Inhibition of Mutant GNAQ Signaling in Uveal Melanoma Induces AMPK-Dependent Autophagic Cell Death

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Abstract
Oncogenic mutations in GNAQ and GNA11 genes are found in 80% of uveal melanoma. These mutations result in the activation of the RAF/MEK signaling pathway culminating in the stimulation of ERK1/2 mitogen-activated protein kinases. In this study, using a siRNA strategy, we show that mutant GNAQ signals to both MEK and AKT, and that combined inhibition of these pathways with the MEK inhibitor selumetinib (AZD6244) and the AKT inhibitor MK2206 induced a synergistic decrease in cell viability. This effect was genotype dependent as autophagic markers like beclin1 and LC3 were induced in GNAQ-mutant cells, whereas apoptosis was the mechanism of cell death of BRAF-mutant cells, and cells without either mutation underwent cell-cycle arrest. The inhibition of MEK/ATK pathways induced activation of AMP-activated protein kinase (AMPK) in the GNAQ-mutant cells. The downregulation of AMPK by siRNA or its inhibition with compound C did not rescue the cells from autophagy, rather they died by apoptosis, defining AMPK as a key regulator of mutant GNAQ signaling and a switch between autophagy and apoptosis. Furthermore, this combination treatment was effective in inhibiting tumor growth in xenograft mouse models. These findings suggest that inhibition of MEK and AKT may represent a promising approach for targeted therapy of patients with uveal melanoma. Mol Cancer Ther; 12(5); 768–76. ©2013 AACR.

Introduction
Mutant GNAQ and GNA11 are constitutive active and have oncogenic properties. Activating mutations at codon 209, and less frequently at codon 183, have been recently described in uveal melanoma (1–3), whereas BRAF mutations are relatively rare (4, 5). Uveal melanoma represents the most common intraocular malignancy. Approximately 95% of ocular melanomas are posterior uveal (ciliary body and choroid) in origin (6), and accounts for a significant rate of deaths. The development of metastasis occurs in 50% of patients with uveal melanomas within 15 years of initial diagnosis, even after treatment and removal of the primary tumor (7). We and other groups have reported that GNAQ-mutant uveal melanoma cells are relatively less sensitive to MEK inhibition than BRAF-mutant uveal or cutaneous melanoma (8, 9). Furthermore, mechanism of resistance with MEK inhibitors has been described in some cancers (10, 11) and more effective therapies are needed for the treatment of uveal melanoma.

G alpha protein subunits, classified into subfamilies by their sequence homology and downstream signals, include Gαq (GNAQ), Gα11, Gα12, Gα13, and Gα12/13. They transduce external signals from G protein-coupled seven transmembrane domain receptors (GPCR) to intracellular signaling pathways (12). GNAQ stimulation leads to activation of phospholipase C-β, which, through the production of inositol trisphosphate and diacylglycerol, elevates intracellular calcium levels and protein kinase C (PKC) activity. It has been reported that GNAQ binds and signals to phosphoinositide-3 kinase (PI3K; refs. 13–15), and that targeting PI3K/AKT pathway while inhibiting MEK/ERK is an effective approach to reduce the proliferation of uveal melanoma (8, 16, 17). However, the mechanisms involved in these processes are still unclear.

Autophagy is a regulated process of degradation and recycling of cellular constituents, participating in the bioenergetic management of starvation (18). During autophagy, parts of the cytoplasm are sequestered into double-membraned vesicles, called autophagosomes, which then fuse with lysosomes and degrading their content. Autophagy is considered cytoprotective, as it can rescue cells under conditions of starvation (19), whereas recent studies confirmed that autophagy also plays a role in cell death (20–22). Here, we showed that mutant GNAQ signals to both ERK and AKT pathways, and that dual inhibition of MEK and AKT induced synergistic inhibition of cell viability of GNAQ-mutant uveal melanoma in vitro and in xenograft tumors. This effect was accompanied by the activation of AMPK and induction of cell death by autophagy. These findings indicate that the combined inhibition of MEK and AKT is a valid therapeutic approach for the treatment of GNAQ-mutant uveal melanoma.
melanoma, and that AMPK is a key regulator in these tumor cells.

Materials and Methods

Cell culture

Mel202, Mel270, Mel290, 92.1 and OCM1A are primary choroidal uveal melanoma and Omm1.3 cells are liver metastasis from Mel270 (23). Omm1.3, Mel202 and Mel270 have been kindly provided by Dr. Bruce Ksander (Harvard Medical School, Boston, MA). OCM1A and 92.1 were from Dr. William Harbour (Washington University, St. Louis, MO). Mel290 and C918 were from Robert Folberg (University of Illinois, Chicago, IL). Uveal melanoma cell lines have been sequenced for the presence of activating mutations in codons 209 (exon 5) and 183 (exon 4) of GNAQ and GNA11 and for BRAF mutations. Omm1.3 and 92.1 cell lines were also authenticated by cytology analysis. Cells were cultured in RPMI medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin, and maintained at 37°C in 5% CO2. Cells were treated with selumetinib (AZD6244, ARRY-142866, AstraZeneca) and MK2206 (supplied by MERCK). Compound C was purchased from Sigma.

Cell viability assays and combination index analysis

Cells were plated in 96-well plates, and treated with the indicated concentrations of drugs. Viability was assessed by counting the cells with condensed and fragmented chromatin using a Nikon Eclipse TE2000-U microscope (Nikon Instruments Inc).

RNAi-mediated gene knockdown

Small-interfering RNA (siRNA) against GNAQ (Gq1, sc-35429), AMPK (sc-45312), and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. The SMARTPool GNAQ siRNA (Gq2) was from Dharmacon (L-008562). Single sequence siRNA was: CAUAAGG-CUCAUGCAACA (Gq3, Life Technologies, s5886). They were transfected using Lipofectamine RNAiMAX reagent (Invitrogen) following the manufacturer’s instructions.

GFP-LC3 transfections

GFP-LC3 was generated as described (24). Cells were grown on slides and transiently transfected with the GFP-LC3 plasmid using FuGENE 6 (Roche Applied Science). Treated and untreated cells were visualized using wide-field fluorescence microscopic images with a Nikon Eclipse TE2000-U microscope (Nikon Instruments Inc).

Immunoblotting

Cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail tablets (Complete Mini, Roche Diagnostics) and 1 mmol/L NaVO₃. Equal amounts of protein were loaded on 4%–12% PAGE gels (Invitrogen). Polyvinylidene difluoride membranes were blocked with 5% nonfat dried milk in PBS buffer containing 0.1% Tween-20 (PBST) and probed with pERK, ERK, pAKT, AKT, pAMPK, AMPK, pAkt, pBAD, p70S6K, mTor, pS6, beclin1, tubulin (Cell Signaling), and LC3 (Novus Biologicals) antibodies.

Animal studies

Severe combined immunodeficient mice (SCID) mice were purchased from Taconic, and used when they were 8-weeks old. A total of 15 × 10⁶ 92.1 cells were inoculated subcutaneously into the right flanks of the nude mice. When tumors reached a volume of approximately 100 mm³ diameter, animals were administered (5/group) vehicle, selumetinib 25 mg/kg orally, MK2206 150 mg/kg orally, alone and in combination. The treatment duration was 3 weeks and the tumor size was measured twice a week. After the fifth treatment, 2 animals from each cohort were sacrificed and the tumors were collected for Western blot analysis. Experiments were carried out under an Institutional Animal Care and Use Committee–approved protocol, and Institutional guidelines for the proper and humane use of animals were followed. Statistical significance was determined by 2-sample Student t tests.

Results

Depletion of mutant GNAQ inhibits multiple pathways

Uveal melanoma cells with GNAQ mutations are sensitive to the MEK inhibitor selumetinib, showing decreased pERK and cell-cycle arrest (9). To further explore signaling pathways downstream of mutant
GNAQ in uveal melanoma cells, GNAQ was downregulated using 2 specific pools of siRNA (Fig. 1). Western blot analysis confirmed decreased levels of GNAQ in all the cell lines, which correlated with a decrease in pERK and pAKT only in the GNAQ-mutant cells, compared to nonspecific control siRNA (Fig. 1A). The downregulation of pERK and pAKT was observed also using single siRNA sequences at different time points up to 72 hours (Supplementary Fig. S1). No changes in pERK and pAKT were detected in cell lines with wild-type GNAQ, and the levels of total ERK and AKT did not change in any cell line (Fig. 1A). Furthermore, downregulation of GNAQ decreased cell viability of the mutant cells only (Fig. 1B).

**Combined MEK and AKT inhibition synergistically suppresses cell viability and induces autophagy in GNAQ-mutant cells**

We hypothesized that combined inhibition of both pathways downstream of mutant GNAQ would induce enhanced antiproliferative effects. We used the MEK inhibitor selumetinib in combination with the AKT inhibitor MK2206 (structures shown in Fig. 1C) and measured cell viability of uveal melanoma cell lines with GNAQ mutation, in comparison with cells carrying a BRAF mutation, which also activates the mitogen-activated protein kinase (MAPK) pathway, or cells without either mutation (WT). Combinatorial treatments inhibited cell viability more effectively than monotherapy in GNAQ and BRAF-mutant cells (Fig. 2A–C). The treatments were less effective in the WT cells as higher doses of the drugs were required for the inhibition of cell viability (Fig. 2D). The combination of selumetinib + MK2206 at increasing doses consistently reduced cell viability in the mutant cells in a synergistic manner by more than 50% “fractional activity” (Fa > 0.5) and with CI values < 1 (Supplementary Fig. S2; ref. 25).

The effect of either drug alone or in combination on cell cycle and apoptosis was evaluated by flow cytometry. All the cell lines showed an arrest in G1 of the cell cycle with selumetinib treatment (Fig. 3A). MK2206 as single agent induced a modest G2 arrest, and the combination was similar to the effects of selumetinib alone. Furthermore, no
significant sub-G1 peak (apoptosis) was detected in the GNAQ-mutant cells with any treatment. A small apoptotic sub-G1 population was present in the BRAF-mutant cells treated with the combination. The WT cells had also a small sub-G1 population that did not change with the treatments. Overall, the decreases in cell growth with the combination therapy could not be accounted for by changes in cell-cycle distribution.

The combination treatment was also the most effective in decreasing colony survival of the GNAQ- and BRAF-mutant cells (Fig. 3B).

Western blot analysis was used to assess drug-mediated target inhibition and the effect on downstream signaling pathways. Selumetinib alone or in combination inhibited pERK in either GNAQ- or BRAF-mutant cell lines (Fig. 3C). In contrast, the WT cell line did not show a significant decrease in pERK levels. MK2206 inhibited pAKT in all the cell lines. Cyclin D1 was downregulated in all the cells by selumetinib and the combination treatment. To determine whether the treatments affected proapoptotic proteins, we examined expression levels of Bim and Bad. As previously reported (26), selumetinib induced Bim expression in all the cell lines, especially in OCM1A and Omm1.3.

Bad integrates survival signaling by MAPK and PI3K/Akt kinase pathways (27). Phosphorylation at ser-136 of Bad was inhibited by MK2206 in the BRAF-mutant and wild-type cells, but not in the GNAQ-mutant cells. Only the combination therapy was effective in decreasing pBad in these cells (Fig. 3C). However, the induction of Bim and the downregulation of pBad were insufficient to induce apoptosis in the GNAQ-mutant cells, as no PARP cleavage was detected at 24 hours (Fig. 3C), or 48 hours (data not shown). In contrast, cleaved PARP was detected in the BRAF-mutant cell line with the combination therapy, suggesting possible alternative ways of cell death in the GNAQ-mutant cells.

We then assessed the mTOR signaling pathway, which is important in the induction of autophagy. Phosphop70s6k and p-pS6 were inhibited by selumetinib and the combination with MK2206 in the mutant BRAF while they were inhibited only by the combination therapy in the GNAQ-mutant cells. Also, the energy sensor AMPK was induced in the GNAQ-mutant cells, as well as the autophagy marker beclin1. In addition, the levels of LC3-II were markedly elevated in the GNAQ-mutant cells, especially with the combination therapy, suggesting that autophagy may represent the major mechanism of cell death in these
cells. Induction of autophagy was further measured by the expression of a GFP-LC3 fusion protein, which localizes to autophagosomal membranes during autophagy. In control untreated cells (ND) or cells treated with single agents, GFP-LC3 localized evenly throughout the cytoplasm with little or no punctuations per cell (Fig. 4A). Both 92.1 and Omm1.3 GNAQ-mutant cells treated with selumetinib + MK2206 showed increased punctate autophagosomal structures, whereas WT cells did not show GFP-LC3 condensation under any condition (Fig. 4A). The percentage of cells that contained 2 or more punctuations is shown in Fig. 4B, indicating that 62 ± 4.0% to 74 ± 4.5% of the cells undergo autophagy with the combination therapy.

Activation of AMPK is essential in the autophagic response of GNAQ-mutant cells

Induction of autophagy in combination-treated GNAQ-mutant cells was accompanied by an increase in pAMPK, LC3 and beclin1. This was confirmed in mutant cells after GNAQ depletion by siRNA (Fig. 5A). pAMPK was upregulated in mutant cells but not in the wild-type cell line (Fig. 5A and Supplementary Fig. S3), showing the regulation of AMPK by the inhibition of mutant GNAQ signaling, and excluding drug unrelated effects. Also, beclin1 and LC3 were upregulated by GNAQ silencing, whereas no PARP cleavage was detected (Fig. 5A).

Activation of AMPK occurs in various cellular stress conditions when the AMP/ATP ratio is elevated, and it has been recently implicated in the induction of autophagy (28). To elucidate the role of AMPK in GNAQ-mutant cells under energetic stress, AMPK expression was silenced by siRNA. Upon treatment with selumetinib + MK2206, the AMPK-depleted cells showed induction of apoptosis by 2 independent methods, PARP cleavage (Fig. 5B) and quantitative fluorescent microscopy (Fig. 5E), in both 92.1 and Omm1.3 cell lines, with a concomitant decrease in beclin1 expression, suggesting that AMPK activation is mainly responsible for the induction of beclin1 and autophagy. The effect of AMPK downregulation was also assessed by flow cytometry (Supplementary Fig. S4). AMPK silencing caused an increase from 51.5% to 76.0% of cells in G1, suggesting a role of AMPK in cell-cycle regulation. With the combination treatment,
there was a further increase in G1 and the induction of a sub-G1 population (Supplementary Fig. S4).

Next, we used compound C, an inhibitor of AMPK, alone or together with selumetinib + MK2206. Compound C inhibited treatment-induced phosphorylation of AMPK and its specific substrate acetyl-CoA carboxylase (Fig. 5C). It also decreased the expression of beclin1 and LC3 and induced PARP cleavage when combined with selumetinib + MK2206. Furthermore, the direct suppression of beclin1 with siRNA induced PARP cleavage in GNAQ-mutant–treated cells (Fig. 5D) and increased apoptotic cells (Fig. 5E). The suppression of both GNAQ and AMPK by siRNA also resulted in induction of PARP cleavage (Supplementary Fig. S5).

AMPK activation thus serves to protect the cells from apoptosis by inducing autophagy. Nevertheless, autophagy is the mechanisms of cell death when inhibiting MEK and AKT in GNAQ-mutant cells. Altogether, these results show that AMPK phosphorylation is induced in response to mutant GNAQ signaling inhibition and it mediates an autophagic response, whereas its inhibition favors cell-cycle arrest in G1 and apoptosis under these conditions.

The combination of selumetinib with MK2206 inhibits growth of xenograft tumors

To test the effects of selumetinib and MK2206 in xenograft mouse models, the GNAQ-mutant cell line 92.1 was subcutaneously injected in mice. Animals were treated with vehicle, selumetinib, and MK2206 alone or in combination for 5 days each week for a total of 3 weeks. By day 21, the combination of selumetinib + MK2206 resulted in significant lower tumor volumes than vehicle or single agents (Fig. 6A). Tumor samples from each group were analyzed by immunoblotting. Tumors from treated mice showed inhibition of pERK and pAKT, and only specimens from mice treated with the combination selumetinib + MK2206 showed induction of pAMPK, beclin1, and LC3 (Fig. 6B), confirming the induction of autophagic markers, as in the in vitro experiments.

Discussion

Most uveal melanoma harbor mutually exclusive mutations of the GNAQ or GNA11 proteins, which lead to the activation of RAF/MEK/ERK pathway. In fact, these tumor cells are sensitive to MEK inhibition (2, 9).
However, MEK inhibitors cause cell-cycle arrest without significant cell death. The PI3K/AKT and mTOR/p70S6K pathways are critical for tumorigenesis, and the targeting of PI3K while inhibiting ERK has been shown to reduce the proliferation of uveal melanoma cells (8, 16, 17). Interestingly, we have shown that the constitutively active mutant GNAQ signals not only to ERK but also to AKT, possibly through PI3K direct binding (29) or cross talk between GPCR and receptor tyrosine kinase (RTK)-mediated signaling pathways (30, 31). We report that inhibition of both MEK and AKT induced a synergistic decrease in cell viability and this is associated with autophagic cell death. This is mediated by the activation of AMPK, a key regulator of energy homeostasis. In contrast, the blockade...
of AMPK by siRNA or using compound C in the setting of inhibition of GNAQ signaling caused downregulation of beclin1, converting autophagy into apoptosis. Recent studies have shown that activation of AMPK induced by metformin disrupted crosstalk between insulin/IGF-1 receptor and GPCR signaling and inhibited cell growth of pancreatic cancer cells (32). AMPK seems to be at the crossroad of multiple signaling pathways, and its activation in uveal melanoma coincided with induction of autophagy.

AMPK is activated under conditions of low intracellular ATP following stresses such as nutrient deprivation, or hypoxia (33), and numerous reports showed that AMPK is involved in the regulation of autophagic proteolysis (28, 34, 35). One of the major downstream signaling pathways regulated by AMPK is the mTOR pathway, through the phosphorylation of TSC2 and inhibition of Raptor (36), thereby impeding protein synthesis (37). In GNAQ-mutant cells, AMPK was activated by the inhibition of both AKT and MEK, with downstream inhibition of p70S6K and only partial downregulation of p-pS6, whereas LC3 and beclin1 were upregulated. Low levels of autophagy are considered a survival mechanism as they allow removal of damaged proteins and mitochondria and help maintain metabolic homeostasis. Conversely, high levels of autophagic activity can lead to cell death and cooperate with apoptosis (20). In addition, excessive stimulation of autophagy through overexpression of beclin1 has been associated with suppression of tumorigenesis, and eventually with cell death (38, 39).

The induction of autophagy in uveal melanoma was genotype-dependent as only GNAQ-mutant cells showed an increase in autophagic markers, as opposed to BRAF-mutant cells and cells without either mutation. Our group also reported that the effect of combined MEK and mTOR kinase inhibition in uveal melanoma cells depended on genotype, as the BRAF-mutant cells were particularly susceptible to these treatments compared to GNAQ-mutant cells (40). This might be due to the complexity of GPCR and G protein-mediated signaling. G proteins have multiple downstream binding partners and various regulatory scaffolding/adaptor and effector proteins that are often cell type specific (41).

The combination therapy selumetinib + MK2206 has been reported to have synergistic effects on the inhibition of lung cancer cells with RAS mutation, compared with single drug treatment, by the enhancement of Bim expression and induction of apoptosis (42). Despite a great increase in Bim expression and inhibition of pBAD in our cells, autophagy was the prevalent pathway of cell death in GNAQ-mutant cells. It has been reported that the treatment with inhibitors of PI3K and MEK induced apoptosis in GNAQ-mutant uveal melanoma cells, even though pAKT was not inhibited by GNAQ knockdown (17). It seems that the direct inhibition of AKT and MEK by specific inhibitors or GNAQ downregulation results in an energy depletion signal rather than an apoptotic stimulus like PI3K inhibition, and further studies are needed to characterize these differences.

The energy sensor AMPK was activated together with autophagic markers in GNAQ-mutant cells when AKT and MEK were inhibited, whereas its inhibition favored apoptosis. Although autophagy is considered a protective mechanism, it still mediated a synergistic decrease in cell viability of GNAQ-mutant cells, confirming that MEK and AKT inhibition is a valid strategy for the treatment of GNAQ-mutant cells. The combination therapy selumetinib + MK2206 is currently in a phase I clinical trial and is well tolerated in patients with advanced metastatic solid tumors (43).

The preliminary results of the single agent phase II study with selumetinib in patients with metastatic uveal melanoma appear promising, with inhibition of pERK, suppression of cyclin D1 and partial radiologic responses (44). Therefore, these results would indicate that combining MEK and AKT inhibitors could represent the next step in developing a novel therapeutic approach for the treatment of patients with this disease.

Disclosure of Potential Conflicts of Interest

A L Flo has a commercial research grant and is a consultant/advisory board member of AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: G. Ambrosini, G.K. Schwartz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Ambrosini, E. Musi, E. de Stanchina, G.K. Schwartz
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Ambrosini, E. Musi, A.L. Ho, G.K. Schwartz
Writing, review, and/or revision of the manuscript: G. Ambrosini, A.L. Ho, G.K. Schwartz
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