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Acquired BRAF inhibitor resistance: a multicenter meta-analysis of the spectrum and frequencies, clinical behavior, and phenotypic associations of resistance mechanisms

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Abstract

Background—Acquired resistance to BRAF inhibitors (BRAFi) is a near-universal phenomenon caused by numerous genetic and non-genetic alterations. In this study, we evaluated the spectrum, onset, pattern of progression, and subsequent clinical outcomes associated with specific mechanisms of resistance.

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Methods—We compiled clinical and genetic data from 100 patients with 132 tissue samples obtained at progression on BRAFi therapy from 3 large, previously published studies of BRAFi resistance. These samples were subjected to whole exome sequencing and/or PCR-based genetic testing.

Results—Among 132 samples, putative resistance mechanisms were identified in 58%, including *NRAS* or *KRAS* mutations (20%), *BRAF* splice variants (16%), *BRAF*^{V600E/K} amplifications (13%), *MEK1/2* mutations (7%), and non-MAPK pathway alterations (11%). Marked heterogeneity was observed within tumors and patients; 18 of 19 patients (95%) with >1 progression biopsy had distinct/unknown drivers of resistance between samples. *NRAS* mutations were associated with vemurafenib use (p=0.045) and intracranial metastases (p=0.036), and *MEK1/2* mutations correlated with hepatic progression (p=0.011). Progression-free survival and overall survival were similar across resistance mechanisms. The median survival after disease progression was 6.9 months, and responses to subsequent BRAF and MEK inhibition were uncommon (2 of 15; 13%). Post-progression outcomes did not correlate with specific acquired BRAFi resistance mechanisms.

Conclusions—This is the first study to systematically characterize the clinical implications of particular acquired BRAFi resistance mechanisms in patients with BRAF-mutant melanoma largest study to compile the landscape of resistance. Despite marked heterogeneity of resistance mechanisms within patients, *NRAS* mutations correlated with vemurafenib use and intracranial disease involvement.

Keywords

BRAF; vemurafenib; dabrafenib; resistance; acquired; MAPK; NRAS; amplification; splice; MEK1; MEK2; meta-analysis; spectrum

Introduction

Nearly half of melanomas harbor a valine to glutamine substitution in codon 600 of the serine-threonine kinase BRAF (1). These alterations confer constitutive activation of the mitogen activated-protein kinase (MAPK) pathway and thereby drive melanoma growth and progression (1, 2). Small molecule inhibitors of mutant BRAF (vemurafenib and dabrafenib) induce tumor regression and improve survival in melanoma patients compared to chemotherapy (3, 4).

The development of acquired resistance to BRAFi, however, is a significant obstacle to effective targeted therapy. To characterize and overcome acquired resistance, studies have identified numerous genetic and non-genetic drivers of resistance involving MAPK pathway reactivation and MAPK-redundant signaling. These include *NRAS* mutations (5), *BRAF*^{V600E/K} amplification (6), alternate splicing of *BRAF* (7), *MEK1/2* mutations (8), PI3K/AKT pathway dysregulation (9, 10), and overexpression of genes including *COT*, *PDGFRβ*, and others (5, 11–13). The translational value of these findings has been clearly demonstrated by successful co-targeting of BRAF and MEK, which has further improved clinical outcomes compared with single-agent BRAFi (14–17).

Efforts to systematically define the spectrum and frequency of these BRAFi-resistance mechanisms have been published recently (18–20). These studies established the high incidence of MAPK-reactivating alterations, but identified intra-patient and intra-tumoral heterogeneity of resistance, and a sizable subset of resistance unaccounted for by genomic profiling alone. Despite the value of these studies, each was limited by sample size to correlate specific mechanisms of resistance with corresponding clinical/biologic behavior. Moreover, the different studies reported discordant frequencies of particular resistance mechanisms (e.g. *NRAS* mutations). Thus, we combined published data from the three largest studies of acquired BRAFi-resistance in 100 melanoma patients to assess the landscape of resistance mechanisms and the corresponding clinical characteristics (18–20).

Methods

Patients and Study Design

Patients (n=100) and progression samples (n=132) were aggregated from previously-published studies conducted under IRB-approved protocols. These studies were led by University Hospital Essen (Essen, Germany) and the Broad Institute (Boston, MA, USA) (18), Melanoma Institute Australia (Sydney, NSW, Australia) (19), University of California, Los Angeles (USA) (20), and collaborators. All patients had advanced *BRAF*^{V600}-mutant melanoma and received vemurafenib or dabrafenib as first-line MAPK-directed therapy through clinical trials or as commercially-available therapy. Nearly all patients experienced tumor regression and subsequent progressive disease, except seven with primary disease progression. Objective responses and disease progression were defined using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 (21). Baseline demographics, treatment duration, overall survival (OS), progression-free survival (PFS), prior therapies, and subsequent treatments were obtained by reviewing medical records and imaging results.

Melanoma Samples

All patients underwent biopsies of melanoma metastases that were present at baseline and enlarged on therapy, or arose during treatment. Most had matched pre-treatment biopsies and some had multiple biopsies obtained upon disease progression. Specimens were stored as FFPE or were snap-frozen per institutional standard operating procedures. Tumor DNA and RNA were extracted per institutional protocols.

Tumor Sequencing

Sequencing was performed by whole exome sequencing (WES in 56% of disease-progression samples or PCR-based sequencing in the remainder. Sequencing of pre-treatment samples corresponding to 107 of the progressing samples was performed (81%), including in all WES samples. Recurrent “hotspot” mutations in *NRAS*, *MEK1*, and *MEK2* were assessed in all 132 progression samples. Quantitative genomic DNA PCR was performed to detect *BRAF*^{V600E/K} amplifications in 120 samples (91%). Alternative splicing of *BRAF* was evaluated by Sanger detection of novel exon–exon boundaries in the cDNAs in 86 progression samples (65%). Recurrent *AKT1* “hotspot” mutations were assessed in all samples, other mutations in the PI3K/AKT pathway were evaluated in the WES samples.

WES data analysis has been previously described (18–20). Analyses performed in particular tumors are shown in Table S1.

Resistance Mechanisms

Mechanisms of acquired BRAFi resistance were limited to molecular alterations that were: 1) detected in the progression sample, 2) not present in the pre-treatment sample, or if baseline tissue was unavailable, prior establishment as a resistance mechanism had been performed, and 3) previously validated to confer BRAFi-resistance *in vitro* (Table S2). Mechanisms proposed in other publications as possible drivers of resistance without pre-clinical validation were not included.

Statistical Analysis

Associations between classes of resistance mechanisms and clinical variables were evaluated using multivariable logistic regression models. We classified resistance mechanisms as the following: 1) *NRAS* or *KRAS* mutations, 2) *BRAF* amplifications, 3) *BRAF* splice variants, 4) *MEK1* or *MEK2* mutations, and 5) non-MAPK alterations. The elastic net method was used for variable selection for building multivariable models. The elastic net is a generalization of the LASSO (least absolute shrinkage and selection operator), which provides variable selection in the $p \gg N$ case without being limited by sample size, and improves performance in the case of potentially correlated explanatory variables (22). We used the elastic net method for prescreening to discard those least contributing variables ignoring the covariance structure due to multiple samples in some patients (23). Following variable selection, generalized linear mixed-effects models (logit link or identity link depending on outcome variable type) were used for coefficient estimates to account for multiple biopsy specimens within patients.

PFS, OS, and survival after progression were calculated using the Kaplan-Meier method. Due to the co-occurrence of alterations among tumor specimens and within patients, each class of resistance mechanism was compared against all other patients using the logrank test. Cox mixed effects models were used to investigate baseline factors that influenced survival. All analyses were conducted using R version 3.1.1.

Results

Patients

We included 100 patients with 132 progression samples. Patients had a median age of 54 years; 70% had AJCC stage IV M1c melanoma, and 17% had brain metastases (Table 1). *BRAF*^{V600E} was present in 87% of patients, and *BRAF*^{V600K/R} in 13%. Vemurafenib was the BRAFi received by 64% of patients and dabrafenib by 36%. The median PFS was 4.7 months (95% CI 3.8–5.6 months); median OS was 12.8 months (95% CI 10.7–15.0 months). The objective response rate was 72%.

Sequencing and identified mechanisms of resistance

Validated mechanisms of resistance were identified in 77/132 progressing tumor samples (58%). Sixty-four samples (48%) had a single identified resistance mechanism, 9 (7%) had

two identified alterations and 4 (3%) had 3 or more (Fig. 1A). Among all progression tumors, *NRAS* or *KRAS* mutations were identified in 26 samples (17% and 2%, respectively), *BRAF*^{V600E/K} amplification in 17 (13%), *BRAF* splice variants in 21 (16%), *MEK1/2* mutations in 9 (7%), and non-MAPK pathway alterations in 14 (11%) (Table 2). Of note, the *BRAF* splice variant was assessed in 65% of samples (n=86) and identified in 24% of these, suggesting that this may be the most common resistance mechanism. Non-MAPK pathway alterations largely occurred in the PI3K-AKT pathway but also included *MITF* amplification, and overexpression of PDGFR/IGF1R. *CDKN2A* deletion and *DUSP4* loss occurred in 3 samples and were not included in further analyses given limited sample size. Several candidate “primary resistance” mechanisms were identified in pre-treatment samples from patients with primary disease progression or short duration of therapeutic benefit and are listed descriptively (Table S1, S3).

Heterogeneity of BRAFi resistance mechanisms

We then investigated the intra-tumor and intra-patient heterogeneity of resistance. Although most progression samples had 0–1 detected alterations, 13 progression tumors, had >1 identified mechanism of resistance (10%) co-occurring in the same sample. We assessed whether specific resistance mechanisms occurred more frequently in isolation or arose concurrently with other alterations (Fig. 1B/C). *NRAS* mutations (20 of 26, 77%), *BRAF* splice variants (18 of 21, 86%), and *MEK1/2* mutations (8 of 9, 89%) usually occurred in isolation. By contrast, *BRAF*^{V600E/K} amplification (11 of 17, 65%) and non-MAPK alterations (7 of 14, 50%) more commonly co-occurred in tumors with other genetic changes (p=0.015). Among MAPK-alterations, *NRAS*, *KRAS*, *MEK1*, and *MEK2* mutations arose in mutually exclusive fashion with each other, whereas *BRAF*^{V600E/K} amplifications overlapped with *NRAS* mutations (n=3), non-MAPK alterations (n=4), and a *MEK2* mutation. *BRAF* splice variants did not overlap with *MEK1/2* mutations or *BRAF* amplifications. These results may suggest that certain mutations play complementary roles in driving resistance (e.g. *BRAF*^{V600E/K} amplifications and non-MAPK alterations) whereas others have redundant signaling functions and are unlikely to co-occur.

To investigate genetic heterogeneity within individual patients (as opposed to individual tumor samples), we evaluated 19 patients who underwent multiple biopsies during or after progression. Of these, 10 (53%) had completely distinct resistance mechanisms between separate biopsies, 4 (21%) had no identified alterations in any biopsy, and only 1 (5%) had the same identified mechanism in all progressing tumors (Fig. 2). Four patients (21%) were found to have some overlapping but not identical alterations, supporting the evolutionary development of resistance (20). Of these 19 patients, 12 had serial tissue acquisition at two distinct time points after progression. On average, earlier progression sample had fewer resistance mechanisms compared to the later sample (mean 0.42 vs. 0.83, p=0.054). These results may suggest that mutational complexity increases over time on BRAFi.

Clinical correlation with mechanisms of resistance

We then investigated whether baseline disease, treatment, and host factors were related to the development of particular resistance mechanisms. We developed multivariable logistic regression models assessing the impact of age, gender, LDH levels, sites of metastatic

disease, drug (vemurafenib vs. dabrafenib), number of prior therapies, and burden of disease (RECIST sum of target lesion diameters). Several associations were identified; *NRAS* mutations occurred more often in patients who received vemurafenib (odds ratio 3.53, $p=0.045$), among patients who had received prior therapies (OR 2.57, $p=0.003$), and in those with brain metastases at baseline (OR 4.57, $p=0.037$). *BRAF*^{V600E/K} amplifications also arose more frequently in pre-treated patients (OR 2.19, $p=0.037$). No clear associations were identified for other resistance mechanisms. The association between *NRAS* mutations and vemurafenib use is consistent with the higher incidence of cutaneous squamous cell carcinomas (cuSCCs) observed with vemurafenib compared with dabrafenib (3, 4, 24, 25). These cuSCCs usually harbor RAS mutations (26), suggesting that these otherwise similar agents may promote distinct resistant subclonal populations.

Next, we explored the timing and pattern of disease progression associated with particular resistance mechanisms. PFS did not significantly vary by group either in multivariable Cox regression models or in univariate analyses (Fig S1). Patients without known resistance mechanisms also had similar PFS. *NRAS* mutations were marginally associated with disease progression in the brain (OR 3.05, $p=0.066$) and negatively correlated with progression in the lungs (OR 0.25, $p=0.036$); *MEK1/2* mutations were associated with hepatic progression (OR 7.61, $p=0.011$; 6 of 9 patients with *MEK1/2* mutations had disease progression in the liver).

Effects of mechanisms of resistance on survival and response to subsequent therapies

We then assessed whether specific mechanisms of resistance influenced clinical outcomes following disease progression. Median survival following progression was 6.9 months (95% CI 5.3 – 8.5 months), and no statistically-significant differences were identified between patients harboring particular resistance mechanisms (Fig S2). OS from the start of treatment was also similar, regardless of the mechanism of resistance identified (Fig S3). Of interest, 6 patients experienced overall survival of > 1000 days (PFS range 300–672 days) and 5 had unknown drivers of resistance (19).

Finally, we assessed the influence of resistance mechanisms on response to subsequent therapy particularly focusing on MAPK-directed treatment. Fifteen patients received subsequent BRAF +MEK inhibition; two experienced partial responses (13%), and no mechanism of BRAFi resistance was identified in either patient. Stable disease was observed in an additional 7 patients (objective response + stable disease in 9 of 15 patients; 60%) and occurred in patients with various resistance mechanisms. Patients with MAPK-only resistance drivers appeared to have more frequent disease stabilization (6 of 8; 75%) compared to patients with non-MAPK alterations (1 of 3, 33%). In addition, 21 patients also received ipilimumab following progression, and no patients responded (Table S3).

Discussion

In this study, we combined previously-published analyses of BRAFi resistance to form the largest cohort (to our knowledge) of 132 BRAFi progression melanoma tissue samples from 100 patients. This large population allowed us to correlate resistance mechanisms with

clinical characteristics and outcomes. This study also provides a comprehensive landscape of the incidence and heterogeneity of genetic changes driving BRAFi-resistance.

Acquired resistance is a major hindrance to effective molecularly-targeted therapy. BRAFi in particular have been linked to a diverse array of genetic changes driving resistance with unclear therapeutic and prognostic implications. Assessing the resistance landscape in a large cohort with integrated clinical data has therefore been a critical need. We conclude that *NRAS* mutations, *BRAF*^{V600E/K} amplifications, *BRAF* splice variants, and various non-MAPK alterations each occur in 11–24% of BRAFi-progression melanomas. We also observed marked tumor heterogeneity; although individual progression samples usually harbored only a single resistance mechanism, nearly all patients with multiple resistance biopsies had distinct or unknown drivers of resistance (95%). Such marked intra-patient genetic heterogeneity likely limited our ability to correlate individual genetic changes with clinical behavior.

Despite this, we identified clinically-relevant associations. First, resistance changes correlated with specific organ involvement in some cases (*NRAS* mutations and brain metastases; *MEK1/2* mutations and hepatic progression). Second, *NRAS* mutations occurred more frequently in vemurafenib-treated patients, suggesting differences in subclonal activation between BRAFi. Third, the similar spectrum of resistance drivers between BRAFi monotherapy and those observed with BRAFi/MEKi therapy suggests that these clinical associations may be relevant for the now-preferred combination (19, 27–29). Finally, the intra-patient heterogeneity and lack of clear associations with post-progression outcomes implies that genetic profiling from a single post-progression biopsy to guide further therapy will be challenging.

Although this study was not designed to identify novel resistance mechanisms, several biologic insights were suggested. Some alterations, including *MEK1*^{P124} and *PTEN* mutations appear to mediate resistance in a complex, context-dependent fashion. For example, pre-existing *MEK1*^{P124} mutations diminished, but did not preclude clinical responses to BRAFi in this study and others (30). Conversely, in pre-clinical models, these mutations robustly mediate BRAFi-resistance (18). Similar dynamic changes should be considered in other drug-resistance states. In addition, patients with serial biopsies following progression had more alterations in the later post-progression sample compared to the earlier sample, potentially suggesting increasing mutational complexity arising on therapy. Finally, we observed that some resistance mechanisms tended to arise in isolation, whereas others tended to co-occur with other drivers, suggesting that particular alterations may differ in their ability to drive resistance *in vivo*.

Exploring the non-genetic and immune features of BRAFi-resistance will be a crucial next step. Despite comprehensive molecular characterization, >40% of tumor progression samples harbored no identified resistance drivers, including several patients with multiple progression samples analyzed by WES. Non-genetic (epigenetic) or transcriptome-based changes likely drive resistance in this substantial cohort. In addition, no patients responded to ipilimumab after BRAFi-failure, corroborating previous findings (31, 32). Thus,

characterization of non-genetic drivers and the immune state of BRAFi-resistance is needed to complement important early findings (33–35).

Our study has several limitations. Sequencing was performed at three institutions with distinct protocols and analysis methods. Although most patients had baseline pre-treatment samples for comparison, nearly 20% did not. In addition, *BRAF* splice variants were assessed in 65% of progression samples. Finally, survival in this cohort was somewhat inferior to that observed in phase II/III BRAFi clinical trials, likely reflecting that progression must have occurred during the study timeframe.

Acquired resistance to targeted therapy remains a major unanswered challenge of cancer therapeutics. This study provides a comprehensive characterization of the genetic landscape of acquired BRAFi-resistance in advanced melanoma. Furthermore, it provides insight into the clinical implications of these alterations and suggests further investigation into immune and epigenetic changes accompanying resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We examined clinical implications of BRAFi-resistance mechanisms in 100 patients
- Mitogen-activated protein kinase pathway mechanisms predominated and overlapped
- Acquired NRAS mutations were associated with vemurafenib use and brain metastases
- MEK1/2 mutations correlated with hepatic disease progression
- Post-progression outcomes were similar across resistance mechanisms

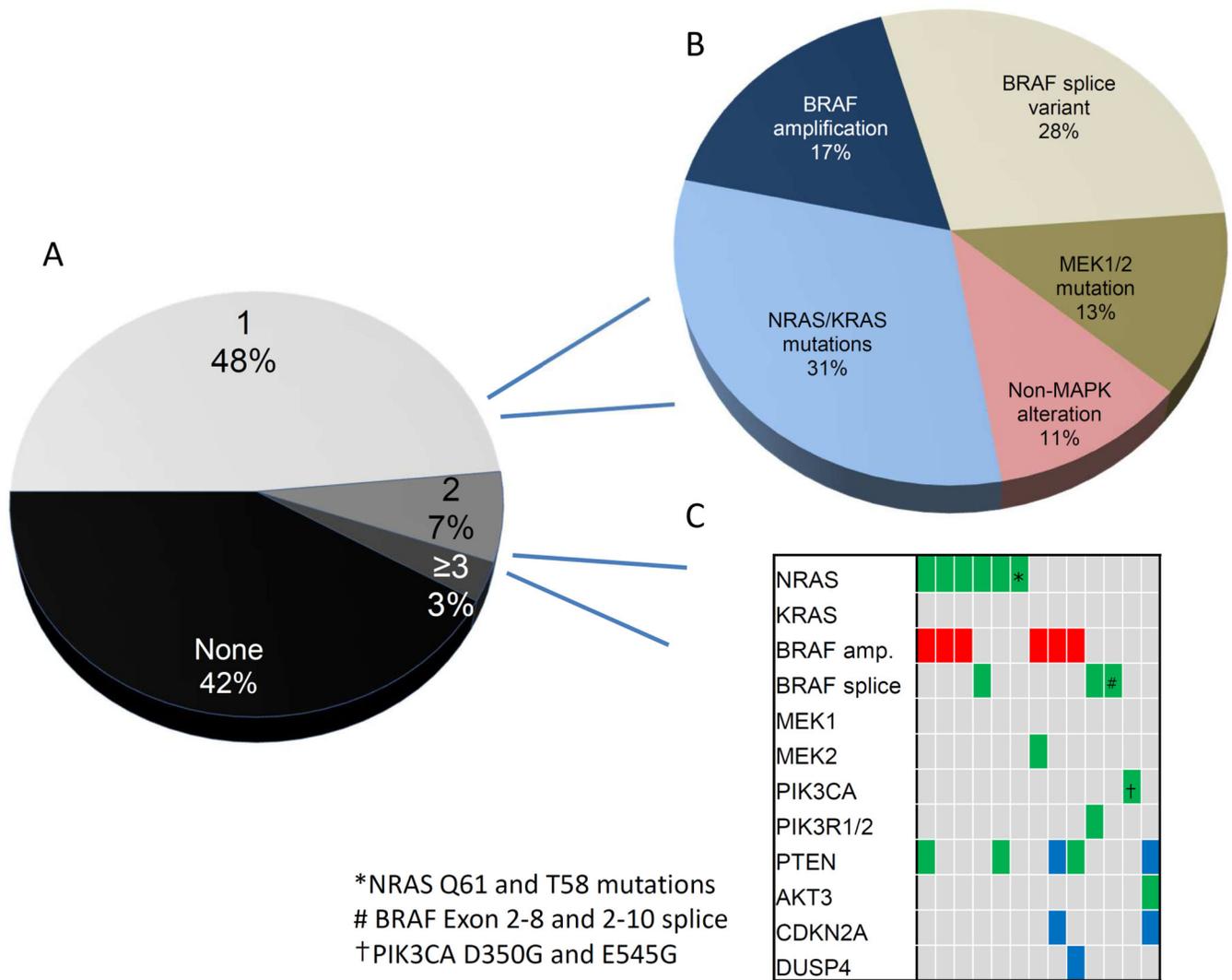


Figure 1. (A) Number of identified resistance mechanisms per progression sample (B) Spectrum of resistance mechanisms in samples with only one identified alteration (C) Spectrum of resistance mechanisms in samples with >1 identified alteration co-occurring in the same sample
 Legend: Green: mutation; Red: amplification; Blue: deletion; ^Distinct *NRAS* mutations identified in different samples

Table 1

Patient demographics

Total Patients (n=100)	Number
Gender	
Male	63
Female	37
Age	54 (median)
ECOG	
0	51
1	42
Stage	
M1a	20
M1b	10
M1c	70
LDH	
Elevated	47
Normal	47
Unknown	6
Disease sites (baseline)	
Brain	17
Lung	52
Liver	52
Skin/Subcutaneous	77
BRAF mutation	
V600E	87
V600K	12
V600R	1
BRAF inhibitor	
Vemurafenib	64
Dabrafenib	36
RECIST sum of disease in target lesions (mm)	97 (median)

Table 2

Mechanisms of resistance and method of testing

	<i>Number</i>	<i>Percent</i>
Total progression samples	132	
Corresponding pre-treatment sample assessed	109	83
Analyses performed		
PCR	132	100
WES	74	56
IHC	10	8
Identified Mechanism	77	58
Single Mechanism per sample	64	48
Two Mechanisms per sample	9	7
3 Mechanisms per sample	4	3
Unknown	55	42
Specific mechanisms*		
<i>NRAS</i> mutation	23	17 (30)
<i>KRAS</i> mutation	3	2 (4)
<i>BRAF</i> splice variants [†]	21	16 (27)
<i>BRAF</i> ^{V600E/K} amplification [‡]	17	13 (22)
<i>MEK1</i> mutation	5	4 (6)
<i>MEK2</i> mutation	4	3 (5)
Other MAPK alterations [#]	3	2 (4)
Non-MAPK alterations	14	11 (18)

* Percentage of total samples; percentage of samples with identified alterations denoted in parentheses

[†] *BRAF* splice variants were assessed in 65% of all samples

[‡] *BRAF* amplifications were assessed in 91% of all samples

[#] Includes acquired *DUSP4* loss and *CDKN2A* deletion.