Oncogenic Mutations in GNAQ Occur Early in Uveal Melanoma

Michael D. Onken,1 Lori A. Worley,1 Meghan D. Long,1 Shenghui Duan,2 M. Laurin Council,2 Anne M. Bowcock,2 and J. William Harbour1

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 UM were analyzed for mutations in 24 potential oncogenes that affect the RAF/MEK/ERK pathway. For GNAQ, a stimulatory αq G-protein subunit which was recently found to be mutated in UM, resequencing was expanded to include 67 primary UM 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 (49%) of 67 primary UM, including 2 (22%) of 9 iris melanomas and 31 (54%) of 58 posterior UM. 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 UM, 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-coupled receptor provide new insights into UM pathogenesis and could lead to new therapeutic possibilities.

Uveal melanoma (UM) is the most common primary intraocular malignant 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 a 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 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.6–9 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 UM,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 αq 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, in accordance with the Declaration of Helsinki. Tumor samples included 67 primary UM (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, we cut the eye in half immediately after enucleation, and collected normal choroid from a location opposite the tumor, before collection of tumor tissue, so that none of the instruments 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.1–13 The technique and results of array-based comparative genomic hybridization (aCGH) were previously described.19 The tech-
niques for generating transcription profile data in two gene arrays (Hu133A: Affymetrix, Santa Clara, CA, and Human Ref8 BeadChip; Illumina, San Diego, CA) have been described.1,19,20

Analysis of Array—CGH Profiles

Genome-wide CGH data were available on 28 primary UM from a previously published study.17 CGH profiles were analyzed using CGHminer software (http://www-stat.stanford.edu/~wp57/CGH-Miner/ provided in the public domain by Stanford University Labs, Stanford, CA). Commercial statistical-analysis software (Excel; Microsoft, Redmond, WA) was used to identify small, discrete regions of DNA gain, defined as one or more contiguous probes with a log\_ratio ≥ 3 SD of the mean for the entire chromosomal arm in at least 15% of tumor samples.

DNA Sequencing

Exon 5 of GNAQ was resequenced by routine methods after polymerase chain reaction amplification of exon 5 with primers: GNAQE5L: 5′-TTCCTAAGTTTGAATGTGC and GNAQE5R: 5′-AGAAGATAGTTCACTCCATCC. This generated a product of 317 bp that included codon 209. Additional candidate oncogenes were resequenced to search for potential nucleic acid substitutions that could serve as activating mutations. Factors used to choose regions to be resequenced included: (1) the locations of reported cancer-related mutations in the Sanger Institute Catalog of Somatic Mutations (http://www.sanger.ac.uk/genetics/CGP/cosmic, provided in the public domain by the Wellcome Trust Sanger Institute Cancer Genome Project, Hinxton, UK), and (2) the locations of catalytic or regulatory domains in the Swiss-Prot Database (http://www.expasy.org/sprot/ provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland). For genes without known domains that would be likely targets for mutation, the entire coding region was resequenced. Primers were designed with Primer 3 software21 to amplify all coding regions as well as exon-intron boundaries. Sequences were analyzed by computer (Sequencher 4.5 software; GeneCodes, Madison, WI). Nonsynonymous 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 University of California Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/). Primer sequences and other details of our sequencing strategy are available on request.

Analysis of Microarray Transcription Profiles

Twenty-nine of the tumors in this study (10 GNAQ wild-type and 19 GNAQ mutant) were analyzed for transcription profile using gene microarray (U133A GeneChip array [10 cases Affymetrix], the BeadChip array [14 cases; Illumina]; or both [5 cases]:).1 The clinical, pathologic, and molecular information, and microarray platforms used for each tumor sample are indicated in Supplementary Table S1. http://www.iovs.org/cgi/content/full/49/12/5230/DC1. Affymetrix data were normalized by robust multichip average (RMA) using RMA-Express (written by Ben Bolstad and available in the public domain at rmaexpress.bmibolstad.com), and Illumina data were normalized by the rank invariant method using (BeadStudio software; Illumina). Principal component analysis (PCA) was performed to study unsupervised tumor clustering with respect to GNAQ status (Spotfire DecisionSite software; Tibco Software, Inc., Palo Alto, CA). 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; provided in the public domain by Stanford University Labs, Stanford, CA). Median centering and t-test statistic were used as analysis parameters, and the false discovery rate was set to zero. Class 1 and 2 tumors were analyzed separately. There were only four Affymetrix class 2 tumors, and so this subset was excluded from the analysis. The three subsets included: Affymetrix class 1 (six GNAQ wild-type and five GNAQ mutant tumors), Illumina class 1 (three GNAQ wild-type and six GNAQ mutant tumors) and Illumina class 2 (three GNAQ wild-type 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.37 The status of extracellular matrix patterns was available on 13 patients from a previous study.22 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 published.17,19,20-25 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 2, status of chromosome 3, and chromosomal arms 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 (MedCalc ver. 9.4.2.0 statistical software; MedCalc Software, Mariakerke, Belgium). Categorical variables were analyzed using the Fisher exact test, and continuous variables by the Mann-Whitney test. Metastasis-free survival was analyzed by the Kaplan-Meier method. P < 0.05 was considered significant.

RESULTS

Identification of Potential Oncogenes

Genome-wide aCGH data were available on 28 primary UM.19 and these data were reanalyzed 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.24 CGHminer analysis identified frequent gains of large regions of 6p and 8q (Fig. 1A), which were known regions of chromosomal gain in UM.25-29 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 nonamplified tumor samples. Thus, we searched for discrete regions of DNA gain, defined as one or more contiguous probes with a log\_ratio ≥ 3 SD 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 5q, corresponding to the location of PIK3R1 (Fig. 1B), the regulatory subunit of phosphatidylinositol 3-kinase, which can activate the RAF/MEK/ERK pathway.30

Resequencing of Potential Oncogenes

Along with PIK3R1, we selected 12 genes from 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 UM in our previously published microarray expression profiles.1,19,20 and in most cases, have known mutations in other cancers (Table 1, Fig. 1C). Resequencing of these genes in 19 primary UM revealed no mutations. We extended our resequencing 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 homologue activating protein ARHGAF1, and the PI3K pathway member PI3KCI. Resequencing of these genes in 19 primary UM also revealed no mutations. Four additional genes were selected...
Mutations in UM

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 CCA (22 cases), CTA (13 cases) and CAT (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 S2, http://www.iovs.org/cgi/content/full/49/12/5230/DC1). 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 Fig. S1, http://www.iovs.org/cgi/content/full/49/12/5230/DC1). 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 Fig. S1). 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 Fig. S1).

DISCUSSION

Activated 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.7,8,31 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 α subunit that couples G-protein coupled receptor signaling to the RAF/MEK/ERK and other intracellular pathways through 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 (Fig. 2). In all cases, mutations in GNAQ occurred at codon 209 (wild-type sequence: CAA).

GNAQ Mutations in UM

Prompted by the finding of mutations in GNAQ, analysis of this gene was expanded to include 67 primary UMs. GNAQ
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.35 As a result, one may expect to find GNAQ mutations in uveal melanocytes of tumor-bearing patients, but not in iris UMs, which are located in the anterior uveal tract. Conversely, BRAF mutations are found in some iris UMs,36 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 pathogenic differences.37

The finding of GNAQ mutations in half of UMs raises the exciting possibility that other important oncogenic 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 resequenced several members of this family (DIRAS2, REMI, 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, KRA5, and NRAS previously have been shown to be free of mutations in UM,6–8 and not analyzed in this study. Similarly, BRAF is frequently mutated in cutaneous melanoma and other cancers, but not in UM.6,7,9,31 so we extended our resequencing to the other RAF family members, BRAF and RARF1. The PI3K pathway is activated in UM,38 and can activate MEK/ERK.30 Thus, we analyzed several members of the PI3K pathway, including PTPN11, PT2K, 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.39–41 Even though our mutational screen revealed no additional oncogenic mutations, this screening 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 an-

### Table 1. Summary of Candidate Oncogenes

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Chromosomal Location</th>
<th>Exons Resequenced</th>
<th>Primary Tumors Analyzed (n)</th>
<th>Tumors with Mutations (n)</th>
<th>RAF/MEK/ERK Pathway</th>
<th>DNA Gain</th>
<th>Mutated in Other Cancers</th>
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Protein kinase C activated by stimulation of phospholipase C-ß.35 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,35 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.52 The finding of GNAQ mutations 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 premalignant nevi.34,35 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.35 As a result, one may 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,36 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 pathogenic differences.37

The finding of GNAQ mutations in half of UMs raises the exciting possibility that other important oncogenic 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 resequenced several members of this family (DIRAS2, REMI, 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, KRA5, and NRAS previously have been shown to be free of mutations in UM,6–8 and not analyzed in this study. Similarly, BRAF is frequently mutated in cutaneous melanoma and other cancers, but not in UM.6,7,9,31 so we extended our resequencing to the other RAF family members, ARAF and RARF1. The PI3K pathway is activated in UMs,38 and can activate MEK/ERK.30 Thus, we analyzed several members of the PI3K pathway, including PTPN11, PT2K, 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.39–41 Even though our mutational screen revealed no additional oncogenic mutations, this screening 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 an-
alyzed and found to harbor no mutations in UM. Future studies should continue to focus on screening for mutations in members of this pathway.

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References