Title: RAS/ PI3K crosstalk and cetuximab resistance in head and neck squamous cell carcinoma

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Conflict of Interest
The authors declare that they have no conflict of interest.

Running title:
Cetuximab resistance and HRAS oncogenic mutations in HNSCC.
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 cetuximab-treated HNSCC patients, we uncover aberrant RAS/MAPK/ERK signaling as a central mediator of cetuximab resistance. In our study, the gene expression signature of C-myc, BCL2, 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.
Abstract

Purpose: Cetuximab, an antibody directed against the epidermal growth factor receptor (EGFR), 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, BCL2, 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 head and neck cancer patients 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.

Keywords

Epidermal growth factor receptor; drug resistance; HRAS signaling; cetuximab; head and neck cancer
**Introduction**

EGFR signaling pathway is commonly activated in HNSCC and represents a validated target for therapy. Grandis et al first demonstrated that EGFR overexpression is very common molecular alteration in HNSCC(1), while 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 which constitutively activate key signaling mediators downstream of EGFR, particularly K-Ras, 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. Whereas K-RAS mutations are rare (approximately 1%) in HNSCC, HRAS mutations appear 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 head and neck cancer patients that can truly benefit from cetuximab. We hypothesized, therefore, that HNSCC with constitutively activated downstream signaling mediators of the EGFR pathway, particularly HRAS, would be refractory to inhibition of the pathway at the receptor level. To define mechanisms of de novo
cetuximab resistance, we studied a series of cetuximab-resistant head and neck squamous cell carcinoma cell lines, including cell lines bearing HRAS or PIK3CA mutations and transgenic mouse model of RAS driven oral tumor development. We combined our findings with studies of tumor specimens from cetuximab-treated HNSCC patients.

Materials and Methods

Patient Population

One hundred eighty tumor specimens were obtained from Attikon Hospital archives after institutional review board approval. All patients provided written informed consent. The clinopathological data of patients are provided in supplementary table S7.

Cell lines, reagents and MTT viability assays

The HRAS mutated squamous head and neck cell line (p.Q61L, homozygous) BB49(9), the PI3KCA mutated squamous head and neck cell line (p.H1047R, heterozygous) Cal-33(10) and the A-431 epidermoid carcinoma cell line were obtained from American Type Tissue Collection (ATCC). The head and neck cancer cell lines UM-SCC1, UM-SCC25, UM-SCC6, UM-SCC11 that harbor wild type HRAS and PI3KCA were obtained from the University of Michigan(11). All cell lines were authenticated by DNA typing and used within 6 months of receipt(12). The mutation status of HRAS and PI3KCA genes was verified by sanger sequencing in all cell lines. Details about cell culture, reagents and MTT viability assays are described in the supplemental materials and methods.

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, Temecula CA) according to the manufacturer’s tissue protocol. The details of HRAS mutation detection are provided in supplemental Materials.

**Lentivirus mediated shRNA silencing of HRAS gene**

The lentiviral PLKO.1 puro vectors (MISSION® shRNA plasmids) encoding the short hairpin RNA #64 (TRCN0000033264) and #65 (TRCN0000033265) that target the HRAS coding sequence were purchased from Sigma-Aldrich (Munich, Germany). The MISSION pLKO.1 puro-non target shRNA plasmid (SHC016-1EA) served as negative control. The specific shRNA sequences and further details are provided in the supplemental 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. Eric Campeau, Addgene, Cambridge, MA, USA) or control empty vector were co-transfected with the packaging plasmids pMD2.G and psPAX2 (Addgene, Cambridge, MA, USA) to 293T cells. Tittrations and infections were performed as previously described(13). Further details are provided in the supplemental 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-Kras\textsuperscript{G12D} knock-in mice (Mouse Genome Informatics ID: MGI: 3837679). In this strain, upon Cre recombination of the loxP sequences, the floxed block is removed and the mouse Kras cDNA containing a G to A nucleotide substitution in codon 12 (GGT ->GAT) is conditionally expressed under the eEF1a1 (eukaryotic translation elongation factor 1 alpha 1) promoter. Genotyping of the eEF1a1-Kras\textsuperscript{G12D} mice was performed by PCR analysis of tail biopsy using specific primers(14). To induce recombination of the loxP sequences, a recombinant adenovirus (rAd) expressing Cre recombinase under the control of the cytomegalovirus (CMV) promoter (Ad-CMV-Cre) (Clontech,Mountain View,
CA) was used for injections in oral tissue. Adeovirus expressing GFP under the control of the CMV promoter (Ad-CMV-GFP) (Clontech, Mountain View, CA) was used as a control. Viruses were propagated in 293A cells and titrated using a plaque assay as previously described(15). All experiments with eEF1a1-KrasG12D mice were performed with approved animal protocols according to the institutional guidelines established by Biomedical Research Foundation of the Academy of Athens (BRFAA).

Western blot analysis and quantitative RT-PCR
Methodology is provided in the supplemental 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 supplemental Materials.

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

Matrigel invasion assay and anchorage-independent growth assay
Methodology is provided in the supplemental Materials.
Results

HRAS is frequently mutated in HNSCC
A total of 180 FFPE HNSCC were evaluated for HRAS mutations. Hotspot HRAS mutations (codon 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 Figure 2, 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 (OSCCs) 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 nM of cetuximab as compared to control treated cells (Supplementary Figure 1, Fig. S1).

To further address the molecular mechanism underlying the connection between activation of RAS/RAF/MAPK and PI3K signaling pathways and cetuximab resistance, we silenced HRAS expression in a panel of cetuximab resistant head and neck cancer cell lines harboring activating mutations in either HRAS (BB49) or PI3KCA genes (Cal-33) along with cetuximab resistant cell lines containing wild type HRAS and PI3KCA genes (UM-SCC11A, UM-SCC6). As shown in Figure 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 flat- shaped phenotype and remodeling of tubulin cytoskeleton (Figure 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 (Figure 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 to 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 figure 1D. These results suggests 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\(^{308}\) and Ser\(^{473}\). The downregulation of phospho-AKT protein levels by HRAS depletion in BB49 and Cal-33 cells in our experiments suggests that HRAS is 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 (Figure 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 Figure 2B, phosphorylation of AKT was substantially decreased in wild type BB49 cells treated with cetuximab, compared to 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 EGF 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 in order 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 to 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 (Figure 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 to 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 (Figure 3A,B). To the contrary, cetuximab treatment of HRAS depleted UM-SCC11A and UM-SCC6 cell lines was not associated with a growth inhibition compared to 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 (Figure 3C). Phospho STAT3 protein levels were not affected by HRAS silencing in both cell lines, whereas were diminished upon treatment of Cal-33 and BB49 cells with 50 nM cetuximab. Inactivation of 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 Figure 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 to 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.

**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 *PI3KCA* 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 nM cetuximab in MTT viability assays. Sequencing analysis of HRAS and PI3KCA genes revealed that both cell lines harbor wild type HRAS and PI3KCA 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 morphological changes were less marked than those observed in HRAS depleted head and neck cancer cells (Figure 4A). As shown in Figure 4B, a robust overexpression of HRAS protein was detected in cell lines infected by lentivirus expressing the oncogenic G12V HRAS, compared to 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 Figure 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 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 due to the G12V HRAS driven activation of MAPK pathway, we compared the expression profile of known nuclear transcriptional targets of MAPK pathway between the G12V HRAS expressing UM-SCC1 and UM-SCC25 cells and their mock infected controls by western blot and qRT-PCR analysis.

Among the transcription factor targets of MAP kinase pathway included in our expression analysis, phospho CREB and phospho Elk1 were found to be significantly upregulated in G12V HRAS expressing cells compared to controls, whereas, total levels of CREB and Elk1 were unaffected by G12V HRAS expression (Figure 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 (HATs) CBP/p300 and the Mediator co-activator 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 MAP Kinase 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 Ser62 by MAPK stabilizes Myc, allowing Myc to activate transcription as a heterodimeric partner with Myc Associated Factor X (Max)\(^{(27, 28)}\). It is worth noting that in our expression analysis, we observed a robust upregulation of phospho and total c-myc protein levels (figure 4C).

Cyclin D1 proto-oncogene associates with its binding partners 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 (RB)\(^{(29)}\). In HNSCC, high cyclin D1 expression is correlated with chemotherapy failure and poor prognosis\(^{(30-32)}\). In response to G12V HRAS driven activation of MAP kinase pathway, we observed a robust upregulation of cyclin D1 protein levels in UM-SCC1 cells while cyclin D1 levels were only slightly upregulated in UM-SCC25 cells (Figure 4C).

Previously, it has been shown that expression of an activated form of KRAS (KRAS\(^{G12D}\)) 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 a eEF1a1 Kras\textsuperscript{G12D} mouse model. In this mouse, the highly expressed eEF1a1 locus (Eukaryotic Translation Elongation Factor 1 Alpha 1,) serves as recipient site for knock-in of the sequence encoding the active oncogenic Kras \textsuperscript{G12D} 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\textsuperscript{7} particles of adenovirus in head and neck tissue (tongue, oral cavity, larynx) of anesthetized eEF1a1 Kras\textsuperscript{G12D} mice. Four weeks after injection of recombinant adenovirus, oral verrucose squamous carcinoma was detected in 20% (4 of 10 mice) of injected mice (Figure 4D).

Microscopically, all examined cases, of RAS induced carcinomas were histologically homogeneous and consisted of large masses of well differentiated epithelium with keratinized squamous cell carcinomas (SCC) with clear evidence of keratinization. Immunostaining analysis showed a strong expression of phosphor-ERK, Cyclin D1 and c-myc, suggesting that these proteins play a pivotal role in cell proliferation of Ras driven cancer.

**G12V HRAS expression profile in HNSCC is associated with an invasive phenotype and with resistance to cetuximab**

Activated HRAS in cooperation with transforming growth factor β (TGFβ) 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 h 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 to their respective mock-infected cells (Fig. 
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 nM 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 Figure 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. Regarding 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 (Figure 5B). We also observed that the levels of antiapoptotic proteins BCL-2 and BCL-XL were significantly increased in G12V HRAS cells, while the expression of proapoptotic Bad protein were unaffected by overexpression of oncogenic HRAS(Figure 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 to their mock infected control cells. MTT viability assays with increasing concentrations of cetuximab (0-50 nM) 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 figure 5C, treatment with 25 nM cetuximab, caused a 55% and 50% growth suppression to the mock infected UM-SCC1 and UM-SCC25 cells, respectively, compared to 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 regarding 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 figure 6A, treatment of UM-SCC1 and UM-SCC25 cells expressing G12V HRAS with 50 nM cetuximab for 72h 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 (Figure 6B). Taken together these data suggest that the expression signature of G12V HRAS regarding c-myc, BCL-XL and Cyclin D1 in HNSCC is not altered by cetuximab treatment.

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

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 (\( p=0.33 \)), TNM
stage (p=0.99), ETOH (p=0.69), Surgery (p=0.21), tobacco use (p=0.99) and subsite (p=0.10) (Table S4, Supplementary Results). HRAS mutation was associated with lower likelihood of attaining response (p=0.046, Fisher's Exact test) to cetuximab-based therapy. Specifically the group of ‘nonmutants’ was more likely to have complete or partial response, as compared to mutants (81.3% versus 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 while median TTP for patients with no HRAS mutation has not been reached (Figure 6C). The median OS was longer for patients without HRAS mutation (HRAS mutants 24 months; HRAS non-mutants, 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 prognostic variables in the regression model: gender, TNM stage, age, tobacco, alcohol use and surgery. Multiple Cox regression analysis examining the relationship between overall survival and HRAS mutation did not show a statistically significant effect (p=0.178), while HRAS mutation was associated with shorter TTP (p=0.022) with a hazard ratio for HRAS mutant tumors of 5.098 (Table S6, 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 HNSCC patients.

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 l(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 HNSCC patients. Herein, by using a combination of cetuximab-resistant cell lines and a transgenic mouse model of RAS driven oral cancer, coupled with analyses of cetuximab-treated HNSCC patients, 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 CR or 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 RAS/RAF/MAPK and PI3K/AKT pathways. PI3K and Ras are pivotal players in signal transduction pathways that co-ordinate proliferation, survival and migration in head and neck cancer cells. Morris et al performed sequencing and high-resolution copy number assessment of core components of PI3K pathway in HNSCC(41). The authors reported that 74% of HNSCC contain activating genetic alterations, mainly copy number alterations, of components of the PI3K pathway. In our model system we found that RAS is required for PI3K activity, even in the presence of active EGFR signaling. Our data suggest that constitutive activation of PI3K is not sufficient to promote its oncogenic activity in the absence of activated RAS in HNSCC cells. In the same context, Rodriguez-Viciana and colleagues, showed that overexpression of a dominant-negative Asn17 Ras mutant that does not interact with PI3K, partly inhibits induction of PIP3 by epidermal and nerve growth factor in cultured PC12 cells(42). To the contrary, a recent study by Chaussade et al, based on in vitro PI3K assays with exogenous H1047R PI3K, revealed that recombinant oncogenic RAS (G12V) does not significantly activate the H1047R PI3K(43). Another recent study by Burke et al., indicated that compared with the wild-type PI3K, mutant H1047R PI3K has higher affinity for lipids and this may explain its higher basal kinase activity compared to the wild-type enzyme(44). It is worth noting, that the strong and clear dependence of H1047R PI3K activity on HRAS interaction observed in our study was based on endogenous levels of H1047R PI3K and HRAS proteins, in a cellular environment with activated EGFR signaling; thus, this may explain the different effects on H1047R PI3K
activity compared to previous in vitro studies. Our findings may have tremendous implications for overcoming resistance to cetuximab in HNSCC. If we pharmacologically modulate the interaction between PI3K and HRAS we will be able to restore sensitivity to cetuximab in cancers bearing genetic alterations of components of PI3K or MAPK/RAF/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 a 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, CyclinD1 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 CyclinD1 and c-myc has pivotal role in head and neck carcinoma with oncogenic RAS signaling. We also identified Bcl-2 and Bcl-XL as antiapoptotic proteins that are significantly overexpressed upon activation of RAS signaling. Interestingly, the gene expression signature of oncogenic RAS regarding C-myc, BCL2, 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 HNSCC patients that are resistant to cetuximab-based therapy and may help guide subsequent treatment. Several agents that target PI3K or RAS signaling are undergoing clinical development. Hence, the findings from the current study can be immediately used to design potential clinical therapies for HNSCC patients. Given the retrospective nature of our studies in patient
samples, these findings need further clinical validation. The frequency and the relationship of HRAS mutation in cetuximab-resistant cancers need to be fully assessed in prospective studies.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflict of interest.
References


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.
Figure Legends

Figure 1. HRAS depletion affects cytoskeletal dynamics and cell proliferation.

(A) Efficiency of HRAS silencing in BB49, Cal-33, UM-SCC6 and UM-SCC11A cells. Western blot analysis of HRAS levels was performed from protein extracts that were made from uninfected (un) cells and cells that were infected with lentivirus expressing shRNA#64 (#64), shRNA#65 (#65), or the scrambled sequence control shRNA (scr).

(B) HRAS depletion affects cytoskeletal dynamics. Representative phase-contrast images of BB49 and Cal-33 cells that were infected with lentivirus expressing shRNA#65 (LV65shRNA), or the scrambled sequence control shRNA (LVscr) and immunofluorescence analysis of β-tubulin fibers.

(C) Effect of HRAS depletion on MAPK, AKT and EGFR signaling in head and neck cell lines BB49 and Cal-33. Protein extracts were made from uninfected (un) cells and from cells that were infected with lentivirus expressing shRNA#64 (#64), shRNA#65 (#65), or the scrambled sequence control shRNA (scr).

(D) Anchorage-independent growth of BB49 and Cal-33 cells following knockdown of HRAS. Representative phase-contrast photomicrographs (10× magnification) depicting the colony growth in soft agar for BB49 and Cal-33 cells that were infected with lentivirus expressing shRNA#65, 7 and 14 days after seeding.
Figure 2. HRAS expression levels affect PI3K activity and phosphorylation of AKT.

(A) Sustained PI3K inhibition decreases AKT phosphorylation in BB49 and Cal-33 cells. BB49 and Cal-33 cells were starved for 24h, treated with 20μM LY294002 for 0, 5 and 10 hours and lysates were evaluated for phospho and total AKT expression by immunoblot.

(B) HRAS depletion is associated with downregulation of phospho AKT levels in BB49 and Cal-33 cells. Wild type (un) and lentivirus infected BB49 and Cal-33 cells expressing either a scrambled shRNA (LVscrshRNA) or the shRNA #65 oligo (LV65shRNA) were either treated with 50 nM cetuximab (+) or not (-). The phospho and total levels of EGFR and AKT were measured by immunoblot analysis of whole protein lysates. The protein expression levels of PI3K were measured in the same immunoblot analysis using a specific antibody against the p110α domain. Actin was used as loading control. H-Ras-GTP protein levels were purified using the GST-Raf-RBD pull-down assay. In brief, 10^7 cells from each cell line were lysed in ice-cold RBD lysis buffer and 500μg of whole protein lysate were incubated with 20μg GST-Raf-RBD glutathione agarose beads for 1 h at 4°C. The HRAS-GTP protein levels were measured by immunoblot analysis using a specific anti HRAS antibody (c-20, Santa Cruz).
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 nM cetuximab for 72h. 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±s.e.m. (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 72h, 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 nM cetuximab (+) for 72h 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 nM cetuximab (+) or not (-). After two 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.
**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 100μM.

(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 eEF1α1 Kras<sup>G12D</sup> mice, 4 weeks after Ad-CMV-Cre injection. H&E staining and immunohistochemistry analysis of oral tumors for pankeratin, phospho-ERK, cyclin D1 and c-Myc.
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) allowed to invade through transwell inserts (8 µm) coated with matrigel either in presence of 50nM cetuximab (+) or not (-). After 24h, non-invading 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 72h, and viable cells were measured by MTT assay and plotted (mean +/- SD) as a percentage of growth relative to untreated controls.
**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 nM cetuximab for 72h (+) 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, cyclinD1 and BCL-xl by oncogenic HRAS is not inhibited by EGFR blockage. Gene expression of c-myc, cyclinD1 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 nM cetuximab for 72h (G12VHRAS+cet) or not (G12VHRAS). Gene expression was normalized to β-actin, and the fold-change was calculated with respect to gene expression in uninfected cells. Data are from three 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) doesn’t show a statistically significant difference in OS (p=0.21).
Figure 2

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(h) 20 μM LY294002

p-AKT (S473)
AKT

B.

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EGFR
p-EGFR
p-AKT (Ser 473)
AKT
p110α (PI3K)
HRAS-GTP
b-actin

BB49
CAL-33
Figure 4

A.

UM-SCC25
LV_EV

UM-SCC25
LV_G12VHRAAS

UM-SCC1
LV_EV

UM-SCC1
LV_G12VHRAAS

B.

EGFR
p-EGFR(Y1068)
p-AKT (Ser 473)
AKT
p-ERK1/2 (Thr202/Tyr204)
ERK1/2
HRAS
b-actin

UM-SCC25
UM-SCC1

C.

Cyclin D1
p-CREB (Ser133)
CREB
p-ATF1(Ser63)
ATF1
p-cMyc (Thr58/Ser62)
c-Myc
p-Elk1 (Ser383)
Elk1
b-actin

UM-SCC25
UM-SCC1

D.

eEF1a1 KrasG12D
oral tumor

H&N
Pankeratin

phospho-ERK
Cyclin D1
c-Myc
Figure 6

A

Cetuximab: (50nM)

EGFR

p-EGFR(Y1068)

c-Myc

CyclinD1

Bcl-xL

b-actin

UM-SCC25  UM-SCC1

B

Fold Change Expression

Cyclin D1  c-Myc  Bcl-xL

UMSCC-1  Empty vector  G12V  HRAS  G12V HRAS+Cet

UMSCC-25  Empty vector  G12V  HRAS  G12V HRAS+Cet

OS

TTP

Cum Survival

Cum Survival

Time to Progression (months)

Overall Survival (months)

HRAS MUTATION

No

Yes

No-censored

Yes-censored
Clinical Cancer Research

RAS/ PI3K crosstalk and cetuximab resistance in head and neck squamous cell carcinoma


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