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ERK-TSC2 signalling in constitutively-active HRAS mutant HNSCC cells promotes resistance to PI3K inhibition

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ABSTRACT

Objectives: The PI3K/AKT/mTOR pathway is frequently altered in head and neck squamous cell cancer (HNSCC), making this pathway a logical therapeutic target. However, PI3K targeting is not universally effective. Biomarkers of response are needed to stratify patients likely to derive benefit and exclude those unlikely to respond.

Materials and methods: We examined the sensitivity of cell lines with constitutively-active (G12V mutant) HRAS and wild-type HRAS to PI3K inhibition using flow cytometry and cell viability assays. We then overexpressed and silenced HRAS and measured sensitivity to the PI3K inhibitor BYL719. Immunoblotting was used to determine activation of the PI3K pathway. MEK and mTOR inhibitors were then tested in HRAS mutant cells to determine their efficacy.

Results: HRAS mutant cell lines were non-responsive to PI3K inhibition. Overexpression of HRAS led to reduced susceptibility to PI3K inhibition, while knockdown improved sensitivity. Immunoblotting revealed suppressed AKT phosphorylation upon PI3K inhibition in both wild-type and HRAS mutant cell lines, however mutant lines maintained phosphorylation of S6, downstream of mTOR. Targeting mTOR effectively reduced viability of HRAS mutant cells and we subsequently examined the ERK-TSC2-mTOR cascade as a mediator of resistance to PI3K inhibition.

Conclusions: HRAS mutant cells are resistant to PI3K inhibition and our findings suggest the involvement of a signalling intersection of the MAPK and PI3K pathways at the level of ERK-TSC2, leading to persistent mTOR activity. mTOR inhibition alone or in combination with MAPK pathway inhibition may be a promising therapeutic strategy for this subset of HNSCC tumors.

Introduction

Phosphoinositide 3-kinase (PI3K)/AKT/mTOR signalling regulates critical tumor cell functions, including cellular metabolism, survival, angiogenesis, growth and migration [1]. Hyper-activation of PI3K signalling is frequently observed in head and neck squamous cell carcinomas (HNSCCs), with nearly 80% of tumors containing amplifications or mutations of *PIK3CA* and numerous additional tumors containing losses of tumor suppressor *PTEN* or amplifications of *EGFR* or *AKT1/2/3* [2–4]. Owing to the prevalence of PI3K-pathway aberrations in HNSCC and the dependency of tumor cells on PI3K signalling for survival and growth, targeting this pathway is an attractive therapeutic strategy for HNSCC patients.

Early clinical studies of PI3K inhibitors alpelisib (BYL719) and buparlisib (BKM120) in HNSCC have shown tolerable toxicity profiles and "on-target" PI3K inhibition [5–7]. However, the clinical efficacy of PI3K inhibitors to date has been limited and not all patients respond [7–9]. The PI3K/AKT/mTOR network contains numerous feedback loops and crosstalk nodes with other pathways, providing innumerable opportunities for circumventing the effects of PI3K inhibition. Studies of the signalling loops and adjacent pathways that counteract PI3K inhibition will help focus the use of PI3K inhibitors for patients likely to achieve maximal benefit. Further, identifying mediators of innate resistance to PI3K inhibition may highlight potentially targetable

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signalling dependencies in these non-responsive tumors that can be exploited for therapy using appropriate inhibitors.

HRAS belongs to the RAS family of GDP/GTP-binding proteins that function as intracellular signal transducers. When bound to GTP, HRAS is active and interacts with various downstream effectors, including RAF, which stimulates a phosphorylation cascade involving, mitogen-activated protein kinase kinases (e.g. MEK1) and extracellular signal-related kinases (*e.g.* ERK1/2) [10]. The RAS-RAF-MEK-ERK (MAPK) signalling pathway plays an integral role in cellular proliferation and survival and is highly interconnected with PI3K/AKT signalling [11]. The RAS isoforms (HRAS, KRAS and NRAS) are frequently altered human cancer, with particular isoforms having relevance in specific cancers [12–14]. Alterations in *HRAS* are most prevalent in HNSCC, observed in ~6% of tumors [2]. In general, aberrations in the RAS isoforms are activating, maintaining RAS in a GTP-bound state by impairing its GTPase activity. As a result, stimuli-independent RAS signalling is perpetuated [12]. RAS alterations have been used to define specific patient subsets in various cancers that respond differently to anti-cancer therapies and/or display distinct clinical features, such as rapidly progressive disease [15–17].

In the present study, we explored the constitutively-active HRAS G12V mutation in HNSCC as a biomarker for non-response to PI3K inhibition. We first established HRAS G12V to be a mediator of intrinsic resistance to PI3K inhibition and secondarily interrogated the mechanism. We observed persistent downstream mTORC1 signalling in G12V mutant cells, despite PI3K blockade. We then explored ERK-mediated TSC2 inactivation and highlight mTOR inhibition, alone or in combination with MAPK pathway inhibition, to be a novel therapeutic susceptibility of HRAS G12V mutant tumor cells [18].

Materials & methods

Cell culture

Cell lines were obtained from the sources listed (**Supp. Table 1**). All cell lines were cultured in DMEM/F12, with 10% fetal bovine serum (GIBCO), penicillin (100 IU/mL; Invitrogen) and streptomycin (100 µg/mL; Invitrogen), unless otherwise stated (**Supp. Table 1**). Cells were maintained in a 37°C humidified atmosphere with 5% CO_2. We previously used short tandem repeat profiling (The Center for Applied Genetics; Toronto) to confirm cell line identities [19]. T24 urinary bladder epithelial cells were used as a model cell line for human cancer cells with an endogenous HRAS mutation at codon 12, as to date there are no established HNSCC cell lines with HRAS mutations at codon 12 or 13 documented, despite the prevalence of these aberrations in primary tumors.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.oraloncology.2018.07.010.

Immunoblotting

Cell lysates were obtained using a radioimmunoprecipitation assay (RIPA) buffer. Cells were washed once in 1x PBS before lysis. Lysates were kept on ice for 15 min, then centrifuged 15 min at 14 000 rpm. Protein concentration was determined using a Bradford assay. Using 4–12% SDS-PAGE, 30 µg of protein was resolved for 1 h (hr) at 200 V (V) in 1x MES buffer. Protein was transferred to a PVDF Blotting Membrane (GE Healthcare) for 1 h, 14 V at room temperature. Membranes were blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich) in 1x TBST. Membranes were then incubated overnight at 4 °C with primary antibodies (**Supp. Table 2**). Of note, owing to the high degree of sequence homology between the RAS isoforms, we used a specific G12V-mutant RAS antibody to detect mutant RAS in our cell lines. Immunoreactive bands were visualized by incubating membranes for 1 h at room temperature with a peroxidase-conjugated anti-rabbit IgG in 5% skim milk/1x TBST. Membranes were visualized following exposure to enhanced chemiluminescence reagent (Luminata™ Crescendo, Western HRP Substrate; Millipore).

Cell viability assays

Cells were seeded in 96-well plates at 2,400 cells/well and cultured overnight. Drugs (**Supp. Table 3**) were then added at the indicated doses. Viability was determined indirectly using the PrestoBlue® Reagent (Thermo Fisher Scientific) at 0 and 72 h following drug treatment on a Synergy™ H4 Hybrid Reader (BioTek) with 560 nm excitation and 590 nm emission wavelengths.

Flow cytometry

To examine the effects of BYL719 on cell cycle, we treated cells with 5 µM BYL719 for 24 h. Three biological replicates were prepared. Prior to harvesting, BrdU (GE Healthcare, cat. RPN201) was added at 1:1000 and incubated with the cells for 2 h. Cells were then trypsinized, pelleted and the supernatant was removed. Cells were suspended in 1xPBS and fixed by adding 95% ethanol drop-wise while vortexing. Cells were then pelleted and resuspended in 2 N HCl, 0.5% Tx-100 drop-wise while vortexing, followed by 0.1 M $Na_2B_4O_7$ (pH 8.5), each for 30 min (mins) to allow permeabilization. Mouse anti-BrdU primary antibody (1:50, BD Biosciences lot. 347580) and FITC-conjugated horse anti-mouse secondary antibody (1:25, Vector Laboratories cat. FI-2000) were added respectively and incubated for 30 min per step, at room temperature and protected from light. Cells were then resuspended in a propidium iodide (PI; Biolegend®; Cat No. 421301) and RNase A (Bioshop Canada Inc., cat. RNA675) solution (PBS with 1% BSA, 0.25 mg/ml PI, 0.25 mg/ml RNAse A) overnight at 4°C, protected from light. Cells were then incubated overnight at 4 °C. Cells were passed through a cell strainer and then DNA content was measured by flow cytometry on a Beckman-Coulter Cytomics™ FC500 flow cytometer with at least 10,000 events counted per test [20].

RNA interference

For RNAi-mediated knockdown of gene expression, cells were seeded at 200,000 cells/well into 6-well dishes in antibiotic-free media and allowed to attach overnight. The next day, Lipofectamine® RNAiMax was used to deliver 30 pmol of either anti-HRAS siRNA (Thermo Fisher Scientific, Cat No.4390824.), anti-ERK1/2 siRNA (Cell Signalling Technology, Cat No.6560) or scrambled siRNA (Thermo Fisher Scientific; Cat No. 4390843) in Opti-MEM®. Media was replaced 24 h post-transfection and cells were allowed to recover for an additional 48 h prior to collection and lysis, or subsequent drug testing. Knockdown was confirmed by immunoblotting and real-time quantitative RT-PCR (qRT-PCR), described below.

For drug testing, cells were seeded into 96-well dishes at 2,400 cells/well. BYL719 was added the next day over a 10-point dose range (0–40 µM) and cells were incubated for 72 h. For each drug concentration, three replicates were completed per cell line. Cell viability was determined as described above. To calculate half-maximal inhibitory concentration (IC_{50}) values, relative fluorescence units (RFU) measures were normalized to the vehicle treatment (DMSO only). IC_{50} values (defined as the concentration at which the normalized RFU reached 50%) were then calculated by non-linear regression.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using AllPrep DNA/RNA Mini Kits (Qiagen). Eluted RNA was reverse transcribed to complementary DNA (cDNA) using QuantiTect Reverse Transcription Kits (Qiagen). qRT-PCR was then performed in 20 µl reactions, using Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific), 200 nM of each primer and