Crizotinib Fails to Enhance the Effect of Radiation in Head and Neck Squamous Cell Carcinoma Xenografts

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Abstract. *Aim: Mesenchymal-epithelial transition factor (MET), a receptor tyrosine kinase, is expressed in head and neck squamous cell carcinomas (HNSCC) and is involved in tumor progression and associated with poor prognosis. MET can be inhibited by crizotinib, a potent ATP-competitive kinase inhibitor. We examined the effects of combining crizotinib and radiation in a pre-clinical HNSCC model. Materials and Methods: Nine HNSCC cell lines were screened for MET expression, copy-number amplification and mutational status. The in vitro effects of crizotinib and radiation were assessed with clonogenic survival assays. MET signaling proteins were assessed with western blot and receptor tyrosine kinase array. Tumor growth-delay experiments with UT-SCC-14 and UT-SCC-15 oral tongue xenografts were used to assess in vivo tumor radiosensitivity. Results: All nine HNSCC cell lines showed a varying degree of MET protein and RNA expression. Increased MET copy number was not present. MET was expressed after irradiation both in vitro and in vivo. Crizotinib alone inhibited phosphorylation of MET and inhibited cell growth in vitro but did not inhibit phosphorylation of downstream signaling proteins: MAPK, AKT or c-SRC. When combined with radiation in vitro, crizotinib demonstrated radiation enhancement in only one cell line. Crizotinib did not enhance the effect of radiation in either UT-SCC-14 or UT-SCC-15 tumors grown as xenografts. Conclusion: MET is overexpressed in HNSCC cell lines, however, crizotinib failed to enhance the radiation response and failed to inhibit MET downstream signaling proteins in this HNSCC model.*

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The rapid development of molecular targeted therapeutics has opened-up new opportunities to explore the interaction of radiation with inhibition of specific cancer-associated pathways. However, identifying the most appropriate pathway to target still remains a challenge. In head and neck squamous cell carcinoma (HNSCC) early success was achieved by targeting the epidermal growth factor receptor (EGFR) pathway (4). Apart from the inhibitors of EGFR and vascular endothelial growth factor (VEGF), very few molecular targeted agents have advanced to phase III clinical trials in combination with radiation (21).

Mesenchymal–epithelial transition factor (MET or c-MET), also known as hepatocyte growth factor receptor, may be a promising target for combination with radiation. MET is a transmembrane receptor tyrosine kinase (RTK) normally activated by its only known ligand, hepatocyte growth factor (5). Phosphorylation of the intracellular domain of MET can activate the PI3K/AKT, RAS/MAPK and STAT cell signaling pathways and when abnormally activated or overexpressed, MET can cause tumor progression, invasion and metastasis (3, 12). We have shown that MET is overexpressed in HNSCC and high expression is associated with poor prognosis and tumor recurrence (1, 2). In other studies, MET inhibition was shown to cause decreased cell proliferation and increased apoptosis in HNSCC cell lines (15, 24).

There exist multiple drugs that can inhibit MET and in particular, crizotinib, a potent ATP-competitive inhibitor of the MET and anaplastic lymphoma kinase (ALK), has been shown to inhibit growth in various HNSCC cell lines (15, 24, 31). The objective of this study was to investigate MET as a therapeutic target and to determine if crizotinib enhances the effect of radiation in a pre-clinical HNSCC model.

Materials and Methods

Cell lines and drug. Nine HNSCC cell lines, provided by Dr. Reidar Grénman (Turku University Hospital, Turku, Finland), were used. Six of the cell lines were derived from patients with oral tongue cancer:

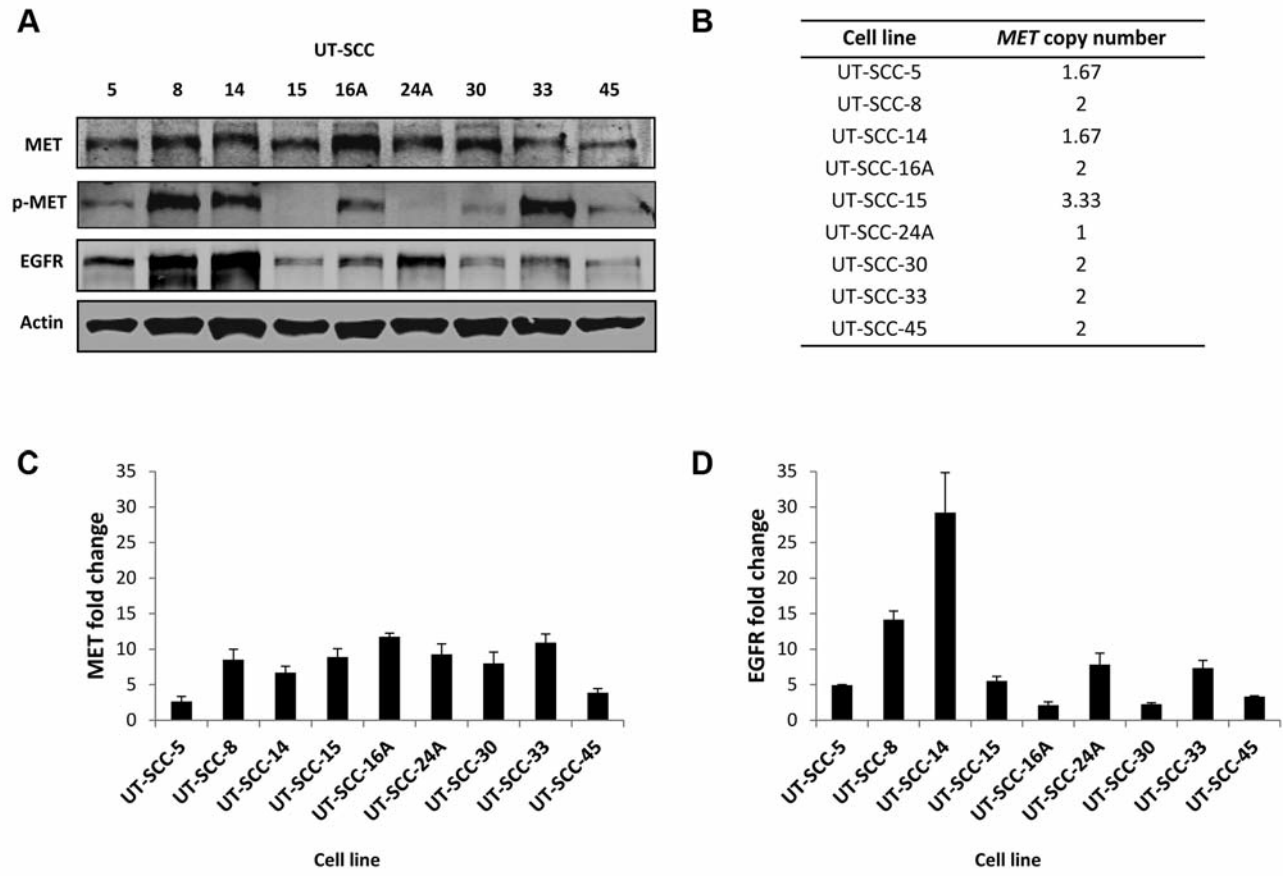

Figure 1. *Mesenchymal-epithelial transition factor (MET) is expressed but not amplified in HNSCC cell lines. Immunoblots (A) show protein expression of MET, phospho-MET (p-MET) and epidermal growth factor receptor (EGFR) in nine cell lines under normal growth conditions. MET is not amplified (B) in the cell lines. qRT-PCR results (C, D) for EGFR and MET RNA show increase expression when normalized to a normal human RNA tongue control.*

UT-SCC-5, UT-SCC-14, UT-SCC-15, UT-SCC-16A, UT-SCC-24 and UT-SCC-30. UT-SCC-8 was derived from a laryngeal cancer, UT-SCC-33 from a mandibular gingiva tumor, and UT-SCC-45 from a tumor of the floor of the mouth. All cell lines are negative for human papillomavirus (HPV) except UT-SCC-45 which is positive for HPV-33 (23). Cell lines were authenticated and kept at low passage number. The radiosensitivity of these cell lines have been reported elsewhere (13, 14, 32).

Cells were cultured and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). Crizotinib, provided by Pfizer, was reconstituted in dimethyl sulfoxide and stored at −70˚C for *in vitro* experiments. For *in vivo* experiments crizotinib was prepared daily in Cremophor EL/ethanol (50:50; Sigma Cremophor EL: 95% ethyl alcohol) and diluted with sterile water. For select *in vitro* experiments cells were stimulated with hepatocyte growth factor (HGF) (R&D Systems, Minneapolis, USA).

Irradiation. Cells were irradiated with a Xstrahl X-ray System, Model RS225 (Xstrahl, UK) at a dose rate of 0.29 Gy/min, tube voltage of 160 kVp, current of 4 mA and filtration with 0.5 mM Al and 0.5 mM Cu. Cells were irradiated (0.5-4 Gy) in 25 cm^2 flasks at 37˚C. Animals were irradiated with a Faxitron Cabinet X-ray System, Model 43855F (Faxitron X-Ray, Wheeling, IL, USA) at a dose rate of 0.69 Gy/min, tube voltage of 160 kVp and current of 4 mA.

Western immunoblot assay. Cells were treated, washed with PBS and protein extracted with lysis buffer. Equal amounts of protein (20 μg) were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane by electroblotting. After blocking, the membrane was incubated with antibodies against MET (1:100; Cell Signaling, Inc.), phospho-MET (Tyr1234/1235) (1:1,000; Cell Signaling, Inc.), EGFR (1:1000; Cell Signaling, Inc.), phospho-AKT (Ser 473) (1:2000; Cell Signaling, Inc.), phospho-p44/42-MAPK (ERK1/2) (Thr 202/Tyr 204) (1:1000; Cell Signaling, Inc.), ALK (1:2,000; Cell Signaling, Inc.) or actin (1:20,000; MP Biomedicals). The membrane was washed and the secondary antibody (IRDye 800CW, 1:20,000; Licor, Lincoln, NB, USA) was applied for 1 hour. The membranes were analyzed with an Odyssey infra-red imaging system (Li-Cor).

Quantitative real time-polymerase chain reaction (qRT-PCR). RNA was extracted from cells using Allprep RNA Mini Kit (Qiagen,

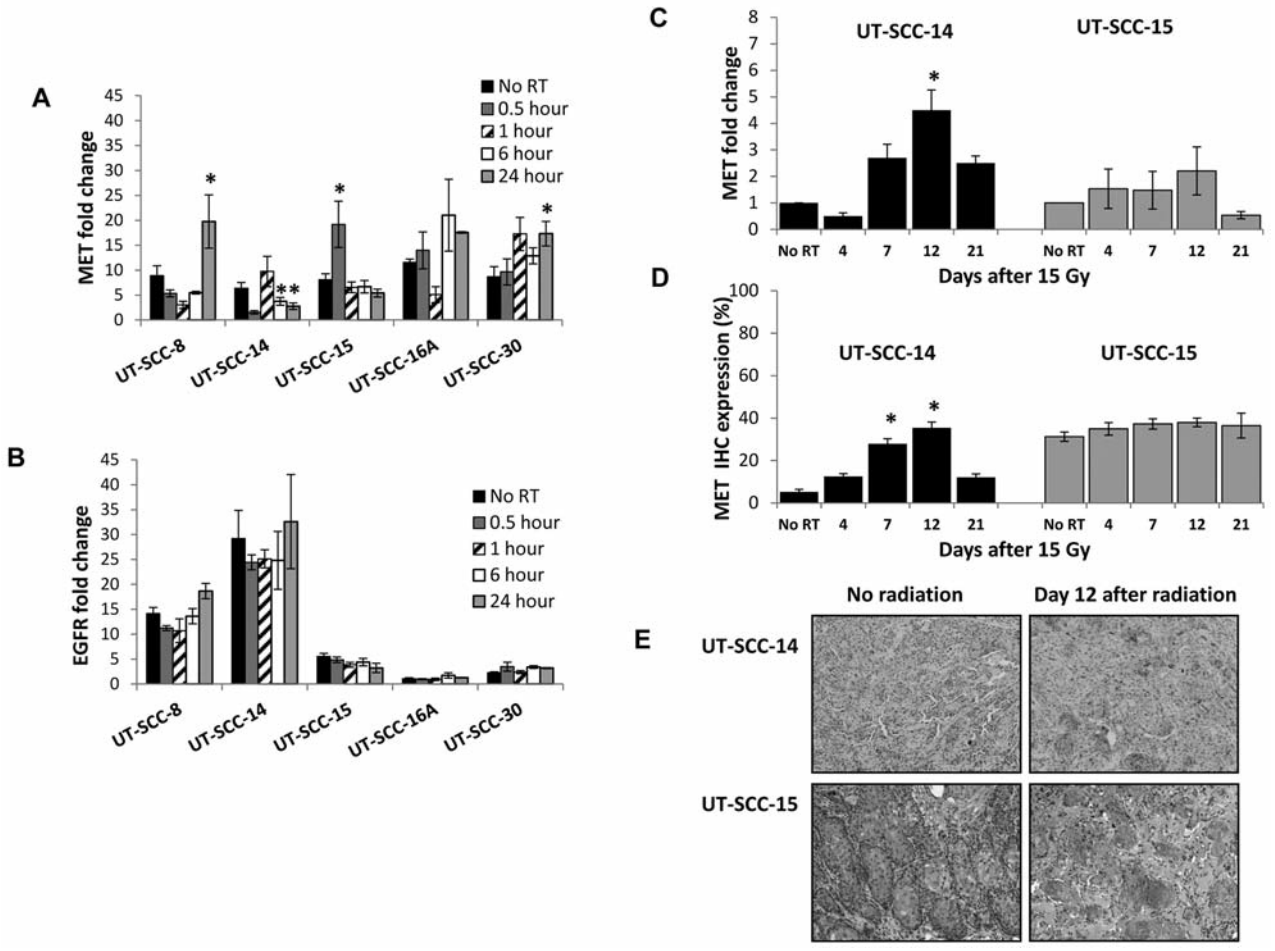

Figure 2. *Radiation alters expression of mesenchymal–epithelial transition factor (MET). Radiation (4 Gy) increased MET RNA expression in five HNSCC cell lines (A). Radiation had little effect on epidermal growth factor receptor (EGFR) expression (B). MET RNA (C) and protein (D) expression were also examined in tumors grown as flank xenografts. Tumors were treated with a single dose of 15 Gy and collected at 4, 7, 12 and 21 days after irradiation and compared to non-irradiated controls. *p<0.05, compared to non-irradiated cells. (E) Shows representative histological sections of MET protein expression from xenografts in mice with and without radiation.*

Valencia, CA, USA) and from xenografts using RNeasy Micro Kit (Qiagen). cDNA was generated and pre-designed TaqMan gene specific primers (Life Technologies, Carlsbad, CA, USA) were used: MET (Hs01565584_m1), EGFR (Hs01076078_m1) and GAPDH (Hs99999905_m1). MET gene expression was quantified using a Mastercycler Realplex System (Eppendorf, Hauppauge, NY, USA); *EGFR* gene expression using the ViiA 7 RT-PCR system (Life Technologies). Samples were normalized to normal human tongue RNA (Cat #540149; Agilent).

DNA-copy number by qRT-PCR. MET copy number variation was determined by the TaqMan® Copy Number Assay (Life Technologies) (Hs04933308_cn). RT-PCR was performed using the 7500 Fast Real-Time PCR System (Life Technologies). Samples were normalized to a positive control known to have two copies of the *MET* sequence.

DNA sequencing. Genomic DNA was extracted by Allprep DNA Mini Kit (Qiagen) and quantified using a Qubit fluorimeter (Life Technologies). For targeted PCR amplification, 10 ng DNA was used, with the Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies) which amplifies 50 oncogenes and tumor-suppressor genes including 2,800 mutations listed in the Catalogue of Somatic Mutations in Cancer (COSMIC) database. (http://cancer.sanger.ac.uk) Barcoded libraries were created and sequencing was completed on an Ion Torrent Personal Genome Machine (Life Technologies) using an Ion 318 Chip with Ion PGM 200 Sequencing Kit. Reads were aligned to the human Hg19 genome using NextGENe sequencing software (SoftGenetics, State College, PA, USA).

Histology and immunohistochemistry. Formalin-fixed paraffin-embedded UT-SCC-14 and UT-SCC-15 tissue were cut (5 μm) and stained with MET antibody (Vector Labs, Burlingame, CA, USA). Images were obtained at ×10 magnification and analyzed in a blinded manner with ImageJ software (http://rsb.info.nih.gov/ij) by converting the image to RGB scale and using the threshold tool to calculate the percentage area positively stained.

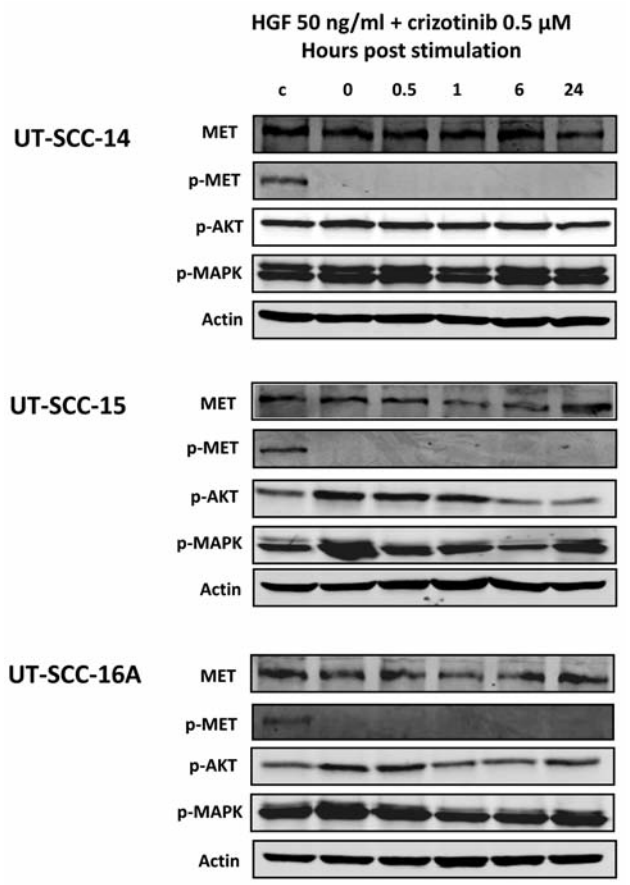

Figure 3. *Effects of crizotinib on mesenchymal-epithelial transition factor (MET) and it's downstream signaling pathway. Immunoblot assay of HGF hepatocyte growth factor (HGF) stimulated and treated UT-SCC-14, UT-SCC-15 and UT-SCC-16A cells shows inhibition of phosphorylated-MET (p-MET) with crizotinib but not inhibition of phosphorylation of downstream proteins AKT and mitogen-activated protein kinase (MAPK).*

Phospho-receptor tyrosine kinase array. The PathScan® RTK Signaling Antibody Array Kit (Cell Signaling) was used to assess activation of 39 different tyrosine kinases. Cell lysates containing 75 μg of protein from the treated cells were incubated on the slide followed by application of the supplied biotinylated detection antibody cocktail. Streptavidin-conjugated DyLight 680™ was then used to visualize the bound detection antibody. Fluorescent intensities were then determined with an Odyssey infra-red imaging system (Li-Cor).

3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For the MTT assay, cells were plated into 96-well plates and allowed to attach overnight. The next day, media were exchanged for media containing different concentrations of crizotinib and the plates returned to the incubator. After an additional 3 days, MTT (5 mg/ml in PBS) was added to each well and the plate returned to the CO_2 incubator for ~5 h. The media containing MTT were then aspirated from the wells, and DMSO was added to dissolve the purple formazan. After 5 min incubation at 37˚C, absorbance readings (at 560 nm and 670 nm) were taken on a Versamax multiplate reader (Molecular Devices, Sunnyvale, CA, USA).

Clonogenic survival assay. Cells were treated with 0.5 μM of crizotinib for 1 hour, irradiated (0-4 Gy) and then plated into flasks containing growth media and 0.5 μM of crizotinib. Colonies were allowed to develop for 10-14 days. Colonies were then stained with crystal violet and colonies were counted, and surviving fractions were calculated. Data was normalized for plating efficiency and survival curves were fitted using the linear-quadratic equation.

Xenograft growth delay assay. After approval by the Animal Care Committee (AL-12-06), xenografts were established in 4-to-6-week old female nude NIH III mice (Charles Rivers Laboratories, Wilmington, MA, USA) by injecting UT-SCC-14 or UT-SCC-15 cells subcutaneously into the flank, at a density of 2×10^6 cells per 100 μl of Matrigel (BD, Franklin Lakes, NJ, USA). Tumor volume was measured three times weekly by digital calipers and calculated using the formula $(\pi ab^2)/6$ (a=largest diameter, b=smallest diameter). When the tumor reached a volume of 200-300 mm^3, animals were randomly assigned to the experimental groups. The end-point of the experiment was when tumors grew to a volume of 3,000 mm^3. Crizotinib was given by oral gavage one hour before radiation treatment. Sham oral gavage consisted of sterile water with Cremophor EL (Sigma-Aldrich, St. Louis, MO, USA)/ethanol). Six or 10 mice were used in each experimental group.

The first UT-14-SCC experiment consisted of four treatment groups: (1) control with sham oral gavage (sterile water with Cremophor EL), (2) crizotinib (12.5 mg/kg) by oral gavage daily (five times per week) for 3 weeks, (3) sham oral gavage followed one hour later by radiation delivered as 2.0 Gy/day (five times per week) for 3 weeks, and (4) crizotinib (12.5 mg/kg) by oral gavage followed one hour later by radiation 2.0 Gy/day (five times per week) for 3 weeks. Ten mice were included in each group.

The second UT-14-SCC experiment consisted of either (1) controls with sham oral gavage, (2) crizotinib alone (25 mg/kg or 50 mg/kg) by oral gavage daily (five times per week) for 2 weeks; (3) radiation alone given in a single 10 Gy fraction or (4) crizotinib (25 mg/kg or 50 mg/kg) by oral gavage daily (five times per week) for 2 weeks followed by radiation given as a single fraction of 10 Gy 1 h after the first dose of crizotinib. Six mice were included in each group.

The experiment involving the UT-15-SCC xenografts consisted of either (1) controls with sham oral gavage, (2) crizotinib alone (25 mg/kg) by oral gavage daily (five times per week) for 2 weeks; (3) radiation alone given in a single 10 Gy fraction or (4) crizotinib (25 mg/kg) by oral gavage daily (five times per week) for 2 weeks) followed by radiation given as a single fraction of 10 Gy 1 h after the first dose of crizotinib. Six mice were included in each group.

Statistical analysis. *In vitro* experiments were repeated three times and statistical analysis was carried out using a Student's *t*-test. Data are presented as the mean±SE. A probability level of a p value of <0.05 was considered significant.

Results

MET is expressed in HNSCC cell lines. Immunoblot analysis demonstrated that the MET protein is expressed in all nine HNSCC cell lines grown under cell culture conditions without HGF stimulation (Figure 1A). *MET* copy number analysis showed that most cells had two copies, indicating amplification of *MET* was not present (Figure 1B). *MET* mRNA expression

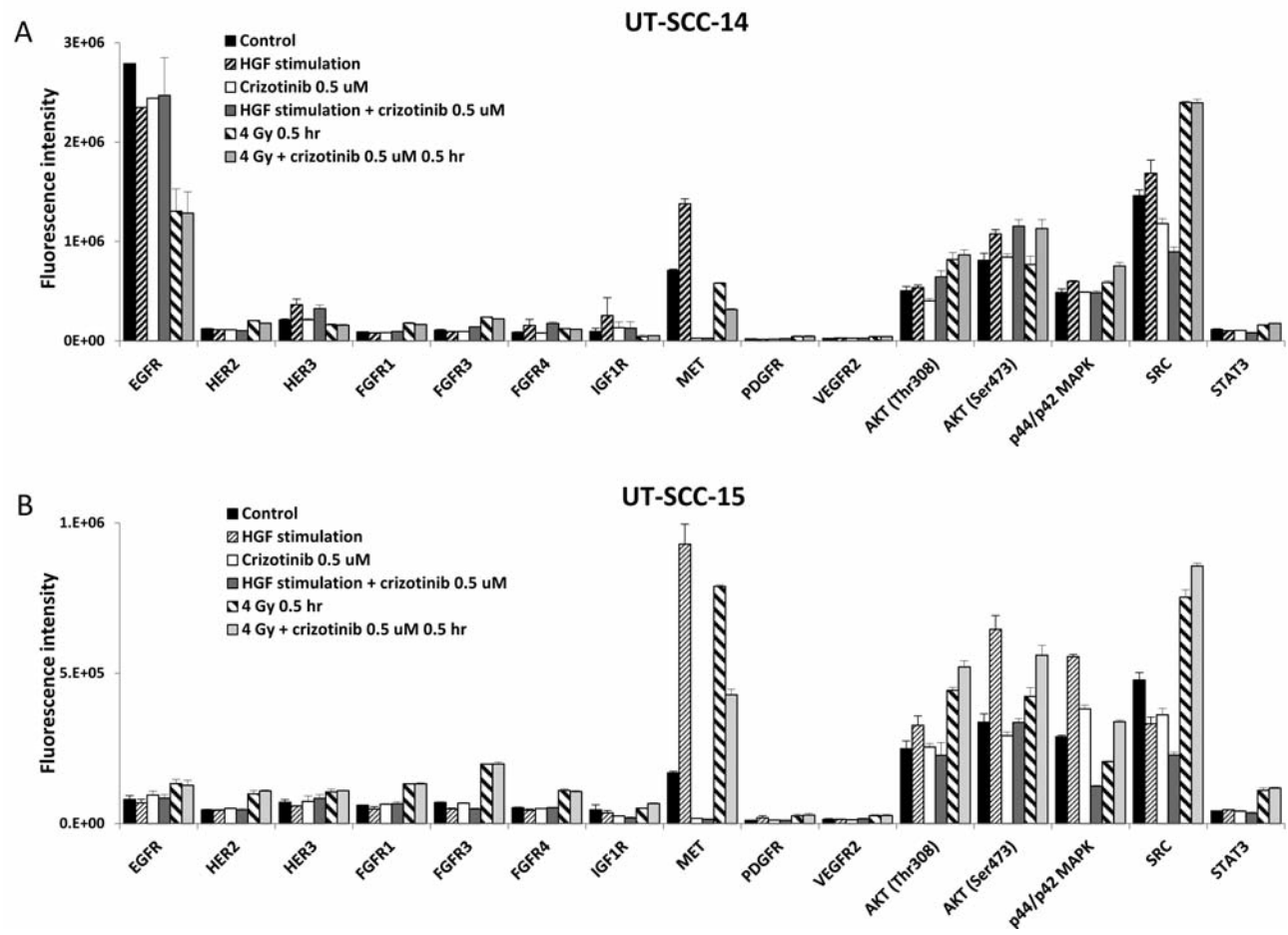

Figure 4. *The effects of crizotinib on different tyrosine kinases. Activation of tyrosine kinases in UT-SCC-14 (A) and UT-SCC-15 (B) cells, when untreated (control), stimulated with hepatocyte growth factor (HGF), treated with 0.5 μM crizotinib, with irradiation alone (RT; 4 Gy for 0.5 h) or irradiation with 0.5 μM crizotinib.*

varied from 3-fold to 12-fold higher than that of a normal tongue control (Figure 1C). *EGFR* mRNA expression varied between the cell lines, with UT-SCC-14 showing a very high expression (Figure 1D). Since crizotinib inhibits both MET and ALK, cell lines were screened for ALK by immunoblot and were found not to express ALK when compared to ALK-expressing control SK-N-BE(2) neuroblastoma cells (data not shown).

Mutation analysis. MET mutation analysis was performed with next-generation sequencing using a cancer panel that includes 18 known *MET* mutations found in the COSMIC database. Out of the nine UT cell lines sequenced, only the UT-SCC-15 line was found to harbor a single *MET* mutation. This mutation (c.1142delA) causes a deletion of adenine which leads to a frame shift. The UT-SCC-14 cell line did not have any *MET* mutations but had a missense mutation in *PI3KCA* (c.1173A>G, p.I391M) and a deletion in phosphatase and tensin homolog (*PTEN*) (c.683delA), which are both downstream of the MET pathway. UT-SCC-15 cells also had a deletion in *PI3KCA* and a fame shift mutation in *PTEN*. There were no *SRC* mutations identified.

MET expression after radiation. To determine if MET is expressed after radiation, five HNSCC cell lines were irradiated with a single dose of 4 Gy and collected at either 0.5, 1, 6 or 24 h after irradiation and then analyzed for MET expression. MET was statistically ($p<0.05$) overexpressed at different time points compared to the control in four out of the five cell lines (Figure 2A). Figure 2B shows EGFR expression did not vary with irradiation.

MET expression was also analyzed in UT-SCC-14 and UT-SCC-15 tumors grown *in vivo* as flank xenografts. Tumors were irradiated with a single dose of 15 Gy and collected at 4, 7, 12 and 21 days after irradiation. MET gene and protein

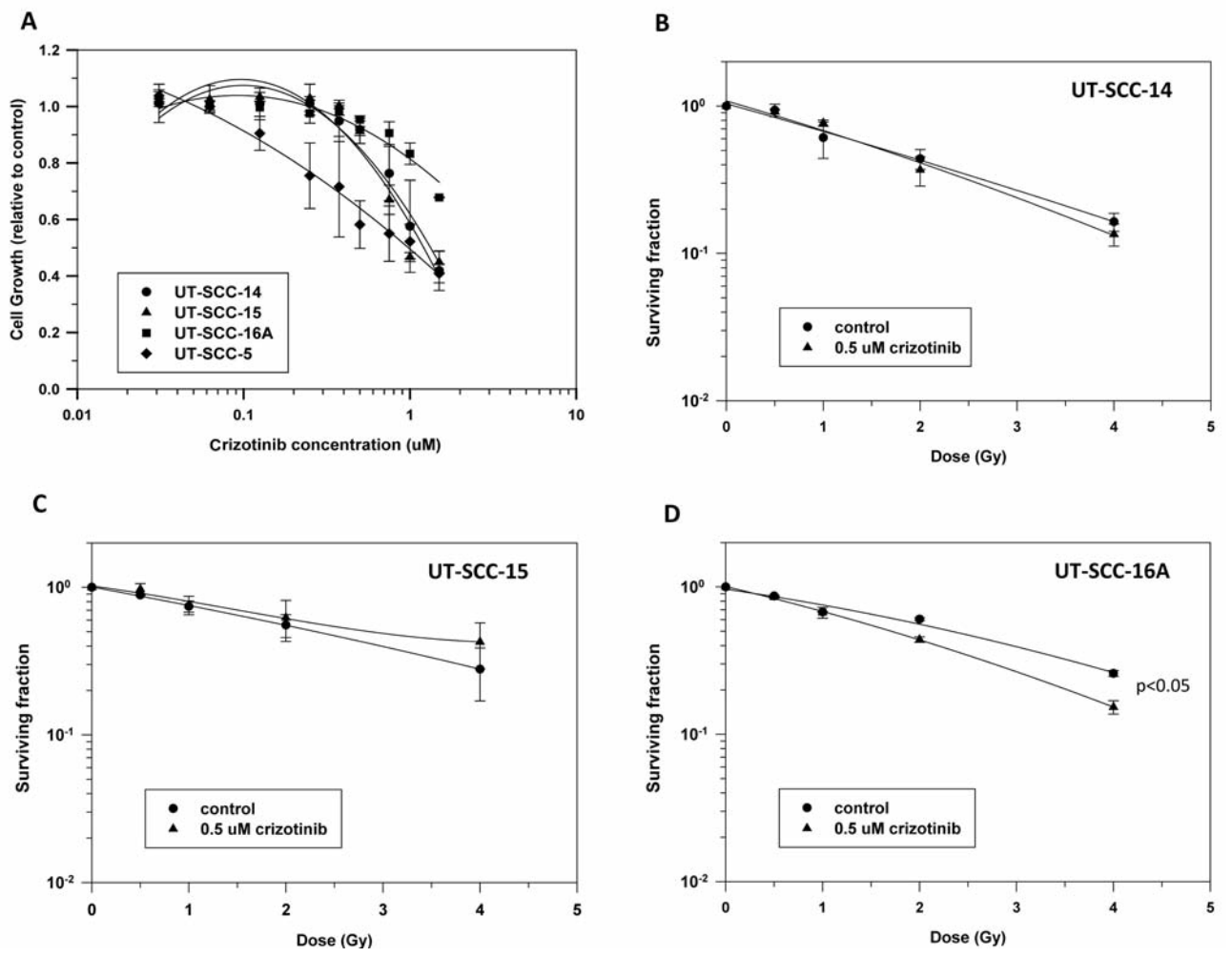

Figure 5. *Effects of crizotinib on in vitro cell growth and in combination with radiation. Cell viability assay results (A) showing dose-dependent cytotoxicity of crizotinib against four HNSCC cell lines. The radiosensitivity effects of crizotinib on (B) UT-SCC-14, (C) UT-SCC-15 and (D) UT-SCC-16A cells. Cell cultures were exposed to 0.5 μM crizotinib for 1 h before irradiation and maintained in the medium after irradiation. Colony-forming efficiency was determined 10 to 14 days later and survival curves were generated after normalizing for the cytotoxicity induced by crizotinib alone. Data are the mean±SE.*

expression were both found to be significantly up-regulated at day 12 (4.5-fold change, $p<0.05$) in the UT-SCC-14 cells after irradiation compared to non-irradiated tumors (Figure 2C and D). In the UT-SCC-15 cells *MET* gene expression was also expressed at day 12, however this was not statistically significant (Figure 2C). Figure 2E shows representative histological sections of MET protein expression from xenografts in mice with and without radiation.

Crizotinib inhibits phosphorylation of MET in vitro. To determine if the MET pathway can be inhibited by crizotinib, cells were stimulated with 50 ng/ml HGF for 15 min and then treated with 0.5 μM of crizotinib. Protein was then collected 0-24 h later. Figure 3 shows that the addition of crizotinib, resulted in inhibition of p-MET in all three cell lines up to 24 h, however, crizotinib did not inhibit phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) or p-AKT (Ser473).

The effects of crizotinib on different tyrosine kinases. UT-SCC-14 and UT-SCC-15 cells were stimulated with or without HGF and treated with crizotinib (0.5 μM), irradiation (4 Gy) or crizotinib (0.5 μM) plus irradiation (4 Gy). Cells grown under normal conditions without HGF, radiation or crizotinib were used as controls. Cell lysates were then collected at 0.5 and 1 h after treatment and phospho-kinase array experiments were performed. HGF activated the MET

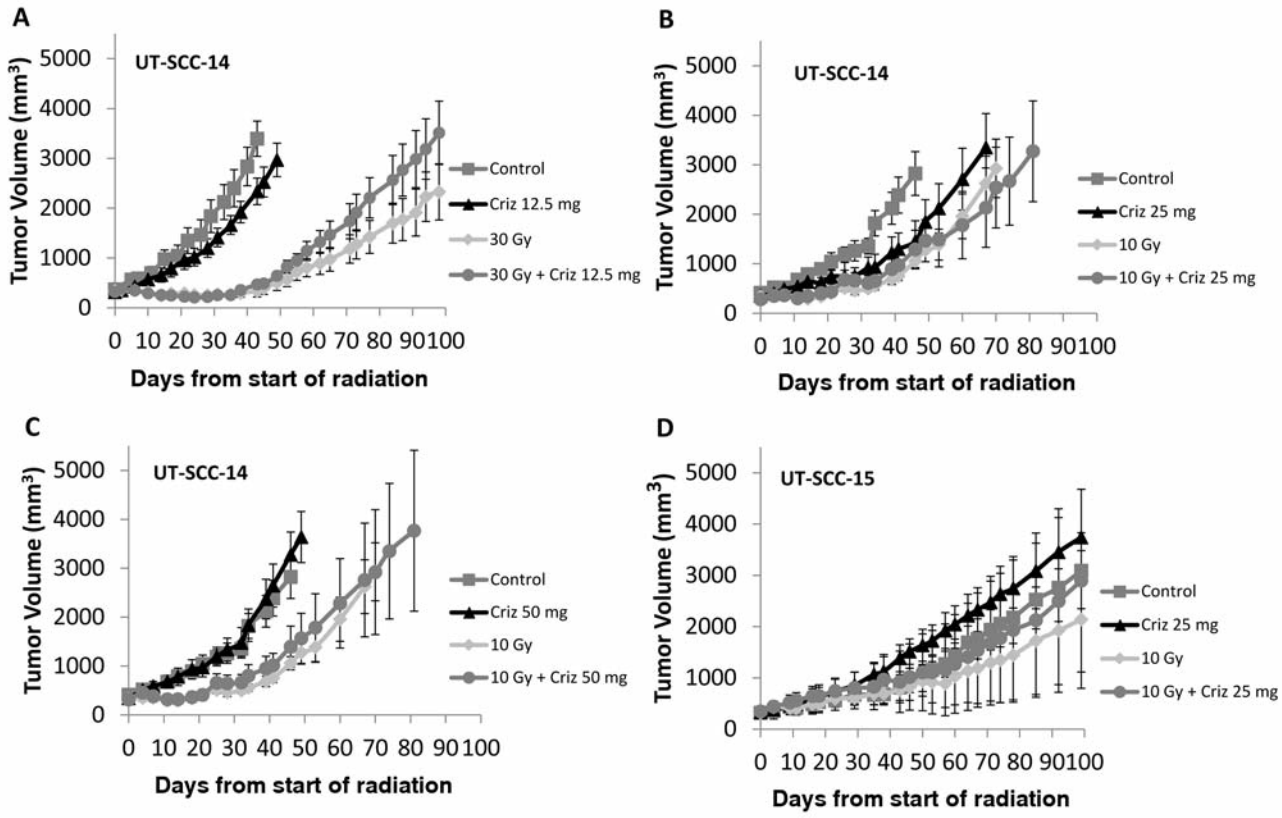

Figure 6. *In vivo effects of crizotinib (Criz) combined with irradiation. The effects of crizotinib and irradiation on UT-SCC-14 and UT-SCC-15 xenograft tumor volume. Animals were treated with either 12.5 mg/kg of crizotinib over three weeks or 25 mg/kg or 50 mg/kg of crizotinib over two weeks with crizotinib given daily by p.o. gavage one hour before irradiation. Radiation was given as either 30 Gy in 15 daily fractions (A) or 10 Gy in a single fraction on day one of crizotinib (B-D). Data are the mean tumor volumes±SE.*

receptor while crizotinib inhibited activation of MET. Radiation alone and radiation plus crizotinib both analyzed at 0.5 h showed activation of MET (Figure 4) and at 1 h this activation returned to baseline (data not shown). UT-SCC-14 cells had high activation of EGFR at baseline and after treatment. UT-SCC-15 cells showed little EGFR activation in both untreated and treated cells. In both cell lines, AKT (Ser473), AKT (Thr308), MAPK (Thr202/Tyr204) and c-SRC showed an increased activation when cells were treated with irradiation alone or crizotinib plus irradiation.

The effects of crizotinib on in vitro cell growth and radiosensitivity. UT-SCC-5, UT-SCC-14, UT-SCC-15 and UT-SCC-16A cell lines were exposed to increasing concentrations of crizotinib for 72 h, after which MTT cell viability assay was performed. Crizotinib inhibited cell growth in a dose-dependent manner in all four cell lines as shown in Figure 5A. Clonogenic survival analysis was performed on three cell lines: UT-SCC-14, UT-SCC-15 and UT-SCC-16A. Cells were exposed to 0.5 μM crizotinib for 1 h before irradiation with drug maintained in the medium after irradiation. As shown in Figure 5B-D, crizotinib enhanced the effect of radiation in UT-SCC-16A cells ($p<0.05$) but not in UT-SCC-14 or UT-SCC-15 cells.

In vivo effects of crizotinb. Out of the nine HNSCC cell lines studied, only UT-SCC-14 and UT-SCC-15 grow well as xenografts. Mice bearing UT-SCC-14 xenografts were randomized into four groups: vehicle, crizotinib (12.5 mg/kg) daily for 15 days, irradiation (30 Gy in 15 fractions), and crizotinib (12.5 mg/kg) daily for 15 days given 1 h before daily irradiation (30 Gy in 15 fractions). The relative tumor growth delay curves are shown in Figure 6. Crizotinib at 12.5 mg/kg per day for 15 days had a small effect on inhibiting tumor growth compared to controls. The time for the tumors to regrow to five times their initial size (1,500 mm^3) was 23.7±7.8 days in the control group compared to 30.4±8.3 days in the group treated with crizotinib alone ($p=0.07$). The addition of crizotinib at 12.5 mg/kg to radiation resulted in an increase in tumor growth compared to radiation alone (Figure 6A). The time for the tumors to regrow to five times their initial size

(1,500 mm^3) was 66.0±10.2 days in the crizotinib and radiation-treated group compared to 92.5±18.9 days in the group treated with radiation alone (p=0.07).

Since MET was expressed after a single dose of 15 Gy (Figure 2) at 7 and 12 days, xenograft experiments using a single 10-Gy dose (to allow for tumor regrowth) with crizotinib given for two weeks were performed. UT-SCC-14 xenografts in mice treated with 10 Gy and 25 mg/kg or 50 mg/kg of crizotinib for 10 days did not show any radiation enhancement effect (Figure 6B-D). Crizotinb at 25 mg/kg led to growth delay compared to controls but a larger dose of 50 mg/kg had no effect. Crizotinib at 25 mg/kg in the UT-SCC-15 xenograft experiments did not lead to any tumor growth delay with or without radiation treatment.

Discussion

We have previously shown that MET is overexpressed in HNSCC primary tumors and high expression is an independent marker of poor prognosis in those treated with concurrent chemoradiation (2). MET is also involved in multiple pathways associated with radiation response, and targeting it in combination with radiation would seem to be an attractive therapeutic strategy. In the present study, we found that crizotinib, a potent inhibitor of MET, in combination with radiation did not enhance the effect of radiation but instead suggested enhanced tumor growth in our *in vivo* models of oral tongue cancer. All cell lines studied demonstrated relatively high MET expression as measured by western blot and RT-PCR but did not exhibit *MET* amplification or ALK expression. Our unexpected results could be due to many reasons, including true lack of *MET* amplification, activation of redundant signaling pathways which compensate for MET inhibition, or from alterations in downstream signaling proteins which are involved in radiosensitization.

From our data, crizotinib inhibited phosphorylation of MET, but failed to inhibit downstream pathway proteins AKT, MAPK and c-SRC. Its failure to inhibit these downstream pathways could be due to activation by alternate RTKs, including EGFR. Our cell lines all express MET but also highly express EGFR. EGFR is co-expressed with MET and both receptor signaling pathways converge on PI3K/AKT and RAS/MAPK (31). Dual inhibition of MET and EGFR has been shown to result in maximum inhibition of p-AKT and p-MAPK, and HNSCC tumor growth, suggesting that MET or EGFR can compensate when phosphorylation of the other receptor is inhibited (15, 31). Furthermore activation of AKT has been shown to be associated with radioresistance and linked to up-regulation of MET in HNSCC (11). AKT remained activated in our study, that could explain why crizotinib failed to affect the radiation response. Others have suggested c-SRC plays a role in MET and EGFR activation and resistance in HNSCC. (25, 26). Sen *et al*. showed sustained MET activation can mediate resistance to c-SRC inhibition and inhibiting c-SRC and MET together has a synergistic effect on preventing growth of oral cancer cells (25). This supports a model in which c-SRC and MET cooperate to maintain cell survival in sensitive HNSCC cells. Increased activation of c-SRC was seen in our cells when treated with crizotinib plus radiation.

Another explanation of why crizotinib did not have a desirable effect could be the absence of *MET* amplification in our cell lines. The effect of crizotinib through MET may rely more on *MET* amplification than MET protein expression. This has been shown by Tanizaki *et al*., who examined crizotinib in lung cancer cells that were positive or negative for *MET* amplification or mutation and found that crizotinib had a marked antitumor action in *MET* amplified lung cancer cells but not in cells without *MET* amplification, including those with a *MET* mutation (28). A recent a phase I trial has also shown that patients with *MET*-amplified NSCLC are sensitive to crizotinib (7). *MET* amplification is also a mechanism of resistance to EGFR therapy (10) and has been shown to be a poor prognostic marker in a variety of cancer types (22). Interestingly, data from The Cancer Genome Atlas (TCGA) shows HNSCC tumors exhibit very low levels of *MET* amplification (8). Analysis of the TCGA data also shows that *MET* mutations are rarely seen in HNSCC (8). Out of our nine HNSCC cell lines, only the UT-SCC-15 cells had a mutation causing a frame shift deletion. However, both cell lines used in our xenograft experiments harbored mutations in *PI3KCA* and *PTEN*, which could explain why crizotinib failed to inhibit AKT or MAPK.

Interestingly, we are the second group to demonstrate increased tumor growth when crizotinib is combined with radiation in MET-expressing cells. Dai *et al*. examined the effects of crizotinib and radiation in three NSCLC cell lines with various ALK and MET status (9). In their ALK-negative, MET-expressing cell line, they observed an *in vitro* radioprotective effect of crizotinib consisting of enhanced survival and reduced apoptosis and an increase in *in vivo* tumor growth when crizotinib was combined with radiation compared to radiation alone (9). In their ALK-positive cells, they saw a synergistic effect of crizotinib and radiation, that is consistent with another report examining ALK-expressing NSCLC cells (27). Tumati *et al*. examined multiple NSCLC cell lines with different expression levels of MET and ALK and found no radiosensitizing effects (29). They suggest that transient inhibition of AKT followed by rebound AKT activation may be a mechanism by which crizotinib renders cells radioresistant (29).

Overall, the role of MET inhibition in combination with radiation remains unresolved. Besides the above mentioned studies on crizotinib, there exist various studies with other MET inhibitors showing radiosensitization in NSCLC, glioblastoma, prostate, gastric and thyroid cancer cell lines (6, 16, 18-20, 30). Some of these studies suggest that MET inhibition results in

DNA double-strand breaks and can synergize with radiation (6, 20). To our knowledge, there currently are no published *in vivo* data showing crizotinib enhances the effect of radiation through inhibition of MET and not through ALK.

While our data did not show any radiation enhancement, the results are nonetheless important and demonstrate the challenges of studying combinations of radiation with molecular targeted agents. Despite the fact that radiation therapy is a common form of treatment in patients with cancer, there is a paucity of clinical trials investigating combinations of radiation and molecular targeted agents (21). There is also a lack and a need for pre-clinical studies of targeted drugs with radiation, including the publishing of negative data to inform the research community (17). When designing pre-clinical radiosensitization studies, it is important to test drugs in well-characterized cell lines and in xenograft models since some drugs have a limited effect *in vitro* but varying effects *in vivo* (17). In addition, validation of pre-clinical work in multiple tumor models, including our own, needs to be performed before translating any findings into the clinic. Our results have raised many interesting questions that need to be further investigated with future work.

In conclusion, we have shown that MET is expressed in HNSCC cell lines but crizotinib did not enhance the anti-tumor effect of radiation in two HPV-negative HNSCC cell lines grown *in vivo*. Instead, there was suggestion of enhanced tumor growth when crizotinib was combined with radiation. Crizotinib inhibited phosphorylation of MET, but did not inhibit phosphorylation of downstream signaling proteins AKT, MAPK or c-SRC. Failure of crizotinib to elicit radiosensitization could be from lack of *MET* amplification or possibly from activation of alternative pathways by other RTKs. Future radiosensitizing studies will need to focus on inhibiting combinations of RTKs, such as of EGFR along with MET, and inhibiting common downstream proteins such as PI3K/AKT/mTOR and c-SRC. MET still remains a therapeutic target of interest since it is highly overexpressed in HNSCC tumors and is associated with aggressive disease; however, the role of crizotinib in combination with radiation in HNSCC remains unclear.

Conflicts of Interest

Part of this work was funded by a grant from Pfizer. The funding source had no involvement in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. TheAuthors declare no other conflicts of interest.

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