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Abstract

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Reference


DOI : 10.1167/iovs.18-26508
PMID : 31247083
Conjunctival Melanoma Targeted Therapy: MAPK and PI3K/mTOR Pathways Inhibition

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Submitted: December 22, 2018
Accepted: May 3, 2019


PURPOSE. To analyze the activity of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinases/mechanistic target of rapamycin (PI3K/mTOR) pathways in benign and malignant conjunctival melanocytic proliferations and explore whether specific inhibitors can suppress growth of conjunctival melanoma (CJM) cells.

METHODS. The presence of a BRAF V600E mutation and activation of ERK, MEK, S6, and AKT were assessed with immunohistochemistry in 35 conjunctival nevi and 51 melanomas. Three CJM cell lines were used: CRMM1, carrying the BRAF V600E mutation; CRMM2, harboring the NRAS Q61L mutation; and T1527A, with a BRAF G616E mutation. WST-1 assays were performed with a BRAF inhibitor ( vemurafenib), two MEK inhibitors ( trametinib, selumetinib), a PI3K inhibitor ( pictilisib), and a dual PI3K/mTOR inhibitor ( dactolisib). The phosphorylation of ERK, MEK, and S6 were tested with western blots and apoptosis with cleaved caspase-3 immunostaining.

RESULTS. A BRAF V600E mutation was detected in 42.6% of nevi and in 35.5% of CJM. MEK and ERK activation were higher in CJM, occurring in 62.9% and 45.7% of the nevi and 90.3% and 96.8% of the CJM, respectively. There was also a significant increase in S6 activation in CJM (90.3%) compared with the nevi (20%). CRMM1 was sensitive to trametinib and the PI5K inhibitors but only marginally to vemurafenib. CRMM2 was moderately sensitive to pictilisib, and T1527A was resistant to all drugs tested.

CONCLUSIONS. The MAPK pathway activity in CJM is increased, not only as a consequence of the BRAF V600E mutation. Targeted therapy may be useful for patients with CJM, especially those with activating BRAF mutations, whereas NRAS-mutated melanomas are relatively resistant.

Keywords: melanoma, conjunctiva, mutations, MAPK pathway, BRAF

Conjunctival melanoma, whose incidence increased from 1990 to 1999 in the United States (0.46/million),1 is associated with a high recurrences rate, ranging from 32% to 62%.2,3 The therapeutic management of metastatic conjunctival melanoma is challenging and metastatic death occurs in 9% to 35% of patients at 10 years postdiagnosis.4–6

Conjunctival and cutaneous melanomas not only share common clinical features, such as metastatic spread through the lymphatic system, but also share some molecular features. The mutation spectrum appears to be similar to cutaneous melanoma, with a majority of cytosine to thymine transitions (C>T)7,8 suggesting a high impact of UV light-induced damage. The high mutation load with ~90,000 mutations in the entire genome is also similar to skin melanoma and contrasts sharply with the low mutation load identified in uveal melanoma.9 Mutations commonly observed in cutaneous melanoma, such as V600E in exon 15 of BRAF or Q61L in exon 3 of NRAS8,11 are also identified in conjunctival melanoma, with BRAF mutations being found in 29% to 55%,12,13 and NRAS mutations in 18% of the cases.12 More recently, NFI mutations could be identified in 33% of the patients.14

Numerous studies have assessed the importance of the mitogen-activated protein kinase (MAPK) pathway in cutaneous melanoma.15 Small-molecule inhibitors, binding to the active conformation of BRAF and blocking access to ATP, disrupt MAPK signaling, reduce proliferation in vitro, and induce tumor regression in vivo in BRAFV600E melanomas.16,17 In patients with metastatic melanoma carrying the BRAFV600E mutation, these inhibitors were associated with an impressive complete or partial tumor response.18–20

Direct MEK targeting downstream of BRAF represents an alternative way to overcome tumor resistance in patients with
**BRAF**-mutated skin melanomas. Trametinib, an allosteric and highly selective MEK inhibitor, significantly improved progression-free survival and overall survival in patients with **BRAF** mutant metastatic melanoma.²²

**NRAS** mutations, usually mutually exclusive with **BRAF** mutations, occur in 20% to 30% of skin melanomas and more commonly in older patients.¹¹,²² The RAS⁵⁶⁴⁶ mutation impairs hydrolysis of guanosine-5′-triphosphate (GTP) to guanosine diphosphate (GDP) and the transition of NRAS from an active to an inactive state. Downstream single inhibition of the MAPK pathway through MEK inhibition,²³ alone or in combination with phosphoinositide 3-kinases/mechanistic target of rapamycin (**PI3K/mTOR**) inhibition,²³ has been attempted in **NRAS**-mutated melanoma. Although in vitro MEK inhibitors appeared more successful than PI3K inhibitors, the combined use of MEK and PI3K reduced cell viability and tumor size in vivo models more efficiently.²⁴

The efficiency of direct **BRAF** inhibition in conjunctival melanoma (**CJM**) has been evaluated in vitro in two studies.²⁵,²⁶ The response to **BRAF** inhibition in advanced metastatic **CJM** has been variable, ranging from a partial regression lasting 1 month²⁷ to significant tumor regression for 38 weeks.²² A complete regression for 38 weeks of an irradiated metastatic **CJM** treated with vemurafenib has also been documented.²⁹

We conducted a study to assess the activity of the MAPK and PI3K/mTOR pathways in conjunctival melanomas tissues in relation to the presence or absence of the oncogenic **BRAF⁶⁰⁰⁰** mutation. We also assessed in vitro the response of conjunctival melanoma cell lines containing defined **BRAF** and **NRAS** mutations to **BRAF**, MEK, PI3K, and dual PI3K/mTOR inhibitors.

**Materials and Methods**

**Patients**

The clinical charts of patients treated for conjunctival melanoma or conjunctival nevi in Jules-Gonin Eye Hospital from 1998 until 2013 where formalin-fixed paraffin-embedded tissue was available were selected. The following clinical information was retrieved from the patients’ charts: age, sex, tumor localization, treatment, recurrences, metastases, death, and length of follow-up. The study was approved by the Swiss Federal Department of Health (authorization 032.0003-48) as well as local ethic committee (protocol 340/15) and adhered to the tenets of the Declaration of Helsinki.

**Tissues**

Formalin-fixed paraffin-embedded tissues were retrieved from the archives of Jules-Gonin Eye Hospital Pathology Laboratory. Sections (5 μm) were cut, and hematoxylin-eosin stained tissues were performed. The sections were incubated with the following anti-human antibodies: anti-phospho-MEK (Ser221, rabbit monoclonal; Cell Signaling, Danvers, MA, USA; dilution 1:200), anti-phospho-ERK (Thr202/Tyr204, rabbit monoclonal; Cell Signaling; dilution 1:600), anti-phospho-AKT (Ser473, rabbit monoclonal, dilution 1:50), and anti-phospho-S6 (Ser235/236, rabbit monoclonal; Cell Signaling; dilution 1:200). Positive controls were found in endothelial cells of conjunctival stroma and conjunctival epithelium for anti-phospho-S6, anti-phospho-MEK, and ERK (Supplementary Fig. S1). For phospho-AKT, a phospho-AKT (Ser 473) slide with LNCaP cells treated with or without a PI3K inhibitor, LY294002, was used. A streptavidin/biotin detection method with 3,3′-diaminobenzidine tetrachloride or 3-amino 9-ethylcarbazole was used for signal detection (Dako Envision System/HRP Dual Link; Dako Agilent, Santa Clara, CA, USA).

The **BRAF** V600E mutation was assessed in the tissues using a **BRAF**-specific monoclonal antibody (clone VE1; mouse monoclonal; Ventana Medical Systems, Oro Valley, AZ, USA) with a BenchMark Ultra platform (Roche Diagnostics, Rotkreuz, Switzerland).

The proportion of positively stained cells was scored in the following manner: 0% to 10% positive cells: score 1; >10% to 50%: score 2; and >50% to 100%: score 3. Three independent observers (APM, MB, and MN) evaluated the immunostained slides and discordant cases were reviewed simultaneously to reach a concordant agreement.

There was a concordance of 75.8%, 88.6%, 95.4%, 93.9%, and 93.9% among the observers and the evaluation of pMEK, pERK, cytoplasmic pAKT, nuclear pAKT, and pS6, respectively.

**Cells**

Conjunctival melanoma cell lines CRM1 and CRM2, established by Gordon Nareyek, University of Essen, Germany, were kindly provided by Martine Jager, University of Leiden with the authorization of use granted from Gordon Nareyek. The cells were cultured in F-12K nutrient mixture, with Kaighn’s modification, containing L-glutamine (Gibco, Life Technologies, Carlsbad, CA, USA), 10% heat-inactivated fetal bovine serum (Greiner Bio-one, Kremsmuenster, Austria) and 2% penicillin/streptomycin (Gibco). T1527A was established in our laboratories (Jules-Gonin Eye Hospital and Ludwig Center for Cancer Research, Switzerland) from a perlimbic conjunctival melanoma extending onto the cornea in a 65-year-old man. These cells were grown in a mixture of 50% of RPMI 1640 modified media (Gibco) and 50% Dulbecco’s modified Eagle’s medium (DMEM) F-12 (nutrient mixture with GlutaMAX; Gibco) supplemented with 10% fetal bovine serum (Greiner Bio-one) and 6 mM N-2-hydroxyethyl-1-piperazine-N’-2-ethanesulfonic acid (HEPES; Gibco). An analysis of the full length of **BRAF** in the original tumor and the derived cell line T1527A revealed the mutation G466E in exon 11 of **BRAF** in both samples (Supplementary Fig. S2). **NRAS** was found to be wild type in both the cell line and the original tumor. In addition, next-generation sequencing of the tumor, performed as in Rivolta et al.,²⁷ did not reveal any mutation in **NFI**. An analysis of HRAS revealed the heterozygous mutation Q61R in the T1527A cell line.

**Reagents**

Vemurafenib (PLX4032, S1267), pictilisib (GDC-0941, S1065), selumetinib (AZD6244, S1008), trametinib (GSK1120212, S2673), and dactolisib (BEZ 235) were purchased from Selleck Chemicals (Huisgen, The Netherlands). All drugs were dissolved in dimethyl sulfoxide (DMSO) to reach a stock concentration of 10 mM.

**WST-1 Assay**

The WST-1 assay, a colorimetric test that was developed to determine the survival and growth of eukaryotic cells in proliferation or to evaluate cytotoxicity,³⁰ was used to evaluate cell viability and calculate the half maximal inhibitory concentration (IC₅₀) of the drugs.

Briefly, cells were seeded in a 96-multiwell plate at a density of 5000 to 6000 cells/well in 200 μl of medium. The following day, cells were treated with increasing concentrations of kinase inhibitors. Twenty four and 72 hours later, Cell Proliferation Reagent WST1 (20 μl) was added to each well,³¹ including the wells that contained only medium (with corresponding diluted
DMSO). Plates were then incubated for 1 hour at 37°C in the dark and analyzed with a microplate reader (Hidex Sense; Hidex, Turku, Finland) at 420 nm versus 650 nm. Experiments were performed in triplicate and repeated three times. IC\textsubscript{50} values were calculated using GraphPad software (GraphPad Software, San Diego, CA, USA).

### Western Blot Analysis
Cells were disrupted in radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 1% sodiumdodecyl sulfate) in the presence of protease and phosphatase inhibitors. Protein concentration was measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).\textsuperscript{32}

Proteins were boiled in Laemmli buffer (62 mM Tris-HCl [6.8 pH], 2% SDS, 10% glycerol, 5% β-mercapto-ethanol, and 0.01% bromphenol blue). After blocking with 5% BSA in PBS for 1 hour at room temperature, membranes were incubated overnight at 4°C with the following primary antibodies: anti-ERK and anti-phospho-ERK (Cell Signaling; catalog no. 4695 and catalog no. 4370S, used at 1:1000); anti-MEK and anti-phospho-MEK (Cell Signaling; catalog no. 9126S and catalog no. 2338S, 1:1000); anti-S6 ribosomal protein and anti-phospho-S6 ribosomal protein (Cell Signaling; catalog no. 2217S and catalog no. 4858 S, 1:1000); and anti-alpha-tubulin (Sigma; catalog no. T5168, 1:4000). Horseradish peroxidase-conjugated anti-rabbit (Dako; P0399) or anti-mouse (Dako; P0447) antibodies, diluted 1:5000, were used as secondary reagents. Visualization of the immunoreactive bands was obtained by a chemoluminescent substrate, Super Signal West Pico Chemiluminescent Substrate (Thermo Fischer Scientific, Waltham, MA, USA) using fusion solo chemiluminescence and optional fluorescence imaging system.

### Indirect Immunofluorescence Experiments
Cells were seeded at a density of 10/well in 1 ml of complete medium in Lab-Tek devices (4 wells, glass slide; Thermo Fisher Scientific). Cells were treated with various kinase inhibitors at their respective IC\textsubscript{50} concentrations. The activation of caspase-3 was evaluated 24 hours later by immunofluorescence with cleaved caspase-3 (catalog no. AP3725a; Abgent, San Diego, CA, USA); dilution 1:300). Cells were observed using a fluorescence microscope, Olympus BX51 (Olympus, Shinjuku, Tokyo, Japan), equipped with 40× and 60× objectives.

### Statistical Analysis
Statistical analysis was performed using JMP 8.0 software (SAS, Cary, NC, United States), GraphPad Prism 5 program (GraphPad Software) and R statistical software, version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria).

Tests of significance were two-tailed, and results were considered as significant at an alpha level of \( P < 0.05 \), and a \( P \) value of 0.05 was considered to be borderline significant. Fisher’s exact tests were used to establish an association between two qualitative variables. Mann-Whitney \( U \) test was used to compare continuous variables.

### Results

#### Clinicopathologic Characteristics
The nevi group (\( n = 35 \)) comprised 13 subepithelial and 22 compound nevi. The male/female patient ratio was 21/14, with a mean age of 36.9 (SD, 21.3 years). None of the nevi occurred in the tarsal conjunctiva and nine nevi arose in the caruncula. None of the nevi recurred (mean follow up, 31.5 months [SD, 29.9 months]).

The melanoma group (\( n = 31 \)) consisted of 15 males and 16 females, with a mean age of 66.25 (SD, 20.4 years). According to the last American Joint Committee on Cancer TNM classification, 12 tumors belonged to the T1, 7 to the T2, and 12 to the T3 category. Melanomas arose mostly from primary acquired melanosis (67.8%) and less frequently from a nevus (19.3%) or de novo (12.9%). The origin of melanoma was determined by a combination of clinical and histopathologic factors.

Mean follow-up was 55 months (SD, 76.3 months). Local recurrences were found in 58.6% of the cases. Metastases developed in 10 patients, of whom 4 died.

### Pathway Activation in the Tissues
The BRAF mutation was detected with immunohistochemistry in 42.6% (15/35) of the nevi and 35.5% (11/31) of the melanomas. In the nevi, BRAF mutation was more frequent in the subepithelial nevi (69%, 6/9) than in the compound nevi (27%, 6/22; Fisher \( P = 0.032 \)). In the melanomas, BRAF mutation was not significantly correlated with any clinicopathologic factor (sex, age, tumor localization, melanoma origin [nevus, primary acquired melanosis, de novo], depth of invasion, proliferative activity, local lymphatic invasion, local recurrence, and metastases; Supplementary Table S1), but our study included only 31 melanomas.

Activation of the MAPK and AKT pathways in nevi and conjunctival melanomas was assessed by immunohistochemistry using antibodies specific for the phosphorylated forms of MEK, ERK, AKT, and S6 proteins, as detailed in the Materials and Methods section. The results are summarized in Table 1 and Figure 1 (nevus) and Figure 2 (melanomas), as well as Supplementary Table S1. Considering as positive those lesions containing at least 10% stained melanocytic cells (i.e., those...
scored as 2 and 3 in our scoring method), cytoplasmic MEK activation was found in 62.9% of the nevi and in 90.3% of the melanomas (Cochran-Armitage test, \( P = 0.0008 \)). ERK phosphorylation was detected both in the nucleus and the cytoplasm in 45.7% of the nevi and in 96.8% of the melanoma cases (Cochran-Armitage test, \( P < 0.0001 \)). There was good correlation between the activation of ERK in the cytoplasm and nucleus (Cochran-Armitage test, \( P < 0.0001 \)). Cytoplasmic pAKT was positive (score 2 or 3) in 94.3% of the nevi and in all melanomas, and similarly, nuclear pAKT was

![Figure 1](image1.png)

**Figure 1.** Activation status of the MAPK and PI3K pathways in a conjunctival subepithelial nevus. A BRAF V600E mutation can be identified with immunohistochemistry (D). There is no significant activation of MEK (B) or ERK (E) or S6 (F), as indicated by immunostaining with antibodies to the respective phosphoproteins. AKT (C) activation can be observed in some cells. (A) Corresponding area demonstrating a subepithelial nevus with pseudoglands containing goblet cells (stain: hematoxylin-eosin). (B) phospho-MEK. (C) phospho-AKT. (D) BRAF\textsuperscript{V600E}. (E) phospho-ERK. (F) phospho-S6. (A-F) Magnification: ×126; insets (B-F): magnification: ×252.

![Figure 2](image2.png)

**Figure 2.** MAPK and PI3K pathways in an advanced conjunctival melanoma. Despite the absence of the BRAF V600E mutation (D), there is an activation of MEK (B) and ERK translocating to the nucleus (E) as well as an activation of AKT (C) and S6 (F). (A) Corresponding area with sheets of pleomorphic, atypical melanoma cells (stain: hematoxylin-eosin). (B) phospho-MEK. (C) phospho-AKT. (D) BRAF\textsuperscript{V600E}. (E) phospho-ERK. (F) phospho-S6. (A-F) Magnification: ×126; insets (B-F) magnification: ×252.
identified in 94.3% of the nevi and in 96.8% of the melanomas. However, the proportion of CJM samples with a more extensive staining (score 3) was higher (80.6%) than observed in nevi (42.9%; Cochran-Armitage test, $P = 0.002$). PS6 was found in 20% of nevi and in 90.3% of melanoma (Cochran-Armitage test, $P < 0.0001$).

A significant correlation was found between the activation of MEK and ERK in both melanomas and nevi (Fisher, $P = 0.022$). There was no correlation between the activation of AKT and the downstream activation of S6.

There was no correlation between the presence of BRAF mutation detected with immunohistochemistry and the activation of MEK, ERK, AKT, and S6.

In the melanomas, there was no correlation between the activation of AKT, S6, MEK, or ERK and the TNM stage, depth of invasion, proliferative activity, local lymphatic invasion or local recurrences, metastases, and death (Supplementary Table S1).

### In Vitro Responses to MAPK and AKT Inhibitors

To evaluate the sensitivity of conjunctival melanoma cells to inhibitors targeting the signaling pathways implicated in tumorigenesis, cultured conjunctival melanoma cell lines containing characterized BRAF and NRAS mutations were treated with different concentrations of vemurafenib (BRAF inhibitor), selumetinib and trametinib (MEK inhibitors), pictilisib (AKT inhibitor), and dactolisib (a dual PI3K/mTOR inhibitor). The effect on viability was measured after 24 and 72 hours of treatment using the WST-1 assay (Fig. 3; Supplementary Fig. S3). Figure 3 shows that the cell lines had different sensitivity to the drugs. IC$_{50}$ values calculated for the inhibitors are summarized in Table 2. CRMM1 was the only cell line sensitive to inhibitors of both MAPK (vemurafenib and trametinib) and AKT (pictilisib and dactolisib) pathways. However, sensitivity to vemurafenib was weak (just above 1 μM), despite the presence of $BRAF^{V600E}$ mutation. CRMM2 was moderately sensitive to pictilisib, whereas T1527A was resistant to all inhibitors tested. Our analysis revealed that T1527A was harboring not only a $BRAF^{G466E}$ mutation but also a $HRAS^{Q61R}$ mutation. The $HRAS^{Q61R}$ mutation was possibly already present at a lower level in the primary tumor (CM3-T) from which it was derived (Supplementary Fig. S2).

To assess the involvement of apoptosis in the drug-induced effect on viability, we asked whether the caspase-3 apoptosis pathway was activated in the more sensitive CRMM1 cells treated with the various inhibitors at IC$_{50}$ concentrations. Immunocytochemistry of cleaved caspase-3 showed cells with increased perinuclear staining following a 24-hour treatment but not in CRMM1 cells without the inhibitors (Supplementary Fig. S5).

#### Table 2. IC$_{50}$ Values of Inhibitors in Conjunctival Melanomas

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Vemurafenib (μM)</th>
<th>Selumetinib (μM)</th>
<th>Trametinib (nM)</th>
<th>Pictilisib (nM)</th>
<th>Dactolisib (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRMM1</td>
<td>1.08</td>
<td>7</td>
<td>9.8</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>CRMM2</td>
<td>&gt;10</td>
<td>10</td>
<td>360</td>
<td>500</td>
<td>1700</td>
</tr>
<tr>
<td>T1527A</td>
<td>&gt;100</td>
<td>&gt;10</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
</tbody>
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IC$_{50}$ values were calculated from viability assays (as shown in Fig. 3) performed on cells treated with the indicated inhibitors for 72 hours. Values are the mean of three independent experiments. IC$_{50}$ values demonstrate that CRMM1 is at least partially sensitive to vemurafenib, trametinib, pictilisib, and dactolisib. CRMM2 is only sensitive to pictilisib and trametinib, whereas T1527a is not sensitive to any of the drugs tested.
FIGURE 4. Effect of BRAF, MAPK, and PI3K/mTOR inhibitor treatment on MEK, ERK, and S6 protein phosphorylation of CJM cells. (A–C) Western blots of lysates from CRMM1 and CRMM2 cells treated with increasing doses of selumetinib, trametinib, and dactolisib, respectively, for 24 hours, showing changes in the phosphorylated forms of downstream substrates (p-ERK, p-MEK, and p-S6). Total levels of the respective proteins are shown as control. α-Tubulin was used as a gel loading control. The figure shows the results of a representative experiment.

Effect of Kinase Inhibitors on Phospho-MEK, -ERK, and -S6 Levels

To understand the different sensitivity of the cell lines tested to the inhibitors, Western blot analyses were first performed to assess the basal levels of phosphorylated (activated) and total ERK, MEK, and S6 proteins in CJM cell lines. All three cell lines displayed a constitutive activation of the above proteins (Supplementary Fig. S4).

To assess whether the responses to inhibitors were pathway-specific, we treated the cell lines harboring activating BRAF and NRAS mutations, that is CRMM1 and CRMM2, with MEKI (trametinib and selumetinib) or dual PI3K-mTORi (dactolisib) for 24 hours and assessed the effects on the activation of MEK, ERK, and S6 with Western blotting. In CRMM1 and CRMM2 (Fig. 4A), the lowest dosage of selumetinib (0.1 μM) greatly inhibited the phosphorylation of both MEK and downstream ERK. Trametinib caused similar concentration-dependent decreases in the phosphorylation of MEK and ERK in the two cell lines. In both cases, levels of total MEK and ERK remained unchanged (Fig. 4B). Phosphorylation of S6 was inhibited by dactolisib in both cell lines (Fig. 4C). Altogether, the drugs showed activity on relevant targets in both cell lines.

DISCUSSION

As a significant proportion of conjunctival melanomas contains BRAF or NRAS mutations, we directly tested in the tumor tissues the activation of the downstream pathways, that is the MAPK and PI3K/mTOR pathways. The BRAF<sup>V600E</sup> mutation was found in 42.6% of the nevi and 35.5% of melanomas. The presence of this BRAF mutation in conjunctival nevi has been documented in 19%<sup>25</sup> to 50%<sup>23</sup> of cases, a lower rate than in cutaneous nevi, where BRAF<sup>V600E</sup> has been identified in 60%<sup>34</sup> to 87.5%<sup>35-37</sup>. Based upon this study and previous reports, a BRAF<sup>V600E</sup> mutation occurs in 26%<sup>25</sup> to 35%<sup>13,26</sup> of conjunctival melanomas. Although a BRAF<sup>V600E</sup> occurred at an earlier age in our study with respect to what has been previously reported in Denmark<sup>3</sup> (62.09 years versus 68.55 years), this was not significant.

Our results demonstrate that the MAPK pathway is constitutively activated in both conjunctival nevi and melanomas. Moreover, the fact that a BRAF<sup>V600E</sup> mutation is less frequent in melanomas than in nevi implies that the increased activation of this pathway identified in our study may not depend only upon a BRAF oncogenic mutation. It is understood that the modulation of the MAPK pathway results from a fine balance between positive and negative regulators. Apart from BRAF<sup>V600E</sup>, the loss of the negative control by NF1 may partially explain this enhanced MAPK activity. In cutaneous melanoma, an NF1 mutation is an important event, identified within 14% of cases by the tumor genome atlas project.<sup>11</sup> Recently, NF1 mutations could be identified in 21 of 65 conjunctival melanomas (33%).<sup>34</sup> Alternatively, the increased MAPK activity may also be directly explained by the presence of other oncogenic mutations, such as NRAS mutations, discovered in 18% of conjunctival melanoma cases.<sup>24</sup> By analogy, in 32 NRAS-mutated cutaneous melanomas, increased activity of the MAPK pathway was observed in more than 60% of cases.<sup>24</sup>

In the conjunctival melanomas, we encountered an almost significantly increased nuclear localization of AKT compared with the nevi, as well as a concomitant increased phosphorylation of S6, suggesting an increased activity of the PI3K/mTOR pathway, which could be partially explained by the presence of an NF1 mutation or a loss of NF1 function.

Considering that an activation of both the MAPK and PI3K/mTOR pathways occurs in conjunctival melanoma, we tested their inhibition in vitro in cell lines with a BRAF<sup>V600E</sup> or an NRAS<sup>G12D</sup> mutation or without these mutations. Direct inhibition of BRAF with vemurafenib in the BRAF<sup>V600E</sup>-mutated cell line CRMM1 resulted in a moderate effect. The IC<sub>50</sub> value of 1.08 μM, calculated for a 72-hour treatment, is very similar to that of previously published data on the same cell line.<sup>25</sup> However, significant inhibition of growth was not seen at 24 hours. Interestingly, live cell imaging of the effect of vemurafenib on BRAF<sup>V600E</sup>-mutated cutaneous melanoma cell lines demonstrated that at this time a significant proportion of the cells was still dividing.<sup>28</sup> Vemurafenib was not effective in the NRAS<sup>G12D</sup>-mutated cell line or the cell line with the BRAF<sup>V600E</sup>-mutated cell line. Our data are in line with the paradoxical MAPK activation and increased ERK phosphorylation observed in cutaneous and conjunctival melanomas with an NRAS mutation and treated with a BRAF inhibitor.<sup>25,39</sup>

Our results demonstrate that the MEK inhibitors trametinib and selumetinib significantly decreased phosphorylation of MEK and ERK in both the BRAF<sup>V600E</sup> and the NRAS<sup>G12D</sup>-mutated cell lines. However, significant growth inhibition after 72 hours was only achieved in the BRAF<sup>V600E</sup> mutant with trametinib. The IC<sub>50</sub> of 10 nM obtained in our study with the BRAF-
in our study, the use of a single drug allowed growth reduction, more in the BRAF-mutated and less the NRAS-mutated cell lines. In a study by Cao et al.,55 both MEK inhibition and AKT inhibition resulted in a synergistic effect in the same cell lines used in our study. Similarly, in cutaneous melanoma, synergistic inhibition of the MAPK and PI3K pathways has been demonstrated in cell lines with an NRAS mutation24 and in BRAF-resistant cell lines.54 In metastatic cutaneous melanoma cell line spheroids, the combination of MEK and AKT inhibition prevented growth and invasion.55 However, in four phase I clinical trials assessing the use of PI3K and MEK inhibitors in solid tumors, the overall response rate was limited to 4.7%, with a disease control rate of 19.2%, although a partial response was noted in a few patients with BRAF- or NRAS-mutated melanomas.56

Over the last years, modulation of the tumor immune microenvironment with checkpoint inhibitors has significantly improved the survival of patients with cutaneous melanoma.57–61 The response to checkpoint inhibitors depends on the local tumor immune microenvironment, that is, the expression of T CD8+62 or PD-L162,62 within the tumor. In conjunctival melanoma, the expression of PD-L1, found in 19% (5/27) of cases, has been associated with a worse tumor-related survival63 and might be an argument for the use of checkpoint inhibitors in advanced conjunctival melanoma, especially considering the relative resistance of some CJM cell lines to MAPK or PI3K/mTOR inhibition.

In conclusion, in this study the increased activity of the MAPK and PI3K/mTOR pathways in conjunctival melanoma tumor progression may not rely solely on the presence of a BRAF mutation. We further demonstrate that the responsiveness of the NRASQ61C and the BRAFQ600E/HRASQ61B conjunctival melanoma cell lines to single MAPK or PI3K inhibition is limited. Altogether, our findings suggest that in advanced conjunctival melanoma, the molecular genetic background of the tumor should be determined to guide the choice of the best therapeutic option. Further investigations into mechanisms of intrinsic resistance and combination targeted therapies should also be pursued.

Acknowledgments

Supported by the “Fond’Action Contre le Cancer” Foundation, Switzerland.

Disclosure: L. El Zaoui, None; M. Bucher, None; D. Rimoldi, None; M. Nicolas, None; G. Kaya, None; R. Pescini Gobert, None; N. Bedoni, None; A. Schalenbourg, None; E. Sakina, None; L. Zografos, None; S. Leyvraz, None; N. Riggi, None; C. Rivolta, None; Aelxandre P. Moulin, None

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