Conjunctival Melanomas Harbor BRAF and NRAS Mutations and Copy Number Changes Similar to Cutaneous and Mucosal Melanomas

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Abstract

Purpose: Conjunctival melanoma is a rare but potentially deadly tumor of the eye. Despite effective local therapies, recurrence and metastasis remain frequent. Once the tumor has metastasized, treatment options are limited and the prognosis is poor. To date, little is known of the genetic alterations in conjunctival melanomas.

Experimental Design: We conducted genetic analysis of 78 conjunctival melanomas, to our knowledge the largest cohort reported to date. An oncogene hotspot array was run on 38 samples, screening for a panel of known cancer-relevant mutations. Thirty tumors were analyzed for genome-wide copy number alterations (CNA) using array-based comparative genomic hybridization. Sanger sequencing of selected target mutations was conducted in all samples.

Results: BRAF mutations were identified in 23 of 78 (29%) tumors. NRAS mutations, previously not recognized as relevant in conjunctival melanoma, were detected in 14 of 78 (18%) tumors. We found CNAs affecting various chromosomes distributed across the genome in a pattern reminiscent of cutaneous and mucosal melanoma but differing markedly from uveal melanoma.

Conclusions: The presence of NRAS or BRAF mutations in a mutually exclusive pattern in roughly half (47%) of conjunctival melanomas and the pattern of CNAs argue for conjunctival melanoma being closely related to cutaneous and mucosal melanoma but entirely distinct from uveal melanoma. Patients with metastatic conjunctival melanoma should be considered for therapeutic modalities available for metastatic cutaneous and mucosal melanoma including clinical trials of novel agents.

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Introduction

Melanoma is a disease with a significant death toll affecting people worldwide (1, 2). Recently, a number of promising treatments targeting specific melanoma subsets have shown an improvement in overall survival in patients with metastatic melanoma (3–7). Accurate classification and genetic characterization of melanoma, including its less frequent subtypes, is critical to allow selection of appropriate treatment modalities.

Ocular melanoma is classified on the basis of anatomic site of origin as conjunctival, anterior uveal (originating in the iris), or posterior uveal (originating in the ciliary body or choroid) melanoma. Conjunctival melanoma accounts for 5% to 10% of all ocular melanomas (8). The population incidence is 0.2 to 0.8 per million (9–13) with studies reporting an increase in incidence (9, 12, 14). It occurs more commonly in Caucasian populations and in middle-aged and elderly individuals (13). The rate of local recurrence is high, between 26%–61% at 5 years and 38%–69% at 10 years (15–19) after diagnosis. Disease-related mortality at 10 years ranges from 13% to 38% (15–19).

Conjunctival melanoma may arise from so-called “primary acquired melanosis” (45%–74%), from preexisting melanocytic nevi (5%–21%), or de novo, without an...
associated lesion (18%–30%; refs. 19–22). Prognostic factors include tumor thickness (Breslow thickness; refs. 10, 16, 18, 23) and tumor location, with higher mortality rates reported for tumors of caruncular, fornical, or palpebral origin (anatomic scheme—Supplementary Fig. S1; refs. 10, 16–18).

Over the past 2 decades, studies have revealed different genetic subsets of melanoma. Cutaneous and uveal melanomas are genetically particularly distinct. Cutaneous melanomas typically harbor activating mutations in BRAF (~50%) or NRAS (~15%; refs. 24, 25) and loss of tumor suppressor genes such as CDKN2A and PTEN (26). Uveal melanomas lack these mutations (27) but are characterized by activating mutations in GNAQ or GNA11 (28, 29) and frequent loss of BAP1 (30).

Conjunctival melanomas have not been well-characterized at the genetic level. Previous genetic studies of conjunctival melanomas evaluated small numbers of tumors, focusing on known cutaneous and uveal melanoma oncogenes. BrafV600E mutations have been reported in 14% to 50% of conjunctival melanomas (27, 31–33). GNAQ or GNA11 mutations have not been detected (28, 29, 34). KIT mutations appear to be rare—one study reported a KIT mutation in 1 of 14 (7%) tumors (35), whereas another found none in 5 tumors (36). To our knowledge, only one study of conjunctival melanomas investigated NRAS mutations and found no mutations in 6 tumors (37). Comprehensive copy number analysis of conjunctival melanoma has only been reported in 2 cases as part of a larger cohort of uveal melanomas (38). An analysis of loss or amplification of selected regions by multiplex ligation–dependent probe amplification was recently reported (31).

The aim of our study was to genetically analyze a large number of conjunctival melanomas by screening for a panel of oncogene hotspot mutations and analyzing genome-wide DNA copy number alterations (CNA).

Materials and Methods

Sample selection and histopathology

 Conjunctival melanoma tumor samples were obtained from patients treated in the Department of Ophthalmology for conjunctival melanoma, as well as from the tissue archives of the Departments of Ophthalmology, Dermatology and Pathology of the University Hospital Essen, Germany, and the Department of Ophthalmology, University Hospital Tübingen, Germany. Tumor slides were reviewed by at least one experienced histopathologist (U. Hillen, F. Grabelius, K. Griewank, or T. Schimming). The study was approved by the local ethics committee of the University of Duisburg-Essen.

DNA isolation

Ten-micrometer-thick sections were cut from formalin-fixed, paraffin-embedded tumor tissues. The sections were deparaffinized and manually microdissected according to standard procedures. gDNA was isolated using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s instructions. DNA allowing genetic analysis was isolated from 78 of 96 tumor samples. Copy number and oncogene screen analyses were conducted in samples that yielded large amounts of DNA. Low DNA yield samples allowed only Sanger sequencing of selected target mutations.

Copy number analysis

Array-based comparative genomic hybridization (CGH) was used to conduct analysis of DNA CNAs. The methods for hybridization and analysis, including GISTIC 2.0 statistical analysis, have been described previously (39–42). Whole-genome amplification was conducted using Sigma’s GenomPlex Single Cell Whole Genome Amplification Kit as described previously (43).

Screen for known oncogene mutations

The screen was run on a Sequenom MassARRAY (Sequenom, Inc.) platform as described technically in detail previously (44). A panel of 164 hotspot mutations in 33 oncogenes (including the following: AKT1-3, ALK, BRAF, CDK4, CTNNB1, EGFR, FGFR2-3, GNAQ, GNA11, IDH1-2, KIT, KRAS, MET, NRAS, PDGFRA, PIK3CA, and RET) was analyzed. As an example, mutations included were those leading to amino acid changes in BRAF for G464, G466, G469, E586, D594, L597, V600 and K601, as well as in KIT for Y553, L576, V559, V560, N566, R634, K642, D816, V825, and N882. A complete list of genes and mutations is listed in Supplementary Table S1.

Direct (Sanger) sequencing

Nestcd PCR was conducted to amplify BRAF exon 11 and 15 and NRAS exon 1 and 2 and sequenced as previously described (45). Sequencing of KIT exons 9, 11, 13, 17, and 18 was conducted similarly. Primers and conditions for KIT as well as all other gene mutations analyzed by single-step
PCR amplification are listed in Supplementary Tables S2–S4. PCR reaction products were purified with the QIAquick PCR Purification Kit (Qiagen) and then used as templates for sequencing in both directions. The sequencing chromatogram files were examined, and mutations were identified using Chromas software (version 2.01, University of Sussex, Brighton, United Kingdom).

**Associations of BRAF and NRAS mutation status with clinical and pathologic parameters**

We investigated associations of mutation status with available clinical and pathologic parameters. The parameters studied are listed in Table 1. We also analyzed, using univariate Cox regression models, associations of mutation status with disease-free, distant metastasis-free, and overall survival; the intervals for each of these parameters were from time of diagnosis of primary conjunctival melanoma to first tumor recurrence, first distant metastases, and death, respectively. Cases in which the specified endpoints were not reached at the time of last follow-up were censored. All statistical analyses were conducted using IBM SPSS Statistics software (version 20.0; International Business Machines Corp.). \( P \leq 0.05 \) was considered statistically significant.

**Results**

**Tumors and patients**

Thirty-eight (49%) tumors occurred in females and 40 (51%) in males, with a median age of 64 years (range, 34–89 years). All samples analyzed were from primary (71) or locally recurrent (7) tumors; no metastatic tumor samples were included. Of samples for which information was available, 52% (34 of 65) originated from primary acquired melanosis (PAM), 29% (19 of 65) from nevi, and 18% (12 of 65) arose de novo. Fifty-eight percent (40 of 69) of patients initially presented with clinical stage I, 25% (17 of 69) with stage II, and 17% (12 of 69) with stage III disease (American Joint Committee on Cancer staging system for conjunctival melanoma, 7th edition, 2010). Seventy percent (48 of 68) of patients received some form of adjuvant treatment (20 ruthenium, 11 proton, 6 mitomycin C, 3 strontium, and 8 cryotherapy). Fifty-three percent (37 of 70) of tumors recurred at least once, and 31% (18 of 58) eventually metastasized. Additional information is listed in Table 1.

**Copy number analysis**

Copy number analysis was conducted on 32 samples from 31 tumors. Eight samples were hybridized with unamplified DNA and 24 samples were hybridized after whole genome amplification of DNA. To ensure that whole-genome amplification did not result in loss of quality, we compared hybridized samples of one tumor before and after DNA amplification—both samples showed identical CNAs (Supplementary Fig. S2).

One copy of the sample hybridized twice, and a sample that was later found to be a uveal metastasis (please see below) were excluded from the analysis. The remaining 30 tumors showed a range of CNAs, including recurrent losses of 1p, 3q, 6q, 8p, 9p, 10, 11q, 12q, 13, 15p, and 16q and recurrent gains of 1q, 3p, 6p, 7, 8q, 11q, 12p, 14p, and 17q (Fig. 1, Supplementary Figs. S3–S5). For comparison, CGH profiles of a similar number of uveal melanomas were analyzed (Fig. 1, Supplementary Figs. S3 and S6). These showed characteristic CNAs (loss of 1p, 3, and 6q as well as gains of 6p and 8q: refs. 46–48).

**Screen for mutations in known oncogenes**

Forty tumors (39 ocular melanomas and a known KIT L576P mutant cutaneous melanoma to serve as a positive control) were analyzed. Six BRAF V600E (T1799A) mutations and 4 NRAS Q61 mutations [3 Q61R (A182G), 1 Q61K (C181A)] were detected. Twenty-one other potential mutations that were detected in at least one run of the assay were reanalyzed by direct sequencing (Supplementary Table S2). Validated mutations included 1 GNA11 Q209L (A626T) mutation, 2 MET mutations/variants R988C (C2962T homozygous) and T1010I (C3069T) and the KIT L576P (T1727C) mutation in the positive control sample. No other mutations were confirmed by direct sequencing.

In a single tumor, an activating GNA11 Q209L mutation was identified and validated. Copy number analysis of the tumor harboring this mutation showed that it was the only tumor showing a complete loss of chromosome 3 (Supplementary Fig. S10). Losses of 1p and 6q and gains of 6p and 8q were also found. Review of the clinical information on this patient revealed that 2 months after removal of the tumor initially diagnosed as conjunctival melanoma, a large amelanotic uveal melanoma affecting the same eye was identified. In light of this information, the conjunctival tumor was interpreted as a metastasis from the uveal melanoma, and the sample was excluded from further analysis.

**BRAF and NRAS mutations**

As the screen indicated that BRAF and NRAS mutations were the most frequent driver mutations in conjunctival melanoma, we conducted direct sequencing of these mutations in all tumors. All 78 tumors included in the study gave high-quality sequencing reads of both BRAF and NRAS (Table 2). We detected BRAF mutations in 23 (29%) tumors and NRAS mutations in 14 (18%) tumors. The mutations in NRAS and BRAF were mutually exclusive. The overwhelming majority of BRAF mutations (\( n = 21; 91\% \)) were V600E (T1799A) mutations; one tumor (4%) harbored a G469A (G1406C) mutation and another tumor (4%) showed a D594G (A1781G) mutation. NRAS mutations were identified in 14 (18%) tumors. They included Q61R (A182G) (\( n = 6; 43\% \)), Q61K (C181A) (\( n = 4; 29\% \)), Q61H (A183C) (\( n = 2; 14\% \)), and Q61L (A182T) (\( n = 2; 14\% \)) mutations.

**KIT alterations**

Gains of the KIT locus on chromosome 4 were detected in 5 of 30 tumors by CGH (Fig. 2). However the hotspot array, which covers the most common activating KIT mutations, detected no mutations in the 38 conjunctival tumors analyzed. In addition, direct sequencing of exon 9, 11, 13, 17, and 18 was conducted in 24 tumors. This included 20 hotspot array-screened and 4 additional tumors. All tumors
Table 1. Associations of \textit{BRAF} and \textit{NRAS} mutation status with clinical and pathologic parameters

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that showed gains of \textit{KIT} by CGH were screened. One tumor (shown in Fig. 2) harbored a T847M (C2540T) mutation, which has not been previously described in cancer. In summary, no known activating \textit{KIT} mutations were found in a total of 42 tumors.

**Copy number changes stratified according to \textit{BRAF} and \textit{NRAS} mutation status**

Copy number profiles were grouped according to presence of a known activating oncogene mutation (Fig. 3; Supplementary Figs. S3 and S7–S9). These groups consisted of 10 \textit{BRAF}-mutant tumors, 7 \textit{NRAS}-mutant tumors, and 13 tumors having neither \textit{NRAS} nor \textit{BRAF} mutation (hereafter designated “wild-type”). In comparison to \textit{NRAS}- or \textit{BRAF}-mutant tumors, wild-type tumors generally had higher numbers of chromosomal alterations. Specific chromosomal alteration frequencies varied between groups, that is, gains of 1q, 3p, and 17q being less common in \textit{BRAF}-mutant than in \textit{NRAS}-mutant or wild-type tumors. Losses of chromosome 10, including the \textit{PTEN} locus 10q23 were particularly prominent in \textit{BRAF}-mutant tumors but also evident in other groups.

All tumor groups (\textit{BRAF}-mutant, \textit{NRAS}-mutant, and wild-type tumors) harbored a number of common alterations: i.e. gains in 1q, 6p, 7, 8q, 11q (proximal to the centromere) and 17q; as well as losses in 6p, 8p, 9p, 10,
11q (distal from the centromere), and 16q. Examples of these, including a gain of CCND1, as well as a loss of CDKN2A are shown in detail in Fig. 2.

**Associations of BRAF and NRAS mutation status with clinical and pathologic parameters**

*BRAF* mutations were significantly more common in tumors involving the caruncle (66% *BRAF* vs. 0% *NRAS* and 33% wild-type; *P* = 0.03) as well as tumors arising from melanocytic nevi (65% *BRAF* vs. 27% *NRAS* and 9% wild-type; *P* < 0.001). There were no other statistically significant associations (Table 1). There were no statistically significant associations of mutation status with disease-free survival or overall survival (Table 3). No statistically significant associations of mutation status with distant metastasis-free survival were found (data not shown).

**Discussion**

To the best of our knowledge, the present study represents the most detailed genetic analysis of conjunctival melanoma to date. The oncogene mutation profile detected is reminiscent of cutaneous melanoma. *NRAS* mutations have not been analyzed in most genetic studies of conjunctival melanomas (27, 31–33). One study failed to detect *NRAS* mutations in 6 tumors (37). Ours is the first study to identify frequent *NRAS* mutations as a relevant oncogene in conjunctival melanoma. The detected frequency of 18% is similar to that found in cutaneous melanoma (24, 25). *BRAF* mutation frequencies reported in previous studies of conjunctival melanoma were 14% to 50% (27, 31–33). Sample bias or technical differences in mutation detection could explain the variations in the reported mutation frequency. We identified *BRAF* mutations in 29% of tumors. In view of the recent development of effective *BRAF* inhibitors, the relatively common occurrence of *BRAF* mutations in conjunctival melanomas is of great therapeutic relevance. Gains of the *KIT* locus on chromosome 4 were detected in 5 of 30 tumors by CGH (Fig. 2). No known activating mutations in *KIT* were found. The T847M (C2540T) mutation we identified in one sample is of unclear significance, as it has not been previously reported in cancer. However, as it is located in the kinase domain of the protein, it could be an activating mutation. Our findings argue that while *KIT* overexpression may be important, clinically relevant *KIT* mutations appear to be rare in conjunctival melanoma. The oncogenic roles of the *MET*Q1010I and R988C mutations/variants detected have been questioned in a recent publication (49). The variants failed to show transforming ability and were also found in healthy individuals, supporting the possibility these variants are actually rare SNPs. Gains of the chromosome 7q locus could support overexpression of *MET* having an oncogenic effect in some tumors. Further studies are required to investigate the oncogenic role of *c-MET* in conjunctival melanoma.

The only tumor harboring a GNA11 Q209L mutation was found to be a uveal melanoma metastasis. The absence of any GNA11 or GNAQ mutations in the bona fide conjunctival melanomas suggests that these mutations are probably very rare in this tumor type.

To our knowledge, the present study is the first to describe results of comprehensive genome-wide copy number analysis of a substantial number of conjunctival melanomas. Uveal melanoma has a very characteristic CGH profile (Fig. 1; Supplementary Figs. S3 and S6), which includes frequent losses of 1p, 3, and 6q and gains of 6p and 8q. The changes seen in chromosomes 6 and 8 as well as losses of 1p are also frequent in cutaneous melanoma (26). However, losses of the entire chromosome 3 are rare in cutaneous tumors (26, 50). Alterations involving other chromosomes are not common in uveal melanoma but are frequently found in cutaneous and mucosal melanoma. They include gains of 1q, 3p, 7, 17q and losses of 9p, 10, 11, and 12q. Alterations we also found present in conjunctival melanomas (Fig. 1; Supplementary Figs. S3 and S5). Indeed, the CNAs observed in our conjunctival melanoma cohort are very similar to those in cutaneous and mucosal melanoma (26, 50, 51) being quite distinct from those of uveal melanoma.

A number of *BRAF*- and *NRAS*-mutant tumors showed gains of their respective oncogenic loci. This was noted in 3 of 10 (30%) of *BRAF*- and 3 of 7 (43%) of *NRAS*-mutant samples, arguing that higher expression levels of the oncoproteins might play a role in tumorigenesis. *BRAF*-mutant tumors showed frequent chromosome 10 and *PTEN* loss, a finding seen in *BRAF*-mutant cutaneous melanomas (50, 51), and supporting the concept that *BRAF* mutant tumors require an additional genetic event leading to the activation of the AKT pathway. This event is not as relevant in *NRAS*-mutant tumors, where the mutation directly leads to downstream AKT activation. CNAs were more frequent in *BRAF*/*NRAS* wild-type tumors, similar to findings in cutaneous melanoma (50). Also, reminiscent of findings in cutaneous and mucosal melanoma, tumors showing gains of the *KIT*
locus lacked both BRAF and NRAS mutations (52). BRAF-mutant, NRAS-mutant, and tumors that were wild-type for BRAF and NRAS tumors shared a considerable number of mutual chromosomal alterations. Particularly prominent examples (reminiscent of cutaneous and mucosal melanomas) are loss of 9p, containing the CDKN2A locus and focal centromere-proximal gains in 11q, including the CCND1 gene (Fig. 2). These findings suggest that independent of the activating oncogene, all conjunctival melanomas share a number of common pathogenic mechanisms.

BRAF-mutant tumors more commonly involved the caruncle and more often arose in association with melanocytic nevi than NRAS-mutant and wild-type tumors. BRAF-mutant tumors less commonly arose in association with PAM. One potential implication of this finding is that BRAF mutations are early events in conjunctival melanomas arising from nevi but not in those arising in the setting of PAM. This is in keeping with our understanding of nevi as clonal proliferations with oncogenic BRAF mutations, unlike PAM. Clinically, the finding of a conjunctival melanoma arising in association with a nevus should indicate a high probability of a BRAF mutation and is therefore likely to be susceptible to BRAF inhibitor therapy. There were no other statistically significant associations between mutation status and clinicopathologic parameters or survival. Studies of large cohorts of patients with conjunctival melanoma with long follow-up will be required to validate these findings.

Our results show that uveal melanoma and conjunctival melanoma are genetically distinguishable, both in terms of CNAs and mutations. In cases where a tumor is clinically or pathologically difficult to classify it could be correctly categorized on the basis of the oncogenic mutation and the copy number profile. GNAQ or GNA11 mutations would strongly favor uveal melanoma, whereas BRAF or NRAS mutations would favor conjunctival melanoma. In
cases where these mutations are not detected, a copy number analysis could be conducted. Monosomy 3 would point toward uveal melanoma, whereas losses in 9p, gains of chromosome 7, or amplifications of the \(\text{CCND1}\) centromere proximal chromosomal areas of chromosome 11 would favor conjunctival melanoma. Genetic analysis could therefore be a useful diagnostic adjunct to help classify tumor type in cases that are otherwise difficult to categorize clinically or pathologically.

Patients with ocular (including conjunctival) melanomas are frequently excluded from melanoma clinical trials targeted at patients with cutaneous or mucosal melanoma. For uveal melanomas, which are genetically very distinct from cutaneous and mucosal melanoma, this may be warranted. However, in the case of conjunctival melanoma, our data indicate that this exclusion is not justified and that they should be grouped with cutaneous and mucosal melanomas. As a consequence, patients with metastatic conjunctival melanoma should be considered for clinical trials of novel therapeutic agents being trialed in patients with advanced cutaneous or mucosal melanoma, including selective BRAF

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**Table 3.** Associations of \(\text{BRAF}\) and \(\text{NRAS}\) mutation status with survival

<table>
<thead>
<tr>
<th>Mutation status</th>
<th>(N)</th>
<th>Mean survival, a mo</th>
<th>Median survival, a mo</th>
<th>Coefficient,b (B)</th>
<th>SEb</th>
<th>Waldb</th>
<th>(P^b)</th>
<th>HR (95% CI)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease-free survival</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT (ref)</td>
<td>33</td>
<td>56.4</td>
<td>42.0</td>
<td>1.00 (ref)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{BRAF})</td>
<td>21</td>
<td>47.9</td>
<td>28.0</td>
<td>0.24</td>
<td>0.36</td>
<td>0.46</td>
<td>0.50</td>
<td>1.27 (0.63–2.56)</td>
</tr>
<tr>
<td>(\text{NRAS})</td>
<td>13</td>
<td>121.5</td>
<td>NR</td>
<td>–0.99</td>
<td>0.54</td>
<td>3.40</td>
<td>0.07</td>
<td>0.37 (0.13–1.06)</td>
</tr>
<tr>
<td>(\text{NRAS or BRAF})</td>
<td>34</td>
<td>84.4</td>
<td>48.0</td>
<td>–0.30</td>
<td>0.33</td>
<td>0.82</td>
<td>0.37</td>
<td>0.74 (0.38–1.42)</td>
</tr>
<tr>
<td>Overall survival</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT (ref)</td>
<td>30</td>
<td>121.6</td>
<td>168.0</td>
<td>1.00 (ref)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{BRAF})</td>
<td>20</td>
<td>170.6</td>
<td>NR</td>
<td>0.08</td>
<td>0.46</td>
<td>0.03</td>
<td>0.86</td>
<td>1.08 (0.44–2.69)</td>
</tr>
<tr>
<td>(\text{NRAS})</td>
<td>12</td>
<td>106.4</td>
<td>104.0</td>
<td>0.26</td>
<td>0.51</td>
<td>0.26</td>
<td>0.61</td>
<td>1.30 (0.48–3.55)</td>
</tr>
<tr>
<td>(\text{NRAS or BRAF})</td>
<td>32</td>
<td>164.0</td>
<td>104.0</td>
<td>0.31</td>
<td>0.44</td>
<td>0.48</td>
<td>0.49</td>
<td>1.36 (0.57–3.23)</td>
</tr>
</tbody>
</table>

Abbreviations: NR, not reached; WT, wild-type for both \(\text{BRAF}\) and \(\text{NRAS}\).

Values derived from aKaplan–Meier method and bunivariate Cox regression.
inhibitors (4, 6), MEK inhibitors (5, 53) and many others in development.

Disclosure of Potential Conflicts of Interest

D. Schadendorf is on the advisory board of or has received honoraria from Roche, Genetech, Novartis, Amgen, GSK, Boehringer Ingelheim, and Merck. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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References


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