Lack of BRAF Mutations in Uveal Melanoma

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Abstract

RAF proteins are serine/threonine kinases that mediate cellular responses to growth signals by activating the mitogen-activated protein kinase pathway. Mutations in the BRAF gene causing a V599E amino acid substitution that enhance the kinase activity have been described in >60% of cutaneous melanomas and premalignant melanocytic lesions. We have investigated the frequency of BRAF mutations at the expression level in melanomas of the uveal tract. None of the 30 metastases and 10 primary uveal melanomas tested expressed the V599E mutation. In contrast, this mutation was expressed by 65% of cutaneous melanoma samples, confirming previous results. In addition, a double mutation resulting in V599K substitution was detected in two suspect uveal metastases of cutaneous melanoma. Analysis of exon 11, the second common site of BRAF mutations, revealed only wild-type sequences in uveal melanomas. Analysis of tumor lysates showed the presence of phosphorylated mitogen-activated protein kinase, kinase, and mitogen-activated protein kinase in 50% of uveal and 100% of cutaneous melanoma metastases. Taken together, these results suggest that although the common BRAF mutations found in cutaneous melanoma do not play a role in tumorigenesis of uveal tract melanocytes, activation of the RAF/mitogen-activated protein kinase pathway may nevertheless play an important role in uveal melanoma.

Introduction

BRAF has been recently reported as a primary target of mutations in cutaneous melanoma (1). BRAF is a serine/threonine kinase in the MAPK2 pathway that transduces signals from RAS family members to MEK1/2 (2). Over 65% of melanomas harbored activating mutations in BRAF. A single transversion in exon 15 (T1796A), leading to a V599E amino acid substitution in the BRAF kinase activation domain, accounted for 90% of all of the detected mutations (1, 3). A similar high frequency of V599E mutations has also been reported in benign nevi, suggesting that deregulation of the RAS/RAF/MAPK pathway plays a role in very early steps of melanocytic neoplasia (4). BRAF mutations have been detected in other human cancers, albeit at lower frequencies (1, 3, 5, 6).

Uveal melanoma is the most common primary intraocular malignancy in adults. It originates from melanocytes of the choroid, ciliary body, and iris, which, together with retinal pigment epithelial cells, constitute pigment producing cells in the eye. Uveal melanocytes, together with their skin counterparts, derive from the neural crest in the body, and iris, which, together with retinal pigment epithelial cells, develop in the optic cup. Despite their common embryological background, uveal and cutaneous melanomas display only partially overlapping cytogenetic abnormalities (7, 8). Although alterations of chromosomes 1 and 6 are common to both tumors, aberrations such as monosomy of chromosome 3 and gain of 8q are typically found in uveal but rare in cutaneous melanomas. Conversely, alterations at the CDKN2 locus on 9p21, common in cutaneous melanoma, are infrequent in uveal tumors (9). Specific genes involved in tumorigenesis of uveal melanocytes are yet to be identified. In addition to different genetic alterations, the two tumors have a different biological and clinical behavior. In contrast to cutaneous melanoma, uveal melanoma spreads almost exclusively via hematogenous route, most usually to the liver. Up to 50% of the patients with choroidal and ciliary body melanoma eventually develop metastases. No standard treatment exists for these patients who have a poor overall 1-year survival rate (10, 11).

To investigate the role of BRAF in the development of uveal melanoma, we have analyzed the frequency of BRAF mutations at the expression level in a series of primary and metastatic tumors. We found that in contrast to cutaneous melanoma, BRAF is not mutated in uveal melanomas.

Materials and Methods

Detection of BRAF Mutations. Frozen samples of 31 metastases from cutaneous melanoma (11 cutaneous, 19 lymph node, and 1 abdominal metastasis) and 30 metastases from uveal melanomas were collected at the Multidisciplinary Oncology Center, University Cantonal Hospital (Lausanne, Switzerland). Twelve uveal melanomas, of which 10 were primary uveal melanomas, were from the Ophthalmology Hospital (Lausanne, Switzerland). RNA was extracted from tumor samples by the guanidinium/CsCl method and cDNA prepared as described previously (12). PCR was performed to amplify exon 15 and flanking exons of BRAF with primers 5'-GTAAGGGCTGCTGACCTGTGATAC-3' (forward, exon 13) and 5'-AAACGACGCTCTCAGGAGGG3' (reverse, exon 18) and Qiagen TaqMix (Qiagen). After 35 cycles of amplification (annealing temperature 55°C), the 621-bp products were purified using a Qiagen PCR purification kit. As a prescreen to detect the presence of the common T1796A mutation, amplified DNA was subjected to TspRI restriction digestion because this mutation abrogates a restriction site present in the wild-type sequence. After incubation with the enzyme (New England Biolabs) at 65°C for at least 4 h, the products were analyzed on 1.8% agarose gels. Special care was taken to directly load the samples at high temperature and in the presence of SDS (0.2%) to avoid reannealing of the restricted products (TspRI leaves a 9 base 3' overhang). cDNAs from cell lines containing the wild-type or the mutated T1796 was always included as controls. Amplified samples were also subjected to automated sequencing (Microsynth, Balgach, Switzerland) using the forward amplification primer, and in some cases, mutations were confirmed by sequencing with the reverse amplification primer. Exon 11 was amplified with primers 5’-AGCTC- CCAATGTGCACTAAAG3’ (forward, exon 8) and 5’-GGAATACCCGTC- GAAGAGTAG3’ (reverse, exon 13) as above. The 466-bp products were sequenced using the forward amplification primer. The majority of uveal melanoma samples was highly pigmented, and high expression levels of differentiation antigens tyrosinase and Melan-A/MART-1 were detected by PCR (12) in all of them.

Western Blot Analyses. Cryostat sections of frozen tumor samples were lyzed in a buffer containing 1% NP40, 150 mM NaCl, 1 mM Na2VO4, 50 mM...
NaF, 2 mM EDTA, 50 mM Tris-HCl (pH 7.5), and a mixture of protease inhibitors (Roche). Lysates (50 μg of protein) were subjected to SDS-PAGE under nonreducing conditions. Separated proteins were transferred to a nitrocellulose membrane (Amersham). Duplicate identical blots were probed with the following antibodies: rabbit polyclonal anti-MEK 1/2 and antimphospho-MEK 1/2 (Cell Signaling Technology); mouse monoclonal antiphospho-ERK-1/2 (Sigma), anti ERK-2 (Santa Cruz Biotechnology); and mouse monoclonal anti-α-tubulin (Sigma) and anti-MART-1 M2-7C10 (a gift from Steven A. Rosenberg). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham), immunocomplexes were detected using an enhanced chemiluminescence detection kit (Amersham) or SuperSignal West Femto (Pierce, for phospho-MEK).

Results and Discussion

A predominant mutation in exon 15 of BRAF (T1796A) has been detected by analysis of genomic DNA from cutaneous melanoma tumors and low passage cultures (1, 3). To confirm the high frequency of the T1796A mutation in cutaneous melanoma at the mRNA level, we amplified a BRAF cDNA fragment containing exon 15 from 31 cutaneous melanoma metastases. Amplified products were first screened by restriction analysis, taking advantage of the fact that the T1796A mutation abrogates a TspRI restriction site present in the wild-type sequence (Fig. 1). Twenty samples (65%) showed loss of the TspRI restriction site. The majority of these cases showed a concomitant expression of the mutated and the wild-type allele (Fig. 1 and data not shown). Direct sequencing confirmed the presence of the T1796A mutation and revealed the presence of an A1798G transition in one sample. The latter mutation, generating a K600E substitution, has also been detected in another malignant melanoma (3) and in a colorectal tumor (5). This mutation may mimic phosphorylation of the adjacent residue S601 that is normally required, together with phosphorylation of T598, for maximal kinase activity. Taken together, these results confirm, in a different experimental setting, the elevated frequency of BRAF mutations at the regulatory phosphorylation site of the kinase domain in cutaneous melanoma (1, 3).

We next analyzed a series of cDNAs from 40 uveal melanomas, including 30 metastases (all at the liver, except for 1 cutaneous and 1 lymph node metastasis) and 10 primary tumors from independent patients (Table 1). In striking contrast to the samples of cutaneous melanoma, none of the uveal melanomas was found to harbor the T1796A mutation. Sequencing of the entire RT-PCR products from tumors in 20 patients confirmed a wild-type sequence over the entire exon 15 and the flanking exons (exons 13–18). Although the T1796A/V599E mutation is greatly predominant in cutaneous melanoma, BRAF mutations have been also detected in the glycine-rich loop of the kinase domain and close to AKT phosphorylation sites encoded by exon 11. The latter mutations seem to be particularly relevant in lung cancer (3). To investigate whether the glycine-rich region is a preferred target for BRAF mutations in uveal melanoma, a sequence spanning exons 9–13 was amplified from 16 of the uveal melanoma tumors and sequenced. All samples showed a wild-type sequence over the entire amplified region. It should be noted that alternatively spliced exons have been described for BRAF such as exon 8b and 10b (the latter expressed specifically in brain; Ref. 13). These exons were not present in the sequenced RT-PCR products. In summary, no mutations were found in the BRAF cDNA encoding the protein kinase domain.

In addition to the above samples, we also analyzed two unusual cases of ocular melanoma. One tumor was diagnosed as a metastasis to the ciliary body from cutaneous melanoma and the second, a melanoma of the orbit, as a suspect metastasis from an unknown primary melanoma (the patient had a previous history of melanoma in a lymph node but no evidence of primary cutaneous melanoma). Surprisingly, both the latter tumors contained a double mutation GT to AA at residues 1795/6 resulting in a V599K amino acid substitution (Fig. 1B). Thus, the only BRAF mutations detected in our series of ocular melanoma occurred in two tumors that were not pathologically confirmed as uveal melanomas. The presence of an identical mutation in these two samples is puzzling, although it could be purely coincidental. The same mutation has been recently detected in two metastatic uveal melanomas but not in primary melanomas or nevi (4).

These results indicate that direct activation of BRAF by mutations in exons 11 or 15 is a rare event in uveal melanoma. Activating BRAF mutations alter the RAS signaling pathway downstream of many growth factor receptors, contributing to cell autonomy from external growth signals. A deregulated RAS/BRAF/MAPK signaling pathway is a hallmark of many tumors (14). Approximately 50% of colorectal tumors bear a mutated KRAS, and recently, BRAF mutations have been detected in this type of cancer at a frequency of 10% (5). More interestingly, mutations in RAS and BRAF were found to be mutually exclusive, suggesting that activation of either gene equally contributes

Table 1  BRAF mutations resulting in V599E substitution in uveal and cutaneous melanoma

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>BRAF (V599E)</th>
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<tr>
<td>Uveal tract melanoma</td>
<td></td>
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<tr>
<td>Primary</td>
<td></td>
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<tr>
<td>Choroid</td>
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<tr>
<td>Choroid/ciliary body</td>
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<tr>
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<td>Cutaneous melanoma</td>
<td></td>
</tr>
<tr>
<td>Metastases</td>
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Fig. 1. Analysis of BRAF mutations in cDNA from melanoma tumors. A, detection of V599E mutations by TspRI restriction of amplified BRAF cDNA. Analysis of cultured cell lines (melanoma cell line Me275, normal human fibroblasts, and melanocytes) and representative tumors (LAU 49, uveal melanoma metastasis; LAU 592 and LAU 599, primary uveal melanomas; LAU588, ocular metastasis from cutaneous melanoma; LAU 270 and LAU 90, cutaneous melanoma metastases) are shown. PCR amplification of cDNA samples and restriction digestion were performed as detailed in “Materials and Methods.” Nonmutated DNA gives rise to two fragments (indicated as w.t.). Presence of undigested DNA (≈600 bp) indicates the presence of mutation. Note the presence of both mutated and nonmutated forms of DNA, even in a cloned cell line (Me275), indicating biallelic expression. B, sequence chromatograms from BRAF exon 15 for representative samples shown in A.
to tumorigenesis. This may also be the case for metastatic melanoma, although the relative mutation frequencies of RAS and BRAF are inverted compared with colon cancer. Indeed, although mutations in RAS genes (codon 61 of NRAS being the prevalent target) are not very frequent in cutaneous melanoma, they were recently found to segregate to the minority of tumors harboring a wild-type BRAF (4). At the light of our results, one may therefore expect a high frequency of RAS mutations in uveal melanoma. However, only wild-type sequences have been reported for codon 61, as well as codon 12 and 13 of NRAS in uveal melanoma samples (15). Similarly, no mutations have been detected in HRA5 or KRAS. Taken together, this indicates that neither RAS nor BRAF common activating mutations initiate tumorigenesis in uveal tract melanocytes. This is in striking contrast to skin-derived melanocytes, where BRAF mutations are already present at high frequency in benign lesions such as common nevi, indicating a role in proliferation (4). Thus, either constitutive activation of the RAS/RAF/MAPK signaling pathway is not a requisite for tumorigenesis of uveal tract melanocytes, or, more likely, other component(s) of the growth signaling pathway, including upstream growth factor receptors and ligands, may be altered.

To address this issue, we analyzed the in vivo status of the MAPK pathway in uveal melanomas. Upon activation by interaction with GTP-bound RAS, RAF proteins phosphorylate MAPK/ERK kinases, called MEK (2). Western blot analyses of lysates of frozen tumor samples showed that half of uveal melanoma metastases expressed phosphorylated MEK-1/2 (Fig. 2, left panel). We next analyzed the activation status of the downstream targets, namely MAPK/ERKs. Phosphorylated ERK-1/2 were detected in all of the samples analyzed (Fig. 2, right panel), irrespective of the BRAF mutation status (the V599E mutation was present in samples Me 260, LAU 108, LAU 431, and LAU 444 but absent in the others). These data confirm recent results demonstrating the presence of a constitutively activated MAPK pathway in cutaneous melanoma (17). Using cultured melanoma cell lines, the authors found that MAPK activation was mediated by both BRAF mutations and autocrine stimulation by growth factors, notably basic fibroblast and hepatocyte growth factors (17). The specific factors (autocrine or paracrine) and pathways leading to activation of MEK and ERK in uveal melanomas remain to be established.

The etiology of uveal melanoma is largely unknown. Solar UV radiation, a well-recognized environmental factor contributing to cutaneous melanoma (18), may play a role in the development of uveal melanoma, although a clear correlation still has to be established (19–21). The lack of the common exon 15 BRAF mutation in uveal melanoma suggests the involvement of a different causative agent from that of cutaneous melanoma.

In summary, the lack of common BRAF mutations in uveal melanomas further differentiates this tumor from its cutaneous counterpart. The presence of an activated MAPK pathway in a fraction of uveal melanoma suggests nevertheless that common pathways may contribute to the transformation of uveal and cutaneous melanocytes. As drugs targeting RAF and downstream kinases are being developed, efforts should be directed at defining the role of the MAPK pathway in the development of uveal melanomas.

Acknowledgments

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References


Fig. 2. Analysis of the status of MAPK signaling pathway proteins in uveal and cutaneous melanoma in vivo. Protein extracts from the indicated tumor samples were analyzed by Western blotting as detailed in “Materials and Methods.” All tumors were metastatic. A lysate from an exponentially growing melanoma cell line (Me 260) was used as positive control. Antibodies recognizing the phosphorylated forms (phospho-MEK and phospho-ERK) or total MEK and ERK proteins were used. Antibodies to α-tubulin were used as loading controls.
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