c-Src Activation Mediates Erlotinib Resistance in Head and Neck Cancer by Stimulating c-Met

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Abstract

**Purpose:** Strategies to inhibit the EGF receptor (EGFR) using the tyrosine kinase inhibitor erlotinib have been associated with limited clinical efficacy in head and neck squamous cell carcinoma (HNSCC). Co-activation of alternative kinases may contribute to erlotinib resistance.

**Experimental Design:** We generated HNSCC cells expressing dominant-active c-Src (DA-Src) to determine the contribution of c-Src activation to erlotinib response.

**Results:** Expression of DA-Src conferred resistance to erlotinib in vitro and in vivo compared with vector-transfected control cells. Phospho-Met was strongly upregulated by DA-Src, and DA-Src cells did not produce hepatocyte growth factor (HGF). Knockdown of c-Met enhanced sensitivity to erlotinib in DA-Src cells in vitro, as did combining a c-Met or c-Src inhibitor with erlotinib. Inhibiting EGFR resulted in minimal reduction of phospho-Met in DA-Src cells, whereas complete phospho-Met inhibition was achieved by inhibiting c-Src. A c-Met inhibitor significantly sensitized DA-Src tumors to erlotinib in vivo, resulting in reduced Ki67 labeling and increased apoptosis. In parental cells, knockdown of endogenous c-Src enhanced sensitivity to erlotinib, whereas treatment with HGF to directly induce phospho-Met resulted in erlotinib resistance. The level of endogenous phospho-c-Src in HNSCC cell lines was also significantly correlated with erlotinib resistance.

**Conclusions:** Ligand-independent activation of c-Met contributes specifically to erlotinib resistance, not cetuximab resistance, in HNSCC with activated c-Src, where c-Met activation is more dependent on c-Src than on EGFR, providing an alternate survival pathway. Addition of a c-Met or c-Src inhibitor to erlotinib may increase efficacy of EGFR inhibition in patients with activated c-Src.

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Introduction

EGF receptor (EGFR) is a receptor tyrosine kinase that is overexpressed in about 90% of head and neck squamous cell carcinoma (HNSCC). Previous studies have shown that overexpression of EGFR in HNSCC correlates with regional lymph node metastasis and poor clinical outcome (1). The contribution of EGFR to proliferation, cell-cycle regulation, invasion, and angiogenesis and its accessible location on the cell surface makes it an attractive target for therapeutic intervention (2). EGFR small-molecule tyrosine kinase inhibitors (TKI), such as gefitinib and erlotinib, are known to selectively inhibit the tyrosine kinase domain of EGFR by competitive binding to the ATP-binding region. Despite frequent overexpression of EGFR in various human cancers, including HNSCC, EGFR-TKIs have shown limited clinical efficacy to date, implicating intrinsic resistance mechanisms (3). There are more than 50 clinical trials currently underway in different disease settings of HNSCC using erlotinib, so understanding intrinsic resistance to erlotinib could help identify a subset of patients most likely to respond. Unlike non–small cell lung cancer (NSCLC), where EGFR-activating mutations confer sensitivity to EGFR-TKIs (4), HNSCC very rarely contain EGFR-activating mutations. Co-activation of several receptor and non–receptor tyrosine kinases has been implicated in the limited response to targeting a single pathway in cancer models (5). However, the precise mechanisms determining EGFR-TKI sensitivity in HNSCC remain incompletely understood.

Elevated expression of the non–receptor tyrosine kinase c-Src and/or increased Src kinase activity has been reported in a wide variety of human cancers, including breast, colon, lung, and head and neck cancers (6–8). Activated c-Src
Translational Relevance

The EGF receptor (EGFR) tyrosine kinase inhibitor, erlotinib, has had limited clinical efficacy as monotherapy treatment to date in head and neck squamous cell carcinoma (HNSCC) but is still under investigation in many clinical trials of different HNSCC disease settings. Our results in HNSCC preclinical models suggest that c-Src activation contributes to erlotinib resistance, but not cetuximab resistance, through ligand-independent activation of c-Met in HNSCC. In the presence of elevated c-Src activation, c-Met activation is more dependent on c-Src than on EGFR, providing an alternate pathway for tyrosine kinase signaling. Expression of phosphorylated c-Src predicts erlotinib resistance in HNSCC cell lines. Dual treatment with erlotinib and either a c-Met or a Src inhibitor may be an effective therapeutic approach for patients with HNSCC whose tumors have high levels of activated c-Src with EGFR expression. Activated c-Src in patient tumors may be a potential marker for erlotinib resistance.

Materials and Methods

Reagents and antibodies

Erlotinib and the highly selective c-Met inhibitor PF04217903 (which lacks inhibitory activity against RON, ALK, and other kinases; ref. 13) were purchased from ChemieTek, dissolved in dimethyl sulfoxide (DMSO), and stored as 10 and 100 mmol/L stock solutions at −20°C for in vitro use. Cetuximab was from Bristol Myers Squibb as a 2 mg/mL solution in sterile saline. PF04217903 was dissolved in water at 25 mg/mL, and erlotinib was dissolved in 20%-trappsol at 4 mg/mL for in vitro use. Lipofectamine 2000 was obtained from Invitrogen. Antibodies for phospho-Src Family (Tyr416), total Src (32G6 monoclonal antibody), phospho-EGFR (Tyr845), phospho-EGFR (Tyr1068), and phospho-Met (Tyr1234/1235) were obtained from Cell Signaling Technology, Inc. Purified mouse anti-EGFR antibody was purchased from BD Biosciences Pharmingen. Total c-Met (C-12) antibody was from Santa Cruz Biotechnology, Inc., and β-tubulin antibody was from Abcam. Secondary antibodies were from Bio-Rad Laboratories.

Cell lines

All HNSCC cell lines were of human origin and obtained from the following sources: 686LN (14), G. Chen (Emory University, Atlanta, GA); FaDu, American Type Culture Collection; UM-SCC-22A, UM-SCC-22B, and UM-SCC1, T. Carey (University of Michigan, Ann Arbor, MI); PCI-15B, T. Whiteside (University of Pittsburgh, Pittsburgh, PA); CAL-33, G. Milano (Centre Antoine-Lacassagne, Nice, France); and HN-5, J. Myers (MD Anderson, Houston, TX). All cell lines were validated by genotyping within 2 months of conducting the experiments (15).

Generation of stable HNSCC cell lines expressing dominant-active c-Src

686LN cells were transfected with a plasmid expressing dominant-active c-Src (DA-Src) carrying a mutation at tyrosine 529 to phenylalanine, which eliminates the inhibitory phospho-tyrosine at position 529. Corresponding control vector, pUSEamp, and the DA-Src plasmids were obtained from Upstate Biotechnology, Inc. Three days after transfection, cells were subjected to selection medium containing 200 μg/mL neomycin for an additional 10 days followed by validation.

Western blotting

Cell lysates were prepared as described previously (16). Forty micrograms of total protein was subjected to SDS-PAGE and immunoblotting as previously reported (17).

Hepatocyte growth factor ELISA

Cell culture media were harvested and analyzed in triplicate by Quantikine Human HGF ELISA (R&D Systems) according to the manufacturer's instructions.

Matrigel invasion assay

Invasion assays were conducted using Matrigel-coated modified Boyden chamber inserts (BD Biosciences). Cells
(1.25 × 10^4) were seeded onto the upper chamber. Both the upper and lower chambers contained drug treatment. The lower chamber also contained 10% FBS which served as a chemoattractant. Cells were incubated for 24 hours at 37°C in a 5% CO2 incubator. Noninvading cells retained in the upper chamber were removed, and the invaded cells were fixed and stained with Hema 3 (Fisher Scientific). Invaded cell number was normalized to cell proliferation (normalized to optical density by MTT assay for each respective treatment group). Six randomly selected fields were counted under the microscope at ×200 magnification. The mean ± SE was calculated from 3 independent experiments.

**MTT assay**

Cell viability was measured using MTT assay. Cells were plated in triplicate (3 × 10^4/well in 24-well plates) overnight followed by drug treatment at various concentrations as indicated in the legends. MTT solution (5 mg/mL of MTT in PBS; Sigma-Aldrich) was added for the appearance of colored formazan product, which was then dissolved in DMSO, and subjected to colorimetric measurement at 570 nm with a plate reader (BioTek Instruments, Inc.). Percentage of growth inhibition was calculated as (OD_vehicle − OD_drug)/OD_vehicle × 100%. For Fig. 5D, cells were plated in 24-well plates in the presence of recombinant human hepatocyte growth factor (HGF; 50 ng/mL) or vehicle control. Cells were subjected to drug treatment in the continued presence of vehicle or HGF for 48 hours before analysis with MTT.

**siRNA transfection**

siRNAs were purchased from Dharmacon. The target sequence for human-specific c-Src siRNA (D-003175-05) was 5’ GAGAACCUGGUGUGCAAAG-3’. c-Met was targeted using a mixture of 4 siRNAs provided as a single reagent (L-003156-00). Control siRNA from Dharmacon (D-001210-01 for c-Src, D-001810-10-05 for c-Met) was used as a control for nonsequence-specific effects. siRNA (300 pmoles) was transfected in HNSCC cell lines using Lipofectamine 2000 according to the manufacturer’s instructions.

**In vivo studies**

Six-week-old female athymic nude mice (nu/nu) were purchased from Jackson Laboratory. Stable cell lines vector-transfected control (VC)–2 or DA-Src-5 were inoculated into the flanks of athymic nude mice (1 × 10^6 cells/tumor) with Matrigel (BD Biosciences). Tumor nodules were palpable on day 5 after inoculation, and mice were randomized into 4 groups. Mice were treated 5 d/wk with 40 mg/kg erlotinib, 15 mg/kg PF04217903, or vehicle (20% trapposil in H2O, Sigma-Aldrich) by oral gavage. In a separate experiment, mice were treated 5 d/wk with 0.03 mg cetuximab intraperitoneally (i.p.) or vehicle control. Tumor size was measured 2 times a week with digital calipers. Tumor volume was calculated as: volume = length × width^2/2. At the end of the treatment period, the animals were killed and tumors were removed and fixed in 10% buffered formalin for immunostaining. Animal care was in strict compliance with the institutional guidelines established by the University of Pittsburgh.

**Immunohistochemistry**

Slides were deparaffinized with xylenes and rehydrated before heat-induced antigen retrieval. Nonspecific binding was blocked for 45 minutes at room temperature. Sections were incubated with primary antibodies for K67 (1:50, GeneTex), phospho-Src (1:100, Santa Cruz Biotechnology), and phospho-Met (1:100, Cell Signaling Technology). The number of apoptotic cells was determined using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore) as described (18). Slides were read and scored for the number of positive cells per 5 fields per sample. Results are reported as the mean ± SE.

**Statistical analysis**

Statistical analyses were conducted using PRISM 4 software (GraphPad), the P values were obtained by the Wilcoxon–Mann–Whitney test or Student t test. Results were considered as statistically significant with a P < 0.05.

**Results**

**Overexpression of activated c-Src confers resistance to erlotinib in HNSCC cells in vitro**

Activation of c-Src is associated with more aggressive tumor behavior in head and neck cancers (8). The contribution of c-Src activation to effectiveness of EGFR targeting in head and neck cancer has not been previously explored. We evaluated the expression of phosphorylated and total EGFR as well as phosphorylated and total c-Src in a panel of 8 HNSCC cell lines and found variable expression of activated EGFR and c-Src with no correlation in expression levels (Fig. 1A). To investigate the consequences of c-Src activation on erlotinib sensitivity, we generated stable head and neck cancer clonal cell lines expressing activated c-Src (by ectopic expression of dominant active c-Src, DA-Src) in an invasive HNSCC cell line that readily forms tumors in xenografts, 686LN, with moderate endogenous c-Src expression and low endogenous activated c-Src (Fig. 1A). These cells also have moderate EGFR and phospho-EGFR expression (Fig. 1A). As shown in Fig. 1B, the DA-Src clones (DA-Src-3 and DA-Src-5) were validated and confirmed by Western blotting to express greater than 3-fold higher levels of activated c-Src when compared with vector control clones, VC-1 and VC-2 (P < 0.05), with unchanged level of total EGFR. Functional activation of c-Src was also confirmed by the increase in Tyr845 phospho-EGFR expression in the DA-Src cell lines, as activated c-Src is known to induce EGFR phosphorylation specifically at amino acid residue Tyr845 (19). A slight increase in EGFR phosphorylation at the Tyr1068 autophosphorylation site was also observed, suggesting that overall levels of EGFR tyrosine phosphorylation are somewhat affected.

To determine the sensitivity of these cells to an EGFR-TKI, VC stable clones and DA c-Src clones were subjected to treatment with erlotinib at increasing concentrations
followed by a cell viability assay. A representative experiment is shown in Fig. 1C. Erlotinib induced dose-dependent growth inhibition of the 686LN vector control clones, VC-1 and VC-2, with an average EC50 of 2.7 μmol/L. Expression of activated c-Src in DA-Src-3 and DA-Src-5 cells was found to confer marked resistance to erlotinib (shift of concentration–response curve to the right), compared with the vector control clones, with an average EC50 of 12.5 μmol/L, a 4.6-fold increase. Cumulative data from multiple independent experiments showed that both the DA-Src-3 and DA-Src-5 clones were 4-fold more resistant to erlotinib than to VC-1 and VC-2 cells (n = 10, P = 0.0004, data not shown). To determine the specificity of c-Src activation to erlotinib sensitivity, the same cells were treated with cisplatin, a common chemotherapeutic agent for the treatment of HNSCC. c-Src activation status did not alter the sensitivity of HNSCC to cisplatin (Supplementary Fig. S1), indicating that increased c-Src activation does not generally contribute to drug resistance. EGFR-TKIs have also been shown to inhibit cancer cell invasion (17), and c-Src is also known to mediate invasion. We have previously shown that HNSCC containing DA-Src are more invasive than vector control cells and the invasive behavior was resistant to treatment with the EGFR-TKI gefitinib (17). We confirmed that the increased invasion seen with DA-Src transfection was also resistant to erlotinib compared with vector control cells (data not shown).

**c-Src activation contributes to erlotinib resistance in vivo**

Next, we sought to examine whether c-Src–mediated resistance to erlotinib was also observed in vivo. 686LN VC-2 and DA-Src-5 cells were injected s.c. into immunocompromised mice and were treated with vehicle (20%
trappsol) or erlotinib (40 mg/kg) by oral gavage after a 5-day period to establish the tumors. The initial pretreatment tumor volumes of all groups were comparable with no statistical differences. Erlotinib treatment reduced the tumor volume established from the VC cells by 68.7% compared with vehicle treatment (average tumor volume \(= 1,215 \pm 320 \text{ mm}^3\) vs. \(382 \pm 32 \text{ mm}^3\), \(n = 14\), \(P = 0.034\); Fig. 2A). However, tumors established from DA c-Src cells were completely insensitive to erlotinib compared with vehicle (average tumor volume \(= 977 \pm 200 \text{ mm}^3\) vs. \(910 \pm 140 \text{ mm}^3\), \(n = 14\), \(P = 0.95\)). These results suggest that c-Src activation also contributes to erlotinib resistance in vivo.

Figure 2. c-Src activation contributes to erlotinib resistance but not cetuximab resistance in vivo. A, VC-2 and DA-Src-5 HNSCC cells were inoculated into the flanks of nude mice. Mice were treated 5 d/wk with either vehicle control (20% trappsol) or 40 mg/kg erlotinib by oral gavage for 4 weeks. Treatment began 5 days after tumor implantation. Results represent the mean tumor volumes \(\pm\) SE of 14 tumors per treatment group. Unpaired Student \(t\) test, *, \(P < 0.05\). B, representative images and quantitative results of immunohistochemical staining of proliferating cells by Ki67 and apoptotic cells by TUNEL assay. The results are presented as the mean number of apoptotic cells or Ki67-positive cells per area \(\pm\) SE. Unpaired Student \(t\) test, **, \(P < 0.005\); ***, \(P < 0.0005\). Scale bar, 50 \(\mu\)m. C, a total of \(1 \times 10^6\) DA-Src-5 HNSCC cells were inoculated into the flanks of nude mice. Mice were treated 2 d/wk with either vehicle control (saline) or 0.03 mg cetuximab i.p. for 13 days. Treatment began 15 days after tumor implantation. Results represent the mean tumor volumes \(\pm\) SE of 7 tumors per treatment group. Unpaired Student \(t\) test, *, \(P < 0.05\).
Differences in tumor volume between VC and DA c-Src cells are most likely due to a lower stromal content in DA-Src tumors.

Extent of apoptosis and cell proliferation in tumor xenografts were examined in formalin-fixed excised tumors (Fig. 2B). Staining of apoptotic nuclei was increased, whereas Ki67 staining was decreased by erlotinib treatment in the VC but not in the DA-Src tumor xenografts. The number of apoptotic and proliferating cells was quantitated in sections from 4 tumors per treatment group showing a significant 4-fold increase in apoptotic cells with erlotinib treatment in the VC xenografts (mean = 177 ± 11 apoptotic cells/field) compared with control treatment in VC xenografts (mean = 43.7 ± 9.2 apoptotic cells/field, P = 0.0007). There was a statistically significant 52.7% decrease in cell proliferation with erlotinib treatment in the VC xenografts (mean = 140.7 ± 40.2 proliferating cells/field) compared with control (mean = 298 ± 19 proliferating cells/field, P = 0.025).

No significant differences in apoptosis or cell proliferation were observed in DA-Src xenografts treated with erlotinib compared with vehicle control. In addition, we observed that the DA-Src tumor xenografts expressed 5.2-fold more phospho-Src than VC xenografts (P < 0.005), irrespective of erlotinib treatment (data not shown).

In parallel experiments, we found that DA-Src cells remained sensitive to the EGFR neutralizing antibody cetuximab (at the EC50 dose for parental cells) with a 57.4% inhibition in tumor volume (P < 0.05; Fig. 2C) compared with a 63.4% inhibition in the VC xenografts. The 686LN parental cells have previously been shown to be sensitive to cetuximab in vivo (20). The in vivo cetuximab response in DA-Src xenografts was consistent with in vitro invasion assays in which cetuximab showed a significant decrease in number of invading cells in the DA-Src cell line (data not shown).

**Activated c-Src induces c-Met phosphorylation in HNSCC cells**

Amplification and overexpression of c-Met has been associated with acquired erlotinib resistance in NSCLC (11). To determine the role of c-Met in c-Src–mediated erlotinib resistance, we assessed the expression of total and phosphorylated forms of c-Met in HNSCC expressing DA-Src. As shown in Fig. 3A, both DA-Src-3 and DA-Src-5 showed a greater than 25-fold increase in phospho-Met, with a very moderate increase in total c-Met expression, when compared with controls. Consistent with our previous findings that HGF is a paracrine factor in HNSCC (10),...
A

DA-Src-5

Tumor volume (mm$^3$)

Days

0 5 10 15 20 25

Vehicle

Erlotinib

PF

PF + Erlotinib

通讯量

0 100 200 300 400 500 600 700

0 10 20 30 40 50 60 70 80 90 100

% Growth inhibition (vs. DMSO control)

B

DA-Src-5

% Growth inhibition (vs. DMSO control)

0.1 μmol/L 0.5 μmol/L

Erlotinib

Erlotinib only

Erlotinib + PF

Erlotinib + dasatinib

C

Tumor volume (mm$^3$)

Days

0 5 10 15 20 25

Vehicle

Erlotinib

PF

PF + Erlotinib

* * * * * * * *

Mean number of apoptotic cells

Control

Erlotinib

PF

PF + Erlotinib

Mean number of Ki67-positive cells

Control

Erlotinib

PF

PF + Erlotinib

* * * * * * * *

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we confirmed by ELISA that the increase in c-Met phosphorylation in DA-Src cells was independent of the c-Met ligand, HGF, because no HGF production was detected (Supplementary Fig. S2). This suggests that activation by phosphorylated c-Src is responsible for the observed c-Met activation, as has been reported previously (21). We also confirmed that in vivo, phospho-Met expression was upregulated in tumor xenografts from DA-Src cells. A 20-fold increase was detected by immunohistochemistry in DA-Src cells compared with VC cells (Supplementary Fig. S3).

As ligand-independent activation of c-Met has been reported for both EGFR (12) and c-Src (21), we examined the extent to which EGFR or c-Src inhibitors modulated phospho-Met in the presence of activated c-Src. In DA c-Src cells, erlotinib treatment resulted in a nonsignificant minimal reduction (10%–15%) of phospho-Met, whereas dasatinib treatment achieved almost a complete reduction of phospho-Met levels (Fig. 3B). Both dasatinib and erlotinib inhibited their respective targets, phospho-Src and phospho-EGFR. To confirm this finding, we examined the extent of inhibition of phospho-Met in 4 HNSCC cell lines from Fig. 1A (CAL-33, UM-SCC-22A, HN5, and PCI-15B) with widely varying erlotinib sensitivity (ref. 20 and Supplementary Table S1), along with the 2 686LN DA-Src clones and the 2 VC clones. We found a significant correlation (P = 0.045) between erlotinib EC50 and the extent to which phospho-Met was inhibited in the presence of dasatinib versus erlotinib, using a concentration that produced complete reduction in phosphorylation of the respective target (c-Src or EGFR). For this determination, the ratio of residual phospho-Met seen in dasatinib-treated cells compared with residual phospho-Met observed in erlotinib-treated cells was calculated (Fig. 3C). The more resistant the cells were to erlotinib, the lower this ratio was. This suggests that in DA c-Src cells and other HNSCC cell lines that are erlotinib-resistant, ligand-independent c-Met activation is coupled to c-Src more so than to EGFR because it is more sensitive to dasatinib than to erlotinib. This can provide an alternative pathway for tyrosine kinase signaling in the presence of an EGFR-TKI.

**Knockdown of c-Met enhances sensitivity to erlotinib**

We next used c-Met siRNA to determine whether knockdown of c-Met in DA-Src cells would increase erlotinib sensitivity. Figure 4A shows that knockdown of c-Met resulted in a 2- to 3-fold increase in erlotinib sensitivity (P < 0.05). Control siRNA–transfected cells showed a 7.6% ± 5.0% and 23.9% ± 2.9% inhibition of cell growth with 0.1 and 0.5 μmol/L erlotinib, respectively, whereas c-Met knockdown resulted in 24.9% ± 0.4% and 40.7% ± 0.5% inhibition, respectively, at these erlotinib concentrations (c-Met vs. control siRNA growth inhibition at 0.1 μmol/L erlotinib, P = 0.040, 0.5 μmol/L erlotinib, P = 0.016). The inset shows an 83.8% inhibition of c-Met expression with siRNA treatment.

We next combined a c-Src or a c-Met inhibitor with erlotinib in the DA c-Src cells and examined cell viability to determine whether the erlotinib response could be enhanced by dual treatment (Fig. 4B). In this experiment, erlotinib alone resulted in a 10.6% ± 3.7% and 14.7% ± 5.6% inhibition of cell growth at 0.1 and 0.5 μmol/L, respectively. The c-Met TKI PF04217903 (13) showed minimal inhibition (10%) of cell proliferation as a single agent up to 5 μmol/L. However, addition of 1 μmol/L PF04217903 resulted in 21.9% ± 3.1% and 31.4% ± 9.0% inhibition in combination with 0.1 and 0.5 μmol/L erlotinib, respectively. This represents a 2-fold increase in sensitivity. Similarly, addition of dasatinib at its EC50 (380 nmol/L) to erlotinib at 0.1 and 0.5 μmol/L in the DA-Src cells resulted in 81.1% ± 0.7% and 81.7% ± 0.3% inhibition of cell growth, respectively, enhancing the effect of erlotinib over 6-fold. A similar trend was observed with addition of dasatinib at its EC50 (30 nmol/L) to 0.1 and 0.5 μmol/L erlotinib with 43.8% ± 1.5% and 58.8% ± 1.9% inhibition of cell growth, respectively (data not shown). These results suggest that erlotinib resistance due to overexpression of activated c-Src can be overcome by addition of a selective c-Met inhibitor to erlotinib or by combining erlotinib with a Src inhibitor.

**c-Met inhibition sensitizes DA-Src tumors to erlotinib in vivo**

If erlotinib resistance is due to activation of c-Src, this suggests the alternate use of a c-Src inhibitor in patients who show high levels of c-Src activation. Results of a recent phase II trial in patients with HNSCC showed that dasatinib as a single agent lacks clinical activity and was associated with toxicity, even at a dose that was inhibiting c-Src (22). Increased activity of dasatinib against HNSCC in a preclinical model has been previously observed when combined with a Met inhibitor in vivo (21), consistent with our in vitro observation that c-Met activation is coupled to overactive c-Src. As combination treatments with dasatinib (including adding dasatinib to either erlotinib or a c-Met inhibitor) might show unacceptable toxicities in patients, we investigated whether the marked c-Met activation conferred by

![Figure 4](https://example.com/figure4.png)

Figure 4. Specific knockdown of c-Met by siRNA in DA-Src cells restores sensitivity to erlotinib. 686LN DA-Src-5 cells were transiently transfected with c-Met or control siRNA (300 pmol) for 48 hours followed by treatment with erlotinib at the indicated doses. Cell viability was assessed by MTT assay after 48 hours. The results from 3 independent experiments are shown. Student t test, *P < 0.05. Knockdown of c-Met was confirmed by Western blotting (inset). B, 686LN DA-Src-5 cells were treated with 0.1 and 0.5 μmol/L erlotinib alone or in combination with PF04217903 (1 μmol/L) or dasatinib (EC50, 380 nmol/L) for 48 hours followed by MTT assay. Results from 3 independent experiments are shown. Student t test, *P = 0.010; **P = 0.003; ***P = 0.0001. C, DA-Src-5 tumor-bearing mice received the following treatments 5 d/wk for 4 weeks by oral gavage: vehicle control, erlotinib (40 mg/kg), PF04217903 (15 mg/kg), or combination. Treatment began 6 days following tumor implantation. Results represent the mean tumor volumes ± SE of 12 tumors per treatment group. Unpaired Student t test, *P = 0.0068 compared with control. D, representative images and quantitative results of immunohistochemical staining of proliferating cells by KI67 and apoptotic cells by TUNEL assay. The results are presented as the mean number of apoptotic cells or KI67-positive cells per area ± SE. Unpaired Student t test, *P < 0.05; **P < 0.005. Scale bar, 50 μm.
DA-Src could serve as an alternate therapeutic target in vivo. We used a tumor xenograft model with the DA-Src-5 cells to test whether the addition of a c-Met inhibitor could improve response to erlotinib. Inhibitors of c-Met have been successfully and safely combined with erlotinib in clinical trials of patients with lung cancer (23). The c-Met inhibitor PF04217903, at the published dose of 15 mg/mL (24), was used alone and combined with erlotinib. Immuno-compromised mice bearing DA-Src-5 xenograft tumors were treated with either vehicle control, erlotinib (40 mg/kg), PF04217903 (15 mg/kg), or erlotinib plus PF04217903 for 5 d/wk for 4 weeks. Initial tumor volumes were similar between all treatment groups. Both erlotinib and PF04217903 alone resulted in no significant difference in tumor volume compared with control (P > 0.05). The combination treatment, however, resulted in a 56.2% decrease in tumor volume compared with control (P = 0.0068; Fig. 4C), comparing favorably to the 68.7% inhibition in tumor volumes seen with erlotinib-sensitive VC controls treated with erlotinib alone (Fig. 2A). This result shows that in vivo erlotinib resistance in c-Src–acti-
vated HNSCC cells can be about 80% overcome by the addition of a c-Met inhibitor.

The mean number of apoptotic and proliferating cells in the tumors at the end of the experiment were assessed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay and Ki67 immunostaining, respectively (Fig. 4D). The maximum number of apoptotic cells and the least amount of proliferating cells were found in the combination treatment group. Apoptotic cells were increased by all treatment groups (erlotinib: 18.0 ± 1.6, PF04217903: 28.2 ± 4.3, combination: 40.7 ± 2.4) compared with the control group (15.2 ± 1.8). The mean number of apoptotic cells in the combination treatment group was significantly higher than the mean number of apoptotic cells in the control (P < 0.0001), erlotinib only (P < 0.0001), and PF04217903 only (P = 0.033) treatment groups. Similar results were observed with the number of proliferating cells. Erlotinib and PF04217903 treatment alone resulted in an 8.5% (P = 0.26) and 26% (P =
0.007) decreases in the number of proliferating cells compared with control. Combination treatment resulted in a 58.5% decrease compared with control (P<0.0001) and was also significantly lower than both single-agent treatment groups. This effect on labeling of proliferative cells compares favorably to that observed with erlotinib alone in VC cell xenografts that are sensitive to erlotinib (52.7% decrease, Fig. 2B).

**Knockdown of c-Src in parental cells reduces c-Met activation and enhances sensitivity to erlotinib, whereas HGF treatment results in erlotinib resistance**

To determine the contribution of endogenous c-Src to c-Met phosphorylation in the absence of an exogenously added c-Src vector, expression of total and phosphorylated c-Met in HNSCC parental cells treated with c-Src siRNA was examined. Transient transfection of c-Src siRNA in 2 representative HNSCC cell lines with moderate endogenous expression of c-Src, 686LN, and FaDu resulted in a significant but partial knockdown of c-Src protein at 48 hours (63% in 686LN and 51% in FaDu; Fig. 5A). The expression of phosphorylated c-Met was almost completely abolished even with this partial knockdown of endogenous c-Src in both 686LN and FaDu cells. This result suggests that endogenous c-Src expression in HNSCC is also associated with c-Met activation in HNSCC. The activation of c-Met is most likely through a ligand-independent mechanism directly mediated by c-Src (21), especially as no HGF is detected in HNSCC parental cell lines (10).

We also tested whether downregulation of endogenous c-Src, with its accompanying reduction in activated c-Met, would enhance the sensitivity of HNSCC parental cells to erlotinib. As shown in Fig. 5B and C, even with the partial knockdown of endogenous c-Src by siRNA, the erlotinib sensitivity of both 686LN and FaDu cells was enhanced at their respective EC50 and sub-EC50 doses of erlotinib. At 48 hours of 0.1 μmol/L erlotinib treatment, transient transfection of c-Src siRNA increased erlotinib sensitivity of 686LN cells by 1.8-fold (63% ± 1.7% growth inhibition vs. 35.0% ± 0.18% growth inhibition with control siRNA; n = 3 experiments, P = 0.003), and a consistent enhancement was also observed with 0.5 μmol/L erlotinib at 48 hours (Fig. 5B). In FaDu cells, which have higher endogenous c-Src and phospho-c-Src, as well as a higher EC50 for erlotinib (2.0 μmol/L) than 686LN cells (0.5 μmol/L), specific knockdown of c-Src resulted in 1.6- and 2.8-fold increases in erlotinib sensitivity at the EC50 (2.0 μmol/L) and sub-EC50 (0.5 μmol/L) concentrations of erlotinib, respectively (Fig. 5C). These results indicate that endogenous c-Src contributes to erlotinib sensitivity in HNSCC.

To determine whether the parental erlotinib-sensitive 686LN cells could also become resistant to erlotinib by activating c-Met directly through HGF exposure, we treated these cells with exogenous human recombinant HGF (Fig. 5D, inset). A shift to the left in the erlotinib concentration response at 48 hours was observed (Fig. 5D) showing that parental cells became more than 2-fold resistant to erlotinib following HGF treatment (EC50 = 2.45 μmol/L without HGF treatment vs. EC50 = 5.98 μmol/L with HGF treatment, P<0.0001). Together, these observations support a role for c-Met activation in erlotinib resistance in HNSCC, which could occur through ligand stimulation as well as through ligand-independent activation.

**Endogenous-activated c-Src is a predictor of erlotinib resistance**

Using the erlotinib EC50 values (ref. 20 and Supplementary Table S1) for the panel of 8 cell lines shown in Fig. 1A, a significant correlation (P = 0.019) was found between endogenous phospho-c-Src expression and erlotinib EC50 (Fig. 6). Cells with higher basal expression of phospho-c-Src have higher erlotinib EC50 values and thus phospho-Src may represent a novel therapeutic biomarker for erlotinib response. In this panel of cell lines, we did not observe any significant correlation between total EGFR, phospho-EGFR, or total c-Src and erlotinib response, and combined consideration of phospho-EGFR with phospho-c-Src did not improve the correlation with erlotinib response seen with phospho-c-Src alone (all P > 0.05, data not shown). There was also no relationship between erlotinib sensitivity and the extent of phosphorylation of EGFR residue Tyr845 (the c-Src-dependent residue). Relationship of phospho-Met to erlotinib sensitivity was also not significant, alone or combined with phospho-c-Src measurement (data not shown), suggesting that extent of c-Src activation is the most important variable in predicting erlotinib sensitivity.

**Discussion**

Constitutive activation of c-Src is observed in HNSCC, where expression of activated Src correlates with aggressive clinical features such as invasive tumor fronts, poor differentiation, and lymph node metastasis (8). The implication of high c-Src activation on treatment selection for HNSCC...
has not been previously addressed. The findings in the present study show that increasing c-Src activation in HNSCC through either a dominant-active construct or via endogenous expression is associated with intrinsic resistance to erlotinib treatment. Expression of the dominant-active c-Src construct did not alter responsiveness to a common chemotherapy, cisplatin, suggesting that the resistance to erlotinib is specific to dependence on its targeted pathway, EGFR, and not to a general change in tumor cell behavior. Ectopic expression of activated c-Src by HNSCC cells conferred resistance to erlotinib treatment in vivo as well as in vitro, implicating c-Src activation in erlotinib resistance in patients with HNSCC. In addition, this resistance is specific to erlotinib, as the EGFR monoclonal antibody cetuximab did not show the same resistance in vivo or in vitro, suggesting that Src activation does not contribute to cetuximab resistance. The connection between c-Src and erlotinib sensitivity was confirmed by specific knockdown of endogenous c-Src, which significantly enhanced erlotinib sensitivity in HNSCC cells that were not engineered to overexpress c-Src. Higher constitutive c-Src activation, assessed by basal phospho-c-Src level, also was a significant indicator of erlotinib resistance in HNSCC cell lines, whereas level of basal phospho-EGFR was not, suggesting that c-Src signaling serves as a means of resistance to EGFR inhibitors. These findings suggest that c-Src activation directly contributes to erlotinib, but not cetuximab, resistance in EGFR-expressing HNSCC cancer cells. The mechanism by which increased c-Src activation occurs in HNSCC has not been fully elucidated. Our recent study of HNSCC mutation profiling in 74 patient tumors revealed the absence of any activating mutations of c-Src in patients with HNSCC (25). Previous studies by us and others showed that upstream signaling components, such as EGFR activation by autocrine ligands (26), other growth factors, or integrin signaling may also activate c-Src in HNSCC (27). Activation of c-Src may lead to an EGFR-independent means of stimulating cell proliferation or survival, mediated, at least in part, by activating c-Met.

Inhibiting c-Src directly is a possible strategy for erlotinib-resistant HNSCC tumors with high c-Src activation. However, results of a recent phase II trial in patients with HNSCC showed that dasatinib lacked clinical efficacy, even though inhibition of c-Src was achieved in patients (22). Moreover, apparent toxicity of dasatinib has been observed in patients with recurrent and/or metastatic HNSCC after platinum-based therapy, illustrating the challenge of c-Src targeting in HNSCC (22). Furthermore, treatment with Src inhibitors in laboratory studies often leads to decreased tumor cell migration and invasion, without affecting cell survival (28). Identifying targets downstream of c-Src that might mediate its responses and are more directly coupled with cell growth and survival could suggest alternative rational and clinically feasible (and possibly less toxic) strategies than dasatinib for HNSCC treatment.

EGFR/c-Met cross-talk signaling has been well-described in several cancer models including HNSCC (12). Extent of activation of c-Met by c-Src has recently been reported to be linked to dasatinib sensitivity in HNSCC cells, and alternate activation of c-Met by EGFR was found to be a factor in dasatinib resistance (21). This observation taken together with our data reported here indicates that the extent to which ligand-independent activation of c-Met is coupled to c-Src, as opposed to being coupled with EGFR, may determine whether sensitivity is linked to erlotinib or dasatinib. Our observations that (i) erlotinib resistance associated with c-Src activation was accompanied by high ligand-independent activation of c-Met and (ii) erlotinib resistance could be overcome by downregulating c-Met or by combining c-Met and EGFR inhibition show that the biologic coupling of c-Met activation to c-Src contributes to erlotinib resistance in HNSCC. This is consistent with the observation that c-Met phosphorylation was largely dependent on c-Src, but not on EGFR, in the DA-cSrc cells. This was further supported by the association we observed between the degree of c-Met activation that was dependent on c-Src compared with EGFR and erlotinib resistance in the HNSCC cell lines.

Further evidence in favor of this hypothesis is the observation that in the absence of exogenously induced c-Src activation, EGFR inhibition resistance could be produced by directly activating the c-Met pathway with its ligand, HGF. In this case, c-Met activation would also not depend upon the EGFR pathway. This is consistent with the idea that c-Met activation can prevent cell death in the presence of an EGFR inhibitor. We also found in lung cancer cells that ligand-independent activation of c-Met required c-Src (29). We did not find evidence for a direct interaction between c-Src and c-Met however, and both gene transcription and prolonged c-Src activation were involved in persistent c-Met activation (29), suggesting that an intermediary protein is involved. Integrins have been postulated to play a role in this mechanism (30).

The correlation we observed between endogenous phospho-c-Src expression and erlotinib sensitivity in HNSCC cell lines suggests that measurement of phosphorylated c-Src may serve as a clinical indicator to help identify patients with erlotinib-resistant HNSCC. Such patients might benefit from dual EGFR and c-Met inhibitor treatment, or dual treatment with either erlotinib and a c-Src inhibitor, or a c-Met inhibitor combined with a c-Src inhibitor. Tolerability of combinations will be an important factor for consideration in clinical settings. In the HNSCC cell line panel, phospho-Met expression was generally higher as erlotinib sensitivity declined but did not reach significance alone as a factor that correlated with erlotinib EC50 or when combined with phospho-c-Src expression. Total endogenous phospho-Met may not predict sensitivity to erlotinib because some phospho-Met may be driven by coupling with EGFR and remains sensitive to EGFR inhibition. Although c-Met is likely not the only target that is phosphorylated by activated c-Src and does not appear from our results to be solely responsible for erlotinib resistance mediated by activated c-Src, addition of a c-Met inhibitor to erlotinib reversed erlotinib resistance by approximately 2-fold in DA-c-Src cells in vitro and restored about 80% of the activity of
erlotinib in these cells in vivo, compared with the empty vector control cells. Reliable measurement of phospho-Src levels in patient tumor tissues may require routine freezing of tumor biopsies, rather than formalin fixation of tissues. The potential use of phospho-c-Src as a biomarker of erlotinib response in patients with HNSCC will require future validation in clinical settings. Currently, tissues from responders and nonresponders from an erlotinib clinical trial are not available for testing.

The addition of a c-Src inhibitor to erlotinib in the DA-Src cells enhanced the overall anti-growth effects of either agent alone, as would be expected. Given the aggressive behavior of HNSCC tumors with high endogenous c-Src activation (8) and the lack of clinical efficacy of the dasatinib single-agent clinical trial in patients with HNSCC (22), our findings may suggest an alternative therapeutic approach (combination EGFR and c-Met inhibition) for patients with HNSCC with activation of c-Src. Combination c-Src and c-Met inhibitors or c-Src and EGFR inhibitors also would be logical choices. Several c-Met inhibitors are currently being tested as monotherapies in head and neck cancer, including foretinib (NCT00725764), which is a dual c-Met-VEGFR2 inhibitor. Cetuximab is another possible choice for patients with activation of c-Src because cells with constitutively activated c-Src remained sensitive to cetuximab. EGFR inhibitors with different mechanisms of action probably have different biomarkers of relative sensitivity. Cetuximab sensitivity may depend more on availability of EGFR for binding by the neutralizing antibody and competition for binding from EGFR ligands such as TGFα and epigen (31, 32). Our results suggest that extent of EGFR downstream signaling events through coupled Src and c-Met may be more predictive of erlotinib sensitivity in head and neck cancer, rather than state of the EGFR itself. Biomarker-guided use of targeted combination treatments is worthy of future clinical investigation in patients with HNSCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Correction: c-Src Activation Mediates Erlotinib Resistance in Head and Neck Cancer by Stimulating c-Met

In this article (Clin Cancer Res 2013;19:380–92), which was published in the January 15, 2013, issue of Clinical Cancer Research (1), the author Dr. Sufi Thomas has been added to the manuscript. Dr. Thomas contributed to the conception and design of the experiments that are outlined in the article.

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The online version has been corrected and no longer matches the print version. The authors regret this error.

Reference

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