Absence of \textit{BRAF} and \textit{NRAS} Mutations in Uveal Melanoma

Frank Cruz III, Brian P. Rubin, David Wilson, Ajia Town, Arin Schroeder, Andrea Haley, Troy Bainbridge, Michael C. Heinrich, and Christopher L. Corless

Oregon Health and Science University (OHSU) Cancer Institute [F. C., A. T., A. S., A. H., T. B., M. C. H., C. L. C.], Departments of Pathology [F. C., C. L. C.], and Ophthalmology [D. W.], and Division of Hematology and Oncology [M. C. H.], OHSU and Portland Veterans Affairs Medical Center, Portland, Oregon 97239, and Department of Anatomic Pathology, University of Washington Medical Center, Seattle, Washington 98195 [B. P. R.]

ABSTRACT

Uveal melanoma (UM) and cutaneous melanoma (CM) differ significantly in their epidemiological, clinical, immunophenotypical, and cytogenetic features, but the molecular basis for these differences has not been delineated. CMs frequently harbor an activating mutation in either \textit{NRAS} or the RAS-regulated kinase \textit{BRAF}, suggesting that either of these oncogenes may increase signaling through the mitogen-activated protein (MAP) kinase pathway and promote melanoma development. The aim of this study was to examine \textit{BRAF} and \textit{NRAS} gene mutations in UM. Genomic DNA from CM and UM was screened for mutations in \textit{BRAF} exons 11 and 15 and \textit{NRAS} exons 1 and 2 using a combination of denaturing-high-performance liquid chromatography and direct sequencing. Mutations in \textit{BRAF} exon 15 were detected in 16 (36.4%) of 44 CMs and 0 (0%) of 62 UMs. The most common mutation in CM was V599E, but a novel point mutation (L596Q) was identified in two cases and an in-frame deletion/insertion (VKSRWK599–604D) was discovered in one case. No \textit{BRAF} exon 11 mutations were observed among seven CMs and nine UMs that were wild-type for exon 15. Mutation of \textit{NRAS} exon 2 was rare in CM [1 (3.7%) of 27] and absent in UM [0 (0%) of 47]. No \textit{NRAS} exon 1 mutations were detected in either type of melanoma. We conclude that UMs arise independent of oncogenic \textit{BRAF} and \textit{NRAS} mutations, an observation that may have implications for therapies targeted to the NRAS-BRAF pathway.

INTRODUCTION

UMs comprise \(\sim 3\%–5\%\) of all melanomas and are thought to arise from neural crest-derived melanocytic precursors similar to the more common cutaneous form of melanoma (1). There are, however, significant epidemiological differences between UM and CM. Although there has been a world-wide increase in CM in recent decades, the incidence of UM has remained unchanged, possibly reflecting a greater role for UV light exposure in the development of CM than in UM (2). Patients with a diagnosis of CM have a 10-fold increased risk of developing a second cutaneous lesion, but they have no additional risk for the development of a UM (3). In addition, CM generally spreads by way of regional lymph nodes, whereas UM shows a marked predilection for metastasis to the liver (4).

The epidemiological differences between UM and CM are paralleled by differences in their immunophenotypic, karyotypic, and gene expression profiles. Both types of tumors commonly express the melanocytic markers HMB-45 and tyrosinase, but expression of S-100 is much more uniform among CMs than among UMs (5, 6). In addition, UMs are negative for the p75 neurotrophin receptor that is readily detected in CMs (7). Karyotypes of UM are often shown to have monosomy 3 and overrepresentation of chromosome 8q, both of which are negative prognostic factors (8–10). In contrast, CMs preferentially show alterations in chromosomes 1, 6, 7, and 10 (11). By cDNA microarray analysis, the gene expression profiles of CM cell lines cluster separately from the majority of CM cell lines and tumor samples (12).

Despite the abundant evidence that UM and CM are distinct tumors, few genotypic differences between these melanoma subtypes have been identified. Functional loss of the CDKN2A (p16\(^{INK4a}\)) tumor suppressor, whether through promotor methylation, mutation, and/or deletion, is observed in a significant fraction of both UMs and CMs (13–17). Likewise, p53 alterations are observed in both melanoma types in association with tumor progression (18–22). Mutations of the CTNNB1 (\(\beta\)-catenin) gene appear to be rare in both UMs and CMs (14, 23).

Mutations in members of the \textit{RAS} gene family have been identified in CMs, although their reported frequency varies over a wide range (24–33). Interestingly, melanocytic tumors develop in the skin of mice expressing activated HRAS, but NRAS appears to be the favored target in human CM (34). There appears to be no information available for the genotypic status of \textit{RAS} genes in UM. Recently, Davies et al. (28) discovered a high frequency of activating mutations in \textit{BRAF} in both CM cell lines and tumor samples. A member of the RAF family of serine-threonine kinases, BRAF is an immediate downstream mediator of RAS signaling and activates the MEK1/2-ERK1/2 (mitogen-activated protein kinase [MAP]) pathway (35). The presence of \textit{BRAF} gene mutations in CM has been confirmed by several groups, and it has been observed that \textit{BRAF} and \textit{NRAS} mutations rarely overlap (26, 28, 32, 36). Thus, activation of either of these signaling molecules may support melanoma growth and survival.

In this study, we compare the type and frequency of mutations in \textit{BRAF} and \textit{NRAS} in samples of UM and CM, using a combination of D-HPLC and direct sequencing. The results demonstrate a significant difference in the oncogenic activation of signaling intermediates between these two melanoma types. In addition, two novel mutations of \textit{BRAF} in CM are described.

MATERIALS AND METHODS

Tissues and Cell Lines. All of the tumor samples used in the study were obtained from the pathology archives of the University Hospital and Casey Eye Institute of OHSU, or the Portland Veterans Affairs Medical Center, or the University of Washington Medical Center, and were handled in accordance with the institutional review board regulations for each of the source institutions. Of the 138 cases of melanoma that were collected for the study, 66 were cases of CM and 72 cases were of UM. The majority of the tumors were formalin fixed and paraffin embedded (\(n = 114\)). Most of the others were formalin fixed but not paraffin embedded (\(n = 21\)). For these cases, a portion of the tissue was processed, embedded in paraffin, sectioned and stained using routine histology protocols so that the tumor purity could be assessed. Also included in the study were fresh-frozen samples of two UMs and one CM obtained from the Tumor Bank of the OHSU Cancer Institute. Finally, formalin-fixed, paraffin-embedded samples of 36 gliomas and 14 ependymomas were collected for the study.

H&E-stained sections (either paraffin or cryosections) of all tumor samples were assessed for quality and purity. In cases in which the tumor cellularity was judged to be >90%, DNA was prepared from shavings taken directly from the corresponding paraffin block or the remaining unembedded tissue. For all

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1 To whom requests for reprints should be addressed, at OHSU Department of Pathology (L471), 3181 SW Sam Jackson Park Road, Portland, OR 97239. E-mail: corlessc@ohsu.edu.

2 The abbreviations used are: UV, uveal melanoma; CM, cutaneous melanoma; HPLC, high-performance liquid chromatography; D-HPLC, denaturing HPLC; OHSU, Oregon Health and Science University; PDGFR\(A\), platelet derived growth factor receptor alpha.
other cases, unstained 5-µm sections were prepared, and areas rich in tumor (as determined by comparison with an H&E-stained section) were scraped into a microcentrifuge tube using a clean scalpel blade. Block shavings or slide scrapings were deparaffinized by serial extractions with xylenes and ethanol and allowed to air-dry at room temperature. DNA was extracted using the Qiagen minikit in accordance with the manufacturer’s recommendations (Qiagen, Valencia, CA).

Melanoma cell lines SK-MEL-2 and SK-MEL-28 were kindly provided by the laboratory of Dr. Molly Kulesz-Martin, OHSU. SK-MEL-2 has been reported to contain an NRAS Q61R point mutation (exon 2), and SK-MEL-28 has been reported as having a BRAF V599E point mutation (exon 15; 28). DNA from these cell lines was extracted using the Qiagen mini-kit, as above. Genomic DNA from the cell line NCI-H1395, reported to contain a reported by Chan et al. (28), who used a heteroduplex detection assay based on capillary electrophoresis. Concerned that methodological differences might be a factor, we examined a series of gliomas and detected mutations at a higher frequency (5.6%; Table 1) than observed by Davies et al. (28), who used a heterogeneous sample set.

RESULTS

D-HPLC was used to screen for BRAF and NRAS mutations in cases of UM and CM because this method is highly sensitive for a variety of mutation types, including point mutations, insertions, and deletions (40). As illustrated in Fig. 1, D-HPLC readily detected the presence of the most common BRAF mutation reported in melanoma, V599E. The sensitivity of the system for detecting this mutation was assessed by serially diluting pure V599E amplimer with pure wild-type exon 15 amplimer (see “Materials and Methods”). The V599E mutation was detectable down to a level of ~20%, which is equivalent to 40% tumor DNA, assuming heterozygosity for the allele (Fig. 1). Because all of the tumor samples included in the study were highly enriched in tumor cells (>90%), this was considered an adequate level of sensitivity.

Because D-HPLC profiling is dependent on heteroduplex formation between wild-type and mutant amplimers, it can miss mutations that are hemizygous or homozygous, such as the BRAF V599E mutation present in the melanoma cell line SK-MEL-28. Two approaches were used to exclude such mutations among the melanoma tissue samples that were studied. First, BRAF exon 15 amplimers from 12 UMs and 12 CMs with wild-type D-HPLC profiles were annealed with SK-MEL-28 amplimer and then were reanalyzed. All of the 24 samples showed the appropriate mutant profile, indicating that the tumor DNA was indeed negative for the V599E mutation. Second, direct DNA sequencing was performed on another 10 UMs and 10 CMs with wild-type D-HPLC profiles, and no mutations were detected. Thus, hemi/homozygous mutations for BRAF mutations were not detected in the melanoma tissue samples.

Among 44 CMs that were analyzed (10 primary, 34 metastatic), 16 had mutations in BRAF exon 15 (14 metastatic, 2 primary; Table 1). The frequency of BRAF mutations was not significantly different between primary and metastatic melanomas (P = 0.70156, Fisher’s exact test). No BRAF exon 11 mutations were detected among seven CMs that were wild-type for exon 15. UMs (61 primary, 1 secondary) were uniformly negative for mutations in BRAF exon 15 (0 of 62; Table 1). Nine UMs screened for exon 11 mutations were likewise negative (0 of 9).

Two independent cases of metastatic CM harbored a novel L596Q missense mutation (Fig. 2A). A novel, in-frame deletion/insertion mutation, VKSRRWK599—604D, was also observed in one primary CM (Fig. 2B). In all cases, the novel mutations were confirmed by reextraction of DNA from the original melanoma sample, followed by repeat amplification, D-HPLC screening, and direct sequencing.

The overall frequency of BRAF exon 15 mutations in our CM samples (36.4%; Table 1) was lower than that reported by Davies et al. (28), who used a heteroduplex detection assay based on capillary electrophoresis. Concerned that methodological differences might be a factor, we examined a series of gliomas and detected BRAF exon 15 mutations at a higher frequency (5.6%; Table 1) than observed by Davies et al. (0 of 15; Ref. 28). It was also higher than that recently reported by Chan et al. (1 of 166; Ref. 41). These findings indicate

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DNA Sequence Analysis. All of the D-HPLC-detected mutants were bidirectionally sequenced on an ABI 310 sequencer using the Big Dye Terminon kit (Applied Biosystems, Inc., Foster City, CA). Novel D-HPLC-detected mutants were confirmed by three methods: (a) reamplification of the exon and repeat D-HPLC analysis; (b) reextraction of DNA from the tumor tissue, reamplification, and repeat D-HPLC analysis; and (c) bidirectional sequence analysis after each analysis by D-HPLC.
that the sensitivity of D-HPLC is at least comparable with, if not better than, other published methodologies.

Previous reports have indicated that NRAS exon 2 mutations are present in a subset of melanomas that lack a BRAF mutation (28, 32). We screened for NRAS exon 2 mutations in 19 CM samples that were wild-type for BRAF exon 15 and found one point mutation [1 (5.3%) of 19; Fig. 3]. No NRAS exon 2 mutations were detected among eight CMs positive for a BRAF mutation (0 of 8). UM samples were completely negative for mutations of NRAS exon 2 (0 of 47). NRAS exon 1 mutations were not observed among the samples of CM (0 of 21) and UM (0 of 22) tested.

**DISCUSSION**

In recent studies of oncogenic mutations of the KIT and PDGFRA tyrosine kinases, we found that the combination of D-HPLC screening and direct sequence confirmation is highly sensitive for a variety of mutation types (40, 42). In the current study, D-HPLC readily detected the common BRAF V599E mutation and uncovered two other novel BRAF mutations. Importantly, the mutations were identified only in CMs, leading to the conclusion that genomic mutation of BRAF does not play a role in the development of UMs. This observation is consistent with epidemiological, immunophenotypical and cytogenetic differences that have been defined between CMs UMs and that, as discussed below, has implications for BRAF-targeted therapies.

On the basis of previous reports, mutations in exon 15 of BRAF are far more common in melanoma than mutations in exon 11, and our findings support this trend (26, 28, 32, 36). However, the overall frequency of BRAF mutations detected in our CM samples (36.4%) was somewhat lower than that published by some other groups. Using a capillary electrophoresis-based heteroduplex detection assay, Davies et al. (28) concentrated primarily on cell lines and short-term cultures of melanoma, in which BRAF mutations were found in 59% and 80% of cases, respectively. Brose et al. (26) observed a similar frequency of BRAF mutations (63%) among 35 melanoma cell lines screened by the same methodology (26). Although it is possible that BRAF mutations are more common in cultured melanoma cells than in melanoma tissue samples (as is the case for CDKN2A mutations (43), this explanation does not fit with the 67% incidence of V599E mutations observed by Pollock et al. (32) in 55 samples of metastatic melanoma analyzed by allele-specific PCR. The study by Pollock et al. (32) also provided evidence that BRAF mutations are acquired very early in melanoma development, so that metastatic lesions are no more likely to have a mutation than primary lesions.

The higher BRAF mutation frequencies observed by other groups

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**Table 1**  Frequency of B-RAF exon 15 mutations in tested tumors

<table>
<thead>
<tr>
<th>B-RAF exon 15 mutation</th>
<th>CMs</th>
<th>UMs</th>
<th>Gliomas</th>
<th>Ependymomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>V599E</td>
<td>13/44 (29.5%)</td>
<td>0/62 (0%)</td>
<td>2/26 (5.6%)</td>
<td>1/14 (7.1%)</td>
</tr>
<tr>
<td>L596Q</td>
<td>2/44 (4.5%)</td>
<td>0/62 (0%)</td>
<td>0/36 (0%)</td>
<td>0/14 (0%)</td>
</tr>
<tr>
<td>Deletion/Insertion</td>
<td>1/44 (2.3%)</td>
<td>0/62 (0%)</td>
<td>0/36 (0%)</td>
<td>0/14 (0%)</td>
</tr>
<tr>
<td>Total</td>
<td>16/44 (36.4%)</td>
<td>0/62 (0%)</td>
<td>2/26 (5.6%)</td>
<td>1/14 (7.1%)</td>
</tr>
</tbody>
</table>

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Fig. 1. D-HPLC elution profiles of BRAF exon 15 amplimers. Progressive dilutions of V599E amplimer with wild-type amplimer reveal a distinct heteroduplex peak at concentrations above 15% V599E allele.
Fig. 2. A, BRAF L596Q mutation. The double-peaked elution profile detected on D-HPLC analysis at denaturing temperature (56.3°C, left) corresponded with the point mutation found on direct DNA sequencing (right). B, BRAF deletion/insertion mutation. D-HPLC elution profile at the nondenaturing temperature (50.0°C) revealed three distinct peaks (left). Direct sequencing of the amplicon confirmed a VKSRWS599–605D deletion/insertion (right).

Fig. 3. NRAS exon 2 point mutation. A double-peaked elution profile detected on D-HPLC analysis at denaturing temperature (59.3°C, left) corresponded with the point mutation Q61R found on direct DNA sequencing (right).
raised concern that our assay was not sufficiently sensitive, and we, therefore, validated our approach in two ways. First, we demonstrated that the V599E mutation was detectable by D-HPLC down to a level of 20% mutant allele, which is at, or below, the level that can be confirmed by direct sequencing. Second, we analyzed a series of gliomas and observed a higher frequency of mutations than that reported by other groups, including Davies et al. (28) In addition, a preliminary analysis of 25 papillary thyroid carcinomas in our laboratory has demonstrated BRAF exon 15 mutations at a frequency (60%) that matches recent reports by two other groups (44, 45). It should also be noted that Lang et al. (36) observed BRAF mutations in only 6 (27%) of 22 metastatic melanomas. It is possible that some of the variation in reported BRAF mutation frequencies reflects population differences (e.g., differences in levels of sun exposure). Whereas the V599E substitution is not typical of a UV-induced mutation, it may still be related to sunburn-associated inflammation. In this regard, it is interesting that UMs, in which BRAF mutations are absent, are not associated with sun exposure (2, 46).

Two CM cases with a novel L596Q mutation in BRAF exon 15 were identified. Other substitutions of this amino acid were previously reported by Davies et al. (28), including L596R (in a primary ovarian tumor) and L596W (in a non-small cell lung carcinoma cell line). The latter isoform of BRAF was shown to have significant transforming activity. Whether L596Q is equally activating remains to be determined.

The other mutation uncovered in our study was an in-frame deletion/insertion in exon 15 (VKSRWK599–604D), which to our knowledge is the first example of this type of mutation to be identified in BRAF. The deletion begins at the valine that is targeted in the common V599E substitution, spans an additional five amino acids, and ends with the insertion of an aspartic acid. This insertion is interesting, because it may generate a BRAF isoform similar to the V599D isoform observed in a melanoma cell line by Brose et al. (26) and in a metastatic melanoma by Pollock et al. (32) It has been suggested that acidic residues [Asp (D) or Glu (E)] substituted at this position may serve as phosphomimetics for two nearby phosphorylation sites (T598 and S601) that are critical to kinase activation (28, 47). However, Pollock et al. (32) also identified two melanoma metastases in which there was a basic amino acid substitution at position 599 (V599K). Clearly, detailed biochemical studies of the growing number of mutant BRAF isoforms identified in human tumors, particularly melanoma, are needed. It is also important that future surveys of human tumors not be restricted to the V599E mutation. In this regard, D-HPLC offers advantages over other screening methodologies because it is sensitive both for point mutations and deletions/insertions.

The reported frequency of NRAS mutations in melanoma ranges from 0 to 69% (24–33). This broad range in mutation frequency may partly reflect variation among populations, but methodological differences probably also play a role. The frequency of NRAS mutations in our CM samples was low (3.7%), but there was no overlap with BRAF mutations. This fits with the common theme in other recent reports on CM to the effect that NRAS and BRAF mutations rarely occur in the same tumor (26, 28, 32). The same trend has been observed for KRAS and BRAF mutations in colon adenocarcinoma, cholangiocarcinoma, papillary thyroid carcinoma, and low-grade micropapillary serous carcinomas of the ovary, supporting the widely held hypothesis that oncogenic activation of either of these key molecules may drive tumorigenesis through the mitogen-activated protein kinase signaling pathway (44, 45, 48, 49).

Recent clinical success with the kinase inhibitor imatinib (STI571; Gleevec) makes BRAF a tempting target for the treatment of melanoma (50). The effectiveness of this drug is limited almost exclusively to tumors in which activation of an imatinib-sensitive kinase (Abel-

son, KIT, PDGFRα, Platelet Derived Growth Factor Receptor Beta) is caused by a genomic mutation (50–54). A RAF kinase inhibitor with activity against BRAF has recently entered clinical trials (55-57). On the basis of the imatinib paradigm, the extent to which tumors such as melanoma are dependent on BRAF signaling may be reflected in their frequency of BRAF mutations, so it is important these mutations be identified and their drug sensitivity studied in vitro. Our data have added to the breadth of BRAF mutations identified in CM, and the new mutant isoforms we have identified can now be examined further. Our data also provide the basis for suggesting that UM, as well as the majority of gliomas and ependymomas, will not be good treatment targets for a RAF kinase inhibitor.

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Note Added in Proof

During the review of this manuscript, similar findings were published by Cohen et al. (Invest Ophthalmol Vis Sci 44:2876–2878, 2003) and by Edmunds et al. (Br J Cancer 88:1403–1405, 2003).

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