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AXL regulates neuregulin1 expression leading to cetuximab resistance in head and neck cancer

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Abstract

Background: The receptor tyrosine kinase (RTK) epidermal growth factor receptor (EGFR) is overexpressed and an important therapeutic target in Head and Neck cancer (HNC). Cetuximab is currently the only EGFR-targeting agent approved by the FDA for treatment of HNC; however, intrinsic and acquired resistance to cetuximab is a major problem in the clinic. Our lab previously reported that AXL leads to cetuximab resistance via activation of HER3. In this study, we investigate the connection between AXL, HER3, and neuregulin1 (NRG1) gene expression with a focus on understanding how their interdependent signaling promotes resistance to cetuximab in HNC.

Methods: Plasmid or siRNA transfections and cell-based assays were conducted to test cetuximab sensitivity. Quantitative PCR and immunoblot analysis were used to analyze gene and protein expression levels. Seven HNC patient-derived xenografts (PDXs) were evaluated for protein expression levels.

Results: We found that HER3 expression was necessary but not sufficient for cetuximab resistance without AXL expression. Our results demonstrated that addition of the HER3 ligand NRG1 to cetuximab-sensitive HNC cells leads to cetuximab resistance. Further, AXL-overexpressing cells regulate NRG1 at the level of transcription, thereby promoting cetuximab resistance. Immunoblot analysis revealed that NRG1 expression was relatively high in cetuximab-resistant HNC PDXs compared to cetuximab-sensitive HNC PDXs. Finally, genetic inhibition of NRG1 resensitized AXL-overexpressing cells to cetuximab.

Conclusions: The results of this study indicate that AXL may signal through HER3 via NRG1 to promote cetuximab resistance and that targeting of NRG1 could have significant clinical implications for HNC therapeutic approaches.

Keywords: AXL, neuregulin1, Head and neck cancer, Cetuximab, Resistance, HER3

Background

Head and neck squamous cell carcinomas (HNSCC) develop from the mucosal lining of the aerodigestive tract. It is estimated that in 2020 there will be over 53,000 new cases of HNSCC in the United States and 11,000 deaths from this disease [1, 2]. HNSCC is a complex heterogeneous disease that arises from various sites including the oral cavity, tongue, pharynx, larynx, and salivary glands. Over 90% of tumors that originate in the oropharyngolaryngeal axis are squamous cell carcinomas. The 5-year relative survival rate for oral cavity and pharynx during 2009–2015 was 65% in the US [2]. The therapy regimen used to treat this cancer typically involves surgery, radiotherapy, chemotherapy, targeted therapy, and/or immunotherapy [3].

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Common systemic agents that are utilized for treatment of HNC patients are monotherapy or combination regimens containing platinums, taxanes, immune checkpoint inhibitors, and cetuximab [4, 5]. Cetuximab is a chimeric monoclonal antibody targeting the epidermal growth factor receptor (EGFR) that was approved by the FDA for treatment of HNC in 2006. EGFR is a member of the HER family of receptor tyrosine kinases (RTKs), and aberrant expression of EGFR has been strongly noted in the etiology of HNSCC [3, 6]. Cetuximab is the only drug that exhibits efficacy in both locally advanced and recurrent/metastatic HNC. It functions by binding the extracellular domain of EGFR with high affinity, blocking ligand binding and stimulating receptor internalization. As an IgG1 class antibody, it also stimulates antibody dependent cell cytotoxicity [7]. Targeting of EGFR with cetuximab has improved overall survival of HNSCC patients when added to radiotherapy or chemotherapy regimens [8, 9]. Despite the clinical benefit of cetuximab treatment, resistance to cetuximab can develop in patients [10]. Thus, investigation of resistant mechanisms can provide new insights to improving treatment strategies in HNSCC.

The RTK AXL is a member of the TAM family of receptors (Tyro, AXL, MER) and has been implicated in the development and progression of many malignancies [11–14]. These studies indicate a role for AXL in cancer cell proliferation, migration, angiogenesis, and metastasis [14–16]. AXL mRNA expression has been correlated with poor disease outcome in HNSCC, indicating a putative role for AXL in the development and/or progression of this disease [17]. Moreover, AXL protein expression levels increased during HNSCC tumor progression [18]. Our laboratory previously reported that AXL expression was significantly associated with higher pathologic grade, distant metastases, and shorter relapse-free survival in HNSCC patients. Many studies have found that AXL can also mediate resistance to anti-EGFR inhibitors which further unveils a role for AXL in therapeutic resistance [11, 13, 19–24].

The RTK HER3 has been shown to be upregulated in many cancer types including HNSCC and has been correlated with invasion and metastasis [25–27]. It has also been reported that membranous HER3 expression is significantly associated with worse overall survival in HNSCC [28, 29]. Our laboratory reported that acquired resistance to cetuximab is accompanied by EGFR-dependent activation of HER3 in non-small cell lung cancer (NSCLC) and HNSCC [19, 30, 31]. We also revealed that genetic silencing of HER3 was able to resensitize cetuximab-resistant clones to cetuximab therapy, suggesting a role for HER3 in driving resistance [32]. This role is also established in a study that demonstrates inhibition of HER3 combined with cetuximab has strong anti-tumor activity in cetuximab-resistant HNSCC patient-derived xenografts [33]. All of these studies underscore the important role of HER3 in therapeutic resistance [26, 34]. Collectively, AXL or HER3 can play important roles in mediating resistance to cetuximab and that targeting of AXL or HER3 can resensitize to cetuximab treatment [31, 35].

Based on our previous studies of AXL and HER3 in the context of cetuximab resistance, we investigated the connection between these two receptors with a focus on understanding their interdependent signaling to promote resistance to cetuximab. A model of AXL overexpression revealed that AXL leads to cetuximab resistance via activation of HER3, but we found that genetic overexpression of HER3 is insufficient for cetuximab resistance. However, exogenous expression of the HER3 ligand neuregulin1 (NRG1) does lead to cetuximab resistance. Further experimentation revealed that AXL can regulate NRG1, at the level of mRNA, to promote cetuximab resistance. Other cetuximab-resistant models have relatively high AXL and NRG1 expression, and targeting of NRG1 in these models is sufficient to overcome cetuximab resistance. Collectively, this data indicates that AXL can signal through HER3 via NRG1 to promote cetuximab resistance and that targeting of NRG1 could have significant clinical implications for HNSCC therapeutic approaches.

Materials and methods

Reagents

Cetuximab (IMC-C225, Erbitux) was purchased from the University of Wisconsin Pharmacy. Gas6 and NRG1 were obtained from R&D systems (Minneapolis, MN). DMSO (MilliporeSigma, St. Louis, MO) was used as the vehicle control in vitro. Human IgG (MilliporeSigma, St. Louis, MO) was the control for cetuximab.

Cell lines and HNSCC PDXs

UMSCC1, UMSCC6, and HNSCC PDXs were obtained from SPORE resources. The HN30 and PCI37A cell lines were a gift from Dr. Ravi Salgia, City of Hope, Duarte, CA and Dr. Jennifer Grandis, UCSF, CA, respectively. All cell lines were cultured in DMEM with 4.5 g/dL glucose, 10% FBS, penicillin (100 units/mL), and streptomycin (100 mg/mL). Cell line identity was confirmed using short tandem repeat analysis and publicly available databases by the TRIP lab at the University of Wisconsin-Madison. STR reference of PCI37A was not available in public, however the genomic integrity remains similar for more than 2 years. Mycoplasma testing was completed through the WiCell Core Service at the University of Wisconsin-Madison. Detailed information about cell

lines and PDXs is listed in Supplemental Materials and Methods.

Plasmids and transfection

Plasmids were prepared and selected as previously described [36]. Transfection was performed using Lipofectamine3000 and Opti-MEM (Life Technology, Carlsbad, CA) according to the manufacturer's instructions [31, 36]. Blasticidin (3ug/mL) was used as the selective antibiotic when maintaining cells.

siRNA transfection

Non-targeting control pool siRNA (Cat#D-001810), SMARTpool siRNA targeting HER3 (Cat#L-003127), AXL (Cat#L-003104) and NRG1 (Cat#L-004608) were purchased from Dharmacon, Inc (Lafayette, CO) and utilized for transfection with Lipofectamine RNAiMAX (Life Technologies) [31]. Successful knockdown was confirmed by immunoblot.

Cell-based assays

Cell Counting Kit-8 (CCK8, Dojindo Molecular Technologies, MD) and crystal violet assays were performed to determine cell proliferation or cell viability as described previously [37]. For the CCK8 assay, equal number of cells were plated on 96-well plates. Following treatment, CCK8 analysis was performed according to the manufacturer's instructions. For the crystal violet assays, equal number of cells were seeded in 6-well plates. Following treatment, the cells were stained with 0.5% crystal violet. Plates were air dried, and dye was eluted with 0.1 M sodium citrate (pH 4.2) and ethanol (1:1). Elution was transferred to 96-well plates, and the absorbance was read at 540 nm to determine cell proliferation. For studies using combination treatment of siRNA and other agents, cells were first transfected with siRNA. Twenty-four hours later, cells were treated with the other agents for an additional 72 h. All treatments were performed in duplicate or triplicate.

Immunoblot analysis

Whole-cell protein was obtained from cells using RIPA buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% Tween-20, 10% glycerol, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM Na3VO4, 1 mM PMSF, 1 mM beta-glycerophosphate (BGP), and 10 μg/ml leupeptin and aprotinin). Immunoblot analysis was performed as previously described [36, 37]. Antibodies were used according to the manufacturer's instructions: AXL (Cell Signaling Technologies, MA (CST) #8661), pAXL/pMerTK/pTyro3 (CST #44463), HER3 (CST #12708), pHER3 Y1197 (CST #4561), AKT (CST #2920), pAKT (CST #4060), NRG1 (CST #2573), GAPDH (CST #2118), α-tubulin (MilliporeSigma #CP06).

RNA isolation, cDNA synthesis and qPCR

RNA isolation, cDNA synthesis and qPCR were performed as previously described [31]. Briefly, total RNA was isolated from the cells using RNeasy kit (Qiagen). The RNA was then quantified by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA was reverse transcribed to cDNA using qScript™ cDNA SuperMix (Quantabio, Beverly, MA). Quantitative real time qPCR was carried out using the Bio-Rad CFX96 real-time PCR system (Bio-Rad, Hercules, CA). Eukaryotic 18S rRNA (4,333,760, Life Technologies) and human ACTB (4,333,762, Life Technologies) were used as the normalization controls. The levels of gene expression were analyzed using the $\Delta\Delta C_t$ method.

Statistical analysis

Statistical analyses were performed using Prism (GraphPad Software, Inc.). Differences between multiple groups were evaluated using a repeated measures ANOVA with a Bonferroni post-hoc test [38]. Differences were considered significant when $P < 0.05$.

Results

AXL leads to cetuximab resistance and increased HER3 activity

Previously, we reported that many cetuximab-resistant HNSCC cell lines exhibited increased expression and activation of AXL relative to cetuximab-sensitive cell lines. In addition, we showed that HNSCC cell lines that expressed AXL were dependent on this receptor for proliferation by using small interfering RNA (siRNA) analysis [35, 37]. Since siAXL impaired the proliferation of cetuximab-resistant HNSCC cells, we first sought to identify if ligand-induced activation of AXL may mediate cetuximab resistance. We stimulated cetuximab-sensitive HN30 cells with Gas6, an AXL ligand, to determine if this would lead to increased resistance to cetuximab. Stimulation of AXL by Gas6 in HN30 cells did result in resistance to cetuximab treatment (Fig. 1A). Immunoblot analysis indicated that HN30 cells stimulated with Gas6 had robust expression and phosphorylation of AXL and HER3 receptors, even in the presence of cetuximab. AKT was also highly phosphorylated by Gas6 stimulation. This result suggests that activation of AXL and HER3 by Gas6 can stimulate the cell proliferation and survival pathway, thereby escaping the inhibitory effects of cetuximab.

To further evaluate whether overexpression of AXL increased HER3 phosphorylation in HNSCC cells, we overexpressed AXL in the cetuximab-sensitive cell lines HN30 (HN30-AXL) and PCI37A (PCI37A-AXL)

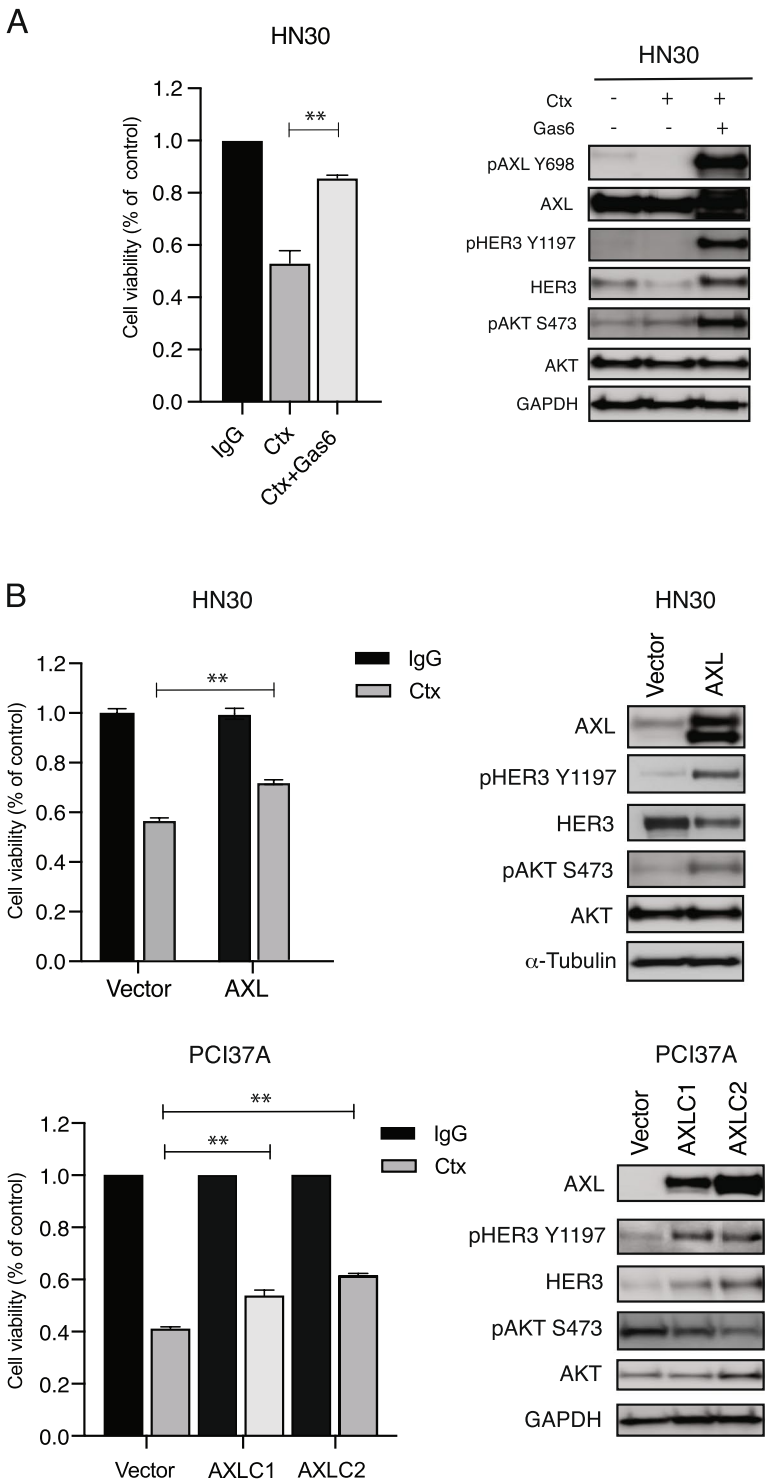

Fig. 1 AXL leads to cetuximab resistance and increased HER3 activity. A: HN30 cells were treated with 100 nM of IgG, 100 nM of cetuximab (Ctx), or combination of Ctx and Gas6 (200 ng/uL) for 72 h, and cell viability was determined by crystal violet assay. Mean values, SEs, and statistical analyses are representative of two independent experiments. $N = 3$, $^{**}P < 0.01$. Whole cell lysates were harvested and fractionated via SDS-PAGE, followed by immunoblotting for the indicated proteins. GAPDH was used as a loading control. B: Cell viability in AXL overexpressed cells was measured via crystal violet assay after 72 h of treatment with cetuximab. Mean values, SEs, and statistical analyses are representative of three independent experiments. $N = 3$, $^{**}P < 0.01$. Whole cell lysates were harvested and fractionated via SDS-PAGE, followed by immunoblotting for the indicated proteins. α-Tubulin and GAPDH were used as loading controls

via stable transfection. Endogenous levels of AXL and HER3 in HN30 and PCI37A cell lines were evaluated via immunoblot (Supplemental Fig. 1). Proliferation assays demonstrated that AXL overexpressing cell lines were significantly resistant to increasing doses of cetuximab compared to vector cells (Fig. 1B). Immunoblot analysis confirmed that total AXL expression was increased in the cells, and it also indicated that phosphorylation of HER3 was increased in HN30-AXL and PCI37A-AXL cells. Collectively, these results suggest that overexpression of AXL leads to cetuximab resistance and increased HER3 activity.

HER3 is necessary for AXL to mediate cetuximab resistance

To determine if HER3 is important in cetuximab-resistant cells whose resistance is caused by AXL overexpression, cell proliferation assays were performed using cetuximab treatment and siRNAs targeting HER3 (Fig. 2A). Cell proliferation of HN30-AXL, PCI37A-AXLC1, and PCI37A-AXLC2 cells was significantly inhibited when treated with the combination of siHER3 and cetuximab compared to either treatment alone. Immunoblot analysis showed that the combination of siHER3 and cetuximab treatment decreased phosphorylation of AKT.

Because siHER3 inhibited the proliferation of HN30-AXL and PCI37A-AXL cells, we next investigated if targeting AXL in cell lines that are *intrinsically* resistant to cetuximab (UMSCC6, UMSCC1) would enhance cetuximab sensitivity and decrease phosphorylation of HER3. Cell proliferation analysis was performed after treatment of UMSCC6 and UMSCC1 HNSCC cells with sinontarget (siNT), cetuximab, siAXL, or the combination of siAXL and cetuximab. The results of these experiments demonstrated that the combination of siAXL and cetuximab had a significant anti-proliferative effect on these cells compared to either treatment alone (Fig. 2B). Immunoblot analysis indicated that phosphorylation of HER3 was inhibited by the combination treatment. These results indicate that HER3 signaling collaborates with AXL to regulate cellular proliferation and response

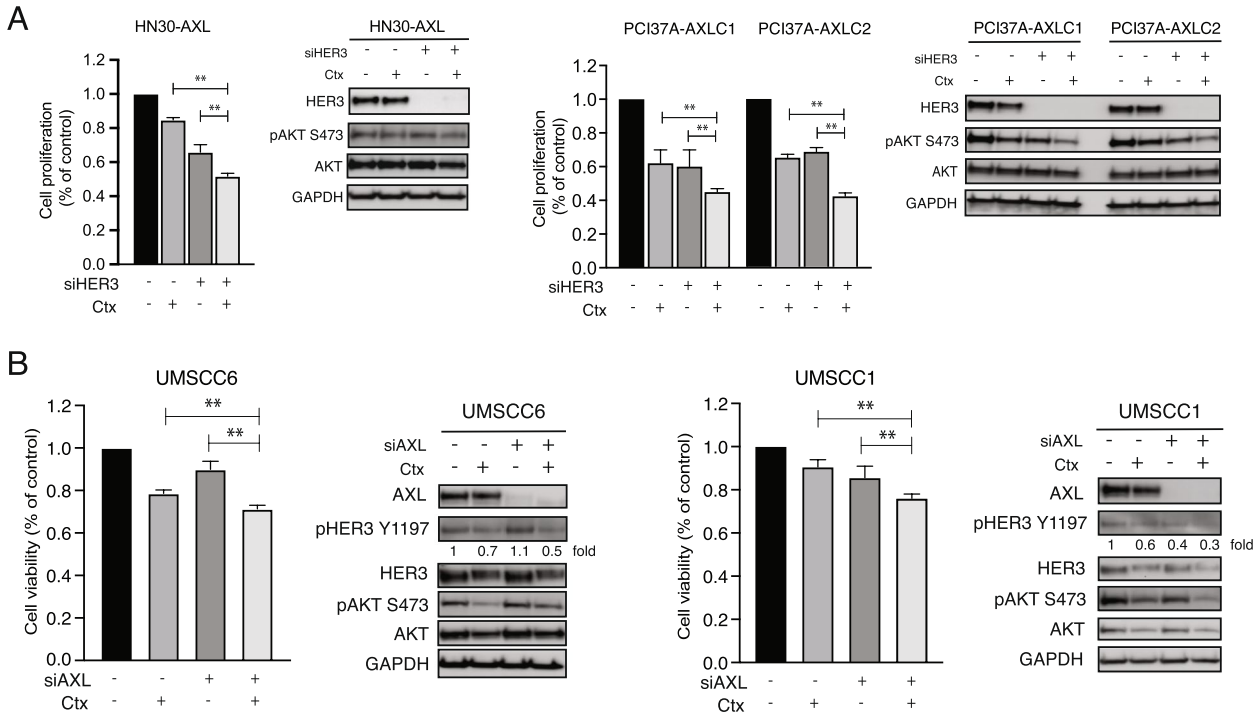

Fig. 2 HER3 is necessary for AXL to mediate cetuximab resistance. **A** HN30-AXL and PCI37A-AXL cells were plated and treated with 30 nM of HER3 siRNA (siHER3) or 30 nM non-target siRNA (siNT). The next day, cells were treated with 100 nM of IgG or 100 nM cetuximab (Ctx) for 72 h. Cell proliferation was measured after drug treatment by CCK8 assay. Mean values, SEs, and statistical analyses are representative of three independent experiments. $N = 5–10$, $^{**}P < 0.01$. Whole cell lysates were collected 24 h after treatment, fractionated by SDS-PAGE and immunoblotted for the indicated proteins. GAPDH was used as a loading control. **B** UMSCC1 and UMSCC6 HNSCC cells were plated and treated with 30 nM of siAXL or 30 nM siNT. The next day, cells were treated with 100 nM of IgG or 100 nM Ctx for 72 h. Cell viability was measured after drug treatment by crystal violet assay. Mean values, SEs, and statistical analyses are representative of three independent experiments. $N = 3$, $^{**}P < 0.01$. Whole cell lysates were collected at 24 h after treatment, fractionated by SDS-PAGE, and immunoblotted for the indicated proteins. GAPDH was used as a loading control

to cetuximab. To further investigate whether the expression of HER3 alone is sufficient to mediate resistance to cetuximab, two HNSCC cell lines that are sensitive to cetuximab and express little or no endogenous HER3 were manipulated to overexpress HER3 and treated with cetuximab for cell proliferation analysis (Fig. 3). Results of this experiment indicated that both HN30 and PCI37A cells remained sensitive to cetuximab treatment even with overexpression of HER3. AXL phosphorylation levels were not increased in HN30-HER3 and PCI37A-HER3 cells. Collectively, these results demonstrate that HER3 overexpression without AXL activation is insufficient for cetuximab resistance.

Exogenous expression of NRG1 leads to cetuximab resistance

We have previously reported that ligand-mediated activation of HER family receptors, especially HER3, could mediate resistance to cetuximab [31, 39]. In this study, however, HER3 expression was necessary but not sufficient for cetuximab resistance without AXL expression. Therefore, we hypothesized that another pathway downstream of AXL must influence expression of HER3 and the HER3 ligand, NRG1. To test this hypothesis, we first stimulated the HN30 and PCI37A cells with NRG1 to assess if this would lead to increased resistance to cetuximab. Addition of NRG1 to HN30 or PCI37A cells did result in resistance to cetuximab (Fig. 4A). Immunoblot analysis indicated that phosphorylation levels of HER3 and AKT were increased in both cell lines after NRG1 stimulation and not inhibited by cetuximab in the presence of NRG1. This result suggested that presence of NRG1 is sufficient to stimulate HER3, leading to regulation of cell proliferation and survival pathways, thus bypassing the inhibitory effects of cetuximab.

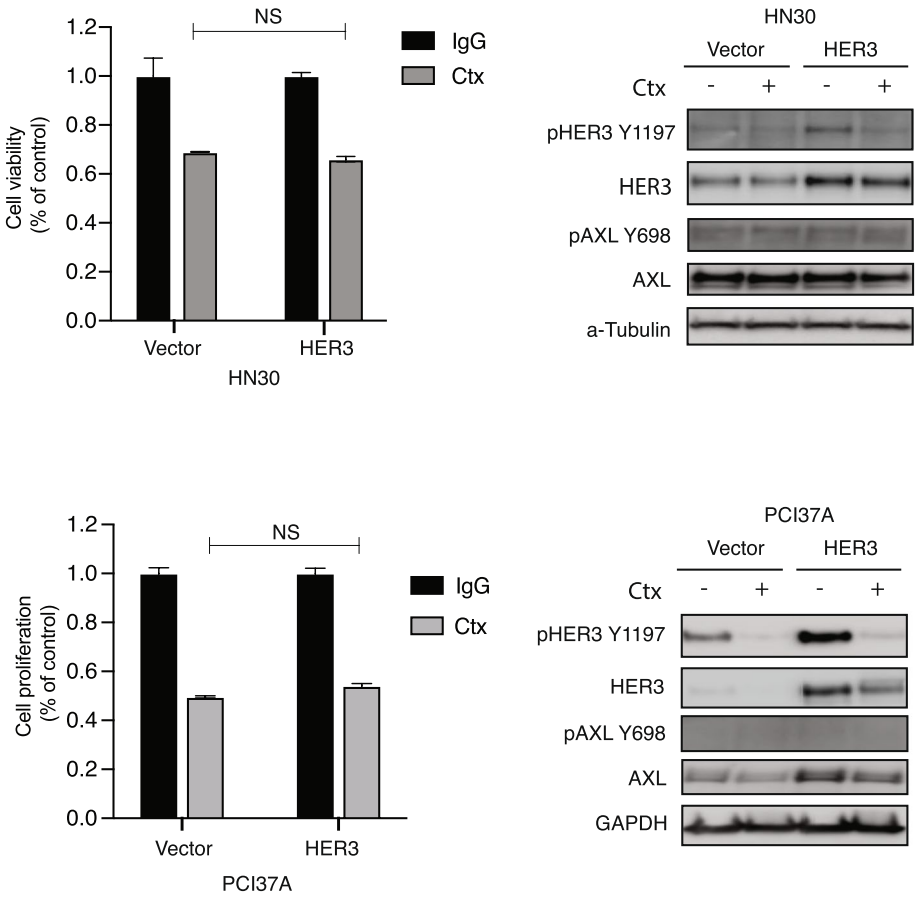

Fig. 3 HER3 overexpression alone is insufficient for cetuximab resistance. A: HN30 and PCI37A cells stably overexpressing HER3 or the pcDNA6.0 vector were treated with 100 nM of cetuximab (Ctx) for 72 h before performing crystal violet assay for HN30 cells and CCK8 assays for PCI37A cells. Whole cell lysate was harvested at 24 h after treatment and subjected to immunoblot analysis following fractionation via SDS-PAGE. GAPDH or α-Tubulin was used as a loading control. Mean values, SEs, and statistical analyses are representative of two or three independent experiments. $N = 3–6$. NS: not significant

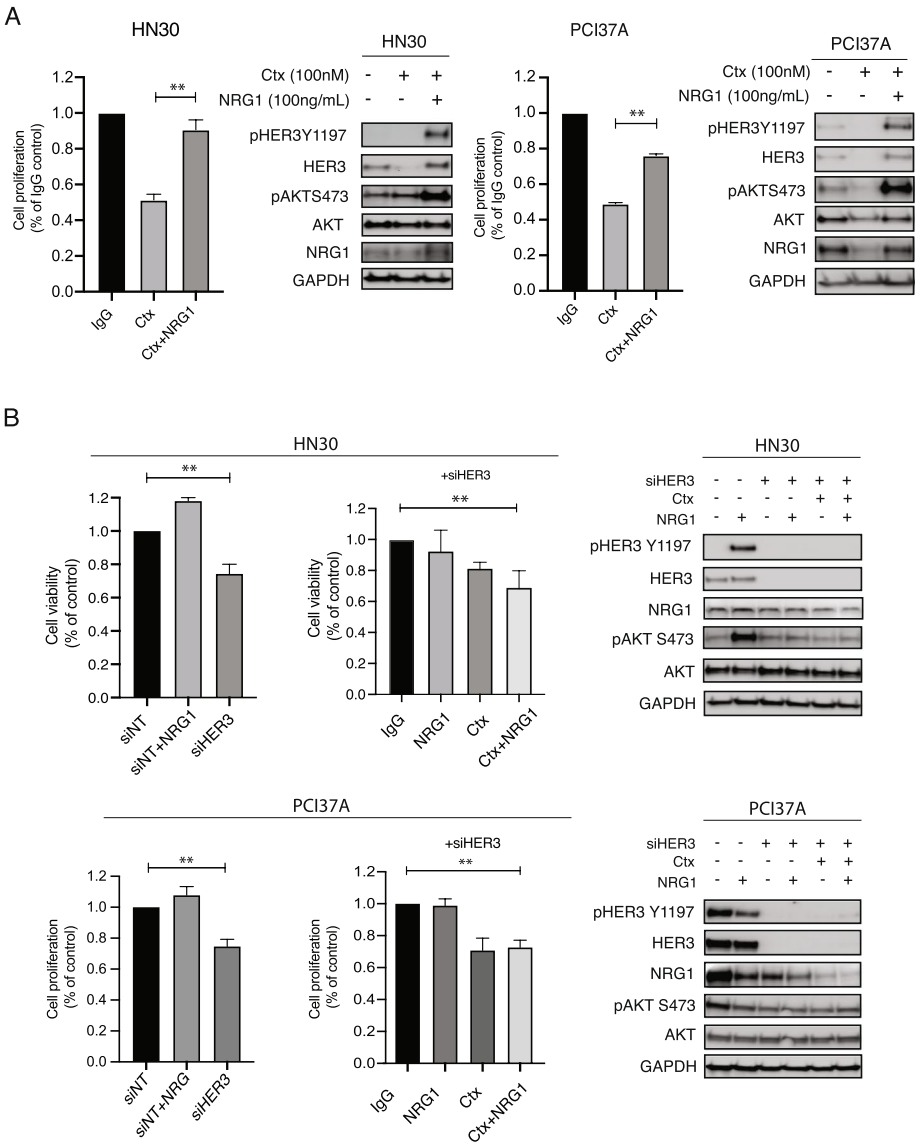

Fig. 4 Exogenous expression of NRG1 leads to cetuximab resistance. **A** HN30 and PCI37A cells were plated and treated with 100 nM of cetuximab (Ctx), 100 ng/mL of NRG1, or the combination of Ctx and NRG1 for 72 h. Cell proliferation was determined by CCK8 assay. Mean values, SEs, and statistical analyses are representative of two independent experiments. $N=6$, $^{**}P<0.01$. Whole cell lysates were harvested at 24 h after treatment and fractionated via SDS-PAGE, followed by immunoblotting for the indicated proteins. GAPDH was used as a loading control. **B** HN30 and PCI37A cells were transfected with 30 nM siHER3 or 30 nM siNT for 24 h before treatment with Ctx (100 nM) or NRG1 (100 ng/ml) for an additional 72 h. Cell viability or cell proliferation was determined by crystal violet assay for HN30 cells and CCK8 assay for PCI37A cells. Mean values, SEs, and statistical analyses are representative of two or three independent experiments. $N=3$–10, $^{**}P<0.01$. Whole cell lysate was harvested at 24 h after treatment and subjected to immunoblot analysis following fractionation via SDS-PAGE. GAPDH was used as a loading control

To determine the importance of HER3 activation in cetuximab resistance, we treated the cetuximab-sensitive cell lines HN30 and PCI37A with siHER3 and cetuximab for 72 h and subsequently stimulated them with exogenous NRG1. Analysis of cell proliferation indicated that both HN30 and PCI37A cells were sensitive to cetuximab after HER3 knockdown and NRG1 stimulation (Fig. 4B). This result further confirms that HER3 activation is necessary for cetuximab resistance.

On the basis of these results, we next evaluated whether AXL stimulates NRG1 to regulate cell proliferation in cetuximab-resistant cell lines. Quantitative PCR (qPCR) and immunoblot analysis were used to analyze NRG1 expression levels in HN30-AXL or PCI37A-AXL

cells compared to the HN30-vector or PCI37A-vector control, respectively (Fig. 5A, Supplemental Fig. 2). The abundance of NRG1 was increased at both the mRNA and protein levels in HN30-AXL and PCI37A-AXL cells.

To expand these findings, HNSCC patient-derived xenografts (PDXs) were evaluated for NRG1 expression levels (Fig. 5B). Seven HNSCC PDXs were previously characterized and evaluated for cetuximab response [40]. PDX samples were harvested from early-passage tumors and evaluated for NRG1 expression by immunoblot analysis. There were three cetuximab-sensitive PDXs (UWSCC-22, UWSCC-34 and UWSCC-36) and four cetuximab-resistant PDXs (UWSCC-1, UWSCC-17, UWSCC-25 and UWSCC-64). In this small cohort, immunoblot analysis showed that the cetuximab-resistant PDXs on average expressed approximately eight-fold more NRG1 than cetuximab-sensitive PDXs (Fig. 5B, $^{*}p < 0.05$). Collectively, these data demonstrate that NRG1 is overexpressed in cetuximab-resistant HNSCC.

AXL regulates NRG1 to lead to cetuximab resistance

Our data indicated that AXL increased HER3 activation, leading to cetuximab resistance. Furthermore, overexpression of AXL increased NRG1 expression levels in cetuximab-resistant cells, and cetuximab-resistant PDXs have more NRG1 expression than cetuximab-sensitive PDXs (Fig. 5). Thus, we hypothesized that NRG1 may be critical for AXL to mediate cetuximab resistance. To test

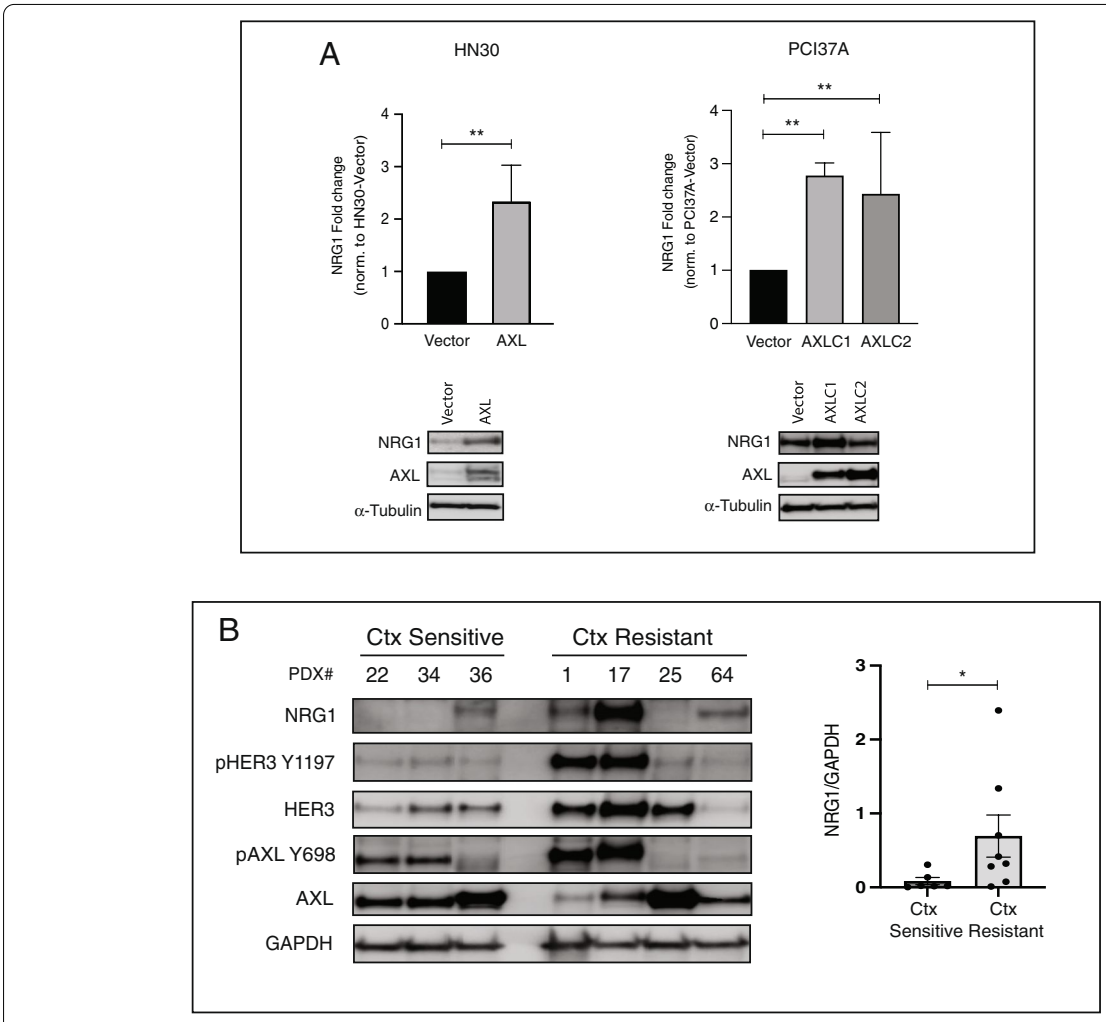

Fig. 5 AXL regulates NRG1. **A** The expression levels of NRG1 in HN30-Vector, HN30-AXL PCI37A-Vector and PCI37A-AXL cells were determined by qPCR and immunoblot analysis. α-Tubulin was used as a loading control. Mean values, SEs, and statistical analyses are representative of three independent experiments. $N = 2$–4. **B** Whole cell lysates were harvested from HNSCC PDX tumors and fractionated via SDS-PAGE, followed by an immunoblot for indicated proteins. GAPDH was used as a loading control. The densitometry values represent the mean ratios of NRG1/GAPDH from two independent experiments. Error bars: SEM ($^{*}P < 0.05$, Mann–Whitney test)

this, we targeted NRG1 by siRNA in PCI37A-AXL cells and treated with cetuximab for 72 h. Cell proliferation assays indicated that loss of NRG1 expression resensitized cells to cetuximab (Fig. 6A). In PCI37A-AXL cells treated with siNRG1 and cetuximab, there was a substantial decrease in phosphorylation of HER3 and AKT. Collectively, these results demonstrated that AXL is signaling through NRG1 to promote cetuximab resistance.

Discussion

The current report presents data suggesting that AXL can signal through HER3 via NRG1 to promote cetuximab resistance in HNC. Notably, our models demonstrated that NRG1 is necessary and sufficient for cetuximab resistance. Further investigation revealed that AXL can regulate the expression of NRG1, thereby promoting resistance to cetuximab. Together these findings suggest that AXL and NRG1 expression could predict patient responses to cetuximab therapy and strengthen the rationale for the use of AXL or NRG1 targeted therapies in HNC treatment strategies.

Previous research by our laboratory and other investigators has demonstrated an increase in expression levels of several receptor tyrosine kinases, including AXL and HER3, after development of resistance to EGFR-targeting [32, 41, 42]. Altered expression of AXL in cancer has been

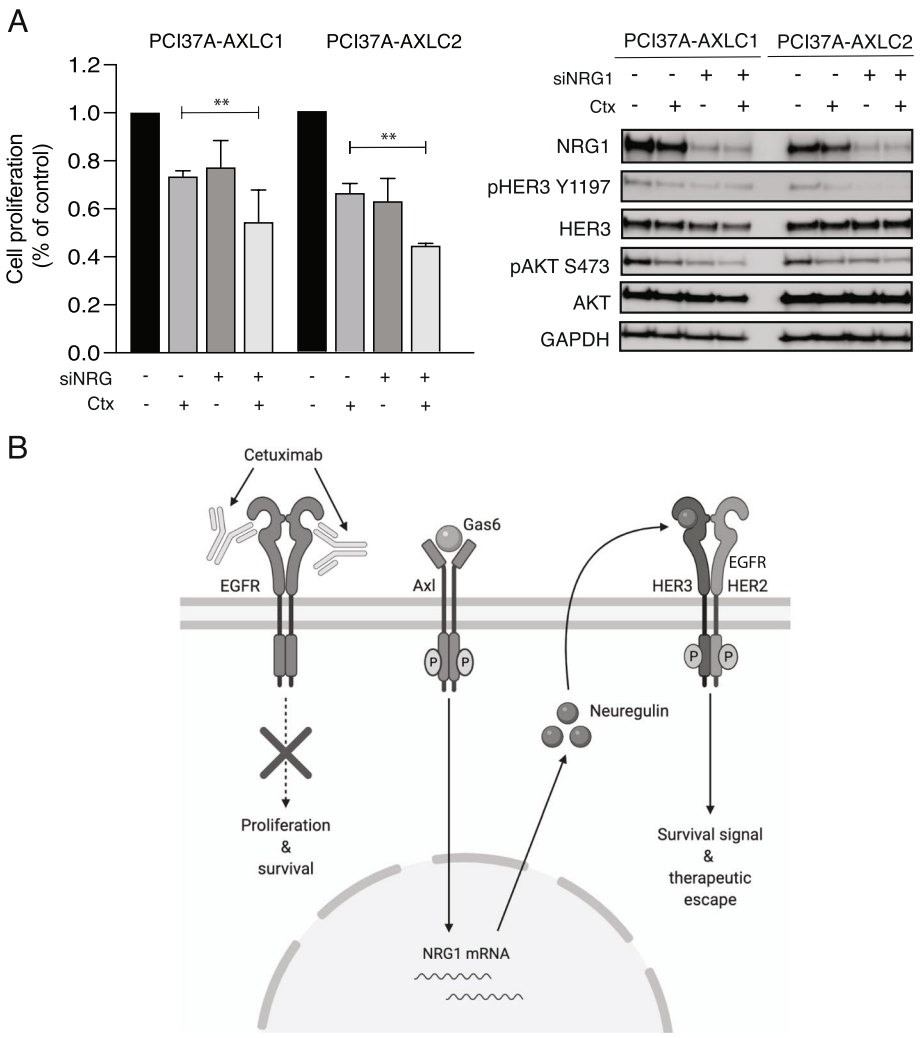

Fig. 6 AXL regulates NRG1 to lead to cetuximab resistance. **A** PCI37A-AXL cells were plated and treated with 30 nM of NRG1 siRNA or 30 nM siNT. The next day, cells were treated with 100 nM of 100 nM of IgG or 100 nM cetuximab (Ctx) for 72 h. Cell proliferation was measured after drug treatment using the CCK8 assay. Mean values, SEs, and statistical analyses are representative of seven independent experiments. $N = 6–10$, $^{**}P < 0.01$. Whole cell lysates were collected at 24 h after treatment, fractioned by SDS-PAGE, and immunoblotted for the indicated proteins. GAPDH was used as a loading control. **B** Proposed model for AXL regulation of NRG1 leading to Ctx resistance

studied as a mechanism of acquired resistance to cetuximab [19, 35] and targeting of AXL has been shown to resensitize cells and tumors to EGFR-targeted therapy [22, 36]. Increased expression of EGFR family member HER3 has also been observed in cetuximab-resistant models leading to development of several EGFR-HER3 co-targeting therapeutic strategies [30, 31, 43–45]. In this study, we found that overexpression of AXL leads to cetuximab resistance and increased HER3 activity (Fig. 1), and that HER3 was necessary for AXL to mediate cetuximab resistance (Fig. 2). We also found that HER3 overexpression alone was insufficient for cetuximab resistance in HNSCC cells (Fig. 3). Immunoblot analysis showed that overexpression of HER3 did not increase AXL activity (Fig. 3). These results suggested that overexpression of both AXL and HER3 may be necessary for cetuximab resistance. Despite wide preclinical success of HER3 targeting to overcome cetuximab resistance [33, 46, 47], the combination of a HER3 inhibitor with cetuximab did not demonstrate clinical success over cetuximab therapy alone [48–50]. Thus, our lab has continued to investigate the signaling role of HER3 discovering that perhaps AXL and NRG1 are of more importance in the cetuximab resistance pathway.

In the current study, we found that the addition of NRG1 to the cetuximab-sensitive HN30 and PCI37A cell lines rendered these cells resistant to cetuximab (Fig. 4A). In line with this data, we previously reported that NRG1 autocrine signaling is a major driver of acquired resistance to cetuximab [31]. We also found that cetuximab-resistant HN30-AXL and PCI37A-AXLC1 cells relatively expressed more NRG1 than the vector control (Fig. 5A) and that cetuximab-resistant PDX tumors have more NRG1 than cetuximab-sensitive PDXs (Fig. 5B, $^{*}p < 0.05$). These findings suggested that AXL could regulate NRG1 in cetuximab-resistant HNSCC cells. In addition, analysis of 89 HPV(+) and 409 HPV(-) primary HNC tumor samples in the TCGA as well as 33 HNC cell lines in Cancer Cell Line Encyclopedia (CCLE) showed a positive correlation between AXL and NRG1 mRNA levels (Supplemental Figure S3). Interestingly, PCI37A-AXLC2 cells did not express more NRG1 compared to vector control despite AXL being overexpressed (Fig. 5A), and cetuximab-resistant PDX tumor (UW-SCC25) did not express NRG1 even though they had AXL expression (Fig. 5B). Many groups have begun investigating NRG1 expression in cancer and how this correlates with therapeutic response with varied results. Meetze et al. found a significant correlation between NRG1 expression and tumor growth inhibition by the HER3 inhibitory antibody AV-203 [51], and another study demonstrated that HER3 inhibition could be quite effective in NRG1-rearranged cancers [52]. Baro et al. also identified upregulation of autocrine NRG1 signaling as a mechanism of cetuximab resistance in HNSCC tumors. Using the HER3 antibody CDX-3379, they were able to overcome cetuximab resistance and enhance tumor growth delay and radiosensitivity [53]. In contrast, one study found that expression of HER3 did not indicate sensitivity to a HER3 antibody or cetuximab. They demonstrated that NRG1 expression along with other EGFR-activation biomarkers correlated with better anti-HER3 response [54]. In this study, knockdown of NRG1 expression by siRNA combined with cetuximab treatment led to diminished cell proliferation by impairing AKT survival signaling in cetuximab-resistant PCI37A-AXL cells (Fig. 6A). Because of the varying results in different cancer models, more investigation of NRG1 expression and cetuximab response must be completed. Additional testing of NRG1 inhibition and resensitization to cetuximab could also be investigated using two high-affinity monoclonal antibodies to NRG1 [55] or an anti-NRG1 antibody that has shown inhibition of tumor growth in preclinical models of pancreatic cancer [56]. Collectively, the data presented within explores a signaling connection between AXL, NRG1, and HER3 in the context of cetuximab resistance in HNC (Fig. 6B).

Conclusions

We have shown that NRG1 expression, more than HER3 expression, is necessary and sufficient for resistance to cetuximab in our models and that the RTK AXL can regulate expression of NRG1. This data corroborates findings that a combination of HER3 and AXL therapy might be more effective than targeting either alone [57]. Further exploration must be done to identify if the co-expression of AXL and NRG1 could be as used as biomarkers to predict resistance to cetuximab or if a targeting strategy for AXL or NRG1 would provide a therapeutic advantage in the setting of resistance.

Abbreviations

CCK8: Cell Counting Kit-8; Ctx: Cetuximab; EGFR: Epidermal Growth Factor Receptor; HNC: Head and Neck cancer; HNSCC: Head and Neck Squamous Cell Carcinomas; NRG1: Neuregulin1; PDXs: Patient-Derived Xenografts; qPCR: Quantitative PCR; RTK: Receptor Tyrosine Kinase; siRNA: Small interfering RNA; siNT: SiNontarget.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-022-09511-6.

Additional file 1. Supplemental materials and methods.

Additional file 2: Figure 1. AXL leads to cetuximab resistance and increased HER3 activity. **Figure 2.** HER3 is necessary for AXL to mediate cetuximab resistance. **Figure 3.** HER3 overexpression alone is insufficient for cetuximab resistance. **Figure 4.** Exogenous expression of NRG1 leads

to cetuximab resistance. **Figure 5.** AXL regulates NRG1. **Figure 6.** AXL regulates NRG1 to lead to cetuximab resistance.

Additional file 3: Supplemental Figure S1. Endogenous protein expression levels of HN30 and PCI37A cells. **Supplemental Figure S2.** AXL mRNA expression in HN30-AXL and PCI37A-AXLC1 and -AXLC2 cells. **Suppemental Figure S3.** Correlation between AXL and NRG1 mRNA expression levels in TCGA HNC primary tumor samples (A) and CCLE HNC cell lines (B).

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Authors' contributions
M.I., N.M., and D.W. designed the study. M.I., N.M., N.W., C.K. and P.L. carried out data acquisition and analysis. M.I., N.M., K.K. and D.W. wrote the manuscript. M.I., N.M., K.K., N.W., C.K., P.L. and C.L. contributed to preparing and making figures. K.K., P.L., C.L., J.B., S.H., R.S., and D.W. contributed to reviewing and editing the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The generation of patient derived xenografts from deidentified tissues has been deemed to be not-human subjects research and exempt from University of Wisconsin-Madison IRB review. All methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare no potential conflicts of interest.

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