Pyrophosphorolysis Detects B-RAF Mutations in Primary Uveal Melanoma

Willem Maat,1 Emine Kilic,2 Gré P. M. Luyten,1 Annelies de Klein,5 Martine J. Jager,1 Nelleke A. Gruis,4 and Pieter A. Van der Velden1,4

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-assy, indicating a low frequency of the mutant allele in primary samples.

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 Ophthal mol 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.

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

Supported by Dutch Cancer Society KWF Grant RUL 2001-2472. Submitted for publication June 15, 2007; revised September 11, 2007; accepted November 26, 2007.

Disclosure: W. Maat, None; E. Kilic, 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.

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 often 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. All 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.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.11,12 The same group also reported B-RAF mutations in cell lines MTK-BR and SP6.13 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.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.
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, -2, 3.2, 4.1, 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. 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 metastases (OMM-1, 24 -2.3, and -2.5) were analyzed for B-RAF mutations. 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.3 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 pyrophosphorylase activity can remove the dideoxy-nucleotide and thereby activate polymerization. The mutant base is resynthesized based on the original template sequence. Since this pyrophosphorylase 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 TaqS (ScienTech, 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, 68°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; Syn-gene 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.5,6,7,8 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.3,11,14,28

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. 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 TaqS (ScienTech, 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, 68°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; Syn-gene 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.

**Table 1. Summary of Published B-RAF Mutation Studies in Uveal Melanoma Cell Lines**

<table>
<thead>
<tr>
<th>Study</th>
<th>B-RAF Mutation Frequency*</th>
<th>Cell Line with B-RAF Mutation</th>
</tr>
</thead>
<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, MKT-BR</td>
</tr>
<tr>
<td>Calipel et al.15</td>
<td>3/3</td>
<td>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>
</tr>
</thead>
<tbody>
<tr>
<td>PAP B-RAF (forward)</td>
<td>5’-GTTTTCGTTTTAATACCTGCAGATATCTTCTCAG-3’</td>
</tr>
<tr>
<td>PAP B-RAF (reverse)</td>
<td>5’-CTCGTGAACCTGGAGCCACCTCCATCGAGATTTC-dd*T-3’</td>
</tr>
<tr>
<td>Sequence BRAF exon 15 (forward)</td>
<td>5’-AATGCTCGTGTGCTCTGATTGAG-3’</td>
</tr>
<tr>
<td>Sequence BRAF exon 15 (reverse)</td>
<td>5’-GCTCTCGGCGCCTGACGCGAATATAG-3’</td>
</tr>
</tbody>
</table>

dd*T: 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.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,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.5,11,16,31,32 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.22,26 Whereas conventional techniques used to detect mutations theoretically have a predicted sensitivity varying between 1:10^1 to 1:10,9 PAP has a predicted sensitivity of 1:10,9 making it suitable for the detection of sporadic muta-
B-RAF
the hypothesis that uveal melanoma display heterogeneity for
ments with OCM-1 and wild-type
we have never detected before. Moreover, dilution experi-
ments before and after PCR. The negative controls furthermore
indicate that this is not the explanation for the positive tumors,
rooms before and after PCR. The negative controls furthermore
suggest that this error rate is limited. Cross contamination as
experiments never resulted in a positive PAP assay and thereby
cause of positive PAP assays is prevented by using separate
assays with normal DNA that we always include in our
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, al-
though 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 muta-
tions 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 (Scheper Eye Institute, Harvard Medical School, Bos-
ton, MA) and June Kan-Mitchell (Karmanos Cancer Institute, Wayne State
University School of Medicine, Detroit, MI) for their gifts of cell lines.

References
1. Egan KM, Seddon JM, Glynn RJ, Gragoudas ES, Albert DM. Epide-
miologic aspects of uveal melanoma. Surv Ophthal. 1988;32:
239–251.
2. Singh AD, Topham A. Incidence of uveal melanoma in the United
MAPK pathway is a common event in uveal melanomas although it
rarely occurs through mutation of BRAF or RAS. Br J Cancer.
7000.
5. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in
7. Satyamoorthy K, Li G, Gerrero MR, et al. Constitutive mitogen-
activated protein kinase activation in melanoma is mediated by
both BRAF mutations and autocrine growth factor stimulation.
8. Uribe P, Wistuba II, Gonzalez S. BRAF mutation: a frequent event in
benign, atypical, and malignant melanocytic lesions of the skin.
10. Zhang BH, Guan KL. Transformation through the MEK/ERK Pathway.
Human Choroidal Melanoma Cells Mediates Cell Proliferation and
Transformation through the MEK/ERK Pathway. Journal of Bio-
logical Chemistry. 2003;278:42409–42418.
pathway is not involved in uveal melanoma. Melanoma Res. 2004;
14:203–205.
lated kinase-dependent proliferation is mediated through the pro-
FIGURE 3. The PAP dilution experiment shows the high specificity and sensitivity of this assay. The B-RAF mutation is still recognizable in the presence of tens of thousands of wild-type templates.

progression or evolution or appear as spontaneous mutations
within the developing tumor. Mutations are found in ex-
ons 11 and 15, but only mutations in the activation domain of
B-RAF such as the V600E are thought to have a selective
advantage. Of interest, the V600E mutation accounts for 92%
of the B-RAF mutations detected in cutaneous melanoma sam-
ple. 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 cutane-
ous melanoma, highlighting the requirement for additional
molecular changes.

In this study, PAP detected B-RAF mutations in uveal mel-
anoma 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, al-
though 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 muta-
tions in uveal melanoma. However, the relative scarcity of the
B-RAF mutation excludes an elemental role for this mutation in
uveal melanoma.

Limitied by the input of genomic DNA, the practical
sensitivity of the assays is lower. For the PAP assay, the prac-
tical sensitivity is at least 1:104 (Fig. 3). Also in our study, direct
sequencing of exon 15 PCR products did not reveal the muta-
tions 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 se-
quencing. 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 poly-
merization 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 sub-
sequently 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-ssays 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 experi-
ments with OCM-1 and wild-type B-RAF genomic DNA illus-
states the sensitivity of the PAP assay (Fig. 5). Under experi-
mental 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 nec-
assary for the TagMan 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

14:203–205.


