AXL is a logical molecular target in head and neck squamous cell carcinoma

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

Purpose: Head and neck squamous cell carcinoma (HNSCC) represents the eighth most common malignancy worldwide. Standard of care treatments for HNSCC patients include surgery, radiation and chemotherapy. Additionally, the anti-epidermal growth factor receptor (EGFR) monoclonal antibody cetuximab is often used in combination with these treatment modalities. Despite clinical success with these therapeutics, HNSCC remains a difficult to treat malignancy. 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 (PDXs), 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 regimes 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. Additionally, 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.

Conclusion: Collectively, this study provides evidence for the role of AXL in HNSCC pathogenesis and supports further pre-clinical and clinical evaluation of anti-AXL therapeutics for the treatment of patients with HNSCC.
**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. In order 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-epidermal growth factor receptor (EGFR) monoclonal antibody, can benefit patients when combined with platinum chemotherapy or radiation (6-8). While 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 (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, we sought to determine if AXL is a functional molecular target in HNSCC, and if targeting AXL could enhance the efficacy of standard treatments used to treat HNSCC patients.
Materials and Methods

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

Antibodies and Compounds. All antibodies used are indicated below: R&D Systems: AXL (for immunoblotting) and pAXL (Y779). Cell Signaling Technology: Phospho-SFK (Y419), pDNA-PK (S216), DNAPK, pAKT (S473), AKT, p-γ-H2AX (S139), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and pan-tyrosine (pan-Tyr). Santa Cruz Biotechnology Inc.: AXL (for immunoprecipitation (IP)), E-Cadherin, Vimentin, and horseradish peroxidase (HRP)—conjugated goat–anti-rabbit IgG, goat–anti-mouse IgG, and donkey–anti-goat IgG. Abcam: EGFR and pEGFR (Y1101). Calbiochem: α-tubulin. R428 was purchased from Selleckchem (Houston, TX, USA). Cetuximab (ICM-225; Erbitux) was purchased from University of Wisconsin Pharmacy. Cisplatin, carboplatin, and camptothecin were purchased from LC Laboratories (Woburn, MA, USA).

Plasmids, transfection, and siRNA technology. Plasmid construction and stable selection of AXL overexpressing cells was described previously (30). Cells were transiently transfected with AXL siRNA (siAXL-1; ON-TARGETplus, SMARTpool #L-003104; GE Dharmacon, Lafayette, CO, USA or siAXL-2; cell signaling AXL siRNA I #6263) or nontargeting siRNA (siNT; ON-TARGETplus Non-targeting Pool, #D-001810; Dharmacon) using Lipofectamine RNAiMAX according to the manufacturer's instructions (Life Technologies, Grand Island, NY, USA). siAXL-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 Supplemental Materials and Methods (30, 32). Crystal violet assays were performed to identify the combinatorial effect of siAXL and radiation.

Apoptosis Assay: HNSCC cell lines were treated with 0.5 uM or 1.0 uM of R428 for 24 hours prior to staining with YO-PRO-1 and propidium iodide according to manufacture’s instructions (Vybrant® Apoptosis Assay Kit #4, YO-PRO®-1/Propidium Iodide, Invitrogen). Uptake of YO-PRO-1 and PI was measured using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo analysis software (TreeStar Inc).

Immunoblot analysis. Whole-cell lysis, immunoprecipitation and western blot analysis was 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 80-90% 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 cultivated in complete medium with/without indicated doses of R428. Alternatively, cells were transfected with siAXL or siNT for 24 hours prior to wound exposure. Photographs of the wound adjacent to reference lines were taken using an Olympus IX51 microscope (10×) 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. 24 hours post therapy cell invasion was measured as per manufacturers instructions.

**γ-H2AX fluorescent assay.** Cells were plated in 96-well dishes and pre-treated with vehicle or R428 (1uM). 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 (1:1000) (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, Sunnyvale, CA, USA). All γ-H2AX fluorescent values were averaged and then normalized to averaged values from vehicle treated cells.

**Cell line xenografts, patient derived xenografts (PDXs), and radiation response.** Cell line xenografts and PDXs 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 (TMA) construction.** HNSCC patients completed written consent in accordance with IRB approval from the University of Chicago (protocol number 10-343-A). See Supplemental Materials and Methods for details.
**Statistical Analysis.** HNSCC patient demographics and clinical characteristics were summarized using descriptive statistics in the Supplemental Materials and Methods. Student’s t-test was employed 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 if AXL could represent a molecular target in HNSCC, AXL expression was evaluated in a panel of 14 HSNCC cell lines (**Figure 1A, top**). These results indicated that 11 of the 14 cell lines expressed AXL, while three cell lines (SCC90, SCC2, and SCC1483) had either low or undetectable AXL protein levels. Since 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 non-targeting siRNA control (siNT) (**Figure 1A, bottom**), and cellular proliferation was measured 72 hours post-transfection. 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, while 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 TMA was stained for AXL via immunohistochemistry (IHC) using a previously validated anti-AXL antibody (30). This TMA contained several early passaged 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 0-4 where 0 represented no staining and 4+ was the most intense staining. Pathologic analysis (by D.Y.) of AXL staining patterns indicated that 82% of the PDXs expressed AXL, where AXL expression remained relatively consistent across early passaged tumors (**Figure 1B**). Four percent of the PDXs were negative for AXL expression. Collectively, these data indicate 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 1 for clinical information of the patient cohort). Pathologic analysis (by M.W.L.) of this cohort indicated that 38% expressed high levels of AXL, while 62% expressed either low or no AXL (Figure 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 (Figure 1C). Using a logistic regression model, the multivariate predictors of elevated AXL expression included: male vs. female gender (odds ratio (OR) 6.38 (95% confidence interval (CI): 1.18, 34.5)), poorly differentiated vs. moderate/well differentiated tumor grade (OR 4.03 (95% CI: 1.05, 15.48)) and presence of distant metastasis (OR 3.58 (95% CI: 1.03, 12.53)) (Supplemental Table 2A). 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 to patients with low AXL (4.3 years (95% CI: 1.4, ∞)) (Figure 1C and Supplemental Table 2B). The hazard ratio for PFS among patients with high vs. 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.** Since 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 uM – 1.0 uM) for 72-96 hours prior to performing proliferation assays. All AXL expressing HNSCC cell lines were significantly growth inhibited with increasing doses of R428 (Figure 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. Further, via evaluation of pan phospho-tyrosine post immunoprecipitation with an anti-AXL antibody, R428 inhibited AXL activation at several doses that also resulted in the most robust anti-proliferative responses (0.5 uM and 1.0 uM).

In addition to influencing cellular proliferation pathways, AXL has been shown to mediate the metastatic potential of cancer cells (26, 27). To determine if AXL regulates the migratory potential of HNSCC cells, wound-healing assays were performed using SCC4, SCC6, SCC104, HN4 and SCC2 cells post AXL inhibition. In this assay, cells were subjected to injury directly after treatment with either 0.1 uM or 0.5 uM R428. Wound length was measured after the wound was first made (0 hr) and at the indicated time points post wound exposure (Figure 2B). SCC4 and SCC6 cells treated with either dose of R428 displayed less wound closure at both time points as compared to 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 uM 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 impact their migratory capacity. To further validate...
the specificity of R428 for AXL, cells were transfected with siNT or siAXL for 24 hours prior to performing wound-healing assays (Supplemental Figure 1). AXL knockdown significantly impacted wound closure in all AXL expressing cell lines tested as compared to cells transfected with siNT.

Next, the invasive potential of the HNSCC cells was measured via Boyden chamber invasion assays 24 hours post R428 treatment (Figure 2C). All AXL expressing cell lines examined were significantly inhibited in their invasive potential when pretreated with 1.0 uM of R428, while HN4 and SCC4 cells were inhibited at lower doses (0.5 uM). Additionally, 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 post treatment with 0.5 uM and 1.0 uM R428 (Supplemental Figure 2). 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 if AXL inhibition could augment the sensitivity of HNSCC cells to standard of care treatments, we first tested if AXL inhibition enhanced HNSCC cell line sensitivity to the platinum based chemotherapies cisplatin and carboplatin (Figure 3A). Evaluation of cellular proliferation 72-96 hours post-treatment 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 (39-41)), the nature of the interaction between R428 and each chemotherapy was evaluated for synergy as described in the Supplemental Materials and Methods. AXL inhibition synergized with both cisplatin and carboplatin in all AXL expressing cell lines, where the ratio of the observed (O) to expected (E) effect was less than 1 (Supplemental Table 3A and 3B). Additionally, siRNA targeting AXL synergized with cisplatin, providing further evidence for the specificity of R428 for AXL inhibition (Supplemental Figure 3A). Collectively, these results demonstrate that AXL inhibition enhances chemotherapy sensitivity in HNSCC cells.

Since AXL expression has been shown to mediate cetuximab resistance (30), we hypothesized that AXL inhibition may improve the efficacy of cetuximab therapy in HNSCC. Therefore, several HNSCC cell lines were treated with vehicle, R428 (1.0 μM), cetuximab (100 nM), or the combination, and cellular proliferation was measured 72 hours later (Figure 3B). Three cell lines (SCC4, SCC6, and SCC104) were resistant to cetuximab, while HN4 was sensitive to cetuximab monotherapy. Importantly, when treated with both R428 and cetuximab, all cell lines demonstrated significant reductions in cellular proliferation as compared to 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 (Supplemental Table 3C). Additional siRNA studies confirmed specificity of R428 for AXL inhibition, as AXL knockdown synergized with cetuximab in all cell lines tested (Supplemental Figure 3B). To further evaluate if 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 to HN4-Vector cells (Figure 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 (SFKs) (42-44). Additionally, 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 (Figure 1A), including SCC1, SCC47, and SCC147T (35). We thus examined if 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 (33)), SCC1, SCC47, SCC147T, and SCC2 were irradiated with 4 Gy after 24 hour pre-treatment with either vehicle or R428 (1 μM). Then, the induction of DNA double strand breaks (DSBs) was examined via gamma-H2AX (γ-H2AX), which is phosphorylated and recruited to sites of DNA damage in response to radiation (Figure 4A). At four hours post irradiation, total γ-H2AX fluorescent intensity per cell was determined via a fluorescent plate reader with image cytometer. This
system allowed for the quantitation 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 to 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 (Figure 4B). In this experiment, an equal number of cells were plated per well and subsequently pre-treated with 0.25 uM R428 for 24 hours prior to XRT exposure. Non-irradiated cells treated with vehicle or R428 demonstrated similar plating efficiency as compared to vehicle treated cells (data not shown). However, all AXL expressing cell lines pre-treated with R428 demonstrated significantly reduced survival following radiation exposure as compared to cells treated with radiation only. The effect of R428 and radiation was determined to be synergistic in all AXL expressing cell lines tested (Supplemental Table 3C). R428 pre-treated SCC2 cells did not demonstrate a reduction in survival as compared to cells treated with radiation only. Finally, AXL knockdown with siRNA prior to radiation exposure resulted in reduced cellular viability, further supporting the AXL specific radiosensitizing effects of R428 (Supplemental Figure 3C).

To investigate the potential molecular mechanisms underlying this enhanced radiation response, the activation of DNA-protein kinase (DNA-PK) and AKT were examined post R428 and radiation therapy (Figure 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 to 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 four hours later (Figure 4D, left). HN4-AXL cells had less γ-H2AX foci indicating that these cells had more repaired DNA DSBs as compared to HN4-Vector cells. Clonogenic survival analyses indicated that HN4-AXL cells had significantly more surviving cells post irradiation as compared to HN4-Vector cells (Figure 4D, middle). Additionally, HN4-AXL cells expressed increased levels of phosphorylated DNA-PK and AKT post irradiation (Figure 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 line 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 and radiation (n=12 mice/24 tumors per group). The radiation group was subjected to four 2 Gy fractions over a period of two 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, while SCC1, SCC47, and SCC147T were resistant (Figure 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 Figure 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 Figure 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 Supplemental 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 Supplemental Table 4 for clinical parameters of patients prior to PDX establishment). For each PDX, dual flank tumors were established in 16 athymic nude mice. When tumors reached approximately 200 mm$^3$, 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) while three were resistant (UW-SCC1, UW-SCC30 and UW-SCC6) (Figure 6). PDXs harvested from early passaged tumors prior to treatment were stained for both AXL and pAXL-Y779 by IHC and staining intensity was scored as described in Figure 1B. Consistent with Figure 5, radiosensitive PDXs expressed low levels of both AXL and pAXL-Y779. In comparison, radiation resistant PDXs had very 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). Additionally, 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 (Figures 2B and 2C). HNSCC cell proliferation was also decreased upon AXL knockdown or kinase inhibition (Figures 1A and 2A), which is contrary to studies in ovarian cancer where only metastatic spread was abrogated post 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 chemotherapy and anti-EGFR therapies in non small cell lung cancer (NSCLC) (9-11, 49), triple-negative breast cancer (TNBC) (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 (Figure 3B and Supplemental Figure 3B). Additionally, AXL inhibition enhanced the anti-proliferative effect of cetuximab in a cetuximab sensitive cell line (HN4) (Figure 3B). These data indicate that dual targeting both AXL and EGFR may provide beneficial anti-tumor
responses irrespective of initial sensitivity to monotherapy. The enhanced efficacy of cetuximab may be due to the suppression of both EMT and SFK activity post AXL inhibition. EMT has been previously implicated in cetuximab resistant HNSCCs, where resistant cells had increased vimentin and decreased E-cadherin levels (50). Additionally, 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 (Figure 3C). This data is 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 (Figures 5 and 6). The correlation between AXL expression and activity in the radiation resistant tumors implies an inherit role for AXL in radiation resistance. This is supported in Figure 4, where AXL inhibition increased γ-H2AX foci and enhanced the sensitivity of HNSCC cells to radiation (Figures 4A and 4B). AXL was further found to regulate the DNA repair pathway via AKT and DNA-PK activity (Figures 4C and 4D). Since 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 outcome 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 non-oropharyngeal 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). While 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 anti-tumor 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 YW327.6S2 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.

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References

Figure Legends

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 nM), siAXL-2 (100 nM) or non-targeting (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 ± s.e.m. *p<0.05, **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 (10x). Pathologic IHC quantitation (by D.Y.) was determined via a categorical scale from 0-4+. (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 (20x). Pathologic IHC quantitation (by M.W.L) was determined via a categorical scale from 0-3+. High AXL expression was significantly associated with shorter median progression free survival (PFS) in HNSCC patients as analyzed by the Kaplan-Meir method. *p<0.05.

Figure 2. AXL mediates HNSCC cell proliferation, migration, and invasion. (A) Cells were treated with R428 at indicated doses for 72-96 hours prior to performing proliferation assays. Proliferation is plotted as a percentage of growth relative to vehicle-treated cells (n=6 in three independent experiments). R428 knockdown of AXL activity was evaluated via IP analysis for pan-tyrosine 24 hours post-treatment. (B) Cells were treated with R428 or vehicle and subsequently subjected to wound exposure. Wound length was imaged and measured after the
wound was first made (0 hr) and at the indicated time points post wound exposure (10x) (n=3-6 replicates in three independent experiments). (C) Cells were plated in a 96-well Boyden chamber and subsequently treated with R428 (indicated doses) or vehicle. 24 hours later, invading cells that had penetrated the bottom side of the chamber were quantified by calcein-AM. Cell invasion was calculated by normalizing fluorescent values of R428 treated invading cells to vehicle controls (n=9 in three independent experiments). All data points are represented as mean ± s.e.m. *p<0.05, **p<0.01.

Figure 3. AXL inhibition increases the sensitivity of HNSCC cells to chemotherapy and cetuximab. (A) Cells were treated with cisplatin (2.0 uM), carboplatin (10 uM), R428 (1.0 uM) or the combination of R428 and each chemotherapy for 72-96 hours before performing proliferation assays. (B) Cells were treated with vehicle, cetuximab (100 nM), R428 (1.0 uM), or the combination for 72 hours before performing proliferation assays. (C) HN4 cells stably overexpressing AXL (HN4-AXL) or pcDNA6.0 Vector (HN4-Vector) were treated with increasing doses of cetuximab (0.1–100 nM) for 72 hours before performing proliferation assays. Whole cell lysate was harvested and subjected to immunoblot analysis. GAPDH was used as a loading control. All proliferation assays are plotted as a percentage of growth relative to vehicle-treated cells (n=6 in three independent experiments). Data points are represented as mean ± s.e.m. *p<0.05, **p<0.01.

Figure 4. Targeting AXL can enhance the efficacy of radiation therapy in HNSCC. (A) Cells were pre-treated with vehicle or R428 (1.0 uM) for 24 hours and then subjected to 4 Gy radiation (XRT). γ-H2AX fluorescence per cell was evaluated via a SpectraMax i3 plate reader.
with MiniMax 300 imaging cytometer four hours post XRT. All γ-H2AX fluorescent values were averaged and normalized to averaged values from vehicle treated cells (n=12 in three independent experiments). (B) Cells were pre-treated with vehicle or R428 and then subjected to indicated doses of radiation: 4 Gy (SCC1, SCC47, and SCC2) or 2 Gy (SCC147T). Clonogenic survival was determined 10-14 days post radiotherapy (n=6 in three independent experiments). (C) Cells were pre-treated with R428 (1.0 uM) for 24 hours prior to receiving 4 Gy XRT. 15 minutes post radiotherapy cells were lysed and processed for immunoblot analysis for indicated proteins. GAPDH was used as a loading control. (D) HN4-Vector and HN4-AXL stable cells were subjected to 4 Gy radiation prior to fixation and γ-H2AX evaluation as in (A). Clonogenic survival analysis and immunoblot analysis were performed as in (B) and (C). Data points are represented as mean ± s.e.m. *p<0.05, **p<0.01.

**Figure 5. AXL is overexpressed and activated in radiation resistant HNSCC cell line xenografts.** Cell line xenografts were established and evaluated for radiation response as described in the Supplemental 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<0.05, **p<0.01. Representative IHC images of AXL and pAXL-Y779 staining from control group tumors are depicted (20x). Pathologic IHC quantitation (by D.Y.) was determined via a categorical scale from 0-4+. NS: not significant.

**Figure 6. AXL is overexpressed and activated in radiation resistant HNSCC PDXs.** PDXs were evaluated for radiation response as described in the Supplemental 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 (20x). Pathologic IHC quantitation (by D.Y.) was determined via a categorical scale from 0-4+. NS: not significant
Figure 1

A. Western blot analysis of AXL expression in various squamous cell carcinoma (SCC) lines.

B. Immunohistochemical analysis showing the effect of AXL knockdown on cell proliferation in PDX tissue microarrays. Images are categorized into low and high AXL score groups.

C. Kaplan-Meier survival curves showing progression-free survival in patients with low and high AXL expression levels.
Figure 2

A

![Graph showing cell proliferation](image)

B

![Images of cell cultures](image)

C

![Graph showing cell invasion](image)
Figure 3

A

Vehicle C platin (2.0 uM)  Carboplatin (10 uM)

HN4-Vector  HN4-AXL

**  **

SCC4  SCC6  HN4  SCC104  SCC2

Vehicle  CTX + R428

**  **

R428

**  **

SCC4  SCC6  HN4  SCC104  SCC2

Vehicle  CTX + R428

**  **

B

**  **

C

HN4-Vector  HN4-AXL

**  **

Veh  0.1nM  1.0nM  10nM  100nM

E-Cadherin  pEGFR-Y1101  EGFR  pSFK-Y419  Vimentin  GAPDH

Cell Proliferation Relative to Vehicle (%)

Cell Proliferation Relative to Vehicle (%)

Cell Proliferation Relative to Vehicle (%)
Figure 6

Radiation Sensitive PDXs

UW-SCC36

% Tumor Growth
Normalized to Control

Day 25  Day 29  Day 35

**  **  **

AXL  pAXL  No Antibody

UW-SCC22

% Tumor Growth
Normalized to Control

Day 21  Day 29  Day 43

**  **  **

AXL  pAXL  No Antibody

Radiation Resistant PDXs

UW-SCC1

% Tumor Growth
Normalized to Control

Day 49  Day 60  Day 69

NS  NS  NS

AXL  pAXL  No Antibody

UW-SCC30

% Tumor Growth
Normalized to Control

Day 55  Day 65  Day 72

NS  NS  NS

AXL  pAXL  No Antibody

UW-SCC6

% Tumor Growth
Normalized to Control

Day 65  Day 80  Day 95

NS  NS  NS

AXL  pAXL  No Antibody
Correction: AXL Is a Logical Molecular Target in Head and Neck Squamous Cell Carcinoma


In the original version of this article (1), three figures are incorrect. Fig. 6 uses the same image to represent “No Antibody” for UW-SCC36 and UW-SCC6; Fig. 2C includes bar graphs that exhibit irregularities, including an improperly scaled y-axis; and Fig. 2B contains duplicate images in two different cases (SCC104 and SCC2). The authors provided the journal with corrected files for Fig. 6, Fig. 2C, and for SCC104 in Fig. 2B; because corrected files were not available for SCC2 in Fig. 2B, the incorrect images were removed from the figure. These errors have been corrected in the latest online HTML and PDF versions of the article. The authors regret these errors.

Reference

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