AXL Is a Logical Molecular Target in Head and Neck Squamous Cell Carcinoma

Toni M. Brand1, Mari Iida1, Andrew P. Stein1, Kelsey L. Corrigan1, Cara M. Braverman1, John P. Coan1, Hannah E. Pearson1, Harsh Baharal1, Tyler L. Fowler1,2, Bryan P. Bednarz2, Sandeep Saha3, David Yang4, Parkash S. Gill5, Mark W. Lingen6, Vassiliki Saloura7, Victoria M. Villaflor7, Ravi Salgia1, Randall J. Kimple1, and Deric L. Wheeler1

Abstract

Purpose: Head and neck squamous cell carcinoma (HNSCC) represents the eighth most common malignancy worldwide. Standard-of-care treatments for patients with HNSCC include surgery, radiation, and chemotherapy. In addition, the anti-EGFR monoclonal antibody cetuximab is often used in combination with these treatment modalities. Despite clinical success with these therapeutics, HNSCC remains a difficult malignancy to treat. Thus, identification of new molecular targets is critical.

Experimental Design: In the current study, the receptor tyrosine kinase AXL was investigated as a molecular target in HNSCC using established cell lines, HNSCC patient-derived xenografts (PDX), and human tumors. HNSCC dependency on AXL was evaluated with both anti-AXL siRNAs and the small-molecule AXL inhibitor R428. Furthermore, AXL inhibition was evaluated with standard-of-care treatment regimens used in HNSCC.

Results: AXL was found to be highly overexpressed in several models of HNSCC, where AXL was significantly associated with higher pathologic grade, presence of distant metastases, and shorter relapse-free survival in patients with HNSCC. Further investigations indicated that HNSCC cells were reliant on AXL for cellular proliferation, migration, and invasion. In addition, targeting AXL increased HNSCC cell line sensitivity to chemotherapy, cetuximab, and radiation. Moreover, radiation-resistant HNSCC cell line xenografts and PDXs expressed elevated levels of both total and activated AXL, indicating a role for AXL in radiation resistance.

Conclusions: This study provides evidence for the role of AXL in HNSCC pathogenesis and supports further preclinical and clinical evaluation of anti-AXL therapeutics for the treatment of patients with HNSCC. Clin Cancer Res; 21(11); 2601–12. ©2015 AACR.

Introduction

With more than 600,000 new cases diagnosed worldwide each year, head and neck squamous cell carcinoma (HNSCC) represents the eighth most common malignancy (1). HNSCC arises from epithelial cells that comprise the mucosal surfaces of the lips, oral cavity, larynx, pharynx, and nasal passages. Classically, these malignancies were highly associated with alcohol and tobacco abuse, but over the past decade, it has been determined that human papillomavirus (HPV) is causally associated with a subset of HNSCCs (2).

Approximately 60% of patients with HNSCC present with locoregionally advanced disease at the time of diagnosis. To achieve the greatest chance for cure, these patients are typically treated with a multimodality approach of systemic chemotherapy, radiation, and surgery (3–5). Advances in molecular targeting of HNSCC have found that cetuximab, an anti-EGFR monoclonal antibody, can benefit patients when combined with platinum chemotherapy or radiation (6–8). Although advances in these treatment modalities have improved patient outcomes, many patients still develop recurrent tumors and distant metastases. Upon relapse, patient survival remains poor. In this manner, the identification of new therapeutic targets is critical.

The receptor tyrosine kinase AXL has now been implicated in the development and progression of many malignancies, including lung (9–14), breast (12, 15–19), ovarian (20), colon (21), head and neck (22), thyroid (23), prostate (24), pancreatic (25), osteosarcoma (26), and Kaposi sarcoma (27). These studies indicate a role for AXL in cancer cell proliferation, migration, angiogenesis, and metastasis (reviewed in refs. 28, 29). Moreover, AXL mRNA expression has been correlated with poor disease outcome in HNSCC (22), indicating a putative role for AXL in the formation and/or progression of this disease. Recent studies have also found that AXL can mediate resistance to anti-EGFR inhibitors, further unveiling a role for AXL in cancer progression (9, 11, 13, 22, 30, 31). In the current study,
**Translational Relevance**

Head and neck squamous cell carcinoma (HNSCC) represents the eighth most common malignancy worldwide. Standard-of-care treatments include surgery, radiation, and chemotherapy. In addition, the anti-EGFR monoclonal antibody cetuximab is commonly used. Despite clinical success with these therapies, HNSCC remains a difficult malignancy to treat. Thus, identification of molecular targets is critical. In the current study, the receptor tyrosine kinase AXL was over-expressed and significantly associated with higher pathologic grade, distant metastases, and shorter relapse-free survival in patients with HNSCC. On the basis of these findings, AX L was evaluated as a molecular target in HNSCC models using the clinically relevant tyrosine kinase inhibitor R428, where AX L targeting enhanced the efficacy of platinum chemotherapy, cetuximab, and radiation. Importantly, AX L was overexpressed and hyperactivated in radiation-resistant in vitro HNSCC models. Collectively, these studies provide rationale for the clinical evaluation of anti-AXL therapeutics for the treatment of patients with HNSCC.

we sought to determine whether AX L is a functional molecular target in HNSCC, and whether targeting AX L could enhance the efficacy of standard treatments used to treat patients with this disease.

**Materials and Methods**

**Cell lines**

All cell lines were obtained from the indicated sources (Supplementary Materials and Methods). The identity of all cell lines was confirmed via short tandem repeat testing.

**Antibodies and compounds**

All antibodies used are as follows: R&D Systems: AX L (for immunoblotting) and pAX L (Y779). Cell Signaling Technology: Phospho-SEK(Y419), pDNA-PK(S5216), DNA-PK, pAKT(S473), AKT, γ-H2AX(S139), GAPDH, and pan-tyrosine (pan-Tyr). Santa Cruz Biotechnology Inc.: AX L (for immunoprecipitation; IP), E-Cadherin, vimentin, and horseradish peroxidase (HRP) antibodies. Abcam: EGFR and pEGFR(Y1101). Calbiochem: α-tubulin. R428 was purchased from Selleckchem. Cetuximab (ICM-225; Erbitux) was purchased from University Biochem: R428 was purchased from Selleckchem.

**Plasmids, transfection, and siRNA technology**

Plasmid construction and stable selection of AX L overexpressing cells were described previously ([30](#)). Cells were transiently transfected with AX L siRNA (siAX L-1; ON-TARGETplus, SMARTpool #L-003104; GE Dharmaco, Lafayette, CO, USA) or siAX L-2; Cell Signaling AX L siRNA i #6263) or nontargeting siRNA (siNT; ON-TARGETplus Non-targeting Pool, #D-001810; Dharmaco) using Lipofectamine RNAiMAX according to the manufacturer's instructions (Life Technologies). siAX L-1 was used for cisplatin, cetuximab, and radiation studies.

**Cell proliferation assay and clonogenic survival assay**

Crystal violet assay and Cell Counting Kit-8 (Dojindo Molecular Technologies) were performed as previously described and in the Supplementary Materials and Methods ([30](#)), [32](#)). Crystal violet assays were performed to identify the combinatorial effect of siAX L and radiation.

**Apoptosis assay**

HNSCC cell lines were treated with 0.5 or 1.0 μmol/L of R428 for 24 hours before staining with YO-PRO-1 and propidium iodide according to the manufacturer's instructions (Vybrant Apoptosis Assay Kit #4, YO-PRO-1/propidium iodide, Invitrogen). Uptake of YO-PRO-1 and PI was measured using a FACScan Calibur flow cytometer (BD Biosciences) and FlowJo analysis software (TreeStar Inc).

**Immunoblot analysis**

Whole-cell lysis, immunoprecipitation, and Western blot analysis were performed as previously described ([30](#)). Enhanced chemiluminescence (ECL) detection system was used to visualize proteins.

**Wound-healing assay**

Cells were plated in 6-well culture plates. Upon 80% to 90% confluence, the cell layer was scratched with a p-200 pipette tip (3 scratches per well, 2–3 wells per treatment). The cells were then incubated in complete medium with/without indicated doses of R428. Alternatively, cells were transfected with siAX L or siNT for 24 hours before wound exposure. Photographs of the wound adjacent to reference lines were taken using an Olympus IX51 microscope (×20) at indicated time points. CellSens Standard 1.9 software (Olympus) was used to digitally measure wound closure; wound closure at each time point was normalized to the averaged scratch width measured at time 0.

**Cell invasion assay**

CultreCoat Low BME Cell Invasion Assay was purchased from R&D Systems. Cells were plated in 96-well CultreCoat plates with either vehicle or R428. Twenty-four hours post therapy, cell invasion was measured as per manufacturer's instructions.

**γ-H2AX fluorescent assay**

Cells were plated in 96-well dishes and pretreated with vehicle or R428 (1 μmol/L). A novel high-throughput irradiator utilizing a 50 kVp X-ray beam spectrum was used to deliver 4 gray (Gy) as previously described ([33](#)), [34](#)). Cells were fixed in 4% paraformaldehyde 4 hours later, permeabilized in 90% methanol, blocked, and incubated with γ-H2AX primary antibody (1:500) overnight. Cells were washed and incubated with FITC-conjugated secondary antibody (1:1,000; Santa Cruz Biotechnology). γ-H2AX fluorescence per cell was evaluated via a SpectraMax i3 plate reader with MiniMax 300 imaging cytometer using SoftMax Pro v6.4 software (Molecular Devices). All γ-H2AX fluorescent values were averaged and then normalized to averaged values from vehicle-treated cells.

**Cell line xenografts, patient-derived xenografts (PDX), and radiation response**

Cell line xenografts and patient-derived xenografts (PDX) were established as previously described ([30](#)), [35](#)), [36](#)). Radiation response was evaluated as described in the Supplementary Materials and Methods.
HNSCC patient cohort and tissue microarray construction

Patients with HNSCC completed written consent in accordance with Institutional Review Board approval from the University of Chicago (Chicago, IL; protocol number 10-343-A). See Supplementary Materials and Methods for details.

Statistical analysis

HNSCC patient demographics and clinical characteristics were summarized using descriptive statistics in the Supplementary Materials and Methods. Student t test was used to evaluate differences in cell proliferation, migration, and invasion. Differences were considered statistically significant if *, P < 0.05; **, P < 0.01.

Results

AXL is expressed in HNSCC cell lines, PDXs, and human tumors

To determine whether AXL could represent a molecular target in HNSCC, AXL expression was evaluated in a panel of 14 HNSCC cell lines (Fig. 1A, top). These results indicated that 11 of the 14 cell lines expressed AXL, whereas three cell lines (SCC90, SCC2, SCC4) did not express AXL.

Figure 1. HNSCC cell lines, PDXs, and human tumors express AXL. A, whole-cell lysate was harvested from 14 HNSCC cell lines and evaluated for AXL expression. GAPDH was used as a loading control. AXL-expressing cell lines were transfected with siAXL-1 (50 nmol/L), siAXL-2 (100 nmol/L), or nontargeting (NT) siRNA for 72 hours before performing proliferation assays (n = 4–6 replicates in three independent experiments). Whole-cell lysate was harvested at the same time to confirm AXL knockdown. Data points are represented as mean ± SEM., **, P < 0.01. B, AXL is differentially expressed in a 22 HNSCC PDX TMA consisting of several early passaged tumors per PDX. Representative images of low and high AXL expressing PDXs are shown (×10). Pathologic IHC quantitation (by D. Yang) was determined via a categorical scale from 0 to 4+.

C, high AXL expression is associated with increased tumor grade in a 63 HNSCC patient cohort. Representative images of low and high AXL expressing patient tumors corresponding to pathologic tumor grade are shown (×20). Pathologic IHC quantitation (by M.W. Lingen) was determined via a categorical scale from 0 to 3+. High AXL expression was significantly associated with shorter median PFS in patients with HNSCC as analyzed by the Kaplan–Meier method. *, P < 0.05; HR, hazard ratio.
and SCC1483) had either low or undetectable AXL protein levels. Because AXL was differentially expressed, the dependency of these cell lines on AXL for proliferation was evaluated with AXL siRNAs. Four AXL-positive cell lines were transfected with a pooled AXL-siRNA (siAXL-1), a second individual AXL siRNA (siAXL-2) or a nontargeting siRNA control (siNT; Fig. 1A, bottom), and cellular proliferation was measured 72 hours posttransfection. Genetic ablation of AXL with either siAXL-1 or siAXL-2 resulted in statistically significant inhibition of cellular proliferation in the AXL-expressing cell lines SCC4, SCC6, SCC104, and HN4, whereas the AXL-negative cell line, SCC2, was unaffected. Collectively, these data indicated that HNSCC cell lines expressed AXL and were dependent on this receptor for proliferation.

To expand these findings, a 22 PDX tissue microarray (TMA) was stained for AXL via IHC using a previously validated anti-AXL antibody (30). This TMA contained several early passed tumors from each PDX to evaluate consistency of protein expression across passages. Each TMA core was scored for AXL expression on a categorical scale from 0 to 4+. Where 0 represented no staining and 4+ was the most intense staining. Pathologic analysis (by D. Yang) of AXL staining patterns indicated that 82% of the PDXs expressed AXL, where AXL expression remained relatively consistent across early passed tumors (Fig. 1B). Four percent of the PDXs were negative for AXL expression. Collectively, these data indicate that AXL is commonly expressed in a clinically relevant model of HNSCC.

To further define the expression status of AXL in HNSCC, 63 HNSCC patient tumors were evaluated for AXL expression by IHC on a categorical scale of 0–3+, where 0–1+ was considered a low AXL score, and 2–3+ was considered a high AXL score (see Supplementary Table S1 for clinical information of the patient cohort). Pathologic analysis (by M.W. Lingen) of this cohort indicated that 38% expressed high levels of AXL whereas 62% expressed either low or no AXL (Fig. 1C). Normal oral tissue from six different patients was stained for AXL, where AXL staining was low or undetectable, indicating increased expression of AXL specifically in tumor cells (Fig. 1C). Using a logistic regression model, the multivariate predictors of elevated AXL expression included: male versus female gender (OR, 6.38; 95% confidence interval [CI], 1.18–34.5), poorly differentiated versus moderate/well differentiated tumor grade (OR, 4.03; 95% CI, 1.03–15.48) and presence of distant metastasis (OR, 3.58; 95% CI, 1.03–12.53; Supplementary Table S2A). The expression of p16, indicative of HPV-associated cancers (37), was not associated with AXL expression. Progression-free survival (PFS) in this cohort was determined by evaluating the number of patients in the low and high AXL groups who experienced a recurrence of their disease or passed away. There was a significant association between PFS and AXL score (P = 0.027), where median PFS was shorter in patients with high AXL (1.0 years; 95% CI, 0.6–∞) as compared with patients with low AXL (4.3 years; 95% CI, 1.4–∞; Fig. 1C and Supplementary Table S2B). The hazard ratio (HR) for PFS among patients with high versus low AXL score was 2.3 (95% CI, 1.1–5.1), indicating a higher probability of death or recurrence in patients with high AXL expression. Collectively, these results indicate that AXL is overexpressed in more aggressive HNSCCs and is related to poor clinical outcome.

AXL inhibition effectively reduces HNSCC cell growth, migration, and invasion

Because several HNSCC cell lines were sensitive to AXL knockdown by siRNA, we hypothesized that these cells would also be sensitive to the AXL tyrosine kinase inhibitor R428. R428 specificity for AXL has been previously evaluated (15), and this agent has now undergone successful phase Ia clinical evaluation (38). HNSCC cells were treated with increasing doses of R428 (0.001–1.0 μmol/L) for 72 to 96 hours before performing proliferation assays. All AXL-expressing HNSCC cell lines were significantly growth inhibited with increasing doses of R428 (Fig. 2A). The AXL-negative cell lines, SCC2 and SCC90, were used as controls, and their proliferation was not significantly altered with increasing doses of R428. Furthermore, via evaluation of pan phosphotyrosine post-immunoprecipitation with an anti-AXL antibody, R428 inhibited AXL activation at several doses that also resulted in the most robust antiproliferative responses (0.5 and 1.0 μmol/L).

In addition to influencing cellular proliferation pathways, AXL has been shown to mediate the metastatic potential of cancer cells (26, 27). To determine whether AXL regulates the migratory potential of HNSCC cells, wound-healing assays were performed using SCC4, SCC6, SCC104, HN4, and SCC2 cell lines post AXL inhibition. In this assay, cells were subjected to injury directly after treatment with either 0.1 or 0.5 μmol/L R428. Wound length was measured after the wounds were made (0 hour) and at the indicated time points post wound exposure (Fig. 2B). SCC4 and SCC6 cells treated with either dose of R428 displayed less wound closure at both time points as compared with vehicle-treated cells (where the wound was completely closed at 24 hours). SCC104 and HN4 cells displayed less wound closure when treated with 0.5 μmol/L of R428 at both displayed time points. The AXL-negative cell line, SCC2, was the least migratory of all HNSCC cell lines tested, and R428 did not have an impact on its migratory capacity.

To further validate the specificity of R428 for AXL, cells were transfected with siNT or siAXL for 24 hours before performing wound-healing assays (Supplementary Fig. S1). AXL knockdown significantly impacted wound closure in all AXL-expressing cell lines tested as compared with cells transfected with siNT.

Next, the invasive potential of the HNSCC cells was measured via Boyden chamber invasion assays 24 hours after R428 treatment (Fig. 2C). All AXL-expressing cell lines examined were significantly inhibited in their invasive potential when pretreated with 1.0 μmol/L of R428, whereas HN4 and SCC4 cells were inhibited at lower doses (0.5 μmol/L). In addition, R428 did not impede the invasive potential of SCC2 cells.
To ensure R428’s effects on migration and invasion were not due to changes in cell death or proliferation, cellular proliferation and apoptosis were measured 24 hours posttreatment with 0.5 and 1.0 μmol/L R428 (Supplementary Fig. S2). This analysis indicated that HNSCC cell proliferation and apoptosis were not significantly altered by increasing doses of R428 at this time point, supporting R428’s specific effects on the migratory and invasive capacity of HNSCC cells. Overall, AXL inhibition effectively reduced proliferation, migration, and invasion of HNSCC cell lines suggesting that AXL may represent a potent molecular target in HNSCC.

AXL inhibition increases the sensitivity of HNSCC cells to chemotherapy and cetuximab

To evaluate whether AXL inhibition could augment the sensitivity of HNSCC cells to standard of care treatments, we first tested whether AXL inhibition enhanced HNSCC cell line sensitivity to the platinum-based chemotherapies cisplatin and carboplatin (Fig. 3A). Evaluation of cellular proliferation 72 to 96 hours posttreatment indicated differential responses to cisplatin and carboplatin monotherapy. However, addition of R428 to either cytotoxic agent led to statistically significant reductions in cell proliferation as compared to cells treated with R428 only. The AXL-negative cell line, SCC2, was the most sensitive to chemotherapy, and R428 did not augment response. Using the fractional product method (described by Chou and Talalay; refs. 39–41), the nature of the interaction between R428 and each chemotherapy was evaluated for synergy as described in the Supplementary Materials and Methods. AXL inhibition synergized with both cisplatin and carboplatin in all AXL-expressing cell lines, where the ratio of the observed

![Figure 3](image-url)
(O) to expected (E) effect was less than 1 (Supplementary Table S3A and S3B). In addition, siRNA-targeting AXL synergized with cisplatin, providing further evidence for the specificity of R428 for AXL inhibition (Supplementary Fig. S3A). Collectively, these results demonstrate that AXL inhibition enhances chemotherapy sensitivity in HNSCC cells.

Because AXL expression has been shown to mediate cetuximab resistance (30), we hypothesized that AXL inhibition may improve the efficacy of cetuximab in HNSCC. Therefore, five HNSCC cell lines were treated with vehicle, R428 (1.0 μmol/L), cetuximab (100 nmol/L), or the combination, and cellular proliferation was measured 72 hours later (Fig. 3B). Three cell lines (SCC4, SCC6, and SCC104) were resistant to cetuximab, whereas HN4 cells were sensitive to cetuximab monotherapy. Importantly, when treated with both R428 and cetuximab, all cell lines demonstrated significant reduction in cellular proliferation as compared with cells treated with R428 only. The resulting effect of both drugs was determined to be synergistic in all cell lines except for HN4, where an additive effect was observed (Supplementary Table S3C). Additional siRNA studies confirmed specificity of R428 for AXL inhibition, as AXL knockout synergized with cetuximab in all cell lines tested (Supplementary Fig. S3B). To further evaluate whether AXL mediates cetuximab response in HNSCC cells, the cetuximab-sensitive cell line HN4 was manipulated to highly overexpress AXL via stable transfection. Cetuximab dose–response proliferation assays demonstrated HN4-AXL cells were statistically more resistant to increasing doses of cetuximab as compared with HN4-Vector cells (Fig. 3C). Immunoblot analysis of HN4-AXL cells indicated increased activation of proteins previously reported to play a role in cetuximab resistance, including increased phosphorylation of EGFR on tyrosine 1101 and src family kinases (SEK, refs. 42–44). In addition, HN4-AXL cells had decreased levels of E-cadherin and increased levels of vimentin, two hallmarks of cells that have undergone epithelial-to-mesenchymal transition (EMT). Taken together, these studies support a role for AXL in cetuximab resistance, and suggest that AXL inhibition can enhance cetuximab sensitivity in HNSCC cells.

**Targeting AXL can enhance the efficacy of radiation therapy in HNSCC**

It is well established that several RTKs play a role in modulating DNA repair pathways and response to radiation therapy (45, 46). However, the role of AXL in radiation response has never been investigated. A previous report has indicated differential radiation responses for several HNSCC cell lines that express AXL (Fig. 1A), including SCC1, SCC47, and SCC147T (35). Therefore, we examined AXL inhibition could augment the sensitivity of these cell lines to radiotherapy. Using a high-throughput X-ray radiation system that delivers the same absorbed dose of ionizing radiation...
to cells plated in a 96-well format (development and characterization; ref. 33). SCC1, SCC47, SCC147T, and SCC2 were irradiated with 4 Gy after 24 hour pretreatment with either vehicle or R428 (1 μmol/L). Then, the induction of DNA double-strand breaks (DSB) were examined via γ-H2AX, which is phosphorylated and recruited to sites of DNA damage in response to radiation (Fig. 4A). At 4 hours postirradiation, total γ-H2AX fluorescent intensity per cell was determined via a fluorescent plate reader with image cytometry. This system allowed for the quantification of multiple replicates at the same time (n = 12 wells per treatment group) while simultaneously eliminating human error in counting γ-H2AX foci. A significant increase in γ-H2AX fluorescent foci was observed in cells treated with R428 and radiation as compared with cells treated with radiation only. The AXL-negative cell line, SCC2, was used as a control, and there was not a significant difference in γ-H2AX between either radiation treatment group.

To further assess the impact of AXL inhibition on radiation response, clonogenic survival assays were performed after exposure to R428 and radiation (Fig. 4B). In this experiment, an equal number of cells were plated per well and subsequently pretreated with 0.25 μmol/L R428 for 24 hours before XRT exposure. Nonirradiated cells treated with vehicle or R428 demonstrated similar plating efficiency (data not shown). However, all AXL-expressing cell lines pretreated with R428 demonstrated significantly reduced survival following radiation exposure as compared with cells treated with radiation only. The effect of R428 and radiation was determined to be synergistic in all cell AXL-expressing cell lines tested (Supplementary Table S3C). R428 pretreated SCC2 cells did not demonstrate a reduction in survival as compared with cells treated with radiation only. Finally, AXL knockdown with siRNA before radiation exposure resulted in reduced cellular viability, further supporting the AXL-specific radiosensitizing effects of R428 (Supplementary Fig. S3C).

To investigate the potential molecular mechanisms underlying this enhanced radiation response, the activation of DNA-protein kinase (DNA-PK) and AKT was examined post R428 and radiation therapy (Fig. 4C). DNA-PK is largely responsible for mediating DNA DSB repair through nonhomologous end joining, and thus, when activated can lead to radiation resistance (47). AKT is an intracellular serine/threonine kinase that directly interacts with DNA-PK to promote DNA DSB repair and cell survival (48). AXL-expressing HNSCC cells treated with R428 and radiation expressed considerably less activated DNA-PK and AKT levels as compared with cells treated with radiation alone. R428 did not augment radiation-induced DNA-PK or AKT activation in the AXL-negative cell line SCC2. Collectively, these data suggest that AXL signaling mediates DNA DSB repair and therefore targeting AXL may enhance the efficacy of radiation therapy.

To further define AXL’s role in radiation response and DNA DSB repair, HN4-Vector and HN4-AXL stable cells were irradiated and γ-H2AX fluorescence was measured 4 hours later (Fig. 4D, left). HN4-AXL cells had less γ-H2AX foci indicating that these cells had more repaired DNA DSBs as compared with HN4-Vector cells. Clonogenic survival analyses indicated that HN4-AXL cells had significantly more surviving cells postirradiation as compared with HN4-Vector cells (Fig. 4D, middle). In addition, HN4-AXL cells expressed increased levels of phosphorylated DNA-PK and AKT postirradiation (Fig. 4D, right). Collectively, these studies support a putative role for AXL in the regulation of DNA repair and resistance to radiation.

AXL is overexpressed in radiation-resistant cell lines and patient-derived xenografts

To expand these findings, the HNSCC cell lines SCC2, SCC22B, SCC90, SCC1, SCC47, and SCC147T were injected into both dorsal flanks of athymic nude mice (n = 24 mice per cell line). Once tumors reached approximately 200 mm³, mice were stratified into two treatment groups: control or radiation (n = 12 mice/24 tumors per group). The radiation group was subjected to four 2 Gy fractions over a period of 2 weeks. After completing the treatment regimen, tumor growth was monitored on a weekly basis to evaluate response to radiation. The results of this experimentation indicated that SCC2, SCC22B, and SCC90 cell line xenografts were sensitive to radiation, whereas SCC1, SCC47, and SCC147T were resistant (Fig. 5). Tumors harvested from mice in the control groups were evaluated for AXL expression and activation by IHC and staining intensity was scored as described in Fig. 1B. On average, the radiosensitive tumors expressed low levels of both AXL and pAXL-Y779 (1+ to 2+), with SCC2 and SCC90 having the lowest levels of staining (consistent with AXL expression levels detected in Fig. 1A). The radioresistant tumors, SCC1, SCC47, and SCC147T, expressed considerably more AXL and pAXL-Y779 (2+ to 4+ staining), especially SCC147T tumors. AXL expression was not associated with the HPV status of the HNSCC cell lines used (see Supplementary Materials and Methods for HPV status of cell lines used).

Next, the radiation responses of five HNSCC PDXs were evaluated for AXL and pAXL-Y779 expression levels (see Supplementary Table S4 for clinical parameters of patients before PDX establishment). For each PDX, dual flank tumors were established in 16 athymic nude mice. When tumors reached approximately 200 mm³, mice were stratified into two treatment groups: control or radiation (n = 8 mice/16 tumors per group). After completing the treatment regimen, tumor growth was monitored to evaluate response to radiation. The results of this experimentation indicated that two PDXs were sensitive to radiation (UW-SCC36 and UW-SCC22), whereas three were resistant (UW-SCC1, UW-SCC30, and UW-SCC6; Fig. 6). PDXs harvested from early passed tumors before treatment were stained for both AXL and pAXL-Y779 by IHC and staining intensity was scored as described in Fig. 1B. Consistent with Fig. 5, radiation-sensitive PDXs expressed low levels of both AXL and pAXL-Y779. In comparison, radiation-resistant PDXs had intense AXL and pAXL-Y779 staining (3+ staining for both markers). In this small PDX cohort (n = 5), HPV status was not associated with AXL expression or radiation response. Taken together, these data demonstrate that AXL is overexpressed and activated in PDXs that are intrinsically resistant to radiation therapy.

Discussion

The current study identifies AXL to be highly expressed and associated with worse clinical outcome in HNSCC. Elevated AXL expression has been identified as a poor prognostic factor for shorter relapse-free survival or overall survival in colon cancer (21), pancreatic cancer (25), and osteosarcoma (26). In addition, AXL expression was prognostic for increased lymph node involvement and/or clinical stage in lung adenocarcinoma (14), ovarian cancer (20), and breast cancer (19). Interestingly, male gender was
associated with high AXL expression in the current study, which is an association that has not been reported in other cancers. Several preclinical studies have highlighted the importance of AXL in regulating the metastatic potential of cancer cells (12, 14, 15, 17–19, 23, 24, 27), which is in agreement with the current findings (Figs. 2B and C). HNSCC cell proliferation was also decreased upon AXL knockdown or kinase inhibition (Figs. 1A and 2A), which is contrary to studies in ovarian cancer where only metastatic spread was abrogated after AXL knockdown (20). In models of breast and lung cancer, cell proliferation was decreased upon AXL inhibition as well, which corresponded to increased chemosensitivity (10, 18, 49). Taken together, targeting AXL may inhibit both proliferation and motility pathways in HNSCC.

AXL has been reported to play a role in resistance to chemotherapies and anti-EGFR therapies in non–small cell lung cancer (NSCLC; refs. 9–11, 49), triple-negative breast cancer (TNBC; refs. 13, 16, 49), and HNSCC (22). In this study, several HNSCC cell lines that were intrinsically resistant to cetuximab were sensitized upon transfection with siAXL or treatment with R428 (Fig. 3B and Supplementary Fig. S3B). In addition, AXL inhibition enhanced the antiproliferative effect of cetuximab in a cMet–positive HNSCC cell line (HN4; Fig. 3B). These data indicate that dual targeting both AXL and EGFR may provide beneficial antitumor responses irrespective of initial sensitivity to monotherapy. The enhanced efficacy of cetuximab may be due to the suppression of both EMT and SFK activity after AXL inhibition. EMT has been previously implicated in cetuximab-resistant HNSCCs, where resistant cells had increased vimentin and decreased E-cadherin levels (50). In addition, SFK activation of EGFR-Y1101 has been implicated in mediating the nuclear translocation of EGFR, a reported mechanism of cetuximab resistance (42, 44). In the current study, HN4 cells stably overexpressing AXL had an increased EMT signature, SFK activity and pEGFR-Y1101, all of which corresponded to increased cetuximab resistance (Fig. 3C). These data are supported by studies identifying a similar AXL regulated EMT signature in erlotinib-resistant HNSCC (22), TNBC (16), and NSCLC cell models (9, 49).

One of the most profound findings of the current study was the identification of AXL overexpression and hyperactivation in radiation-resistant HNSCC cell line xenografts and PDXs (Figs. 5 and 6). The correlation between AXL expression and activity in the radiation-resistant tumors implies an inherent role for AXL in radiation resistance. This is supported in Fig. 4, where AXL inhibition increased γ-H2AX foci and enhanced the sensitivity of HNSCC cells to radiation (Fig. 4A and B). AXL was further found to regulate the DNA repair pathway via AKT and DNA-PK activity (Fig. 4C and D). Because AKT and DNA-PK mediate DNA repair, their increased activity has been indicative of radioresistant cancer cells (47, 48); thus, targeting AXL may have radiosensitizing effects in HNSCC. Collectively, these studies are the first to identify AXL as a mediator of radiation response in HNSCC.

HPV infection has been shown to play a causal role in the development of a subset of HNSCCs (2). Importantly, patients with HPV-positive HNSCC demonstrate significantly improved survival outcomes with standard-of-care treatments (37, 51). One mechanism underlying the improved outcome of the HPV-positive population has been attributed to their increased sensitivity to radiation therapy (37, 52). However, there are several important molecular differences driving oncogenesis in HPV-positive versus HPV-negative HNSCCs that likely underlie the differential treatment response observed (53, 54). In the current study, among the 63 patient cohort, AXL expression was not associated with HPV positivity (as determined by p16 IHC). Although no correlation was determined, it is important to note that approximately 27% of the patients in this cohort had oropharyngeal cancer (anatomic area including the tonsils, base of tongue, soft palate, and lateral/posterior pharyngeal walls). Considering the oropharynx represents the site with the greatest proportion of HPV-associated cancers that are accurately defined by p16 expression (55), it would be important in the future to specifically evaluate the relationship between HPV status and AXL staining in patients with oropharyngeal cancers and compare the results.
to patients with nonoropharyngeal malignancies. In this manner, further research is required to determine whether there is a significant relationship between HPV status and AXL expression/function in HNSCC.

Several anti-AXL therapeutics are currently being evaluated for movement into clinical trials. R428, licensed as BGB324, has now undergone successful phase Ia clinical evaluation in healthy volunteers, where it was deemed safe and well tolerated (38). Although R428 is greater than 100 times more selective for AXL than several other tyrosine kinases (such as the insulin receptor, EGFR, and HER2), we cannot rule out the possibility that the antitumor responses observed in the current study were solely due to AXL inhibition (15). However, the use of both AXL siRNAs and the AXL-negative cell line, SCC2, throughout this study supports the specificity for AXL inhibition by R428. Several neutralizing anti-AXL monoclonal antibodies have also been designed, including YW327.652 and MAb173 (13, 27, 30); however, these therapies are still undergoing preclinical evaluation. To date, R428 is the most clinically advanced anti-AXL therapeutic, and thus, further evaluation of its benefit in HNSCC is warranted.

Figure 6. AXL is overexpressed and activated in radiation-resistant HNSCC PDXs. PDXs were evaluated for radiation response as described in the Supplementary Materials and Methods. Tumor growth was plotted as a percentage of averaged vehicle-treated tumor volumes at the last three time points of the study; **, $P < 0.01$. Representative IHC images of AXL and pAXL-Y779 staining in early passaged PDXs are shown (×20). Pathologic IHC quantitation (by D. Yang) was determined via a categorical scale from 0 to 4+. NS, not significant.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: T.M. Brand, D.L. Wheeler, A.P. Stein, R. Salgia, R.J. Kimple


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.M. Brand, M. Iida, A.P. Stein, K.L. Corrigan, C. Braverman, J.P. Coan, T.L. Fowler, D. Yang, M.W. Lingen, V. Saloura, V.M. Villaflor, R. Salgia, R.J. Kimple


Writing, review, and/or revision of the manuscript: T.M. Brand, D.L. Wheeler, M. Iida, A.P. Stein, T.L. Fowler, P.S. Gill, M.W. Lingen, V.M. Villaflor, R. Salgia, R.J. Kimple

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.M. Brand, D.L. Wheeler, H. Baharz, P.S. Gill

Study supervision: T.M. Brand, D.L. Wheeler, B.P. Bednarz

Other (data acquisition): H.E. Pearson

References


Acknowledgments

The authors acknowledge the University of Wisconsin Medical Radiation Research Center for their assistance during this project.

Grant Support

This work was supported by the Clinical and Translational Science Award (CTSA) program, through the NIH National Center for Advancing Translational Sciences (NCATS) grant UL1TR000427 (KL2TR000428), grant RSG-10-193-01-TBG from the American Cancer Society (to D.L. Wheeler), and grant 8W1XWH-12-1-0467 from United States Army Medical Research and Materiel Command (to D.L. Wheeler). CA160639 (to R.J. Kimple), and the NIH/NCI P30 CA14520 (UW Comprehensive Cancer Center Grant). T.L. Fowler was supported in part by the University of Wisconsin Science and Medicine Graduate Research Scholars program.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 13, 2014, revised February 5, 2015; accepted February 27, 2015; published OnlineFirst March 12, 2015.
28. Linner B, Keating AK, Epp HS, Graham DK. TAM receptor tyrosine
kinases: biologic functions, signaling, and potential therapeutic targeting in
P, et al. Ad kinase as a key target for oncology: focus on small molecule
AXL mediates resistance to cetuximab therapy. Cancer Res 2014;74:
5152–64.
inhibitor NPS-1034 exerts efficacy against lung cancer cells resistant to
EGFR kinase inhibitors because of MET or AXL activation. Cancer Res
Human epidermal growth factor receptor 3 (HER3) blockade with U3-
1287/AMG580 enhances the efficacy of radiation therapy in lung and head
33. Fowler TL, Fullerson RK, Micka JA, Kimpel RJ, Bednarz BP. A novel high-
throughput irradiator for in vitro radiation sensitivity biosassays. Phys Med
Biol 2014;59:1459–70.
34. Fowler TL, Bailey AM, Bednarz BP, Kimpel RJ. High-throughput detection
of DNA double-strand breaks using image cytometry. BioTechniques
Enhanced radiation sensitivity in HPV-positive head and neck cancer. Cancer Res
2013;73:6791–800.
Development and characterization of HPV-positive and HPV-negative
head and neck squamous cell carcinoma tumorigrafts. Clin Cancer Res
significance of p16(INK4a) and human papillomavirus in patients with
oropharyngeal cancer treated on TROG 02.02 phase III trial. J Clin Oncol
BCG324, a selective small molecule Ad kinase inhibitor to overcome EMT-
associated drug resistance in carcinomas: therapeutic rationale and early
clinical studies [abstract]. In: Proceedings of the 105th Annual Meeting of
the American Association for Cancer Research, 2014 Apr 5–9; San
39. Chou TC. Theoretical basis, experimental design, and computerized sim-
ulation of synergism and antagonism in drug combination studies. Phar-
40. Chou TC. Drug combination studies and their synergy quantification using
AXL Is a Logical Molecular Target in Head and Neck Squamous Cell Carcinoma

Toni M. Brand, Mari Iida, Andrew P. Stein, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-2648

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2015/03/13/1078-0432.CCR-14-2648.DC1

Cited articles
This article cites 54 articles, 26 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/21/11/2601.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/21/11/2601.full.html#related-urls

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.