Identification of Unique MEK-Dependent Genes in GNAQ Mutant Uveal Melanoma Involved in Cell Growth, Tumor Cell Invasion, and MEK Resistance

Grazia Ambrosini¹, Christine A. Pratilas², Li-Xuan Qin³, Madhavi Tadi², Oliver Surriga¹, Richard D. Carvajal¹, and Gary K. Schwartz¹

Abstract

Purpose: Metastatic uveal melanoma represents the most common intraocular malignancy with very poor prognosis and no effective treatments. Oncogenic mutations in the G-protein α-subunit q and 11 have been described in about 85% of uveal melanomas and confer constitutive activation. Multiple signaling pathways are induced as a consequence of GNAQ/11 activation, which include the MEK/ERK kinase cascade. We analyzed the transcriptional profile of cell lines treated with a mitogen-activated protein (MAP)/extracellular signal–regulated (ERK) kinase (MEK) inhibitor to identify gene targets of activated GNAQ and to evaluate the biologic importance of these genes in uveal melanoma.

Experimental Design: We conducted microarray analysis of uveal melanoma cell lines with GNAQ mutations treated with the MEK inhibitor selumetinib. For comparison, we used cells carrying BRAFV600E and cells without either mutation. Changes in the expression of selected genes were then confirmed by quantitative real-time PCR and immunoblotting.

Results: We found that GNAQ mutant cells have a MEK-dependent transcriptional output and identified a unique set of genes that are downregulated by MEK inhibition, including the RNA helicase DDX21 and the cyclin-dependent kinase regulator CDK5R1 whereas Jun was induced. We provide evidence that these genes are involved in cell proliferation, tumor cell invasion, and drug resistance, respectively. Furthermore, we show that selumetinib treatment regulates the expression of these genes in tumor tissues of patients with metastatic GNAQ/11 mutant uveal melanoma.

Conclusions: Our findings define a subset of transcriptionally regulated genes by selumetinib in GNAQ mutant cells and provide new insights into understanding the biologic effect of MEK inhibition in this disease.

Introduction

The RAS/RAF/MEK/ERK pathway is often activated by genetic alterations in upstream signaling molecules, such as RAS and BRAF. Hyperactivation of this signaling cascade results in dysregulated cell proliferation and malignant transformation of a number of human tumors (1). Recently, the presence of somatic activating mutations in the heterotrimeric G-protein α-subunit q (GNAQ) and 11 (GNA11) in 46% and 30% of primary uveal melanomas, respectively, has been shown (2–4). These mutations occur in codon 209 in the RAS-like domain, resulting in loss of GTPase activity and constitutive activation. Approximately 85% of ocular melanomas arise from melanocytes within the uveal tract, which consists of the iris, ciliary body, and choroid of the eye (5), and account for a significant rate of deaths. Uveal melanoma is biologically distinct from cutaneous melanoma, as recurrent chromosomal abnormalities have been detected in chromosomes 3 and 8 (6), as well as a very high tendency to metastasize to the liver (7). NRAS mutations have not been detected in uveal melanoma (8), and BRAF mutations are considered rare (9, 10); however, activation of the mitogen-activated protein kinase (MAPK) pathway by mutant GNAQ/11 appears to be critical for the development of this disease (2). G-protein α-subunits transduce external signals from 7-transmembrane domain G-protein–coupled receptor (GPCR) to intracellular signaling pathways. The canonical target of GNAQ/11 stimulation is phospholipase C-β, which, in turn, stimulates inositol lipid signaling [i.e., calcium and protein kinase C (PKC)], in response to mitogenic GPCR agonists (11, 12). The constitutively active mutant GNAQ and GNA11 have been reported to activate the extracellular signal–regulated (ERK)
MEK Regulated Genes in Uveal Melanoma with GNAQ Mutation

Translational Relevance

Uveal melanoma is the most common primary intraocular malignancy in adults. Metastasis occurs frequently and there are no effective therapies. Recently, it has been shown that 85% of uveal melanomas have oncogenic mutations in the GNAQ/11, which activate the mitogen-activated protein kinase (MAPK) pathway. Here, we analyzed the transcriptional profile of GNAQ mutant cell lines treated with selumetinib, an MAP/extracellular signal–regulated (ERK) kinase (MEK) inhibitor, currently in clinical trial for uveal melanoma. We found that these cells have a MEK functional activation signature and a unique set of MEK-dependent genes involved in proliferation, cell invasion, and drug resistance. These genes are also regulated in pre- and posttreatment tumor biopsies obtained from patients with uveal melanoma treated with selumetinib, and their expression may correlate to clinical activity. Our findings provide new insights into the biologic effect of selumetinib in this disease and may have profound implications for the clinical development of MEK inhibitors in uveal melanoma.

pathway (2, 4), and knockdown of mutant GNAQ in uveal melanoma cells resulted in MAPK inhibition, reduced growth, and induced apoptosis (2). However, the transcriptional output of ERK signaling downstream of mutant G-proteins is not well characterized. Targeting MAP/ERK kinase (MEK) with allosteric small-molecule inhibitors has been reported to be effective in suppressing cell growth and, in some cells, inducing apoptosis. In particular, melanoma, thyroid, and non–small-cell lung cancer with mutant BRAFV600E have been shown to be sensitive to MEK inhibitors (13–15). In a large number of tumor types treated with the MEK inhibitor selumetinib (AZD6244), Dry and colleagues have reported transcriptional signatures that predict MEK addiction or drug resistance (16). In another study, using microarray analysis of cells treated with a MEK inhibitor, Pratillas and colleagues have found genes, including members of the dual specificity phosphatase and sproty gene families, that were differentially regulated by MEK inhibition in BRAFV600E cells but not in receptor tyrosine kinase–driven tumor cells with similarly elevated levels of pERK (17). They also showed that BRAFV600E cells have elevated ERK-dependent transcriptional output and disabled feedback inhibition of RAF/MEK signaling. Here, we report that uveal melanoma cells with GNAQ mutations are highly sensitive to MEK inhibition with selumetinib. Expression microarray analysis identified a MEK-dependent transcriptional profile that is, in part, similar to that of BRAFV600E melanoma cells. In addition, we identified several genes unique to GNAQ mutant cells, which are involved in proliferation and tumor cell invasion. Furthermore, pre- and posttreatment tumor biopsies from an ongoing clinical trial of selumetinib in patients with uveal melanoma indicate that these genes are transcriptionally regulated and may correlate with clinical benefit.

Materials and Methods

Cell culture

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). OCM3, 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. Two cell lines had Q209L mutation (92.1, Mel202), whereas Omm1.3 and Mel270 had Q209P mutation. None had GNA11 mutations. Cells were cultured in RPMI medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin and maintained at 37°C in 5% CO2. Cells were treated with selumetinib (AZD6244, graciously supplied by AstraZeneca).

Cell viability assays

Cells were plated in 96-well plates and treated with the indicated concentrations of selumetinib, PD0325901, or dimethyl sulfoxide (DMSO) in triplicates. Viability was assessed after 4 days of treatment using the Cell Counting Kit 8 (CCK8) from Dojindo Molecular Technologies according to the manufacturer’s instructions. Survival is expressed as a percentage of untreated cells. The calculations of combination index values were conducted using the CompuSyn software (CompuSyn; ref. 18).

Microarray data analysis

Cells were treated in triplicate with 250 nmol/L selumetinib, or 0.01% DMSO as control, for 8 hours. Following RNA extraction with TRIzol reagent (Invitrogen), cDNA was synthesized in the presence of oligo(dT)24-T7 from Genset Corp. cRNA was prepared using biotinylated UTP and CTP and was hybridized to Human HT-12 oligonucleotide Illumina arrays in triplicates. We used a single array slide for each cell line to minimize the effect of experimental artifact. Differential expression analysis was conducted to identify genes whose expression is affected by the treatment by comparing the posttreatment expression versus the pretreatment expression. The array data were log2-transformed and quantile-normalized. For each cell line, gene expression was compared between time points using the empirical Bayesian method and the R LIMMA package. An empirical Bayes t test was applied to each gene (19). Linear models and empirical Bayes methods were used for assessing differential expression in microarray experiments and a P value cutoff of 0.0001 was used to select differentially expressed genes. There are about 47,000 markers on the Illumina array and 5 genes are expected by chance to have a P value<0.0001. Data are deposited at GEO accession no. GSE33655.
**Immunoblotting**

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail tablets (Roche Diagnostics) and 1 mmol/L Na$_2$VO$_4$. Equal amounts of protein were loaded on 4% to 12% PAGE gels (Invitrogen). Polyvinylidene difluoride (PVDF) membranes were blocked with 5% nonfat dried milk and probed with pERK, ERK, cyclin D1, CDK5R1 (p35), c-Jun, and α-tubulin (Cell Signaling); SPRY2, DUSP6, ETV5, and DDX21 (Abcam).

**Quantitative real-time PCR**

Reverse transcription of 1 μg of RNA was done using the SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR (qRT-PCR) assays were done on the 7300 Real Time PCR System (Applied Biosystems). TaqMan gene expression assays, which include gene-specific probe primer sets (Applied Biosystems), were used to detect the indicated genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)/hypoxanthine phosphoribosyltransferase (HPRT) mRNA. The relative expression of each gene was calculated by the ΔΔC$_T$ method.

**RNA interference–mediated gene knockdown**

The list of siRNA is reported in the Supplementary Methods. siRNAs were transfected using Lipofectamine RNAiMAX reagent (Invitrogen). After transfections, cells were counted using a Nexcelom cell counter and plated in 96-well plates for cell viability assays. The statistical significance of the experimental results was determined by the 2-sided t test.

**Tissue sample collection and analysis**

Matched tumor biopsies were collected from patients with metastatic uveal melanoma (clinicaltrials.gov; no. NCT01143402) before and after 14 days of selumetinib. Tissue sample collection and analysis. Cells from each genetically defined subgroup of uveal melanoma (3 cell lines with GNAQQ209L/P, 1 line with BRAFV600E, and 1 cell line WT for both) were treated with 250 nmol/L selumetinib or vehicle for 8 hours. Genes whose change upon MEK inhibition exceeded defined statistical thresholds for all 3 GNAQQ209L/P cell lines were considered significant and defined the ERK-dependent transcriptional output of GNAQQ209L/P uveal melanoma cells. A total number of 387 genes met the significance level of $P < 0.0001$ in the GNAQQ209L/P cells. Identical parameters were used to define the set of genes regulated differentially by MEK inhibitor in the BRAFV600E and WT cell lines, and a direct comparison was conducted to evaluate common genes among the genetic subgroups. Of the 387 genes determined to have changed significantly in response to selumetinib, 308 were differentially expressed only in GNAQQ209L/P cells, whereas 29 overlapped with BRAFV600E MEK-dependent genes, 42 overlapped with genes meeting significance in WT cells, and 8 genes were common to all 3 groups (Fig. 1B; Venn diagram). The top 19 genes with highest significance and fold change common to all GNAQQ209L/P cells before and after treatment with selumetinib are displayed in a heatmap (Fig. 1D). A list of P values and fold change of representative genes for each group (GNAQQ209L/P-only and overlap groups) are shown in Table 1. Of note, several of the genes that were shared between GNAQQ209L/P and BRAFV600E cells were previously described ERK targets, such as CCND1, transcription factors ETV5, MYC, and genes involved in the feedback inhibition of MEK/ERK signaling, that is, dual specificity phosphatase 6 (DUSP6), and sprouty family members SPRY2, SPRY4. These genes are key components of MEK signatures.

**Expression profile of GNAQQ209L/P cells compared with BRAFV600E MEK signature**

To identify the transcriptional profile of MEK inhibition in GNAQQ209L/P cells and potential novel targets of GNAQQ209L/P signaling, we used microarray gene expression analysis. Cells from each genetically defined subgroup of uveal melanoma (3 cell lines with GNAQQ209L/P, 1 line with BRAFV600E, and 1 cell line WT for both) were treated with 250 nmol/L selumetinib or vehicle for 8 hours. Genes whose change upon MEK inhibition exceeded defined statistical thresholds for all 3 GNAQQ209L/P cell lines were considered significant and defined the ERK-dependent transcriptional output of GNAQQ209L/P uveal melanoma cells. A total number of 387 genes met the significance level of $P < 0.0001$ in the GNAQQ209L/P cells. Identical parameters were used to define the set of genes regulated differentially by MEK inhibitor in the BRAFV600E and WT cell lines, and a direct comparison was conducted to evaluate common genes among the genetic subgroups. Of the 387 genes determined to have changed significantly in response to selumetinib, 308 were differentially expressed only in GNAQQ209L/P cells, whereas 29 overlapped with BRAFV600E MEK-dependent genes, 42 overlapped with genes meeting significance in WT cells, and 8 genes were common to all 3 groups (Fig. 1B; Venn diagram). The top 19 genes with highest significance and fold change common to all GNAQQ209L/P cells before and after treatment with selumetinib are displayed in a heatmap (Fig. 1D). A list of P values and fold change of representative genes for each group (GNAQQ209L/P-only and overlap groups) are shown in Table 1. Of note, several of the genes that were shared between GNAQQ209L/P and BRAFV600E cells were previously described ERK targets, such as CCND1, transcription factors ETV5, MYC, and genes involved in the feedback inhibition of MEK/ERK signaling, that is, dual specificity phosphatase 6 (DUSP6), and sprouty family members SPRY2, SPRY4. These genes are key components of MEK signatures.
described in other tumor cells (16, 17), and we confirmed their decline in expression (mRNA and protein levels) after selumetinib treatment, using immunoblotting and qRT-PCR (Fig. 2). Following exposure to selumetinib for up to 24 hours, pERK levels were determined by immunoblotting (Fig. 2A). In the less sensitive WT cell line, pERK was slightly downregulated at 2 hours and quickly rebounded at later time points, whereas its inhibition was more complete in cells with BRAFV600E and GNAQQ209L/P mutations. Basal expression levels of ETV5, DUSP6, and SPRY2 proteins were almost undetectable in the WT cell line, whereas they were highly expressed in the mutant cells (both BRAFV600E and GNAQQ209L/P), confirming elevated transcriptional output of MEK signaling in these cells (17). Cyclin D1 was durably downregulated in BRAFV600E cells, whereas its expression rebounded in GNAQQ209L/P and WT cells at later time points. The induction of pMEK did not occur in BRAFV600E cells, in accordance with the reported abrogation of negative feedback between ERK and RAF proteins by BRAFV600E (17, 20). The microarray results of these MEK-dependent genes were also confirmed by qRT-PCR in 3 GNAQQ209L/P cell lines, where these genes were suppressed (Fig. 2B–D). The high basal expression of these transcripts and their downregulation by selumetinib confirmed that the MEK/ERK pathway is active in GNAQQ209L/P cells and that the transcriptional events described in GNAQQ209L/P are at least, in part, similar to reported MEK functional activation signatures. For example, Pratilas and colleagues reported a signature of 52 MEK-dependent genes in BRAFV600E cells (17), of which 19 are represented among our 345 genes (5.5%) and are significantly overenriched ($P < 0.0001$).

Figure 1. Selumetinib inhibits cell viability of BRAFV600E and GNAQQ209L/P cell lines. A, cells with wild-type GNAQ/BRAF (C318, Mel290), mutant GNAQ (92.1, Omm1.3, Mel270, Mel202), and BRAFV600E (OCM3, OCM1A) were treated with increasing concentrations of selumetinib (0, 25, 50, 100, 250, 500, 1,000 nmol/L) and analyzed for cell viability on day 5, calculated as percentage of untreated controls. Data are representative of 3 independent experiments. Bars, mean ± SD. B, immunoblots of pERK and pMEK in response to increasing concentration of selumetinib in cells with different mutational status. Microarray results: C, Venn diagram summarizing differentially expressed genes in selumetinib-treated GNAQQ209L/P cell lines (green circle), BRAFV600E (red circle), and WT cells (blue circle), with corresponding overlapping genes as indicated. D, heatmap representation of top 19 genes identified as meeting the statistical threshold (see Materials and Methods) for significant change in expression in all the cell lines and with fold change > 2 in GNAQQ209L/P cell lines, in response to 250 nmol/L selumetinib (gray bar), or DMSO as control for 8 hours, in triplicate. One replicate of the Mel290 cell line was excluded as it did not cluster with the other 2 replicates. Cell lines with individual replicates are columns, genes are rows.
From this list, we selected 3 genes, 2 downregulated induced upon treatment in GNAQQ209L/P cells, whereas levels of DDX21 and CDK5R1 (Fig. 3A–C). Also, Jun was inhibition over time but not in the BRAFV600E and WT cell lines significantly change in WT cells (Fig. 3B and C). In the BRAFV600E MEK inhibition regulates unique genes in GNAQQ209L/P cells. In contrast, Jun protein expression was high in cells, whereas it was quickly downregulated in BRAFV600E cells. In terms of protein expression, the change in c-Jun (induction in GNAQQ209L/P, suppression in BRAFV600E, and no change in WT) was similar to selumetinib, but

| Table 1. List of differentially expressed genes in response to selumetinib |
|---------------------------------|-----------------|-----------------|-----------------|
| Genotype                        | Probe ID        | Gene name       | P values        | Log2 FC       |
| GNAQ^{Q209L/P}                  | ILMN_1730928    | CDK5R1          | <10^-11         | -1.96         |
| GNAQ^{Q209L/P}                  | ILMN_1735461    | DDX21           | <10^-7 to 10^-11| -1.73         |
| GNAQ^{Q209L/P}                  | ILMN_1806023    | Jun             | <10^-3 to 10^-12| +1.64         |
| GNAQ^{Q209L/P}                  | ILMN_1748476    | NOP58           | <10^-8 to 10^-9 | -1.49         |
| GNAQ^{Q209L/P}                  | ILMN_2330267    | ABCE1           | <10^-6 to 10^-10| -1.51         |
| GNAQ^{Q209L/P}                  | ILMN_1806106    | GNL3            | <10^-8 to 10^-10| -1.50         |
| GNAQ^{Q209L/P}                  | ILMN_1776577    | DSCC1           | <10^-5 to 10^-9 | -1.41         |
| GNAQ^{Q209L/P}                  | ILMN_1674302    | PPAT            | <10^-9 to 10^-11| -1.37         |
| GNAQ^{Q209L/P}                  | ILMN_2221564    | LYAR            | <10^-9 to 10^-10| -1.36         |
| GNAQ^{Q209L/P}                  | ILMN_2067708    | TFB2M           | <10^-7 to 10^-10| -1.35         |
| GNAQ^{Q209L/P}                  | ILMN_1683120    | UNG             | <10^-8 to 10^-10| -1.34         |
| GNAQ^{Q209L/P}                  | ILMN_1739222    | ETV5            | <10^-13 to 10^-14| -2.34         |
| GNAQ^{Q209L/P}                  | ILMN_2089329    | SPRY2           | <10^-12 to 10^-15| -2.33         |
| GNAQ^{Q209L/P}                  | ILMN_2396020    | DUSP6           | <10^-9 to 10^-12| -1.94         |
| GNAQ^{Q209L/P}                  | ILMN_1688480    | CCND1           | <10^-11 to 10^-12| -1.81         |
| GNAQ^{Q209L/P}                  | ILMN_1729691    | SLC16A6         | <10^-8 to 10^-12| -1.80         |
| GNAQ^{Q209L/P}                  | ILMN_2086105    | SPRY4           | <10^-10 to 10^-13| -1.61         |
| GNAQ^{Q209L/P}                  | ILMN_2329914    | SPRY1           | <10^-6 to 10^-10| -1.50         |
| GNAQ^{Q209L/P}                  | ILMN_1737184    | CDC7            | <10^-6 to 10^-10| -1.20         |
| GNAQ^{Q209L/P}                  | ILMN_1677765    | LRP8            | <10^-7 to 10^-10| -1.20         |
| GNAQ^{Q209L/P}                  | ILMN_1680618    | MYC             | <10^-6 to 10^-10| -1.10         |
| GNAQ^{Q209L/P}                  | ILMN_1744147    | OSBPL8          | <10^-7 to 10^-10| -1.56         |
| GNAQ^{Q209L/P}                  | ILMN_1782459    | CEBPZ           | <10^-7 to 10^-8 | -1.35         |
| GNAQ^{Q209L/P}                  | ILMN_2131523    | SACS            | <10^-6 to 10^-9 | -1.34         |
| GNAQ^{Q209L/P}                  | ILMN_1687978    | PHDHA1          | <10^-5 to 10^-10| -1.33         |
| GNAQ^{Q209L/P}                  | ILMN_1733356    | PREI3           | <10^-5 to 10^-10| -1.11         |

NOTE: List of most significant genes regulated by selumetinib with >2-fold change (average for all GNAQ^{Q209L/P} cells, log2 scale) in GNAQ^{Q209L/P} cells only, or shared with BRAF^{V600E} and WT cells. A range of P values for each group of cell lines is shown. Log2 FC = average fold change for 3 GNAQ^{Q209L/P} cell lines.
suppression of CDK5R1 and DDX21 was observed in both GNAQQ209L/P and BRAFV600E cell lines (Supplementary Fig. S3A). Interestingly, PD0325901 inhibited pERK in the WT cell line but this resulted in essentially no reduction in cell viability, nor a decrease in cyclin D1 expression. Overall, these results suggest that all the effects of selumetinib on transcriptional expression in uveal melanoma cells may not be shared by other MEK inhibitors. This will require further investigation.

MEK-dependent GNAQQ209L/P-specific genes mediate cell proliferation and cell migration

Of the genes uniquely downregulated by selumetinib in GNAQQ209L/P cells, we focused on DDX21 and CDKR51. DDX21 is a member of RNA helicase family, which contains a motif (DExD/H) highly conserved from bacteria to humans (21). They are multifunctional proteins involved in RNA unwinding and play a role in transcription regulation (22). To test whether DDX21 is involved in the antiproliferative effects of selumetinib, we carried out gene knockdown experiments. Three GNAQQ209L/P mutant cell lines, as well as BRAFV600E and WT cells, were transiently transfected with 2 different DDX21-specific or control siRNAs (Fig. 4A, bottom; Supplementary Fig. S4A). Depletion of DDX21 reduced cell viability of all the cell lines, indicating a role for DDX21 in proliferation of these cells. Furthermore, DDX21 downregulation contributes to the antiproliferative effects of selumetinib in these cells. Interestingly, DDX21 downregulation did not affect migration of the cell lines (data not shown).

In contrast, DDX21 downregulation did not affect migration of the cell lines (data not shown).

CDK5R1 encodes p35, a specific activator of the serine/threonine kinase CDK5, which plays a crucial role in central nervous system development and maintenance (23). A role for CDK5 and p35 in cell migration has also been reported (24, 25). Seeking to establish a possible functional role underlying the observed differences in CDK5R1 expression, we assessed the impact of knockdown of this protein in uveal melanoma cells using 2 different siRNAs. CDK5R1 siRNA did not significantly inhibit cell viability (Supplementary Fig. 4C). All 3 GNAQQ209L/P cell lines depleted of CDK5R1 (Fig. 4B, bottom) showed a decrease in cell migration (Fig. 4B, top) compared with cells with control siRNA. The WT cells (Fig. 4B), as well as the BRAFV600E (Supplementary Fig. 6), showed no inhibition of migration with siRNA to CDK5R1, whereas GNAQ siRNA inhibited migration of the GNAQQ209L/P cell line Omm1.3 (Supplementary Fig. 6). Interestingly, selumetinib inhibited migration of both GNAQQ209L/P and BRAFV600E, but not the WT cells (Supplementary Fig. 6), suggesting that selumetinib may have effects on the cell migration of BRAFV600E cells, but this effect is independent of CDK5R1.

Jun expression is related to sensitivity to MEK inhibition

c-Jun is a component of the AP-1 transcription complex regulated by c-Jun-NH2-kinase (JNK), and it is involved in a number of cell responses, such as cell proliferation and cell death (26). c-Jun was upregulated in GNAQQ209L/P cells after selumetinib treatment, showing a differential
regulation compared with cells with BRAFV600E. Knockdown of c-Jun by 2 different siRNAs (Fig. 4C; Supplementary Fig. S7A) significantly increased the antiproliferative effects of selumetinib in GNAQQ209L/P cell lines (Fig. 4D; Supplementary Fig. S7B), suggesting that c-Jun induction may be involved in mechanisms of resistance to MEK inhibition. In contrast, c-Jun did not seem to play a role in WT and BRAFV600E cells as its knockdown did not alter the sensitivity to the selumetinib when compared with control siRNA (Fig. 4D; Supplementary Fig. S7B).

**Validation of GNAQQ209L/P-specific ERK transcriptional output in tumor tissues**

To assess the clinical efficacy of MEK inhibition on uveal melanoma, we are conducting a phase II clinical trial of selumetinib versus temozolomide in patients with metastatic uveal melanoma (clinicaltrials.gov; no. NCT01143402). Matched tumor biopsies are collected to examine target modulation between baseline and day 14 from patients with GNAQ/11 mutations receiving selumetinib. Results from 3 representative biopsy pairs are shown (Fig. 5A). The results of all the patients in the trial will be reported separately. Sustained inhibition of pERK and suppression of cyclin D1 were observed in patients A and B on day 14, but not in patient C. This correlated to best clinical response by Response Evaluation Criteria in Solid Tumors (RECIST; ref. 27): partial response in liver metastases in patient A (Fig. 5B), stable disease in patient B, and progression of disease in patient C. Furthermore, DDX21 was downregulated by selumetinib in patients A and C, CDK5R1 decreased in patient A and B, whereas they could not be detected in patients B and C, respectively. c-Jun expression increased in patient C (Fig. 5A). A similar trend has been seen in other patients treated on this clinical trial. These preliminary results are consistent with our in vitro studies indicating the existence of unique subset of genes in GNAQQ209L/P cells that are regulated by selumetinib and the expression of which could have an impact on clinical outcome.

**Discussion**

Uveal melanoma represents the most common intraocular malignancy. However, there are no effective treatments for this aggressive disease. Selumetinib is the only MEK inhibitor in clinical trials currently in the United States for patients with uveal melanoma. Here, we report that cells with GNAQQ209L/P mutations are sensitive to MEK inhibition by selumetinib, and sensitization was associated with a MEK-dependent gene expression profile. Some features of this profile are overlapping with that elicited in BRAFV600E uveal melanoma cells and other cell types (16), which
supports the MEK dependence of GNAQ<sub>Q209L/P</sub> cells. This gene profile includes the dual-specificity phosphatases (DUSP4/6), the sprouty homologues (SPRY1/2/4), which are known transcriptional targets of the ERK pathway involved in negative feedback regulation of ERK. The Ets variant transcription factor ETV5 was also regulated by MEK inhibition, along with cell-cycle division–associated protein 7 (CDCA7), the proto-oncogene MYC, and the solute carrier family 16, member 6 (SLC16A6).

Additional features of the MEK profile were identified as specific for GNAQ<sub>Q209L/P</sub> cells. A number of genes suppressed in GNAQ<sub>Q209L/P</sub> cells by selumetinib, such as LYAR, NOP58, GNL3, and PPAT, were reported as nuclear proteins involved in cell growth and tumorigenesis (28–31). DDX21 was recently identified as a novel biomarker for colorectal cancer (32), whereas CDK5 and CDK5R1 (p35) to increase malignant progression and invasion of pancreatic cancer cells (35). It is plausible that mutant GNAQ acts similarly to mutant K-RAS, as CDK5R1 expression was in fact elevated in the GNAQ<sub>Q209L/P</sub> cells compared with cells with other genetic backgrounds. Furthermore, it has been reported that CDK5 negatively regulates JNK3 activity and its target c-Jun, to prevent apoptosis in developing neurons (36). This implicates a possible interaction between these proteins in promoting survival of uveal melanoma cells.

Jun was upregulated after selumetinib treatment in the GNAQ<sub>Q209L/P</sub> cells only. Depending on the cell type and drug treatment, c-Jun and Jun kinase have been implicated in both pro- and antiapoptotic responses (37). In cutaneous melanoma cells, active ERK induces c-Jun expression (38). In contrast, c-Jun was induced by MEK inhibition of CDK5 and CDK5R1 (p35).
in GNAQ \(^{Q209L/P}\) uveal melanoma cells, suggesting a differential regulation of the ERK/JNK pathway. G-protein-mediated signaling is complex and involves multiple downstream binding partners and various regulatory scaffolding/adaptor and effector proteins [12]. For example, PKC is a target of GNAQ activation, and it might be involved in feedback regulation of c-Jun when ERK is inhibited. The upregulation of c-Jun could represent an alternative route to cell proliferation, which would explain the relative lower sensitivity to selumetinib of GNAQ \(^{Q209L/P}\) cells as compared with BRAF \(^{V600E}\) cells. Interestingly, increased expression of c-Jun has also been reported in colorectal cancer cells with K-RAS or BRAF mutations after acquired resistance to selumetinib [39]. We showed that the antiproliferative effect of selumetinib can be enhanced by suppressing c-Jun in the GNAQ \(^{Q209L/P}\) cells. This would suggest that targeting c-Jun in the presence of MEK inhibition would result in enhanced antitumor effects and may prevent selumetinib resistance. In conclusion, our findings define a unique molecular profile of MEK inhibition by selumetinib in uveal melanoma cells with mutant GNAQ and point to a set of transcriptionally modified genes that could have an impact on the activity of this agent in this disease.

Disclosure of Potential Conflicts of Interest
C.A. Pratilas is a consultant/advisory board member for Roche. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: G. Ambrosini, C.A. Pratilas, G.K. Schwartz
Development of methodology: G. Ambrosini, G.K. Schwartz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Ambrosini, O. Surriga, R.D. Carvajal, G.K. Schwartz
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.A. Pratilas, L.-X. Qin, M. Tadi, G.K. Schwartz
Writing, review, and/or revision of the manuscript (i.e., reporting or organizing data, constructing databases): G. Ambrosini, G.K. Schwartz
Study supervision: G. Ambrosini, G.K. Schwartz

Grant Support
This work was supported by Melanoma Research Alliance and Cycle for Survival funds. C.A. Pratilas was supported by the NIH grant # K08-127350. The costs of publication of this article were Defrayed in part by the payment of page charges. This article must therefore be hereby marked this fact.

Received December 2, 2011; revised April 18, 2012; accepted April 26, 2012; published OnlineFirst May 1, 2012.

References
event in uveal melanomas although it rarely occurs through mutation of BRAF or RAS. Br J Cancer 2005;92:2032–8.
Identification of Unique MEK-Dependent Genes in GNAQ Mutant Uveal Melanoma Involved in Cell Growth, Tumor Cell Invasion, and MEK Resistance

Grazia Ambrosini, Christine A. Pratilas, Li-Xuan Qin, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-3086

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/05/01/1078-0432.CCR-11-3086.DC1

Cited articles
This article cites 38 articles, 16 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/18/13/3552.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/18/13/3552.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.