Pyrophosphorolysis Detects B-RAF Mutations in Primary Uveal Melanoma

Willem Maat, Emine Kilic, 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 TP-3 (V600E) and in six primary uveal melanomas. The V600K mutation was detected in one primary uveal melanoma, and -3 (V600E) and in six primary uveal melanomas. The fatal within a year of onset of symptoms. any effective treatment for such metastases, they are usually through hematogenic spread to the liver. Since there is hardly large uveal melanoma will develop metastatic disease, mostly 

CONCLUSIONS. Because 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.

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In contrast to these findings, B-RAF mutations have been reported only rarely in uveal melanoma (Table 1). In 2003, Calipel et al.11 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.3,11,12 The same group also reported B-RAF mutations in cell lines MKT-BR and SP6.5.13 To our knowledge, only one B-RAF mutation (V600E) has been described in a primary uveal melanoma,14 whereas several studies reported a lack of B-RAF mutations in cell lines and primary tumors.3,12,15–19 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.20,21 To detect mutations in a background of normal DNA, we used pyrophosphorolysis-activated polymerization (PAP)22 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.
**Calipel et al.** 11 2/4 OCM-1, TP-31

The ethical principles for medical research involving human subjects. The protocol followed the tenets of the current version of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1964; 2000). A V600E-positive colon carcinoma cell line (HT29) was used to extract DNA from the cell line before by conventional sequencing. The fact that these mutations are V600E mutations.

**Materials and Methods**

**Cell Lines and Primary Uveal Melanoma Specimens**

In total, 11 cell lines derived from primary uveal melanomas (92.1, 30 OCM-1, -3, and -8, Mel-202, -270, -285, -290) and uveal melanoma metastases (OMM-1, -2.3, and -2.5) were analyzed for BRAF 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. 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. Archival 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 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. 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.24), 0.3 µL (10 picomoles/µL) of each primer (Eurogentec Nederland BV, Maastricht, The Netherlands), 0.75 µL Klen TaqS (SciencTech, 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 1X 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; Syn-gene Europe, Cambridge, UK). After gel electrophoresis, DNA bands were excised from the gel, purified with a gel extraction kit (Nucleogene Europe, Cambridge, UK). Direct sequencing with specific primers (Invitrogen, Breda, The Netherlands) (Table 2) was used for screening for the BRAF mutation in all cell lines and primary tumors.

**Results**

Numerous BRAF mutations have been detected in primary cutaneous melanomas and their cell lines. In contrast, an extremely low frequency of BRAF mutations in uveal melanoma cell lines and primary tumors has been reported, whereas in vitro studies suggest that the BRAF pathway plays a role in uveal melanoma cell growth. Applying PAP on 11 uveal melanoma cell lines and 45 primary uveal melanomas, we detected several BRAF 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. Several investigators have questioned the choroidal origin of cell line OCM-1 based on the presence of the BRAF V600E mutation, which is definitely related to cutaneous melanoma. 

**Table 1. Summary of Published BRAF Mutation Studies in Uveal Melanoma Cell Lines**

<table>
<thead>
<tr>
<th>Study</th>
<th>BRAF Mutation Frequency*</th>
<th>Cell Line with BRAF Mutation</th>
</tr>
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<tbody>
<tr>
<td>Calipel et al.11</td>
<td>2/4</td>
<td>OCM-1, TP-31</td>
</tr>
<tr>
<td>Kilic et al.12</td>
<td>1/11</td>
<td>OCM-1</td>
</tr>
<tr>
<td>Zuidervaart et al.3</td>
<td>1/10</td>
<td>OCM-1</td>
</tr>
<tr>
<td>Calipel et al.13</td>
<td>3/3</td>
<td>OCM-1, SP6.5</td>
</tr>
<tr>
<td>This study</td>
<td>2/11</td>
<td>OCM-1, OCM-3</td>
</tr>
</tbody>
</table>

* All are V600E mutations.

**Table 2. Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>PAP BRAF (forward)</td>
<td>5’-GTTTTCCTTTAATCTACTGATGATGATGTTCTCATAG-3’</td>
</tr>
<tr>
<td>PAP BRAF (reverse)</td>
<td>5’-CTGTTTGAACTGATGAGGAGGGCCACTGACACAGATTTTC-3’</td>
</tr>
<tr>
<td>Sequence BRAF exon 15 (forward)</td>
<td>5’-AAGCTTTGCTGACTGATGATGATGTTCTCATAG-3’</td>
</tr>
<tr>
<td>Sequence BRAF exon 15 (reverse)</td>
<td>5’-GGCTGAATTTCTTACTGATGATGATGTTCTCATAG-3’</td>
</tr>
</tbody>
</table>

ddT: dideoxy-nucleotide (ddNTP).
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.

consists of a GT→AA substitution at position 1798-1799 and is also located in the serine/threonine kinase domain of B-RAF.\textsuperscript{5} 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,\textsuperscript{5} 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.\textsuperscript{3,11,16,31,32} 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.\textsuperscript{22,26} Whereas conventional techniques used to detect mutations theoretically have a predicted sensitivity varying between 1:10\textsuperscript{4} to 1:10\textsuperscript{5}, PAP has a predicted sensitivity of 1:10\textsuperscript{9},\textsuperscript{9} making it suitable for the detection of sporadic mutations.
B-RAF mutations and sensitivity of this assay. The B-RAF mutation is still recognizable in the presence of tens of thousands of wild-type templates.22,35 Limited by the input of genomic DNA, the practical sensitivity of the essays is lower. For the PAP essay, the practical sensitivity is at least 1:10^4 (Fig. 3). Also in our study, direct sequencing of exon 15 PCR products did not reveal the mutations found with the PAP-assay suggesting a minor frequency for the mutant allele, apart from the V600E mutation in cell lines OCM-1 and -3, which could be detected by direct sequencing. Although the reverse primer in our PAP-assay is blocked at the 3-prime end, which first must be removed to start polymerization, the forward primer will start DNA polymerization each cycle, independent of the B-RAF genotype. Because of the intrinsic error rate of the forward polymerase reaction, theoretically, an adenine can be misincorporated at position 1799. This erroneously synthesized copy can subsequently serve as a template for the blocked primer and falsely start a PCR reaction. However, the control assays that we performed indicate that the positive PAP-assays with primary tumors are not likely to be explained by polymerase artifacts. The assays with normal DNA that we always include in our experiments never resulted in a positive PAP assay and thereby suggest that this error rate is limited. Cross contamination as cause of positive PAP assays is prevented by using separate rooms before and after PCR. The negative controls furthermore indicate that this is not the explanation for the positive tumors, and the latter specifically applies to the V600K mutation that we have never detected before. Moreover, dilution experiments with OCM-1 and wild-type B-RAF genomic DNA illustrates the sensitivity of the PAP assay (Fig. 5). Under experimental conditions a few mutant copies can be detected in the presence of tens of thousands of wild-type copies and supports the hypothesis that uveal melanoma display heterogeneity for B-RAF mutations. Unfortunately, it is not possible to quantify the number of B-RAF mutants in a tumor sample with a real time approach because PAP is inhibited by fluorescent dyes and the polymerase lacks the 5'→3' exonuclease activity necessary for the TaqMan approach (ABI). That to date only PAP is able to detect B-RAF mutations in primary uveal melanoma may indicate 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.20,21 The role of these sporadic mutations remains unclear. It may be that the observed B-RAF mutations represent a sign of tumor progression or evolution or appear as spontaneous mutations within the developing tumor.34,35 Mutations are found in exons 11 and 15, but only mutations in the activation domain of B-RAF such as the V600E are thought to have a selective advantage.36 Of interest, the V600E mutation accounts for 92% of the B-RAF mutations detected in cutaneous melanoma samples.3 However, Pollock et al.6 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.

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**References**

13. Calipel A, Mouriaux F, Glotin AL, et al. Extracellular signal-regulated kinase-dependent proliferation is mediated through the pro-