**AXL Mediates Resistance to Cetuximab Therapy**

Toni M. Brand\(^1\), Mari Iida\(^1\), Andrew P. Stein\(^1\), Kelsey L. Corrigan\(^1\), Cara M. Braverman\(^1\), Neha Luthar\(^1\), Mahmoud Toulany\(^2\), Parkash S. Gill\(^3\), Ravi Salgia\(^4\), Randall J. Kimple\(^1\), and Deric L. Wheeler\(^1\)

---

**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 (Ctx\(^R\) cells). Using RNAi methods and novel AXL-targeting agents, we found that AXL activation stimulated cell proliferation, EGFR activation, and MAPK signaling in Ctx\(^R\) cells. Notably, EGFR directly regulated the expression of AXL mRNA through MAPK signaling and the transcription factor c-Jun in Ctx\(^R\) 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;* 1–13. ©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 stimulate 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 (Ctx\(^S\)) 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 (Ctx\(^R\)) 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, Ctx\(^R\) 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 Ctx\(^R\) cells. Further analysis indicated that Ctx\(^R\) cells had increased dependency on AXL for cellular proliferation, EGFR activation, and MAPK signaling. AXL activity...
was also examined in tumors harvested from *de novo*–acquired Ctx\(^6\) 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 Harbor, MI) and maintained in 10% FBS in Dulbecco’s Modified Eagle Medium (DMEM) with 1% penicillin and streptomycin. The development of Ctx\(^6\) cells has been previously described (30–32). All Ctx\(^6\) cell lines were validated for cetuximab response as described in Supplementary Materials and Methods. A XL mRNA expression, and pAXL-Y779 expression were determined, A XL, cells were treated with pervanadate (0.12 mmol/L Na\(_3\)VO\(_4\) in 0.002% H\(_2\)O\(_2\)) for 2 minutes before cell lysis, a method previously described (10). EGFR and TGF\(\alpha\) ligands were added to growth media 45 minutes before lysis.

**Immunoblot analysis**

Whole-cell lysis was performed as previously described (31, 33). Enhanced chemiluminescence (ECL) detection system was used to visualize proteins. For detection of phosphorylated AXL, cells were treated with pervanadate (0.12 mmol/L Na\(_3\)VO\(_4\) in 0.002% H\(_2\)O\(_2\)) for 2 minutes before cell lysis, a method previously described (10). EGFR and TGF\(\alpha\) ligands were added to growth media 45 minutes before lysis.

**Immunoprecipitation**

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.

**Cell proliferation assay**

Crystal violet assay and Cell Counting Kit-8 (Dojindo Molecular Technologies) were performed as previously described (31, 35). Cellular proliferation was measured 72 hours after siRNA or drug treatment.

**Flow cytometric analysis**

Cells were processed as previously described (36) and analyzed using a FACScalibur flow cytometer (BD Biosciences). Propidium iodide was added to each sample at a final concentration of 5 mg/mL. Histogram analysis was performed using FlowJo software (TreeStar Inc.).

**Plasmids, transfection, and stable cell line construction**

pDONR223-AXL (Plasmid 23945) was purchased from Addgene and subcloned into the *Bam*H1/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, \(2^{\Delta C_t}\) values were compared between AXL and 18S, where the change in crossing threshold \((\Delta C_t) = C_t(AXL) - C_t(18S)\) and \(\Delta C_t = \Delta C_t(HC1, HC4, or HC8) - \Delta C_t(HC)\) or \(\Delta C_t = \Delta C_t(NT) - \Delta C_t(AXL)\).

**Cetuximab-resistant cell line xenografts and PDXs**

Ctx\(^6\) cell line xenografts were established as previously described (31), and HNSCC PDXs were established and evaluated for cetuximab response as described in Supplementary Materials and Methods.

**Statistical analysis**

Student *t* tests were used to evaluate differences in proliferation, AXI 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
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
the transcription factor c-Jun (38). Because CtxR 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 CtxR 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 CtxR clones.

**CtxR cells are sensitive to anti-AXL monoclonal antibody and TKI therapies**

Because CtxR 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 CtxR cell proliferation (Fig. 3A). CtxR clones were significantly growth inhibited upon treatment with increasing doses of MAb173, whereas CtxS HP cells were less sensitive. In addition, the growth-inhibitory effects of CtxR 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 CtxR 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 CtxR clones (Fig. 4A). All CtxR 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
**AXL and Resistance to Cetuximab**

**A**

<table>
<thead>
<tr>
<th>HP-Vector</th>
<th>HP-AXL</th>
</tr>
</thead>
<tbody>
<tr>
<td>AXL</td>
<td>AXL</td>
</tr>
<tr>
<td>pEGFR-Y1068</td>
<td>pEGFR-Y1068</td>
</tr>
<tr>
<td>EGFR</td>
<td>EGFR</td>
</tr>
<tr>
<td>p-cRAF</td>
<td>p-cRAF</td>
</tr>
<tr>
<td>cRAF</td>
<td>cRAF</td>
</tr>
<tr>
<td>pMAPK</td>
<td>pMAPK</td>
</tr>
<tr>
<td>MAPK</td>
<td>MAPK</td>
</tr>
<tr>
<td>p-rpS6</td>
<td>rpS6</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td>α-Tubulin</td>
</tr>
</tbody>
</table>

**B**

![Graph showing cell proliferation](image)

**C**

<table>
<thead>
<tr>
<th>pAXL-Y779</th>
<th>AXL</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEGFR-Y1068</td>
<td>pEGFR-Y1068</td>
</tr>
<tr>
<td>EGFR</td>
<td>EGFR</td>
</tr>
<tr>
<td>p-cRAF</td>
<td>p-cRAF</td>
</tr>
<tr>
<td>cRAF</td>
<td>cRAF</td>
</tr>
<tr>
<td>pMAPK</td>
<td>pMAPK</td>
</tr>
<tr>
<td>MAPK</td>
<td>MAPK</td>
</tr>
<tr>
<td>p-rpS6</td>
<td>rpS6</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td>α-Tubulin</td>
</tr>
</tbody>
</table>

**D**

<table>
<thead>
<tr>
<th>IgG tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG-2</td>
</tr>
<tr>
<td>IgG-3</td>
</tr>
<tr>
<td>IgG-1</td>
</tr>
<tr>
<td>IgG-4</td>
</tr>
<tr>
<td>CTX^0-4</td>
</tr>
<tr>
<td>CTX^0-3</td>
</tr>
<tr>
<td>CTX^0-2</td>
</tr>
<tr>
<td>CTX^0-1</td>
</tr>
<tr>
<td>CTX^0-6</td>
</tr>
</tbody>
</table>

**E**

<table>
<thead>
<tr>
<th>IgG tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAXL-Y779</td>
</tr>
<tr>
<td>No antibody</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CTX^0 tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEGFR-Y1068</td>
</tr>
<tr>
<td>EGFR</td>
</tr>
<tr>
<td>α-Tubulin</td>
</tr>
</tbody>
</table>
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, Gas6, was not overexpressed in Ctx8 clones and did not drive resistance in HP cells (data not shown). Collectively, these data suggest that EGFR ligands 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. Acquired resistance was observed after approximately 30 days of cetuximab exposure in 6 of the cetuximab-treated mice (tumors denoted as Ctx8-1 to Ctx8-5, and Ctx8-7), at which point there was marked tumor growth in the presence of continued cetuximab therapy (Fig. 5D). One mouse was continued on cetuximab for 90 days until a significant increase in tumor growth was observed (Ctx8-6). Once tumors reached 2,000 mm3, they were harvested and processed for immunoblot analysis (Fig. 5D) and IHC (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: control (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-SCC32) 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.
detected in UW-SCC36 tumors. Collectively, these data demonstrate that AXL is overexpressed and activated in PDXs that are intrinsically resistant to cetuximab therapy.

**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 CtxR 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 CtxR clones remained resistant (Fig. 7A). Analysis of HNSCC CtxR 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 CtxR cell proliferation, cells were transfected with siAXL or NT siRNA and proliferation assays were performed. Loss of AXL expression resulted in a significant inhibition in cellular proliferation (20%–25%) in HNSCC CtxR clones, while parental SP cells were nonresponsive (Fig. 7C). The growth-inhibitory effects of siAXL in HNSCC CtxR clones were statistically decreased compared with the effect on SP cells (P < 0.01). Furthermore, all HNSCC CtxR 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 CtxR tumors.

Cetuximab resistance is challenging to study due to the lack of access to patient tissue upon relapse. To model CtxR mechanisms that may occur in humans, several models of acquired resistance were established via prolonged exposure of CtxR cells to cetuximab (30–32). These models indicated that CtxR clones and tumors had increased expression and dependency on the EGFR (30–32). In this study, AXL was found to activate EGFR in CtxR 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 CtxR clones and tumors (Figs. 2C and 5D), a finding was that EGFR–AXL association in CtxR tumors.
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 Ctx R 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 CtxS parental cells, this regulation did not reduce EGFR activity (Fig. 2F), suggesting that EGFR and AXL are not coupled in CtxS 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...
AXL and Resistance to Cetuximab

AXL protein

EGFR

L

MAPK

c-JUN

RAF

AXL mRNA

Cetuximab resistance

Figure 8. Model for AXL and EGFR cooperation in cetuximab resistance. 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.

Discussion

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.S. 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.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.M. Brand, A.P. Stein, K.L. Corrigan, C. Braverman, N. Luthar, R. Salgia

Analysis and interpretation of data (e.g., statistical analysis, biosistatistics, computational analysis): T.M. Brand, M. Iida, A.P. Stein, M. Toulany, K.L. Corrigan, R. Salgia, R.J. Kimple, D.L. Wheeler

Writing, review, and/or revision of the manuscript: T.M. Brand, M. Iida, A.P. Stein, K.L. Corrigan, M. Toulany, P.S. Gill, R. Salgia, R.J. Kimple, D.L. Wheeler

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.M. Brand, M. Iida, D.L. Wheeler

Study supervision: T.M. Brand, R. Salgia, R.J. Kimple, D.L. Wheeler

www.aacrjournals.org Cancer Res; 2014 OF11

Downloaded from cancerres.aacrjournals.org on January 6, 2018. © 2014 American Association for Cancer Research.
Grant Support

This study was supported by grant UL1TR000427 from the Clinical and Translational Science Award program (to D.L. Wheeler), through the NIH National Center for Advancing Translational Sciences, grant RSC 10-193-01-TBG from the American Cancer Society (to D.L. Wheeler), grant W81XWH-12-1-0467 from United States Army Medical Research and Materiel Command (to D.L. Wheeler), University of Wisconsin Carbone Cancer Center Cancer Center Support Grant P30 CA014520 (to D.L. Wheeler) and Mary Kay Foundation grant MSN152261 (to D.L. Wheeler).

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

References

AXL Mediates Resistance to Cetuximab Therapy

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

Cancer Res  Published OnlineFirst August 18, 2014.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-0294

Supplementary Material  Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/07/23/0008-5472.CAN-14-0294.DC1

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, use this link
http://cancerres.aacrjournals.org/content/early/2014/08/19/0008-5472.CAN-14-0294. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.