Genetic and Chemical Targeting of Epithelial-Restricted with Serine Box Reduces EGF Receptor and Potentiates the Efficacy of Afatinib

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Abstract

EGF receptor (EGFR) is elevated in more than 90% of head and neck squamous cell carcinoma (HNSCC). However, a majority of patients with HNSCC do not respond to anti-EGFR therapeutics. Insensitivity to EGFR inhibitors may be due to kinase-independent actions of EGFR and/or activation of Her2. Strategies to reduce EGFR and Her2 protein levels in concert may be an optimal approach to enhance the efficacy of current anti-EGFR molecules. In this study, knockdown of epithelial-restricted with serine box (ESX) decreased EGFR and Her2 promoter activity, expression, and levels. ESX was elevated in primary HNSCC tumors and associated with increased EGFR and Her2. Genetic ablation of ESX decreased EGFR and Her2 levels and enhanced the antiproliferative effects of EGFR/Her2 tyrosine kinase inhibitors (TKI), lapatinib and afatinib. Biphenyl isoxazolidine, a novel small-molecule ESX inhibitor, reduced EGFR and Her2 levels and potentiated the antiproliferative efficacy of afatinib. Single-agent biphenyl isoxazolidine retarded the in vivo tumorigenicity of CAL27 cells. Importantly, the combination of biphenyl isoxazolidine and afatinib was significantly superior in vivo and resulted in a 100% response rate with a 94% reduction in tumor volume. Targeting EGFR/Her2 levels with an ESX inhibitor and EGFR/Her2 kinase activity with a TKI simultaneously is a highly active therapeutic approach to manage HNSCC. Our work provides evidence to support the further development of ESX inhibitors as an adjuvant to enhance the response rate of patients with HNSCC to current anti-EGFR/Her2 therapeutics.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer with an annual incidence of approximately 600,000 cases worldwide (1). A well-recognized genetic alteration in HNSCC is the dysregulation of EGF receptor (EGFR). EGFR is almost universally over-expressed and elevated EGFR expression is associated with inferior clinical outcome in patients with HNSCC (2–5). Although elevated EGFR is a frequent event, only a small proportion, around 5% to 15%, of patients with HNSCC responds to single-agent anti-EGFR therapy, suggesting that blocking EGFR tyrosine kinase-dependent activity and/or downstream signaling is insufficient for optimal clinical response (6). A potential explanation for the low response rate to EGFR inhibitors may be due to the kinase-independent actions of EGFR. A recent study showed that EGFR mediates cell survival by controlling autophagy independent of EGFR kinase activity (7). EGFR can translocate from the cell membrane to the nucleus to regulate the transcription of genes involved in cell proliferation and survival (8, 9). Alternatively, tolerance to EGFR inhibitors may be due to a compensatory mechanism resulting in activation of other EGFR family members, in particular Her2 and Her3 (10).

The ETS transcription factor family is intimately involved in tumorigenesis through direct regulation of genes critical for angiogenesis, apoptosis, invasion, and proliferation (11). Epithelial-restricted with serine box (ESX), a member of the ETS transcription factor family, is exclusively expressed in terminally differentiated epithelial cells, suggesting that ESX may play a role in controlling cell differentiation (12, 13). ESX was reported to be over-expressed in lung and breast carcinomas, in part, through gene amplification (14, 15). Ectopic expression of ESX was sufficient to transform MCF12A mammary epithelial cells resulting in an increase in cell invasion, cell motility, and anchorage-independent growth (16). Similarly, ESX enhanced cell migration in MCF10A mammary epithelial cells (17). In ZR-75-1 and MCF7 breast carcinoma cells, ESX was...
predominantly localized in the nucleus and short hairpin RNA (shRNA)-mediated ablation of ESX was sufficient to
decrease cell proliferation and anchorage-independent
growth (18). ESX was shown to transactivate the Her2
promoter through direct promoter occupancy at a discrete
ETS transcriptional response element (19). Interestingly,
overexpression of Her2 enhanced ESX promoter activity
in MCF7 cells and chemical inhibition of Her2 decreased
ESX promoter activity in SKBR3 Her2-positive breast
carcinoma cells (20). These observations indicate that a
positive ESX/Her2 feedback loop may be critical to pro-
mote tumorigenesis.

To date, the role of ESX in HNSCC has not been exa-
mined. Our work showed that ESX regulates EGFR and
Her2 promoter activity, expression, and levels in HNSCC.
ESX is associated with elevated EGFR and Her2 in HNSCC
tumor specimens. Genetic ablation of ESX reduced EGFR
and Her2 levels and inhibited cell proliferation, invasion,
migration, and clonogenic survival. A novel small-mole-
cule ESX mimic, biphenyl isoxazolidine, showed antitu-
mor activity as monotherapy and potentiated the efficacy
of afatinib, an irreversible EGFR/Her2 tyrosine kinase
inhibitor (TKI), in vitro and in vivo. Our work shows, for
the first time, that ESX modulates EGFR and is a druggable
target in HNSCC.

Materials and Methods

Cell lines

SCC15, SCC25, and CAL27 cells were purchased from
American Type Culture Collection (ATCC). SCC15 and
SCC25 cells were grown in a 1:1 mixture of Ham's F-12 and
Dulbecco’s Modified Eagle Medium (DMEM) supple-
mented with 10% FBS, 0.4 μg/mL hydrocortisone, 2 mmol/L
l-glutamine, 100 mg/mL streptomycin, and 100 U/mL
penicillin. CAL27 cells were grown in DMEM supple-
mented with 10% FBS, 2 mmol/L l-glutamine, 100 mg/mL
streptomycin, and 100 U/mL penicillin. Cell lines were
authenticated using short tandem repeat profiling (ATCC).

Generation of CAL27/shRNA-ESX cells

CAL27 cells were transduced with shRNA-control or
shRNA-ESX (10:1 MOI, pGIPZ Lentiviral shRNAmir;
Open Biosystems) and selected in puromycin to generate
polycyonal CAL27/shRNA-control and CAL27/shRNA-
ESX cells.

Western blot analysis

Cells were washed in ice-cold PBS and lysed in buffer
containing 1% Triton X-100, 50 mmol/L HEPES, pH 7.4,
10% glycerol, 137 mmol/L NaCl, 10 mmol/L NaF, 100
mmol/L Na3VO4, 10 mmol/L Na4P2O7, 2 mmol/L EDTA,
10 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfo-
nylfluoride. Whole-cell lysates were mixed with Laemmli
loading buffer, boiled, separated by SDS-PAGE, and trans-
ferred to a nitrocellulose membrane. Subsequently, immu-
noblot analysis was conducted using an ESX-specific
antibody (GenWay Biotech), an EGFR-specific antibody (Cell
Signaling Technology), a Her2-specific antibody (Santa
Cruz Biotechnology), or a glyceraldehyde-3-phosphate
dehydrogenase (GAPDH)-specific antibody (Sigma).

Cell proliferation

Cell proliferation was assessed using the CCK-8 reagent
to detect metabolically active cells (Dojindo Inc.). The
absorbance at 450 nm was quantitated using a microplate
reader (Molecular Devices). Dose–response curves and
IC50 values were generated using GraphPad Prism 4.0
(GraphPad Software).

Cell invasion and migration

Cell invasion was determined as described from the
Cell Invasion Assay Kit (Chemicon International). Cells
were harvested and resuspended in serum-free medium.
An aliquot (1 × 104 cells) of the prepared cell suspension
was added to the top chamber and 10% FBS was added to
the bottom chamber. After 24 hours, noninvading cells
were gently removed from the interior of the inserts with
a cotton-tipped swab. Invasive cells were stained and
visualized. Cell migration was determined using the
wound-healing assay. Cells were seeded and allowed to
grow until confluence. Confluent monolayers were scrat-
tched using a sterile pipette tip, washed, and incubated in
complete medium.

Clonogenic survival

SCC15/shRNA-control, SCC15/shRNA-ESX, CAL27/
shRNA-control, and CAL27/shRNA-ESX cell were trea-
ted with lapatinib or afatinib for 72 hours. CAL27 cells
were treated with biphenyl isoxazolidine, lapatinib, afat-
inib, biphenyl isoxazolidine + lapatinib, or biphenyl
isoxazolidine + afatinib for 72 hours. Cell were harvested
and resuspended in complete growth media. Cells were
seeded onto 60 cm2 dishes and allowed to grow until
visible colonies formed (10–14 days). Cell colonies were
fixed with cold methanol, stained with 0.25% crystal violet
in 25% methanol, washed, and air-dried.

EGFR and Her2 promoter activity

Cells were cotransfected with an EGFR or Her2 pro-
moter-Renilla luciferase vector and a CMV-Firefly lucif-
erase vector (SwitchGear Genomics) using FuGENE HD
(Promega). After 24 hours, cells were washed with PBS,
lysed in passive lysis buffer, and measured for Renilla/
Firefly luciferase activities in a luminometer using the
Dual-Light System (Applied Biosystems). EGFR and Her2
promoter Renilla luciferase activities were normalized
with Firefly luciferase activities to control for transfection
efficiency.

Gene expression analysis of primary HNSCC tumors

Sixteen primary tumors were collected at The Ohio
State University James Cancer Hospital (Columbus, OH)
from patients with HNSCC at the time of surgical resec-
tion between 1997 and 2000. All tissues were diagnosed
histologically as HNSCC by a board-certified pathologist.
Written informed consent, as required by the Institutional Review Board, was obtained from all patients. Collected samples were stored immediately in liquid nitrogen at −80°C until analysis. Total RNA was isolated from the frozen tumors with TRIzol (Invitrogen). Expression of ESX, EGFR, and Her2 was determined using the Applied Biosystems 7900HT Fast Real-Time PCR System with validated TaqMan gene expression assays (Applied Biosystems). Gene expression was normalized to GAPDH using theΔΔCt method.

**In vivo tumorigenicity and efficacy**

For the *in vivo* tumorigenicity study, CAL27/shRNA-control or CAL27/shRNA-ESX cells (1 × 10⁶ cells) mixed with Matrigel (1:1) were implanted into the flanks of 6- to 8-week-old female athymic nude mice (National Cancer Institute, Frederick, MD). Tumors were measured and resected for analysis at 18 days postimplantation. For the *in vivo* efficacy study, CAL27 cells (1 × 10⁶ cells) mixed with Matrigel (1:1) were implanted into the flanks of 6- to 8-week-old female athymic nude mice (National Cancer Institute). Mice with palpable tumors (~50 mm³) were randomly assigned to 4 experimental groups: vehicle (intratumoral injection and oral gavage), biphenyl isoxazolidine (100 μg/mouse intratumoral injection, 5× week), afatinib (0.4 mg/mouse oral gavage, 5× week), and biphenyl isoxazolidine (100 μg/mouse intratumoral injection, 5× week), afatinib (0.4 mg/mouse oral gavage, 5× week), or biphenyl isoxazolidine + afatinib. Tumors were measured using a digital caliper and tumor volumes were calculated using the formula: tumor volume = length × width × height × 0.5. Any mouse with a tumor volume equal to or greater than 1,000 mm³ was euthanized and removed from the study. All animal work was conducted in accordance with and approved by the Institutional Animal Care and Use Committee at the Ohio State University.

**Immunohistochemical analysis**

Resected tumors were fixed in 10% formalin and paraffin-embedded. Slides were incubated in citrate buffer (pH 6.0) for antigen retrieval and immunohistochemical staining was carried out using Peroxidase Histostain-Plus Kit (Invitrogen) according to the manufacturer’s protocol. ESX antibody (LifeSpan Biosciences Inc.) was used at a 1:500 dilution, EGFR antibody (Millipore) was used at a 1:10 dilution, Her2 antibody (Santa Cruz Biotechnology) was used at a 1:100 dilution, pEGFR-Y1173 antibody (Millipore) was used at 1:100 dilution, and pHer2-Y1221/1222 antibody (Cell Signaling Technology) was used at 1:100 dilution. Slides were counterstained with hematoxylin and coverslipped using glycerin.

**Statistical analysis**

Data were analyzed by two-tailed Student t test. P values less than 0.05 were considered significant.

**Results**

**ESX regulates EGFR and Her2 in HNSCC**

It is clear that EGFR is almost universally increased in HNSCC; however, in contrast to other carcinomas, EGFR amplification is low in this patient population (21–23). EGFR mRNA expression was shown to be elevated in HNSCC tumors compared with mucosal specimens from normal volunteers, suggesting that the predominant mechanism of EGFR dysregulation in HNSCC may be at the transcriptional level (24). *In silico* analysis identified multiple putative ESX-binding sites containing the GGAA core sequence in the EGFR promoter; −146 to −149, −256 to −259, −270 to −273, −433 to −436, −458 to −461, −468 to −471, and −609 to −611. This observation suggests that ESX may directly hyperactivate the EGFR promoter to drive EGFR expression. As shown in Fig. 1A, ESX is elevated in SCC15 and CAL27 HNSCC cells compared with oral epithelial cells (NOE). SCC15 and CAL27 cells have higher levels of EGFR and Her2, suggesting an association between ESX and EGFR/Her2 in HNSCC. shRNA-mediated ablation of ESX resulted in a decrease in EGFR and Her2 protein levels and mRNA expression in CAL27 cells (Fig. 1B and C). Genetic knockdown of ESX reduced EGFR promoter activity by 83% (*P < 0.01) in CAL27 cells (Fig. 1D). Consistent with published literature, Her2 promoter activity was suppressed by 56% (*P < 0.01) in CAL27/shRNA-ESX cells compared with shRNA-control cells. Next, we determined whether ESX is associated with EGFR and Her2 in primary tumors from previously untreated patients with HNSCC. A considerable range (0.00007–0.04310) in ESX mRNA expression in primary HNSCC tumors (*n = 16) was observed (Fig. 1E). We decided to stratify ESX expression into two groups: low and high ESX. Eight patients with the highest ESX expression were binned into the high ESX group and 8 patients with the lowest ESX expression were binned into the low ESX group. ESX expression was 0.02 ± 0.005 and 0.002 ± 0.001 for the high and low ESX group, respectively. The high-ESX HNSCC patients had a dramatic 11-fold increase in ESX expression compared with the low-ESX HNSCC patients (*P = 0.0012). In comparison with the low-ESX patients, the high-ESX patients had a 93% (*P < 0.05) increase in EGFR expression and an 86% (*P < 0.04) increase in Her2 expression (Fig. 1F). Pearson analyses showed a significant correlation between ESX and EGFR (*P = 0.029) and ESX and Her2 (*P = 0.004). These results indicate that ESX is elevated and associated with EGFR and Her2 in HNSCC.

**Genetic ablation of ESX reduces EGFR and Her2 levels and inhibits tumorigenicity in vitro and in vivo**

We examined the effect of targeting ESX on the tumorigenicity of HNSCC cells *in vitro* and *in vivo*. Genetic ablation of ESX decreased the proliferation and invasion of CAL27 cells by 42% and 67%, respectively (Fig. 2A and B). Moreover, ESX-deficient CAL27 cells were less motile than shRNA-control cells (Fig. 2C). *In vivo* tumorigenicity of CAL27 cells in athymic nude mice was retarded (99% inhibition, *P < 0.05, n = 7) with ESX knockdown (Fig. 2D). CAL27/shRNA-control cells had a mean tumor volume of 471 mm³ and CAL27/shRNA-ESX cells had a mean tumor volume of 286 mm³. Immunoblot
and immunohistochemical analyses showed that CAL27/shRNA-ESX tumors had lower ESX, EGFR, and Her2 levels than CAL27/shRNA-control tumors (Fig. 2F and G). These results indicate that targeting ESX is sufficient to dampen the tumorigenicity of HNSCC cells.

**Genetic ablation of ESX potentiates the efficacy of EGFR/Her2 TKIs**

EGFR is almost universally elevated in HNSCC, however, only a minority of patients with HNSCC respond to anti-EGFR therapies. Tolerance to current EGFR inhibitors may be due several reasons, including insufficient clinical dose to achieve maximal therapeutic response, kinase-independent actions of EGFR, and activation of other EGFR family members, namely Her2. Therefore, strategies to reduce EGFR and Her2 protein levels in concert may be an attractive approach to enhance the efficacy of anti-EGFR therapeutics. Our work showed that EGFR and Her2 levels are decreased with ESX knockdown, suggesting that ESX-deficient HNSCC cells may be more responsive to...
EGFR/Her2 TKIs, lapatinib and afatinib (Fig. 3A). As shown in Fig. 3B, ESX-deficient CAL27 cells were more sensitive to the antiproliferative effects of lapatinib and afatinib. CAL27/shRNA-control cells had an IC50 dose of 11.9 μmol/L for lapatinib and 2.3 μmol/L for afatinib. In contrast, the IC50 dose was 4.9 μmol/L for lapatinib and 0.7 μmol/L for afatinib in CAL27/shRNA-ESX cells. Similar results were obtained for SCC15/shRNA-control and SCC15/shRNA-ESX cells. Genetic ablation of ESX reduced clonogenic survival by 73% in CAL27 cells and 59% in SCC15 cells (P < 0.01; Fig. 3C). Clonogenic survival was inhibited by 73% and 72% with lapatinib and 89% and 69% with afatinib in CAL27/shRNA-control and SCC15/shRNA-control cells, respectively (P < 0.01). In addition, CAL27/shRNA-ESX and SCC15/shRNA-ESX cells were more responsive to EGFR/Her2 TKIs than shRNA-control cells. In fact, afatinib almost completely ablated the survival of CAL27/shRNA-ESX (98% inhibition) and SCC15/shRNA-ESX (99% inhibition) cells.

**Biphenyl isoxazolidine, a ESX mimetic, potentiates the efficacy of afatinib in vitro and in vivo**

ESX interacts with multiple coactivator proteins to regulate gene transcription. Med23, the most well-characterized ESX coactivator, interacts with ESX to regulate Her2 transcription. An eight-amino acid (137-SWIIELLE-146) α-helical region in ESX was reported to mediate the interaction between ESX and Med23 (25). Tryptophan 138 was shown to be essential for the specificity of the ESX-Med23 interaction (25). In addition, nuclear magnetic resonance spectroscopy suggests that W138 along with I139, I140, L142, and L143 form a hydrophobic surface along an amphipathic helix that interacts with Med23 (25). The binding interaction between ESX and Med23 was
shown to be disrupted with a small-molecule α-helix mimic of ESX, wrenchnolol (26). Wrenchnolol decreased Her2 expression and inhibited cell proliferation of SKBR3 Her2-positive breast carcinoma cells (26). Our group designed and synthesized a novel α-helix ESX mimic, biphenyl isoxazolidine, to block the interaction between ESX and Med23 (27). Similar to wrenchnolol, biphenyl isoxazolidine decreased Her2 expression and inhibited cell proliferation of SKBR3 cells (27).

To assess whether ESX is a druggable target in HNSCC, we examined the efficacy of biphenyl isoxazolidine (Fig. 4A) in CAL27 cells. In Fig. 4B, a dose-dependent decrease in EGFR and Her2 levels in CAL27 cells was observed in response to biphenyl isoxazolidine. Cell proliferation was inhibited with biphenyl isoxazolidine with an IC50 of 46.8 μmol/L (Fig. 4C). In contrast, biphenyl isoxazolidine (50 μmol/L) had no effect on the proliferation of normal human IMR90 fibroblasts (Fig. 4D). Cell invasion and migration were dramatically suppressed in CAL27 cells using sub-IC50 doses of biphenyl isoxazolidine (Fig. 4E and F). Next, we determined whether biphenyl isoxazolidine can potentiate the efficacy of EGFR/Her2 TKIs. CAL27 cells were treated with lapatinib or afatinib at various concentrations with and without an IC50 dose of biphenyl isoxazolidine (Fig. 5A). Single-agent lapatinib and afatinib inhibited the proliferation of CAL27 cells after 24 hours of treatment with an IC50 of 11.8 μmol/L for lapatinib and 1.5 μmol/L for afatinib. Importantly, the combination of biphenyl isoxazolidine and lapatinib or afatinib (IC50 dose) decreased cell proliferation by 90% or more in CAL27 cells. Similarly, the combination treatments were more efficacious than single-agent treatments.
for clonogenic survival (Fig. 5B). Single-agent biphenyl isoxazolidine, lapatinib, and afatinib blocked clonogenic survival by 49%, 45%, and 72%, respectively (P < 0.01). In contrast, clonogenic survival was reduced by 89% with biphenyl isoxazolidine and lapatinib and completely ablated (100%) with biphenyl isoxazolidine and afatinib. Our work shows that targeting EGFR/Her2 levels and EGFR/Her2 kinase activities simultaneously is a highly active therapeutic regimen to eliminate HNSCC cells.

Finally, the in vivo efficacy of biphenyl isoxazolidine as single agent and in combination with afatinib was assessed in a preclinical mouse model of HNSCC (Fig. 6A). CAL27 cells (1 x 10⁶) were implanted into the flank of 8-week-old athymic nude mice and tumors were allowed to develop without treatment. At 3 weeks after tumor cell implantation, mice with established tumors were randomly assigned to 4 treatment arms; vehicle, biphenyl isoxazolidine (100 μg; 5× week; intratumoral injection), afatinib (0.4 mg; 5× week; oral gavage), or biphenyl isoxazolidine and afatinib. Biphenyl isoxazolidine was well tolerated as monotherapy and in combination with afatinib. Mice in the single-agent biphenyl isoxazolidine arm and the combination treatment arm did not display abnormal behavior nor had significant weight loss compared with the vehicle arm. Single-agent biphenyl isoxazolidine was blocked tumor growth by 51% (n = 10, P < 0.05) and single-agent afatinib suppressed tumor growth by 87% (n = 10, P < 0.01). The combination of biphenyl isoxazolidine and afatinib was the most active and blocked tumor growth by 94% (n = 10, P < 0.01). It should be noted that the mean tumor volume was 2.1-fold higher for the single-agent afatinib arm compared with the
combination treatment arm (43 mm³ vs. 20 mm³; Fig. 6A, right). Importantly, the antitumor efficacy of the combination treatment arm was statistically superior to either single-agent biphenyl isoxazolidine ($P < 0.01$) or single-agent afatinib ($P < 0.01$).

Total and phosphorylated EGFR and Her2 levels were assessed in the tumors after treatment. As shown in Fig. 6B, intratumoral levels of total and phosphorylated EGFR and Her2 were decreased following treatment with single-agent biphenyl isoxazolidine or afatinib compared with vehicle. Total EGFR levels were similar between tumors in the combination and single-agent treatment arms. In contrast, the combination of biphenyl isoxazolidine and afatinib showed a further reduction in total and phosphorylated Her2 levels than biphenyl isoxazolidine or afatinib monotherapy. Interestingly, afatinib treatment altered the cellular localization of phosphorylated EGFR from the cytosol to the nucleus in a majority of the residual tumor cells. In the combination treatment arm, phosphorylated EGFR was not detected in the nucleus but in the cytosol of most of the tumor cells albeit at a lower intensity than the vehicle treatment arm.

**Discussion**

It is well recognized that EGFR dysregulation is a frequent event in HNSCC. Elevated EGFR is associated with increased local recurrence and decreased disease-free survival in patients with HNSCC (2–5). EGFR mRNA expression was reported to be increased by 69-fold in 92% of HNSCC tumors compared with normal mucosa (24). In addition, in 10 HNSCC cell lines, a 77-fold increase in EGFR mRNA expression was observed without any evidence of EGFR gene amplification or rearrangement (24). In line with this finding, several independent reports showed that EGFR amplification is infrequent and ranges between 10% and 15% in primary HNSCC tumors (21–23). These studies indicate that the major mechanism of EGFR overexpression remains to be elucidated and may be at the transcriptional level. In this study, we revealed that ESX is elevated in HNSCC and modulates EGFR. Genetic ablation of ESX decreased EGFR promoter activity, mRNA expression, and protein levels. High ESX correlated with high EGFR expression in HNSCC tumors further confirming the link between these two genes. Our study shows, for the first time, that ESX is associated with EGFR, suggesting a novel mechanistic basis for EGFR dysregulation in HNSCC.
Monotherapy with EGFR inhibitors, such as cetuximab, a humanized anti-EGFR antibody, or TKIs, such as gefitinib, lapatinib, and erlotinib, has yielded modest activity in patients with HNSCC to date. These clinical observations provide evidence that targeting EGFR at the cell membrane and/or EGFR kinase-dependent activity may not be robust enough to reach the maximal anticancer therapeutic response. A combination of two different yet complementary approaches to target the EGFR family members, reduction of EGFR/Her2 levels with ESX inhibition and suppression of EGFR/Her2 kinase activity with TKIs, may result in greater therapeutic efficacy. Our results showed that this is the case. ESX-deficient HNSCC cells were more responsive to the effects of EGFR/Her2 TKIs, lapatinib and afatinib, than shRNA-control HNSCC cells. Moreover, combination treatment of biphenyl isoxazolidine and afatinib was more active than either single-agent treatment in vitro and in vivo. An appropriate method to assess tumor response is to use Response Evaluation Criteria in Solid Tumors (RECIST) criterion established for clinical trials for anticancer therapeutics. Using RECIST guidelines, the combination biphenyl isoxazolidine and afatinib arm had a significantly higher response rate than single-agent afatinib (100% vs. 50% response rate; P < 0.01, n = 10). A 100% partial response rate was observed for the combination arm, whereas single-agent afatinib showed 50% partial response, 40% stable disease, and 10% progressive disease. These results show that biphenyl isoxazolidine enhances...
the response rate of afatinib in HNSCC. Our immunohistochemical analyses showed that the combination treatment resulted in lower levels of total and phosphorylated Her2 than either single-agent biphenyl isoxazolidine or afatinib. Afatinib reduced intratumoral phosphorylated EGFR levels; however, it promoted the nuclear accumulation of phosphorylated EGFR in the residual tumor cells, which suggests that a potential mechanism for afatinib resistance may be due to enhanced nuclear-dependent actions of EGFR. Interestingly, phosphorylated EGFR was not nuclear but cytosolic in most of the residual tumor cells in the combination treatment arm. Our findings provide initial evidence that biphenyl isoxazolidine may potentiate HNSCC cells to respond to afatinib by preventing afatinib-induced nuclear accumulation of phosphorylated EGFR and/or further reducing the levels of total and phosphorylated Her2.

It is interesting that the concentration of biphenyl isoxazolidine needed to inhibit cell invasion and migration is much lower than that required to inhibit cell proliferation. This observation argues that the EGFR/Her2 gene dosage threshold to modulate cell invasion, migration, and proliferation is different; cell migration and invasion requires a higher level of EGFR/Her2 than cell proliferation. Moreover, our work shows that inhibition of cell invasion and migration mediated by biphenyl isoxazolidine is due to a general decrease in cell viability but most likely due to dampening of signal transduction pathways specific to cell morphology, invasion, and motility. It is clear that EGFR and Her2 control transduction pathways specific to cell morphology, invasion, and proliferation. It is clear that EGFR and Her2 control transduction pathways specific to cell morphology, invasion, and motility. It is clear that EGFR and Her2 control transduction pathways specific to cell morphology, invasion, and motility. However, it is possible that a yet to be identified ESX-regulated gene may contribute to ESX modulation of the tumor cell phenotype in HNSCC. Additional research using a systems biology approach will be necessary to delineate the signaling hubs modulated by ESX to drive cell invasion, migration, and proliferation with temporal and gene dosage resolution.

In summary, our data showed that ESX is overexpressed and regulates EGFR in HNSCC. Biphenyl isoxazolidine, a small-molecule ESX mimic, was shown as an active anticancer therapeutic and potentiated the anti-tumor effects of EGFR TKIs. These results reveal that targeting ESX is a novel approach to enhance the efficacy of anti-EGFR/Her2 therapeutics in HNSCC. Further development of ESX mimics as a novel small-molecule platform to target EGFR and Her2 levels is warranted.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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