Degradation of Epidermal Growth Factor Receptor Mediates Dasatinib-Induced Apoptosis in Head and Neck Squamous Cell Carcinoma Cells


*Graduate Institute of Pharmacology, National Taiwan University College of Medicine, Taipei, Taiwan; †Department of Oncology, National Taiwan University Hospital, Taipei, Taiwan; ‡Division of Oncology and Hematology, Department of Internal Medicine, Far-Eastern Memorial Hospital, Taipei, Taiwan; §National Center of Excellence for Clinical Trial and Research, National Taiwan University Hospital, Taipei, Taiwan; ¶Medical Research, National Taiwan University Hospital, Taipei, Taiwan; #Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan

Abstract

Epidermal growth factor receptor (EGFR) is an important oncoprotein that promotes cell growth and proliferation. Dasatinib, a bcr-abl inhibitor, has been approved clinically for the treatment of chronic myeloid leukemia and demonstrated to be effective against solid tumors in vitro through Src inhibition. Here, we disclose that EGFR degradation mediated dasatinib-induced apoptosis in head and neck squamous cell carcinoma (HNSCC) cells. HNSCC cells, including Ca9-22, FaDu, HSC3, SAS, SCC-25, and UMSCC1, were treated with dasatinib, and cell viability, apoptosis, and underlying signal transduction were evaluated. Dasatinib exhibited differential sensitivities against HNSCC cells. Growth inhibition and apoptosis were correlated with its inhibition on Akt, Erk, and Bcl-2, irrespective of Src inhibition. Accordingly, we found that down-regulation of EGFR was a determinant of dasatinib sensitivity. Lysosome inhibitor reversed dasatinib-induced EGFR down-regulation, and c-cbl activity was increased by dasatinib, indicating that dasatinib-induced EGFR down-regulation might be through c-cbl-mediated lysosome degradation. Increased EGFR activation by ligand administration rescued cells from dasatinib-induced apoptosis, whereas inhibition of EGFR enhanced its apoptotic effect. Estrogen receptor α (ERα) was demonstrated to play a role in Bcl-2 expression, and dasatinib inhibited ERα at the pretranslational level. ERα was associated with EGFR in dasatinib-treated HNSCC cells. Furthermore, the xenograft model showed that dasatinib inhibited HSC3 tumor growth through in vivo down-regulation of EGFR and ERα. In conclusion, degradation of EGFR is a novel mechanism responsible for dasatinib-induced apoptosis in HNSCC cells.

Neoplasia (2012) 14, 463–475

Abbreviations: HNSCC, head and neck squamous cell carcinoma; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; E2, estradiol; ER, estrogen receptor

Address all correspondence to: Ching-Chow Chen, PhD, Graduate Institute of Pharmacology, National Taiwan University College of Medicine, No. 1, Jen-Ai Rd, Section 1, Taipei 10018, Taiwan. E-mail: chingchowchen@ntu.edu.tw

© 2012 Neoplasia Press, Inc. All rights reserved 1522-8002/12/$25.00 DOI 10.1596/ne.12300
Introduction
Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK) that belongs to the ErbB family. Once activated, EGFR transduces signaling through the Ras-MAPK and PI3K-Akt pathways to promote cellular growth, proliferation, and survival [1]. Overexpression of EGFR is frequently found in epithelial cancers. Studies in head and neck squamous cell carcinoma (HNSCC) have shown that 80% to 100% of tumors have a high level of EGFR expression, which is correlated with advanced stage of disease and poor prognosis [2-4]. EGFR-targeted therapy, including monoclonal antibody and small molecules, has led to advance of cancer treatment. Cetuximab, a monoclonal antibody against EGFR, demonstrates its clinical activity and has been approved to be the first molecular targeted therapy for HNSCC [5,6].

Estrogen receptor (ER) is a nuclear receptor localized in both cytoplasm and nucleus. ER exerts its nuclear activity by directly binding to DNA or interacting with other transcription factors [7]. In addition, ER has rapid cytoplasmic actions by interacting with other growth factor pathways through kinase cascade, Ca2+, and other second messengers, which regulate gene transcription, cell proliferation, and cell survival [8,9]. There are two different forms of the estrogen receptor, ERα and ERβ. ERα expresses in endometrium, breast, ovary, and hypothalamus, whereas ERβ in kidney, brain, bone, heart, lung, intestinal mucosa, prostate, and vascular endothelium [10]. ERα is well known to be closely associated with breast cancer carcinogenesis and prognosis and serves as an important therapeutic target [11]. The significance of ERα signaling in lung, esophagus, and HNSCC has also been reported [12-14]. In HNSCC, ERα signaling interacts with EGFR to enhance cell growth and invasion and confers poor clinical outcome [14].

HNSCC is a heterogeneous disease composed of oral, oropharyngeal, hypopharyngeal, and laryngeal squamous cell carcinoma. It is closely associated with alcohol, betel nut, and cigarette. In Taiwan, betel nut chewing is a problem of public health, and the incidence of oral cancer is higher than that of Western countries. Despite aggressive treatment, recurrence or metastasis is common, and most patients will die within 1 year of disease recurrence [15]. Thus, development of new therapies is urgent.

Dasatinib is a protein kinase inhibitor targeting bcr-abl, the hallmark of chronic myeloid leukemia, and has been clinically approved to treat chronic myeloid leukemia [16]. Dasatinib also exhibits a broad spectrum of protein kinase inhibition including c-kit, platelet-derived growth factor receptor, ephA2, and Src. Because Src is an oncprotein closely associated with solid tumor progression, metastasis, and poor outcome, dasatinib has been reported to inhibit the growth, migration, and invasion of non-small cell lung cancer (NSCLC) and HNSCC cells [17-19]. However, dasatinib exerts limited activities against NSCLC and HNSCC in clinical trials despite consistent Src inhibition [20,21], implicating that there are mechanisms beyond Src inhibition responsible for the efficacy of dasatinib. In our study, degradation of EGFR was seen in dasatinib-sensitive but not dasatinib-resistant HNSCC cells. The role of EGFR was further validated in dasatinib-induced apoptosis. In addition, Erα played a role and was correlated with EGFR to regulate dasatinib-induced apoptosis. We demonstrated a novel mechanism responsible for dasatinib-induced apoptosis in HNSCC.
with 2% paraformaldehyde in PBS for 15 minutes and permeabilized with 1% Triton X-100 in PBS for 15 minutes. Cells were incubated with anti-EGFR antibody (dilution, 1:100) and anti-lysosomal-associated membrane protein 1 (LAMP-1) antibody (dilution, 1:100) for 1 hour. Secondary antibody was applied to anti-EGFR antibody for 1 hour. For labeling DNA, cells were stained with 4',6-diamidino-2-phenylindole (10 μg/ml) for 20 minutes. Cells were imaged on a Leica SP5 confocal microscope with ×40 oil objective (Leica, Buffalo Grove, IL). Laser lines of 488 and 543 nm were used for sequential excitation. Brightness and contrast of the images were adjusted in PhotoShop CS (Adobe Systems Incorporated, San Jose, CA).

Preparation and Infection of shEGFR-Expressing Lentivirus

Briefly, 6 μg of pCMV-dR8.91, 3 μg of pMD2.G, and 9 μg of pLKO-shLuciferase and pLKOshEGFR were co-transfected into HEK293T cells containing Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The supernatants containing infectious shLuciferase or shEGFR1 lentivirus were collected on day 3 after transfection and stored at −80°C. For lentivirus infection, 2 × 10⁵ HNSCC cells were infected with shLuciferase or shEGFR1 lentivirus at a multiplicity of infection of 1. The short hairpin RNA (shRNA) sequence of EGFR is CCGGGCTGCTCTGAAATCTCCTTTACTCTGAGTAAAGGAGATTTCAGAGCAGCTTTTTG. The shRNA sequence of luciferase is CCGGCAAATCACAGAATCGTCGTATGAGTAAAGGAGATTTCAGAGCAGCTTTTTG. For lentivirus infection, 6 μg of pMD2.G, and 9 μg of pLKOshLuciferase were cotransfected into HEK293T cells containing Infecton, San Jose, CA).

Real-time Polymerase Chain Reaction

Total RNA was isolated from HNSCC cells using TRIzol reagent (Life Technologies, Camarillo, CA). Reverse transcription reaction was performed using 2 μg of total RNA, which was reverse transcribed into complementary DNA using oligo dT primer. Real-time polymerase chain reaction (PCR) was performed with complementary DNA samples using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers were as follows: EGFR (forward primer, 5′-TTCTTCCCAATGCTCTGAAT-3′; reverse primer, 5′-GGTTCAGGCTGTAT-GTGTAT-3′), ERα (forward primer, 5′-CTCTAACTTTGCTCTT-ACAGC 3′; reverse primer, 5′-TCG-GTTCGGC ATGATGAACT-3′), and actin (forward primer, 5′-CCAACGC-CAGAAGATGA-3′; reverse primer, 5′-TCCATCACAGTGCAGT-3′). Data were normalized by the actin housekeeping gene detection.

Subcutaneous Ectopic Xenograft Tumor Model

Eighteen male NCr athymic nude mice (4 weeks of age) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). At the age of 6 weeks, 3 × 10⁶ HSC3 cells (suspended in 0.1 ml of PBS and mixed with 0.1 ml of Matrigel) were inoculated subcutaneously in the rear left flank. Dasatinib was dissolved in citrate buffer (pH = 3.1). When tumors reached 100 mm³, mice were randomized to receive oral dasatinib (n = 9, 80 mg/kg) or vehicle (n = 9, citrate acid) 5 d/wk for 4 weeks. Tumor volume is calculated using the formula V (mm³) = ab² / 2, where a is the length and b is the width of the tumor.

Results

Differential Sensitivities of HNSCC Cells to Dasatinib

We first evaluate the growth-inhibitory effect of dasatinib on six HNSCC cell lines including Ca9-22, FaDu, HSC3, SAS, SCC-25, and UMSCC1. By MTT assay, median inhibitory concentrations (IC₅₀) ranged from 280 nM to more than 10 μM (Table 1). Dasatinib exhibited a significant growth-inhibitory effect on Ca9-22, HSC3, SCC-25, and UMSCC1 cells but not FaDu and SAS cells (Figure 1A). Therefore, six HNSCC cells are classified into dasatinib-sensitive and -resistant cells by their IC₅₀ (Table 1).

The sub-G₁ fraction was analyzed by cell cycle analysis to determine the apoptotic effect induced by dasatinib after 48 hours of treatment. Dasatinib induced apoptosis in Ca9-22, HSC3, and SCC-25 cells, whereas this was not observed in UMSCC1, FaDu, and SAS cells (Figure 1B). Similar results were seen in the down-regulation of Bcl-2 and cleavage of pro-apoptotic 3 in Ca9-22, HSC3, and SCC-25 but not in UMSCC1, FaDu, and SAS cells (Figure 1C).

The expression of proapoptotic BAX and BAK proteins and antiapoptotic Bcl-2, Mcl-1, and Bcl-XL proteins was further examined to explore the mechanism of dasatinib-induced apoptosis. The decreased expression of Bcl-2 by dasatinib in sensitive HSC3 was seen (Figure 1D), whereas the level of proapoptotic and other antiapoptotic proteins was not affected. In dasatinib-resistant SAS cells, all test proteins were not affected.

Inactivation of Akt and Erk but Not Src Correlated with Dasatinib Sensitivity

The interaction of Src and RTK is well characterized that RTK may act both upstream and downstream of Src [23]. P13k-Akt and Ras-MAPK(Erk) pathways, which regulate cellular growth, proliferation, and survival, are downstream of RTK [24]. Therefore, the activation of Src (phosphor-Src, p-Src, Y416), Akt (phosphor-Akt, p-Akt, S473), and Erk (phosphor-Erk, p-Erk, T202/Y204) was evaluated after treatment with dasatinib for 24 hours. Inactivation of Src as well as Akt and Erk in a dose-dependent manner was seen in dasatinib-sensitive cells; however, only Src was inactivated in dasatinib-resistant cells (Figure 2).

Down-regulation of EGFR Correlated with Dasatinib Sensitivity

Given that inhibition of Akt and Erk was correlated with dasatinib sensitivity, we examined the activities of membrane RTKs. EGFR and c-MET are well-known membrane RTKs expressed in HNSCC, which are closely associated with carcinogenesis and tumor progression [14,25]. The activation of EGFR (phosphor-EGFR, p-EGFR, Y1068) was inhibited in sensitive but not resistant cells, and similar results were also seen in the expression of EGFR (Figure 3, A and B). However, the

<table>
<thead>
<tr>
<th>Cell Name</th>
<th>Site of Origin</th>
<th>IC₅₀ (μM)</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMSCC1</td>
<td>Mouth floor</td>
<td>0.28</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Ca9-22</td>
<td>Gingiva</td>
<td>0.45</td>
<td>Sensitive</td>
</tr>
<tr>
<td>SCC-25</td>
<td>Tongue</td>
<td>0.62</td>
<td>Sensitive</td>
</tr>
<tr>
<td>HSC3</td>
<td>Tongue</td>
<td>0.78</td>
<td>Resistant</td>
</tr>
<tr>
<td>FaDu</td>
<td>Pharynx</td>
<td>&gt;10</td>
<td>Resistant</td>
</tr>
<tr>
<td>SAS</td>
<td>Tongue</td>
<td>&gt;10</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

Table 1. Phenotype Classification of HNSCC Cells.
Dasatinib Downregulated EGFR through Lysosomal Degradation

It is well known that c-cbl mediates EGFR ubiquitination followed by its internalization and degradation in lysosome [26]. Proteasome has also shown to play a role in EGFR degradation [27,28]. By confocal microscopy, the expression of LAMP-1 (green), which is closely related to late endosome activity [29], was increased by dasatinib in sensitive HSC3 but not resistant SAS cells (Figure 3C). Dasatinib-induced EGFR down-regulation was reversed by NH₄Cl (lysosome inhibitor) but not lactacystin (proteasome inhibitor) in sensitive HSC3 and Ca9-22 cells (Figure 4A, lanes 4 and 5). C-cbl activity (phosphor-774, Y774) was increased within 4 hours by dasatinib (Figure 4C), indicating that c-cbl–mediated lysosome degradation might play a role in dasatinib-induced EGFR degradation.

EGFR Played a Role in Dasatinib-Induced Apoptosis

We further examined the role of EGFR as well as its downstream signaling in dasatinib-induced apoptosis. Epidermal growth factor (EGF) has been reported to activate EGFR and subsequently induce EGFR degradation [29]. In sensitive HSC3 and Ca9-22 cells, dasatinib down-regulated EGFR and inactivated its downstream Akt and Erk. Enhancement of EGFR activation (p-EGFR, Y1068) by the addition of EGF (10 ng/ml) abolished dasatinib-induced apoptosis as well as its inactivation of Akt and Erk (Figure 5, A and B, lanes 3 and 4). Nevertheless, inhibition of EGFR activation by erlotinib (3 μM) enhanced dasatinib-induced apoptosis as well as its inactivation of Akt and Erk in Ca9-22 and HSC3 cells (Figure 5, C and 5D, lanes 3 and 4). Knockdown of
Figure 2. Effect of dasatinib on Src, Akt, Erk, and Bcl-2. Inactivation of Src, Akt, and Erk in dasatinib-sensitive and -resistant cells treated with dasatinib at the indicated doses for 24 hours. Upper panel, Western blot analysis of HNSCC cells. Representative of three independent experiments. Lower panel, Immunoblots were quantitated to determine the ratio of p-Src to actin, p-Akt to actin, and p-Erk to actin.
EGFR by shRNA also enhanced dasatinib-induced apoptosis and its inhibition on Akt and Erk in HSC3 cells (Figure 5E, lanes 3 and 4).

In dasatinib-resistant SAS cells, dasatinib neither downregulated EGFR nor inactivated the downstream Akt and Erk (Figure 5E, lanes 1 and 3). However, after EGFR was knocked down by shEGFR, dasatinib could induce inactivation of Akt and Erk and apoptosis (Figure 5E, lanes 3 and 4). Taken together, EGFR plays a role in dasatinib-induced apoptosis.

**Figure 3.** Down-regulation of EGFR by dasatinib correlates with the sensitivity of dasatinib. (A, B) Expression of EGFR and downstream Akt and Erk in dasatinib-sensitive (A) and -resistant (B) cells treated with dasatinib 1 μM for indicated intervals. Upper panel, Western blot analysis of HNSCC cells. Representative of three independent experiments. Lower panel, Immunoblots were quantitated to determine the ratio of EGFR to actin. (C) Evaluation of EGFR and LAMP-1 expression by fluorescence confocal microscopy. HNSCC cells were treated with DMSO or dasatinib 1 μM for 24 hours.
ERα Was Correlated with EGFR to Regulate Dasatinib-Induced Apoptosis

ERα has been reported to regulate Bcl-2 expression through binding to estrogen response element in the promoters of Bcl-2 gene [30,31]. Because dasatinib downregulated Bcl-2 in some HNSCC cells (Figure 1C), its effect on ERα was examined. Dasatinib downregulated ERα in sensitive but not resistant cells (Figure 6A). The mechanism of ERα down-regulation was further investigated. Cycloheximide, an mRNA translation inhibitor, decreased ERα level in a time-dependent manner, which was reversed by the addition of dasatinib (Figure 6B), indicating that dasatinib increased ERα stability. The effect of dasatinib on ERα mRNA transcription was further examined. Real-time PCR showed that dasatinib decreased ERα mRNA level in sensitive cells but not resistant cells, indicating that dasatinib-induced

---

**Figure 4.** Mechanism of dasatinib-induced EGFR down-regulation. (A) Effect of lysosome inhibitor (NH₄Cl, 20 mM) and proteasome inhibitor (lactacystin, 10 μM) on dasatinib-induced EGFR down-regulation in HSC3 and Ca9-22 cells. Lower panel, Western blot analysis of HNSCC cells. Upper panel, Immunoblots were quantitated to determine the ratio of EGFR to actin. Column, mean (n = 3); bar, SD; *P < .05 by paired Student’s t test. Representative of three independent experiments. (B) Effect of dasatinib on EGFR mRNA transcription in HSC3 and SAS cells treated with dasatinib 1 μM for designated intervals. (C) Effect of dasatinib on c-cbl activity in HSC3 and Ca9-22 cells. Representative of three independent experiments.
ERα down-regulation was attributed to the inhibition of ERα mRNA transcription (Figure 6C).

The role of ERα in dasatinib-induced apoptosis was examined. Addition of estradiol (E2) to dasatinib-treated Ca9-22 cells increased Bcl-2 level and rescued cells from apoptosis (Figure W1A). The correlation between EGFR and ERα was further examined. In sensitive cells, addition of E2 to Ca9-22 cells partially reversed dasatinib-induced EGFR down-regulation (Figure 6D, upper panel), and activation of

Figure 5. Role of EGFR in dasatinib-induced apoptosis. (A, B) Effect of EGF addition to dasatinib (1 μM)-induced apoptosis and inactivation of Akt and Erk in sensitive HSC3 (A) and Ca9-22 (B) cells. Cells were treated with dasatinib (1 μM, 0 hour) with or without EGF (10 ng/ml, 24 hours [15 minutes before lysate collection]). (C, D) Effect of EGFR inhibition by erlotinib (3 μM) on dasatinib (1 μM)-induced apoptosis and inactivation of Akt and Erk in sensitive HSC3 (C) and Ca9-22 (D) cells. (E, F) Effect of EGFR knockdown in sensitive HSC3 (E) and resistant SAS (F) cells treated with dasatinib (1 μM). Cells were transfected with control shRNA or EGFR shRNA for 72 hours. Cells were prepared for Western blot analysis after 24 hours of dasatinib treatment and for sub-G1 analysis after 48 hours of treatment. Column, mean (n = 3); bar, SD; *P < .05 by paired Student’s t test. Representative of three independent experiments.
Figure 6. Correlation of ERα and EGFR in dasatinib-treated HNSCC cells. (A) Expression of ERα in HNSCC cells treated with dasatinib at 1 μM for designated intervals. (B) Protein stability of ERα. Left, Expression of ERα in HSC3 cells treated with cycloheximide (10 μg/ml) alone or in combination with dasatinib (1 μM) for designated intervals. Right, Immunoblots were quantitated to determine the ratio of ERα to actin. (C) Effect of dasatinib (1 μM) on ERα mRNA transcription in dasatinib-sensitive HSC3 and -resistant SAS cells. Columns, mean (n = 3); bars, SD; *P < .05 by paired Student’s t test. (D) Correlation of EGFR and ERα. Upper panel, Effect of E2 (1 nM) addition on dasatinib (1 μM)-induced EGFR down-regulation and EGF (100 ng/ml) addition on dasatinib-induced ERα down-regulation in Ca9-22 cells. Lower panel, Effect of EGFR inhibition by erlotinib (1 μM) or shRNA on ERα in SAS cells treated with dasatinib (1 μM). Cells were treated with indicated agents for 24 hours. Immunoblots were quantitated to determine the ratio of EGFR to actin and ERα to actin.
Dasatinib Degrades EGFR in HNSCC Cells

Lin et al.
Neoplasia Vol. 14, No. 6, 2012

Figure 7. In vivo effect of dasatinib on xenograft nude mice. (A) Antitumor effect of dasatinib on HSC3 tumors. Line, mean (n = 9); bars, SE; *P < .05 by unpaired Student’s t test. (B) Left panel, Photograph of representative nude mice treated with vehicle or dasatinib. Right panel, Comparison of tumor weight in two groups after the 4-week treatment. Column, mean (n = 9); bars, SE; *P < .05 by unpaired Student’s t test. (C) Expression of EGFR and ERα in vehicle- or dasatinib-treated HSC3 tumors. Left, Western blot analysis of EGFR, ERα, and p-Src in HSC3 tumors. The homogenates of four representative tumors treated with vehicle or dasatinib (80 mg/kg) for 28 days were analyzed. Right, Immunoblots were quantitated to determine the ratio of EGFR to actin, ERα to actin, and p-Src to actin. Column, mean (n = 4); bars, SE; *P < .05 by unpaired Student’s t test; NS, not significant. (D) Schematic illustration of the effect of dasatinib-induced EGFR and ERα down-regulation.

EGFR by EGF stimulation reversed dasatinib-induced ERα down-regulation (Figure 6D, upper panel). Similarly, inhibition of EGFR activation by erlotinib enhanced dasatinib-induced ERα down-regulation in sensitive HSC3 cells (Figure W1B). Although ERα was not affected by dasatinib in resistant SAS cells, it was inhibited by dasatinib in combination with erlotinib or EGFR shRNA (Figure 6D, lower panel). All these results indicate that ERα is correlated with EGFR in dasatinib-treated HNSCC cells.
Effect of Dasatinib on HNSCC Xenograft Tumor

To confirm possible clinical implications, the in vivo effect of dasatinib on HNSCC xenograft tumors was assessed. HSC3 cells were subcutaneously injected into dorsal part of nude mice. Vehicle (citrate buffer) or dasatinib at a dose of 80 mg/kg was orally given 5 d/wk for 4 weeks. Dasatinib significantly inhibited HSC3 tumor growth (Figure 7, A and B; unpaired Student’s t test, P < .05). Ulcerative wound and eschar formation were observed in dasatinib-treated tumors, which might be attributed to treatment-related tumor necrosis. All mice tolerated this treatment well without significant toxicity and had stable body weights. In addition, dasatinib-treated HSC3 tumors showed inhibition of Src and down-regulation of EGFR and ERα (Figure 7C, Student t test, P < .05), indicating that both EGFR and ERα played critical roles in the antitumor effect of dasatinib in vivo.

Discussion

Molecular targeting therapy has become a relevant strategy in cancer treatment within the past decade, in which proper patient selection by predictive biomarkers leads to the success. For example, overexpression of HER2 on trastuzumab treatment of breast cancer, distinct EGFR mutation on gefitinib treatment of NSCLC, and wild type k-ras on cetuximab treatment of colorectal cancer have been the paradigms of predictive biomarkers for molecular targeted therapy [32–34]. Dasatinib exerts limited clinical activity in unselected recurrent HNSCC patients (0% objective response and 16.7% stable disease) despite consistent Src inhibition [20,21]. We found that degradation of EGFR mediated dasatinib-induced apoptosis in HNSCC cells. In addition, ERα was associated with EGFR in dasatinib-treated HNSCC cells. Our study not only demonstrated a new mechanism responsible for dasatinib-induced apoptosis in HNSCC cells but also provided a molecular basis to explore the dasatinib-sensitive cellular feature and predictive biomarker. Complexities of cell signaling in advanced cancer including recurrent HNSCC may result in the activation of feedback mechanisms. Therefore, the combination of molecular targeted therapy is a rational approach. We found that EGFR was differentially degraded by dasatinib, providing the basis for combination of dasatinib with EGFR inhibitor to treat HNSCC. Dasatinib, in combination with cetuximab, was well tolerated in patients with solid tumors [35], and clinical trial is ongoing. A pharmacodynamic study shows that Src activity is inhibited by dasatinib (at a dosage of 100 mg/d) rapidly but recovered within 6 to 12 hours [21]. Because dasatinib 100 mg/d is well tolerated [21], dose escalation is a possible strategy to overcome the recovery of Src activity.

Akt and Erk are signal hubs from membrane RTK [24]. Because Akt and Erk were inactivated by dasatinib, the activation of EGFR and c-MET, well-known RTKs in HNSCC [14,25], was examined. Dasatinib not only inhibited EGFR activation but also downregulated EGFR expression in sensitive but not resistant cells. Its inhibition on c-MET was variable and not correlated with the sensitivity. Sen et al. [36] reported that inhibition of c-MET activation was the determinant of dasatinib in HNSCC, and they found the contribution of EGFR to c-MET activation. The association of EGFR and c-MET has also been reported in NSCLC, in which MET amplification leads to acquired resistance of gefitinib and potentiates EGFR action independent of the hepatocyte growth factor [37,38]. The association between EGFR and c-MET in HNSCC cells deserves further investigation.

It is well known that c-cbl–mediated EGFR ubiquitination promotes its degradation after ligand-induced activation [29]. We found that late endosome activity was increased by dasatinib in sensitive cells, and lysosome but not proteasome inhibitor reversed dasatinib-induced EGFR degradation. Therefore, lysosome might play a role in dasatinib-induced EGFR degradation. In addition, c-cbl activity was increased by dasatinib, indicating that c-cbl–mediated lysosome pathway might be responsible for dasatinib-induced EGFR degradation.

Cisplatin or gemcitabine has been reported to degrade EGFR in sensitive HNSCC cells [39,40]. Increase in cisplatin- or gemcitabine-induced cytotoxicity is seen with EGF addition, which enhances EGFR degradation, whereas cytotoxicity is decreased with the addition of gefitinib or erlotinib, which attenuates EGFR degradation [39,40]. By inhibiting EGFR degradation, addition of bortezomib decreases the effect of cetuximab plus radiotherapy in HNSCC cells [41]. Our recent work also shows that HDAC inhibitors induced cytotoxicity in colorectal cancer cells through down-regulation of EGFR [42]. Therefore, degradation of EGFR plays a critical role in anticancer treatment. The status of cell cycle also has an impact on the effect of chemotherapy in combination with EGF or EGFR inhibitor (gefitinib or erlotinib). Because gemcitabine cytotoxicity is S phase–dependent and EGFR inhibitor induces G1 arrest [43], gemcitabine cytotoxicity is enhanced by the addition of gefitinib after gemcitabine but is attenuated by gefitinib before gemcitabine [43]. EGF can accelerate cell cycle progression to potentiate gemcitabine cytotoxicity [43]. In the present study, cell cycle acceleration by EGF addition reversed apoptosis induced by dasatinib, which induces G1 arrest [19], whereas erlotinib inducing G1 arrest enhanced the effect of dasatinib. Erlotinib also inhibits EGFR activity, and EGF addition activates EGFR, indicating that EGFR activity rather than EGFR per se might determine dasatinib-induced apoptosis.

Up-regulation of antia apoptotic proteins might play a role to protect HNSCC cells from dasatinib-induced apoptosis. We found that dasatinib induced growth inhibition but not apoptosis in UMSCC1 cells, in which inactivation of Akt and Erk but up-regulation of Bcl-2 were seen. Up-regulation of Bcl-2 might play a protective role from apoptosis despite inactivation of Akt and Erk in UMSCC1 cells. In addition, Src inhibition in some HNSCC cells leads to activation of STAT3, which upregulates its downstream antia apoptotic Bcl-XL and Mcl-1 proteins [44–46]. In our study, dasatinib did not affect the expression of Bcl-XL or Mcl-1 in either sensitive HSC3 or resistant SAS cells, indicating that STAT3 activation might not occur in these cells.

We found a negative correlation between Bcl-2 expression and dasatinib-induced apoptosis. Bcl-2 has been reported to be regulated by ERα through its nuclear action [30,31]. Although protein stability of ERα was increased by dasatinib, ERα was downregulated by dasatinib through inhibition of mRNA transcription. The increased ERα protein stability might be attributed to dasatinib-induced Src inhibition because ERα degradation was promoted by Src activation [47]. Addition of estrogen upregulated Bcl-2 and attenuated apoptosis, validating the role of ERα in dasatinib-induced apoptosis. The crosstalk between ERα and RTK family receptors is best characterized in breast cancer. HER2 activation leads to the resistance of hormonal therapy, and ERα signaling attenuates the antitumor effect of HER2 inhibition [48]. Recently, estrogen has been reported to be associated with early and late carcinogenesis of HNSCC, and ERα works in concert with EGFR to promote cellular growth and invasion and confers poor prognosis of HNSCC [14,49]. We found that ERα was correlated with EGFR in dasatinib-treated HNSCC cells, supporting the association between EGFR and ERα in HNSCC.

In conclusion, we demonstrate that degradation of EGFR mediates dasatinib-induced apoptosis in HNSCC cell. Our study also discloses a new mechanism responsible for apoptosis, which provides a
molecular framework to explore the predictive biomarker of dasatinib in HNSCC.

References


Figure W1. (A) Effect of E_2 (1 nM) addition on dasatinib (1 μM)-induced apoptosis and Bcl-2 down-regulation in sensitive Ca9-22 cells. Cell lysates were prepared for Western blot analysis after 24 hours of treatment, and cells were prepared for sub-G_1 analysis after 48 hours of treatment. Columns, mean (n = 3); bars, SD; *P < .05 by paired Student’s t test. (B) Effect of EGFR inhibition by erlotinib (1 μM) on dasatinib (1 μM)-induced ER down-regulation in HSC3 cells for 24 hours.