RAS/PI3K Crosstalk and Cetuximab Resistance in Head and Neck Squamous Cell Carcinoma

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Abstract

Purpose: Cetuximab, an antibody directed against the EGF receptor, is an effective clinical therapy for patients with head and neck squamous cell cancer (HNSCC). Despite great clinical promise, intrinsic or acquired cetuximab resistance hinders successful treatment outcomes but little is known about the underlying mechanism.

Experimental Design: To study the role of oncogenic HRAS in cetuximab resistance in HNSCC, the frequency of oncogenic HRAS mutations was determined in a cohort of 180 genomic DNAs from head and neck cancer specimens. We also used a combination of cetuximab-resistant cell lines and a transgenic mouse model of RAS-driven oral cancer to identify an oncogenic RAS-specific gene expression signature that promotes cetuximab resistance.

Results: Here, we show that activation of RAS signaling leads to persistent extracellular signal-regulated kinase 1/2 signaling and consequently to cetuximab resistance. HRAS depletion in cells containing oncogenic HRAS or PIK3CA restored cetuximab sensitivity. In our study, the gene expression signature of c-MYC, BCL-2, BCL-XL, and cyclin D1 upon activation of MAPK signaling was not altered by cetuximab treatment, suggesting that this signature may have a pivotal role in cetuximab resistance of RAS-activated HNSCC. Finally, a subset of patients with head and neck cancer with oncogenic HRAS mutations was found to exhibit de novo resistance to cetuximab-based therapy.

Conclusions: Collectively, these findings identify a distinct cetuximab resistance mechanism. Oncogenic HRAS in HNSCC promotes activation of ERK signaling, which in turn mediates cetuximab resistance through a specific gene expression signature.

Introduction

The EGF receptor (EGFR) signaling pathway is commonly activated in head and neck squamous cell carcinoma (HNSCC) and represents a validated target for therapy. Grandis and colleagues first demonstrated that EGFR overexpression is very common molecular alteration in HNSCC (1), whereas further work revealed that the intensity of its expression is linked to reduced survival (2, 3). EGFR activation triggers signal transduction cascade that includes activation of the Ras/Raf/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)–Akt signaling pathway (4–6).

Cetuximab is chimeric IgG1-human antibody directed against the extracellular domain of EGFR, blocking ligand binding to receptor and has been approved in the United States for treatment of HNSCC. Cetuximab, however, has objective response rate of only 13% when used as a single agent. There is therefore clear need for biomarkers predictive of response to cetuximab to maximize likelihood of response while minimizing toxicities.

One of the mechanisms of cetuximab resistance may be the presence of mutations that result in constitutive activation of EGFR-mediated signaling. In colon cancer, mutations that constitutively activate key signaling mediators downstream of EGFR, particularly KRAS, have been associated with cetuximab resistance. Activating point mutations in genes encoding the Ras subfamily of small GTP-binding proteins contribute to the formation of a large proportion of human tumors. The identification of Ras-related resistance mechanisms to EGFR inhibitors remains critical to the clinical management of patients with head and neck cancer. Although KRAS mutations are rare (approximately 1%) in HNSCC, HRAS mutations seem more common, whereas the reverse is true for several other malignancies (7, 8).

Understanding the mechanisms of cetuximab resistance may help delineate the subgroup of patients with head and neck cancer that can truly benefit from cetuximab. We
Translational Relevance
Cetuximab has been approved for treatment of head and neck squamous cell carcinoma (HNSCC). Despite great clinical promise, however, only 13% of patients respond to cetuximab when used as a single agent. Herein, by using a combination of cetuximab-resistant cell lines and a transgenic mouse model of RAS-driven oral cancer combined with analyses of patients with cetuximab-treated HNSCC, we uncover aberrant RAS/MAPK/ERK signaling as a central mediator of cetuximab resistance. In our study, the gene expression signature of C-myc, BCL-2, BCL-XL, and cyclin D1 upon activation of MAPK signaling was not altered by cetuximab treatment, suggesting that this signature may have a pivotal role in cetuximab resistance of RAS activated HNSCC. We further showed that repression of HRAS expression restores the ability of cetuximab to suppress the growth of head and neck squamous cancer cells containing activating mutations in HRAS, pointing to a promising resistance fighting approach.

Genomic DNA extraction and HRAS mutation detection
A total of 180 genomic DNAs from head and neck cancer samples were analyzed in this study. Genomic DNA was extracted from 10-μm paraffin-embedded sections of the tumor samples. Slides were microscopically examined and tumor areas were marked and carefully dissected under microscopic observation. DNA extraction from dissected material was performed using the EX-WAX DNA Extraction Kit (Millipore) according to the manufacturer's tissue protocol. The details of HRAS mutation detection are provided in Supplementary Materials.

Lentivirus-mediated short hairpin RNA silencing of HRAS gene
The lentiviral PLKO.1 puro vectors (MISSION shRNA plasmids) encoding the short hairpin RNA (shRNA) plasmid (SHC016-1EA) served as negative control. The specific shRNA sequences and further details are provided in the Supplementary Materials and Methods.

Lentivirus-mediated overexpression of G12V HRAS
The lentivirus vector pLenti CMV RasV12 Neo (w108-1) encoding the G12V HRAS (plasmid 22259, created by Prof. E. Campeau, Addgene) or control empty vector were cotransfected with the packaging plasmids pMD2.G and psPAX2 (Addgene) to 293T cells. Titrations and infections were performed as previously described (13). Further details are provided in the Supplementary Materials and Methods.

Mouse breeding and adenovirus infection
To study the expression signature of oncogenic RAS in an in vivo murine model of head and neck cancer, we used the eEF1a1-KrasG12D knock-in mice (Mouse Genome Informatics ID: MGI: 3837679). In this strain, upon Cre recombination of the loxP sequences, the floxed box is removed and the mouse Kras cDNA containing a G to A nucleotide substitution in codon 12 (GGT → GAT) is
Western blot analysis and quantitative reverse transcription (RT)-PCR

Methodology is provided in the Supplementary Materials.

GST-Raf-1 RBD pull-down assay

The levels of active, GTP-HRAS were determined by GST-Raf-1RBD pull-down assay. The methodology of GST-Raf-1RBD pull-down assay is provided in the Supplementary Materials.

Confocal microscopy

For confocal microscopy, cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.5% Triton X-100 for 10 minutes, and incubated with an α-tubulin mouse monoclonal antibody conjugated to Alexa Fluor 488 fluorescent dye (DM1A clone; Cell Signaling; 1:200 dilution) Further details are provided in the Supplementary Materials.

Matrigel invasion assay and anchorage-independent growth assay

Methodology is provided in the Supplementary Materials.

Results

HRAS is frequently mutated in HNSCC

A total of 180 Formalin-Fixed, Paraffin-Embedded (FFPE) HNSCC were evaluated for HRAS mutations. Hotspot HRAS mutations (codons 12, 13, and 61) were found in 11 of 180 (6.67%) specimens. In addition, rare heterozygous HRAS mutations were detected in codon 14(V14M), 17(S17I), 54(D54G), and 63(E63K) (Supplementary Fig. S2). The overall frequency of HRAS mutations in our cohort of HNSCCs was 9.44% (Supplementary Results, Table S3), which is relatively higher to the reported frequency (8%) of HRAS mutations in HNSC in COSMIC mutation database (16).

Recently, Pickering and colleagues in whole exome sequencing of 38 oral squamous cell carcinomas reported that 9% of their samples harbor HRAS alterations (17).

HRAS silencing reduces the proliferation capacity of head and neck cancer cells

MTT viability assays showed that cell lines containing mutations in HRAS (BB49) or PIK3CA (Cal-33) displayed robust cetuximab-resistant phenotype when challenged with 50 nmol/L of cetuximab as compared with control treated cells (Supplementary Fig. S1).

To further address the molecular mechanism underlying the connection between activation of RAS/RAF/MAPK and PI3K/AKT pathways in cetuximab resistant cancer cells, we silenced HRAS expression in a panel of cetuximab-resistant head and neck cancer cell lines harboring activating mutations in either HRAS (BB49) or PIK3CA genes (Cal-33). As shown in Fig. 1A, silencing experiments revealed that HRAS protein levels were considerably downregulated (>90%) by shRNA#65 oligo whereas, HRAS silencing using shRNA#64 oligo was less efficient. HRAS silencing was associated with an altered morphology, which is characterized by a flattened phenotype and remodeling of tubulin cytoskeleton (Fig. 1B). The phenotype of lentivirus infected cell lines, confirmed 100% efficiency of HRAS silencing and selection in our approach and also verified that the molecular profile of HRAS-depleted cell lines is homogeneous. As expected, phospho-ERK1/2 protein levels were substantially reduced in cells following HRAS silencing, whereas EGFR, phospho-EGFR (Y1068), AKT, and ERK1/2 protein levels were not affected (Fig. 1C). Interestingly, downregulation of HRAS levels was associated with decreased levels of phosphorylated AKT, which is consistent with previously reported data showing that RAS and PI3K/AKT signaling pathways are highly interconnected (18–20). MTT viability assays showed that silencing of HRAS in BB49, Cal-33, UM-SCC11A, and UM-SCC6 was associated with a lower rate of proliferation (approximately, 20% lower) compared with uninfected or control infected cells (data not shown). However, HRAS-depleted cells were still able to proliferate and we did not observe any degree of apoptotic cell death or senescence. The ability of cells harboring low RAS levels to proliferate even with a low rate was also confirmed by soft agar assays as shown in Fig. 1D. These results suggest that in HRAS-deficient BB49 and Cal-33 cells, ERK-independent signaling pathways can still maintain a basal proliferation capacity.

Cross-talk between RAS/MAPK and PI3K/AKT pathways in HNSCC

Activation of PI3K recruits AKT to plasma membrane where AKT is phosphorylated at Thr^{473} and Ser^{547} (21). The downregulation of phospho-AKT protein levels by HRAS depletion in BB49 and Cal-33 cells in our experiments suggests that HRAS is an important regulator of PI3K activity in both cell lines. To investigate whether phosphorylation of AKT is exclusively mediated by PI3K, we treated BB49 and Cal-33 cells with the PI3K inhibitor LY294002. Treatment with LY294002 inhibitor diminished the phospho-AKT levels in both cell lines (Fig. 2A), suggesting that HRAS depletion in BB49 and Cal-33 cells is associated with strong inhibition of PI3K.

To investigate the role of HRAS and EGFR on PI3K activation in head and neck cell lines with oncogenic HRAS or PI3KCA, we treated the wild type and the low RAS-containing BB49 and Cal-33 cell lines with cetuximab and PI3K activity was assessed by measuring phospho-AKT protein levels. Interestingly, as shown in Fig. 2B, phosphorylation of AKT was substantially decreased in wild-type BB49 cells treated with cetuximab, compared with cetuximab untreated cells, suggesting that EGFR blockade leads to complete PI3K inhibition in these cells despite the presence of activated HRAS. This finding supports previous data showing that the ability of oncogenic mutant RAS by itself to drive PI3K membrane localization and activation is probably dependent on the signaling input from EGFR receptor (21). The fact that HRAS depletion in these cells can cause a complete PI3K inhibition as well, even in the...
presence of activated EGFR, provides evidence that the activation of wild-type PI3K in these cells requires the concurrent activation of HRAS and EGFR proteins. Wild-type Cal-33 cells harbor wild-type HRAS and the activated mutation H1047R in PIK3CA gene. Interestingly, in cetuximab untreated Cal-33 cells, HRAS depletion caused a complete inhibition of PI3K in the presence of activated EGFR, suggesting that oncogenic PI3K (H1047R) requires the direct interaction with HRAS to exhibit aberrant activity.

When parental Cal-33 cells were treated with cetuximab, PI3K was still able to promote the phosphorylation of AKT to some extent (p-AKT levels were less than 30% compared with the levels in untreated cells). These data suggest that oncogenic PI3K maintains a low level of activity in presence of HRAS even if EGFR is not activated. As expected, no phosphorylated AKT was detected when we treated HRAS-depleted Cal-33 cells with cetuximab (Fig. 2B).

**HRAS silencing in cetuximab-resistant head and neck cancer cell lines containing oncogenic HRAS or PI3K, restores their sensitivity to cetuximab**

Cetuximab treatment was associated with a complete growth inhibition of HRAS-depleted BB49 and Cal-33 cell lines compared with cetuximab treated control infected and parental BB49 and Cal-33 cell lines, suggesting that EGFR blockade, in addition to AKT/MAPK abrogation following HRAS depletion, is sufficient to suppress the growth of HNSCC (Fig. 3A and B). To the contrary, cetuximab treatment of HRAS-depleted UM-SCC11A and UM-SCC6 cell lines was not associated with a growth inhibition compared
with the control infected and parental UM-SCC11A and UM-SCC6 cell lines, suggesting that cetuximab resistance mechanisms that are HRAS/PI3K independent cannot be overcome by AKT/MAPK abrogation following HRAS depletion or inhibition.

Biochemical analysis of HRAS-depleted BB49 and Cal-33 cells and their control infected cells, revealed that the proliferation capacity of HRAS-depleted cells is mainly maintained from EGFR-dependent signaling pathways such as the STAT3 pathway (Fig. 3C). Phospho-STAT3 protein levels were not affected by HRAS silencing in both cell lines, whereas these were diminished upon treatment of Cal-33 and BB49 cells with 50 nmol/L cetuximab. Inactivation of the STAT3 pathway by cetuximab treatment was associated with growth suppression of BB49 and Cal-33 cells, only when combined with AKT/MAPK abrogation, following HRAS silencing, suggesting that the proliferation capacity in these cells is regulated by a fine balance between EGFR, RAS/MAPK, and PI3K/AKT signaling pathways.

The effect of cetuximab in viability of HRAS-depleted Cal-33 cells was also confirmed by colony-formation assay. As shown in Fig. 3D, cetuximab treatment did not inhibit the anchorage-independent growth of Cal-33 cells and had no effect on the number of soft agar colonies. However, the size of colonies in cetuximab-treated cells was substantially reduced compared with untreated, which is in accordance to the finding that EGFR inhibition decreases by approximately 10% the proliferation rate of Cal-33 cells on MTT viability assay. As expected, HRAS depletion, in combination with cetuximab treatment caused a complete inhibition of anchorage-independent growth of Cal-33 cells.
Figure 3. HRAS silencing in combination with cetuximab treatment suppress the growth of BB49 and Cal-33 cells. A, cetuximab growth response of HRAS-depleted BB49 and Cal-33, UM-SCC6, and UM-SCC11A cell lines. Uninfected (un) and lentivirus-infected BB49 and Cal-33 cells expressing either a scrambled shRNA (scr) or the shRNA #65 oligo (LV_65) were treated with 50 nmol/L cetuximab for 72 hours. Growth was measured using the MTT viability assay and plotted as a percentage of growth relative to the uninfected (un) cells of each cell line. Data points are represented as mean ± SEM (n = 3). B, cetuximab sensitivity in HRAS-depleted head and neck cell lines. Lentivirus-infected BB49 and Cal-33 cells expressing either a scrambled shRNA (scr) or the shRNA #65 oligo were treated with cetuximab at the indicated concentrations for 72 hours, and viable cells were measured by MTT assay and plotted (mean ± SD) as a percentage of growth relative to untreated controls. C, EGFR signaling promotes STAT3 activation in BB49 and Cal-33 cells. Uninfected (un) and lentivirus infected BB49 and Cal-33 cells expressing either a scrambled shRNA (scr) or the shRNA #65 oligo (LV_65) were either treated with 50 nmol/L cetuximab (+) for 72 hours or not (−). Phospho and total levels of STAT3 were measured by immunoblot analysis. Actin was used as loading control. D, HRAS depletion in combination with cetuximab treatment inhibits the colony formation of Cal-33 cells in soft agar assay. Uninfected (un) and lentivirus-infected Cal-33 cells expressing either a scrambled shRNA (scr) or the shRNA #65 oligo (LV_65) were either treated with 50 nmol/L cetuximab (+) or not (−). After 2 weeks of growth, the colonies were stained with crystal violet and quantified by light microscopy. The relative growth of colonies for each cell line is provided by representative phase-contrast photomicrographs (10× magnification) 10 days after seeding.
Oncogenic HRAS activates MAPK and AKT signaling pathways in HNSCC

As described above, HRAS depletion in cetuximab-resistant cell lines with oncogenic mutations in HRAS or PIK3CA restored their sensitivity to cetuximab. In a next step, we asked whether overexpression of oncogenic G12V HRAS in cetuximab-sensitive cell lines can lead to specific molecular signature that is associated with cetuximab resistance. Among HNSCC cell lines tested for cetuximab resistance, UM-SCC1 and UM-SCC25 cell lines were found to be cetuximab sensitive and their growth was suppressed by more than 50% in the presence of 50 nmol/L cetuximab in MTT viability assays. Sequencing analysis of HRAS and PIK3CA genes revealed that both cell lines harbor wild-type HRAS and PIK3CA genes (data not shown).

In this direction, the cetuximab-sensitive cell lines UM-SCC1 and UM-SCC25 were either mock infected, or infected with a lentivirus expressing the oncogenic G12V HRAS protein. G12V HRAS–expressing cell lines exhibit a more rounded shape; however, the cellular morphologic changes were less marked than those observed in HRAS-depleted head and neck cancer cells (Fig. 4A). As shown in Fig. 4B, a robust overexpression of HRAS protein was detected in cell lines infected by lentivirus expressing the oncogenic G12V HRAS, compared with wild-type or mock-infected cell lines.

As expected, overexpression of activated HRAS was associated with activation of MAPK and AKT signaling pathways. As shown in Fig. 4B, we observed a marked activation of p42Erk2 and a marked upregulation on the levels of phospho AKT in UM-SCC1 and UM-SCC25 cancer cells with oncogenic HRAS overexpression.

The gene expression signature upon activation of the MAPK pathway by oncogenic RAS in HNSCC

Upon activation, MAPKs phosphorylate and control the activity of key nuclear proteins, which in turn can regulate gene expression. To identify the group of downstream transcriptional factors that their expression was upregulated because of the G12V HRAS–driven activation of the MAPK pathway, we compared the expression profile of known nuclear transcriptional targets of the MAPK pathway between the G12V HRAS–expressing UM-SCC1 and UM-SCC25 cells and their mock infected controls by Western blot and qRT-PCR analyses.

Among the transcription factor targets of the MAPK pathway included in our expression analysis, phospho-CREB and phospho-Elk1 were found to be significantly upregulated in G12V HRAS–expressing cells compared with controls, whereas total levels of CREB and Elk1 were unaffected by G12V HRAS expression (Fig. 4C). Elk1 is one of the best-studied targets of the ERK cascade as ERKs phosphorylate Elk1 in several sites increasing its transactivation potential mostly by allowing increased interaction with other cofactors such as the histone acetyl-transferases (HAT) CBP/p300 and the mediator coactivator complex (22, 23).

Phosphorylation of CREB is regulated by ERK5, a MAPK distantly related to ERK1/2 that is also activated by activated HRAS and has been implicated as important for cellular survival in cultured cells (24–26). CREB within the cell is believed to bind the DNA regulatory element CRE, either as a homodimer or as a heterodimer, with ATF-1. Interestingly, phosphorylated ATF-1 protein levels were significantly reduced upon G12V HRAS overexpression, suggesting that nuclear ATF-1/CREB heterodimers are probably replaced by CREB homodimers on the CRE binding transcriptional activator complexes.

Another direct target of MAPK signaling is the product of proto-oncogene v-Myc Myelocytomatosis Viral Oncogene Homolog (c-myc). Previous studies have shown that phosphorylation of Myc at Ser 58 and Ser 62 by MAPK stabilizes Myc, allowing Myc to activate transcription as a heterodimeric partner with Myc-associated factor X (Max; refs. 27 and 28). It is worth noting that in our expression analysis, we observed a robust upregulation of phospho and total c-myc protein levels (Fig. 4C).

Cyclin D1 proto-oncogene associates with its binding partner cyclin-dependent kinase 4 and 6 (CDK4 and CDK6), and forms active complexes that promote cell-cycle progression by phosphorylating and inactivating the retinoblastoma protein (29). In HNSCC, high cyclin D1 expression is correlated with chemotherapy failure and poor prognosis (30–32). In response to G12V HRAS–driven activation of the MAPK pathway, we observed a robust upregulation of cyclin D1 protein levels in UM-SCC1 cells, whereas cyclin D1 levels were only slightly upregulated in UM-SCC25 cells (Fig. 4C).

Previously, it has been shown that expression of an activated form of KRAS (KRASG12D) in mice models initiates squamous tumour formation in the oral epithelium as well as in the skin (33–36). To validate the specific role of c-myc and cyclin D1 overexpression in cell proliferation of RAS-driven squamous cell carcinoma in head and neck cancer, we used an eEF1a1KrasG12D mouse model. In this mouse, the highly expressed eEF1a1 locus, serves as recipient site for knock-in of the sequence encoding the active oncogenic KrasG12D cDNA. The tissue-specific and inducible control of oncogene expression is achieved by the Cre/LoxP technology (14).

Activation of oncogenic KRAS expression was achieved by removing the floxed block using recombinant adenovirus-expressing cre recombinase (Ad-CMV-Cre). More specifically, we delivered Cre with intra-oral submucosal injections of 2.5 × 10^7 particles of adenovirus in head and neck tissue (tongue, oral cavity, larynx) of anesthetized eEF1a1KrasG12D mice. Four weeks after injection of recombinant adenovirus, oral verrucous squamous carcinoma was detected in 20% (4/10 mice) of injected mice (Fig. 4D). Microscopically, all examined cases of RAS-induced carcinomas were histologically homogeneous and consisted of large masses of well-differentiated epithelium with keratinized SCC with clear evidence of keratinization. Immunostaining analysis showed a strong expression of phospho-ERK, cyclin D1, and c-myc, suggesting that these proteins play a pivotal role in cell proliferation of Ras-driven cancer.
Figure 4. Effect of oncogenic RAS expression in cell proliferation. A, bright field microscopic images of UM-SCC1 and UM-SCC25 cells that were either mock infected (empty vector, LV_EV) or infected with a lentivirus expressing G12V HRAS (LV_G12VHRAS). Scale bar represents 100 μmol/L. B, effect of G12V HRAS expression in MAPK and AKT signaling pathways. Protein lysates from UM-SCC1 and UM-SCC25 cells that were uninfected (un), mock infected (LV_EV), or infected with a lentivirus expressing G12V HRAS (LV_G12VHRAS) were used for Western blot analysis of the indicated proteins. Actin expression served as loading control. C, G12V HRAS signature in expression profile of transcription factors. Protein lysates from UM-SCC1 and UM-SCC25 cells that were uninfected (un), mock infected (LV_EV), or infected with a lentivirus-expressing G12V HRAS (LV_G12VHRAS) were used for Western blot analysis of the indicated proteins. Actin expression served as loading control. D, gross appearance of oral tumors developed in eEF1a1 Kras<sup>G12D</sup> mice 4 weeks after Ad-CMV-Cre injection. Hematoxylin and eosin staining and immunohistochemistry analysis of oral tumors for pankeratin, phospho-ERK, cyclin D1, and c-Myc.
G12V HRAS expression profile in HNSCC is associated with an invasive phenotype and with resistance to cetuximab

Activated HRAS in cooperation with TGF-β has been implicated in tumor progression, invasion and metastasis, inducing an EMT phenotype in squamous cell carcinoma (37). Therefore, we evaluated the effect of G12V HRAS signaling on cell invasion, using matrigel-coated transwell culture chambers. After 48 hours of incubation, invading cells, attached to the membrane were stained and counted. We observed that forced expression of G12V HRAS in both UM-SCC1 and UM-SCC25 cell lines did indeed stimulate cell invasion compared with their respective mock-infected cells (Fig. 5A). To investigate whether the G12V HRAS-driven invasive phenotype of UM-SCC1 and UM-SCC25 cells is also cetuximab resistant, the invasion assays were repeated in presence of 50 nmol/L cetuximab. As a result, the mock-infected cells from UM-SCC1 and UM-SCC25 cell lines failed to survive or invade to the matrigel matrix, consistent with our data that these cell lines are cetuximab sensitive. To the contrary, cetuximab-treated UM-SCC1 and UM-SCC25 cells expressing G12V HRAS were cetuximab resistant and capable of invading to the matrigel matrix. As shown in Fig. 5A, the number of membrane-attached cells from UM-SCC1 and UM-SCC25 cell lines expressing G12V HRAS is not affected by cetuximab treatment, suggesting that oncogenic RAS signaling can maintain the proliferation, survival, and invasion potential in HNSCC. About the expression of biomarkers that are associated with cell–cell or cell–matrix interactions and survival, we observed a slight reduction of E-cadherin and a marked overexpression of CD44v6 levels in G12V HRAS cells (Fig. 5B). We also observed that the levels of antiapoptotic proteins BCL-2 and BCL-XL were significantly increased in G12V HRAS cells, whereas the expression of proapoptotic Bad protein were unaffected by overexpression of oncogenic HRAS (Fig. 5B).

To further study the cetuximab resistance of UM-SCC1 and UM-SCC25 cells expressing G12V HRAS, we compared the growth between G12V HRAS–overexpressing cells and their relative mock-infected controls in different cetuximab concentrations by MTT viability assay. In these experiments, when cetuximab was not added in the culture medium, the growth of G12V HRAS–expressing UM-SCC25 and UM-SCC1 cells was only 2% and 5% increased, respectively, compared with their mock-infected control cells. MTT viability assays with increasing concentrations of cetuximab (0–50 nmol/L) in the medium did not cause any significant growth suppression of G12V HRAS–expressing UM-SCC25 and UM-SCC1 cells. To the contrary, we observed marked growth suppression in mock-infected cells, consistent with our observation that the parental cell lines were cetuximab sensitive. As shown in Fig. 5C, treatment with 25 nmol/L cetuximab caused a 55% and 50% growth suppression to the mock-infected UM-SCC1 and UM-SCC25 cells, respectively, compared with their G12V HRAS–expressing cells.

Overall, these findings indicate that head and neck cancer cells with oncogenic RAS signaling exhibit an aggressive phenotype, which is characterized by cetuximab resistance and enhanced invasion potential.

The gene expression signature of G12V HRAS about c-myc, BCL-XL, and cyclin D1 in HNSCC is not altered by cetuximab treatment

In a next step, we asked whether the expression profile of G12V RAS signaling in HNSCC is altered by cetuximab treatment. More specifically, we investigated whether the protein and mRNA expression of c-myc and cyclin D1, as key drivers of proliferation, and of BCL-XL, as key driver of antiapoptosis, and survival in G12V HRAS–expressing cells is affected by EGFR signaling inhibition. As shown in Fig. 6A, treatment of UM-SCC1 and UM-SCC25 cells expressing G12V HRAS with 50 nmol/L cetuximab for 72 hours did not alter the protein expression profile of c-myc, cyclin D1, and BCL-XL. Quantitative real-time PCR analysis confirmed that the gene expression profile of c-myc, cyclin D1, and BCL-XL was unaffected by cetuximab treatment in cells with oncogenic HRAS expression and also revealed that c-myc upregulation is mediated by both posttranslational modifications (phosphorylation on threonine 58 and serine 62) and transcriptional activation (Fig. 6B). Taken together, these data suggest that the expression signature of G12V HRAS about c-myc, BCL-XL and cyclin D1 in HNSCC is not altered by cetuximab treatment.

HRAS mutation is associated with de novo resistance in patients with cetuximab-treated HNSCC

On the basis of our in vitro findings demonstrating a role for HRAS mutation in causing cetuximab resistance, we sought to determine whether this mechanism also mediates clinical cetuximab resistance. We evaluated the clinical impact of de novo HRAS mutation in a cohort of 55 patients (HRAS wild-type n = 48; HRAS mutant n = 7) who had been treated with cetuximab-based chemoradiotherapy. Hotspot HRAS mutations (codon 12, 13, and 61) were detected in 7 of 55 (12.7%) primary HNSCC specimens. There was no evidence for association between HRAS mutation and age (P = 0.60), sex (P = 0.33), tumor–node–metastasis (TNM) stage (P = 0.99), ETOH (P = 0.69), surgery (P = 0.21), tobacco use (P = 0.99), and subsite (P = 0.10; Supplementary Table S4 and Supplementary Results). HRAS mutation was associated with lower likelihood of attaining response (P = 0.046, Fisher exact test) to cetuximab-based therapy. Specifically, the group of "nonmutants" was more likely to have complete or partial response, as compared with mutants (81.3% vs. 42.9%, respectively). Time to progression (TTP) was significantly longer for patients without HRAS mutation (P = 0.053, log rank test). Specifically, the median TTP for HRAS mutants was 30 months whereas median TTP for patients with no HRAS mutation has not been reached (Fig. 6C). The median OS was longer for patients without HRAS mutation (HRAS mutants 24 months; HRAS nonmutants, 48 months, P = 0.21). Using the Cox proportional hazards model, we carried out multivariable analysis to assess the prognostic value of HRAS mutation for TTP and OS. We included the following
**A**

Cetuximab: (50 nmol/L) – + Cetuximab: (50 nmol/L) – +

UM-SCC1

UM-SCC25

**B**

UN  LV  LV_G12VHRAS

BCL-XL

BCL2

Bad

E-cadherin

CD44v6

β-Actin

UM-SCC25 UM-SCC1

**C**

Cell viability (% of control)

Cetuximab (nmol/L)

UM-SCC1 LV_G12VHRAS

UM-SCC1 LV_EV

UM-SCC25 LV_G12VHRAS

UM-SCC25 LV_EV
prognostic variables in the regression model: gender, TNM stage, age, tobacco, alcohol use, and surgery. Multiple Cox regression analysis examining the relationship between OS and HRAS mutation did not show a statistically significant effect ($P = 0.178$), whereas HRAS mutation was associated with shorter TTP ($P = 0.022$) with a HR for HRAS mutant tumors of 5.098 (Supplementary Table S6 and Supplementary Results).

Collectively, these clinical studies further support our in vitro studies and demonstrate that HRAS mutation is associated with de novo resistance to cetuximab-based therapy in patients with HNSCC.

**Discussion**

Cetuximab is a validated target for therapy in HNSCC. Cetuximab has been approved in combination with radiotherapy in locally advanced HNSCC and in recurrent metastatic setting based on results of pivotal phase III studies I (38, 39). Despite its great clinical promise, the majority of HNSCC patients do not respond to cetuximab.

Findings from studies of drug resistance to EGFR-targeted therapies have been applied to develop the next generation of clinical trials in lung cancer (40). In contrast, there has been limited exploration of mechanisms of resistance to cetuximab in patients with HNSCC. Herein, by using a combination of cetuximab-resistant cell lines and a transgenic mouse model of RAS-driven oral cancer, coupled with analyses of patients with cetuximab-treated HNSCC, we uncover aberrant RAS/MAPK/ERK signaling as a central mediator of cetuximab resistance in HNSCC. In our study, we found a high incidence of HRAS mutations in HNSCC (9.44%). HRAS mutation status was associated with lower likelihood of attaining complete response (CR) or partial response (PR) to treatment and significantly reduced TTP in a cohort of locally advanced HNSCC treated with cetuximab-based therapy. In multivariate analysis, HRAS mutation status retained its prognostic significance for TTP after adjustment for well-characterized prognostic indicators.

Another interesting observation in our study was the molecular crosstalk between the RAS/RAF/MAPK and PI3K/AKT pathways. PI3K and Ras are pivotal players in squamous cell carcinoma, which is characterized by strong expression of phospho-ERK, cyclin D1, and c-myc. Consistent with these data, overexpression of G12V HRAS in UM-SCC1 and UM-SCC25 HNSCC cell lines that harbor wild-type HRAS and PI3K revealed that overexpression of cyclin D1 and c-myc has pivotal role in head and neck carcinoma with oncogenic RAS signaling. We also show that overexpression of G12V HRAS is associated with HRAS resistance to cetuximab.

Our findings may have tremendous implications for overcoming resistance to cetuximab in cancers bearing genetic alterations of components of PI3K or MAPK/RAS signaling pathways, which represent the vast majority of HNSCC.

Another important finding of our study is that the proliferation capacity of HRAS-depleted CAL33 and BB49 cells is mainly maintained from EGFR-dependent signaling pathways such as STAT3 and therefore, EGFR blockade could be combined with RAS inhibition for effective treatment of HNSCC containing aberrations in PI3K or RAS signaling pathways. In our study, we used eEF1a1 KrasG12D transgenic mouse as model for Ras-driven oral tumor development. Activation of oncogenic KrasG12D in oral cavity of this mouse promotes squamous cell carcinoma, which is characterized by strong expression of phospho-ERK, cyclin D1, and c-myc. Consistent with these data, overexpression of G12V HRAS in UM-SCC1 and UM-SCC25 HNSCC cell lines that harbor wild-type HRAS and PI3K revealed that overexpression of cyclin D1 and c-myc has pivotal role in head and neck carcinoma with oncogenic RAS signaling. We also

![Figure 5](https://www.aacrjournals.org/clinica/images/clinica/1078/1078-0432.CCR-13-2721-F5.jpg)

**Figure 5.** Effect of G12V HRAS expression promotes cell survival, invasion, and resistance to cetuximab. A, effect of G12V HRAS expression on the cell invasion capacity. UM-SCC1 and UM-SCC25 cells that were either mock infected (empty vector, LV_EV) or infected with a lentivirus-expressing G12V HRAS (LV_G12VHRAS) were allowed to invade through transwell inserts (8 μm) coated with matrigel either in presence of 50 mmol/L cetuximab (─) or not (─). After 24 hours, noninvading cells were removed with cotton swabs and invading cells on the reverse side of the filter were fixed, stained, and photographed under a light microscope. B, G12V HRAS signature in expression profile of proteins that promote cell survival and invasion. Protein lysates from UM-SCC1 and UM-SCC25 cells that were uninfected (un), mock infected (LV_EV), or infected with a lentivirus-expressing G12V HRAS (LV_G12VHRAS) were used for Western blot analysis of the indicated proteins. Actin expression served as loading control. C, cetuximab resistance is associated with G12V HRAS expression. UM-SCC1 and UM-SCC25 cells that were mock infected (LV_EV) or infected with a lentivirus-expressing G12V HRAS (LV_G12VHRAS) were treated with cetuximab at the indicated concentrations for 72 hours, and viable cells were measured by MTT assay and plotted (mean ± SD) as a percentage of growth relative to untreated controls.
identified BCL-2 and BCL-XL as antiapoptotic proteins that are significantly overexpressed upon activation of RAS signaling. Interestingly, the gene expression signature of oncopgenic RAS about c-MYC, BCL-2, BCL-XL, and cyclin D1 was not altered by cetuximab treatment, suggesting that this signature may have a pivotal role in cetuximab resistance of RAS-activated HNSCC. One of the limitations of our study was that we studied a single HNSCC cell line with HRAS mutation and one cell line with PIK3CA mutation.

Our findings are directly relevant to patients with HNSCC that are resistant to cetuximab-based therapy and may help guide subsequent treatment. Several agents that target PI3K

Figure 6. G12V HRAS molecular phenotype is associated with cetuximab resistance in HNSCC. A, G12V HRAS expression signature is not altered by cetuximab treatment. UM-SCC1 and UM-SCC25 cells that were uninfected (un), mock infected (LV_EV), or infected with a lentivirus expressing G12V HRAS (LV_G12VHRAS) were either treated with 50 nmol/L cetuximab for 72 hours (+) or not (−) and analyzed by immunoblotting to detect the protein levels of c-myc, cyclin D1, and BCL-XL. Actin expression was used as loading control. B, transcriptional activation of c-myc, cyclin D1, and BCL-XL by oncopgenic HRAS is not inhibited by EGFR blockage. Gene expression of c-myc, cyclin D1, and BCL-XL was examined with qRT-PCR analysis of RNA harvested from UM-SCC1 and UM-SCC25 cells that were uninfected (un), mock infected (empty vector), or infected with a lentivirus expressing G12V HRAS and either treated with 50 nmol/L cetuximab for 72 hours (G12V HRAS+cet) or not (G12V HRAS). Gene expression was normalized to β-actin, and the fold-change was calculated with respect to gene expression in uninfected cells. Data are from 3 individual experiments and are expressed as the mean ± SD. C, Kaplan–Meier survival analyses for TTP and OS. The comparison of TTP between patients with no HRAS mutation (blue line, n = 48) and HRAS mutants (green line, n = 7) shows a statistically significant better prognosis for patients with no HRAS mutation (P = 0.04). The comparison of OS between patients with wild-type HRAS (blue line, n = 48) and HRAS mutants (green line, n = 7) does not show a statistically significant difference in OS (P = 0.21).
or RAS signaling are undergoing clinical development. Hence, the findings from this study can be immediately used to design potential clinical therapies for patients with HNSCC. Given the retrospective nature of our studies in patient samples, these findings need further clinical validation. The frequency and the relationship of HIRAS mutation in cetuximab-resistant cancers need to be fully assessed in prospective studies.

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

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References


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44. Hon WC, Berndt A, Williams RL. Regulation of lipid binding underlies the activation mechanism of class IA PI3-kinases. Oncogene 2012;31:3655–66.
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