Overexpression of DDX43 Mediates MEK Inhibitor Resistance through RAS Upregulation in Uveal Melanoma Cells

Grazia Ambrosini, Raya Khanin, Richard D. Carvajal, and Gary K. Schwartz

Abstract

The majority of uveal melanomas carry oncogenic mutations in the G proteins GNAQ and GNA11, with consequent activation of the MAPK pathway. Selective MEK inhibitors, such as selumetinib, have shown clinical benefit in uveal melanoma. However, mechanisms of drug resistance limit their efficacy in some patients. Analysis of MEK inhibitor–resistant uveal melanoma cell lines revealed the induction of RAS protein expression and activity. This effect was mediated by the RNA helicase DDX43, which was remarkably overexpressed in these cells. Depletion of DDX43 in MEK inhibitor–resistant cells decreased RAS proteins and inhibited ERK and AKT pathways. On the contrary, ectopic expression of DDX43 in parental uveal melanoma cells induced RAS protein levels and rendered cells resistant to MEK inhibition. Similar to DDX43 depletion, downregulation of KRAS, HRAS, and NRAS inhibited downstream pathways in the resistant cells, overcoming mutant GNAQ signaling. We also analyzed the expression of DDX43 in liver metastases of patients with uveal melanoma by RT-PCR, and found a significant overexpression of DDX43 in patients who did not benefit from selumetinib therapy. In conclusion, DDX43 induces RAS protein expression and signaling, mediating a novel mechanism of MEK inhibitor resistance. The detection of DDX43 in patients with uveal melanoma could lead to more targeted therapies for this disease.

Introduction

Uveal melanoma accounts for approximately 5% of all melanomas. Most arise from melanocytes within the uveal tract, which consists of the iris, ciliary body, and choroid of the eye (1). This type of melanoma has a very high tendency to metastasize to the liver (2). As opposed to cutaneous melanoma, BRAF and RAS mutations are absent or extremely rare in uveal melanoma (3). Rather, activating mutations in G-protein α-subunits, GNAQ or GNA11, have been detected in nearly 80% of primary uveal melanomas (4–6), and mediate the activation of the MAPK pathway (4). The prognosis of patients with metastatic uveal melanoma is poor with a median 1-year survival rate of less than 30% (7, 8). Nearly all patients with uveal melanoma die due to metastatic disease and there are limited therapeutic options (9, 10). We have reported that the small-molecule MEK inhibitor, selumetinib, can inhibit pERK and cyclin D1, resulting in decreased viability of uveal melanoma cell lines (11). Furthermore, in patients with uveal melanoma, selumetinib treatment can result in tumor shrinkage, and the sustained inhibition of pERK may be predictive of benefit (12). Recently, our group reported the results of a randomized phase II clinical trial of selumetinib versus temozolomide in patients with metastatic uveal melanoma (13). These results indicated an improvement in the response rate and a significant median progression-free survival. Thus, selumetinib is the first drug in medical oncology to ever show a clinical benefit in patients with this disease. Despite these promising results, "de novo" and "acquired" resistance to this drug and other MEK inhibitors remains a major clinical problem (14). For example, resistance to selumetinib has been described in colorectal cancer cells carrying BRAF and RAS mutations, where resistance is mediated by the amplification of the driving oncogene (15, 16). KRAS overexpression has also been implicated in resistance to MEK inhibition in colon carcinoma (17). RAS activation has been described as a mechanism of acquired resistance to BRAF inhibitors in cutaneous melanoma with BRAF mutation, due to receptor tyrosine kinase (RTK) activation (18) or NFI loss (19). In uveal melanoma with GNAQ/GNA11 mutations, the mechanisms of resistance to MEK inhibitors are unknown and understanding this process will prove critical to develop more effective therapies for the treatment of this disease.
DDX43, also called HAGE, is a member of the DEAD-box family of ATP-dependent RNA helicases. It was first identified as a cancer/testis antigen, and it is highly expressed in many tumor types compared with normal tissues (20). In particular, DDX43 is overexpressed in 50% of acute myeloid leukemias (21), and its expression was associated with advanced disease and poor prognosis (22). These helicases browse RNA molecules and promote the dissociation of the RNA from ribonucleoproteins (23). In this way, they support processes such as gene transcription and posttranscriptional regulation, including pre-mRNA splicing, translation initiation/elongation, and RNA degradation (24–26). Their altered expression levels have also been implicated in tumor initiation, progression, and maintenance (27).

Recently, DDX43 was found highly expressed in melanoma (28), and it promoted protein expression of NRAS by unwinding of mRNA secondary structures, and enhancing NRAS translation (29). Here, we report that the expression of DDX43 is elevated in uveal melanoma cells with acquired resistance to selumetinib and in patients with metastatic uveal melanoma who did not benefit from selumetinib treatment. Furthermore, we demonstrated that DDX43 regulates RAS protein expression and AKT activation. Selumetinib-resistant cells became sensitive to AKT inhibition, suggesting alternative strategies for the treatment of patients with uveal melanoma with high DDX43 expression and resistance to MEK inhibitors.

Materials and Methods

Cell lines and reagents

The GNAQ (Q209P)-mutant cell lines, Omm1.3 and Mel270, were kindly provided by Dr. Bruce Ksander (Harvard Medical School, Boston, MA) in 2009. The cells have been sequenced for the presence of mutations in codons 209 (exon 5) and 183 (exon 4) of GNAQ and GNA11. A karyotype test was also performed for the cell line in 2012, confirming the authenticity of the cell line (28), and it promoted protein expression of NRAS by unwinding of mRNA secondary structures, and enhancing NRAS translation (29). Here, we report that the expression of DDX43 is elevated in uveal melanoma cells with acquired resistance to selumetinib and in patients with metastatic uveal melanoma who did not benefit from selumetinib treatment. Furthermore, we demonstrated that DDX43 regulates RAS protein expression and AKT activation. Selumetinib-resistant cells became sensitive to AKT inhibition, suggesting alternative strategies for the treatment of patients with uveal melanoma with high DDX43 expression and resistance to MEK inhibitors.

Cell viability assays

Cells were plated in 96-well plates, and treated with the indicated concentrations of drugs. Viability was assessed after 4 days of treatment using the Cell Counting Kit 8 from Dojindo Molecular Technologies according to the manufacturer’s instructions. Cell viability is expressed as a percentage of untreated cells.

Immunoblotting and RAS-GTP pull-down assays

The cells and the biopsy tissues were lysed in RIPA buffer supplemented with protease inhibitor cocktail tablets (Roche Diagnostics) and 1 mmol/L NaVO₃. Total protein concentration of the lysates was measured by BCA assay (Bio-Rad), and equal amounts of protein were loaded on 4% to 12% PAGE gels (Invitrogen). Polyvinylidene difluoride membranes were blocked with 5% non-fat dried milk in PBS buffer containing 0.1% Tween-20 (PBST) and probed with antibody for pERK, ERK, pAKT, pan-AKT, pRB, c-Jun, cyclin D1, tubulin (Cell Signaling Technology), DDX43 (Abcam), KRAS, HRAS (Abnova), and NRAS (Santa Cruz Biotechnology). GTP-bound RAS was measured using the RAF RAS-binding domain (RBD) pull-down and Detection Kit (Thermo Scientific), as instructed by the manufacturers.

RNAi-mediated gene knockdown

DDX43-1 and -2 siRNA were from OriGene. siRNA against GNAQ (sc-35429), KRAS (sc-35731), NRAS (sc-36004), HRAS (sc-29340), and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. They were transfected using Lipofectamine RNAiMAX reagent (Life Technologies). The human DDX43 cDNA clone in pCMV6-XL5 was obtained from OriGene.

Tissue sample collection and qRT-PCR

Matched tumor biopsies were collected from patients with metastatic uveal melanoma (clinicaltrials.gov, # NCT01143402). The protocol was approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center (New York, NY), and all patients signed informed consent forms. Total RNA was extracted from flash-frozen specimens and cell lines using TRIzol (Life Technologies). The resultant cDNA was used in RT-PCR reactions using an iCycler (Bio-Rad) with predesigned TaqMan Gene expression Assays for DDX43 and GAPDH genes (Life Technologies). The resulting C₅ values were averaged, the targets were interpolated from the standard curves and normalized to GAPDH. The results are shown as DDX43/GAPDH ratio and analyzed with the Wilcoxon Mann–Whitney test for two-sided P value.

Results

GNAQ-mutant cell lines with acquired resistance to selumetinib display differential expression of RAS and DDX43 and deregulated MAPK/AKT pathways

To explore mechanisms of drug resistance, we generated MEK inhibitor-resistant uveal melanoma cell lines. The GNAQ-mutant cell lines, Omm1.3 and Mel270, which are sensitive to selumetinib treatment (34), were chronically exposed to increasing doses of selumetinib. The resulting cell lines, Rs-Omm1.3 and Rs-Mel270, showed
that IC50 were at least 10-fold higher than their parental cells, Omm1.3 and Mel270, in cell viability assays (Fig. 1A). The cells also showed cross resistance to the MEK inhibitors PD0325901 and GSK1120212 (Supplementary Fig. S1). We then examined the expression levels and phosphorylation status of different components of the ERK pathway after MEK inhibition. In parental cells Omm1.3 and Mel270, increasing doses of selumetinib for 24 hours caused a decrease in pERK, cyclin D1, and pRb (Fig. 1B), whereas c-Jun and pAKT were slightly increased. In Rs-Omm1.3 and Rs-Mel270, pERK was also inhibited by the treatments (Fig. 1B), even though the signal seems to rebound over time (Supplementary Fig. S2). Furthermore, the downstream signaling was deregulated in these cells, with sustained expression of cyclin D1, pRb, and high expression of c-Jun and pAKT, independently of treatments (Fig. 1B).

We next analyzed the expression of RAS in all the cell lines. The parental cells expressed low levels of RAS, which slightly increased with higher doses of selumetinib. In contrast, RAS protein levels were elevated in the resistant cells regardless of treatments (Fig. 1B).

To test whether RAS protein expression corresponded to increased activity, we used RAF-RBD pull-down assays. In the parental Omm1.3 and Mel270 cells, basal RAS expression and activity were relatively low and temporarily induced by selumetinib without changes in RAS expression (Fig. 1C). This activation of RAS is possibly mediated by the downregulation of the Sprouty proteins by ERK inhibition, as reported (35). We have also shown that GNAQ-mutant cells have elevated expression of Spry2 that is downregulated by selumetinib (34). In contrast, both RAS expression and activity were elevated in Rs-Omm1.3 and Rs-Mel270 cell lines, and
remained active even when selumetinib was removed for 24 hours (Fig. 1C). These results show that the selumetinib-resistant cells have sustained activation of the RAS, ERK, and AKT pathways.

To determine whether the increase of RAS protein corresponded to higher RAS mRNA levels, we performed RT-PCR from parental and MEK inhibitor–resistant cells with or without treatment. We did not find any difference in RNA expression for KRAS or NRAS (data not shown). Rs-Omm1.3 and Rs-Mel270 cells were sequenced for MEK, KRAS, NRAS, HRAS mutations and these were negative. FISH analysis was performed to exclude gene amplification of the oncogene GNAQ or RAS proteins. Assays to detect activation of RTK were also negative.

It has been reported that the DEAD-box RNA helicase antigen DDX43 regulates NRAS protein expression through unwinding of duplex RNA in melanoma cells (29). Thus, we analyzed the expression of DDX43 in uveal melanoma cells, and found a remarkable upregulation of DDX43 in the MEK inhibitor–resistant cells, compared with low or no expression in their parental cells (Fig. 1D). Treatments with selumetinib did not further increase DDX43 expression either with increasing doses (Fig. 1D) or over time (Supplementary Fig. S2). The mRNA expression of DDX43 was analyzed by RT-PCR in all the cell lines, showing a 9-fold and 7.6-fold increase in Rs-Omm1.3 and Rs-Mel270 cells, compared with the respective parental cell lines (Fig. 1E).

**DDX43 mediates the expression of RAS and resistance to selumetinib**

To determine whether DDX43 was involved in MEK inhibitor resistance, DDX43 was overexpressed in the selumetinib-sensitive uveal melanoma parental cell lines, Omm1.3 and Mel270. In these cells, DDX43 induced the protein levels of KRAS, HRAS, NRAS, and increased...
pAKT (Fig. 2A). Surprisingly, the levels of pERK did not change, possibly because the pathway is regulated by mutant GNAQ through PKC activation, as reported (36). Nevertheless, higher expression of RAS has important implications as it regulates multiple pathways, including the PI3K/AKT pathway (37, 38). Next, we tested whether DDX43 affected the response to selumetinib. DDX43-expressing uveal melanoma cells were more resistant to selumetinib treatment compared with cells transfected with an empty vector (V) in viability assays (Fig. 2B), suggesting that DDX43 may mediate mechanisms of resistance to MEK inhibition through RAS and AKT activation.

We also evaluated the effect of DDX43 depletion in the MEK inhibitor–resistant cells by using two siRNA sequences (siDDX43-1, Fig. 2C; and siDDX43-2, Supplementary Fig. S3). Silencing of DDX43 affected the response to selumetinib. DDX43-expressing uveal melanoma cells were more resistant to selumetinib treatment compared with cells transfected with an empty vector (V) in viability assays (Fig. 2B), suggesting that DDX43 may mediate mechanisms of resistance to MEK inhibition through RAS and AKT activation.

**Figure 3.** RAS proteins regulate cell signaling in MEK inhibitor–resistant cells. A, selumetinib-resistant Rs-Omm1.3 (left) and Rs-Mel270 (right) cells were transfected with siRNA for KRAS, HRAS, and NRAS and analyzed for downstream signaling, that is, pMEK, pERK, pAKT, and c-Jun by immunoblotting. B, the cells were also transfected with GNAQ siRNA and analyzed for downstream signaling. C, KRAS and HRAS are necessary for survival of MEK inhibitor–resistant uveal melanoma (UM) cells. Rs-Omm1.3 and Rs-Mel270 cells depleted of each RAS protein or GNAQ were tested in cell viability assays after 72 hours of siRNA transfection. "*, P < 0.0001 and **, P < 0.001 for comparison of cells transfected with siKRAS and siHRAS versus control siRNA, for both cell lines.

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DDX43 depletion corresponded to a reduction in RAS activity in both MEK inhibitor–resistant cell lines (Fig. 2D). Most importantly, the depletion of DDX43 caused inhibition of cell viability of the MEK inhibitor–resistant cells, and this effect was further enhanced by the treatment with selumetinib (Fig. 2E), suggesting that DDX43 is required for MEK inhibitor resistance.

To confirm that the effects of DDX43 depletion are mediated by RAS downregulation, each RAS protein was silenced by gene-specific siRNA. KRAS and HRAS knockdown caused downregulation of pMEK, pERK, and pAKT in both cell lines (Fig. 3A), whereas NRAS did not. c-Jun was downregulated by KRAS and NRAS, whereas HRAS regulated expression of c-Jun only in Rs-Mel270. We have reported that GNAQ silencing inhibits pERK and pAKT, and decreases cell viability of the parental GNAQ-mutant uveal melanoma cells (31). We also tested the contribution of mutant GNAQ on downstream signaling pathways in the resistant cells. GNAQ downregulation induced minimal effects on downstream pathways (Fig. 3B). Similar to DDX43 downregulation, KRAS and HRAS depletion decreased cell viability of the selumetinib-resistant cell lines, whereas NRAS had partial effects (Fig. 3C). GNAQ depletion did
not affect cell viability of Rs-Omm1.3 and Rs-Mel270 cells (Fig. 3C), further demonstrating that RAS proteins become the dominant mediators of cell signaling in these cells.

**AKT inhibition inhibits cell viability of selumetinib-resistant GNAQ-mutant cells**

Elevated DDX43 and RAS expression mediated the activation of downstream survival pathways, especially pAKT (Figs. 1B and 2A). Thus, we assessed the effects of AKT inhibition in uveal melanoma cell lines. The AKT inhibitor MK2206 (AKTi) inhibited AKT phosphorylation in parental and MEK inhibitor–resistant cells (Fig. 4A). In cell proliferation assays, AKT inhibition had minimal effects in the parental cells, Omm1.3 and Mel270 (Fig. 4B), while it greatly inhibited cell viability of the resistant cell lines, Rs-Omm1.3 and Rs-Mel270 (Fig. 4B), suggesting that in these cells the DDX43/RAS-mediated activation of AKT is necessary for cell survival, and its inhibition may be a means of overcoming MEK inhibitor resistance.

**DDX43 is highly expressed in liver metastasis of patients with uveal melanoma**

To determine the relevance of DDX43 expression in patients with uveal melanoma, we assessed the expression of DDX43 by RT-PCR in liver metastasis of patients with uveal melanoma carrying GNAQ or GNA11 mutations who were treated on the phase II study of selumetinib. We analyzed biopsies from 14 patients, 6 of whom had partial response or stable disease for ≥16 weeks, defined as “responders” (R) according to RECIST, and 8 patients who had disease progression before 16 weeks with no evidence of radiological response, defined as “nonresponders” (NR). The “responder” patients had low expression of DDX43 (Fig. 5A). In contrast, DDX43 was highly expressed in the “nonresponder” group, and there was a statistically significant association with poor outcome ($P = 0.045$). The tissues from six representative patients were also analyzed by immunoblotting for protein expression. The patients who responded to therapy had complete inhibition of pERK with selumetinib treatment, whereas the “nonresponders” showed none or minimal inhibition (Fig. 5B). The protein levels of DDX43 in biopsy tissues were low in “responders,” whereas it was highly expressed in “nonresponders.” Similarly, levels of total KRAS and NRAS were elevated in the “nonresponder” group, while HRAS was not detectable in these specimens.

**Discussion**

The RAS/RAF/MEK/ERK pathway is often activated by genetic alterations in upstream signaling molecules, such as RAS and BRAF in cutaneous melanoma, and GNAQ/11 in uveal melanoma (4, 5). Hyperactivation of
this signaling cascade results in dysregulated cell proliferation and malignant transformation. Targeting this pathway with MEK inhibitors has shown activity in different types of cancers (39, 40), including uveal melanoma (13). However, resistance to MEK inhibitors is common and undermines the efficacy of these treatments (41, 42). Resistance to MEK inhibitors has been extensively described in cutaneous melanoma with BRAF and RAS mutations (43). Also in colon carcinoma, MEK inhibition leads to gene amplification of RAS and activation of downstream pathways (16). Activated RAS has been linked to MEK inhibitor resistance as it drives the activation of both the PI3K-AKT and MEK-ERK pathways (37). Here, we found that in uveal melanoma cell lines, the sustained inhibition of MEK leads to increased expression of DDX43, and consequent induction of RAS and downstream pathways. Furthermore, using RT-PCR, we found that DDX43 is overexpressed in patients who did not respond to selumetinib treatment, confirming the in vitro findings. Altered expression of DDX43 has been reported in human cancers (27, 44). In particular, DDX43 was required for cell proliferation of malignant melanoma-initiating cells by promoting the expression of NRAS through RNA unwinding (29). However, this is the first time that DDX43 has been linked to MEK inhibitor resistance through the regulation of RAS. Interestingly, it was reported that MEK inhibitor-resistant colon carcinoma cells showed increased KRAS expression, which represented a potential mechanism of MEK inhibitor resistance (17). However, the modalities of KRAS protein induction remained to be established.

Recently, Lith and colleagues have reported that RAF inhibitors cause relief of negative feedback with activation of RAS and rebound of ERK activity, without changes in RAS expression (35). We have shown a similar activation of RAS, which was induced by MEK inhibition in the parental cells. However, this effect was sustained in uveal melanoma selumetinib-resistant cells, where the expression of both DDX43 and RAS was elevated. DDX43 silencing decreased RAS proteins and downstream signaling, i.e., pERK and pAKT, making DDX43 a mediator of MEK resistance that could represent a class effect to all MEK inhibitors. Litt and colleagues reported an increase in c-Jun in colorectal cancer cells with acquired resistance to selumetinib (16). Indeed, c-Jun was highly expressed in MEK inhibitor-resistant cells, and it could be downregulated by knockdown of DDX43 and RAS, but not GNAQ, further confirming the importance of RAS signaling in these cells.

In our cells, DDX43 mediated "acquired" resistance to the MEK inhibitor, as its expression increased after long-term treatments. However, DDX43 seemed to mediate intrinsic resistance to selumetinib in patients with uveal melanoma with elevated expression at baseline. It was reported that DDX43 is highly expressed in chronic myeloid leukemia due to gene demethylation (22), and correlated with poorer prognosis in terms of cytogenetic response to drug treatments. Similar changes in DDX43 gene methylation may occur in uveal melanoma.

DDX43 has been also identified as a tumor-specific gene expressed in sarcoma (20), and it has been associated with poor clinical outcome in patients with breast cancer (45). Recently, several studies have provided new insights on RNA helicases and their emerging roles in cell signaling. For example, DDX3, another member of this family, was found to regulate the Wnt–β-catenin network in a genome-wide siRNA screen in human embryonic kidney cells (46). Another report showed DDX5 as a regulator of alternative splicing of HRAS (47).

Our results provide evidence that DDX43 increased the expression and activation of RAS in uveal melanoma cells with GNAQ mutation, and mediated resistance to MEK inhibitors.

Finally, these findings would indicate that DDX43 may have predictive value in determining who will most benefit from a MEK inhibitor therapy in this disease.

Disclosure of Potential Conflicts of Interest

R.D. Carvajal is a consultant/advisory board member for 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.): R.D. Carvajal, G.K. Schwartz

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