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Axl mediates acquired resistance of head and neck cancer cells to the epidermal growth factor receptor inhibitor erlotinib

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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 (TKIs; eg. erlotinib) have been disappointing in HNC. To investigate the mechanisms mediating resistance to these agents, we developed a 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 miRNAs, and elevated vimentin expression. Phosphorylated RTK profiling identified Axl activation in HN5-ER cells. Growth and migration of HN5-ER cells was blocked with a specific Axl inhibitor, R428, and R428 re-sensitized 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.
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, 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 (eg. cetuximab) or small molecule EGFR tyrosine kinase inhibitors (TKIs, eg. erlotinib or gefitinib) have shown that these agents have only modest activity in recurrent or advanced HNC. While most HNCs are inherently resistant to EGFR inhibition, approximately 5-15% of HNC patients experience an initial anti-tumor 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), while 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), while 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 demonstrated that inhibition of Signal Transducer and Activator of Transcription 3 (STAT3) activity sensitizes HNC cells to EGFR inhibition (9), while 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 (miRNAs) 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 pro-inflammatory signaling, with increased expression and secretion of IL-6 and IL-8 and elevated STAT3 activity. Finally, we demonstrate 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. Terrance Johns (Monash Institute of Medical Research, Australia) and 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 (ATCC). Cells were cultured in Dulbecco’s Modified Eagles Medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS) at 37°C in 5% CO2.

Erlotinib (LC Laboratories; Massachusetts) was prepared as a 23 mM stock solution in 96% (v/v) dimethyl sulfoxide (DMSO; Sigma-Aldrich). R428 (Symansis, California) was prepared as a 10 mM stock solution in DMSO. Gefitinib (Selleck...
Chemicals) was prepared as a 20 mM stock solution in DMSO. Tocilizumab (Roche) was supplied as a 20 mg/ml stock solution. Ruxolitinib (LC Laboratories; Massachusetts) was prepared as a 33 mM 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 (Austin, Texas). Axl siRNAs (Hs_AXL_3 Catalog no. SI00131355, Hs_AXL_9 Catalog no. SI00605304, Hs_AXL_10 Catalog no. SI00605311 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 100X 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 µM over 3 months, maintained in 12.5 µM 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 performed as described (17). Briefly, to determine the sensitivity of HN5 and HN5-ER cell lines to erlotinib, gefitinib and/or R428, 5.0 x 10^3 cells were seeded per well in 96 well plates, and fresh media containing erlotinib or R428 added 24 h after cell plating. Cell viability was measured 3 d after addition of erlotinib/R428/gefitinib using the CellTitre 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega) and a FLUOstar OPTIMA microplate reader.
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 µM). Cell viability was measured 3 d after addition of erlotinib or gefitinib and/or R428 as above.

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 d and then treated with an ineffective concentration of erlotinib (10 µM) for a further 2 d, after which cell viability was determined.

**miRNA precursor and siRNA transfections**

miRNA transfection experiments were performed as described (18). Briefly, HN5-ER cells were seeded in 6-well plates at a density of 5.0 x 10⁵ cells per well and transfected with 30 nM miR-34a or miR-NC for 24 h prior to RNA extraction or protein extraction and immunoblotting as outlined below.

For transfections with Axl siRNA, 2.5 x 10⁵ cells per well were seeded in 6-well plates and transfected with 10 nM Axl siRNA or AllStars negative control siRNA (NC siRNA) for 3 d prior to RNA extraction or protein extraction and immunoblotting as outlined below.

**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 µM R428, 5 µM erlotinib, a combination of 5 µM R428 and 5 µM erlotinib, or DMSO for 4 h prior to preparing cytoplasmic protein extracts for immunoblotting as outlined below.

To assess the effect of R428 on HN5 cells, cells were cultured overnight in DMEM + 0.5% FBS, and then treated with 10 µM R428 or DMSO for 4 h, prior to protein extraction and immunoblotting as outlined below.

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 µM ruxolitinib for 24 h, or 1 µM tocilizumab for 3 d, prior to protein extraction and immunoblotting as outlined below.

Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from HN5 and HN5-ER cell lines with TRIzol reagent (Invitrogen). For 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 performed on a Corbett Rotor-Gene 6000 thermocycler (QIAGEN) using QuantiTect SYBR Mix (QIAGEN) and validated QuantiTect primer assays (QIAGEN) for EGFR (QT00085701), AXL (QT00067725), CDH1 (QT00080143), CDH2 (QT00063196), PTGS2 (QT00040586), ZEB1 (QT01888446), IL-6 (QT00083720), IL-8 (QT00000322), ALAS1 (QT00073122) and GAPDH (QT01192646). Expression of EGFR, AXL, CDH1, CDH2, PTGS2, ZEB1, IL-6 and IL-8 mRNA was determined relative to ALAS1 and GAPDH mRNA using the 2\(^{-\Delta\Delta Ct}\) method (19).
For RT-qPCR analysis of miRNA expression, reverse transcription and PCR were carried out using a TaqMan miRNA assay kit (Applied Biosystems) for hsa-miR-34a-5p (miR-34a; Life Technologies, Assay ID 000426), miR-200a, miR-200b, miR-200c (Life Technologies, Assay IDs 000502, 002251, 002300) and RNU44 small nucleolar RNA (U44 snRNA) (Life Technologies, Assay ID 001094) with a Corbett Rotor-Gene 6000 thermocycler (Qiagen) according to manufacturer’s instructions. Expression of mature miR-34a, miR-200a, miR-200b, or miR-200c miRNAs relative to U44 snRNA was determined using the $2^{-\Delta\Delta Ct}$ method.

**Protein extraction and immunoblotting**

Cytoplasmic protein extracts were prepared from cell lines and immunoblotting performed as described (18). For detection of vimentin, cell lysates were sonicated. Polyvinylidene difluoride (PVDF) membranes (Roche) were probed with anti-EGFR rabbit monoclonal antibody (1:5000, Abcam ab52894-100), anti-phospho-EGFR (Tyr1173) goat polyclonal antibody (1:750, Santa Cruz Biotechnology sc-12351), anti-Akt rabbit polyclonal antibody (1:1000, Cell Signaling Technology 9272), anti-phospho-Akt (Ser473) rabbit monoclonal antibody (1:500, Cell Signaling Technology 4060S), anti-E-cadherin rabbit monoclonal antibody (1:1000, Cell Signaling Technology 3195S), anti-Vimentin mouse monoclonal antibody (1:1000, Cell Signaling Technology 3390), anti-Axl rabbit monoclonal antibody (1:1000, 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 (1:1000, Cell Signaling Technology 4904), anti-phospho-STAT3 (Tyr705) rabbit monoclonal antibody (1:1000, Cell Signaling Technology...
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9145), anti-CD44 mouse monoclonal antibody (1:1000, Cell Signaling Technology 3570), anti-NF-κB p65 (RELA) rabbit monoclonal antibody (1:1000, Cell Signaling Technology 8242), anti-phospho-NF-κB p65 (P-RELA) rabbit monoclonal antibody (1:1000, 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-2020) were used prior to detection with an ECL Plus Western Blotting Detection System (GE Healthcare). Quantitation of specific proteins of interest was performed using Quantity One software (Bio-Rad).

226 **Phospho-receptor tyrosine kinase (P-RTK) array**

227 P-RTK arrays (R&D Systems ARY001) were performed according to manufacturer’s instructions using 500 µg of HN5 or HN5-ER protein. P-RTKs of interest were quantitated using Quantity One software (Bio-Rad).

229 To confirm the inhibitory action of R428 on Axl phosphorylation, HN5 and HN5-ER cells were cultured overnight in DMEM + 0.5% FBS, and then treated with 10 µM R428 for 2 h, prior to protein extraction and P-RTK array analysis as above.

231 **P-Axl, IL-6 and IL-8 enzyme-linked immunosorbent assay (ELISA)**

232 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 x 10^5 cells/well in 6 well plates (total volume of 1.5 ml), and media harvested 48 h 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 x 10^4 cells/well into the upper chamber of CIM-plate 16 xCELLigence plates (Roche 05665817001). DMEM + 20% FBS was used in the lower chambers as a chemoattractant, while serum-free media (SFM) was the control for no cell migration. Cell migration into the lower chamber was measured over 24 h and expressed as a baseline cell index relative to SFM control migration.

For some studies, HN5-ER cells (3.0 x 10^5 cells/well) in 6 well plates were pre-treated with 5 µM R428 or DMSO for 4 h, and cell migration assessed as described above.

**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 performed with three biological replicate samples for each cell line by the Australian Genome Research Facility.
(AGRF; Australia) using HumanHT-12 v4 array chips (Illumina). Data normalization was performed by AGRF and data analyzed as described previously (17). Parametric two-tailed Student’s $t$-test was used to calculate significance of variation, fold change was calculated as mean ratio. Probes with an unadjusted p-value $<0.05$ and an absolute fold change $>1.5$ or more were defined as being differentially expressed between HN5-ER and HN5 cells. Microarray expression data has 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 software was used to define functional pathways affected by the acquisition of erlotinib resistance.

HCC 827 NSCLC microarrays (GEO accession number: GSE38121, (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 Ingenuity Pathway Analysis 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 ± standard deviation (SD). Statistical significance was calculated using the Student’s t-test (two-tailed, unpaired) and the level of significance was set at p<0.05. Statistical analysis of RT-qPCR data was performed using GenEx software (MultiD), and normality of data confirmed using the Kolmogorov-Smirnov test (KS 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, that 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 perform Cox regressions with ties addressed by Efron’s method and produce Kaplan-Meier survival curves, with all deaths used as an end point. 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 (RPKM) 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 utilized 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 six months. Microscopic analysis indicated that while 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 (Figure 1A). We measured erlotinib sensitivity using cell titre assays, and confirmed that HN5-ER cells had >200-fold higher resistance to erlotinib than HN5 cells (Figure 1B; HN5 EC\(_{50}\) erlotinib = 0.1 μM, HN5-ER EC\(_{50}\) erlotinib >20 μM). We also found that HN5-ER cells were cross-resistant to the EGFR TKI gefitinib (Supplementary Figure 1A).

Interestingly, HN5-ER cells maintained their erlotinib resistance and mesenchymal cell morphology when grown for several weeks in erlotinib-free media (Supplementary Figure 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 (Figure 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 expression but expressed vimentin, confirming their mesenchymal cancer cell phenotype. We confirmed these findings by analysing expression of EGFR, Akt, P-Akt and E-cadherin in an independent pool of erlotinib-resistant HN5 cells (HN5-ER2) by immunoblotting (Supplementary Figure 2), and observed changes in expression and activity of these molecules consistent with those in HN5-ER cells. We next used TaqMan microRNA (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 (Figure 1D), consistent with their EMT phenotype. As increased cell migration, invasion and metastasis is a hallmark of mesenchymal tumor cells (26), we performed xCELLigence real-time cell migration studies (27) and found that HN5-ER cells had an increased rate of migration compared to parental HN5 cells (Figure 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 phospho-RTK (P-RTK) arrays to simultaneously measure the activity (tyrosine phosphorylation) of 42 RTKs known to be associated with cancer. These experiments identified several RTKs with altered activity between HN5 and HN5-ER cells (Figure 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, non-small cell lung cancer (NSCLC), and glioblastoma multiforme (28). We confirmed the increase in Axl phosphorylation in HN5-ER cells by densitometry (Figure 2B), and also using ELISA assays specific for P-Axl with protein extracts from HN5 and HN5-ER cells (Figure 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 (Figure 2D). We also found that Axl was abundantly expressed in FaDu HNC cells that are resistant to erlotinib (Figure 2D; (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 (3’-UTR) (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.
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, suggesting that miR-34a might also act as a negative regulator of EMT in this system (Figure 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 μM) or vehicle (DMSO). We found that restoring miR-34a expression to HN5-ER cells sensitized them to erlotinib (Figure 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 Figure 3A). Cell titre assays indicated that both HN5-ER and HN5 cell lines were sensitive to R428 (Figure 3A; HN5-ER EC$_{50}$ R428 = 1.4 μM, HN5 EC$_{50}$ R428 = 1.3 μM). Parental HN5 cells are EGFR-dependent and thus highly sensitive to EGFR inhibitors, but lack
detectable expression of Axl (Figure 2D). R428 inhibits EGFR with >100-fold lower selectivity than for Axl (30), and we found that treatment of HN5 cells with R428 (10 μM) for 4 h caused a significant reduction in P-EGFR levels (Supplementary Figure 3B), demonstrating 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 performed cell titre assays in which cells were treated with sub-effective concentrations of R428 (ranging from 0.1-1 μM) in the presence or absence of an ineffective concentration of erlotinib (10 μM). Erlotinib alone did not significantly reduce cell viability, nor did R428 in the absence of erlotinib (Figure 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 μM 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 Figure 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 (Figure 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 μM; Supplementary Figure 4), 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 Figure 5A and 5B), and using cell titre assays we found that Axl knockdown increased the sensitivity of HN5-ER cells to erlotinib (Supplementary Figure 5C). Finally, as we observed an
increased rate of migration of HN5-ER cells compared with HN5 cells (Figure 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 h period, with the measured cell index for these samples being equivalent to the serum-free media (SFM; no migration) controls (Figure 3D). These results suggest that Axl may be a valid therapeutic target in HNCs that are resistant to EGFR inhibitors such as erlotinib, and that Axl inhibition can restore erlotinib sensitivity and block tumor cell migration.

A pro-inflammatory 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 performed comparative microarray analysis of HN5 and HN5-ER cells. We refined the lists of genes with differential expression between HN5-ER and HN5 cells by assigning a cut off for upregulation or downregulation of at least 1.5 fold and with a significance of p<0.05 (Figure 4A and 4B). Cluster analysis of differentially expressed genes between HN5-ER and HN5 cells (Figure 4C) identified 247 mRNAs with significantly higher expression in HN5-ER than in HN5 cells (Supplementary Table 1), and 626 mRNAs with significantly lower expression in HN5-ER than in HN5 cells (Supplementary Table 2). We used Ingenuity Pathway Analysis (IPA) software to assign biological significance to the genes upregulated and downregulated with acquired erlotinib resistance, based on annotated functional pathways (Supplementary Table 3). 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
457 (invasion of cells)”, “Cellular growth and proliferation (proliferation of cells)”,
458 “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
pro-inflammatory, pro-metastatic phenotype.

463 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, IL-8 (pro-inflammatory 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 (Figure 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 metastasis in various epithelial cancers,
including HNC (31, 32), we performed 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 (Figure 4E), a finding
that is consistent with the respective ~100- and ~300-fold increase in IL-6 and IL-8
mRNA levels in HN5-ER cells (Figure 4D). We also observed increased STAT3
expression and activity (P-STAT3) in HN5-ER cells (Figure 4F), suggesting that IL-6
may be driving pro-survival 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-κB activity between
HN5 and HN5-ER cells, but did not detect elevated phosphorylation of RELA (NF-
κB p65 subunit) in HN5-ER cells (Supplementary Figure 6A), possibly because both EGFR and Axl may contribute to downstream NF-κB signaling. To investigate the regulation of NF-κB activity in HN5-ER cells in more detail, we transfected HN5-ER cells with miR-34a or miR-NC, or with Axl siRNAs or NC siRNA, and analyzed P-RELA levels by immunoblotting (Supplementary Figure 6B). We did not observe a change in P-RELA activity with miR-34a transfection, suggesting that NF-κB 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 (Figure 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 μM for 4 h) 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 (Figure 4G), suggesting that Axl contributes to elevated STAT3 activity. Furthermore, we treated HN5-ER cells with two agents that inhibit the IL-6/STAT3 signaling axis: tocilizumab (1 μM for 3 d), a monoclonal antibody against interleukin-6 receptor (IL-6R) (35) or ruxolitinib (3 μM for 24 h), a small molecule inhibitor of Janus kinase 1/2 (JAK1/2) (36). Both of these agents inhibited STAT3 activity in HN5-ER cells (Figure 4H) without altering Axl expression (Supplementary Figure 6C), 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 pro-inflammatory signaling
(Figure 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 head and neck cancer and non-small cell lung cancer

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 827 NSCLC cells and two 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 three erlotinib-resistant HNC and NSCLC cell lines (HN5ER, HCC 827 ER1, HCC 827 ER2) (Figure 6). To assign biological significance to these altered gene sets, we assigned them to functional pathways using IPA software (Supplementary Table 4). 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 appear to be in common between HNC
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 utilized 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 was available for 302 HNC patients (demographics are detailed in Table 1). When data was adjusted for age, gender and smoking status, Axl mRNA expression was significantly correlated with poor survival (Figure 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 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 TGFβ-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). While 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 appears 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. Firstly, Axl expression is correlated with metastasis and poor prognosis across multiple tumor types (53-55). Secondly, Axl activation promotes oncogenic processes such as cell growth, migration, invasion, survival and angiogenesis (28). Thirdly, targeted inhibition of Axl expression or activity reduces expression of EMT-associated molecules (eg. Slug, Snail, Twist) and decreases tumor cell invasion (54, 56). Lastly, 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 (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 activation, cell invasion and pro-inflammatory 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 (Figure 2D). Several possibilities exist that may account for this effect: (i) a low but detectable level of P-Axl is found in HN5 cells (Figure 2A and 2B), (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
coworkers 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 (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 demonstrated 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 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 Snail1, 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 HN5-ER cells relative to HN5 cells, nine are known to promote EMT and are predicted or experimentally validated
targets of miR-34a (Figure 7); these include: Axin2 (63), Axl (29), CA9 (64),
CXCL10 (65), FOSL1 (66), FUT8 (67), GAS1 (68), KLF6 (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 demonstrated 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, Austin, Texas).

Our study identified a putative role for pro-inflammatory 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 (Figure 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 express 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 pro-inflammatory mediator COX-2 in HN5-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 (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 anti-tumor 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 HN5-ER cells. Our findings implicate pro-inflammatory 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 (Figure 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.

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Perth Hospital Medical Research Foundation fellowship.

ABBREVIATIONS
The abbreviations used are: miRNA, microRNA; mRNA, messenger RNA; HNC,
head and neck cancer; RT-qPCR, reverse transcriptase quantitative polymerase chain
reaction; 3′-UTR, 3′-untranslated region; miR-NC, negative control miRNA; SD,
standard deviation; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KS test,
Kolmogorov-Smirnov test; EGFR, epidermal growth factor receptor; IGF-1R, insulin-
like growth factor 1 receptor; P-EGFR, phosphorylated EGFR, P-Akt, phosphorylated
Akt; ELISA, enzyme-linked immunosorbent assay; TKI, tyrosine kinase inhibitor;
RTK, receptor tyrosine kinase; P-RTK, phosphorylated RTK; EMT, epithelial-
mesenchymal transition; NSCLC, non-small cell lung cancer; HN5-ER, erlotinib
resistant HN5 cells; snRNA, small nucleolar RNA; IPA, Ingenuity Pathway Analysis software; IL-6, interleukin-6; IL-8, interleukin-8.
REFERENCES


60. Bullock MD, Sayan AE, Packham GK, Mirnezami AH. MicroRNAs: critical regulators of epithelial to mesenchymal (EMT) and mesenchymal to epithelial transition (MET) in cancer progression. Biol Cell. 2012;104:3-12.


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><strong>All cases</strong></td>
<td></td>
<td></td>
<td>334 (100%)</td>
</tr>
<tr>
<td>Axl evaluated</td>
<td>153 (50.5%)</td>
<td>150 (49.5%)</td>
<td>303 (90.7%)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>109 (36%)</td>
<td>113 (37.2%)</td>
<td>240 (71.9%)</td>
</tr>
<tr>
<td>Female</td>
<td>45 (14.9%)</td>
<td>36 (11.9%)</td>
<td>94 (28.1%)</td>
</tr>
<tr>
<td><strong>Age at diagnosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 59</td>
<td>66 (21.8%)</td>
<td>61 (20.1%)</td>
<td>142 (42.5%)</td>
</tr>
<tr>
<td>60-69</td>
<td>50 (16.5%)</td>
<td>59 (19.5%)</td>
<td>118 (35.3%)</td>
</tr>
<tr>
<td>&gt; 70</td>
<td>38 (12.5%)</td>
<td>29 (9.6%)</td>
<td>73 (22%)</td>
</tr>
<tr>
<td><strong>Smoking status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smoker</td>
<td>48 (15.8%)</td>
<td>52 (17.2%)</td>
<td>113 (33.8%)</td>
</tr>
<tr>
<td>Quit smoking ≤ 15 years ago</td>
<td>54 (17.8%)</td>
<td>31 (10.2%)</td>
<td>92 (27.6%)</td>
</tr>
<tr>
<td>Quit smoking &gt; 15 years ago</td>
<td>22 (7.3%)</td>
<td>25 (8.3%)</td>
<td>56 (16.8%)</td>
</tr>
<tr>
<td>Lifelong non-smoker</td>
<td>24 (7.9%)</td>
<td>37 (12.2%)</td>
<td>63 (18.9%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>6 (2%)</td>
<td>4 (1.3%)</td>
<td>10 (3%)</td>
</tr>
<tr>
<td><strong>Alcohol consumption</strong></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>42 (13.9%)</td>
</tr>
<tr>
<td>2-3 drinks/day</td>
<td>22 (7.3%)</td>
<td>12 (4%)</td>
<td>36 (11.9%)</td>
</tr>
<tr>
<td>≥ 4 drinks/day</td>
<td>21 (6.9%)</td>
<td>23 (7.6%)</td>
<td>50 (16.5%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>90 (29.7%)</td>
<td>92 (30.4%)</td>
<td>195 (64.4%)</td>
</tr>
<tr>
<td><strong>Cancer stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>7 (2.3%)</td>
<td>3 (1%)</td>
<td>15 (4.5%)</td>
</tr>
<tr>
<td>II</td>
<td>34 (11.2%)</td>
<td>28 (9.2%)</td>
<td>68 (20.4%)</td>
</tr>
<tr>
<td>III</td>
<td>29 (9.6%)</td>
<td>38 (12.5%)</td>
<td>75 (22.5%)</td>
</tr>
<tr>
<td>IV</td>
<td>84 (27.7%)</td>
<td>80 (26.4%)</td>
<td>176 (52.7%)</td>
</tr>
<tr>
<td><strong>Survival status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Living</td>
<td>88 (29%)</td>
<td>95 (31.4%)</td>
<td>210 (62.9%)</td>
</tr>
<tr>
<td>Deceased</td>
<td>66 (21.8%)</td>
<td>54 (17.8%)</td>
<td>124 (37.1%)</td>
</tr>
</tbody>
</table>

Clinicopathologic features relative to Axl mRNA expression levels in HNC
Table 2: Functional pathways altered with acquired erlotinib resistance in head and neck cancer 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>306/7483 (4%)</td>
<td>Increased</td>
<td>5.56x10^{-21}</td>
</tr>
<tr>
<td>Cellular movement (invasion of cells)</td>
<td>60/3046 (2%)</td>
<td>Increased</td>
<td>1.50x10^{-11}</td>
</tr>
<tr>
<td>Cellular growth and proliferation (proliferation of cells)</td>
<td>127/5733 (2%)</td>
<td>Increased</td>
<td>6.20x10^{-9}</td>
</tr>
<tr>
<td>Cell death (apoptosis of tumor cell lines)</td>
<td>81/4535 (2%)</td>
<td>Decreased</td>
<td>1.17x10^{-5}</td>
</tr>
<tr>
<td>Inflammatory response (inflammation)</td>
<td>57/1142 (5%)</td>
<td>Increased</td>
<td>2.45x10^{-5}</td>
</tr>
<tr>
<td>Cancer (head and neck cancer)</td>
<td>47/818 (6%)</td>
<td>Increased</td>
<td>1.45x10^{-4}</td>
</tr>
</tbody>
</table>

A series of cancer-associated pathways altered in HNC cells with acquired resistance to erlotinib, including functions related to tumorigenesis, head and neck cancer, cell growth and death, and inflammation were identified using Ingenuity Pathway Analysis (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.
Table 3: Functional pathways altered with acquired erlotinib resistance in both head and neck cancer and non-small cell lung cancer 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 x 10^-10)</td>
<td>27/75 (p=4.70 x 10^-2)</td>
</tr>
<tr>
<td>Head and neck cancer</td>
<td>10/95 (p=3.29 x 10^-3)</td>
<td>7/75 (p=1.27 x 10^-2)</td>
</tr>
<tr>
<td>Cell movement of tumor cell lines</td>
<td>19/95 (p=9.4 x 10^-8)</td>
<td>7/75 (p=3.88 x 10^-2)</td>
</tr>
<tr>
<td>Apoptosis of tumor cell lines</td>
<td>16/95 (p=3.68 x 10^-3)</td>
<td>10/75 (p=3.86 x 10^-2)</td>
</tr>
<tr>
<td>Proliferation of tumor cell lines</td>
<td>19/95 (p=2.1 x 10^-3)</td>
<td>N.E.</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>6/95 (p=1.83 x 10^-2)</td>
<td>N.E.</td>
</tr>
</tbody>
</table>

N.E., no enrichment of functional pathway among that gene set.

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). 95 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.
FIGURE LEGENDS

Figure 1. Acquired erlotinib resistance in HNC cells is associated with development of an EMT phenotype, altered EGFR pathway signaling, and increased cell migration.

(A) Photomicrograph of HN5 (top) and HN5-ER (bottom) cells at 100x total magnification. (B) Cell titre analysis of erlotinib sensitivity of HN5 and HN5-ER cells. Cells were seeded in 96-well plates, treated with the EGFR inhibitor erlotinib (final concentration 0-100 \( \mu \)M), and the half maximal effective concentration (EC\(_{50}\)) of erlotinib was determined for each cell line 3 d after the addition of erlotinib. Data are normalized to the lowest concentration of erlotinib. (C) Immunoblotting analysis comparing expression and activity of EGFR signaling pathway molecules (EGFR, P-EGFR, Akt, P-Akt, ERK1/2, P-ERK1/2) and expression of EMT markers (E-cadherin, vimentin) between HN5 and HN5-ER cells. \( \beta \)-actin is a loading control. (D) TaqMan miRNA RT-qPCR analysis of miR-200a/b/c expression in HN5 and HN5-ER cells. Data was normalized to U44 snRNA expression and expressed relative to HN5. (E) Real time xCELLigence analysis of migration (represented by cell index) of HN5 (black) and HN5-ER (red) cells. Serum free media (SFM; no migration) controls are included. Error bars represent standard deviations. Data are representative of three independent experiments. *, p<0.05; **, p<0.01, HN5-ER vs HN5.

Figure 2. Increased expression and activity of Axl and decreased miR-34a expression in HN5-ER cells.

(A) Phosphorylated RTK (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 is 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 was normalized to U44 snRNA expression and expressed relative to HN5. (F) Immunoblotting analysis of Axl and E-cadherin expression in HN5-ER cells 3 d after transfection with either synthetic miR-34a, a negative control synthetic miRNA (miR-NC), or mock transfection (vehicle, LF2000). β-actin is a loading control. (G) Cell titre analysis of HN5-ER cells transfected with miR-34a (grey bars) or miR-NC (black bars) (0.5-1.0 nM) or LF2000 (white bars) for 3 d and then treated for a further 3 d with either erlotinib (10 μM) or vehicle (DMSO). Data is expressed relative to LF2000/DMSO-treated HN5-ER cells. Error bars represent standard deviations. Data are representative of three 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.

Figure 3. Axl inhibitor R428 inhibits HN5-ER cell growth and migration and restores erlotinib sensitivity.

(A) Cell titre analysis of sensitivity of HN5 and HN5-ER cells to R428. Cells were seeded in 96-well plates, treated with the Axl inhibitor R428 (final concentration 0-100 μM) for 3 d, and the half maximal effective concentration (EC₅₀) of R428 was determined for each cell line. Data are normalized to the lowest concentration of
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(B) Cell titre analysis of HN5-ER cells measured 3 d after treatment with an ineffective concentration of erlotinib (10 μM) or vehicle (DMSO) and increasing concentrations (0-1.0 μM) of the Axl inhibitor R428. Grey bars indicate the absence of erlotinib, black bars indicate the presence of erlotinib. (C) Immunoblotting analysis of Akt expression and activity of HN5 and HN5-ER cells treated with sub-effective concentrations of erlotinib only, R428 only, and the combination of each drug (as per (B)). β-actin is a loading control. (D) Real time xCELLigence analysis of migration (represented by cell index) of HN5-ER cells that were pre-treated with R428 (black) or vehicle (DMSO; red). Serum free media (SFM; no migration) controls are included for each treatment (vehicle/SFM: blue; R428/SFM: green).

Error bars represent standard deviations. Data are representative of three independent experiments. *, p<0.001, R428/erlotinib vs vehicle/erlotinib.

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 heat map (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. Data were normalized to ALAS1 and GAPDH mRNA expression and expressed relative to HN5. (E) ELISA analysis of IL-6 (left) and IL-8 (right) secretion into cell culture media. Data were expressed relative to HN5. (F) Immunoblotting analysis of STAT3, P-STAT3 and CD44 expression in HN5 and HN5-ER cells. β-actin is included as a loading control. (G) Immunoblotting analysis of P-STAT3, STAT3, P-Akt, and Akt levels in HN5-ER cells treated with
R428 (5 μM for 4 h). β-actin is included as a loading control. (H) Immunoblotting analysis of P-STAT3 and STAT3 levels in HN5-ER cells treated with tocilizumab (1 μM for 3 d) or PBS, or with ruxolitinib (3 μM for 24 h) or DMSO. β-actin is included as a loading control. Error bars represent standard deviations. Data are representative of three independent experiments. *, p<0.0001, HN5-ER vs HN5.

**Figure 5. Increased pro-inflammatory signaling in HNC cells with acquired erlotinib resistance.**

IPA software was used to map and combine differentially expressed genes associated with acquired erlotinib resistance into a custom merged pathway showing components of canonical NF-κB, IL-6, IL-8 and IFN-γ signaling, including Axl, IL-6, IL-8 and COX-2. The density of red shading represents the fold-change upregulation of a given gene upon acquisition of erlotinib resistance.

**Figure 6. A common signature of acquired erlotinib resistance between HNC and NSCLC.**

Venn diagram comparisons are shown between the HN5/HN5-ER microarray study herein, and the NSCLC study of Zhang and coworkers: HCC 827/HCC 827 ER1/HCC 827 ER2 (20). Genes commonly upregulated or downregulated with acquired erlotinib resistance in both HNC (gray) and NSCLC (blue) models are indicated by the intersection of each dataset and are listed in Supplementary Table 4.

**Figure 7. 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 ten-year follow up period after initial surgery. HR, hazard ratio.

Figure 8. Model for miR-34a, Axl, and IL-6 in the regulation of EMT and EGFR inhibitor resistance in HNC.

Acquired resistance of HNC cells to erlotinib is associated with elevated Axl expression and activity, increased synthesis and secretion of IL-6, and reduced levels of miR-34a. Loss of miR-34a permits increased expression of a set of proven or predicted miR-34a target genes that confer an EMT phenotype, including Axl, AXIN2, CA9, CXCL10, FOSL1, FUT8, GAS1, KLF6 and PODXL. Axl and IL-6 activate Akt, NF-κB and STAT3 signaling, which in turn promotes EMT, erlotinib resistance, metastasis and expression of pro-inflammatory cytokines. Finally, increased levels of IL-6 may induce EMT via suppression of miR-200c expression.

Boxes indicate findings in HN5-ER cells presented in this manuscript. Numbers in figure correspond to references for that association.
Figure 1

A) HN5 and HN5-ER cell morphologies under phase contrast microscopy.

B) Graph showing the relative number of viable cells against Log10 erlotinib concentration. HN5 EC50 = 0.1 µM, HN5-ER EC50 > 20 µM.

C) Western blot analysis showing the expression levels of EGFR, P-EGFR, Akt, P-Akt, ERK1/2, P-ERK1/2, E-cadherin, vimentin, and β-actin in HN5 and HN5-ER cells.

D) Graph indicating the relative miR-200 expression (normalized to U44 snRNA) in HN5 and HN5-ER cells. HN5-ER shows significant differences compared to HN5.

E) Graph depicting the cell index over time (h) for HN5, HN5-ER, and HN5-ER (SFM) conditions.
Figure 3

Panel A: Graph showing the relative number of viable cells against Log$_{10}$ R428 [µM].

- HN5 EC$_{50}$ = 1.4 µM
- HN5-ER EC$_{50}$ = 1.3 µM

Panel B: Bar graph illustrating the relative number of viable cells with varying concentrations of R428 and erlotinib.

Panel C: Western blot images comparing HN5-ER with vehicle, erlotinib, R428, and erlotinib + R428 treatments, showing P-Akt, Akt, and β-actin proteins.

Panel D: Graph showing cell index over time (h) for different treatments:
- HN5-ER / vehicle
- HN5-ER / R428
- HN5-ER / vehicle / SFM
- HN5-ER / vehicle / SFM

Legend:
- Red line: HN5-ER / vehicle
- Black line: HN5-ER / R428
- Blue line: HN5-ER / vehicle / SFM
- Green line: HN5-ER / vehicle / SFM
Figure 4
Genes upregulated with acquired erlotinib resistance

HNC NSCLC

152 95 1591

Genes downregulated with acquired erlotinib resistance

HNC NSCLC

551 75 1083
Figure 7

Follow up (months)

Percent survival (%)

HR = 1

HR = 1.66, p = 0.007
AXIN2, CA9, CXCL10, FOSL1, FUT8, GAS1, KLF6, PODXL

miR-34a

 ↓

 Axl

 ↑

 IL-6

 ↓

 IL-6R/JAK

 ↑

 miR-200c

 |  
\( (30) \) |

 |  
\( (74) \) |

 |  
\( (73) \) |

 |  
\( (25) \) |

 |  
\( (63-70) \) |

 |  
\( (9) \) |

 |  
\( (36-75) \) |

 |  
\( (14) \) |

 P-Akt

 ↑

 P-STAT3

 EMT

 metastasis

 EGFR inhibitor resistance

Figure 8