

Absence of *BRAF* and *NRAS* Mutations in Uveal Melanoma

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ABSTRACT

Uveal melanoma (UM) and cutaneous melanoma (CM) differ significantly in their epidemiological, clinical, immunophenotypic, and cytogenetic features, but the molecular basis for these differences has not been delineated. CMs frequently harbor an activating mutation in either *NRAS* or the RAS-regulated kinase *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 *BRAF* and *NRAS* gene mutations in UM. Genomic DNA from CM and UM was screened for mutations in *BRAF* exons 11 and 15 and *NRAS* exons 1 and 2 using a combination of denaturing high-performance liquid chromatography and direct sequencing. Mutations in *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 *BRAF* exon 11 mutations were observed among seven CMs and nine UMs that were wild-type for exon 15. Mutation of *NRAS* exon 2 was rare in CM [1 (3.7%) of 27] and absent in UM [0 (0%) of 47]. No *NRAS* exon 1 mutations were detected in either type of melanoma. We conclude that UMs arise independent of oncogenic *BRAF* and *NRAS* mutations, an observation that may have implications for therapies targeted to the *NRAS*-*BRAF* pathway.

INTRODUCTION

UMs² comprise ~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 often show 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 UM 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^{INK4}) tumor suppressor, whether through promoter 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 *CTNFB1* (*β-catenin*) gene appear to be rare in both UMs and CMs (14, 23).

Mutations in members of the *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 *RAS* genes in UM. Recently, Davies *et al.* (28) discovered a high frequency of activating mutations in *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 *BRAF* gene mutations in CM has been confirmed by several groups, and it has been observed that *BRAF* and *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 *BRAF* and *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 *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

Received 5/13/03; accepted 6/30/03.

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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; PDGFRA, platelet derived growth factor receptor alpha.

other cases, unstained 5- μ m 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. An *NRAS* G12A mutation previously identified in our laboratory in a sample of acute myelogenous leukemia was available as a clone in plasmid PC3. Genomic DNA from the cell line NCI-H1395, reported to contain a *BRAF* G468A mutation, was obtained from the ATCC (37). All of the reported mutations were confirmed upon PCR amplification and direct sequencing, as described below. PCR amplimers of these various mutations were used as controls for D-HPLC assays.

PCR Amplifications. It has been reported that the melanin pigment present in melanomas may copurify with DNA and inhibit DNA polymerase activity in PCR reactions. To reduce this inhibitory effect, BSA (Sigma A3059) was added to PCR mixtures (30 μ g BSA/50 μ l reaction), as described previously (38, 39). After an initial denaturing hold at 95 degrees for 60 s, purified tumor DNA (250 ng) was subjected to 42 cycles of PCR (94°C for 30 s, 56°C for 30 s, 72°C for 30 s) using the High Fidelity PCR system (Roche, Indianapolis, IN), followed by a final extension hold at 72°C for 7 min. Primer pairs used for amplifications were as follows. *BRAF* exon 11, forward (F)-TCTGTTTG-GCTTGACTTGACTT; *BRAF* exon 11, reverse (R)-CGAACAGTGAATAT-TTCCTTTGAT; *BRAF* exon 15, F-TGCTTGCTCTGATAGGAAAATG; *BRAF* exon 15, R-AGCATCTCAGGGCCAAAAAT; *NRAS* exon 1, F-GAT-GTGGCTCGCAATTAAC; *NRAS* exon 1, R-CCGACAAGTGAGAGACAGGA; *NRAS* exon 2, F-ATTGAACCTCCCTCCCTCC; *NRAS* exon 2, R-GTGGTAACCTCATTCCCA.

Negative controls were included in each set of amplifications. After amplification, the added BSA in the PCR reactions was removed using the High Pure PCR Product Purification kit (Roche, Indianapolis, IN) in accordance with the manufacturer's recommendations. This step was necessary to prevent excess protein loading during D-HPLC analysis of the PCR products. With the BSA protocol, the overall success rate for amplification of melanoma DNA extracted from paraffin-embedded tissue was >70%.

DNA from gliomas and ependymomas was purified and amplified by the same methods as above, with the following exceptions: (a) no BSA was added to the PCR reaction and, therefore, post-PCR purification was not necessary; (b) after an initial denaturing hold at 95°C for 60 s, the tumor DNA (250 ng) was subjected to 45 cycles of PCR (94°C for 60 s, 56°C for 60 s, 72°C for 60 s), with a final extension hold at 72°C for 7 min.

Mutation Screening by D-HPLC. Aliquots (40 μ l) of the PCR products were screened for mutations on a Transgenomic WAVE HPLC system (Transgenomic, Inc., Omaha, NE) run at partially denaturing temperatures (58.0°C for *BRAF* exon 11, 56.3°C for *BRAF* exon 15, 60.3°C for *NRAS* exon 1, and 59.3°C for *NRAS* exon 2). In the course of optimizing the D-HPLC assay for the detection of the *BRAF* V599E mutation, it was discovered that the SK-MEL-28 cell line is either hemizygous or homozygous for this mutation (28). This cell line provided a convenient source of pure V599E allele and was used to determine the sensitivity of D-HPLC for the V599E mutation, as follows. *BRAF* exon 15 amplimers of SK-MEL-28 DNA were mixed with various amounts of sequence-confirmed wild-type amplimer, as judged by peak areas of UV₂₆₀ absorbance in pilot D-HPLC elutions. Serial dilutions were calculated using the formula, $F = (AX)(100)/(AX + \alpha B - BX)$, where F = the desired fraction percentage SK-MEL-28, A = SK-MEL-28 relative peak area, X = microliters of SK-MEL-28 sample, α = total volume of loaded sample, and B = wild-type relative peak area. The annealed SK-MEL-28/wild-type DNA mixtures were then reanalyzed by D-HPLC to provide an estimate of the percentage mutant DNA detectable.

The discovery that SK-MEL-28 is hemi/homozygous for the *BRAF* V599E mutation raised the possibility that this might also be true in tissue samples of melanoma, which would then appear to be wild-type when analyzed by D-HPLC (false negative). Therefore, *BRAF* exon 15 amplimers from melano-

mas with apparent wild-type D-HPLC profiles were secondarily annealed to equivalent amounts of SK-MEL-28 DNA (determined by relative peak areas), as follows: 95°C for 5 min, 64°C for 7 min, 44°C for 30 s, 24°C for 30 s, and 4°C for 5 min. The annealed mixtures were then reanalyzed by D-HPLC.

DNA Sequence Analysis. All of the D-HPLC-detected mutants were bidirectionally sequenced on an ABI 310 sequencer using the Big Dye Terminator 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.

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/homozygosity for *BRAF* mutations was 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, VKSRWK599-604D, was also discovered 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

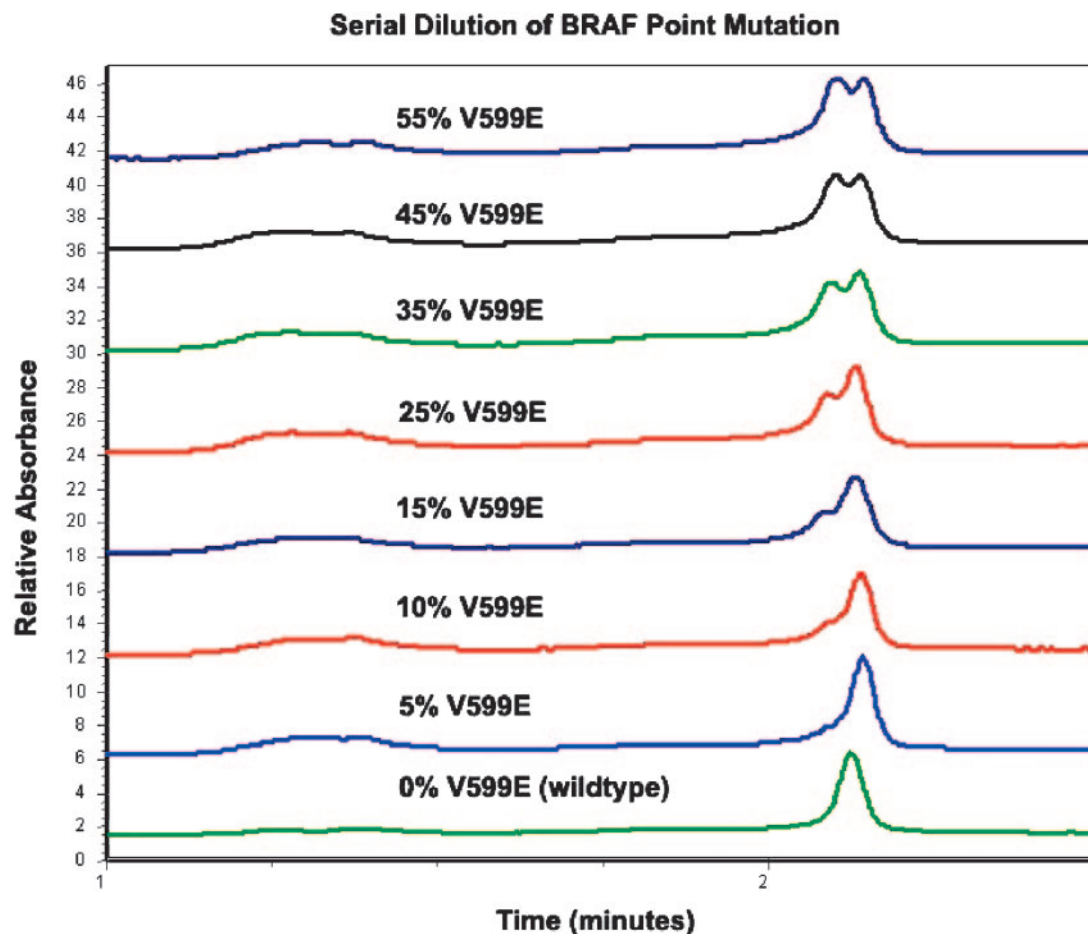


Fig. 1. D-HPLC elution profiles of *BRAF* exon 15 amplimers. Progressive dilutions of V599E amplimer with wild-type amplimer reveal a distinct heteroduplex peak at concentrations above 15% V599E allele.

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, immunophenotypic and cytogenetic differences that have been defined between CMs UMs and that, as discussed below, has implications for *BRAF*-targeted therapies.

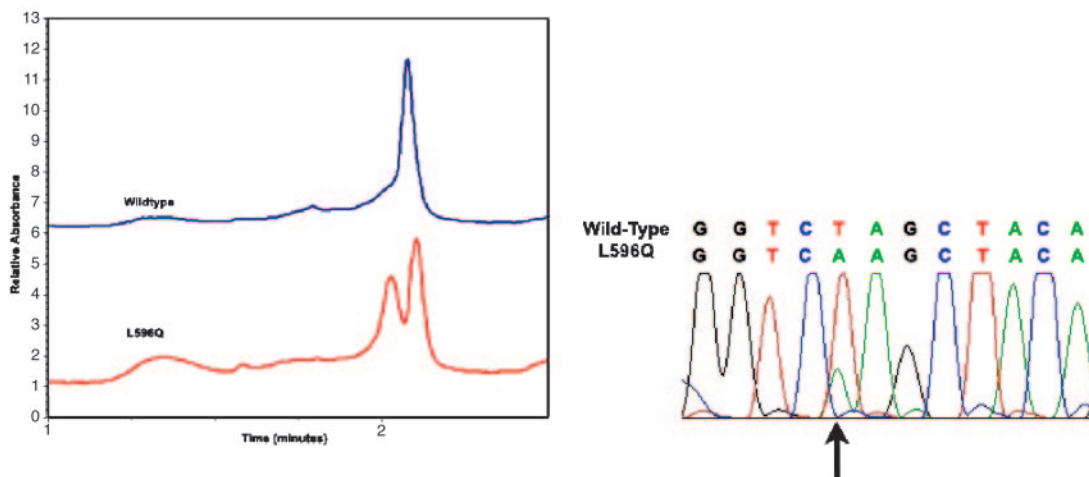
On the basis of previous reports, mutations in exon 15 of *BRAF* are far more common in melanoma than mutations in exon 11, and our findings support this trend (26, 28, 32, 36). However, the overall frequency of *BRAF* mutations detected in our CM samples (36.4%) was somewhat lower than that published 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

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

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

A BRAF Exon 15 Point Mutation



B BRAF Exon 15 Deletion

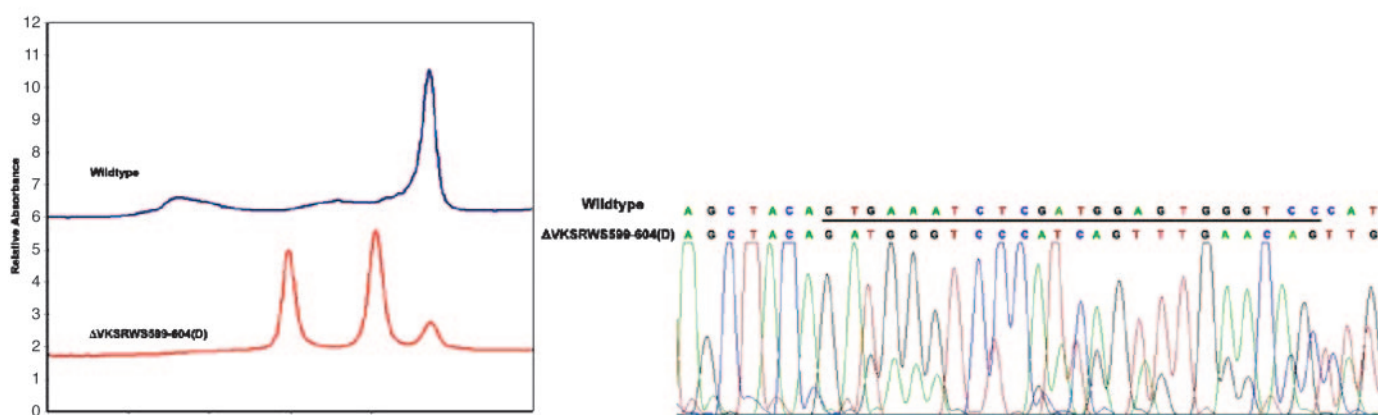


Fig. 2. *A*, *BRAF* L596Q mutation. The double-peaked elution profile detected on D-HPLC analysis at denaturing temperature (56.3°C, *left*) corresponded with the point mutation found on direct DNA sequencing (*right*). *B*, *BRAF* deletion/insertion mutation. D-HPLC elution profile at the non-denaturing temperature (50.0°C) revealed three distinct peaks (*left*). Direct sequencing of the amplicon confirmed a VKSRWS599–605D deletion/insertion (*right*).

NRAS Point Mutation

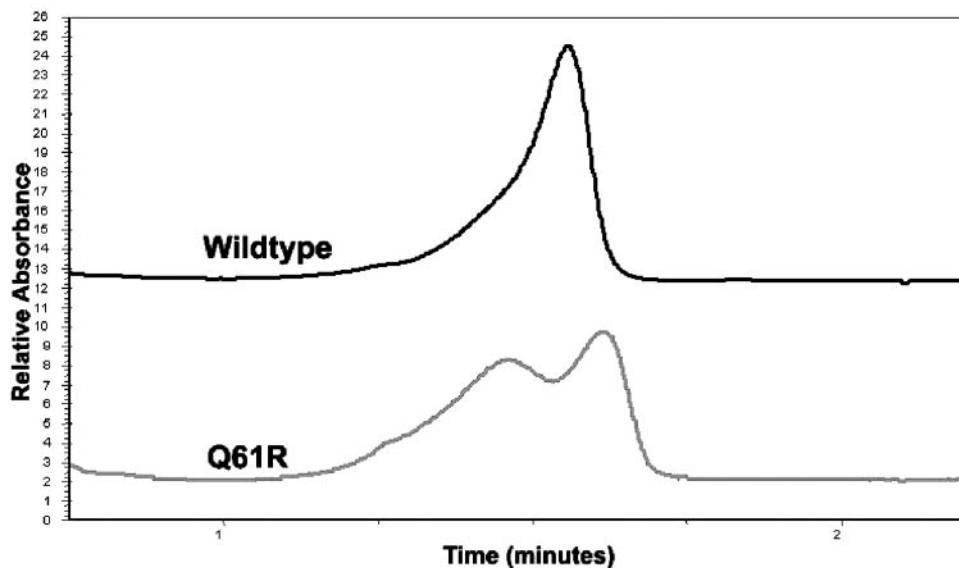


Fig. 3. *NRAS* exon 2 point mutation. A double-peaked elution profile detected on D-HPLC analysis at denaturing temperature (59.3°C, *left*) corresponded with the point mutation Q61R found on direct DNA sequencing (*right*).

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 glial tumors 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 L596V (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 colonic 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, PDGFRA, 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.

ACKNOWLEDGMENTS

We thank Blythe Scherrer for her excellent help in producing the Figures. We are also grateful to Linda Jauron-Mills, Carolyn Gendron, and Laura McGreevy for their exceptional technical assistance.

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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