Axl Mediates Acquired Resistance of Head and Neck Cancer Cells to the Epidermal Growth Factor Receptor Inhibitor Erlotinib

Keith M. Giles\(^1\), Felicity C. Kalinowski\(^1\), Patrick A. Candy\(^1,2,3\), Michael R. Epis\(^1\), Priscilla M. Zhang\(^1\), Andrew D. Redfern\(^1,2,3\), Lisa M. Stuart\(^1\), Gregory J. Goodall\(^4,5,6\), and Peter J. Leedman\(^1,3\)

Abstract

Elevated expression and activity of the epidermal growth factor receptor (EGFR) is associated with development and progression of head and neck cancer (HNC) and a poor prognosis. Clinical trials with EGFR tyrosine kinase inhibitors (e.g., erlotinib) have been disappointing in HNC. To investigate the mechanisms mediating resistance to these agents, we developed an HNC cell line (HN5-ER) with acquired erlotinib resistance. In contrast to parental HN5 HNC cells, HN5-ER cells exhibited an epithelial–mesenchymal (EMT) phenotype with increased migratory potential, reduced E-cadherin and epithelial-associated microRNAs (miRNA), and elevated vimentin expression. Phosphorylated receptor tyrosine kinase profiling identified Axl activation in HN5-ER cells. Growth and migration of HN5-ER cells were blocked with a specific Axl inhibitor, R428, and R428 resensitized HN5-ER cells to erlotinib. Microarray analysis of HN5-ER cells confirmed the EMT phenotype associated with acquired erlotinib resistance, and identified activation of gene expression associated with cell migration and inflammation pathways. Moreover, increased expression and secretion of interleukin (IL)-6 and IL-8 in HN5-ER cells suggested a role for inflammatory cytokine signaling in EMT and erlotinib resistance. Expression of the tumor suppressor miR-34a was reduced in HN5-ER cells and increasing its expression abrogated Axl expression and reversed erlotinib resistance. Finally, analysis of 302 HNC patients revealed that high tumor Axl mRNA expression was associated with poorer survival (HR = 1.66, \(P = 0.007\)). In summary, our results identify Axl as a key mediator of acquired erlotinib resistance in HNC and suggest that therapeutic inhibition of Axl by small molecule drugs or specific miRNAs might overcome anti-EGFR therapy resistance.

Mol Cancer Ther; 12(11); 2541–58. ©2013 AACR.

Introduction

Head and neck cancer (HNC) is a leading cause of worldwide cancer death (1). Many HNC patients present with locally advanced or metastatic disease, and despite advances in surgery, radiotherapy, and chemotherapy the prognosis for such patients remains poor (2). The epidermal growth factor receptor (EGFR) receptor tyrosine kinase (RTK) is overexpressed in more than 80% of HNCs, and is associated with a poor clinical outcome (3), and promotes tumor growth, metastasis, treatment resistance, and angiogenesis, making it an attractive therapeutic target. Disappointingly, clinical trials to date with anti-EGFR monoclonal antibodies (e.g., cetuximab) or small molecule EGFR tyrosine kinase inhibitors (TKI, e.g., erlotinib or gefitinib) have shown that these agents have only modest activity in recurrent or advanced HNC. Although most HNCs are inherently resistant to EGFR inhibition, approximately 5% to 15% of HNC patients experience an initial antitumor response (4), with minor shrinkage or disease stabilization that rarely persists beyond a few months, presumably because tumors rapidly acquire EGFR inhibitor resistance.

Understanding the mechanisms that mediate EGFR inhibitor resistance in HNC might allow better selection of patients most likely to benefit from anti-EGFR therapy, and may identify novel strategies to improve the efficacy of these agents. This is of particular importance given the large number of active trials investigating anti-EGFR agents such as erlotinib in various HNC settings. A number of research studies have investigated the inherent and acquired resistance to EGFR inhibition in HNC, but
the precise mechanisms remain poorly understood. EGFR tyrosine kinase domain mutations determine EGFR inhibitor sensitivity in non–small cell lung cancer (NSCLC), but do not occur in HNC (5), whereas KRAS mutations associated with EGFR inhibitor resistance in colorectal cancer are rarely found in HNC (6). Other studies suggested that EGFR gene amplification and the levels of activated ErbB2 and total ErbB3 may determine gefitinib sensitivity in HNC cell lines (7), whereas ErbB2 inhibition sensitizes HNC cells to cetuximab (8), implying that combined RTK blockade might be an effective approach to overcome EGFR inhibitor resistance. A recent study showed that inhibition of STAT3 activity sensitizes HNC cells to EGFR inhibition (9), whereas EGFR-independent activation of Akt has been associated with inherent and acquired gefitinib resistance in HNC cell lines (10, 11), supporting the concept of sustained Akt activity and tumor progression being mediated through compensatory RTK signaling. Finally, mounting evidence has implicated the process of epithelial–mesenchymal transition (EMT), a state of altered cell morphology and migration, in EGFR inhibitor resistance in a range of solid tumors, including HNC and NSCLC (12, 13), and EMT has itself been associated with increased Akt signaling in HNC (14).

Recognizing that a small subset (5–15%) of unselected HNC patients experience transient clinical responses to EGFR inhibitors, we reasoned that the inevitable treatment failure and disease progression in these cases results from tumors rapidly acquiring resistance to anti-EGFR drugs. We generated and characterized an erlotinib-resistant HNC cell line (HN5-ER) to investigate the molecular mechanisms associated with erlotinib resistance in HNC. We show that erlotinib-resistant HNC cells exhibit a classical EMT phenotype with increased migratory potential, loss of epithelial markers and gain of mesenchymal markers, reduced expression of microRNAs (miRNA) functionally associated with EMT, and increased expression and activation of Axl, an RTK known to promote tumor growth, metastasis, and treatment resistance. Importantly, a small molecule selective inhibitor of Axl, R428, reduced growth and migration of erlotinib-resistant cells, and restored their sensitivity to erlotinib. Microarray analyses identified alterations in several cancer-associated gene expression programs that were associated with acquired erlotinib resistance in HNC, including activation of proinflammatory signaling, with increased expression and secretion of interleukin (IL)-6 and IL-8 and elevated STAT3 activity. Finally, we show a role for the tumor suppressor miRNA miR-34a in the regulation of Axl expression, EMT, and EGFR inhibitor resistance in HNC cells. Together, our findings identify novel targets to develop strategies to circumvent this resistance and improve the efficacy of anti-EGFR therapy in HNC.

Materials and Methods

HN5 cell line, cell culture and reagents, cell imaging

HN5 cells (15) were provided by A/Prof. T. Johns (Monash Institute of Medical Research, Australia) in 2009; their identity was verified by short tandem repeat (STR) profiling at CellBank Australia (Children’s Medical Research Institute, Westmead, Australia). STR profiling also confirmed the parental HN5 origin for HN5-ER cells. The HNC cell line FaDu (16) was derived from a pharyngeal carcinoma from a 56-year-old male and was obtained from the American Type Culture Collection. Cells were cultured in DMEM (Invitrogen) with 10% FBS at 37°C in 5% CO2.

Erlotinib (LC Laboratories) was prepared as a 23 mmol/L stock solution in 96% (v/v) dimethyl sulfoxide (DMSO; Sigma-Aldrich). R428 (Symansis) was prepared as a 10 mmol/L stock solution in DMSO. Gefitinib (Selleck Chemicals) was prepared as a 20 mmol/L stock solution in DMSO. Tocilizumab (Roche) was supplied as a 20 mg/mL stock solution. Ruxolitinib (LC Laboratories) was prepared as a 33 mmol/L stock solution in DMSO. Synthetic miR-34a (hsa-miR-34a-5p; Catalog no. AM17100, Assay ID PM11030) and miR-negative control (miR-NC; negative control #1, Catalog no. AM17110) miRNA precursor molecules were sourced from Ambion. Axl siRNAs (Hs_AXL_3 Catalog no. SI00131355, Hs_AXL_9 Catalog no. SI0065304, Hs_AXL_10 Catalog no. SI0065311, and Hs_AXL_12 Catalog no. SI02626743) and AllStars negative control siRNA (Catalog no. 1027280) were obtained from Qiagen.

Cells were photographed with an Olympus IX71 inverted microscope using an Olympus DP70 Digital Camera System at ×100 magnification.

Generation of an erlotinib-resistant HN5 subline, HN5-ER

HN5 cells were cultured in increasing concentrations of erlotinib to a concentration of 12.5 μmol/L over 3 months, maintained in 12.5 μmol/L erlotinib for 6 months, and tested to confirm they had stably acquired erlotinib resistance. The erlotinib-resistant HN5 cell subline was designated HN5-ER.

Drug sensitivity and cell viability assays

Assays were conducted as described (17). Briefly, to determine the sensitivity of HN5 and HN5-ER cell lines to erlotinib, gefitinib, and/or R428, 5.0 × 103 cells were seeded per well in 96-well plates, and fresh media containing erlotinib or R428 added 24 hours after cell plating. Cell viability was measured 3 days after addition of erlotinib/R428/gefitinib using the CellTitre 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega) and a FLUOstar OPTIMA microplate reader (BMG Labtech). EC50 values for erlotinib and R428 were calculated for each cell line using GraphPad Prism (GraphPad software).

To assess the efficacy of combining R428 and erlotinib or gefitinib in HN5-ER cells, an ineffective concentration of erlotinib or gefitinib was used (7.5 or 10 μmol/L). Cell viability was measured 3 days after addition of erlotinib or gefitinib and/or R428 as earlier.

To assess the efficacy of combining miR-34a and erlotinib in HN5-ER cells, cells were transfected with miR-34a...
or miR-NC for 3 days and then treated with an ineffective concentration of erlotinib (10 μmol/L) for a further 2 days, after which cell viability was determined.

**miRNA precursor and siRNA transfections**

miRNA transfection experiments were conducted as described (18). Briefly, HN5-ER cells were seeded in 6-well plates at a density of 5.0 × 10⁵ cells per well and transfected with 30 nmol/L miR-34a or miR-NC for 24 hours before the RNA extraction or protein extraction and immunoblotting as outlined later.

For transfections with Axl siRNA, 2.5 × 10⁵ cells per well were seeded in 6-well plates and transfected with 10 nmol/L Axl siRNA or AllStars negative control siRNA (NC siRNA) for 3 days before RNA extraction or protein extraction and immunoblotting as outlined later.

**Drug treatments for immunoblotting studies**

For characterization of the effect of combined erlotinib and R428 treatment on HN5-ER cells, cells were cultured overnight in DMEM + 0.2% FBS, and then treated with either 5 μmol/L R428, 5 μmol/L erlotinib, a combination of 5 μmol/L R428, and 5 μmol/L erlotinib, or DMSO for 4 hours before preparing cytoplasmic protein extracts for immunoblotting as outlined later.

To assess the effect of R428 on HN5 cells, cells were cultured overnight in DMEM + 0.5% FBS, and then treated with 10 μmol/L R428 or DMSO for 4 hours, before protein extraction and immunoblotting as outlined later.

To assess the effect of ruxolitinib or tocilizumab on HN5-ER cells, cells were cultured overnight in DMEM + 0.2% FBS, and then treated with DMSO, or 3 μmol/L ruxolitinib for 24 hours, or 1 μmol/L tocilizumab for 3 days, before protein extraction and immunoblotting as outlined later.

**Reverse transcription and quantitative PCR**

Total RNA was extracted from HN5 and HN5-ER cell lines with TRIzol reagent (Invitrogen). For reverse transcription and quantitative PCR (RT-qPCR) analysis of mRNA expression, 0.5 μg of total RNA was reverse transcribed into cDNA using a QuantiTect Reverse Transcription Kit (QIAGEN). Real-time PCR was conducted on a Corbett Rotor-Gene 6000 thermocycler (QIAGEN) using QuantiTect SYBR Mix (QIAGEN) and validated QuantiTect primer assays (QIAGEN) for EGF-R (QT00085701), AXL (QT00067725), CDH1 (QT00080143), CDH2 (QT00063196), PTGS2 (QT00040586), ZEB1 (QT01888446), IL-6 (QT00083720), IL-8 (QT00000322), ALAS1 (QT00073122), and GAPDH (QT00100608) with a Ct method (19).

**Phospho-RTK array**

Phospho-RTK (P-RTK) arrays (R&D Systems ARY001) were probed with anti-EGFR rabbit monoclonal antibody (1:5,000; Abcam ab52894-100), anti-phospho-EGFR (Tyr1173) goat polyclonal antibody (1:750; Santa Cruz Biotechnology sc-12351), anti-Akt rabbit polyclonal antibody (1:1,000; Cell Signaling Technology 9272), anti-phospho-Akt (Ser473) rabbit monoclonal antibody (1:500; Cell Signaling Technology 4606S), anti-E-cadherin rabbit monoclonal antibody (1:1,000; Cell Signaling Technology 3195S), anti-Vimentin mouse monoclonal antibody (1:1,000; Cell Signaling Technology 3390), anti-Axl rabbit monoclonal antibody (1:1,000; Cell Signaling Technology 8661), anti-ERK1/2 rabbit polyclonal antibody (1:750; Cell Signaling Technology 9102), anti-phospho-ERK1/2 (Thr202/Tyr204) rabbit polyclonal antibody (1:750; Cell Signaling Technology 9101), anti-STAT3 rabbit monoclonal antibody (1:1,000; Cell Signaling Technology 3904), anti-phospho-STAT3 (Tyr705) rabbit monoclonal antibody (1:1,000; Cell Signaling Technology 9156), anti-CD44 mouse monoclonal antibody (1:1,000; Cell Signaling Technology 3570), anti-NF-κB p65 (REL) rabbit monoclonal antibody (1:1,000; Cell Signaling Technology 8242), anti-phospho-NF-κB p65 (P-REL) rabbit monoclonal antibody (1:1,000; Cell Signaling Technology 3033), or anti-β-actin mouse monoclonal antibody (1:15,000; Abcam ab6276-100). Secondary horseradish peroxidase linked anti-rabbit-IgG (1:10,000, GE Healthcare NA934V), horseradish peroxidase linked anti-mouse-IgG (1:10,000, GE Healthcare NA931V), and horseradish peroxidase linked anti-goat-IgG antibodies (1:10,000; Santa Cruz Biotechnology sc-2000) were used before the detection with an ECL Plus Western Blotting Detection System (GE Healthcare).

Quantitation of specific proteins of interest was conducted using Quantity One software (Bio-Rad).
μmol/L R428 for 2 hours, before the protein extraction and P-RTK array analysis as earlier.

**P-Axl, IL-6, and IL-8 ELISA**

P-Axl expression in HN5 and HN5-ER cells was quantitated using a DuoSet IC Intracellular Human Phospho-Axl ELISA Kit (R&D Systems DYC2228-2) and an ELISA Development System Troubleshooting Pack (R&D Systems TSP01-B), according to manufacturer’s instructions. Samples were run in duplicate with 17 μg of cytoplasmic protein extract added per well of the 96-well ELISA plate. IL-6 or IL-8 secretion by HN5 and HN5-ER cells was quantitated using a Hu IL-6 Chemiluminescence ELISA Kit (Life Technologies KHC0069) or an IL-8 Human ELISA Kit (Abcam ab100575), according to manufacturer’s instructions. HN5 and HN5-ER cells were seeded at a density of 6.5 × 10^5 cells/well in 6-well plates (total volume of 1.5 mL), and media harvested 48 hours after plating, cleared by centrifugation, and analyzed in triplicate for IL-6 and IL-8 secretion.

**Cell migration assay**

Migration of HN5 and HN5-ER cells was measured using an xCELLigence real-time migration assay system (Roche). HN5 or HN5-ER cells were plated at 3.0 × 10^4 cells/well into the upper chamber of CIM-plate 16 xCELLigence plates (Roche 0566817001). DMEM + 20% FBS was used in the lower chambers as a chemoattractant, whereas serum-free media (SFM) was the control for no cell migration. Cell migration into the lower chamber was measured over 24 hours and expressed as a baseline cell index relative to SFM control migration. For some studies, HN5-ER cells (3.0 × 10^5 cells/well) in 6-well plates were pretreated with 5 μmol/L R428 or DMSO for 4 hours, and cell migration assessed as described earlier.

**cDNA microarray expression profiling of HN5 and HN5-ER cells**

Total RNA was extracted from HN5 and HN5-ER cells using TRIzol reagent (Life Technologies), and its quantity and integrity of extracted RNA confirmed using a 2100 Bioanalyzer (Agilent Technologies) before samples were deemed suitable for microarray analysis. Gene expression profiling was conducted with 3 biological replicate samples for each cell line by the Australian Genome Research Facility (AGRF) using HumanHT-12 v4 array chips (Illumina). Data normalization was conducted by AGRF and data analyzed as described previously (17). Parametric 2-tailed Student t test was used to calculate significance of variation, fold change was calculated as mean ratio. Probes with an unadjusted P < 0.05 and an absolute fold change >1.5 or more were defined as being differentially expressed between HN5-ER and HN5 cells. Microarray expression data have been deposited in the Gene Expression Omnibus (GEO) under Accession Number GSE49135.

**Microarray data analysis**

Clustering, volcano, and scatter plots that showed the distributions and correlations of differential gene expression across the HN5/HN5-ER cell line microarrays were produced from normalized data using the R “graphics” package. A heat map was produced with the R “gplots” package that showed a comparison of significant differential gene expressions across the HN5/HN5-ER cell line microarrays. Ingenuity Pathway Analysis (IPA) software was used to define functional pathways affected by the acquisition of erlotinib resistance.

HCC 827 NSCLC microarrays (GEO accession number: GSE38121; ref. 20) contained information on gene expression upon acquisition of erlotinib resistance in HCC 827 cell lines, and were processed using the R “affy” and “limma” packages. Genes that were differentially expressed in both erlotinib-resistant cell lines (HCC 827 ER1, HCC 827 ER2) relative to the parental HCC 827 cell line were identified and compared to our HN5/HN5-ER expression data. Venn diagrams comparing differential gene expression related to erlotinib resistance in both HNC and NSCLC were produced using the R “vennDiagram” package.

Overrepresentation of a set of predicted and validated miR-34a target genes involved in EMT among all genes upregulated in HN5-ER cells was determined using IPA software. TargetScan (Version 6.0: November 2011) was used for miR-34a target predictions.

**Statistics and investigation of Axl in HNC patients**

Results are presented as mean ± SD. Statistical significance was calculated using the Student t test (2-tailed, unpaired) and the level of significance was set at P < 0.05. Statistical analysis of RT-qPCR data was conducted using GenEx software (MultiD), and normality of data confirmed using the Kolmogorov–Smirnov test.

To investigate the clinical significance of Axl mRNA overexpression, we analyzed clinicopathologic and tumor RNA-seq expression data from 334 HNC patients as shown in Table 1, which were downloaded from The Cancer Genome Atlas (TCGA; https://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp; accessed June 2013). Matched clinical and mRNA expression data was available for 302 HNC patients. The prognostic significance of Axl overexpression was assessed using the R packages “Survival” and “Graphics” to conduct Cox regressions with ties addressed by Efron’s method and produce Kaplan–Meier survival curves, with all deaths used as an endpoint. To avoid confounding, analyses were adjusted for age, gender, and smoking status. The validity of proportional hazards assumptions were assessed by scaled Schoenfeld residuals (21). Dichotomous high and low Axl mRNA expression levels were determined using a median cut point and the reads per kilobase per million mapped reads method (22).
Results

An erlotinib-resistant HN5 cell subline (HN5-ER) with an epithelial–mesenchymal phenotype and increased cell motility

To investigate the mechanisms associated with acquired EGFR inhibitor resistance in HNC, we used HN5 cells, as they have EGFR gene amplification (23) and are EGFR-dependent and highly sensitive to EGFR inhibition in vitro and in vivo (24). To select for acquired erlotinib resistance, HN5 cells were exposed to gradually increasing concentrations of erlotinib for a period of 6 months. Microscopic analysis indicated that although parental HN5 cells (HN5) exhibited a distinctive epithelial morphology, with tight cell–cell junctions, HN5-ER cells possess a mesenchymal, fibroblast-like morphology with increased cell spreading (Fig. 1A). We measured erlotinib sensitivity using cell titer assays, and confirmed that HN5-ER cells had >200-fold higher resistance to erlotinib than HN5 cells (Fig. 1B; HN5 EC\textsubscript{50} erlotinib = 0.1 μmol/L, HN5-ER EC\textsubscript{50} erlotinib >20 μmol/L). We also found that HN5-ER cells were cross-resistant to the EGFR TKI gefitinib (Supplementary Fig. 1A). Interestingly, HN5-ER cells maintained their erlotinib resistance and mesenchymal cell morphology when grown for several weeks in erlotinib-free media (Supplementary Fig. 1B), suggesting that they had acquired stable resistance to erlotinib.

Previous reports indicate that the inherent resistance of several different tumor types to EGFR inhibitors, including erlotinib, is associated with EMT, where mesenchymal tumor cells are typically more resistant to EGFR inhibition than epithelial tumor cells (13). To gain an insight into the signaling mechanisms associated with acquired erlotinib resistance, we analyzed the expression and activity of components of the EGFR signaling pathway together with several established EMT-related markers in HN5 and HN5-ER cells (Fig. 1C). These studies revealed that acquired resistance to erlotinib was associated with reduced EGFR expression and activity (P-EGFR), increased Akt expression and activity (P-Akt), and reduced ERK1/2 activity (P-ERK1/2). Consistent with their epithelial phenotype, HN5 cells had strong E-cadherin expression but did not express detectable levels of vimentin, an established mesenchymal marker. Conversely, HN5-ER cells lacked E-cadherin protein.

Table 1. Clinical information for TCGA HNC patient cohort

<table>
<thead>
<tr>
<th>Patient feature</th>
<th>High Axl (n)</th>
<th>Low Axl (n)</th>
<th>Total (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases</td>
<td>334 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axl evaluated</td>
<td>153 (45.5%)</td>
<td>150 (45.5%)</td>
<td>303 (90.7%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>109 (32.8%)</td>
<td>113 (34.1%)</td>
<td>240 (71.9%)</td>
</tr>
<tr>
<td>Female</td>
<td>45 (13.7%)</td>
<td>36 (10.9%)</td>
<td>84 (24.1%)</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;59</td>
<td>66 (20.1%)</td>
<td>61 (20.1%)</td>
<td>127 (38.1%)</td>
</tr>
<tr>
<td>60–69</td>
<td>50 (15.6%)</td>
<td>59 (18.0%)</td>
<td>109 (33.0%)</td>
</tr>
<tr>
<td>&gt;70</td>
<td>38 (12.5%)</td>
<td>29 (9.4%)</td>
<td>67 (20.5%)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smoker</td>
<td>48 (15.8%)</td>
<td>52 (17.2%)</td>
<td>100 (30.6%)</td>
</tr>
<tr>
<td>Quit smoking &lt;15 years ago</td>
<td>54 (17.8%)</td>
<td>31 (10.2%)</td>
<td>85 (25.4%)</td>
</tr>
<tr>
<td>Quit smoking &gt;15 years ago</td>
<td>22 (7.3%)</td>
<td>25 (8.3%)</td>
<td>47 (14.4%)</td>
</tr>
<tr>
<td>Lifelong nonsmoker</td>
<td>24 (7.9%)</td>
<td>37 (12.2%)</td>
<td>61 (18.6%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>6 (2.0%)</td>
<td>4 (1.3%)</td>
<td>10 (3.0%)</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–1 drinks/day</td>
<td>21 (6.9%)</td>
<td>22 (7.3%)</td>
<td>43 (13.0%)</td>
</tr>
<tr>
<td>2–3 drinks/day</td>
<td>22 (7.3%)</td>
<td>12 (4.0%)</td>
<td>34 (10.6%)</td>
</tr>
<tr>
<td>≥4 drinks/day</td>
<td>21 (6.9%)</td>
<td>23 (7.6%)</td>
<td>44 (13.3%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>90 (29.7%)</td>
<td>92 (30.4%)</td>
<td>182 (54.3%)</td>
</tr>
<tr>
<td>Cancer stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>7 (2.3%)</td>
<td>3 (1.0%)</td>
<td>10 (3.1%)</td>
</tr>
<tr>
<td>II</td>
<td>34 (11.2%)</td>
<td>28 (9.2%)</td>
<td>62 (18.5%)</td>
</tr>
<tr>
<td>III</td>
<td>29 (9.6%)</td>
<td>38 (12.5%)</td>
<td>67 (20.2%)</td>
</tr>
<tr>
<td>IV</td>
<td>84 (27.7%)</td>
<td>80 (26.4%)</td>
<td>164 (49.2%)</td>
</tr>
<tr>
<td>Survival status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Living</td>
<td>88 (29.4%)</td>
<td>95 (31.4%)</td>
<td>183 (54.8%)</td>
</tr>
<tr>
<td>Deceased</td>
<td>66 (21.8%)</td>
<td>54 (17.8%)</td>
<td>120 (36.1%)</td>
</tr>
</tbody>
</table>

NOTE: Clinicopathologic features relative to Axl mRNA expression levels in HNC.
expression but expressed vimentin, confirming their mesenchymal cancer cell phenotype. We confirmed these findings by analyzing expression of EGFR, Akt, P-Akt, and E-cadherin in an independent pool of erlotinib-resistant HN5 cells (HN5-ER2) by immunoblotting (Supplementary Fig. S2), and observed changes in expression and activity of these molecules consistent with those in HN5-ER cells. We next used TaqMan miRNA RT-qPCR assays to measure the levels of miR-200a, miR-200b, and miR-200c in HN5-ER cells, as this miRNA family has been reported to promote epithelial differentiation and its expression is reduced with EMT, tumor progression, and EGFR inhibitor resistance (25). These experiments showed significantly lower expression of miR-200a/b/c in HN5-ER cells compared with HN5 cells (Fig. 1D), consistent with their EMT phenotype. As increased cell migration, invasion, and metastasis is a hallmark of mesenchymal tumor cells (26), we conducted xCELLigence real-time cell migration studies (27) and found that HN5-ER cells had an increased rate of migration compared to parental HN5 cells (Fig. 1E). Taken together, our findings indicate that acquired resistance of HNC cells to erlotinib is associated with development of an EMT phenotype, an increased level of oncogenic Akt activity despite a reduced dependence on EGFR signaling, and increased cell migration.

**Acquired erlotinib resistance is associated with increased Axl expression and activity, and decreased miR-34a expression**

We hypothesized that compensatory activation of other RTKs allows HN5-ER cells to maintain oncogenic Akt signaling in the presence of erlotinib. We used P-RTK arrays to simultaneously measure the activity (tyrosine phosphorylation) of various RTKs in HN5 and HN5-ER cells.
phosphorylation) of 42 RTKs known to be associated with cancer. These experiments identified several RTKs with altered activity between HN5 and HN5-ER cells (Fig. 2A). We observed decreased activity of ErbB2, ErbB3, VEGFR1, IGF1R and increased activity of EphA7 and Axl in HN5-ER cells. The increased Axl activity in HN5-ER cells was of particular interest in light of previous reports showing that Axl mediates EMT, chemotherapeutic resistance, cell migration and invasion, and metastasis in breast cancer, NSCLC, and glioblastoma multiforme (28). We confirmed the increase in Axl phosphorylation in HN5-ER cells by densitometry (Fig. 2B), and also using ELISA assays specific for P-Axl with protein extracts from HN5 and HN5-ER cells (Fig. 2C). Next, we compared total Axl protein expression between HN5 and HN5-ER cells with immunoblotting assays, and observed a substantial increase in total Axl levels in HN5-ER cells (Fig. 2D). We also found that Axl was abundantly expressed in FaDu HNC cells that are resistant to erlotinib (Fig. 2D; ref. 17).

As an inverse association between the levels of Axl and the tumor suppressor miRNA miR-34a has been reported in NSCLC, colorectal cancer, and breast cancer cell lines, and miR-34a was found to repress Axl expression by its direct binding to the Axl mRNA 3′-untranslated region (29), we used TaqMan miRNA assays to compare miR-34a levels between HN5 and HN5-ER cells. We found miR-34a levels were significantly lower in HN5-ER cells than in HN5 cells (Fig. 2E). Next, we determined whether restoring miR-34a expression to HN5-ER cells following transient transfection with synthetic miR-34a could alter Axl levels. These experiments showed that addition of miR-34a decreased Axl expression, confirming Axl as a miR-34a target in HNC cells; we also observed a concomitant increase in E-cadherin expression (Fig. 2F), suggesting that miR-34a might also act as a negative regulator of EMT in this system (Fig. 2F).

Finally, to determine the functional significance of a loss of miR-34a expression to erlotinib resistance, we transfected HN5-ER cells with miR-34a or miR-NC, followed by treatment with an ineffective concentration of erlotinib (10 μmol/L) or vehicle (DMSO). We found that restoring miR-34a expression to HN5-ER cells sensitized them to erlotinib (Fig. 2G). Together, these results indicate that the acquisition of erlotinib resistance in HNC is associated with increased Axl expression and activity, and suggest that Axl overexpression and erlotinib resistance may in part be due to a decrease in miR-34a levels.

**An Axl-specific inhibitor, R428, reduces HN5-ER cell viability, reverses acquired erlotinib resistance, and blocks cell migration**

To investigate the functional significance of increased Axl expression and activity, we treated HN5-ER cells with R428 (Rigel Pharmaceuticals; now BerGenBio BFB324), a selective small molecule Axl inhibitor that is in clinical development for the treatment of cancers (30). We first confirmed that R428 inhibited Axl activity (P-Axl) in HN5-ER cells using P-RTK arrays (Supplementary Fig. 3A). Cell titer assays indicated that both HN5-ER and HN5 cell lines were sensitive to R428 (Fig. 3A; HN5-ER EC_{50} R428 = 1.4 μmol/L, HN5 EC_{50} R428 = 1.3 μmol/L). Parental HN5 cells are EGFR-dependent and thus highly sensitive to EGFR inhibitors, but lack detectable expression of Axl (Fig. 2D). R428 inhibits EGFR with >100-fold lower selectivity than for Axl (30), and we found that treatment of HN5 cells with R428 (10 μmol/L) for 4 hours caused a significant reduction in P-EGFR levels (Supplementary Fig. 3B), showing that Axl and EGFR may be targets of R428 in HNC cells. To determine whether Axl promotes resistance of HN5-ER cells to erlotinib, we conducted cell titer assays, in which cells were treated with subeffective concentrations of R428 (ranging from 0.1–1 μmol/L) in the presence or absence of an ineffective concentration of erlotinib (10 μmol/L). Erlotinib alone did not significantly reduce cell viability, nor did R428 in the absence of erlotinib (Fig. 3B). However, the combination of erlotinib and R428 yielded a significant and dose-responsive inhibition of cell viability. As a control, we confirmed the sensitivity of HN5-ER cells to Axl inhibition by treatment with 5 μmol/L R428, observing a >95% reduction in cell viability (data not shown). To further investigate the combined growth inhibitory effect of erlotinib and R428 on HN5-ER cell signaling pathways, we evaluated P-Akt levels in cells treated with the same ineffective concentrations of erlotinib/DMSO, R428/DMSO, and erlotinib/R428 used in Fig. 3B. Each drug on its own caused a modest reduction in P-Akt levels, but a much greater reduction in P-Akt was observed when erlotinib was combined with R428 (Fig. 3C), suggesting that R428 could reverse the acquired resistance of HNC cells to erlotinib and thus re sensitize them to this agent. Similarly, we found that R428 sensitized HN5-ER cells to an ineffective concentration of gefitinib (7.5 μmol/L; Supplementary Fig. S4), supporting the role of Axl in mediating resistance to EGFR inhibition. To confirm this finding, we used RNAi to deplete HN5-ER cells of Axl expression (Supplementary Fig. S5A and S5B), and using cell titer assays we found that Axl knockdown increased the sensitivity of HN5-ER cells to erlotinib (Supplementary Fig. SSC). Finally, as we observed an increased rate of migration of HN5-ER cells compared with HN5 cells (Fig. 1E), and as Axl promotes cancer cell migration, invasion, and metastasis (28), we tested the effect of R428 on HN5-ER cell migration. Our results indicate that R428 completely blocked HN5-ER cell migration over a 24 hour period, with the measured cell index for these samples being equivalent to the SFM (no migration) controls (Fig. 3D). These results suggest that Axl may be a valid therapeutic target in HNC, which are resistant to EGFR inhibitors such as erlotinib, and that Axl inhibition can restore erlotinib sensitivity and block tumor cell migration.

**A proinflammatory gene signature is associated with acquired erlotinib resistance in HN5-ER cells**

To gain further insight into the molecular mechanisms driving acquired EGFR inhibitor resistance in HNC, we conducted comparative microarray analysis of HN5 and...
Figure 2. Increased expression and activity of Axl and decreased miR-34a expression in HN5-ER cells. A, P-RTK analysis of protein lysates from HN5 (top) and HN5-ER (bottom) cells. Spots of interest are boxed and numbered as follows: 1, positive control; 2, P-EGFR; 3, P-ErbB2; 4, P-ErbB3; 5, P-EphA7; 6, P-VEGFR1; 7, P-IGF-1R; 8, P-Axl. B, densitometry analysis of P-Axl pixel density based on P-RTK profiling of HN5 and HN5-ER protein lysates. Mean pixel density across duplicate spots is expressed relative to HN5 cells. C, ELISA quantitation of P-Axl expression in HN5 and HN5-ER cell lysates. Data are expressed relative to HN5 cells. D, immunoblotting analysis of Axl expression in HN5, HN5-ER, and FaDu cells. β-Actin is included as a loading control. E, TaqMan miRNA RT-qPCR analysis of miR-34a expression in HN5 and HN5-ER cells. Data were normalized to U44 snRNA expression and expressed relative to HN5. F, immunoblotting analysis of Axl and E-cadherin expression in HN5-ER cells 3 days after transfection with either synthetic miR-34a, a negative control synthetic miRNA (miR-NC), or mock transfection (vehicle, LF2000). β-Actin was used as a loading control. G, cell titer analysis of HN5-ER cells transfected with miR-34a (gray bars) or miR-NC (black bars; 0.5–1.0 nmol/L) or LF2000 (white bars) for 3 days and then treated for a further 3 days with either erlotinib (10 μmol/L) or vehicle (DMSO). Data are expressed relative to LF2000/DMSO-treated HN5-ER cells. Error bars represent standard deviations. Data are representative of 3 independent experiments. *, P < 0.02, HN5-ER vs. HN5; **, P < 0.01, HN5-ER vs. HN5; †††, P < 0.001, miR-34a/erlotinib vs. miR-NC/erlotinib; ‡‡‡‡, P < 0.01, miR-34a/erlotinib vs. miR-34a/erlotinib.
HN5-ER cells. We refined the lists of genes with differential expression between HN5-ER and HN5 cells by assigning a cutoff for upregulation or downregulation of at least 1.5-fold and with a significance of \( P < 0.05 \) (Fig. 4A and B). Cluster analysis of differentially expressed genes between HN5-ER and HN5 cells (Fig. 4C) identified 247 mRNAs with significantly higher expression in HN5-ER than in HN5 cells (Supplementary Table S1), and 626 mRNAs with significantly lower expression in HN5-ER than in HN5 cells (Supplementary Table S2). We used IPA software to assign biological significance to the genes upregulated and downregulated with acquired erlotinib resistance, based on annotated functional pathways (Supplementary Table S3). Pathways that were significantly enriched for genes altered between HN5 and HN5-ER cells predicted the activation of terms that include: “Cancer (tumorigenesis),” “Cellular movement (invasion of cells),” “Cellular growth and proliferation (proliferation of cells),” “Inflammatory response (inflammation),” and “Cancer (head and neck cancer),” and decreased activation of pathways that included “Cell death (apoptosis of tumor cell lines)” (Table 2). Therefore, in addition to alterations in cell growth and survival, these findings suggest that acquired erlotinib resistance in HNC is associated with a proinflammatory, prometastatic phenotype.

To confirm our microarray findings, we used RT-qPCR to measure the mRNA expression of a panel of genes that were differentially expressed between HN5 and HN5-ER cells: E-cadherin (epithelial marker), N-cadherin and ZEB1 (mesenchymal markers), COX-2, IL-6, and IL-8 (proinflammatory cytokines), EGFR, and Axl. These studies confirmed both the direction of change and significance for each of these differentially expressed genes based on the microarray data (Fig. 4D). As the cytokines IL-6 and IL-8 were strongly upregulated at the mRNA level in HN5-ER cells, and are associated with EMT and...
Figure 4. Differential gene expression between HN5 and HN5-ER cells. Following microarray data normalization (see Materials and Methods), volcano (A) and scatter (B) plots, and a heatmap (C) were generated showing the distributions, correlations, and replicate consistency of genes with significantly altered expression between HN5 and HN5-ER samples. D, microarray validation by RT-qPCR analysis of E-cadherin, N-cadherin, COX-2, EGFR, Axl, ZEB1, IL-6, and IL-8 mRNA expression in HN5 and HN5-ER cells. (Continued on the following page.)
metastasis in various epithelial cancers, including HNC (31, 32), we conducted ELISA studies to compare IL-6 and IL-8 secretion from HN5 and HN5-ER cells. HN5-ER cells secreted significantly higher levels of IL-6 and IL-8 into cell culture media than HN5 cells (Fig. 4E), finding that is consistent with the respective ~100- and ~300-fold increase in IL-6 and IL-8 mRNA levels in HN5-ER cells (Fig. 4D). We also observed increased STAT3 expression and activity (P-STAT3) in HN5-ER cells (Fig. 4F), suggesting that IL-6 may be driving prosurvival STAT3 signaling in these cells. This was of particular interest as STAT3 activation has recently been shown to promote resistance to EGFR inhibitors in HNC (9). Using immunoblotting, we compared NF-xB activity between HN5 and HN5-ER cells, but did not detect elevated phosphorylation of RELA (NF-xB p65 subunit) in HN5-ER cells (Supplementary Fig. 6A), possibly because both EGFR and Axl may contribute to downstream NF-xB signaling. To investigate the regulation of NF-xB activity in HN5-ER cells in more detail, we transfected HN5-ER cells with miR-34a or miRNA, or with Axl siRNAs or NC siRNA, and analyzed P-RELA levels by immunoblotting (Supplementary Fig. S6B). We did not observe a change in P-RELA activity with miR-34a transfection, suggesting that NF-xB activity is not entirely dependent upon miR-34a or its downstream target Axl. We also observed that relative to HN5 cells, HN5-ER cells have increased expression of CD44 (Fig. 4F), a marker associated with elevated levels of IL-6 in erlotinib resistant, mesenchymal NSCLC cells (33). As R428 has been shown to suppress IL-6 expression in breast cancer cells (30), and Axl inhibition by RNAi impairs IL-6 expression and STAT3 activity in prostate cancer cells (34), we treated HN5-ER cells with R428 (5 μmol/L for 4 hours) to assess the significance of Axl upregulation on STAT3 signaling by immunoblotting. We found that R428 blocked expression of both P-STAT3 and P-Akt in HN5-ER cells (Fig. 4G), suggesting that Axl contributes to elevated STAT3 activity. Furthermore, we treated HN5-ER cells with 2 agents that inhibit the IL-6/STAT3 signaling axis: tocilizumab (1 μmol/L for 3 days), a monoclonal antibody against IL-6 receptor (IL-6R; ref. 35) or ruxolitinib (3 μmol/L for 24 hours), a small molecule inhibitor of Janus-activated kinase 1/2 (36). Both of these agents inhibited STAT3 activity in HN5-ER cells (Fig. 4H) without altering Axl expression (Supplementary Fig. S6C), suggesting that inhibitors of IL-6/STAT3 signaling could be evaluated alone or together with therapies targeting Axl to circumvent EGFR inhibitor resistance in HNC. Together, our findings identify changes in cellular pathways associated with inflammation, growth, metastasis, and cell death in HN5-ER cells, and suggest that elevated proinflammatory signaling (Fig. 5) is a feature of erlotinib-resistant HNC cells, which are CD44 positive and have increased secretion of IL-6 and IL-8, elevated COX-2 expression, and activation of STAT3 signaling.

A common gene signature of acquired erlotinib resistance between HNC and NSCLC

A recent report described activation of Axl in EGFR-mutant NSCLC cells with acquired erlotinib resistance (20), a finding that is consistent with our observations in HNC and suggests that common mechanisms of erlotinib resistance might exist between HNC and NSCLC. To address this issue, we integrated our HNC microarray data with that from the above mentioned NSCLC study, which compared gene expression between parental HCC

---

### Table 2. Functional pathways altered with acquired erlotinib resistance in HNC cells

<table>
<thead>
<tr>
<th>Functional pathways</th>
<th>Number of molecules affected</th>
<th>Predicted activation state</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer (tumorigenesis)</td>
<td>306/7,483 (4%)</td>
<td>Increased</td>
<td>5.56 × 10^{-21}</td>
</tr>
<tr>
<td>Cellular movement (invasion of cells)</td>
<td>60/3,046 (2%)</td>
<td>Increased</td>
<td>1.50 × 10^{-11}</td>
</tr>
<tr>
<td>Cellular growth and proliferation (proliferation of cells)</td>
<td>127/5,733 (2%)</td>
<td>Increased</td>
<td>6.20 × 10^{-9}</td>
</tr>
<tr>
<td>Cell death (apoptosis of tumor cell lines)</td>
<td>81/4,535 (2%)</td>
<td>Decreased</td>
<td>1.17 × 10^{-5}</td>
</tr>
<tr>
<td>Inflammatory response (inflammation)</td>
<td>57/1,142 (5%)</td>
<td>Increased</td>
<td>2.45 × 10^{-5}</td>
</tr>
<tr>
<td>Cancer (HNC)</td>
<td>47/818 (6%)</td>
<td>Increased</td>
<td>1.45 × 10^{-4}</td>
</tr>
</tbody>
</table>

**NOTE:** A series of cancer-associated pathways altered in HNC cells with acquired resistance to erlotinib, including functions related to tumorigenesis, HNC, cell growth and death, and inflammation were identified using IPA software. The total number of molecules upregulated or downregulated in HN5-ER cells relative to HN5 cells is indicated as a proportion of the total number of genes associated with that function, as is the predicted activation state of that pathway in HN5-ER cells.
827 NSCLC cells and 2 sublines with acquired erlotinib resistance: HCC 827 ER1 and HCC 827 ER2. Our analysis identified 95 genes whose expression was upregulated, and 75 genes whose expression was downregulated, across all 3 erlotinib-resistant HNC and NSCLC cell lines (HN5-ER, HCC 827 ER1, HCC 827 ER2; Fig. 6). To assign biological significance to these altered gene sets, we assigned them to functional pathways using IPA software (Supplementary Table S4). We found that acquired erlotinib resistance in HNC and NSCLC is associated with a common overrepresentation of genes involved with biological terms that included “Cancer,” “Head and neck cancer,” “Cell movement of tumor cell lines,” “Apoptosis of tumor cell lines,” “Proliferation of tumor cell lines,” and “Inflammatory response” (Table 3). Expression of Axl, IL-6, and IL-8 was also increased in HN5-ER, HCC 827 ER1, and HCC 827 ER2 cells relative to their parental HNC and NSCLC cell lines. Therefore, some of the functional alterations that mediate acquired resistance to erlotinib seem to be in common between HNC and NSCLC.

**Axl mRNA expression predicts survival in HNC patients**

Overexpression of Axl is associated with a worse prognosis in various cancers, including NSCLC, breast, and pancreatic cancer, and acute myeloid leukemia (28). We used publicly available gene expression data from The Cancer Genome Atlas (TCGA) with matching clinical information to investigate the prognostic value of Axl mRNA expression in HNC. At the time of preparation of this manuscript, matched data were available for 302 HNC patients (demographics are detailed in Table 1). When data were adjusted for age, gender, and smoking status, Axl mRNA expression was significantly correlated with poor survival (Fig. 7), with a 66% increased incidence of death in HNC patients with high tumor Axl mRNA expression (HR = 1.66, P = 0.007). This finding suggests that Axl may be associated with treatment resistance and progression of HNC.

**Discussion**

There is considerable evidence linking EMT to cancer progression, metastasis, and EGFR inhibitor resistance in multiple tumor types (12, 37–41). Our results confirm that the acquisition of erlotinib resistance in HN5-ER cells is associated with an EMT phenotype with fibroblastic cell morphology, increased cell migration, loss of E-cadherin, and miR-200 family miRNA expression, and gain of vimentin and N-cadherin. Interestingly, HN5-ER cells have strong expression of CD44 and reduced EGFR levels; this combination of markers was recently associated with a subpopulation of HNC cells that are highly resistant to treatment with radiation, cisplatin, cetuximab, and gefitinib (42). We also observed increased Akt activity, but reduced ERK1/2 activity, in HN5-ER cells. The latter finding was unexpected as TGF-β-induced EMT in H358 NSCLC cells is reported to promote EGFR-independent activation of ERK1/2 signaling (43), and SCC-1 HNC cells with acquired resistance to cetuximab, gefitinib, or erlotinib display elevated ERK1/2 and Akt activity (10). The precise mechanisms responsible for the increased Akt expression in HN5-ER cells are unclear, but Akt gene amplification and overexpression has been described in ovarian, pancreatic, and breast cancers (44–46).
Elevated Akt activity promotes tumorigenesis and is inversely associated with E-cadherin expression and patient survival in many cancers, including HNC (47), and the combination of a novel Akt inhibitor, MK-2206, with erlotinib synergistically inhibits growth of NCI-H292 NSCLC cancer cells and tumor xenografts (48), implying that targeted inhibition of Akt activity represents a potential strategy for the management of EGFR inhibitor-resistant HNCs.

We used P-RTK arrays to identify RTKs that compensated for loss of EGFR dependence and promoted Akt activity in the context of acquired erlotinib resistance, and reasoned that these RTKs could be targeted therapeutically in EGFR inhibitor-resistant HNC. The decreased activation of EGFR, ErbB2, and ErbB3 in HN5-ER cells was consistent with findings in mesenchymal NSCLC cells resistant to EGFR inhibition (43), whereas the reduced activity of VEGFR1 and IGF-1R in HN5-ER cells is in contrast with reports suggesting that these RTKs mediate resistance to anti-EGFR therapies in colon, prostate, breast, lung, and epidermoid cancer cells (49, 50). Although ligand-independent activation of c-Met has been shown to promote erlotinib resistance in HNC (51), we did not observe altered c-Met activity in HN5-ER cells. The significance of increased EphA7 activity in HN5-ER cells is unclear; the role of Eph signaling in cancer seems to be complex and loss of EphA7 expression is reported in certain cancers (52). Several lines of evidence support a role for elevated Axl expression and activation in cancer progression. First, Axl expression is correlated with metastasis and poor prognosis across multiple tumor types (53–55). Second, Axl activation promotes oncogenic processes such as cell growth, migration, invasion, survival, and angiogenesis (28). Third, targeted inhibition of Axl expression or activity reduces expression of EMT-associated molecules (e.g., Slug, Snail, Twist) and decreases tumor cell invasion (54, 56). Finally, Axl confers resistance to targeted and chemotherapeutic cancer drugs (57, 58), including ErbB-2 inhibitors in breast cancer (59) and erlotinib in NSCLC (20). A number of strategies have been developed to inhibit Axl clinically (reviewed in ref. 28). R428 is an orally bioavailable, potent, and selective small molecule Axl TKI that inhibits several Axl-dependent processes in breast cancer cells, including Akt

### Table 3. Functional pathways altered with acquired erlotinib resistance in both HNC and NSCLC cells

<table>
<thead>
<tr>
<th>Functional pathway</th>
<th>Upregulated genes in HNC-ER and NSCLC-ER</th>
<th>Downregulated genes in HNC-ER and NSCLC-ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>58/95 ($P = 3.03 \times 10^{-15}$)</td>
<td>27/75 ($P = 4.70 \times 10^{-2}$)</td>
</tr>
<tr>
<td>HNC</td>
<td>10/95 ($P = 3.29 \times 10^{-3}$)</td>
<td>7/75 ($P = 1.27 \times 10^{-3}$)</td>
</tr>
<tr>
<td>Cell movement of tumor cell lines</td>
<td>19/95 ($P = 9.4 \times 10^{-6}$)</td>
<td>7/75 ($P = 3.88 \times 10^{-6}$)</td>
</tr>
<tr>
<td>Apoptosis of tumor cell lines</td>
<td>16/95 ($P = 3.68 \times 10^{-3}$)</td>
<td>10/75 ($P = 3.86 \times 10^{-2}$)</td>
</tr>
<tr>
<td>Proliferation of tumor cell lines</td>
<td>19/95 ($P = 2.1 \times 10^{-3}$)</td>
<td>N.E.</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>6/95 ($P = 1.83 \times 10^{-2}$)</td>
<td>N.E.</td>
</tr>
</tbody>
</table>

NOTE: Gene expression signatures related to acquired erlotinib resistance that were common to both HNC and NSCLC were assigned biological significance using IPA software, with a focus on functional pathways that were identified initially in erlotinib-resistant HNC (Table 2). Ninety-five genes were commonly upregulated, and 75 genes were commonly downregulated, with acquired erlotinib resistance in HNC and NSCLC. The proportion of each gene set implicated in these pathways is indicated in the table. Abbreviation: N.E., no enrichment of functional pathway among that gene set.
activation, cell invasion, and proinflammatory cytokine production, and represses EMT and angiogenesis and extends survival in mice bearing metastatic breast tumors (30). In HN5-ER cells, R428 reduced growth, blocked migration, and restored erlotinib sensitivity. We also observed that parental HN5 cells were growth inhibited by R428; this was unexpected given the apparently undetectable expression of Axl protein in HN5 cells in immunoblotting studies (Fig. 2D). Several possibilities exist that may account for this effect: (i) a low but detectable level of P-Axl is found in HN5 cells (Fig. 2A and B), (ii) R428 inhibits EGFR and other related RTKs with >100-fold less selectivity than for Axl (30), and thus the apparent sensitivity of HN5 cells to R428 could result from this "off-target" inhibition of P-EGFR in a highly EGFR-dependent cell line, and (iii) the HN5 cell line contains a small mesenchymal subpopulation of highly tumorigenic HN5-ER cells. In support of the latter hypothesis, Yao and colleagues identified erlotinib-resistant mesenchymal-like cells within NSCLC cell lines and tumors before erlotinib treatment (33), suggesting that acquired erlotinib resistance might arise at least in part from selection for this mesenchymal subpopulation. Our findings emphasize the role of Axl in promoting EMT, cell migration, and erlotinib resistance in HNC, and support the rationale for therapeutic blockade of Axl in EGFR inhibitor-resistant tumors, including HNC. More broadly, our results suggest that EGFR inhibitor resistance may arise via one or more distinct mechanisms, underscoring the need to identify relevant biomarkers to properly select cancer patients for appropriate targeted therapies.

Functional roles for specific miRNAs in the regulation of EMT are well established (reviewed in ref. 60). In view of reports showing that miR-34a expression is reduced in NSCLC, colorectal, and breast cancer cell lines due to promoter methylation, that Axl is a miR-34a target molecule (29), and that miR-34a is part of a miRNA expression signature associated with epithelial morphology and erlotinib sensitivity in lung cancer cells (61), we showed loss of miR-34a in HN5-ER cells and found that restoring miR-34a expression blocked Axl expression, increased levels of E-cadherin, and sensitized cells to erlotinib. Decreased AXIN2, CA9, CXCL10, FOSL1, FUT8, GAS1, KLF6, PODXL levels in Axl mRNA overexpression predicts poor survival in HNC. Kaplan-Meier plot of age-, gender-, and smoking status-adjusted survival for 302 HNC patients according to median tumor Axl mRNA expression over a 10-year follow up period after initial surgery.
expression of miR-34a in colon, breast, and lung cancer cells is thought to result, in part, from loss of wild-type p53 function, and is associated with increased expression of molecules known to promote EMT, including Snail, β-catenin, LEF1, and Axin2 (62). We propose that a loss of miR-34a facilitates coordinate expression of a set of target genes that maintain a mesenchymal cancer cell phenotype associated with EGFR inhibitor resistance and metastasis. Among the genes we found upregulated in HNSC-ER cells relative to HN5 cells, 9 are known to promote EMT and are predicted or experimentally validated targets of miR-34a (Fig. 7); these include Axin2 (63), Axl (29), CA9 (64), CXCL10 (65), FOSE1 (66), FUT8 (67), GAS1 (68), KLK6 (69), and PODXL (70). Therefore, miR-34a may oppose EMT via the coordinate downregulation of a network of miR-34a target mRNAs that promote EMT, metastasis, and EGFR inhibitor resistance. Therapeutic upregulation of miR-34a could reverse the metastatic and EGFR inhibitor-resistant EMT phenotype in tumors, and may ultimately improve treatment responses and patient survival. This may be a particularly attractive strategy given the showed feasibility of delivery and efficacy of miR-34a in preclinical tumor models (71), and the recent commencement of a phase I clinical trial of miR-34a in cancer patients (May 2013; Mirna Therapeutics).

Our study identified a putative role for proinflammatory signaling in erlotinib resistance in HNC, whereby elevated synthesis and secretion of IL-6 and IL-8 may activate STAT3 signaling and target gene expression to promote inflammation, cell survival, and metastasis (Fig. 5). IL-6 is known to promote EMT and metastasis in HNC (32), and its expression may be induced by NF-κB signaling, which in turn also activates the STAT3 pathway (72). Interestingly, IL-6 secretion is regulated by Axl signaling in animal tumor models (30), STAT3 activity is increased in SCC-1 HNC cells with acquired resistance to EGFR inhibitors (10), and may suppress expression of miR-200 miRNAs (73), and increased IL-6 secretion promotes erlotinib resistance in mesenchymal NSCLC cells (33). IL-6 may also induce EMT by blocking expression of miR-200c in breast cancer cells (74). IL-8 induces EMT and activation of NF-κB (75) and Akt activation in HNC, where it is associated with a poor prognosis (31). Low expression of IL-8 is significantly associated with longer overall survival in colorectal cancer patients treated with cetuximab (76), suggesting that IL-8 levels might also have prognostic value in HNC patients receiving anti-EGFR therapy. Finally, we also observed increased expression of the proinflammatory mediator COX-2 in HNSC-ER cells. COX-2 is overexpressed in a variety of human tumors, including HNC (77), where it is implicated in cancer growth and progression and is associated with a worse clinical outcome (reviewed in ref. 78). COX-2 inhibition has been proposed as a strategy to overcome resistance of HNCs to EGFR inhibitors (79), with combinations of EGFR TKIs and COX-2 inhibitors producing synergistic antitumor effects in preclinical studies with HNC (80). COX-2 induces a mesenchymal, metastatic phenotype in NSCLC by repressing expression of E-cadherin and promoting expression of genes such as Snail, ZEB1, and IL-6 (81), suggesting that it might in part maintain the EMT phenotype of HNSC-ER cells. Our findings implicate proinflammatory mediators, including IL-6, IL-8, and COX-2, in driving EMT, STAT3 signaling, and EGFR inhibitor resistance in HNC, and suggest that targeted inhibition of IL-6, IL-8, and/or COX-2 could sensitize CD44 positive, mesenchymal HNC cells to anti-EGFR therapies.

Despite EGFR being widely expressed in HNC, EGFR inhibitors have produced disappointing clinical results. A thorough understanding of the mechanisms behind the inherent and acquired resistance of HNCs to anti-EGFR therapy is required to better select patients most likely to benefit from these treatments and to design new strategies to improve clinical responses. Based on our findings with an in vitro model of erlotinib resistance in HNC, we propose a model (Fig. 8) in which EMT, EGFR inhibitor resistance, and metastasis in HNC are mediated in part by increased expression/activity of Axl, increased synthesis and secretion of IL-6, and reduced expression of miR-34a. Inhibition of Axl activity with R428 decreased cell proliferation, blocked migration, and restored erlotinib sensitivity. There are prognostic and therapeutic implications of these molecules for the use of EGFR-targeted therapies in HNC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: K.M. Giles, P.A. Candy, C.J. Goodall, P.J. Leedman
Development of methodology: K.M. Giles, F.C. Kalinowski, P.A. Candy, P.M. Zhang, L.M. Stuart
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.M. Giles, F.C. Kalinowski, P.A. Candy, M.R. Epis, L.M. Stuart, C.J. Goodall, P.J. Leedman
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.M. Giles, F.C. Kalinowski, P.A. Candy, M.R. Epis, A.D. Redfern, P.J. Leedman
Writing, review, and/or revision of the manuscript: K.M. Giles, F.C. Kalinowski, P.A. Candy, M.R. Epis, A.D. Redfern, P.J. Leedman
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F.C. Kalinowski, P.A. Candy, M.R. Epis, A.D. Redfern
Study supervision: K.M. Giles, P.J. Leedman

Acknowledgments
The authors thank D. Beveridge for helpful discussion about the manuscript and V. Matthews for technical advice with ELISA experiments.

Grant Support
This work was supported by the National Health and Medical Research Council (NHMRC; Grant Number 634375; P.J. Leedman and K.M. Giles), the University of Western Australia Research Development Award scheme (K.M. Giles), and a Royal Perth Hospital Medical Research Foundation fellowship (K.M. Giles).

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 March 6, 2013; revised August 15, 2013; accepted August 17, 2013; published OnlineFirst September 11, 2013.
References


Giles et al.


Axl Mediates Acquired Resistance of Head and Neck Cancer Cells to the Epidermal Growth Factor Receptor Inhibitor Erlotinib

Keith M. Giles, Felicity C. Kalinowski, Patrick A. Candy, et al.