR. Blockade of SFKs using dasatinib in this report and PP2 or Src siRNAs in other published reports suggest that SFK phosphorylation of the EGFR^{Y845} may be a critical step in nuclear translocation of the EGFR [18].

The use of radiation and the EGFR molecular targeting agent cetuximab has represented one of the most recent advances in the treatment of locally advanced HNSCC. However, biological investigations have suggested that both radiation and cetuximab can lead to nuclear EGFR accumulation and this accumulation may play a role in resistance to cetuximab and radiation. Our data suggests that cetuximab and radiation treatment of HNSCC lines results in the phosphorylation of the EGFR^{Y845}, which may be necessary for nuclear translocation of the EGFR. Likewise, dasatinib clearly blocked translocation of EGFR to the nucleus in HNSCC cell lines. Collectively these findings suggest that dasatinib can limit EGFR translocation to the nucleus and may enhance radiotherapy plus cetuximab.

MATERIAL AND METHODS

Cell culture and compounds

HT29, SK-CO-1, SW480, H226, A549 and Calu-3 cells were obtained from American Type Culture Collection (Manassas, VA). UM-SCC1 and UM-SCC6 cells were provided by Dr. Thomas E. Carey (University of Michigan), and SCC1483 cells were provided by Dr.

Jennifer Grandis (University of Pittsburgh, Pittsburgh, PA). The cells were maintained in McCoy's 5a (HT29), Minimum Essential Medium Eagle (SK-CO-1, Calu-3), RPMI-1640 (H226), F-12K Nutrient Mixture (A549), Leibovitz's L-15 (SW480) or Dulbecco's Modification of Eagle's Medium (three SCC lines). The cells were maintained with 10% FBS (Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin; 1 µg/mL hydrocortisone for SCC lines. Cells were incubated at 37 °C in 5% CO₂ except for SW480 was cultured in 100% air. All media was purchased from Mediatech (Manassas, VA). Cetuximab was purchased from Bristol-Myers Squibb (Princeton, NJ). Dasatinib was kindly provided by Bristol-Myers Squibb. All other chemicals were purchased from Sigma (St. Louis, MO).

Immunoblotting analysis

Whole cell protein lysates were isolated with lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Tween-20, 10% glycerol, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 10 µg/ml of leupeptin and aprotinin). Nuclear fractions were performed as described previously [11]. Protein concentrations were determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA). Western blotting was performed as described previously [27]. All antibodies were purchased from the following commercial sources: anti-EGFR, HRP-conjugated goat-anti-rabbit IgG and goat-anti-mouse IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Phospho-EGFR (Tyr845), SRC family Kinase (SFK), phospho-SFK (Y416) and Histone H3 (Cell Signaling Technology, Beverly, MA), α-Tubulin (Calbiochem, San Diego, CA) and phospho-tyrosine (4G10) (Millipore, Billerica, MA).

Immunoprecipitation (Co-IP)

Cell lysates containing 0.2 mg of protein were incubated at 4 °C overnight with 2 µg of anti-EGFR antibody followed by 30 µl of protein A/G agarose beads (Santa Cruz Biotechnology) for 2h. The immunoprecipitates were pelleted and washed three times with lysis buffer. The captured immunocomplexes were then boiled in 2× SDS sample buffer for 5 min and subjected to immunoblot analysis.

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The abbreviations used are

cEGFR	cytoplasmic epidermal growth factor receptor
CRC	colorectal cancer
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FBS	fetal bovine serum
FDA	food and drug administration
HNSCC	head and neck squamous cell carcinoma
mAb	monoclonal antibody
nEGFR	nuclear epidermal growth factor receptor
NSCLC	non-small cell lung cancer

PCNA	proliferating cell nuclear antigen
p-Tyr	phospho-tyrosine
RTK	receptor tyrosine kinase
SCC	squamous cell carcinoma
SFK	Src-family kinases
TKI	tyrosine kinase inhibitor

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Figure 1. Cetuximab induces EGFR translocation to the nucleus

A) Lung (H226, A549, Calu-3), Colon (HT29, SW480, SK-CO-1), and HNSCC (SCC1, SCC6, SCC1483) tumor cells were treated with or without 400 nM of cetuximab for 24 hours. Nuclear fractions were obtained, fractionated by SDS-PAGE and immunoblotted for the indicated proteins. **B)** SCC1, SCC6, SCC1483 cells were treated with increasing concentrations of cetuximab for 24 hours. Nuclear fractions were obtained, fractionated by SDS-PAGE and immunoblotted for the indicated proteins. **C)** SCC1, SCC6, SCC1483 cells were treated with cetuximab (400 nM) for the indicated times. Nuclear fractions were obtained, fractionated by SDS-PAGE and immunoblotted for the indicated proteins. **D)** Time course of cetuximab-induced EGFR nuclear translocation. SCC1, SCC6, SCC1483 cells were treated with cetuximab (400 nM) for the indicated times. Nuclear fractions were obtained, fractionated by SDS-PAGE and immunoblotted for EGFR. Bands were quantitated using ImageJ and presented graphically as fold increase relative to untreated control. Histone H3 and α-Tubulin were used as loading and purity controls for the nuclear and cytoplasmic fractions, respectively.

Figure 2. Radiation induces EGFR translocation to the nucleus

A) SCC1, SCC6, SCC1483 cells were irradiated with 4Gy. At the indicated times, nuclear fractions were obtained, fractionated by SDS-PAGE and immunoblotted for the indicated proteins. **B)** Comparison of time courses of EGFR nuclear translocation respectively induced by EGF, cetuximab and radiation. SCC1, CC6, SCC1483 cells were treated with or without EGF (200ng/ml), cetuximab (400nM) and radiation (4Gy) for indicated times. Nuclear fractions were obtained, fractionated by SDS-PAGE and immunoblotted for EGFR. Bands were quantitated using ImageJ and presented graphically as fold increase relative to untreated control. Histone H3 and α -Tubulin were used as loading and purity controls for the nuclear and cytoplasmic fractions, respectively.

Figure 3. Cetuximab treatment increases phosphorylation of the EGFR in HNSCC tumor cells
A) SCC1, SCC6, SCC1483 cells were treated with or without 100ng/ml EGF for 30 minutes. The EGFR was immunoprecipitated from whole cell lysate and blotted for the indicated proteins. **B)** SCC1, SCC6, SCC1483 cells were treated with or without cetuximab (400nM) for 24 hours. The EGFR was immunoprecipitated from whole cell lysate and blotted for the indicated proteins. **C and D)** SCC1, SCC6, SCC1483 cells were treated with or without cetuximab (400nM) for 24 hours. Cytoplasmic and nuclear fractions were obtained, fractionated by SDS-PAGE and immunoblotted for the indicated proteins. Histone H3 and α -Tubulin were used as loading and purity controls for the nuclear and cytoplasmic fractions, respectively.

Figure 4. Src family kinases mediate cetuximab-induced EGFR translocation to the nucleus
 SCC1, SCC6, SCC1483 cells were pretreated with dasatinib (25nM) for 24 hours followed by cetuximab (400nM) treatment for 24 hours. Cytoplasmic and nuclear fractions were obtained, fractionated by SDS-PAGE and iblotted for the indicated proteins. Histone H3 and α -Tubulin were used as loading and purity controls for the nuclear and cytoplasmic fractions, respectively.

Figure 5. Src family kinase mediates radiation-induced EGFR translocation to the nucleus
 SCC1, SCC6, SCC1483 cells were pretreated with or without dasatinib (25 nM) for 24 hours before irradiation with 4Gy. 30 minutes after radiation, cytoplasmic and nuclear fractions were obtained, fractionated by SDS-PAGE and immunoblotted for the indicated proteins. Histone H3 and α -Tubulin were used as loading and purity controls for the nuclear and cytoplasmic fractions, respectively.

Figure 6. Dasatinib blocks cetuximab and radiation-induced EGFR nuclear translocation

A) SCC1, SCC6, SCC1483 cells were pretreated with or without cetuximab (400 nM) for 1 hour prior to irradiation with 4Gy and harvested after 24 hours. Protein was extracted and immunoblotted for the indicated proteins. **B)** SCC1, SCC6, SCC1483 cells were pretreated with or without cetuximab (400 nM) and dasatinib (25nM) for 1 hours prior to irradiation with 4Gy and harvested after 24 hours. Protein was extracted and immunoblotted for the indicated proteins. Cytoplasmic and nuclear fractions were obtained, fractionated by SDS-PAGE and immunoblotted for the indicated proteins. Histone H3 and α -Tubulin were used as loading and purity controls for the nuclear and cytoplasmic fractions, respectively.