Predictive Biomarkers and Personalized Medicine

BRAF Inhibitor Resistance Mechanisms in Metastatic Melanoma: Spectrum and Clinical Impact

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Abstract

Purpose: Multiple BRAF inhibitor resistance mechanisms have been described, however, their relative frequency, clinical correlates, and effect on subsequent therapy have not been assessed in patients with metastatic melanoma.

Experimental Design: Fifty-nine $BRAF^{V600}$-mutant melanoma metastases from patients treated with dabrafenib or vemurafenib were analyzed. The genetic profile of resistance mechanisms and tumor signaling pathway activity was correlated with clinicopathologic features and therapeutic outcomes.

Results: Resistance mechanisms were identified in 58% progressing tumors and BRAF alterations were common. Gene expression analysis revealed that mitogen-activated protein kinase (MAPK) activity remained inhibited in 21% of resistant tumors, and the outcomes of patients with these tumors were poor. Resistance mechanisms also occurred in pretreatment biopsies and heterogeneity of resistance mechanisms occurred within patients and within tumors. There were no responses to subsequent targeted therapy, even when a progressing tumor had a resistance mechanism predicted to be responsive.

Conclusions: Selecting sequential drugs based on the molecular characteristics of a single progressing biopsy is unlikely to provide improved responses, and first-line therapies targeting multiple pathways will be required. *Clin Cancer Res; 20(7); 1965–77. ©2014 AACR.*

Introduction

The serine/threonine kinase BRAF is constitutively activated via mutations in 40% to 60% of cutaneous melanomas. The valine substitution at residue 600 accounts for more than 90% of BRAF mutations and causes the RAS-independent activation of the mitogen-activated protein kinase (MAPK) cascade (1, 2). Potent inhibitors of $BRAF^{V600}$-mutant protein, dabrafenib and vemurafenib, have produced response rates of 50% to 60%, and prolong the progression-free (PFS) and overall survival (OS) of patients with $BRAF^{V600E}$ melanoma, compared with dacarbazine (3, 4). Dabrafenib also causes regression of brain metastases in patients with $BRAF^{V600E/K}$ melanoma (5).

Despite this activity, 50% of the patients treated with dabrafenib or vemurafenib develop disease progression 6 to 7 months after starting the treatment (6, 7). Multiple mechanisms of acquired resistance have been described, including elevated expression of the kinases CRAF, COT1, or mutant BRAF (8–11), activating mutations in *N-RAS*, *MEK1*, or *AKT1* (12–14), aberrant splicing of *BRAF* (15), activation of phosphatidylinositol-3-OH kinase (PI3K) via the loss of PTEN (16), and persistent activation of receptor tyrosine kinases, including platelet-derived growth factor receptor β (PDGFRβ), insulin-like growth factor IR (IGF-IR), and EGF receptor (EGFR; refs. 12, 17, 18). Interactions between melanoma tumors, and their microenvironment can also elicit innate resistance to BRAF inhibitors and stromal-derived hepatocyte growth factor has been shown to activate the receptor tyrosine kinase MET along with MAPK and PI3K signaling in melanoma cells (19, 20).

The relative frequency of these resistance mechanisms and correlation with clinical outcome to BRAF inhibitor therapy is poorly understood. No single study has analyzed all known mechanisms of resistance in a single patient cohort nor correlated them with clinicopathologic features or outcomes. Previous studies indicate that approximately 32% of patients progressing on vemurafenib therapy have melanomas expressing BRAF splice variants (15), 23% have melanomas with activating *N-RAS* mutations (10, 15, 21),

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Translational Relevance

Heterogeneity of BRAF inhibitor resistance mechanisms is common between patients, within patients, and within individual tumors. Re-activation of mitogen-activated protein kinase (MAPK) signaling in resistant tumors is common and prognostic, and resistant tumor cells can exist before therapy. These results suggest that adaptive clinical trials in metastatic melanoma, which involve the selection of sequential targeted drugs based on the molecular profiling of a single progressing biopsy, are unlikely to provide durable responses. Anticipating the emergence of multiple resistance mechanisms by initiating drug treatments targeting multiple pathways may have more success than an adaptive sequential approach.

and a further 31% have melanoma metastases with *BRAF* copy-number gains (10). The robustness of these proportions in resistance to dabrafenib, the extent of resistant heterogeneity within patients, and the clinical correlates of each resistance mechanism remain to be established. We analyzed 59 tumors; 38 progressing (Prog) and 21 matched pretreatment melanoma tumors from 30 patients with BRAF^{V600}-mutant melanoma receiving dabrafenib or vemurafenib. The genetic profile of resistance mechanisms and tumor signaling pathway activity was correlated with clinicopathologic features and therapeutic outcomes.

Materials and Methods

Patients and BRAF inhibitor therapy

Patients included in this study had BRAF^{V600}-mutant metastatic melanoma, had not received prior MAPK inhibitors or immunotherapy, and were treated with either dabrafenib (150 mg twice daily or total daily dose of at least 300 mg daily) or vemurafenib (960 mg twice daily) as part of a clinical trial (refs. 3–5, 22, 23; including NCT01378975) or an access program. All patients had a progressing melanoma metastasis (Prog) resected that was classified as either (i) a newly identified metastasis that arose during treatment, (ii) a preexisting metastasis, that initially responded and subsequently progressed on BRAF inhibitor therapy, or (iii) a preexisting metastasis that never responded. Where available, a matched pretreatment melanoma tissue sample obtained before commencing BRAF inhibitor was included in the analysis.

Clinical outcome was assessed using best objective response, PFS, and OS from commencement of BRAF inhibitor. The best objective response and PFS was determined using RECIST (24) for patients on clinical trials. For patients not on a clinical trial or those without measurable disease at treatment commencement, the treating physician determined disease progression and categorized the best objective response as "response" ($\geq$30% reduction in tumor burden) or "no response" (<30% reduction). Informed

consent was obtained for each patient under approved Human Research Ethics Committee protocols.

Melanoma tissue samples

Fresh melanoma samples were macrodissected by a pathologist and enriched tumor portions were snap frozen. Frozen tumor sections were cut, stained with hematoxylin and eosin (H&E), and scored for the following parameters: % nontumor cells, % necrosis, degree of pigmentation, predominant cell shape, and cell size of the most cellular portion of tumor (25). Tumor foci were carefully macrodissected using the marked frozen section as a guide to meet minimum criteria for tumor cell content (>80%) and amount of necrosis (<30%). High-percentage tumor content was verified by examining postmacrodissection frozen section slides. Total tumor RNA and DNA was extracted as described previously (25). A short-term Prog-derived cell culture was used to analyze resistance mechanisms in 1 patient (patient 27).

Resistance screen

A reverse transcription (RT-PCR) resistance screen was used to examine the expression of BRAF splice variants, the complete coding sequence of *MEK1*, *MEK2*, and *N-RAS* cDNAs and the 5′ half of the *AKT1* cDNA. This region of *AKT1* encompassed amino acids 1 to 200, which includes the common activating E17K (COSMIC; 84% of tumor-associated *AKT1* mutations) and the BRAF inhibitor resistance allele Q79K (14). *BRAF* relative copy number was determined by quantitative PCR (qPCR) using the Corbett Rotor-Gene 6000 as previously described (11). The allele frequencies of the wild-type and V600E-mutant BRAF were also assessed using allele-specific PCR in Prog tumors showing BRAF copy-number gains. The allele-specific primers (26) showed similar PCR efficiencies (i.e., serial dilution standard curves had equivalent slopes; data not shown) and relative *BRAF* V600:E600 allele frequencies were calculated from the change in cycle threshold.

Reverse transcription reactions were performed using the SuperScript III First-Strand Synthesis Kit (Invitrogen) with the oligo dT primer. The *MEK1*, *MEK2*, *N-RAS*, and *AKT1* gene products were each amplified from cDNA using Taq polymerase (Fisher Biotec) and *BRAF* cDNA was amplified with Pfx polymerase (Life Technologies). PCR products were purified using QIAquick PCR Purification Kit (Qiagen) followed by Sanger sequencing on the 3730xl DNA Analyser (AGRF). Amplification and sequencing primers are listed in Supplementary Table S7. The identity of Prog mutations was confirmed using an independent RT-PCR product, and where available, Prog-associated alterations were examined in matched pretreatment melanoma samples.

Gene expression analysis

Gene expression analysis was performed on matched pretreatment ($n = 21$) and Prog ($n = 29$) biopsies derived from 21 patients, using the Sentrix HumanHT-12 v.4.0 Expression BeadChip (Illumina). Two responding (i.e., shrinking) melanoma samples taken early during BRAF

inhibitor therapy (EDT; 3–15 days after therapy initiation; patients 5 and 10) were also included in these gene expression analyses. Gene expression data were normalized using the cubic spline function. Rank ordering of gene expression data was carried out using the linear model for microarray module (LimmaGP) in GenePattern and analyses was performed using gene set enrichment analysis in preranked mode (GSEA preranked; refs. 27, 28). Two established transcriptional signatures of MAPK activation (12, 29) were included and analyzed as part of the c6.all.v4.0 oncogenic gene set. A false discovery rate (FDR) $q < 0.1$ was used for comparisons between Prog melanoma tumors and matched pretreatment samples. Box-plots were used to illustrate the distribution of MAPK activation scores (mean $\log_2$-transformed expression of MAPK activation gene transcripts). Medians and interquartile ranges were applied to summarize the distributions, and the Mann–Whitney U test was used to determine the differences between the pretreatment and Prog tumors.

The microarray platform and data have been submitted to the Gene Expression Omnibus public database at the National Center for Biotechnology Information, following the minimum information about microarray gene experiment guidelines. The accession number is GSE50509.

Immunohistochemistry

All immunohistochemistry (IHC) staining was performed on a Dako Autostainer Plus (Dako), using the EnVision FLEX, High pH visualization system (K8024; Dako) according to the manufacturer's protocol. Sections (4 μm) were baked at 58°C for 60 minutes and then underwent deparaffinization and antigen retrieval using the heat-induced epitope retrieval method using EnVision FLEX Target Retrieval Solution (High pH) in a PT link unit (Dako). Slides were incubated with the primary antibodies IGF-IR 1:200 (CS-3027; Cell Signaling Technology), PDGFRβ 1:50 (CS-3169; Cell Signaling Technology), and pAKT Ser473 1:75 (CS-3787; Cell Signaling Technology) for 45 minutes at room temperature. Antibody detection was performed using the Dako EnVision FLEX HRP (Dako) and visualized using 3,3′-diaminobenzidine (Dako), and the slides were counterstained with Harris's hematoxylin.

H&E and immunostained slides were evaluated twice by two independent observers (A.M. Menzies and R.A. Scolyer) blinded to clinical and resistance data. Expression in Prog tumors was determined relative to the matched pretreatment sample, and between Prog samples, using a four-tier semiquantitative scale of staining intensity in tumor cells (0, no staining; 1, weakly positive staining; 2, moderately positive staining; 3, strongly positive staining). For IGF-IR and PDGFRβ, membranous staining was scored, and for pAKT, both nuclear and cytoplasmic staining was scored.

Cell culture, constructs, and lentivirus transductions

SKMel28 and A375 melanoma cells were obtained from Prof. P. Hersey (Kolling Institute, University of Sydney, New South Wales) and short-term cultures were established from a subset of patients as previously described (30). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and glutamine (Gibco-BRL) and cultured in a 37°C incubator with 5% CO_2. Stocks of dabrafenib (supplied by Active Biochem) were made in dimethyl sulfoxide (DMSO). Cell authentication was confirmed using the StemElite ID System from Promega. Lentiviruses were produced in HEK293T cells as described previously (31). Cells were infected using a multiplicity of infection of 1 to 5 to provide an efficiency of infection above 90%. Wild-type and mutant FLAG-tagged MEK1 and MYC-tagged MEK2 constructs were each cloned into the *pCDH-CuO-MCS-EF1-CymR-PURO* and *pCDH-CuO-MCS-IRES-copGFP* lentiviral vectors, respectively.

Western blotting

Total cellular proteins were extracted and analyzed as previously described (30). Western blots were probed with the following antibodies: total ERK (137F5; Cell Signaling Technology), phosphorylated ERK (E-4; Santa Cruz Biotechnology), MYC (A-14; Santa Cruz Biotechnology), FLAG (Sigma-Aldrich), MEK1/2 (L38C12; Cell Signaling Technology), ATM (D2E2; Cell Signaling Technology) and β-actin (AC-74; Sigma-Aldrich).

Clonogenic assays

Approximately 96 hours posttransduction, melanoma cells were seeded at 25, 000cells per well onto 6-well culture plates and allowed to grow for 24 hours, followed by the desired treatment. Cells were then washed twice with ice-cold PBS and fixed with ice-cold methanol for 1 minute. After aspirating methanol from plates, 0.1% crystal violet (ScienceLab.com, Inc.) solution was added and the plate was incubated at room temperature for 5 minutes. Distilled water was used to rinse the plate. Colony-forming assays were performed at least twice in triplicate.

Statistical methods

Univariate time-to-event analyses were conducted with the Kaplan–Meier method together with the log-rank test for significant differences between groups. Multivariate times to event analyses were conducted with Cox regression and the forward stepwise variable selection method (inclusion criteria, $P < 0.01$). Clinical features were tested for association with categorical groupings of resistance mechanisms using the Fisher exact test and the Mann–Whitney U test as appropriate. Two-tailed P values less than 0.05 were considered significant for all tests. Analyses of clinical statistics were carried out with the IBM SPSS Statistic 21.0 software package.

Results

Patients and melanoma tissue samples

We analyzed 59 fresh-frozen melanoma tumor samples derived from 30 patients with BRAF[V600]-mutant metastatic melanoma who received either dabrafenib (22 of 30; 73%) or vemurafenib (27%; Table 1); 38 were Prog metastases and 21 were matched pretreatment tumors. All patients

Table 1. Patient, treatment outcome, and resistance characteristics

Patient	Age/sex	BRAF genotype	M stage	Drug	RECIST response category[a]	RECIST% response	PFS (weeks)	OS (weeks)	Prog No.	Site	Type of Prog lesion	Resistance mechanism	MAPK activity[c]
1	51/M	V600E	C	Dab	PR	−71	96.0	203.6[e]	1	SQ	New	MEK1[E203K]	+
2	40/F	V600E	C	Dab	PR	−61	56.7	113.7[e]	1	SQ	New	N-RAS[Q61K]	+
3	59/M	V600K	C	Dab	PR	−32	46.9	89.4	1	SQ	New	Unknown	+
									2	SQ	New	Unknown	+
4	31/M	V600K	C	Dab	PR	−89	39.4	103.9[e]	1	Bowel	New	BRAF exon 2-10Δ	+
5	59/M	V600K	C	Dab	SD	−14	32.0	37.0	1	SQ	Na	Unknown	+
6[b]	67/M	V600E	A	Dab	PR	−42	32.0	65.4	1	LN	New	BRAF amplification	na
7	71/M	V600E	C	Dab	PR	−62	31.4	44.0	1	LN	Existing—RES	BRAF exon 4-8Δ	+
8	75/F	V600E	C	Dab	PR	−67	30.1	80.7	1	SQ	Existing—RES	Unknown	+
										SQ	Existing—RES	Unknown	+
9	59/F	V600E	C	Dab	PR	−63	29.9	135.9	1	SQ	Existing—RES	BRAF exon 2-10Δ	+
10	33/F	V600E	A	Dab	PR	−60	29.9	124.9	1	SQ	New	IGF-IR	−
									2	SQ	New	BRAF exon 4-8Δ	+
11	39/F	V600K	C	Dab	RES	—	17.0	124.9	1	SQ	Na	Unknown	−
									2	Brain	New	AKT1[Q79K]	+
12	40/M	V600K	C	Dab	PR	−71	16.9	77.6	1	SQ	New	Unknown	+
13	31/M	V600E	C	Dab	SD	−5	16.7	30.6	1	SQ	Na	Unknown[d]	−
14	63/M	V600E	C	Dab	SD	−26	16.0	45.7	1	Brain	Existing—RES	BRAF amplification	+
15	40/F	V600E	C	Dab	PR	−43	16.0	22.0	1	SQ	Existing—RES	BRAF exon 2-10Δ & N-RAS[Q61K]	na
16	51/M	V600K	A	Dab	PR	−32	16.0	87.6	1	SQ	existing—RES	Unknown	na
17[g]	17/M	V600E	C	Dab	RES	—	13.4	90.0[e]	1	Lung	Existing—RES	Unknown	+
18	60/M	V600E	C	Dab	SD	−25	12.1	28.4	1	Bowel	Existing—RES	Unknown	−
									2	SQ	New	MEK1[K57E]	+
19	58/M	V600E	C	Dab	PR	−43	10.6	24.7	1	LN	Existing—RES	Unknown	na
20	67/M	V600K	C	Dab	RES	—	9.0	28.6	1	SQ	New	Unknown	na
21	55/F	V600E	C	Dab	No RES	—	8.3	28.6	1	Brain	Existing—no RES	Unknown	−
22	75/M	V600K	C	Dab	No RES	—	7.0	39.0[e]	1	Bowel	Existing—no RES	BRAF exon 2-10Δ & 2-8Δ	na
23	60/F	V600E	C	Vem	PR	−44	32.0	32.9	1	SQ	Existing—RES	BRAF exon 2-8Δ	+
24	72/M	V600E	C	Vem	PR	−53	29.9	94.3	1	SQ	Existing—RES	N-RAS[G13R]†	+
25	80/M	V600K	C	Vem	PR	−58	24.9	73.6[e]	1	SQ	Na	Unknown	−
26	66/M	V600E	C	Vem	PR	−68	23.6	46.7	1	SQ	New	Unknown	na
27	53/F	V600E	C	Vem	PR	−48	23.6	87.9[e]	1	SQ	New	BRAF exon 2-8Δ	na

Table 1. Patient, treatment outcome, and resistance characteristics (Cont'd)

Patient	Age/sex	BRAF genotype	M stage	Drug	RECIST response category[a]	RECIST% response	PFS (weeks)	OS (weeks)	Prog No.	Site	Type of Prog lesion	Resistance mechanism	MAPK activity[c]
28	84/M	V600R	A	Vem	RES	—	22.0	45.4[e]	1	SQ	Existing—RES	*BRAF* exon 2-8Δ	+
									2	SQ	Existing—RES	*BRAF* exon 4-8Δ	+
									3	SQ	Existing—RES	*BRAF* exon 2-8Δ	+
									4	SQ	Existing—RES	*BRAF* exon 4-8Δ	+
29	52/F	V600E	C	Vem	PR	-54	16.0	103.6[e]	1	SQ	na	*BRAF* amplification	na
30	65/M	V600E	C	Vem	SD	-17	15.3	32.1	1	Lung	Existing—RES	*MEK2*[F57C]	+

Abbreviations: Dab, dabrafenib; Vem, vemurafenib; PR, partial response, SD, stable disease; RES, response; SQ, subcutaneous; LN, lymph node; na, data not available.
[a]Patients without prospective RECIST assessments or measurable disease at baseline, response categorized as response (RES) or no response (no RES).
[b]Because of high melanin content in Prog-derived RNA, only qPCR analyses of *BRAF* copy number was performed on this patient Prog tumor.
[c]MAPK activity was determined using GSEA of whole transcriptome data comparing matched pretreatment (n = 21) and Prog (n = 29) biopsies. + indicates re-activated and – indicates inhibited.
[d]An *AKT1* polymorphism (D46E; rs146875699) was identified in the Prog and matching pretreatment tumors of patient 13.
[e]Alive at the time of analysis.
[f]This Prog from patient 24 displayed two distinct subclones by p-ERK IHC (39).
[g]Patient 17 Pre and Prog tumors had nonsense mutations in the *PTEN* and *CDKN2A* genes.

were on BRAF inhibitor at the time of Prog biopsy except patients 27 and 29, who had ceased vemurafenib 10 and 18 days before resection, respectively. The median time to resection of the Prog lesion was 32.5 weeks (range, 8.4–150.7). Prog tumors included 14 newly identified metastases, 17 preexisting and initially responding tumors, two tumors that never responded to treatment, and five subcutaneous Progs not categorized (Table 1). Twenty patients had *BRAF*[V600E] (67%), 9 had *BRAF*[V600K] (30%), and 1 had *BRAF*[V600R] melanoma (3%). Nearly all (28 of 30; 93%) patients experienced tumor regression with treatment. The median PFS was 22.0 weeks [95% confidence interval (CI), 13.0–31.0] and the median OS was 80.7 weeks (95% CI, 46.0–115.4; Table 1).

Resistance profile

A resistance mechanism was identified in 22 of 38 (58%) Prog tumors (Table 1; Fig. 1A); *BRAF* splice variants were detected in 12 (32%), oncogenic *N-RAS* mutations in three (8%), *BRAF* amplification in three (8%), *MEK1/2* mutations in three (8%), an *AKT1* mutation in one (3%), and increased levels of IGF-IR in one (3%) Prog tumor(s). Twenty-nine Prog tumors underwent gene set enrichment analysis (GSEA) of gene expression; six (21%) showed loss of MAPK activity signatures, i.e., MAPK pathway signaling was inhibited in these progressing lesions compared with the matched pretreatment melanoma, and 23 (79%) showed re-activation of MAPK signaling (Table 1, Fig. 1B).

BRAF splice transcripts and BRAF amplification. Alternative *BRAF* splice transcripts were detected in 12 of 38 (32%) Prog tumors (Supplementary Figs. S1 and S2). A single full-length *BRAF* RT-PCR product was present in matched pretreatment samples from 9 of these 12 patients. Full-length *BRAF* was usually heterozygous for wild-type and mutant transcripts, indicating correct BRAF splicing from both alleles in the pretreatment sample (Supplementary Fig. S1). In contrast, the Prog-specific shorter *BRAF* transcripts were always homozygous for *BRAF*[V600] and lacked exons 2 to 10 (n = 3 Prog), exons 2 to 8 (n = 4 Prog), or exons 4 to 8 (n = 4 Prog), with one tumor having two shorter transcripts (lacking exons 2–10 and 2–8; Supplementary Figs. S1 and S2). Immunoblot analyses of one Prog-derived short-term culture (patient 9; Prog lacking exons 2–10) using the V600E-specific BRAF antibody (32) confirmed expression of the full-length BRAF[V600E] protein and the expected 41 kDa BRAF mutant truncated variant (Supplementary Fig. S1). *BRAF* copy-number gains were detected by qPCR in three of 38 Prog samples (8%; 2- to 4-fold copy-number increase; Table 1) and allele-specific PCR confirmed that the mutant allele was amplified relative to the wild-type *BRAF* DNA (Supplementary Fig. S3).

Oncogenic mutations in N-RAS, MEK1, MEK2, and AKT1. Oncogenic mutations in *N-RAS* (G13R or Q61K) were identified in three of 38 (8%) Prog samples (Fig. 1A). An *N-RAS*[P185S] mutation was identified in the Prog and matched pretreatment biopsies derived from patient 4. This missense mutation has been reported in the 1000 Genomes project (33), has a frequency of 0.01% in European
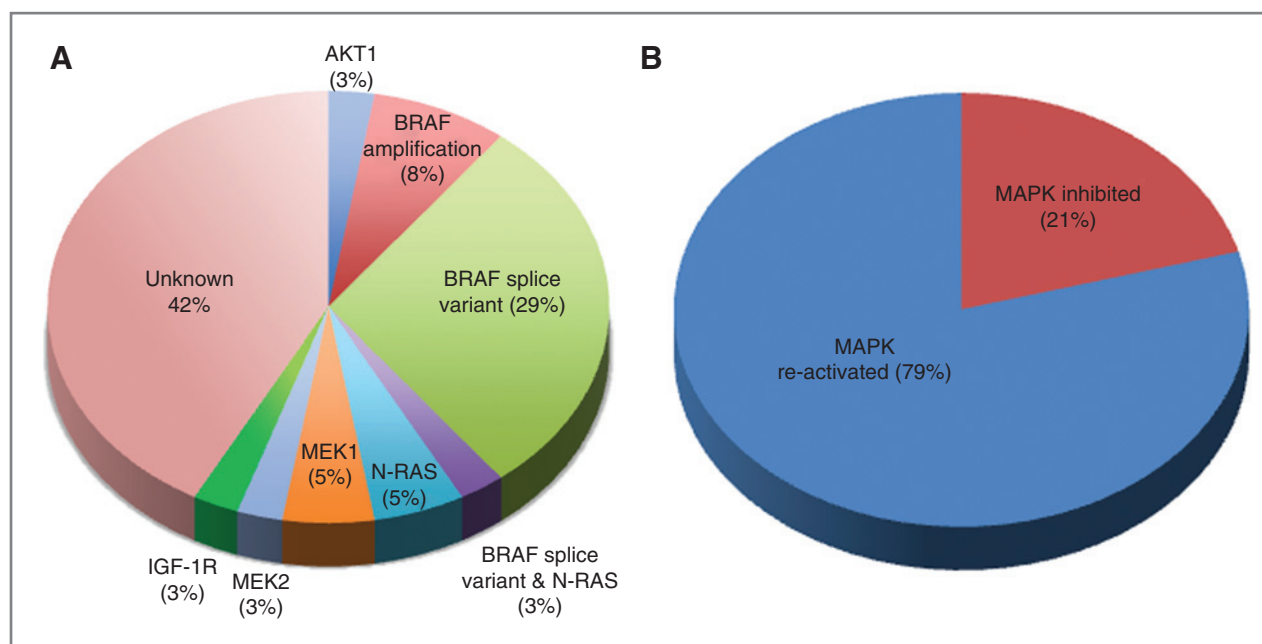
Figure 1. BRAF inhibitor resistance mechanisms. A, mechanisms of resistance in individual Prog tumors ($n = 38$). B, Prog tumor MAPK activation status relative to the pretreatment tumor, as determined by GSEA of gene expression data ($n = 29$).

American populations, and was excluded as a resistance mechanism.

Five MEK1 (K57E, I111S, P124S, G176S, and E203K) and one MEK2 (F57C) mutations were identified in seven Prog tumors (Supplementary Figs. S2 and S4). The I111S, P124S, and G176S MEK1 mutants and the F57C MEK2 mutant occurred in the pretreatment tumors; I111S and P124S have been shown not to preclude clinical response (34). Functional analyses confirmed that MEK1^{K57E} and MEK2^{F57C} mutants restored extracellular signal–regulated kinase (ERK) activation in the presence of dabrafenib, whereas MEK1^{G176S} did not alter melanoma cell sensitivity to dabrafenib (Fig. 2). On the basis of these results, and previous reports (34, 35), we assigned MEK1^{K57E}, MEK1^{E203K}, and MEK2^{F57C} as drivers of resistance (3 of 38; 8%; Fig. 1A). Finally, as reported previously, one Prog tumor (2%; patient 11) carried an AKT1^{Q79K}-activating mutation that was not present in the pretreatment tumor (Fig. 1A; ref. 36). An AKT1^{A102V} mutation was detected in the Prog and matching pretreatment tumor derived from patient 5. This AKT1 missense mutation has not been associated with AKT1 activation in cancer (COSMIC) and because the Prog displayed MAPK re-activation compared with the pretreatment biopsy (Table 1), we did not analyze the activity of this mutation.

Mutations affecting MAPK, PI3K, and cell-cycle pathways. We sequenced the exomes of four matched Pre and Prog melanoma pairs, including two with unknown mechanisms of resistance (Supplementary Table S1). All tumors retained the BRAF^{V600E} mutation (data not shown) and we identified a series of Prog-specific variants in each tumor pair (Supplementary Table S2). Of these, three high-priority candidates were identified on the basis of sequence conservation (PhyloP), predicted functional impact (SIFT), and disease association (Supplementary Table S2). The MEK1^{E203K} and N-RAS^{G13R} mutations were identified in Prog tumors derived from patients 1 and 24, respectively (Supplementary Table S2) and were also detected in our targeted screen (Table 1). The third candidate variant was the pathogenic ATM^{R337C} (37) mutation, which we confirmed by capillary sequencing (Supplementary Fig. S5). Suppression of ATM expression using a highly specific short hairpin RNA (shRNA), however, did not detectably alter the response of two BRAF-mutant melanoma cells to dabrafenib (Supplementary Fig. S5), and ATM was not attributed as a resistance mechanism. Additional analyses of our exome data did not reveal Prog-specific nonsynonymous mutations affecting any of the RAS, RAF, AKT, mTOR, JAK, STAT, or PI3K genes (data not shown). We identified a heterozygous nonsense mutation affecting PTEN (K221ter) and an inactivating CDKN2A/p16^{INK4a} mutation (R80ter) in both the Pre and Prog tumors derived from patient 17 (Supplementary Table S2).

mRNA expression analyses and IHC. The activity of the MAPK pathway was examined using two established gene transcription signatures, indicative of persistent MAPK activation (12, 29). Six Prog tumors (6 of 29; 21%) showed loss of MAPK activation signatures, i.e., MAPK signaling was inhibited using both MAPK activation gene sets in these progressing lesions compared with the pretreatment tumor (Fig. 3; Supplementary Table S3). Five of these six MAPK-inhibited Prog tumors had no established mechanism of resistance (Table 1). A class comparison of the six MAPK-inhibited Prog tumors versus the 23 MAPK re-activated Prog tumors revealed loss of metastatic-associated gene sets, diminished epithelial–mesenchymal transition, reduced

Figure 2. *MEK1*^{K57E} and *MEK2*^{F57C} confer melanoma cell resistance to dabrafenib. A, SKMel28 melanoma cells were stably transduced with the indicated constructs. Cell lysates were analyzed for the indicated proteins 4 hours after incubation with dabrafenib at 0, 10, 50, and 100 nmol/L concentrations. Ectopically expressed MEK1 and MEK2 proteins were tagged with the FLAG and MYC epitopes, respectively. B, transduced SKMel28 cells were seeded at low density and 24 hours after seeding were treated with the indicated concentrations of dabrafenib every 72 to 96 hours. Colonies were stained with crystal violet 12 days post transduction. Photographs are representative of at least two independent transduction experiments. wt, wild-type.
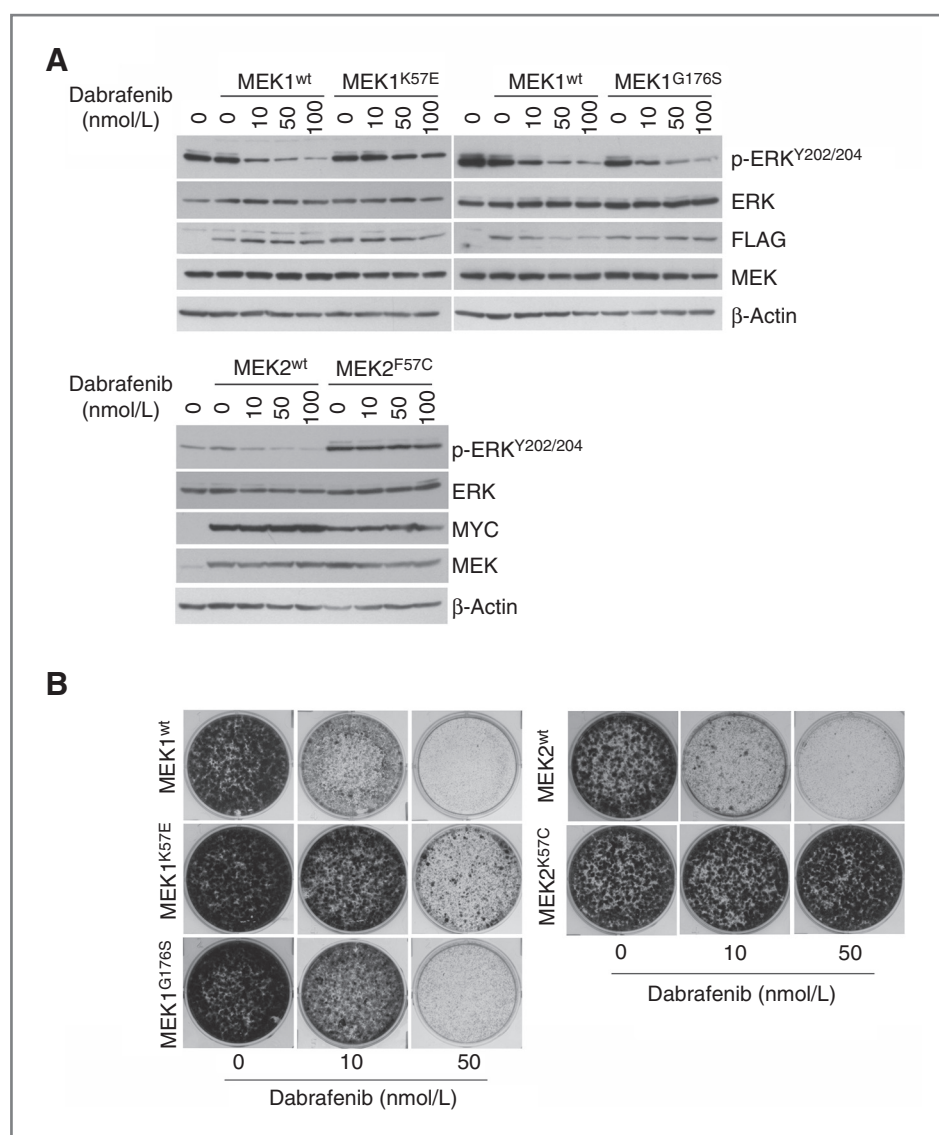
MAPK activity score, and reduced proliferative signatures in the MAPK-inhibited class (Supplementary Table S4).

Expression of CRAF, COT1, and PDGFRβ is upregulated at the transcript and protein levels, in a subset of BRAF inhibitor–resistant melanomas (8, 9, 12). CRAF and COT1 were not substantially increased (>3-fold) in any of the 29 Prog melanoma samples analyzed for mRNA expression, relative to the matched pretreatment sample (Supplementary Fig. S6). PDGFRβ mRNA was upregulated in two Prog tumors with unknown resistance mechanisms (both from patient 3; Supplementary Fig. S6), but this was associated with increased stromal, rather than tumor-specific PDGFRβ expression (Supplementary Fig. S7). This suggests that tumor PDGFRβ protein overexpression was not conferring resistance in these Prog samples.

Tumors from the 6 patients with a MAPK-inhibited Prog tumor were examined for IGF-IR, PDGFRβ, and pAKT expression. No Prog (or pretreatment) tumors expressed

PDGFRβ in melanoma cells. IHC analysis of IGF-IR showed increased expression in three of the six MAPK-inhibited Prog samples (patient 11 Prog 1; patient 13; and patient 10 Prog 1), relative to the matched pretreatment tumors (data not shown). Elevated IGF-IR was not associated with a corresponding increase in pAKT (17) in patient 11 and patient 13 Prog tumors, and hence, IGF-IR activation was not assigned as their resistance mechanism. In patient 10, the MAPK-inhibited Prog 1 tumor stained positive for p-AKT compared with the MAPK re-activated Prog 2 tumor, and thus, activation of IGF-IR/PI3K/AKT was associated with BRAF inhibitor resistance in the Prog 1 tumor (Supplementary Fig. S8).

Clinical correlates of resistance mechanisms and MAPK signature

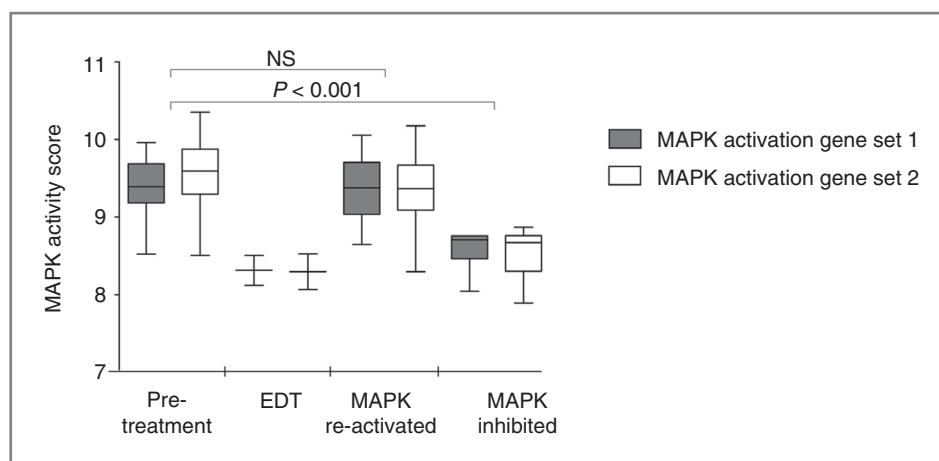Patient clinicopathologic factors and treatment outcomes were correlated with resistance characteristics

Figure 3. Loss of MAPK activation gene sets occurs in early during treatment (EDT) responding melanoma tumors and in a subset of BRAF inhibitor–resistant Prog metastases. Box plots showing significant differences in MAPK activity (mean $\log_2$-transformed expression of MAPK activation gene transcripts) between pretreatment ($n = 21$) and MAPK-inhibited Prog tumors ($n = 6$). NS, no significant differences in comparisons between pretreatment and MAPK-reactivated Prog ($n = 23$) tumors. Statistical comparisons between pretreatment and EDT MAPK activity scores were not performed because of small EDT sample size ($n = 2$). MAPK activation gene set 1 derived from (24) and gene set 2 from ref. (14).

identified in the initial resected Prog tumor. Patients with multiple Progs had subsequent lesions resected after the PFS endpoint, at a median of 18.5 weeks (range, 3.1–47.8) after progression.

No association was observed between specific resistance mechanisms and clinical outcome variables, including the category and degree of best RECIST response (Fig. 4A) or PFS (Fig. 4B; $P > 0.05$). Similarly, there were no associations observed between the MAPK activity (re-activated versus inhibited) and clinicopathologic factors, including age, sex, American Joint Committee on Cancer (AJCC) Metastases (M) stage (38), LDH, BRAF genotype, baseline RECIST sum of diameters, ECOG, the category and degree of best response (Fig. 4C), and OS (all $P > 0.05$; Table 2). However, patients with Prog tumors showing persistent MAPK inhibition had shorter PFS (Fig. 4D; median 16.7 weeks vs. 30.1 weeks; $P = 0.01$) compared with those with MAPK re-activated tumors. In multivariate PFS analysis, MAPK inhibition (HR, 7.88; 95% CI, 1.95–31.78; $P = 0.004$) and lactate dehydrogenase (LDH; HR, 3.66; 95% CI, 1.11–12.05; $P = 0.033$) remained significant ($n = 20$) when adjusting for additional known prognostic factors, including ECOG, M stage, presence of brain metastasis, and BRAF genotype.

The median time to resection of MAPK-inhibited Progs was shorter than MAPK re-activated Progs ($n = 21$; 17.6 vs. 35.0 weeks; $P = 0.036$). The behavior of the Prog tumor before resection (newly emerging, preexisting and initially responded, and preexisting but never responded) did not associate with the MAPK activation signature.

Heterogeneity of resistance mechanisms

Six patients had multiple independent Prog tumors biopsied during the course of their BRAF inhibitor therapy. In 4 of these patients, the lesions showed heterogeneity of known resistance mechanisms (Supplementary Table S5).

Patient 28 had four Prog tumors expressing various BRAF splice variants. Patients 10 and 18 each had one Prog showing MAPK inhibition (elevated IGF-IR in patient 10 Prog) and one Prog showing MAPK re-activation associated with a BRAF splice variant or $MEK1^{K57E}$. Patient 11 had one Prog with an AKT1 mutation, and another Prog with no identified resistance mechanism (Supplementary Table S5).

Heterogeneity of resistance was also observed within four Prog tumors. As previously reported, a single Prog tumor from patient 24 consisted of two morphologically distinct subclones, one with an N-RAS^{G13R} mutation and low p-ERK staining and the second with elevated p-ERK reactivity but no established resistance mechanism (39). A single Prog expressed two BRAF splice variants (patient 22), another coexpressed a BRAF splice variant and an N-RAS mutation (patient 15) and the Prog tumor with an $AKT1^{Q79K}$ mutation (patient 11; second Prog) showed MAPK signature re-activation, suggesting that a second unidentified MAPK reactivating mechanism of resistance was also present (Table 1).

BRAF inhibitor resistance mechanism and response to subsequent MAPK inhibitors

Seven of the 30 patients were subsequently treated with a MEK inhibitor or combined BRAF and MEK inhibitors after progression on single-agent BRAF inhibitor, and there were no RECIST responses (Supplementary Table S6) including patients with $MEK1^{E203K}$ mutation (patient 1), BRAF amplification (patient 6), BRAF splice variant (patient 10), and N-RAS mutation (patient 24). The lack of clinical response to subsequent MEK inhibitor therapy is inconsistent with data demonstrating that melanoma cell lines expressing BRAF amplification (10) or BRAF splice variants (Supplementary Fig. S9) are responsive to downstream MEK inhibition alone or in combination with a BRAF inhibitor.
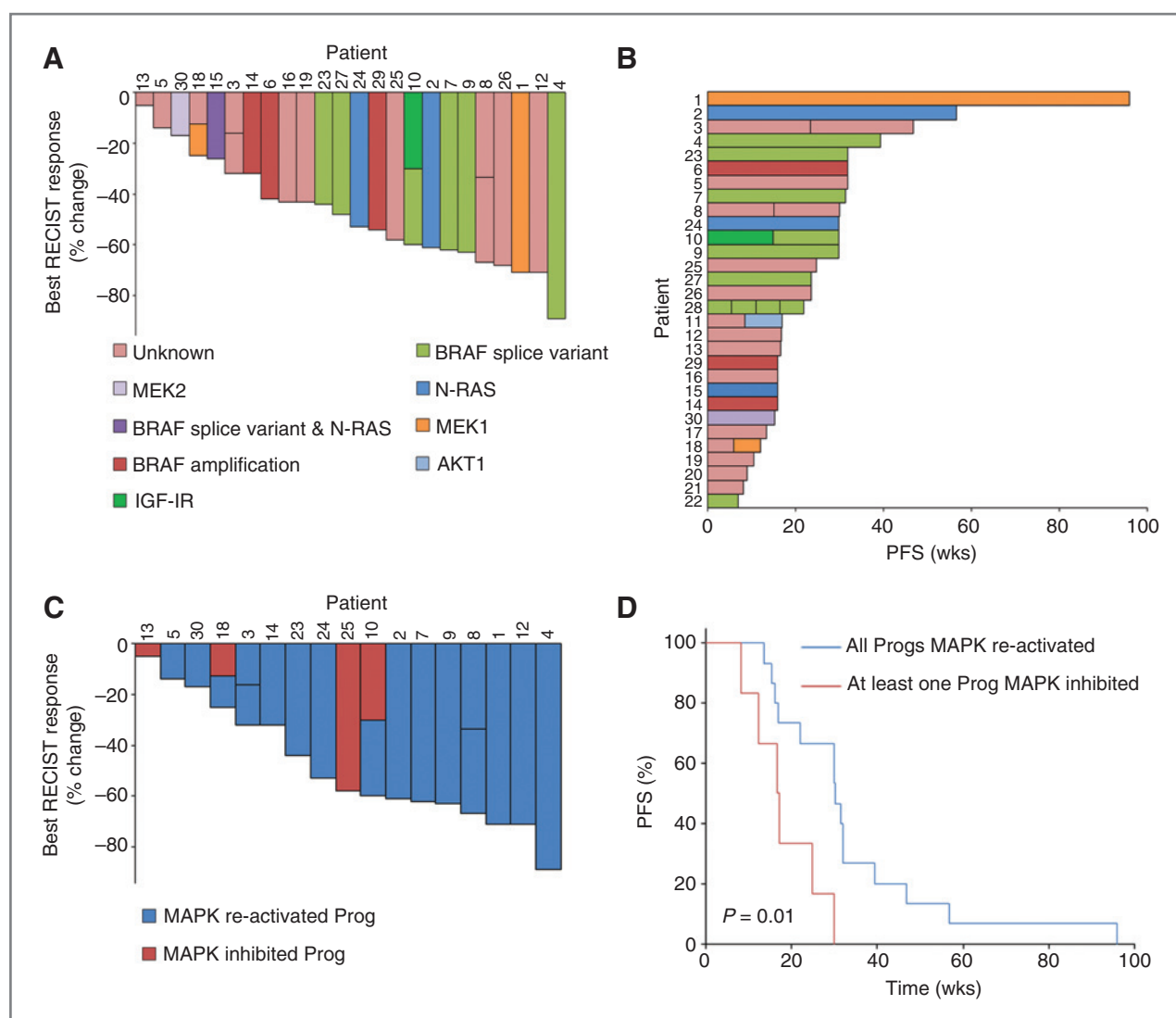
Figure 4. BRAF inhibitor resistance mechanisms and clinical correlates. A, best overall RECIST response by mechanism of resistance ($n = 24$). Six patients were excluded, as they did not have RECIST assessments. Patients with multiple Prog biopsies are shown by a divided bar, and the first biopsied Prog is closest to the x-axis. B, PFS by mechanism of resistance ($n = 30$). Patients with multiple biopsies are shown by a divided bar, and the first biopsied Prog is closest to the y-axis. C, best overall RECIST response by MAPK activity of Prog tumors ($n = 17$). Four patients were excluded, as they did not have RECIST assessments. Patients with multiple biopsies are shown by a divided bar, and the first biopsied Prog is closest to the x-axis. D, PFS by MAPK activity level of Prog tumors ($n = 21$).

Discussion

Our study confirms that MAPK signaling had been restored in 79% of melanoma tumors resistant to dabrafenib or vemurafenib. No association was observed between the type of resistance mechanism and clinicopathologic factors, response rates, or survival outcomes. Patients with MAPK re-activated Prog tumors, however, showed improved PFS, compared with patients with MAPK-inhibited resistant melanomas. This may reflect the partial cytostatic activity of BRAF inhibitors continued beyond progression in MAPK-dependent resistant melanomas (30). Despite this, 7 patients progressing on BRAF inhibitor failed to respond to subsequent therapy involving a MEK inhibitor

(Supplementary Table S6), and at least 3 of these patients had melanomas with mechanisms predicted to respond to MEK inhibition (10, 12). The lack of MEK inhibitor and combination BRAF and MEK inhibitor clinical activity in patients previously treated with BRAF inhibitors (40, 41) is most likely due to the heterogeneous nature of resistance. We identified intertumoral heterogeneity of resistance in all patients with multiple, independent progressing tumors with at least one identified resistance mechanism. We also observed evidence of heterogeneity within four Prog metastases (39). Although multiple mechanisms of resistance may occur within a single tumor cell, this has not been formally demonstrated and current data confirm

Table 2. Patient clinical characteristics according to MAPK activity status of Prog tumors

Feature	Value	MAPK inhibited		MAPK re-activated		
		N	%	N	%	P
Age (y)	Median (range)	47 (31–80)	—	59 (16–84)	—	0.340
Sex	F	3	50%	4	27%	0.299
	M	3	50%	11	73%	
BRAF genotype	V600E	4	67%	10	67%	0.701
	V600K/R	2	33%	5	33%	
AJCC M stage	M1a	1	17%	1	7%	0.500
	M1c	5	83%	14	93%	
ECOG	0	4	67%	7	47%	0.367
	1	2	33%	8	53%	
LDH	Normal	4	67%	9	64%	0.664
	Elevated	2	33%	5	36%	
RECIST SoD (mm)[a]	Median (range)	129 (10–142)	—	83 (23–317)	—	1.000
Brain metastases	Absent	5	83%	11	73%	0.550
	Present	1	17%	4	27%	
Best response	Response[c]	3	50%	3	20%	0.198
	No response[d]	3	50%	12	80%	
Degree of best response[b]	Median	−42% (−60 to −5%)	—	−61% (−89 to −14%)	—	0.202
PFS (wks)	Median (95% CI)	16.7 (10.9–22.5)	—	30.1 (21.2–39.1)	—	0.010
OS (wks)	Median (95% CI)	30.6 (0.0–88.3)	—	89.4 (68.4–110.4)	—	0.360
Total patients		**6**	**100%**	**15**	**100%**	—

Abbreviation: SoD, sum of diameters.
[a]RECIST SoD data only available for 4 patients with MAPK inhibited Progs and 14 patients with MAPK re-activated Progs.
[b]Degree of best response data only available for 4 patients with MAPK inhibited Progs and 13 patients with MAPK re-activated Progs.
[c]RECIST complete/partial response or physician assessed as response where no RECIST performed.
[d]RECIST stable/progressive disease or physician assessed as no response where no RECIST performed.

intratumoral heterogeneity of resistance. For instance, only one of the two phenotypically distinct nodules within a vemurafenib-resistant tumor (patient 24) contained mutant N-RAS. Similarly, two subclonal N-RAS mutations were identified within a single vemurafenib-resistant metastasis (42), and multiple mechanisms of resistance were detected, but did not coexist, in single-cell derived dabrafenib-resistant melanoma clones (43).

Additional data, including the identification of an activating MEK1[F57C] mutation before initiation of dabrafenib therapy and the appearance of multiple, independent resistant melanomas support current models that heterogeneous tumor clones exist before treatment initiation (44). Consequently, progression occurs when resistant tumor subclones expand under the selective pressure of BRAF inhibition and repopulate the lesion. These results suggest that adaptive clinical trials in metastatic melanoma, which involve the selection of sequential targeted drugs, based on the molecular characteristics of a single progressing biopsy, are unlikely to provide durable responses.

The relative frequency of resistance mechanisms identified in our cohort is not always consistent with previous estimates. We observed BRAF splice variants in 31% of patients and these splice variants were identical to the

variants reported in vemurafenib-treated patients (32%) (15). Mutations in N-RAS, identified in approximately 23% of patients with acquired resistance to vemurafenib (10, 15, 21), were far less common in our patient cohort (8%). The lower frequency of activating N-RAS mutations in dabrafenib-treated patients may correlate with the observation that patients on dabrafenib therapy develop fewer RAS-dependent squamous cell carcinomas, compared with vemurafenib-treated patients (45). It has been suggested that increased selectivity of dabrafenib for mutant BRAF over wild-type RAF may limit paradoxical activation of MAPK signaling (45). Paradoxical MAPK activation is dependent on RAS activity and occurs when BRAF inhibitors bind and transactivate wild-type CRAF (46–48). Vemurafenib displays similar potency against BRAF and CRAF (49), and both these kinases regulate MAPK activity in melanoma cells coexpressing BRAF[V600E] and mutant N-RAS (50). Thus, activation of MAPK signaling may be more pronounced in response to vemurafenib, rather than dabrafenib, in N-RAS–mutant melanoma cells.

We observed BRAF copy-number gains in 8% of patients. This compares to 27% of patients from a vemurafenib-treated cohort (10). Whether reported frequencies of BRAF amplification and N-RAS mutations in BRAF inhibitor–

resistant melanomas reflect study sample size, the distribution of biopsy sites (71% of metastases in this study were subcutaneous lesions), or genuine differences between various BRAF inhibitors requires validation.

We found *MEK1* and *MEK2* mutations in 7 of 38 Prog melanoma samples, but assigned resistance to only 3 (8%); *MEK1*[K57E], *MEK1*[E203K], and *MEK2*[F57C] confer resistance to BRAF inhibitors (13, 35). The contribution of *MEK1* and *MEK2* alterations to BRAF inhibitor resistance has been uncertain, because the complete coding regions are not routinely screened (10, 21) and *MEK1* mutations are commonly seen in both matched pretreatment and progressing tumor samples. Functional analyses reported here and by others demonstrated that a subset of *MEK* mutations do not preclude a clinical response (34). We identified 1 patient with a *MEK2*[F57C] mutation in the pretreatment and progressing melanomas and this patient had a poor PFS (15.3 weeks) and OS (32.1 weeks). *MEK2*[F57C] has been observed as a germline mutation in patients with cardio-facio-cutaneous syndrome, does not require RAF for MEK activation (51), and confers resistance to dabrafenib. Exome sequence data also identified heterozygous, nonsense mutations affecting the *PTEN* and *CDKN2A* genes in the baseline and progressing tumors of patient 17. Genetic alterations affecting *PTEN* and *CDKN2A* are associated with poor BRAF inhibitor responses (21, 52), and patient 17 had a poor PFS (13.4 weeks).

A significant proportion (21% in this study) of BRAF inhibitor–resistant melanomas do not display MAPK reactivation, and increased IGF-IR in one such tumor is consistent with an ERK-independent resistance mechanism (17). Most of these MAPK-inhibited tumors have unknown mechanisms of resistance and show loss of metastatic, EMT, and proliferative transcriptome signatures.

Several investigators have reported heterogeneity of BRAF inhibitor resistance mechanisms in subcutaneous and nodal melanoma metastases (12, 39, 42). These data indicate that selecting an effective secondary treatment, after progression on BRAF inhibitor therapy, based on the examination of a single progressing biopsy is unlikely to be effective. Accordingly, although a substantial proportion of patients with dabrafenib- and vemurafenib-resistant melanoma have melanomas expressing a MEK inhibitor–sensitive BRAF slice variant (this report; ref. 15), the MEK inhibitor trametenib displayed minimal clinical activity as a sequential therapy either as single agent (40), or combined with dabrafenib (41). Perhaps a more accurate and complete genetic profile of patient tumors can be defined by analyzing circulating tumor cells or circulating tumor-derived DNA.

Combination therapies targeting single pathways, such as BRAF plus MEK inhibitors, are also unlikely to provide long-term disease control (53), because single mutations, such as those activating *N-RAS*, confer partial cross-resistance (43, 44). Anticipating the emergence of multiple resistance mechanisms by initiating drug treatments targeting multiple pathways may have more success than an adaptive sequential approach (44). For example, the simultaneous inhibition of MAPK and PI3K/mTOR activity effectively killed BRAF inhibitor–resistant melanoma cell models (54), and this strategy is being investigated in patients with BRAF-mutant melanoma (NCT01616199 and NCT01512251). Alternatively, the addition of immunotherapies (e.g., antibodies against PD1 or PD-L1) may enhance the T-cell responses induced early during treatment with BRAF inhibitors (55, 56) and prolong clinical responses, and clinical trials using this approach are being planned.

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BRAF Inhibitor Resistance Mechanisms in Metastatic Melanoma: Spectrum and Clinical Impact

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