Published in final edited form as:
Invest Ophthalmol Vis Sci. 2008 December ; 49(12): 5230–5234. doi:10.1167/iovs.08-2145.

Oncogenic mutations in *GNAQ* occur early in uveal melanoma

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Abstract

Purpose—Early/initiating oncogenic mutations have been identified for many cancers, but such mutations remain unidentified in uveal melanoma (UM). An extensive search for such mutations was undertaken, focusing on the RAF/MEK/ERK pathway, which is often the target of initiating mutations in other types of cancer.

Methods—DNA samples from primary UMs were analyzed for mutations in 24 potential oncogenes that affect the RAF/MEK/ERK pathway. For *GNAQ*, a stimulatory α_q G-protein subunit which was recently found to be mutated in uveal melanomas, re-sequencing was expanded to include 67 primary UMs and 22 peripheral blood samples. *GNAQ* status was analyzed for association with clinical, pathologic, chromosomal, immunohistochemical and transcriptional features.

Results—Activating mutations at codon 209 were identified in *GNAQ* in 33/67 (49%) primary UMs, including 2/9 (22%) iris melanomas and 31/58 (54%) posterior UMs. No mutations were found in the other 23 potential oncogenes. *GNAQ* mutations were not found in normal blood DNA samples. Consistent with *GNAQ* mutation being an early or initiating event, this mutation was not associated with any clinical, pathologic or molecular features associated with late tumor progression.

Conclusions—*GNAQ* mutations occur in about half of UMs, representing the most common known oncogenic mutation in this cancer. The presence of this mutation in tumors at all stages of malignant progression suggests that it is an early event in UM. Mutations in this G-protein provide new insights into UM pathogenesis and could lead to new therapeutic possibilities.

Keywords

uveal melanoma; oncogene; mutation; cancer genetics

INTRODUCTION

Uveal melanoma (UM) is the most common primary intraocular malignancy and the second most common form of melanoma. Several of the late genetic events in tumor progression and metastasis have been identified in UM, such as the loss of chromosome 3 and the switch from class 1 (low metastatic risk) to class 2 (high metastatic risk) gene expression profile.[1-3] In contrast, virtually nothing is known about the early, initiating events in uveal melanocytes leading to malignant transformation and development of a clinically detectable tumor. This

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Disclosure: **M.D. Onken**, None; **L.A. Worley**, None; **M.D. Long**, None; **S. Duan**, None; **M.L. Council**, None; **A.M. Bowcock**, None; **J.W. Harbour**, None.

deficiency stands in contrast to many other forms of cancer, including cutaneous melanoma, where early oncogenic mutations have been well characterized.

Perhaps the most common signaling pathway affected by early oncogenic mutations is the RAF/MEK/ERK pathway, where mutations in *BRAF*, *NRAS*, *HRAS* and *KIT* lead to constitutive activation of the RAF/MEK/ERK pathway, which in turn stimulates the transcription of pro-proliferative genes such as *CCND1*, *JUN* and *MYC*.[4, 5] Curiously, mutations in these genes are extremely rare in UM.[6-8] Nevertheless, there is strong evidence that mutations affecting the RAF/MEK/ERK pathway are present in UM, since *MEK*, *ERK* and *ELK* are constitutively activated in these tumors.[9, 10] Further, the RAF/MEK/ERK pathway target *CCND1*, which encodes cyclin D1, is overexpressed in most UMs,[11, 12] and leads to hyperphosphorylation and inactivation of the retinoblastoma tumor suppressor (Rb) in UM.[13, 14] Since amplification of *CCND1* is rare in UM,[15] *CCND1* overexpression is most likely mediated transcriptionally by activation of the RAF/MEK/ERK pathway.

These lines of evidence implicating the RAF/MEK/ERK pathway as a likely location of early or initiating oncogenic mutations in UM prompted us to screen a large number of potential oncogenes in this pathway. This search included 24 genes and was guided by oncogenomic data from comparative genomic hybridization, transcription profiling and *in silico* gene ontology analysis of public databases. Mutations in *GNAQ*, a stimulatory α_q subunit of heterotrimeric G-proteins that was recently found to be mutated in UM, [16] were found in half of the tumor samples, and the spectrum of *GNAQ* mutations suggested that this may be an early event in UM pathogenesis.

METHODS

Preparation of RNA and DNA

This study was approved by the Human Studies Committee at Washington University, and informed consent was obtained from each subject. Tumor samples included 67 primary UMs (9 iris tumors and 58 posterior tumors). Normal DNA samples from peripheral blood were prepared from 22 patients with *GNAQ*-mutant tumors, as previously described.[17] Normal uveal melanocytes were available in two patients with *GNAQ*-mutant tumors (MM86 and MM101) who had undergone enucleation. To collect melanocytes, the eye was cut in half immediately after enucleation, and normal choroid collected from a location opposite the tumor. This was done before collection of tumor tissue so that none of the instruments had touched the tumor. Melanocytes were then cultured from the choroid sample as previously described, [18] and DNA was obtained from these samples for *GNAQ* sequencing. Tumor tissue was then obtained, snap frozen and prepared for RNA and DNA analysis as previously described.[1, 19] The technique and results of array-based comparative genomic hybridization (aCGH) were previously described.[19] The techniques for generating transcription profile data using Affymetrix Hu133A and Illumina Human Ref8 BeadChip® arrays were previously described.[1, 19, 20]

Analysis of array-CGH profiles

Genome-wide CGH data were available on 28 primary UMs from a previously published study. [17] CGH profiles were analyzed using CGHminer software (http://wwwstat.stanford.edu/~wp57/CGH-Miner). Microsoft Excel was used to identify small, discrete regions of DNA gain, defined as one or more contiguous probes with a log2ratio ≥ 3 standard deviations of the mean for the entire chromosomal arm in at least 15% of tumor samples.

DNA sequencing

Exon 5 of *GNAQ* was re-sequenced by routine methods following polymerase chain reaction amplification of exon 5 with primers: GNAQE5L: 5'-TTCCCTAAGTTTGTAAGTAGTGC and GNAQE5R:5'-AGAAGTAAGTTCACTCCATTCC. This generated a product of 317 bp that included codon 209. Additional candidate oncogenes were re-sequenced to search for potential nucleic acid substitutions that could serve as activating mutations. Factors used to choose regions to be re-sequenced included: (1) the locations of reported cancer-related mutations in the Sanger Institute Catalogue of Somatic Mutations (http://www.sanger.ac.uk/genetics/CGP/cosmic), and (2) the locations of catalytic or regulatory domains in the Swiss-Prot Database (http://www.expasy.org/sprot/). For genes without known domains that would be likely targets for mutation, the entire coding region was re-sequenced. Primers were designed with Primer3 software to amplify all coding regions as well as exon-intron boundaries. Sequences were analyzed with Sequencher 4.5 software (GeneCodes, Madison, WI, USA). Non-synonymous nucleotide changes were screened to rule out known single nucleotide polymorphisms (SNPs) by querying the alignment of the altered region with the reference DNA sequence in the UCSC genome browser (http://genome.ucsc.edu/). Primer sequences and other details of our sequencing strategy are available upon request.

Analysis of microarray transcription profiles

Twenty-nine of the tumors in this study (10 *GNAQ*-wildtype and 19 *GNAQ* -mutant) were previously analyzed for transcription profile using the Affymetrix U133A GeneChip® array (10 cases) and/or the Illumina BeadChip® array (14 cases) or both (5 cases).[1] The clinical, pathologic and molecular information, and microarray platforms used for each tumor sample are indicated in Supplementary Table 1. Affymetrix data were normalized by Robust Multichip Average (RMA) using RMAExpress (rmaexpress.bmbolstad.com), and Illumina data were normalized by the rank invariant method using BeadStudio® software (Illumina). Principal component analysis (PCA) was performed using Spotfire DecisionSite® software (http://www.spotfire.com) to study unsupervised tumor clustering with respect to *GNAQ* status. Significance Analysis of Microarrays (SAM) was used to identify genes that were differentially expressed between tumors with and without *GNAQ* mutation (http://www-stat.stanford.edu/~tibs/SAM). Median centering and t-test statistic were used as analysis parameters, and the false discovery rate was set to zero. Class 1 and class 2 tumors were analyzed separately. There were only four Affymetrix class 2 tumors, so this subset was excluded from the analysis. The three subsets included: Affymetrix class 1 (six *GNAQ*-wildtype and five *GNAQ*-mutant tumors), Illumina class 1 (three *GNAQ*-wildtype and six *GNAQ*-mutant tumors) and Illumina class 2 (three *GNAQ*-wildtype and six *GNAQ*-mutant tumors). SAM was performed using the Wilcoxon nonparametric method.

Chromosome 3 status and extracellular matrix patterns

Chromosome 3 status was available on 42 of the tumors from a previous study using single nucleotide polymorphisms to detect loss of heterozygosity across the entire chromosome.[17] The status of extracellullar matrix patterns was available on 13 patients from a previous study.[21] No additional cases in this cohort were available for this analysis.

Statistical analysis

The patients in this study included a well-characterized cohort of 42 UM patients for whom clinical, pathologic, chromosomal and transcriptional data have been previously published.[17, 19, 20, 22] These data included: age, gender, tumor diameter and thickness, ciliary body involvement, histologic cell type, depth of scleral invasion, metastasis, patient outcome, transcription profile class 1 or class 2, status of chromosomes 3, 6p, 8p and 8q, and

immunohistochemical staining status for β-catenin, E-cadherin and cytokeratin-18. These parameters were analyzed for association with *GNAQ* status using MedCalc® version 9.4.2.0 statistical software (MedCalc Software, Mariakerke, Belgium). Categorical variables were analyzed using Fisher exact test, and continuous variables by Mann-Whitney test. Metastasis-free survival was analyzed by the Kaplan-Meier method. A P-value < 0.05 was considered significant.

RESULTS

Identification of potential oncogenes

Genome-wide aCGH data were available on 28 primary UMs,[19] and these data were re-analyzed to search for regional DNA amplifications that could signify the location of oncogenes. Similar techniques previously have been used successfully to identify, among many other examples, *MITF* as an oncogene in cutaneous melanoma.[23] CGHminer analysis identified frequent gains of large regions of chromosomes 6p and 8q (Figure 1A), which are known regions of chromosomal gain in UM.[24-28] CGHminer also detected occasional gains across chromosome 20. However, no small, discrete regions of gain were identified on these or other chromosomal arms. Amplification of an oncogene in a subset of tumors has commonly been used as a means of identifying activating mutations in that oncogene in other, non-amplified, tumor samples. Thus, we searched for discrete regions of DNA gain, defined as one or more contiguous probes with a log2ratio ≥ 3 standard deviations of the mean for the entire chromosomal arm in at least 15% of tumor samples. Using this technique, a small region of DNA gain was identified on chromosome 5q, corresponding to the location of *PIK3R1* (Figure 1B), the regulatory subunit of phosphatidylinositol 3-kinase, which can activate the RAF/MEK/ERK pathway.[29]

Re-sequencing of potential oncogenes

Along with *PIK3R1*, we selected 12 genes from chromosomes 6p, 8q, and 20 that are known to play a role in activating the RAF/MEK/ERK pathway, exhibited significant expression in uveal melanocytes and UMs in our previously published microarray expression profiles,[1, 19, 20] and in most cases, have known mutations in other cancers (Table 1 and Figure 1C). Re-sequencing of these genes in 19 primary UMs revealed no mutations. We extended our re-sequencing to include seven more oncogenes: two RAF family members (*ARAF* and *RAF1*), the parallel Ras effector *RASIP1*, two additional genes that are closely linked to activation of RAF (*DIRAS2* and *RAPGEF1*), the RAS homolog activating protein *ARHGAP1*, and the PI3K pathway member *PIP5KL1*. Re-sequencing of these genes in 19 primary UMs also revealed no mutations. Four additional genes were selected because of a known association with a melanoma phenotype (*EDG5*, *GNAQ*, *GRM1* and *PTPN11*). Mutations were found in *GNAQ*, but not in *EDG5*, *GRM1* or *PTPN11* (Figure 2). In all cases, mutations in *GNAQ* occurred at codon 209 (wildtype sequence: CAA).

GNAQ mutations in uveal melanoma

Prompted by the finding of mutations in *GNAQ*, analysis of this gene was expanded to include 67 primary UMs. *GNAQ* mutations were found in 49% (33/67) of tumors, including 22% (2/9) iris UMs and 54% (31/58) of posterior UMs. Mutant sequences included C<u>C</u>A (22 cases), C<u>T</u>A (13 cases) and CA<u>T</u> (one case). Normal DNA samples from peripheral blood were available from 22 patients with *GNAQ*-mutant primary tumors, and none of these harbored *GNAQ* mutations. Normal uveal melanocytes surrounding the primary tumor were available in two patients with *GNAQ*-mutant tumors (MM86 and MM101), and neither melanocyte samples showed *GNAQ* mutations.

GNAQ mutations and tumor progression

GNAQ mutations exhibited several properties that would be expected for an early or initiating oncogenic event. First, *GNAQ* mutations did not occur preferentially in tumors with clinical, pathologic or immunohistochemical features indicative of advanced tumor progression (Supplementary Table 2). Second, *GNAQ* mutations did not correlate with the degree of chromosomal aneuploidy, which is often used as a surrogate measure of temporal tumor progression (P=0.498)(Supplementary Figure 1). Third, there was no correlation between *GNAQ* mutation and class 2 gene expression profile, which is perhaps the most accurate indicator of advanced tumor progression.[1, 19, 20] For this analysis, 30 tumors that were previously profiled for gene expression were analyzed with respect to *GNAQ* mutation status. Unsupervised analysis using PCA showed no clustering of tumors based on *GNAQ* status (Supplementary Figure 1). SAM was used to identify genes that were differentially expressed in tumors harboring *GNAQ* mutations. Consistent with the PCA results, SAM revealed no genes that were consistently differentially expressed between tumors with and without *GNAQ* mutations (Supplementary Figure 1).

DISCUSSION

Activating oncogenic mutations affecting the RAF/MEK/ERK pathway are pervasive in cutaneous melanomas and other forms of cancer, but have rarely been found in UM.[7, 8, 30] Mutation of *GNAQ* at codon 209, which occurs in about half of UMs, represents the first common oncogene mutation in UM and provides important new insights into UM pathogenesis. GNAQ is a heterotrimeric GTP-binding protein alpha subunit that couples G-protein coupled receptor signaling to the RAF/MEK/ERF and other intracellular pathways through protein kinase C activated by stimulation of phospholipase C-beta.[31] Codon 209 maps to the catalytic domain of GNAQ, which is involved in GTPase activity. Mutation of this codon inactivates the catalytic domain, preventing hydrolysis of GTP and locking GNAQ in its active, GTP-bound state. This mutation leads to melanocyte proliferation in mice,[32] and can cooperate with other oncogenes to transform melanocytes.[16] Constitutive activation of GNAQ mimics growth factor signaling in sensitive cells through activation of the RAF/MEK/ERK pathway and leads to transcriptional activation of cell cycle genes such as *CCND1*. This could explain the frequent overexpression of cyclin D1 in UMs.[12] The finding of *GNAQ* mutation as a common and early mutational event in UM could pave the way for novel targeted therapies aimed at inhibiting the GNAQ protein product or other members of the pathway.

In many cancers, mutations in the RAF/MEK/ERK pathway are thought to be early or initiating events in tumorigenesis. For example, *BRAF* mutations occur very early in cutaneous melanoma, and are even present in benign and pre-malignant nevi.[33, 34] Similarly, the absence of correlation between *GNAQ* mutation and clinical, pathologic, immunohistochemical and genetic indicators of tumor progression, and the presence of the mutation in tumors at all stages of progression, would support the placement of *GNAQ* mutation as an early event in UM tumorigenesis.

GNAQ mutations were not found in normal DNA from patients bearing *GNAQ*-mutant tumors. This was an important finding, as it indicated that the *GNAQ* mutations were acquired somatically and were not present in the germline. A potential effect of *GNAQ* mutations could be the creation of an expanded pool of morphologically normal but abnormally proliferating melanocytes, as occurred in the mouse model of *GNAQ* mutation.[32] As a result, one might expect to find *GNAQ* mutations in uveal melanocytes of tumor-bearing eyes. However, in two patients with *GNAQ*-mutant tumors from whom we were able to obtain uveal melanocytes, no *GNAQ* mutations were found. *GNAQ* mutations were more common in UMs located in the posterior uveal tract (ciliary body and choroid) compared to iris UMs, which are located in the anterior uveal tract. Conversely, *BRAF* mutations are found in some iris UMs,[35] but not in

posterior UMs. These findings would support the long-held notion that iris UMs and posterior UMs have not only clinical, but also pathogenetic differences.[36]

The finding of *GNAQ* mutations in half of UMs raises the exciting possibility that other important oncogene mutations will be found in the other UMs. The role of GNAQ in activating the RAF/MEK/ERK pathway would suggest that future searches for early oncogenic mutations in UM should focus on genes in this pathway. We screened 23 other potential oncogenes in this pathway. Members of the RAS superfamily of small GTPases are commonly mutated in cutaneous melanoma and other cancers, so we re-sequenced several members of this family (*DIRAS2*, *REM1*, *GEM*, *RAB2A*, *RAB22A* and *RAB23*), as well as positive effectors of RAS signaling (*DIRAS2*, *RAPGEF1* and *RASIP1*), the RAS homolog GTPase activating protein *ARHGAP1*, and the serine/threonine protein kinase *PAK7*, which is an effector of RAS homolog *RAC/CDC42* GTPases. *HRAS*, *KRAS* and *NRAS* previously have been shown to be free of mutations in UM,[6-8] so these were not analyzed here. Similarly, *BRAF* is frequently mutated in cutaneous melanoma and other cancers, but not in UM,[6, 7, 9, 30] so we extended our re-sequencing to the other RAF family members, *ARAF* and *RAF1*. The PI3K pathway is activated in UMs [37] and can activate MEK/ERK.[29] Thus, we analyzed several members of the PI3K pathway, including *PTPN11*, *PTK2*, *PTK6*, *PIK3R1* (the regulatory subunit of PI3K), and *PIP5KL1*. We also analyzed *GRM1* and *EDG5*, which are G-protein coupled receptors that interact with GNAQ and are associated with melanoma phenotypes.[38-40] Even though our mutational screen revealed no additional oncogenic mutations, this screen was valuable in narrowing the search for oncogenic mutations in future studies. To this list can be added the GNAQ-associated genes *GNA12−15*, *GNAS* and *ENDRB*, which were previously analyzed and found to harbor no mutations in UM.[16] Future studies should continue to focus on screening for mutations in members of this pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank Rachel Donaldson and Li Cao for assistance with DNA sequencing.

Supported by grants (to J.W.H.) from the National Eye Institute (R01 EY1316905), Barnes-Jewish Hospital Foundation, Kling Family Foundation, Tumori Foundation, Research to Prevent Blindness, Inc., and Horncrest Foundation, and to the Department of Ophthalmology and Visual Sciences from a Research to Prevent Blindness, Inc. and the NIH Vision Core Grant P30 EY 02687.

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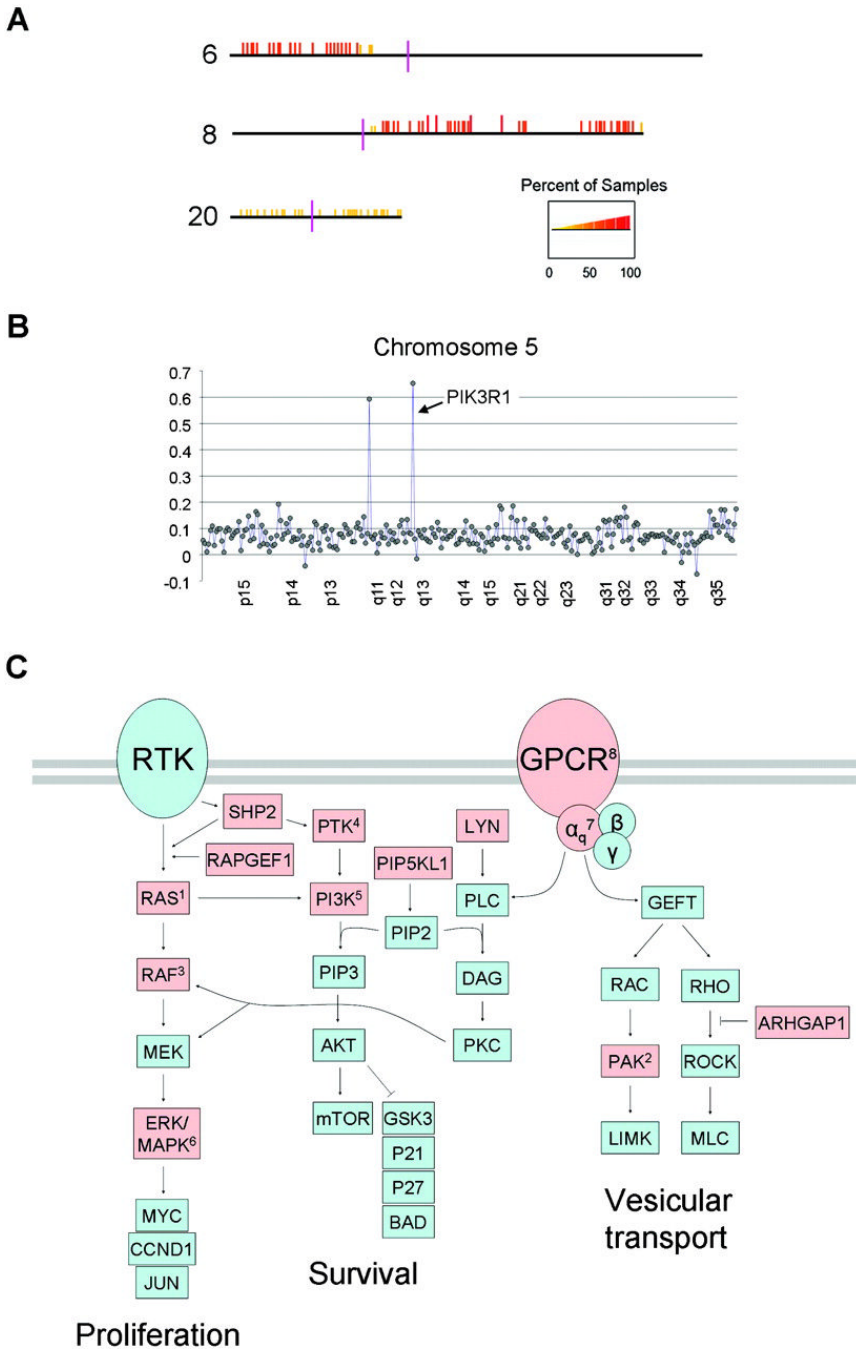

Figure 1.

Regions of chromosomal gain identified by CGH. (**A**) CGHminer result for sixteen class 1 and twelve class 2 tumors. DNA gains (indicated by orange and red vertical bars) with respect to chromosomal position (horizontal lines) on chromosomes 6p, 8q and, to a lesser extent, 20p and 20q. The p-arms are depicted to the left, and the q-arms to the right of the centromeres (vertical purple bars). The percent of samples showing DNA gain is indicated by the scale at the bottom. (**B**) CGH tracing of chromosome 5, showing two peaks with a mean log2ratio ≥ 3 standard deviations above the mean for the chromosomal arm. The larger peak at 5q13.1 corresponded to the location of PIK3R1. The other smaller peak did not correspond to a coding region. (**C**) Pathways that affect RAF/MEK/ERK activation. Arrows indicate stimulatory

interactions, and T-bars indicate inhibitory interactions. Abbreviations: RTK, receptor tyrosine kinase; GPCR, G-protein coupled receptor. Other abbreviations are official gene symbols. Notations of specific genes analyzed in this study: [1]Ras superfamily of small GTPases: DIRAS2, REM1, GEM, RAB2A, RAB22A, RAB23 (HRAS, KRAS and NRAS were previously analyzed); [2]PAK7; [3]ARAF, RAF1 and RASIP1 (BRAF was previously analyzed); [4]PTK2 and PTK6; [5]PIK3R1 regulatory subunit; [6]MAPK13 and MAPK14; [7]GNAQ; [8]GRM1. Red shapes indicate genes that were re-sequenced in this study.

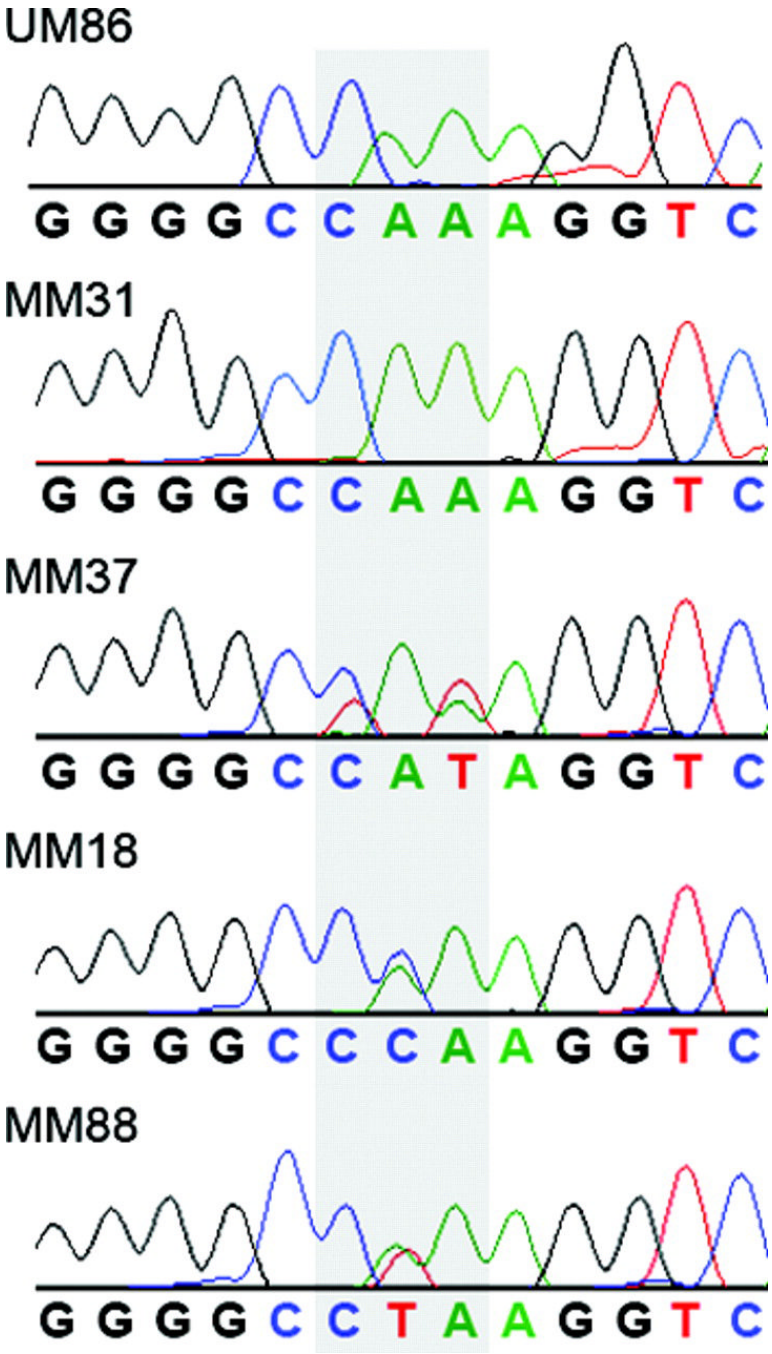

Figure 2.
Representative sequence tracings for GNAQ surrounding codon 209 (shaded). UM86, normal uveal melanocyte sample; MM31, uveal melanoma with wildtype sequence (CAA); MM37, MM18 and MM88, uveal melanomas with the three mutant sequences, as indicated.

Table 1

Summary of Candidate Oncogenes

Gene Symbol	Chromosomal Location	Exons Resequenced	Primary Tumors Analyzed	Tumors with Mutations	RAF/MEK/ERK Pathway	DNA Gain	Mutated in Other Cancers	Pigmentation or Melanoma Phenotype
ARAF	Xp11.4-p11.2	9, 12	19	0	X		X	
ARHGAP1	11p12-q12	11	19	0	X		X	
DIRAS2	9q22.2	1	19	0	X			
EDG5	19p13.2	2	16	0	X			X
GEM	8q13-q21	1−3	19	0	X	X		
GNAQ	9q21	5	67	36	X		X	X
GRM1	6q24	1−8	16	0	X			X
LYN	8q13	12	19	0	X	X	X	
MAPK13	6p21.31	1−11	19	0	X	X	X	
MAPK14	6p21.3-p21.2	1−9	19	0	X	X	X	
PAK7	20p12	10	19	0	X	X	X	
PIK3R1	5q13.1	8	19	0	X	X		
PIP5KL1	9q34.11	3	19	0	X		X	
PSKH2	8q21.2	2	19	0	X	X	X	
PTK2	8q24-qter	19	19	0	X	X	X	
PTK6	20q13.3	1, 7	19	0	X	X	X	
PTPN11	12q24	3−4, 13	16	0	X		X	X
RAB2	8q12.1	1, 2, 4	19	0	X	X		
RAB23	6p11	2−6	19	0	X	X		
RAB22A	20q13.32	1−3	19	0	X	X		
RAF1	3p25	6, 9	19	0	X		X	
RAPGEF1	9q34.3	19	19	0	X		X	
RASIP1	19q13.33	3	19	0	X			
REM1	20q11.21	1−3	19	0	X	X	X	