Lyn Kinase Mediates Cell Motility and Tumor Growth in EGFRvIII-Expressing Head and Neck Cancer

Sarah E. Wheeler¹, Elena M. Morariu³, Joseph S. Bednash⁴, Charlton G. Otte¹, Raja R. Seethala⁵, Simion I. Chiosea⁵, and Jennifer R. Grandis¹,⁵

Abstract

**Purpose:** EGF receptor variant III (EGFRvIII) has been detected in several cancers in which tumors expressing this truncated growth factor receptor show more aggressive behavior. The molecular mechanisms that contribute to EGFRvIII-mediated tumor progression that are amenable to targeted therapy are incompletely understood. The present study aimed to better define the role of Src family kinases (SFKs) in EGFRvIII-mediated cell motility and tumor growth of head and neck squamous cell carcinomas (HNSCC).

**Experimental Design:** HNSCC models expressing EGFRvIII were treated with dasatinib, a pharmacologic inhibitor of SFKs.

**Results:** SFK inhibition significantly decreased cell proliferation, migration, and invasion of EGFRvIII-expressing HNSCC cells. Administration of dasatinib to mice bearing EGFRvIII-expressing HNSCC xenografts resulted in a significant reduction of tumor volume compared with controls. Immunoprecipitation with anti-c-Src, Lyn, Fyn, and Yes antibodies followed by immunoblotting for phosphorylation of the SFK activation site (Y416) showed specific activation of Lyn kinase in EGFRvIII-expressing HNSCC cell lines and human HNSCC tumor specimens. Selective inhibition of Lyn using siRNA decreased cell migration and invasion of EGFRvIII-expressing HNSCCs compared with vector control cells.

**Conclusions:** These findings show that Lyn mediates tumor progression of EGFRvIII-expressing HNSCCs in which strategies to inhibit SFK may represent an effective therapeutic strategy.

Introduction

EGF receptor variant III (EGFRvIII) is the most common EGFR alteration in many cancers including head and neck squamous cell carcinoma (HNSCC). EGFRvIII lacks exons 2 to 7, is constitutively active, and is absent in normal tissue (1, 2). EGFRvIII expression has been shown to contribute to increased cell survival, proliferation, motility, invasiveness, and treatment resistance (3–5). EGFRvIII has been most extensively characterized in glioma where Src family kinases (SFKs) have been implicated in the EGFRvIII oncogenic phenotype. Abrogation of SFK signaling in an in vivo EGFRvIII-positive glioma xenograft model significantly reduces EGFRvIII-mediated tumorigenesis (6). Further studies in glioma found the SFKs, Fyn and c-Src, to be key mediators in EGFRvIII signaling (7).

SFKs have been implicated in many normal cellular functions such as cell adhesion, migration, proliferation, survival, angiogenesis, and differentiation where deregulation of these pathways contributes to tumorigenesis, tumor progression, and metastasis of cancers expressing wild-type EGFR (8). SFKs are rarely mutated in cancer (8) and are activated in response to stimulation of several cellular factors including platelet-derived growth factor receptor (PDGFR), EGFR, insulin-like growth factor (IGF)-1R, G-protein–coupled receptors (GPCR), cytokine receptors, integrins, and cell adhesion complexes (9). Activated c-Src is common in colorectal and breast cancers and elevated levels of c-Src protein have been reported in several cancers including colon, breast, endometrial, ovarian, pancreatic, and HNSCCs (8). c-Src has been reported to be activated in HNSCCs compared with levels in normal mucosa where p-SFK expression correlates with invasiveness and lymph node metastasis (10). Aberrant c-Src activation has been shown to contribute to HNSCC progression and metastasis (11, 12). SFK blockade inhibited proliferation in several tumor models including breast cancer, HNSCCs, prostate cancer, and glioma (11, 13–15). Treatment of cancer cell lines with an SFK inhibitor or siRNA
Head and neck squamous cell carcinoma (HNSCC) expressing the constitutively active altered EGFR variant III (EGFRvIII) protein is resistant to treatment with cetuximab in preclinical models and a recently reported clinical trial. EGFRvIII oncogenic signaling in HNSCCs is incompletely understood and further elucidation of the pathways contributing to EGFRvIII-mediated tumor progression may allow for identification of druggable targets to overcome cetuximab resistance. Here, we present preclinical in vitro and in vivo evidence that Lyn is the primary Src family kinase activated by EGFRvIII in HNSCCs, which can be targeted with dasatinib with resulting antitumor effects.

Translational Relevance

Head and neck squamous cell carcinoma (HNSCC) expressing the constitutively active altered EGFR variant III (EGFRvIII) protein is resistant to treatment with cetuximab in preclinical models and a recently reported clinical trial. EGFRvIII oncogenic signaling in HNSCCs is incompletely understood and further elucidation of the pathways contributing to EGFRvIII-mediated tumor progression may allow for identification of druggable targets to overcome cetuximab resistance. Here, we present preclinical in vitro and in vivo evidence that Lyn is the primary Src family kinase activated by EGFRvIII in HNSCCs, which can be targeted with dasatinib with resulting antitumor effects.

Materials and Methods

Cell lines, reagents, and cell culture

Cal33 (site of origin: tongue) and UMSCC1 (SCC1; site of origin: oral cavity) cells were a kind gift from Dr. Gerard Milano (Centre Antoine-Lacassagne, Nice, France) and Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI), respectively. FaDu (site of origin: pharynx) and 293T cells were purchased from the American Type Culture Collection. All cells were maintained in Dulbecco’s Modified Eagle’s medium (DMEM; Mediatech Inc.) with 10% heat-inactivated FBS (Invitrogen). UMSCC1 cells were supplemented with 0.4 µg/mL hydrocortisone (Invitrogen) and FaDu cells were supplemented with 1% nonessential amino acids (NEAA; Invitrogen). Cells were incubated at 37°C in the presence of 5% CO₂. All cell lines were genotyped by short tandem repeat (STR) profiling by the AmpFISTR Profiler PCR Amplification Kit (Applied Biosystems).

EGFRvIII-transfected HNSCC cells (Cal33vIII) and vector control–transfected HNSCC cells (Cal33control) have been previously described (19). EGFRvIII was subcloned into the pMSCV Neo plasmid (Clontech). EGFRvIII plasmid DNAs were a kind gift from Dr Frank Furnari (Ludwig Institute for Cancer Research, La Jolla, CA). UMSCC1 and FaDu cells were infected with vector alone (MSCV) or EGFRvIII vector (vIII). Briefly, 293T cells were plated at 80% to 90% confluency in a 10-cm dish and reverse transfected with Lipofectamine 2000 (Invitrogen), manufacturer’s plasmids and parent vector or EGFRvIII plasmid overnight. Fresh media was placed on the cells after 16 hours and virus produced over 48 hours. Target cells were plated in 10-cm dishes at 25% confluency 16 hours before treatment to allow cells to adhere. Viral supernatant was collected, centrifuged, filtered, supplemented with polybrene, and placed on target cells for 72 hours. Viral supernatant was replaced with complete media for 24 hours and cells were selected with 0.5 mg/mL G418 (Invitrogen) for 72 hours. The resulting population of cells were maintained under selection pressure and tested for EGFRvIII expression via reverse transcription polymerase chain reaction (RT-PCR) as described previously (19, 22). Briefly, total RNA was isolated from HNSCC cell lines by the RNeasy kit (Qiagen) according to directed against c-Src abrogated tumor cell invasion and migration (12, 14, 15). In HNSCCs, c-Src, Lyn, Fyn, and Yes are expressed at detectable levels in cell lines and tumors (16). Given the paucity of EGFRvIII cancer cell models and the difficulty of detecting EGFRvIII in human tumors, few studies have elucidated the role of SFK in cancers characterized by EGFRvIII expression.

The role of SFK in EGFRvIII-expressing HNSCCs has not been explored, however, studies in wild-type (wt)EGFR-only HNSCCs have found that SFK can mediate proliferation, invasion, and migration through various pathways (12). Glioma expressing EGFRvIII (as compared with wtEGFR) preferentially signals through the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways (17, 18), and we have shown previously that inhibition of the PI3K/Akt pathway reduces cell proliferation but has no effect on cell motility or invasion in EGFRvIII-expressing HNSCCs (19). In wtEGFR-expressing HNSCC SFK inhibition reduced cell motility and invasion by regulating downstream cell adhesion molecules such as FAK (12). SFK is part of the focal adhesion complex which functions to link integrins to the cytoskeleton. In this complex, SFK is involved in FAK activation (at tyrosines 576/577 and 861) and with other proteins, SFK promotes cell motility by turnover of the focal adhesion. Reduced cell motility is observed through SFK inactivation by c-Src tyrosine kinase (20). FAK also contains an autophosphorylation site (tyrosine 397) and when autophosphorylated creates a binding site for SFK via the SH2 domain of SFK which activates SFK by displacing the inhibitory phosphorylation at Y527 (21).

EGFRvIII is expressed in 17% to 42% of HNSCCs, always in conjunction with wtEGFR (22–24). HNSCC cells expressing EGFRvIII have been shown to be resistant to apoptosis by cisplatin in vitro and cetuximab tumor inhibition in vivo (22). A phase III clinical trial with the anti-EGFR monoclonal antibody cetuximab combined with radiation prolonged overall survival but did not alter the incidence of metastasis (25). We have shown previously that EGFRvIII-expressing HNSCC cells are resistant to cetuximab-mediated inhibition of cell motility and invasion (19). A recent report of a phase II trial of cetuximab in combination with docetaxel in recurrent or metastatic HNSCCs found that EGFRvIII expression was associated with reduced progression-free survival (24). In EGFRvIII-expressing glioma, genetic, and chemical inhibition of SFKs in several xenograft models have shown decreased tumor growth and metastasis compared with controls (6, 7). The role of SFK in EGFRvIII-expressing HNSCCs has not been defined. We undertook the present study to determine the contribution of SFK in EGFRvIII-expressing HNSCCs, where SFK targeting could represent an alternative therapeutic strategy in the setting of EGFRvIII-mediated cetuximab resistance. Identification of alternative therapeutic targets in the setting of EGFRvIII may improve treatment responses.
the manufacturer’s protocol. Total RNA (0.5 μg) was reverse transcribed and amplified using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen). For EGFRvIII (primers previously described; ref. 22) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) the following primers and conditions were used: GAPDH: forward, 5'-TGGATTGCTTACATGGGTG-3' and reverse, 5'-GTGAAGTCCGAGTCAAC-3'. Reverse transcription was carried out for 30 minutes at 50°C followed by 2 minutes at 94°C. PCR amplification was carried out for 40 cycles of 94°C one minute, 67°C one minute, and 72°C one minute followed by a final amplification at 72°C for 5 minutes. To detect only wtEGFR, primers were designed in the exon 2 to 7 region of EGFR. Primers were as follows: forward, 5'-ACAAGCTCAAGCATGTTGGCA-3'; reverse, 5'-GGCAGACGGCAGTCGCTC-3'; conditions were as earlier with denaturation at 62°C.

BCR-Abl/Src inhibitor dasatinib (Das) was a kind gift from Bristol-Myers Squibb. Cetuximab was purchased from the research pharmacy at the University of Pittsburgh Cancer Institute.

**Immunoblotting**

Cell lines and tumor pieces were lysed in detergent containing 1% NP-40, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 mg/mL leupeptin, and 1 mg/mL aprotinin, and protein levels were determined by the Bio-Rad Protein Assay Method (Bio-Rad Laboratories). Total protein (40 μg) were separated on 8% SDS-PAGE and transferred to nitrocellulose membranes with the semidry transfer machine (Bio-Rad Laboratories). Membranes were blocked with Odyssey blocking buffer (Li-Cor Biosciences), probed with primary and subsequently secondary antibodies and visualized with Odyssey Infrared Imaging System (Li-Cor Biosciences) according to manufacturer’s instructions. Quantification of Western blotting was conducted with the Li-Cor Odyssey system to record the near-infrared (NIR) signal according to the manufacturer’s instructions. Primary antibodies used for blotting included β-actin, phospho-Akt (Ser473), Akt, phospho-Src (Y416), Lyn, Fyn, c-Src, and Yes from Cell Signaling Technology (also used for immunoprecipitations); Src B-12 from Santa Cruz Biotechnology; EGFR from BD Transduction Laboratories; and anti-phospho-Src Y416 clone 9A6 from Millipore. Anti-EGFRVIII antibody 4-5H was a kind gift from Dr. Careen Tang (Georgetown University, Washington D.C.). Secondary antibodies used for blotting included goat anti-rabbit IRDye 680 or goat anti-mouse IRDye 800CW (Li-Cor Biosciences).

**Proliferation assays**

To assay proliferation 5,000 cells per well were plated in triplicate in a black-walled 96-well plate and allowed to adhere overnight. Following adhesion, cells were treated with dimethyl sulfoxide (DMSO; control) or 100 nmol/L dasatinib for 72 hours and subsequently assayed with CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to manufacturer’s instructions. Briefly, 100 μl CellTiter-Glo reagent was added to each well and the plate was rocked gently for 2 minutes and the luminescent signal allowed to stabilize for 10 minutes before the plate was read on a VictorV 1420 multilabel counter with Wallac 1420 software (Perkin Elmer). Values were normalized to DMSO vector control cells and plotted in GraphPad Prism (version 4.03; GraphPad Prism Software, Inc.).

**Matrigel invasion assay and cell migration assay**

Cell invasion was evaluated in vitro with Matrigel-coated semipermeable modified Boyden inserts with a pore size of 8 μm (Becton Dickinson/Biocoat). Cell migration was evaluated in vitro using semipermeable modified Boyden inserts with a pore size of 8 μm (Becton Dickinson/Biocoat). For both assays, cells were plated in duplicate at a density of 1.3 × 10^4 cells per well in serum-free media in the insert. At the same time, cells were plated in 24-well plates to serve as loading and cell viability controls. Both the insert and the holding well were subjected to the same medium composition (DMEM with 7 ng/mL EGF; Sigma-Aldrich) with the exception of serum. The insert contained no serum, whereas the lower well contained 10% FBS that served as a chemoattractant. After 24 hours of treatment at 37°C in a 5% CO2 incubator, the cells in the insert were removed by wiping gently with a cotton swab. Cells on the reverse side of the insert were fixed and stained with Hema 3 (Fisher Scientific) according to the manufacturer’s instructions. Cells plated in 24-well plates were subjected to MTT assays, and the cell numbers across the groups were normalized. The number of invading or migrating cells was adjusted accordingly.

**In vivo studies**

Nu/nu athymic nude mice (Harlan Sprague-Dawley) were injected subcutaneously with 5 × 10^6 cells per flank suspended in 100 μL serum free media with Cal33control and Cal33vIII in opposing flanks. Tumor volumes were measured in 2 dimensions with vernier calipers and calculated using the formula: \((\text{length} \times \text{width}^2) \times 0.52\). At the end of the study, mice were killed by cervical dislocation under anesthesia; the tumors surgically excised and snap-frozen in dry ice. Tumors were allowed to develop and 10 days after inoculation tumors were measured and stratified randomization conducted dividing the mice into 2 groups of 8 mice per group. Mice were treated with either 80 mmol/L citric acid in PBS by oral gavage or dasatinib (50 mg/kg daily) by oral gavage. All studies involving animal use and care were in strict compliance with institutional guidelines established by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

**Immunoprecipitation**

HNSCC cells were plated at 50% confluency in a 10-cm dish for 48 hours and harvested as indicated in immunoblotting. For immunoprecipitation cells were serum starved for 24 hours following cell adhesion. One microgram of whole-cell lysate from cell lines or 200 ng from patient specimens, 2 to 4 μg antibody and 40 μL Protein G beads (Millipore) were combined and allowed to rotate overnight.
Beads were pelleted the following day by centrifugation and washed 3 times with fresh lysis buffer. Beads were resuspended in 1× Western blot loading dye and boiled for 8 minutes before loading onto a 10% SDS-PAGE. Lyn, Fyn, c-Src, and Yes antibodies were used (Cell Signaling Technology) for immunoprecipitations and subsequent immunoblotting.

**Patient tissues**

Tumors were obtained from patients with HNSCCs with tumors in the oral cavity, oropharynx, hypopharynx, or larynx who provided written informed consent. Tissues were collected under the auspices of a tissue bank protocol approved by the University of Pittsburgh Institutional Review Board. Tissues were evaluated by a pathologist to confirm more than 70% tumor composition and fresh frozen for further studies. RNA was isolated and assayed for the presence of EGFRvIII by RT-PCR as previously described (19). EGFRvIII bands were excised from the agarose gel, purified by the QIAquick gel extraction kit according to manufacturer's protocol (Qiagen) and sent for standard Sanger sequencing at the University of Pittsburgh Genomics and Proteomics Core Laboratories.

**siRNA transfections**

The siRNA sequences targeting Lyn human mRNA (sense, AUUGUGGAACGCAAAGUCCUU; antisense, GGGAUCUUUGCUUUUCGCAAUUIUU; Sigma-Aldrich) were transfected into HNSCC cells for target silencing. The nontargeting siRNA (D-001210-01: sense, 5'-UAAGCGACUAACAGCUUAU-3'; antisense, 5'-UUGAACUUUGUAGUUGCCGCUAUIU-3'; Thermo Scientific Dharmacon) was used as a control. The siRNA or transfections were conducted with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. HNSCC cells were transfected with 800 pmol of siRNA or nontargeting control siRNA per 10-cm dish. The transfection medium was replaced with complete media after 4 to 6 hours of transfection and cells incubated for 48 hours before plating for invasion and migration assays or harvested for Western blotting.

**Statistical analysis**

For migration and invasion studies, the statistical significance of differences in the number of invading or migrating cells was assessed using Wilcoxon–Mann–Whitney exact test. Statistical analysis and graphs were created in GraphPad Prism (version 4.03; GraphPad Prism Software, Inc.).

**Results**

**EGFRvIII is expressed in engineered HNSCC cell lines**

It has been previously reported that EGFRvIII is present in 17% to 42% of HNSCCs (22–24). EGFRvIII is generally lost in vitro for unknown reasons, consequently EGFRvIII mechanistic studies must be conducted on exogenously transfected model systems (26). We previously reported engineering the Cal33 HNSCC cell line to stably express EGFRvIII (19). For the present study, we also stably expressed EGFRvIII constructs in 2 additional HNSCC cell lines, FaDu and UMCC1. Differential transfection and infection efficiencies exist between cell lines and can affect the level of expression of exogenously introduced constructs. EGFRvIII expression in these cells was confirmed by immunoblots for whole-cell lysates with an EGFRvIII antibody and by RT-PCR of isolated RNA (Fig. 1).

**SFKs mediate proliferation, invasion, and migration in EGFRvIII-expressing HNSCC cells**

We previously reported that expression of EGFRvIII enhances HNSCC cell motility and invasion in vitro and tumor growth in vivo (22, 27). Previous reports show that EGFRvIII does not always increase proliferation in vitro but consistently augments tumor growth in vivo (28, 29). In addition, in EGFRvIII-expressing HNSCC cell cetuximab treatment did not abrogate cell motility or invasion as it did in control cells (19). EGFRvIII has been most studied in glioma where SFKs have increased phosphorylation downstream of EGFRvIII compared with parental cell lines and mediate EGFRvIII-induced cell motility and tumor growth (7). We assessed SFK activation in EGFRvIII-expressing HNSCCs by immunoblotting for phosphorylation at the activation site (Y416). All 3 EGFRvIII-expressing cell lines and vector control cells showed phosphorylation at Y416, which was abrogated by treatment with the SFK inhibitor dasatinib (Fig. 2A). Basal phosphorylation levels of SFKs varied among the HNSCC cell lines used. To assess the role of SFKs in EGFRvIII-expressing HNSCC cell proliferation, cells were treated with dasatinib and assayed for cell proliferation as indicated in materials and methods (Fig. 2B). We found that dasatinib significantly inhibited cell proliferation in EGFRvIII-expressing and vector control cells compared with vehicle-treated cells (P < 0.0001). These results indicate that SFK are activated in vector control- and EGFRvIII-expressing HNSCCs where treatment with the SFK inhibitor dasatinib abrogates proliferation.

We previously reported that EGFRvIII increases cell motility in vitro in HNSCCs where EGFRvIII cells are resistant to...
Dasatinib is known to inhibit several other kinases in addition to SFKs (30) and EGFRvIII has been shown to preferentially signal through several pathways including PI3K/Akt and MAPK in glioma (17, 18). To verify that dasatinib abrogated downstream signaling pathways involved in cell proliferation and motility, we conducted immunoblots of dasatinib-treated EGFRvIII-expressing HNSCC cells to evaluate phosphorylation of the active sites of MAPK, Akt, and FAK. EGFRvIII HNSCC cell lines treated with dasatinib showed reduced phosphorylation of Akt and MAPK (Fig. 3A). EGFRvIII-expressing HNSCC cells exposed to dasatinib also showed reduced FAK phosphorylation at tyrosine 576/577 and tyrosine 861. Both of these phosphorylation sites are phosphorylated by active SFK. As anticipated, phosphorylation of FAK at the autophosphorylation site tyrosine 397 was unchanged following dasatinib treatment of EGFRvIII-expressing HNSCC cells. This site, when autophosphorylated creates a binding site for SFK via the SH2 domain of SFK which activates SFK by displacing the inhibitory phosphorylation at Y527 (ref. 21; Fig. 3B). Phosphorylation of Akt, MAPK, and FAK was significantly reduced by dasatinib in HNSCC cells expressing EGFRvIII as well as vector control cells (STAT3 was also evaluated,
It appears that biochemically dasatinib is equally effective in EGFRvIII and control HNSCC cells.

**Dasatinib inhibits tumor growth of EGFRvIII-expressing HNSCC xenografts**

We previously reported that EGFRvIII-expressing HNSCC xenografts grow more rapidly than controls and are relatively insensitive to cetuximab, the only U.S. Food and Drug Administration approved molecular targeted therapy for HNSCCs (22). Because dasatinib was effective at abrogating proliferation, migration, invasion, and signaling in EGFR-vIII-expressing cells, we hypothesized that inhibition of SFK would show antitumor effects in a nonmetastatic subcutaneous model of EGFRvIII-expressing xenografts in vivo. To test this hypothesis, we inoculated nude mice with vector control- or EGFRvIII-expressing HNSCC cells (Cal33) and initiated treatment when tumors were palpable and of equal volume. Mice were treated with vehicle control or dasatinib to assess the effects of SFK inhibition on tumor volume. EGFRvIII-expressing HNSCC xenografts showed increased tumor volumes over HNSCC xenografts derived from vector-transfected control cells (P = 0.019 on day 20, Fig. 4A). In HNSCC xenografts derived from vector-transfected control cells only expressing wtEGFR, SFK inhibition failed to significantly reduce tumor volume compared with vehicle control treatment (P = 0.19 on day 20, Fig. 4B). However, in EGFRvIII-expressing xenografts, SFK inhibition significantly reduced tumor volume (P = 0.047 on day 20, Fig. 4C; P = 0.02 on day 13, Supplementary Fig. S3A), indicating that SFK may represent a plausible therapeutic target in HNSCCs expressing EGFRvIII. These results were also validated in a second HNSCC xenograft model (FaDu, Supplementary Fig. S3B and S3C). When cetuximab was combined with dasatinib, EGFRvIII-expressing tumor volumes were lower than with dasatinib alone (Supplementary Fig. S3D). Dasatinib did not significantly increase the efficacy of cetuximab in xenografts derived from vector control cells (data not shown). To verify that SFKs were durably inhibited by dasatinib treatment we immunoblotted lysates prepared from control and treated xenografts and probed for phosphorylated SFK. We found that phosphorylated SFK at the activation site was significantly decreased in dasatinib-treated xenografts expressing EGFRvIII and vector control (P = 0.0003, Fig. 4D). We also found no difference in the phosphorylation of SFKs at tyrosine 416 between vector control- and EGFRvIII-expressing xenografts (Fig. 4D).

**Lyn kinase mediates migration and invasion in EGFRvIII-expressing HNSCCs**

Assessment of SFK phosphorylation at the active site (Y416) or pharmacologic inhibition of SFK cannot distinguish the precise role of individual SFKs. Reports in glioma-expressing EGFRvIII indicate that Fyn and c-Src are key effectors of EGFRvIII signaling (7). We therefore sought to determine which SFKs were activated in EGFRvIII-expressing HNSCCs. Previous studies in HNSCC expressing wtEGFR indicated that Lyn, Fyn, c-Src, and Yes are all expressed in HNSCCs (16). We therefore immunoprecipitated Lyn, Fyn, c-Src, or Yes followed by immunoblotting...
with SFK Y416 and total SFK protein antibodies. We found that Lyn was the only SFK showing increased phosphorylation in EGFRvIII-expressing HNSCC cells \( (P = 0.019, \text{Fig. 5A}) \). Lyn, while expressed, was not phosphorylated and c-Src and Yes were not differentially phosphorylated in HNSCC cells expressing EGFRvIII or wtEGFR. Increased phosphorylation of Lyn in EGFRvIII-expressing HNSCC cells was also detected in 2 other head and neck cell lines expressing EGFRvIII and 3 separate clones of the Cal33 EGFRvIII-expressing cell line (Fig. 5B, SCC1, \( P = 0.03 \); Cal33, \( P = 0.01 \); Supplementary Figs. S4 and S5).

To confirm that increased phosphorylation of Lyn in EGFRvIII-expressing HNSCCs was indeed a clinically relevant phenomenon we screened a cohort of 52-patient HNSCC tumors for EGFRvIII expression via RT-PCR followed by sequencing. We found that 12 of 52 (23%) tumors expressed detectable levels of EGFRvIII (Fig. 5C). From this cohort we chose tumors based on tissue availability to conduct Lyn immunoprecipitation. Because of the high level of consensus in the active site sequence of SFKs, there is no pLyn antibody of sufficient specificity for immunohistochemical analysis. Of 22 specimens of patients with HNSCCs, 10 tumors harbored EGFRvIII and 12 tumors without EGFRvIII expression had sufficient tissue for immunoprecipitation. Immunoblotting with p-SFK Y416 and total Lyn protein showed a modest but significant increase in phosphorylation of Lyn in patient tumors with EGFRvIII expression compared with wtEGFR-only tumors \( (P = 0.035, \text{Fig. 5D}) \) showing that phosphorylation of Lyn is increased in EGFRvIII-expressing human HNSCCs.

We next examined the effects of Lyn knockdown on the EGFRvIII phenotype of cell motility to evaluate the hypothesis that phosphorylation of Lyn is a key signaling intermediate for EGFRvIII-mediated invasion and migration. We treated SCC1 HNSCC cells for 48 hours with Lyn siRNA \( (\text{Fig. 6A, siRNA specificity; Supplementary Fig. S6}) \) and subjected them to migration and invasion assays in vitro. We found that Lyn siRNA significantly reduced invasion and migration in EGFRvIII-expressing cells \( (P = 0.014 \text{ for both invasion and migration}) \) and vector control cells \( (P = 0.014, \text{Fig. 6B and C}) \). These results were confirmed in a second HNSCC cell line (FaDu, Supplementary Fig. S7). In SCC1 EGFRvIII-expressing cells the percentage of inhibition in migration (68.5%) and invasion (67.2%) was significantly greater than the percentage of inhibition of vector control–expressing cells (migration 43.2%, invasion...
46.7%; $P = 0.029$ for both) indicating that Lyn is a likely intermediate in EGFRvIII-mediated cell motility.

Discussion

EGFRvIII is reportedly more tumorigenic than wtEGFR (1, 3, 18) despite the fact that there is no difference in the cytoplasmic signaling domain of wtEGFR and EGFRvIII. Differential activation of EGFRvIII signaling pathways compared with wtEGFR has been reported. Altered oncogenic phenotypes may be attributed to differential signaling kinetics due to EGFRvIII retention at the plasma membrane which results in low level constitutive signaling (31). EGFRvIII expression is found in 17% to 42% of HNSCCs (22, 23) in addition to other tumor types including glioma, breast, lung (2, 32), and prostate cancers (33). EGFRvIII has been shown to contribute to increased oncogenicity through various signaling pathways (34) and a recent study indicates that patients with HNSCCs with EGFRvIII have reduced disease control rates and shortened progression-free survival when treated with a cetuximab-containing regimen (24). The mechanisms through which EGFRvIII expression increases oncogenicity and confers cetuximab resistance are incompletely understood.

EGFRvIII in glioma has been shown in vitro and in human tumor samples to show constitutive activation of the PI3K/Akt pathway (17, 35). Blockade of this pathway has been shown to reduce the EGFRvIII-enhanced oncogenic phenotype (17). We previously reported that in EGFRvIII-expressing HNSCCs, inhibition of the PI3K/Akt pathway reduces cell proliferation but has no effect on cell motility or invasion (19). In the present study, Akt phosphorylation was detected in EGFRvIII-expressing HNSCC cells and was abrogated by SFK inhibition (Fig. 3). There are conflicting reports on the role of MAPK signaling in EGFRvIII-expressing models where some studies report activation (18, 36) and others fail to suggest a role (29). In this study, we found that MAPK phosphorylation in EGFRvIII-expressing HNSCC cells was significantly reduced by SFK inhibition (Fig. 3).

Both STAT3 and SFKs have been implicated as key mediators in the EGFRvIII oncogenic phenotype in glioma. In glioma specimens there was a significant correlation between activated STAT3 levels and EGFRvIII (but not wtEGFR; ref. 37). We have previously shown that STAT3 is a SFK-dependent mediator of EGFR-stimulated growth in vitro (38), decreased apoptosis (39), and increased tumor growth in vivo (40). SFKs have been shown to contribute to invasion in EGFRvIII-expressing gliomas where genetic and molecular inhibition of SFKs reduced invasion (7). In HNSCCs expressing only wtEGFR, it has been reported that SFK inhibition using dasatinib reduces cell motility and...
invasion by regulating downstream cell adhesion molecules such as FAK (12). In the present study, we found that dasatinib significantly decreases proliferation, invasion, and cell motility of vector control- and EGFRvIII-expressing HNSCC cells (Fig. 2). Total phosphorylated SFK levels at baseline do not appear to correlate with the biologic effects of dasatinib. This has also been noted previously in HNSCC cells expressing only wtEGFR (12). We also detected reduced Akt and MAPK phosphorylation in addition to decreased FAK phosphorylation by dasatinib treatment (Fig. 3). p-FAK Y397, which is the FAK autophosphorylation site and is a docking site for SFKs appeared to be unchanged with SFK inhibition. Dasatinib treatment of HNSCC cells by Johnson and colleagues also showed no change in p-FAK Y397 levels (12). This is not surprising as integrins and several RTKs activate this site (Y397) which can then recruit SFKs (21).

While invasion and cell motility were significantly inhibited by SFK blockade, it appears that there are other mechanisms that contribute to EGFRvIII-expressing HNSCC-mediated invasion and cell motility as cell motility was not entirely abrogated in the presence of SFK inhibition. Persistent phosphorylation of FAK at Y397 may contribute to invasion and migration even in the setting of dasatinib treatment.

Dasatinib is known to inhibit SFKs as well as Abl, c-kit, PDGFR, and EphA2 (30, 41). Studies in HNSCCs show that treatment with imatinib (an inhibitor of Abl, c-kit, and PDGFR) did not affect cell-cycle progression or apoptosis (42, 43). EphA2 is inhibited by dasatinib but dasatinib inhibition of EphA2 activation levels did not correlate with the effects of dasatinib on cell-cycle progression or apoptosis (12). It is therefore likely that not all of the effects observed from dasatinib treatment in HNSCCs are due to SFK inhibition.

In glioma several studies have evaluated the effects of altered SFK activity on EGFRvIII-expressing tumors. Genetic disruption of c-Src using a dominant-negative approach in EGFRvIII-expressing glioma xenografts decreased tumor growth rates and significantly increased the efficacy of EGFRvIII-specific antibody treatment (6). Dasatinib has also showed antitumor effects in glioma model systems. In an endogenously expressing EGFRvIII in vivo model, dasatinib has been shown to inhibit growth and increase survival and apoptosis (7). SFK inhibition through dasatinib treatment of HNSCC xenografts has been primarily reported in the context of downstream signaling markers and the effects of dasatinib on tumor growth in vivo or metastasis are still being defined (44). We found that in vivo treatment with dasatinib had a more profound inhibitory effect on tumor volumes in EGFRvIII-expressing HNSCC xenografts compared with tumors derived from vector control cells expressing wtEGFR only (Fig. 4). This is in agreement with a recent report that a wtEGFR HNSCC orthotopic xenograft model treated with dasatinib alone had no effect on tumor volume (45). The cause of the discrepancy between in vitro and in vivo efficacies in wtEGFR-expressing HNSCCs is as yet unknown but may be related to differences in drug exposure (in vitro cells receive a single dose for 24–72 hours whereas in vivo dosing occurs daily over weeks). These results indicate that tumor growth of EGFRvIII-expressing tumors is, at least in part, by SFK signaling. In glioma, EGFRvIII did not appear to initiate differential expression of specific SFKs but rather preferentially activated Fyn and c-Src compared with Lyn, Yes, Hck, and Blk (7). Lyn has been implicated as a key signaling mediator in several solid cancers including prostate (46), glioma (47), and breast (48). Further experiments showed that while Fyn, c-Src, and

Figure 6. Knockdown of Lyn inhibits EGFRvIII-mediated HNSCC migration and invasion. A, UMSCC1 vector and EGFRvIII-expressing cells were treated with Lyn siRNA for 48 hours and immunoblotted to confirm Lyn knockdown. B, UMSCC1 vector control- (open bars) and EGFRvIII-expressing (closed bars) cells were assayed for cell motility in the presence of nontargeting siRNA or Lyn siRNA. The experiment was repeated 4 times and assessed for significance by the Mann–Whitney test, *P = 0.014. C, UMSCC1 vector control- (open bars) and EGFRvIII-expressing (closed bars) cells were assayed for cell invasion in the presence of nontargeting siRNA or Lyn siRNA. The experiment was repeated 4 times and assessed for significance by the Mann–Whitney test, *P = 0.014. NTC, nontargeting control.
Yes were phosphorylated equally in EGFRvIII and vector control–expressing cells, Lyn phosphorylation was increased in EGFRvIII cells and human HNSCC tumors expressing EGFRvIII. It is therefore plausible that the increased phosphorylation of Lyn in EGFRvIII-expressing cells contributes to the greater efficacy of SFK inhibition to EGFRvIII-expressing HNSCCs in vivo. Evaluation of patient HNSCC samples showed increased phosphorylation of Lyn in EGFRvIII-expressing HNSCCs. Selective targeting of Lyn expression using siRNA inhibited the migration and invasion of EGFRvIII-expressing HNSCC cells, implicating a functional role of increased Lyn activation (Fig. 6). To our knowledge, this is the first report of a specific role for Lyn in HNSCCs. These results suggest that therapeutic agents that selectively target Lyn could be effective in this cancer (49). The Lyn/BCR-ABL inhibitor bafetinib is currently under investigation in phase II clinical trials for patients with prostate cancer, B-cell chronic lymphocytic leukemia, and brain tumors (50). To the best of our knowledge there are limited studies in HNSCCs that have evaluated lymph node and distant metastasis. In a wtEGFR orthotopic mouse model (44) with dasatinib treatment no metastasis of any type was identified in control or treatment groups. Further investigation of SFK/Lyn inhibition in metastatic orthotopic mouse models is warranted.

The majority of reports on EGFRvIII in cancer conclude that EGFRvIII contributes to increased tumor growth and poor prognosis. In HNSCCs, EGFRvIII expression has been associated with cetuximab resistance in preclinical models and now in a human HNSCC cohort (24). Therapeutic strategies that target SFK, specifically Lyn, may be effective alternative strategies in these patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.E. Wheeler, J.R. Grandis
Development of methodology: S.E. Wheeler, S.I. Chiosea, R.R. Seethala
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.E. Wheeler, J.S. Bednash, C.G. Otte, R.R. Seethala, S.I. Chiosea
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.E. Wheeler, E.M. Morariu, C.G. Otte
Writing, review, and/or revision of the manuscript: S.E. Wheeler, S.I. Chiosea, J.R. Grandis
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E.M. Morariu, R.R. Seethala, S.I. Chiosea
Study supervision: S.E. Wheeler, J.R. Grandis

Grant Support
The study was supported by P50CA097190, R01CA098372, I01CA84968, Bristol-Myers Squibb, and the American Cancer Society (to J.R. Grandis), 1F31DE020223 (to S.E. Wheeler).

Received September 27, 2011; revised March 12, 2012; accepted March 27, 2012; published OnlineFirst April 6, 2012.

References


Clinical Cancer Research

Lyn Kinase Mediates Cell Motility and Tumor Growth in EGFRvIII-Expressing Head and Neck Cancer

Sarah E. Wheeler, Elena M. Morariu, Joseph S. Bednash, et al.

Clin Cancer Res  Published OnlineFirst April 6, 2012.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-11-2486
Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2012/04/05/1078-0432.CCR-11-2486.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.