AXL Mediates Resistance to Cetuximab Therapy

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Abstract

The EGFR antibody cetuximab is used to treat numerous cancers, but intrinsic and acquired resistance to this agent is a common clinical outcome. In this study, we show that overexpression of the oncogenic receptor tyrosine kinase AXL is sufficient to mediate acquired resistance to cetuximab in models of non–small cell lung cancer (NSCLC) and head and neck squamous cell carcinoma (HNSCC), where AXL was overexpressed, activated, and tightly associated with EGFR expression in cells resistant to cetuximab (CtxR cells). Using RNAi methods and novel AXL-targeting agents, we found that AXL activation stimulated cell proliferation, EGFR activation, and MAPK signaling in CtxR cells. Notably, EGFR directly regulated the expression of AXL mRNA through MAPK signaling and the transcription factor c-Jun in CtxR cells, creating a positive feedback loop that maintained EGFR activation by AXL. Cetuximab-sensitive parental cells were rendered resistant to cetuximab by stable overexpression of AXL or stimulation with EGFR ligands, the latter of which increased AXL activity and association with the EGFR. In tumor xenograft models, the development of resistance following prolonged treatment with cetuximab was associated with AXL hyperactivation and EGFR association. Furthermore, in an examination of patient-derived xenografts established from surgically resected HNSCCs, AXL was overexpressed and activated in tumors that displayed intrinsic resistance to cetuximab. Collectively, our results identify AXL as a key mediator of cetuximab resistance, providing a rationale for clinical evaluation of AXL-targeting drugs to treat cetuximab-resistant cancers. Cancer Res; 74(18): 5152–64. ©2014 AACR.

Introduction

The TAM family of receptor tyrosine kinases (RTK) is composed of three family members: Tyro-3 (Sky), AXL (Ark or Ufo), and MerTK. Cognate ligand binding to TAM receptors on the cell surface leads to receptor dimerization, kinase domain activation, and auto/trans-phosphorylation of tyrosine residues located on each receptor’s cytoplasmic tail (1). The activation of TAM receptors stimulates PI3K/AKT and Ras/Raf/Mek/Erk (MAPK) signaling cascades, leading to increased cell survival, proliferation, migration, invasion, and angiogenesis (1–4).

TAM family overexpression and activation have been observed in many human cancers (1–11). Recently, the AXL receptor has been implicated in cancer cell resistance to anti-EGFR tyrosine kinase inhibitors (TKI; refs. 12–17) and other chemotherapeutics (10, 15, 18). Collectively, these data indicate that AXL functions as a potent oncogene that can modulate resistance to conventional and targeted cancer therapies.

Cetuximab is an anti-EGFR monoclonal antibody that has shown efficacy in treating head and neck squamous cell carcinoma (HNSCC), metastatic colorectal cancer (mCRC), and non–small cell lung cancer (NSCLC; refs. 19–26). Unfortunately, clinical studies indicate that most patients who initially respond to cetuximab eventually acquire resistance (27–29). To understand the mechanisms of acquired resistance, we previously created a model in which the cetuximab-sensitive (CtxS) NSCLC cell line NCI-H226 was treated with increasing doses of cetuximab for a period of six months until resistant single cell clones emerged (30). Analysis of cetuximab-resistant (CtxR) clones demonstrated that the expression of EGFR and its activation was dramatically increased because of dysregulated EGFR internalization and degradation without mutation of the receptor (30). Overall, CtxR cells remained highly addicted to the EGFR signaling network (30–32).

On the basis of these previous findings, we investigated whether the AXL receptor played a role in cetuximab resistance. Examination of in vitro NSCLC and HNSCC models of acquired resistance indicated that AXL was highly overexpressed and activated in CtxR cells. Further analysis indicated that CtxR cells had increased dependency on AXL for cellular proliferation, EGFR activation, and MAPK signaling.
was also examined in tumors harvested from de novo–acquired Ctx\textsuperscript{R} NCI-H226 xenografts, where AXL was highly activated and associated with the EGFR. Finally, AXL was overexpressed and hyperactivated in HNSCC patient-derived xenografts (PDX) that were intrinsically resistant to cetuximab therapy. Collectively, this work indicates that AXL plays a role in cetuximab resistance and provides rationale for the clinical evaluation of anti-AXL therapeutics for the treatment of cetuximab resistant cancers.

Materials and Methods

Cell lines and development of acquired resistance

The human NSCLC cell line NCI-H226 was purchased from ATCC and maintained in 10% FBS in RPMI-1640 (Mediatech Inc.) with 1% penicillin and streptomycin. The HNSCC cell line UM-SCC1 was provided by Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI) and maintained in 10% FBS in Dulbecco’s Modified Eagle Medium (DMEM) with 1% penicillin and streptomycin. The development of Ctx\textsuperscript{R} cells has been previously described (30–32). All Ctx\textsuperscript{R} cell lines were validated to express wild-type (WT) EGFR by sequencing.

Antibodies

All antibodies were purchased from commercial sources as indicated below:

- RKD Systems: AXL (for immunoblotting) and pAXL-Y779.
- Cell Signaling Technology: pAXL-Y702, pEGFR-Y1068, pMAPK (T202/Y204), MAPK, p-ERK1/2 (T202/Y204), AXL (for immunoprecipitation), and horseradish peroxidase (HRP).
- GAPDH, Santa Cruz Biotechnology Inc.: pEGFR-Y1068, pAXL-Y702, pMAPK (T202/Y204), MAPK, p-ERK1/2 (T202/Y204), AXL (for immunoprecipitation), and horseradish peroxidase (HRP).
- Actin, anti-goat IgG, Life Technologies: AXL (for immunoprecipitation), and donkey–anti-mouse IgG, horseradish peroxidase (HRP).
- Anti-AXL (Santa Cruz Biotechnology), cetuximab, or IgG antibodies (Santa Cruz Biotechnology) were used.

Material and methods

R428 was purchased from Selleckchem and MAb173 was produced in the laboratory of Dr. Parakash Gill (Department of Medicine and Pathology, University of Southern California, Los Angeles, CA). Cetuximab (ICM-225; Erbitux) was purchased from University of Wisconsin Pharmacy. EGF was purchased from Millipore and TGF\textbeta was produced in the laboratory of Dr. Parkash Gill (Department of Medicine and Pathology, University of Southern California, Los Angeles, CA). Cetuximab (ICM-225; Erbitux) was purchased from University of Wisconsin Pharmacy. EGF was purchased from Millipore and TGF\textbeta was purchased from Sigma-Aldrich.

Flow cytometric analysis

Cells were processed for immunoprecipitation as previously described (34). Five-hundred micrograms of protein, 2 μg of anti-AXL (Santa Cruz Biotechnology), cetuximab, or IgG antibody (Santa Cruz Biotechnology) were used.

Plasmids, transfection, and stable cell line construction

pDONR223-AXL (Plasmid 23945) was purchased from Addgene and subcloned into the BamHI/EcoRI restriction sites of the pcDNA6.0 expression vector (Life Technologies). Stable transfection was performed using Lipofectamine LTX and Opti-MEM I (Life Technology) commencing 48 hours after transfection via 6 μg/mL blasticidin to the growth media. Single-cell clones were chosen for expansion and validation for AXL expression.

cDNA synthesis and qPCR

Total RNA and cDNA synthesis were prepared as previously described (34). All reactions were performed in triplicate. To determine the normalized value, 2\textsuperscript{-ΔΔCt} values were compared between AXL and 18S, where the change in crossing threshold (ΔC\textsubscript{T}) = C\textsubscript{T}(AXL) – C\textsubscript{T}(18S) and ΔΔC\textsubscript{T} = ΔC\textsubscript{T}(AXL, HCA1, HCA4, or HCB) – ΔC\textsubscript{T}(HC1, HC4, or HC8) or ΔΔC\textsubscript{T} = ΔC\textsubscript{T}(NT) – ΔC\textsubscript{T}(AXL).

Statistical analysis

Student t tests were used to evaluate differences in proliferation, AXL mRNA expression, and pAXL-Y779 expression levels by IHC. Differences were considered statistically significant if * P < 0.05.
Results

**AXL is overexpressed and activated in a model of acquired resistance to cetuximab**

The NSCLC CtxR clones HC1, HC4, and HC8 have been previously shown to be resistant to increasing doses of cetuximab as compared to the CtxS NCI-H226 parental cell line HP (30, 31). Analysis of CtxR clones HC1, HC4, and HC8 demonstrated that all clones expressed increased AXL mRNA and protein as compared to HP cells (Fig. 1A). Furthermore, AXL exhibited increased phosphorylation on tyrosine 702 and 779 in all CtxR clones. In addition, MAPK and AKT pathways were hyperactivated and there was increased expression and phosphorylation of the transcription factor c-Jun in CtxR clones. Moreover, plasma membrane levels of AXL were detected via flow cytometry, where CtxR cells had approximately 50% to 80% more surface AXL expression as compared to HP cells (Fig. 1B). Collectively, these data demonstrate that AXL is overexpressed and activated in established clones with acquired resistance to cetuximab.

**AXL and EGFR cooperate in CtxR clones to sustain proliferation via MAPK and c-Jun**

CtxR clones are known to be highly dependent on EGFR for proliferation (30–32). To determine whether AXL also plays a role in CtxR cell proliferation, proliferation assays were performed 72 hours after transfection with a pooled siAXL or siNT (Fig. 2A). Loss of AXL expression resulted in statistically significant inhibition of proliferation (25%–35%) in all three CtxR clones. As compared with parental HP cells, the CtxR clones demonstrated significantly greater decreases in proliferation after AXL knockdown (P < 0.01). Analysis of CtxR clones after AXL knockdown demonstrated that EGFR activation was severely diminished at both tyrosine 1068 and 1173, autophosphorylation sites responsible for recruiting Grb2 and Shc (Fig. 2B; ref. 37). In addition, the activation of c-Raf, p44/42 MAPK, AKT, and ribosomal protein S6 (rpS6) were diminished in all CtxR clones upon AXL knockdown, whereas the activation of these molecules were relatively unchanged or slightly increased in HP cells (Fig. 2B). Interestingly, ablation of HER2 or HER3 receptors, previously shown to be hyperactivated in CtxR cells (30), did not affect the phosphorylation of EGFR at either tyrosine site (Fig. 2B, inset). Collectively, these data demonstrate that CtxR clones are dependent on AXL for cellular proliferation via EGFR activation and downstream signaling.

To determine whether AXL and EGFR were physically associated in CtxR clones, coimmunoprecipitation experiments were performed and indicated that AXL was associated with EGFR in all CtxR clones but not parental cells (Fig. 2C). EGFR and AXL cooperation was further analyzed by reciprocally knocking down EGFR expression with siRNA (Fig. 2D). EGFR knockdown

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### Figure 1.

The RTK AXL and its downstream effector molecules are overexpressed in cetuximab resistant cells. A, whole-cell lysate was harvested from the CtxS parental cell line (HP) and three CtxR cell clones (HC1, HC4, and HC8) followed by immunoblotting for the indicated proteins. GAPDH was used as a loading control. Total AXL protein expression was quantitated using ImageJ software. AXL mRNA expression was detected by qPCR and normalized to AXL expression in HP cells (n = 3 in three independent experiments). 18S was used as an endogenous control. B, surface level AXL expression was detected by flow cytometry and normalized to HP. IgG-stained cells were used as a background control (n = 3 in two independent experiments). Data, mean ± SEM. **,** P < 0.01.

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led to a loss of total AXL protein and mRNA expression in CtxR clones and parental HP cells, as well as diminished activation of c-Raf, p44/42 MAPK, AKT, rpS6, and c-Jun. To examine whether EGFR regulation of AXL was contingent on MAPK or AKT signaling directly, we alternatively knocked down p44/42 MAPK or AKT1 with siRNA (Fig. 2E). This experiment indicated that knockdown of p44/42 MAPK led to a loss of AXL mRNA and protein expression, whereas AKT1 did not regulate AXL expression. These results suggest that EGFR regulates AXL expression specifically through MAPK signaling.

Previous studies indicated that the AXL promoter contains binding motifs for AP-1 family transcription factors, in which phorbol myristate acetate (PMA) stimulation of leukemia cells led to increased AXL expression through MAPK signaling to

Figure 2. Cetuximab-resistant cells depend on AXL and its cooperation with EGFR. A, cells were transfected with siAXL or siNT for 72 hours before performing proliferation assays. Proliferation is plotted as percentage of growth relative to NT-transfected cells (n = 6 in three independent experiments). B, cells were incubated with siAXL or NT siRNA for 72 hours before harvesting whole-cell lysate and immunoblotting for the indicated proteins. GAPDH was used as a loading control. Phosphorylation of EGFR on tyrosine 1068 and 1173 were quantitated using ImageJ software. Inset, cells were transfected with siRNA against HER2, HER3, or NT siRNA for 72 hours before harvesting whole-cell lysate. GAPDH was used as a loading control. C, 500 μg of whole-cell lysate was subjected to immunoprecipitation (IP) analysis with cetuximab (IP:EGFR), anti-AXL (IP:AXL), or anti-IgG (IP:IgG) antibody followed by immunoblotting (IB) for either AXL or EGFR. IgG heavy chain staining from the IB:AXL blot was used as a loading control. D, whole-cell lysate and mRNA were harvested from CtxR clones 72 hours after transfection with EGFR siRNA (D), MAPK and AKT1 siRNAs (E), c-Jun siRNA (F), or NT siRNA. GAPDH was used as loading control for protein. In D, AXL protein expression was quantitated using ImageJ software. AXL mRNA expression was detected by qPCR and normalized to AXL expression in siNT-transfected cells (n = 3 in three independent experiments). 18S was used as an endogenous control. Data, mean ± SEM. *, P < 0.05; **, P < 0.01.
the transcription factor c-Jun (38). Because Ctx^R clones were found to overexpress c-Jun (Fig. 1A), we hypothesized that c-Jun may function downstream of MAPK to regulate AXL mRNA expression. To investigate this, c-Jun was knocked down with siRNA (Fig. 2F), leading to an approximate 35% to 55% decrease in AXL mRNA levels. Moreover, there was a loss of AXL protein expression, which appeared similar to the levels detected after EGFR or MAPK knockdown (Fig. 2D and E). Importantly, this led to a loss of EGFR activation in Ctx^R clones, but not in parental HP cells, indicating that AXL is required for EGFR activation and subsequent signaling in the resistant setting. Collectively, these data indicate that AXL expression and subsequent EGFR activation are regulated through the MAPK/c-Jun signaling pathway in Ctx^R clones.

Ctx^R cells are sensitive to anti-AXL monoclonal antibody and TKI therapies

Because Ctx^R clones were sensitive to AXL knockdown by siRNA, we hypothesized that these cells would also be sensitive to anti-AXL therapeutics. First, we tested the ability for the anti-AXL monoclonal antibody MAb173 to inhibit Ctx^R cell proliferation (Fig. 3A). Ctx^R clones were significantly growth inhibited upon treatment with increasing doses of MAb173, whereas Ctx^S HP cells were less sensitive. In addition, the growth-inhibitory effects of Ctx^R clones were statistically decreased from the effect on HP cells when treated with 50 and 100 μg/mL of MAb173 (P < 0.01). Consistent with previous studies (9), MAb173 induced AXL degradation (Fig. 3B). Interestingly, total EGFR protein levels were reduced upon MAb173 treatment of Ctx^R clones, in addition to loss of MAPK signaling. MAb173 did not affect the activation of EGFR or MAPK signaling in HP cells.

Next, the small-molecule TKI R428, which has greater than 100-fold selectivity for AXL as compared with EGFR or Tyro and 50-fold greater affinity than Mer (39), was tested for therapeutic benefit in Ctx^R clones (Fig. 4A). All Ctx^R clones demonstrated robust antiproliferative effects upon treatment with 0.8 and 1 μmol/L of R428, whereas HP cells were less sensitive.
sensitive at these concentrations. In addition, the growth-inhibitory effects of CtxR clones were statistically decreased from the effect on HP cells when treated with 0.8 and 1 μmol/L of R428 (P < 0.01). Analysis of CtxR clones after treatment, via pan-tyrosine, demonstrated that AXL phosphorylation was inhibited with 1.0 μmol/L of R428, the same dose that elicited antiproliferative responses (Fig. 4B). In addition, R428 treatment led to a loss of EGFR phosphorylation on tyrosine 1068 and MAPK signaling, whereas these targets were relatively unaffected in HP cells (Fig. 4C). Interestingly, both MAb173 and R428 did not influence the apoptosis pathway in CtxR clones (data not shown), indicating that AXL more predominantly activates growth-promoting pathways in resistant cells.

**AXL activation and overexpression confers cetuximab resistance in vitro and in vivo mouse xenograft models**

To confirm the role of AXL in cetuximab resistance, AXL was stably overexpressed in the CtxR parental cell line HP (Fig. 5A). Immunoprecipitation analysis of HP-AXL stable cells indicated that AXL was phosphorylated on tyrosine 779, resulting in
increased phosphorylation of EGFR and downstream MAPK signaling. Cetuximab dose–response proliferation assays demonstrated that HP-AXL cells were statistically more resistant to cetuximab as compared with HP-Vector cells (P < 0.01; Fig. 5B). HC4 cells served as a cetuximab-resistant control in these experiments. These data demonstrate that the stable overexpression of AXL can confer resistance to cetuximab in a Ctx8 cell line, supporting a putative role for AXL in the development of cetuximab resistance.

We previously reported that Ctx8 clones overexpressed EGFR ligands (36); however, whether EGFR ligands influenced cetuximab resistance through regulating AXL activity and/or association with the EGFR was not investigated. Therefore, HP cells were stimulated with two EGFR ligands, EGF or TGFα, and subsequently measured for AXL activation, association with the EGFR, and cetuximab response (Fig. 5C). Analysis of HP cells after ligand stimulation indicated that both ligands led to increased AXL activation and association with the EGFR (detected by immunoprecipitation analysis). In addition, incubation with either ligand resulted in increased resistance to cetuximab. Interestingly, the ligand for AXL, Gashi, was not overexpressed in Ctx8 clones and did not drive resistance in HP cells (data not shown). Collectively, these data suggest that AXL may influence cetuximab resistance through stimulating AXL activation and association with the EGFR.

To further analyze the role of AXL in cetuximab resistance, we developed de novo tumors with acquired resistance to cetuximab in vivo (31, 32). To develop de novo–acquired resistance, the Ctx8 cell line NCI-H226 was inoculated unilaterally into the dorsal flank of 11 athymic nude mice (Fig. 5D). Once tumors reached approximately 100 mm3, 4 mice were treated with IgG control antibody (1 mg/mouse) and 7 mice were treated with cetuximab (1 mg/mouse) by intraperitoneal injection twice weekly. Tumors treated with IgG grew rapidly (tumors denoted as IgG-1 to IgG-4 in Fig. 5D), whereas all cetuximab-treated tumors displayed initial growth control. Interestingly, these tumors with acquired resistance to cetuximab as compared with HP-Vector cells (P < 0.01; Fig. 5E). To detect the levels of total and activated AXL (Y779), immunoprecipitation analysis was performed from tumor lysates. Strikingly, a double banding pattern for total AXL was observed in all Ctx8 tumors, whereas a single AXL band was observed in the IgG-treated tumors. The upper band corresponds to a shift in AXL molecular weight due to the presence of phosphorylated AXL, which was detected by the phospho-AXL-Y779 antibody (Fig. 5D, arrows). In addition, AXL was associated with EGFR only in the Ctx8 tumors by immunoprecipitation (Fig. 5D). Analysis of whole-cell lysate indicated that EGFR was also highly activated (indicated by tyrosine 1068 phosphorylation) in the Ctx8 tumors that expressed the highest levels of pAXL-Y779. IHC analysis of IgG versus Ctx8 tumors revealed that Ctx8 tumors had statistically significant increases in pAXL-Y779 staining (Fig. 5E). Collectively, these data demonstrate that AXL overexpression and/or activation plays a role in acquired resistance to cetuximab in vitro and in vivo.

To expand these findings to a more clinically relevant model system, we determined whether there was a correlation between cetuximab response and AXL expression in PDXs established directly from surgically resected HNSCCs. Six PDXs were established from patients who had not received prior cetuximab therapy (see Supplementary Table S1 for clinical characteristics of patients before surgery). For each PDX, dual flank tumors were established in 16 athymic nude mice. When tumors reached approximately 200 mm3, the mice were stratified into two treatment groups (vehicle-treated) and cetuximab (n = 8 mice/16 tumors per group). After completing the treatment regimen, tumor growth was monitored to evaluate response to therapy. Overall, there were three cetuximab-sensitive PDXs (UW-SCC36, UW-SCC22, and UW-SCC52) and three cetuximab-resistant PDXs (UW-SCC1, UW-SCC17, and UW-SCC25; Fig. 6).

PDXs harvested from early-passaged tumors before treatment were evaluated for AXL expression and activation by IHC analysis (Fig. 6). The cetuximab-sensitive PDXs had low levels of AXL and pAXL-Y779 staining, with UW-SCC36 having nearly absent expression of both markers. In comparison, the three cetuximab-resistant PDXs expressed 1.8- to 2.5-fold increases in pAXL-Y779 expression, and 2.5- to 4.3-fold increases in total AXL expression as compared with the staining intensity.
AXL plays a role in acquired resistance to cetuximab in HNSCC

To further investigate whether AXL plays a more global role in acquired resistance to cetuximab, we developed a model of acquired resistance to cetuximab using the Ctx^S parental cell line UM-SCC1 (30). This resulted in a parental SCC1 cell line (SP) and three cetuximab-resistant clones (SP7, SP8, and SP11). SP cell growth was inhibited upon treatment with increasing doses of cetuximab, while the three HNSCC Ctx^R clones remained resistant (Fig. 7A). Analysis of HNSCC Ctx^R clones indicated that all clones had increased steady-state expression of AXL as compared with SP (Fig. 7B). In addition, each clone demonstrated increased activation of c-Raf, p44/42 MAPK, AKT, rpS6, and c-Jun (Fig. 7B). To determine whether AXL influenced HNSCC Ctx^R cell proliferation, cells were transfected with siAXL or NT siRNA and proliferation assays were performed. Loss of AXL expression resulted in a significant inhibition of cellular proliferation (20%–25%) in HNSCC Ctx^R clones, while parental SP cells were nonresponsive (Fig. 7C). The growth-inhibitory effects of siAXL in HNSCC Ctx^R clones were statistically decreased compared with the effect on SP cells ($P < 0.01$). Furthermore, all HNSCC Ctx^R clones expressed diminished activation of EGFR (by tyrosine 1068 phosphorylation) as well as MAPK and AKT signaling pathways upon AXL knockdown, whereas the activation of these molecules was relatively unchanged or slightly increased in SP cells. Collectively, these data suggest that AXL plays a role in acquired resistance to cetuximab in HNSCC.

Discussion

Cetuximab is a commonly used anti-EGFR monoclonal antibody that has demonstrated efficacy in treating in HNSCC, mCRC, and NSCLC (19–26). Although cetuximab treatment has yielded clinical benefit, both intrinsic and acquired resistance are common outcomes. Recently, a novel mutation was identified in the EGFR (S492R) that mediates resistance to cetuximab (40); however, resistance also occurs in the WT setting. Multiple mechanisms of cetuximab resistance exist, including upregulation of EGFR ligands (41), nuclear translocation of EGFR (36), oncogenic shift to vascular endothelial growth factor receptor-1 (VEGFR-1; ref. 42), and constitutive activation of downstream signaling molecules such as KRAS (43) and c-Src (44). This study is the first to describe a role for AXL in mediating cetuximab resistance in the setting of wild type (WT) EGFR, and thus provides rationale for the development and use of anti-AXL therapeutics for treatment of Ctx^R tumors.

Cetuximab resistance is challenging to study due to the lack of access to patient tissue upon relapse. To model Ctx^R mechanisms that may occur in humans, several models of acquired resistance were established via prolonged exposure of Ctx^S cells to cetuximab (30–32). These models indicated that Ctx^R clones and tumors had increased expression and dependency on the EGFR (30–32). In this study, AXL was found to activate EGFR in Ctx^R clones, whereas HER2 and HER3 receptors did not, suggesting that AXL is a key mediator of EGFR activity in the resistant setting. Furthermore, EGFR and AXL were associated in Ctx^R clones and tumors (Figs. 2C and 5D), a finding previously reported in triple-negative breast cancers (TNBC; ref. 14), tumors that are intrinsically resistant to cetuximab. Interestingly, EGF mediated AXL-induced signaling pathways in TNBC, whereas Gas6 did not (14), similar to our findings in Fig. 5C. Another novel finding was that EGFR signaling led to increased AXL mRNA expression in Ctx^R.

detected in UW-SCC36 tumors. Collectively, these data demonstrate that AXL is overexpressed and activated in PDXs that are intrinsically resistant to cetuximab therapy.
clones. The regulation of AXL mRNA was contingent on MAPK and c-Jun because knockdown of either decreased AXL expression (Fig. 2E and F).

These data support a positive-feedback loop that occurs in EGFR-dependent CtxR cells (Fig. 8). In this model, resistance is characterized by increased EGFR ligand production, dimerization, and transactivation of AXL and EGFR. This interaction results in hyperactivated MAPK/c-Jun signaling, upregulation of AXL mRNA expression, and maintenance of constitutive EGFR activation and cetuximab resistance. The de novo CtxR cell line xenografts support this model, as CtxR tumors expressed increased total and activated AXL (especially as compared with IgG-1 and IgG-2). Although c-Jun was capable of regulating AXL mRNA expression in CtxR parental cells, this regulation did not reduce EGFR activity (Fig. 2F), suggesting that EGFR and AXL are not coupled in CtxR cells.

Because of limited availability of patient tissue after cetuximab failure, the expression status of AXL and pAXL-Y779 was evaluated in intrinsically resistant HNSCC PDXs. PDXs are clinically relevant cancer models because they accurately maintain many aspects of the parental tumor, including its histology, gene expression profile, copy number variance, and metastatic patterns (45, 46). In this study, total and activated AXL were highly overexpressed in HNSCC PDXs that were resistant to cetuximab (Fig. 6). The strong correlation between AXL and cetuximab resistance observed in the PDXs supports

Figure 7. AXL mediates acquired resistance to cetuximab in HNSCC. A, CtxR cell clones (SP7, SP8, and SP11) and the CtxR parental cell line (SP) were treated with increasing doses of cetuximab (1, 10, and 100 nmol/L) for 72 hours before performing proliferation assays. Proliferation is plotted as a percentage of growth relative to vehicle-treated cells (n = 5 for three independent experiments). B, whole-cell lysate was harvested from cells followed by immunoblotting for the indicated proteins. α-Tubulin was used as a loading control. Total AXL protein expression was quantitated using ImageJ software. C, cells were incubated with siAXL or nontargeting (NT) siRNA for 72 hours before performing proliferation assays or isolation of whole-cell lysate and immunoblotting for indicated proteins. α-Tubulin was used as a loading control. Proliferation is plotted as a percentage of growth relative to NT-transfected cells (n = 3 for three independent experiments). Data, mean ± SEM. **, P < 0.01.
Cetuximab resistance is characterized by increased AXL mRNA and protein expression, EGFR activation, and MAPK pathway signaling. In CtxR cells, increased EGFR ligand (L) production leads to AXL and EGFR association and transactivation. This results in MAPK and c-Jun signaling and subsequent increases in AXL transcription. Increases in AXL mRNA result in elevated AXL protein levels and maintenance of EGFR activation and signaling. This positive feedback loop results in the constitutive activation of both AXL and EGFR in CtxR cells and thereby mediates cetuximab resistance.

The mechanistic work performed in this study and suggests that AXL may mediate both intrinsic and acquired resistance to cetuximab.

To date, AXL has been identified to play a role in resistance to EGFR TKIs in NSCLC (16), HNSCC (13), and TNBC (14). In NSCLC, AXL was overexpressed and activated in EGFR-mutant erlotinib-resistant cells, where AXL inhibition resensitized tumor cells to erlotinib (13, 16). In this study, AXL inhibition was sufficient to inhibit the growth of CtxR clones, but did not resensitize CtxR clones to cetuximab (data not shown). This likely occurred because AXL inhibition robustly decreased EGFR activation; thus, cetuximab provided no further benefit. Although AXL inhibition led to robust antiproliferative effects in CtxR clones, cell growth was not completely arrested, suggesting that other RTKs may influence resistance. Previous work from our laboratory and others suggests that signaling emanating from HER2:HER3 heterodimers play a role in resistance to anti-EGFR agents (30, 47). Thus, targeting AXL and either HER2 or HER3 may result in even more robust antiproliferative responses because EGFR signaling could be abrogated through AXL inhibition and HER2:HER3 signaling could be blocked with anti-HER2 or HER3 agents. Ultimately, this approach may lead to a complete loss of HER family signaling capabilities and serve as a powerful strategy for the treatment of CtxR cancers.

With increasing evidence supporting the role of AXL in resistance to anti-EGFR agents, the development of anti-AXL therapeutics is essential. In this study, two novel anti-AXL therapeutics were tested: MAb173, an anti-AXL–neutralizing monoclonal antibody, and R428, a selective small-molecule AXL TKI. In previous studies, researchers demonstrated that AXL was hyperactivated in Kaposi sarcoma and that MAb173 induced AXL endocytosis and degradation (9). In addition to AXL, total EGFR expression was decreased upon MAb173 treatment of CtxR cells (Fig. 3B), supporting the existence of AXL and EGFR heterodimers and the utility of this antibody in the setting of cetuximab resistance. Furthermore, EGFR was not degraded in MAb173-treated HP cells, which lack AXL and EGFR association (Fig. 2C). The anti-AXL TKI R428 has also shown antitumorigenic effects in multiple cancer models, including breast cancer (14, 39) and HNSCC (13). The differences in growth inhibition observed between MAb173 and R428 may result from off-target effects of R428, leading to more robust antiproliferative responses. R428 has now entered phase 1 clinical trials, whereas MAb173 is still undergoing preclinical testing.

Overall, AXL plays a key role in tumor growth, metastasis, angiogenesis, and resistance to anti-EGFR agents (12–17). In addition, AXL inhibition has been shown to enhance the efficacy of standard chemotherapy regimens (10, 15, 18). With AXL at the forefront, Tyro and Mer receptors also influence parameters of tumor biology (1, 4). In fact, both Tyro and Mer receptors were differentially overexpressed in the current CtxR models (unpublished data), promoting further research on the global role of TAM receptors in cetuximab resistance. Collectively, the studies herein have strong potential for translation into future clinical trials and therapies for patients with cetuximab-resistant tumors.

Disclosure of Potential Conflicts of Interest
P.A. Gill is CSO of Vasgene Therapeutics and has ownership interest (including patents) in the same. No potential conflicts of interest were disclosed by the other authors.

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Grant Support

This study was supported by grant ULTR000427 from the Clinical and Translational Science Award program (to D.I. Wheeler), through the NIH National Center for Advancing Translational Sciences, grant RSG-10-193-01-TBG from the American Cancer Society (to D.I. Wheeler), grant W81XWH-12-1-0461 from United States Army Medical Research and Materiel Command (to D.I. Wheeler), University of Wisconsin Tomah Cancer Center Center Grant Support Grant P30 CA04520 (to D.I. Wheeler) and Mary Kay Foundation grant MSN152261 (to D.I. Wheeler).

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Received February 5, 2014; revised June 11, 2014; accepted June 30, 2014; published OnlineFirst August 18, 2014.

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