Pyrophosphorolysis Detects B-RAF Mutations in Primary Uveal Melanoma

Willem Maat, Emine Kılıç, Gré P. M. Luyten, Annelies de Klein, Martine J. Jager, Nelleke A. Gruis, and Pieter A. Van der Velden

PURPOSE. Mutations in the genes that control cell proliferation in cutaneous melanoma are generally uncommon in uveal melanoma. Despite the absence of known activating mutations, the RAF-MEK-ERK, or mitogen-activated protein kinase (MAPK), pathway is usually activated in uveal melanoma. An assay with increased potential to identify mutations is now available, and this study was therefore conducted to reanalyze uveal melanoma cell lines and primary tumors for this mutation.

METHODS. Eleven uveal melanoma cell lines and 45 primary uveal melanomas were analyzed for mutations in exon 15 of the B-RAF gene by using pyrophosphorolysis-activated polymerization (PAP). Mutations were validated by sequencing of the PAP product.

RESULTS. B-RAF mutations were detected in cell lines OCM-1 and -3 (V600E) and in six primary uveal melanomas. The V600K mutation was detected in one primary uveal melanoma, for which the V600E assay turned out to be sensitive as well. Direct sequencing of the exon 15 PCR product did not reveal the mutations found with the PAP-assay, indicating a low frequency of the mutant allele in primary samples.

CONCLUSIONS. Of the very sensitive PAP technology, B-RAF mutations were found in cell lines and primary uveal melanomas, which suggests that they may occasionally play a role in the activation of the MAPK pathway in uveal melanoma and indicates a higher prevalence of B-RAF mutations in uveal melanoma than was reported earlier. However, the relative scarcity of the B-RAF mutation excludes an elemental role for this mutation in uveal melanoma. (Invest Ophthalmol Vis Sci. 2008;49:23-27) DOI:10.1167/iovs.07-0722

Uveal melanoma is a rare neoplasm arising from melanocytes in the eye, with an incidence rate of approximately six to eight new cases per million per year among Caucasians. Approximately 50% of patients who have a medium to large uveal melanoma will develop metastatic disease, mostly through hematogenic spread to the liver. Since there is hardly any effective treatment for such metastases, they are usually fatal within a year of onset of symptoms.

Compared with cutaneous melanoma, little is known about the molecular pathogenesis of uveal melanoma, and the role of different tumor pathways is less defined. Cutaneous melanoma shares the same embryonic origin and similar histologic features, but the mutations that activate the major oncogenic pathway in cutaneous melanoma, have not been found in uveal melanoma.

The RAF-MEK-ERK or mitogen-activated protein kinase (MAPK) pathway is of great importance in the development of many types of cancer, as well as in melanocytic neoplasia. In cutaneous melanocytes, activation of this pathway has been shown to occur by a variety of mechanisms, including endocrine and autocrine growth factor stimulation and mutation of the RAS and RAF genes. Activation of the MAPK pathway has also been reported in uveal melanoma, although it only rarely occurs through mutations in B-RAF or RAS. All B-RAF mutations in cutaneous pigmented neoplasms occur within the kinase domain, and the most frequently found mutation in B-RAF consists of a 1799T→A transversion in exon 15, although various other mutations have been described in this exon. This T1799A mutation is located in the serine/threonine kinase domain of B-RAF, resulting in a valine-to-glutamic acid substitution at position 600 (the National Center for Biotechnology Information [NCBI; Bethesda, MD] GenBank re-named the V599E mutation based on newly available sequence data; accession number NM_004333.2; hereafter referred to as B-RAF V600E), leading to a constitutive activation of proliferation signaling.

In contrast to these findings, B-RAF mutations have been reported only rarely in uveal melanoma (Table 1). In 2003, Calipel et al. analyzed four primary uveal melanoma cell lines for mutations in the B-RAF gene and reported the presence of the V600E mutation in uveal melanoma cell lines OCM-1 and TP-31, of which the mutation in OCM-1 was confirmed in two studies by other groups. The same group also reported B-RAF mutations in cell lines MKT-BR and SP6.5. To our knowledge, only one B-RAF mutation (V600E) has been described in a primary uveal melanoma, whereas several studies reported a lack of B-RAF mutations in cell lines and primary tumors. A possible explanation for the apparent lack of B-RAF mutations is that uveal melanomas are genetically heterogeneous, and therefore mutations are not present in each cell, similar to our findings in previous studies on the heterogeneous distribution of monosomy of chromosome 3 and methylation of RASSF1A. To detect mutations in a background of normal DNA, we used pyrophosphorolysis-activated polymerization (PAP) to test whether B-RAF mutations are indeed present in uveal melanoma and to test tumor heterogeneity. We set out to screen exon 15 of the B-RAF gene in uveal melanoma cell lines and primary tumors with PAP and showed that with PAP it is possible to amplify specifically minute amounts of mutant DNA in a background of wild-type DNA, and that, consequently, it has a high sensitivity for mutations present in just a small number of tumor cells.

From the Departments of 1Ophthalmology and 2Dermatology, Leiden University Medical Center (LUMC), Leiden, The Netherlands; and the Departments of 3Ophthalmology and 4Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands.

Supported by Dutch Cancer Society KWF Grant RUL 2001-247. Submitted for publication June 15, 2007; revised September 11, 2007; accepted November 26, 2007.

Disclosure: W. Maat, None; E. Kılıç, None; G.P.M. Luyten, None; A. de Klein, None; M.J. Jager, None; N.A. Gruis, None; P.A. Van der Velden, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Pieter A. van der Velden, Department of Dermatology, Skin Research Lab, Leiden University Medical Center (LUMC), PO Box 9600, 2300 RC Leiden, The Netherlands; velden@lumc.nl.


Copyright © Association for Research in Vision and Ophthalmology
In total, 11 cell lines derived from primary uveal melanomas (92.1, 23 OCM-1, -3 and -8; Mel-202, -270, -285, -290) and uveal melanoma metastases (OMM-1, 24 -2.3, and -2.5) were analyzed for B-RAF mutations. OMM-2.3 and -2.5 were derived from separate tumor nodules in the liver of the same patient from whom cell line Mel-270 was obtained and thus represent a progression model.25 All melanoma cell lines were cultured in RPMI 1640 medium (Invitrogen-Gibco, Paisley, Scotland, UK) supplemented with 3 mM l-glutamine (Gibco), 2% penicillin-streptomycin and 10% FBS (Hyclone, Logan, UT). All cell cultures were incubated at 37°C in a humidified 5% CO2 atmosphere. Archived frozen tumor specimens of primary uveal melanoma came from 37 consenting patients who attended the Leiden University Medical Center and from eight patients attending the Erasmus Medical Center (Rotterdam, The Netherlands). All tumors were primary lesions with a tumor diameter greater than 12 mm and a prominence greater than 6 mm, and they had metastases (OMM-1, 24 -2.3, and -2.5) were analyzed for B-RAF mutations. All tumors were primary lesions with a tumor diameter greater than 12 mm and a prominence greater than 6 mm, and they had not been treated before enucleation. The validity of the diagnosis of uveal melanoma was confirmed histologically in all cases. The research protocol followed the tenets of the current version of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1964; ethical principles for medical research involving human subjects).

**DNA Extraction**

A column-based extraction kit (Genomic tip 100/G; Qiagen Benelux BV, Venlo, The Netherlands) was used to extract DNA from the cell lines and frozen tumor material according to the kit manufacturer’s guidelines. A V600E-positive colon carcinoma cell line (HT29) was used as the control.7 DNA concentrations were determined with a spectrophotometer (model ND-1000; NanoDrop Technologies Inc., Wilmington, DE).

**PAP and Sequence Analysis**

In the PAP reaction, primers are used that contain a dideoxy-nucleotide (ddNTP) at their 3’ terminus and hence cannot be extended. Primers are shown in Table 2. A polymerase with pyrophosphorolysis activity can remove the dideoxy-nucleotide and thereby activate polymerization. The mutant base is resynthesized based on the original template sequence. Since this pyrophosphorolysis activity is dependent on double-stranded DNA, only primers that perfectly match the template will be activated (Fig. 1).

Because the PAP products are based on resynthesis, mutations can be validated by sequence analysis. Amplification was performed on a standard thermal cycler (MJ Research, Watertown, MA) in a final volume of 25 μL containing 5 μL 5 × PAP buffer (prepared as described by Liu et al.26), 0.3 μL (10 picomoles/μL) of each primer (Eurogentec Nederland BV, Maastricht, The Netherlands), 0.75 μL Klen Taq® (SciGen Tech, St. Louis, MO), 16.65 μL H2O, and 2 μL DNA sample. Amplification was initiated by hot start, followed by 50 cycles at 94°C for 15 seconds, 60°C for 40 seconds, 64°C for 40 seconds, and 72°C for 40 seconds. The product was electrophoresed through a standard 2% agarose gel in 1× TBE (0.09 M Tris-borate, 0.002 M EDTA; pH 8.2). The gel was stained with ethidium bromide for UV photography by a charge-coupled device camera (G:BOX Chemi; Syngene Europe, Cambridge, UK). After gel electrophoresis, DNA bands were excised from the gel, purified with a gel extraction kit (Nucleospin Extract II; Macherey-Nagel GmbH & Co., Düren, Germany) and sequenced on a DNA sequencing system (Prism 3700 Applied Biosystems [ABI], Foster City, CA). Direct sequencing with specific primers (Invitrogen, Breda, The Netherlands) (Table 2) was used for screening for the B-RAF mutation in all cell lines and primary tumors.

### RESULTS

Numerous B-RAF mutations have been detected in primary cutaneous melanomas and their cell lines.3,5,7,27 In contrast, an extremely low frequency of B-RAF mutations in uveal melanoma cell lines and primary tumors has been reported, whereas in vitro studies suggest that the B-RAF pathway plays a role in uveal melanoma cell growth.3,5,11,14,28 Applying PAP on 11 uveal melanoma cell lines and 45 primary uveal melanomas, we detected several B-RAF mutations. In cell line OCM-1, the V600E mutation was detected, confirming results from previous studies (Table 1). Of interest, cell line OCM-3 seems to harbor the same V600E mutation. Both cell lines were derived from the same laboratory, and their melanocytic origin has been demonstrated.29 Several investigators have questioned the choroidal origin of cell line OCM-1 based on the presence of the B-RAF V600E mutation, which is definitely related to cutaneous melanoma.12,24,30 Cell lines OCM-1 and -3 showed different HLA-types and confirmed that they were indeed derived from different patients and were not interchanged (data not shown). The presence of the mutation found in OCM-3 has not been reported before, as far as we know, even though this cell line was analyzed in our own laboratory by conventional sequencing.2 The fact that direct sequencing reveals a homozygous genotype for the B-RAF mutation indicates that all cells contain the V600E mutation.

In primary uveal melanomas, the V600E mutation in the B-RAF gene, which consists of a T→A transversion at position 1799 and results in a valine-to-glutamic acid substitution, was detected in 6 (13%) of 45 primary tumors. The other mutation uncovered in our study was the rarely reported V600K mutation in one primary uveal melanoma sample. This mutation

---

### Table 2. Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP B-RAF (forward)</td>
<td>5'-GTGTGCTTCTTTACCTAAGCACAATATGTTGCAGTCCATG-3'</td>
</tr>
<tr>
<td>PAP B-RAF (reverse)</td>
<td>5'-CCTTTGCAACTCTGGTTGGGAGCCCATTCAGAATGTTTC-3'</td>
</tr>
<tr>
<td>Sequence BRAF exon 15 (forward)</td>
<td>5'-AATCTGTGTTGATGCTGCTGATAGG-3'</td>
</tr>
<tr>
<td>Sequence BRAF exon 15 (reverse)</td>
<td>5'-GGCTGAAATTCTGCTACCCCAAAAAGT-3'</td>
</tr>
</tbody>
</table>

ddT: dideoxy-nucleotide (ddNTP).
consists of a GT→AA substitution at position 1798-1799 and is also located in the serine/threonine kinase domain of B-RAF. Results were confirmed by sequencing of the PAP product after purification from the electrophoresis gel (Fig. 2). Our primers were not designed to detect occurrences of V600D or V600R mutations. We did not detect any differences in tumor size, location, cell type or patient survival between tumors with and without the detected V600E mutation.

**DISCUSSION**

That in this study we detected B-RAF mutations, whereas other studies, including our own, reported the absence of mutations may be explained by the techniques used. PCR in combination with direct sequencing or ligase-detection reaction and mutation assay (Mutector; Biomol, Hamburg, Germany) were the techniques used to detect mutations in previous studies. However, these techniques are less sensitive than PAP, especially in samples with a low abundance of mutations in the presence of excess amounts of wild-type DNA in the tumor. Whereas conventional techniques used to detect mutations theoretically have a predicted sensitivity varying between 1:10^4 to 1:10^5, PAP has a predicted sensitivity of 1:10^-9, making it suitable for the detection of sporadic muta-

**FIGURE 1.** (A) B-RAF exon 15 sequence and primers used in PAP experiments. (B) Situation in which the primer with a dideoxy nucleotide (dd*T) at its 3' end does not match with the normal template and hence will not be removed by the phosphorolytic activity of Klen Taq and prevent amplification. (C) Situation in which the primer with dd*T-3' perfectly matches with the mutant template. The phosphorolytic activity of Klen Taq removes the dideoxy nucleotide and the primer initiates polymerization. The mutant base is resynthesized based on the template and thereby provides a means of validation by sequencing of the PAP product.

**FIGURE 2.** Sequence analysis of the PAP products showing the sequence of wild-type B-RAF exon 15 (A), the V600E mutation (B), and the V600K mutation (C) in primary uveal melanoma DNA samples.
time because PAP is inhibited by fluorescent dyes. The number of B-RAF mutants in a tumor sample with a real time approach indicates that cells with mutations are very rare in these tumors and may imply that mutations in B-RAF are not likely to drive uveal melanoma development and also adds further proof for the proposed heterogeneity in uveal melanoma. Of interest, the V600E mutation accounts for 92% of the B-RAF mutations detected in cutaneous melanoma samples. However, Pollock et al. reported the presence of B-RAF mutations in 82% of cutaneous nevi, demonstrating that B-RAF activation alone is insufficient for the development of cutaneous melanoma, highlighting the requirement for additional molecular changes.

In this study, PAP detected B-RAF mutations in uveal melanoma cell lines, as well as in primary tumor samples. The PAP assay is sensitive enough to detect a single mutant sequence in excess of wild-type DNA sequences. Based on this assay, we conclude that B-RAF mutations occur in uveal melanoma, although the clinical relevance of such mutations in a minor percentage of cells has to be determined. Our data reveal that B-RAF mutation frequency in uveal melanoma is higher than earlier anticipated and add to the rarely reported B-RAF mutations in uveal melanoma. However, the relative scarcity of the B-RAF mutation excludes an elemental role for this mutation in uveal melanoma.

Acknowledgments

The authors thank Rolf Vossen (Department of Human and Clinical Genetics, LUMC, The Netherlands) for providing the PAP construct, and Bruce R. Ksander (Scheepens Eye Institute, Harvard Medical School, Boston, MA) and June Kan-Mitchell (Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI) for their gifts of cell lines.

References


13. Calipel A, Mouriaux F, Glotin AL, et al. Extracellular signal-regulated kinase-dependent proliferation is mediated through the protein B-RAF. The B-RAF mutation is still recognizable in the presence of tens of thousands of wild-type templates.


