Inhibition of Rapamycin-Induced AKT Activation Elicits Differential Antitumor Response in Head and Neck Cancers


Abstract

The PI3K/AKT/mTOR pathway is an important signaling axis that is perturbed in majority of cancers. Biomarkers such as pS6RP, GLUT1, and tumor FDG uptake are being evaluated in patient stratification for mTOR pathway inhibitors. In the absence of a clear understanding of the underlying mechanisms in tumor signaling, the biomarker strategy for patient stratification is of limited use. Here, we show that no discernible correlation exists between FDG uptake and the corresponding Ki67, GLUT1, pS6RP expression in tumor biopsies from patients with head and neck cancer. Correlation between GLUT1 and pS6RP levels in tumors was observed but elevated pS6RP was noticed even in the absence of concomitant AKT activation, suggesting that other downstream molecules of PI3K/AKT and/or other pathways upstream of mTOR are active in these tumors. Using an ex vivo platform, we identified putative responders to rapamycin, an mTOR inhibitor in these tumors. However, rapamycin did not induce antitumor effect in the majority of tumors with activated mTOR, potentially attributable to the observation that rapamycin induces feedback activation of AKT. Accordingly, treatment of these tumors with an AKT inhibitor and rapamycin uniformly resulted in abrogation of mTOR inhibition-induced AKT activation in all tumors but failed to induce antitumor response in a subset. Phosphoproteomic profiling of tumors resistant to dual AKT/mTOR inhibitors revealed differential activation of multiple pathways involved in proliferation and survival. Collectively, our results suggest that, in addition to biomarker-based segregation, functional assessment of a patient’s tumor before treatment with mTOR/AKT inhibitors may be useful for patient stratification. Cancer Res; 73(3); 1–10. ©2013 AACR.

Introduction

Head and neck cancer (HNC) is the most frequently occurring malignant tumor in South Asian countries. Despite significant improvements in radiotherapy, chemotherapy, and surgical procedures, the 5-year survival rate (~40%–50%) for this malignancy has not improved in the last few decades (1). HNCs are highly heterogeneous, containing a large number of genetic alterations rendering them refractory to specific targeted drugs. The PI3K/AKT/mTOR pathway is an important nodal signaling axis that is deregulated in about 90% of solid cancers and impacts local recurrence and survival (2, 3). Research into mechanism of action of this protein in initiation, progression, and resistance to therapy in cancer is still evolving (4). mTOR associates with either raptor or rictor to form 2 fundamentally unique complexes: mTORC1 and mTORC2 (3, 5). The mTOR-containing complexes have contrasting sensitivities to rapamycin (an mTOR inhibitor) as well as differential upstream and downstream effectors (6). Despite the robust activity in a variety of model systems, mTOR inhibitors exhibit a more modest antitumor activity in a subset of patients (7). Previous findings indicate that inhibition of mTORC1 (i.e., mTOR/raptor complex) with rapamycin or its derivatives induces feedback activation of the AKT survival pathway in various types of cancer cells, tumor specimens, and in patients with HNCs after exposure to mTOR inhibitor (8, 9). Hyperactivation of AKT is associated with resistance to apoptosis, increased cell growth, cell proliferation, and cell metabolism (10). Studies in Drosophila (11, 12) and mammalian systems have shown that the regulation of this feedback loop is mediated by insulin signaling (13, 14). Besides S6K1, another marker assessed extensively in relation to mTOR signaling in solid cancers is glucose transporter-1 (GLUT1; refs. 15, 16). GLUT1 has been extensively looked at in the context of hypoxia-inducible factor (HIF)-1α, another downstream effector of mTOR (17). Although GLUT1 expression is a common...
feature in patients with HNCs, the prognostic value of this parameter along with the \(^{18}\text{F}\)-2-fluoro-2-deoxy-D-glucose (FDG) uptake as assessed by positron emission tomography (PET) has not been specifically evaluated with respect to mTOR status in HNCs. Clinical development of mTOR inhibitors has prompted us to understand the prevalence of tumor dependence on the mTOR axis. Our data show that while mTOR inhibition activates AKT feedback loop in all the tumors, sensitivity to rapamycin treatment was observed in only a subpopulation of tumors. Further cotargeting the rapamycin-insensitive tumors with rapamycin and MK-2206 (an allosteric inhibitor of AKT; ref. 18) did not enhance the antitumor efficacy of rapamycin in the majority of tumors, indicating that the mTOR pathway despite aberrant activation may not be the only critical node to target in these primary HNC tumors. Targeted phosphoproteomic characterization of these tumors revealed deregulation of several pathways with known anti-apoptotic/prosurvival signaling nodes that could explain the observed resistance.

Materials and Methods

Collection of human tumors

Fresh tumor tissues (n = 22) were collected from patients with HNCs immediately after biopsy procedure, with due informed consent, under Institutional Review Board-approved protocols from Mazumdar-Shaw Cancer Centre, Bangalore, India. Patients who had previously received neoadjuvant chemotherapy and/or radiotherapy were excluded from the study. The primary treatment-naïve tumors evaluated as part of this study were predominantly late stage (stage III and above) with 77% of the patients detected with nodal involvement (Supplementary Table S1). Tumors were predominantly of oral and oropharyngeal origin (~55%), whereas tumors of laryngeal (~27%) and hypopharyngeal (18%) origin were in the minority. The majority of the patients were male (85%) and average age at presentation was less than 65 years (77%). The tumor samples were transported to the laboratory at 4°C in appropriate transport buffer within 30 minutes after resection for ex vivo studies and molecular and pathologic evaluation.

Treatment and clinical examination

The patients enrolled in the study had the standard clinical and radiologic evaluation before biopsy. During baseline visit, PET-CT was conducted and standardized uptake value (SUV) assessed. Tissue viability and histopathologic diagnosis were confirmed by routine histological and immunohistochemistry (IHC)-based examination as per standard protocol for staging and grading.

Drugs and chemicals

CCK-8 assay reagent (Dojindo); rapamycin (Sirolimus) and AKT inhibitor, MK-2206 (Selleck Chemicals), were purchased, dissolved in dimethyl sulfoxide (DMSO), and stored in aliquots as per the recommendations from manufacturers.

Tumor explant culture and drug treatment

For ex vivo analysis of tumor response to targeted drugs, uniform tumor sections were maintained in quadruplicates in 48-well plates in media supplemented with 20% FBS (Invitrogen), 1× GlutaMAX (Invitrogen), and appropriate concentration of antibiotics/antimycotics (Invitrogen; ref. 19). Tumor sections were treated with DMSO, MK-2206 (1 μmol/L), rapamycin (Sirolimus; 10 μmol/L), or combination of rapamycin and MK-2206 for 72 hours. In combination group, tumors were treated with rapamycin for 8 hours followed by incubation for another 64 hours with MK-2206. Medium was changed every 24 hours. The tissue slices at baseline time (T₀) and thereafter harvested at 72 hours (T₇₂) were assessed for cell viability and submitted for histopathologic evaluation.

Tumor viability study

Tumor cell viability was assessed by Cell Counting Kit-8 (CCK-8; Dojindo) using CCK-8 reagent as per manufacturer’s recommendations. One-tenth volume of CCK-8 solution was added to each well and incubated at 37°C for 3 hours in a humidified CO₂ chamber. Absorbance was measured at 450 nm. Samples at baseline (T₀) were used as calibrators to normalize intersample variation in absorbance readings. Tissue viability of drug-treated samples was expressed as a percentage of viability relative to DMSO-treated controls.

Immunohistochemical analysis

Tumors were fixed in 10% buffered formalin and embedded in paraffin. About 5-μm sections were stained with hematoxylin and eosin (H&E). Antigen retrieval was done in Vector Antigen Unmasking Solution (citrate-based, Vector Laboratories) by exposure to microwave heating for 30 minutes. Quenching of endogenous peroxidase was done, and protein blocking was carried out at room temperature for 1 hour with 10% goat serum. Sections were incubated with primary antibody at below mentioned conditions followed by incubation with horseradish peroxidase-conjugated secondary antibody (SignalStain Boost IHC Detection Reagent; Cell Signaling Technology) for 1 hour at room temperature. Chromogenic development of signal was done using 3,3′-diaminobenzidine (DAB Peroxidase Substrate Kit; Vector Laboratories). Tissues were counterstained with hematoxylin (Papanicolaous solution 1α; Merck). Rabbit monoclonal phospho-AKT (Ser473; D9E) and phospho-AMPKα (Thr172; clone 40H9, Cell Signaling Technology) were used at 1:50 and 1:100 dilutions, respectively, for overnight incubation at 4°C. Rabbit monoclonal phospho-S6 ribosomal protein (pS6RP; Ser235/236; D57.2.2E) and phospho-PRAS40 (Thr246, C77D7; Cell Signaling Technology) were used at 1:200 dilution for overnight incubation at 4°C; rabbit polyclonal GLUT1 (Abcam) at a 1:200 dilution was used for 1-hour incubation at room temperature; rabbit polyclonal Ki67 (Vector Laboratories) was used at 1:600 dilution for 1 hour at room temperature. Induction of apoptosis was detected by staining for cleaved caspase-3 using polyclonal anti-cleaved caspase-3 (Asp175) antibody (rabbit polyclonal, Cell Signaling Technology) at 1:600 dilution for 1 hour at room temperature. Matched IgG isotype control (rabbit monoclonal IgG, DA1E, and mouse monoclonal IgG1, G3A1, Cell Signaling Technology) were used for each primary antibody. Each slide was
individually examined by 2 experts, and scoring/grading was conducted as per H-score formula described previously (20).

**Western blotting**

SDS-PAGE–separated protein samples were transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with primary antibodies against pS6RP (Ser235/236, Cell Signaling Technology), GLUT1 (Abcam), and Tubulinβ (Imgenex) and detected by Enhanced chemiluminescence (Amersham, GE Lifesciences).

**Reverse-phase protein array**

PathScan RTK Signaling Antibody Array Kit (Cell Signaling Technology) was used according to the manufacturer’s instructions to simultaneously detect 28 receptor tyrosine kinases (RTK) and 11 downstream signaling nodes. Tissue slices were lysed with 1× cell lysis buffer (Cell Signaling Technology), and protein estimation was carried out by modified Lowry method (Bio-Rad’s DC Protein Assay). Diluted lysates were added to each well in the slide at the concentration of 1 mg/mL, and chemiluminescent signals were captured using digital imaging system. Spot intensity was quantified using image analysis software.

**Real-time PCR**

Total RNA was isolated from head and neck squamous cell cancer tissues (5 mg) using an RNeasy Micro Kit (Qiagen). About 1 μg of total RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol (See Supplementary Tables S2 and S3 for primer information). Each PCR was carried out in 20 μl reaction mix, containing 10 μl of SYBR select master mix (Applied Biosystems), 200 nmol/L primers, and 100 ng of cDNA. The amplified products were run on 1% agarose gel to verify the correct product size—HIF-1α (145 bp). All the samples were normalized using 18S rRNA as the control gene, and the relative fold change was calculated against a control head and neck tissue for HIF-1α.

**Statistical analysis**

One-way ANOVA, linear regression, and Spearman coefficient of correlation were analyzed using GraphPad Prism version 5 for Windows.

**Results**

Expression of GLUT1 correlates with mTOR pathway activation in primary human head and neck tumors

PI3K/AKT/mTOR signaling axis is deregulated in HNCs. We evaluated mTOR activation by the IHC analysis of pS6RP in HNC tumor samples (n = 22) at baseline (Fig. 1A). To understand whether the pS6RP expression is reflective of activation of AKT in these tumors, we conducted pAKT IHC (Fig. 1A). Of the 22 samples assessed, 9 samples exhibited elevated levels of pS6RP (score is ≥3), 4 samples had intermediate level of expression (score is >1–<3), and 9 samples had null or low expression of pS6RP (score is ≤1; Fig. 1B). However, elevated levels of pS6RP expression were observed in tumors lacking endogenous aberrant pAKT activation, indicating that mTOR activation was not contingent upon pAKT status in these tumors (Fig. 1A). GLUT1 is one of the downstream effectors of mTOR signaling, and we wanted to understand whether GLUT1 expression is deregulated in these tumors. Various cellular patterns of GLUT1 staining were observed within individual tumors, including surface and cytoplasmic expression (Fig. 2B and data not shown). On the basis of the intensities and number of GLUT1-positive cells, tumors were divided into 3 groups: high expression (score ≥4), medium expression (score = 1–3), and low/null expression group (score ≤1). Activated AKT, S6RP, and GLUT1 are expressed primarily in these tumors as compared with adjacent normal tissues (Fig. 1 and Supplementary Fig. S1). On comparing the expression levels of these 2 biomarkers, we observed that

![Figure 1](image-url)
tumors exhibiting high pS6RP expression also expressed elevated levels of GLUT1, and tumors with medium or low/null levels of pS6RP had a similar pattern of GLUT1 expression, indicating that a linear correlation exists between expression of pS6RP and GLUT1 (Fig. 2A and B). This was also independently confirmed by Western blotting experiments (Fig. 2D). To delineate whether the GLUT1 expression is linked to HIF-1α, a downstream effector of mTOR, we assessed baseline levels of HIF-1α in these tumors. Our data indicate that the observed levels of GLUT1 correlates positively with HIF-1α levels in these tumors (Fig. 2C). A large body of literature links GLUT1 expression to FDG uptake, and we wanted to assess whether FDG uptake of these tumors could be used as an independent indicator of the mTOR activation status. Toward this end, we compared the FDG uptake with pS6RP and GLUT1 expression levels. Of the 22 patients evaluated, no discernible correlation emerged between the levels of FDG uptake (available from the PET-CT data) and the pS6RP or GLUT1 expression status (Supplementary Fig. S2A–S2D). Similarly when the proliferation status was compared with FDG uptake, it did not evince any statistical correlation (Supplementary Fig. S2A and S2B). These data collectively suggest that neither FDG uptake by the tumors nor the proliferation status is indicative of mTOR pathway activation status, whereas pS6RP and GLUT1 are reliable indicators of the same in HNCs (Fig. 2A).

Induction of AKT activation by rapamycin in primary head and neck tumors

Having established the mTOR activation status in these primary tumors, we assessed their functional response to the mTOR inhibitor rapamycin using the ex vivo tumor explant platform. The tumors having low/null levels of pS6RP (9 of 22) were not sensitive to rapamycin treatment as expected, because the mTOR axis was not deregulated in these tumors (data not shown). We observed contrasting sensitivities to rapamycin treatment in mTOR deregulated tumors expressing high/medium levels of pS6RP (13 of 22 tumors), wherein only a small subset of tumors (15%, 2 of 13) were responsive. These tumors had decreased cell viability and proliferation coupled with increased caspase-3 activation on treatment with rapamycin compared with the DMSO control (Fig. 3A–C). The majority of tumors (11 of 13) were insensitive to rapamycin, as this treatment affected neither the tumor cell viability nor their proliferation (Fig. 4A–C), indicating that mTOR inhibition was not sufficient to mediate tumor inhibition. The induction of the feedback activation of AKT (Figs. 3 and 4 and data not shown).
shown) along with a reduction in GLUT1 expression (Supplementary Fig. S3A) was observed in all pS6RP-positive tumors treated with rapamycin.

Abrogating rapamycin-induced feedback activation of AKT by MK-2206 does not enhance antitumor effect in the majority of tumors. Activation of AKT and concomitant attenuation of growth-inhibitory effects of mTOR inhibitors is largely mediated through IRS-1 signaling (21). Cotargeting the AKT feedback loop, in addition to mTOR pathway, has been suggested as a strategy to overcome the loss of inhibitory effect of mTOR inhibitors. To know whether the abrogation of mTOR inhibition-mediated negative feedback enhanced antitumor effect, we treated tumors with rapamycin followed by MK-2206 in an ex vivo explant setting. In 18% of rapamycin-insensitive tumors, tumor cell viability dropped significantly (Fig. 5A) along with a reduction in GLUT1 expression (Supplementary Fig. S3A) compared with rapamycin, but this did not translate to tumor-inhibitory efficacy in the majority of the patient tumors. These observations collectively suggest that cotargeting mTOR and AKT activation loop does not uniformly induce antitumor response, as tumor dependence on this pathway for survival and maintenance varies in these primary HNCs.

Deregulation of multiple pathways linked to proliferation, angiogenesis, and apoptosis in mTOR activated tumors resistant to combination treatment

To elucidate putative mechanism(s) of resistance observed in the nonresponders to dual treatment, we conducted reverse-phase protein array (RPPA) profiling of these tumors at baseline (Fig. 6D and E). Our data indicated that many of these activated receptors and their nodal downstream molecules such as EGF receptor (EGFR) and related proteins were expressed at much higher levels in this subset than in the responder tumors. Other RTKs such as M-CSFR and c-KIT that may drive growth and proliferation were also expressed at elevated levels in the nonresponders. Overexpression of pSTAT3 was observed in these tumors (Fig. 6D and E), and previous reports have shown that elevated levels of pSTAT3 result in anti-apoptotic and proliferative response. These tumors were further assessed for expression of specific genes implicated in apoptosis, such as BCL2. Data indicate that the nonresponders have very high levels of the anti-apoptotic effector BCL2 than responders (Supplementary Fig. S4A). Total

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Fig. 3. A subset of tumors with activated mTOR are sensitive to rapamycin treatment. A, tumor sections were cultured with DMSO control or rapamycin (Rapa) for 3 days, and cell viability was determined by WST assay. Cell viability is presented as percentage score with corresponding DMSO control. Data are mean of triplicates ± SE. Significance (\(P < 0.01\)) was calculated by ANOVA. B, three days after culture with DMSO (left column) or rapamycin (right column)-treated tumor sections were fixed and embedded in paraffin. Tumor sections were stained with antibodies against pS6RP (second row), pAKT (S473; top row), proliferation marker Ki-67 (third row), and cleaved caspase-3 (Ac-Cas-3; bottom row). Image magnification, ×20 and inset, ×40. C, tumor sections stained with antibodies against cleaved caspase-3, and positive and negative cells were counted. Percentage of caspase-3–positive cells was calculated and plotted in all treatment groups. Data represent mean of triplicates ± SE. Significance (\(P < 0.05\)) was calculated by ANOVA.
EGFR expression levels were also elevated in the subset of nonresponders (Supplementary Fig. S4B). The expression levels of selected proteins in the activated pathways were further confirmed by IHC, wherein elevated levels of total EGFR, pEGFR, and pSTAT3 were observed in nonresponsive samples (Supplementary Fig. S4C). Collectively, these data indicate that there are diverse mechanisms that drive tumor maintenance and progression with multistep intrinsic deregulation contributing towards resistance to a variety of anti-neoplastic agents in HNCs.

Discussion

Carcinogenesis is a complex mechanism known to be orchestrated by multistep genetic changes. Recent studies indicate that there is an intrinsic pathway bias for the response and resistance to chemotherapy and targeted therapy. Despite several advances in the understanding of disease progression in a setting such as HNCs, the overall survival is severely limited. The PI3K/AKT/mTOR pathway is perturbed in a majority of cancers (10), and our data indicate a similar finding in the HNCs. While a majority of the tumors had elevated/intermediate levels of pS6RP expression, this expression did not correlate with activation of AKT. In the tumors that had high/intermediate levels of pS6RP, a linear correlation with pAKT expression was not present hinting at activation of other upstream effectors of mTOR.

To understand the consequence of activation of mTOR pathway and its effect on other downstream effectors such as GLUT1, we investigated the expression status of GLUT1 and its relation to HIF-1α levels. Our data suggested the existence of a positive correlation with respect to expression of pS6RP with GLUT1 and GLUT1 with HIF-1α in clinical biopsies from patients with HNCs. On the basis of our data as well as other published findings linking GLUT1 to mTOR pathway (22), it may potentially be used as a biomarker for mTOR status in HNCs. However, a study with larger number of patients with HNCs is required for the use of GLUT1 as a biomarker for mTOR activation in clinical setting. Research aimed at examining the significance of GLUT1 expression and FDG uptake in a variety of solid cancers has been carried out with conflicting results (23, 24). However, the metabolic status of a tumor and GLUT1 expression in oral carcinomas has not been systematically analyzed. The significance, if any, of FDG uptake in the context of mTOR activation status in HNCs need to be investigated further. Our findings are in agreement with Hong and Lim, wherein we observed that GLUT1 expression status of the tumors and the FDG uptake in the corresponding patients with HNCs do not correlate (25), and comparison of the SUV data with either the Ki-67 or pS6RP failed to yield any statistical correlation. These data indicate that the FDG uptake by tumors, while used by many as a prognostic indicator of survival, would have limited potential for use as a marker of GLUT1 expression or status of mTOR activation and proliferation. Tumors with elevated levels of pS6RP, a downstream target of mTOR, were inhibited by the use of rapamycin in transgenic models (15, 26). Our observations while in complete...
Concordance with the fact that pS6RP level is indicative of mTOR status suggest that the potential value of pS6RP as a prognostic indicator of response to mTOR inhibitors is rather limited. To further assess the functional response of these tumors to mTOR inhibitors, we used an ex vivo tumor explant culture system to delineate the dependence of these tumors on the mTOR. This explant system provides a platform to evaluate drug response of individual tumor tissues while preserving the native tumor microenvironment (19). This system is especially relevant to evaluate drug response of tumors to AKT/mTOR pathway inhibitors as it has been shown that epithelial–stromal interactions significantly alter drug response (27) and that tumor microenvironment is crucial for studying tumor biology (28). Using this model, we found that only a small subset of tumors with elevated/intermediate levels of pS6RP were responsive to rapamycin (15%), suggesting mTOR is the driver of these HNCs as reported in other tumors (16). Tumors with low/null expression of pS6RP (indicative of the absence of an activated target mTOR) as expected failed to respond to this mTOR inhibitor.

Clinically, it has been observed that mTOR inhibitor performance was modest at best and resulted in disease progression at worst and a variety of mechanisms have been described to explain this. The discovery of a "feedback loop culminating in AKT activation" initiated upon treatment with rapamycin has prompted further clinical investigations and in some cases can explain the attenuation seen in antitumor activity of rapamycin (9). Recent findings indicate that the suppression of mTOR/S6K1 inhibition–mediated AKT activation is triggered via IGF1R/P13K signaling to AKT (21, 29). As multiple signaling pathways impinge on the mTOR network, rationally designed cotargeting strategy for clinical management of HNCs is required to regulate tumor growth, recurrence, and resistance. We evaluated the relative merits of the cotargeting approach, by assessing the response of HNC tumors to the combined rapamycin and MK-2206 treatment in ex vivo setting. The combination therapy did not evince any significant improvement over the single agent in a subset of tumors (41%) that were in the low/null category for pS6RP and GLUT1 (data not shown). These findings are in line with previously published reports that indicate these inhibitors are efficacious only in the presence of persistently activated target (15). However, in the samples that had elevated levels of pS6RP (59%; indicative of persistent activation of the mTOR network), only 15% tumors were sensitive to rapamycin. The feedback activation of AKT observed on treatment with rapamycin

Figure 5. Targeted ablation of rapamycin-mediated upregulation of AKT signaling is key for antitumor effect in a subset of rapamycin-insensitive tumors. Tumor sections were cultured with DMSO or rapamycin (Rapa) or inhibitor of AKT (MK-2206) and combination of rapamycin and MK-2206 for 3 days. A, cell viability was measured by WST assay and presented as percentage score with corresponding DMSO control. Data are mean of triplicates ± SE. Significance (*, P < 0.05) was calculated by ANOVA. B, after 3 days of culture with DMSO or Rapa or MK-2206 or Rapa + MK-2206 (as indicated), tumor sections were embedded in paraffin, and 5-μm tumor sections were stained with H&E, antibodies against pS6RP, pAKT, pPRAS40, Ki67, and cleaved caspase-3 (Ac-Cas-3) as indicated. Image magnification, ×20 and insets, ×40. C, number of Ki-67-positive and -negative cells was counted in the entire sections (triplicate), and percentage of Ki67-positive cells was calculated in all treatment groups and plotted. Data represent mean of triplicates ± SE, and significance (*, P < 0.01) was calculated by ANOVA. D, number of activated caspase-3-positive and -negative cells was counted in the entire sections (triplicate), and percentage of activated caspase-3-positive cells was plotted in treatment and control groups. Data represent mean of triplicates ± SE. Significance (*, P < 0.05) was calculated by ANOVA.
Apoptotic cells and phosphorylation of several RTKs (30). The Bcl2 family plays a major role in the biology of HNC (31), and elevated levels of BCL2 observed in the resistant tumors (Supplementary Fig. S4) could potentially impact mTOR inhibitors via cross talk with parallel pathways as evidenced, in part, by the absence of apoptosis induction on treatment with this combination (Fig. 6C). Another plausible mechanism of resistance to mTOR inhibitors elucidated in squamous cell carcinomas is that rapamycin treatment induced transactivation of EGFR (32). Our data indicate that multiple pathways involved in survival and proliferation are aberrantly activated in the tumors nonresponsive to dual treatment. Overexpression of the EGFR family of proteins specifically EGFR along with increased levels of STAT3 is observed at baseline in this subset (Supplementary Fig. S4C). Cross-talk between these potential pathways to activate prosurvival signaling has been implicated in resistance to therapy in HNCs (33, 34) and could potentially contribute to the resistance to therapy observed in these tumors. In the context of nonresponders, we hypothesize that overexpression of c-KIT could play a role in promoting tumor resistance to therapy observed in these tumors. In the context of nonresponders, we hypothesize that overexpression of c-KIT could play a role in promoting tumor

Figure 6. Cotargeting mTOR and AKT using pharmacologic inhibitors ablate rapamycin-induced feedback activation of AKT but does not elicit antitumor efficacy in majority of rapamycin-insensitive HNC tumors. Tumor sections were cultured with DMSO (vehicle control), rapamycin (Rapa), AKT inhibitor (MK-2206), or rapamycin and MK-2206 combination (Rapa + MK-2206) for 3 days. A, cell viability was determined by WST assay, and percentage of cell viability was calculated. Shown here are viability data (mean of triplicates ± SE). B, after WST assays, tumors were fixed and embedded in paraffin. Tumors sections were stained with H&E (top row), cell proliferation marker (Ki-67), apoptotic marker (Ac-Cas-3), and antibodies against pS6RP, pAKT, and pPRAS as indicated. All images were taken at ×20 and insets were taken at ×40 magnification. C, numbers of Ki67-positive and -negative cells and number of activated caspase-3-positive and -negative cells were counted in all sections of all groups (in triplicates). Percentage of Ki67- and caspase-3-positive cells was calculated and plotted. Data are mean ± SE, and statistical analysis was conducted by ANOVA. D, targeted phosphoprotein expression profiling was carried out using total proteins extracted from Rapa + MK-2206 sensitive (bottom) and resistant (top) tumors at baseline. Spots are in duplicate, and each pair corresponds to a specific phosphoprotein (pEGFR and pSTAT3). E, intensity of each phosphoprotein was calculated by densitometry analysis on this phosphoproteomic arrays and plotted.

was ablated on addition of MK-2206 (in the combination treatment group) in all the 13 mTOR-activated tumors. Interestingly, in 82% of rapamycin-insensitive tumors, the MK-2206 and rapamycin combination did not exert a significant antitumor effect despite abrogation of signaling arising from feedback activation of AKT on rapamycin treatment. Recent reports based on cell line and clinical response biomarker analysis indicate that the rapamycin-mediated AKT activation observed is actually common and greater in rapamycin-sensitive cells and tumors than in rapamycin-insensitive cells (21). These findings point to the fact that the feedback loop activation of AKT, while not a marker of resistance, may actually function as a pharmacodynamic indicator of rapamycin activity (21). There are several possible mechanistic scenarios that could potentially explain this observation. Development of resistance to mTORC1 inhibitors has been correlated to the presence of different feedback loops initiated upon mTOR inhibition, and it has been shown that AKT inhibition induces expression and phosphorylation of several RTKs (30). The Bcl2 family plays a major role in the biology of HNC (31), and elevated levels of BCL2 observed in the resistant tumors (Supplementary Fig. S4) could potentially impact mTOR inhibitors via cross talk with parallel pathways as evidenced, in part, by the absence of apoptosis induction on treatment with this combination (Fig. 6C). Another plausible mechanism of resistance to mTOR inhibitors elucidated in squamous cell carcinomas is that rapamycin treatment induced transactivation of EGFR (32). Our data indicate that multiple pathways involved in survival and proliferation are aberrantly activated in the tumors nonresponsive to dual treatment. Overexpression of the EGFR family of proteins specifically EGFR along with increased levels of STAT3 is observed at baseline in this subset (Supplementary Fig. S4C). Cross-talk between these potential pathways to activate prosurvival signaling has been implicated in resistance to therapy in HNCs (33, 34) and could potentially contribute to the resistance to therapy observed in these tumors. In the context of nonresponders, we hypothesize that overexpression of c-KIT could play a role in promoting tumor
proliferation and thereby mediate resistance (35). We have looked at other intersecting pathways that are commonly deregulated in HNCs and a variety of other indications by profiling key genes such as HRAS, KRAS, PIK3CA, BRAF, and LKB1 (data not shown and Supplementary Table S2). Results showed that none of the primary tumors had mutations in HRAS, KRAS, PIK3CA, and BRAF but for 5 tumors that had mutations only in LKB1 (Supplementary Fig. S3A), a tumor suppressor gene implicated in aberrant mTOR activation via AMPK pathway (36, 37). We report here a novel LKB1 mutation (Ala-Val) in exon 5 (at codon 206) that was detected in 55% of nonresponders to combination therapy (Supplementary Table S2 and Supplementary Fig. S3A and S3B). It is reported that the presence of an Ala-Thr mutation in exon 5 (at codon 205) implicated the loss of AMPK activation in HNCs (38), however, the functional significance of the novel mutation is unknown.

The collective observations from this study warrant further detailed characterization of the various pathways that are intrinsically deregulated in these HNCs and provide a rationale for clinical development of mTOR inhibitors in varying combinations. While co-targeting or synchronized combinatorial therapy is a powerful approach to maximize varying combinations. While co-targeting or synchronized combinatorial therapy is a powerful approach to maximize...


**Cancer Research**


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