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A novel AKT1 mutant amplifies an adaptive melanoma response to BRAF inhibition

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Abstract

BRAF inhibitor (BRAFi) therapy leads to remarkable anti-melanoma responses, but the initial tumor shrinkage is commonly incomplete, providing a nidus for subsequent disease progression. Adaptive signaling may underlie early BRAFi resistance and influence the selection pattern for genetic variants causing late, acquired resistance. We show here that BRAFi (or BRAFi+MEKi) therapy in patients frequently led to rebound p-AKT levels in their melanomas early on treatment. In cell lines, BRAFi treatment led to rebound levels of RTKs (including PDGFRβ), PIP_3, pleckstrin homology domain (PHD) recruitment, and p-AKT. PTEN expression limited this BRAFi-elicited PI3K-AKT signaling, which could be rescued by introduction of a mutant AKT1

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(Q79K) kown to confer acquired BRAFi resistance. Functionally, AKT1 Q79K conferred BRAFi resistance via amplifying BRAFi-elicited PI3K-AKT signaling. Additionally, MAPK pathway inhibition enhanced clonogenic growth dependency on PI3K or AKT. Thus, adaptive or genetic upregulation of AKT critically participates in melanoma survival during BRAFi therapy.

Keywords

BRAF; MEK; PI3K; AKT; melanoma; BRAF inhibitors

Introduction

About 50% of metastatic melanomas harbor BRAF V600 mutations, most commonly a V600E substitution (1), which constitutively hyper-activate the MAPK pathway and result in oncogene addiction. Therapy with a BRAF inhibitor (BRAFi) or its combination with a MEK inhibitor (MEKi) leads to rapid and high rates of clinical responses (2, 3), but the initial tumor shrinkage is typically partial, providing niduses for eventual disease progression caused by acquired drug resistance (4). It is unclear what adaptive signal(s) underlies initial incomplete tumor responses and whether a deficiency in such adaptive signaling might favor genetic selection for its upregulation.

We have recently shown that a majority of melanomas with late or acquired resistance to a BRAFi display molecular or genetic alterations which lead to MAPK pathway reactivation (5–8). However, a significant minority of disease progressing melanomas harbor genetic lesions which upregulate the PI3K/PTEN/AKT pathway (9). These genetic alterations include non-synonymous, gain-of-function mutations in positive regulators (*PIK3CA*, *AKT1* and *AKT3*) and loss-of-function mutations (*PIK3R2*, *PTEN*, *PHLPP1*) and gene deletion (*PTEN*) in negative regulators. While some somatic variants (e.g., PIK3CA E545G, AKT1/3 E17K) harbor known mechanisms of action, the structure-functional consequences of others (e.g., PIK3R2 N561D, AKT1 Q79K, PTEN M134del) are novel.

Given that the landscape of mechanisms of acquired BRAFi resistance reflect the essential survival functions of MAPK and PI3K/PTEN/AKT signaling pathways in *BRAF* mutant melanoma, we explored the roles of these pathways in acute or adaptive resistance. Recent works (10, 11) have demonstrated how targeted inhibition of the MAPK pathway can lead to adaptive alterations in signal transduction crosstalk/feedback and transcriptional output. Here we show that, in clinical melanoma biopsy specimens, MAPK pathway inhibition elicited a pervasive rebound in AKT activation. In cell line work, this rebound in AKT activation could be traced to enhanced PIP_3 generation and was antagonized by wild type PTEN. Moreover, the PHD class of AKT mutants, including the novel AKT1 Q79K mutant, displayed enhanced affinity for PIP3, dramatically amplified the BRAFi-induced upregulation of PI3K-AKT signaling in the presence of PTEN, and as such conferred BRAFi resistance in a cell context-dependent manner. These data thus reveal key insights into the dynamic evolutionary continuum of BRAFi resistance in that early adaptive events in response to MAPK inhibitors, which limit the initial efficacy, shape subsequent mechanisms of acquired resistance, which further augment adaptive responses and lead to tumor re-growth.

Results

Although AKT has been implicated in BRAFi resistance, the timing of its upregulation during BRAFi therapy is not clear. To determine whether MAPK pathway targeting results in a rebound increase in p-AKT in the tumors of patients treated with a BRAFi (or BRAFi

+MEKi), we assessed the p-AKT levels in melanoma tumors biopsied early on-treatment (days 4 to 25) relative to their patient-matched baseline (pre-treatment) tumors by immunohistochemistry (IHC) (Fig. 1A) using a validated antibody (Supplementary Fig. S1) and quantifying signals via image analysis (Supplementary Fig. S2). These early on-treatment biopsies took place during a period of time to objective tumor responses, prior to or at maximal tumor responses. Such patient-matched (and in most cases also tumor site-matched) melanoma samples (n = 9 patients; clinical data in Supplementary Table S1) are critical for this analysis since baseline p-AKT levels, assessed in parallel technically, varied over a considerable range among tumors from distinct patients. Importantly, in the majority of tumors biopsied early on-treatment (seven of nine), p-AKT increased upon treatment. We also examined the PDGFRβ levels by IHC as this receptor tyrosine kinase (RTK) has previously been correlated with BRAFi resistance (5, 12). Induction of PDGFRβ expression upon treatment was observed in five out of seven tumors (Supplementary Fig. S3) which displayed a relative increase in p-AKT levels but was not observed in the two tumors which did not display a relative p-AKT increase, consistent with our observations in cell lines (see below). Interestingly, as Patient #1 developed disease progression (via mutant NRAS) on BRAFi, he was treated with BRAFi+MEKi (BRIM7 clinical trial). This combinatorial treatment (day 15) was also associated with PDGFRβ induction (Supplementary Fig. S3). In preliminary work with five sets of transcriptomes for patient-matched pre- and early on-treatment (days 6 to 22; BRAFi or BRAFi+MEKi) melanomas, we observed *PDGFRβ* mRNA induction by MAPK pathway inhibition in five of five patients (fold increase from 1.4 to 2.9, FDR adjusted p-value ≤ 0.05) (unpublished data). Thus, inhibition of the MAPK pathway in ^{V600}*BRAF* mutant melanoma was associated with a tumor-associated rebound PDGFRβ and p-AKT induction in the majority of patients.

As in *BRAF* mutant melanoma tumors, *BRAF* mutant human melanoma cell lines displayed a wide range of basal p-AKT levels at exponential growth densities (Supplementary Fig. S4). We have previously shown that certain *BRAF* mutant human melanoma sub-lines chronically treated with a BRAFi (or some cases of short-term cultures derived from BRAFi-resistant melanoma tissues in patients) upregulated the expression and tyrosine phosphorylation levels of two key receptor tyrosine kinases (RTKs), namely, PDGFRβ and EGFR, as well as the levels of p-AKT. More recent work on triple-negative breast cancer cell lines uncovered a negative feedback of the MAPK pathway (via MYC-mediated transcriptional repression) on RTK expression (13). To assess the timing of p-AKT induction in response to BRAF (or MAPK pathway) inhibition, we treated *BRAF* mutant melanoma cell lines with a BRAFi (vemurafenib), a MEKi (AZD6244), or an ERKi (FR180204). Inhibitor treatment for 48h consistently led to an induction of p-AKT (Thr308) levels, suggesting crosstalk at a level below ERK signaling (Fig. 1B). MAPK pathway inhibition (by BRAFi, MEKi, or ERKi) for 48h led to concomitant c-Myc downregulation, RTK/PDGFRβ (but not EGFR, see below) overexpression, and p-AKT and p-CRAF upregulation, correlating with an induction of PDGFRβ but not EGFR mRNA levels (data not shown).

While EGFR tyrosine-phosphorylation can transiently increase within hours of BRAFi treatment (14), its protein expression level was not induced until after prolonged (weeks) BRAFi treatment coinciding with drug-tolerant persisting (but slowly-cycling; unpublished data) cell sub-populations entering a proliferative phase (Supplementary Fig. S5A and B). Interestingly, while BRAFi induced p-AKT level in M229 at 48h and 72h, co-treatment with a PDGFRβ inhibitor, sunitinib, or an EGFR inhibitor, gefitnib, reduced (but not completely abolished) this BRAFi-induced p-AKT level, with the strongest effect observed with the co-treatment of BRAFi with both sunitinib and gefitnib (Supplementary Fig. S5C). This suggests that these RTKs either contribute independently to p-AKT induction or can compensate for each other's kinase inhibition. After chronic vemurafenib treatment, drug-

resistant, *BRAF* mutant melanoma sub-lines (M229 R5, M238 R1), which over-express both wild type PDGFRβ and EGFR, could be re-sensitized to vemurafenib by co-treatment with sunitinib and gefitnib, suggesting a BRAFi-induced dependency on RTK activities (Supplementary Fig. S5D). Accordingly, BRAFi treatment induced apoptosis in M229 and M238 cell lines (Supplementary Fig. S6A) and the cell-surface expression of PDGFRβ (Supplementary Fig. S6B). In fact, cell-surface PDGFRβ expression was higher in the live sub-populations compared to the dead sub-populations (Supplementary Fig. S6C), and the live sub-populations over time were also more strongly enriched with PDGFRβ-positive cells (Supplementary Fig. S6D). These data suggest that BRAF inhibition specifically (and MAPK pathway inhibition in general) up-regulates RTK expression, which contributes to adaptive AKT signaling. We then traced the BRAFi-induced p-AKT levels to an induction of lipid/membrane-associated phosphatidyl (3,4,5)-triphosphate (PIP_3) levels (Fig. 1C). This increase in PIP_3 levels correlated with an increase in cell surface recruitment of the PHD (Fig. 1D), as visualized by PHD-GFP fusion protein expressed at similar levels (Supplementary Fig. S7). Thus, BRAFi treatment led to rebound RTK upregulation, PIP_3 membrane accumulation, PHD membrane recruitment, and AKT activation.

These studies in melanoma tissues and cell lines (Fig. 1) support the notion that BRAF inhibition leads to early, adaptive AKT signaling. In a subset of melanoma tissues with late, acquired BRAFi resistance, we recently uncovered mutations, including gain-of-function *AKT1* and *AKT3* mutations, which upregulate the PI3K-AKT pathway and confer BRAFi resistance (9), identifuying the PI3K-AKT pathway as another core drug escape pathway in addition to MAPK reactivation. In this setting of late, acquired BRAFi resistance, the detection frequency of MAPK reactivation molecular alterations was greater than that of PI3K-PTEN-AKT-upregulating alterations, raising the possibility this pattern might be shaped by early pathway responses to BRAFi therapy (Fig. 2A). In other words, lack of a robust early adaptive p-AKT tumor response to BRAFi treatment would eventually provide a selective growth advantage for (potentially preexisting) genetic sub-clones which harbor PI3K-AKT-upregulating alterations. Thus, the prevalence of adaptive AKT upregulation early during BRAFi therapy would be inversely correlated with the prevalence of genetic variants which upregulate AKT later during the acquisition of acquired resistance. To understand further this potential mechanistic link between early and late resistance, we hypothesized that a preexisting determinant of AKT activation (e.g., PTEN mutation/ expression status) also restricts the magnitude of BRAFi-elicited rebound p-AKT levels, but the presence of a gain-of-function AKT mutant, for instance, would lift or rescue this restriction via signal amplification.

To test this hypothesis, we took advantage of two gain-of-function AKT mutants (AKT1 E17K and Q79K) identified specifically in melanomas with acquired BRAFi resistance (but not in their patient-matched pre-treatment melanomas) and shown to be capable of conferring BRAFi resistance (9). We first confirmed that both AKT1 mutants are associated with upregulated levels of activation-associated phosphorylation (Fig. 2B). We then tested the structure prediction (9) that the mechanism of AKT1 Q79K gain-of-function is based on its mutant PHD displaying enhanced PIP3 binding and membrane recruitment, as has been shown for the mutant PHD of AKT1 E17K first described in breast cancer (15). In agreement with our structure-based prediction, an AKT1 PHD containing the Q79K mutation and fused to GFP localized to the cell surface independently of serum stimulation, in contrast to WT PHD-GFP and similar to a PHD-GFP fusion protein containing a known AKT1 activating mutation, E17K (Fig. 2C) (15). Moreover, the increased recruitment of Q79K PHD-GFP to the cell surface was also less sensitive to PI3K inhibition by LY294002 when compared to the WT PHD-GFP in the presence of serum stimulation, suggesting that AKT1 Q79K may be hyper-responsive to cell surface membrane recruitment by low levels of PIP_3.

To assess the baseline (no BRAFi treatment) activation status of AKT1 Q79K in the context of *BRAF* mutant human melanoma cell lines with varying PTEN expression (Supplementary Fig. S4), we derived stable cell lines expressing either the vector or AKT1 Q79K in a doxycycline-repressible manner (Fig. 2C). Upon induction of expression for 2 days, similar FLAG-tagged AKT1 Q79K expression levels were achieved in all cell lines (Fig. 2C), resulting in an expression level of the Flag-tagged AKT1 (upper band) comparable to that of the endogenous AKT (lower band). Notably, in the absence of BRAF inhibition, activation-associated phosphorylation of FLAG-AKT1 Q79K (upper band) was greater than that of the endogenous WT AKT1 (lower band) in each cell line. This was most evident in the PTEN WT-expressing line M229 where the endogenous p-AKT level was very low, despite the total AKT levels being similar across all cell lines. The p-AKT1 Q79K level was lowest in the cell line (M229) with the lowest basal endogenous p-AKT level, indicating that, while the AKT1 Q79K mutant is more readily phosphorylated and activated compared to the WT, its maximal phosphorylation still requires upstream signal activation (i.e., PIP_3 generation). Introduction of WT PTEN into the PTEN non-expressing cell lines WM2664 and M249 (Supplementary Fig. S4) suppressed the endogenous p-AKT level (Fig. 2D; data not shown). Upon restoration of PTEN expression and under a lowered PIP_3 environment, the AKT1 PHD mutants (E17K, Q79K) were much more activated than AKT1 WT, i.e., AKT1 PHD mutants were much more sensitive to limiting PIP_3 levels (Fig. 2E).

We then hypothesized that BRAFi treatment, by increasing PIP_3 levels, would provide the necessary upstream signal to maximally activate AKT1 Q79K, enabling the mutant rescue the negative effect of PTEN and to amplify the BRAFi-induced rebound p-AKT (Thr308) level. Consistent with prior experiments (Fig. 1B), treatment of M229 (low basal p-AKT), M238 (intermediate basal p-AKT), and WM2664 (high basal p-AKT) with the BRAFi vemurafenib induced endogenous p-AKT Thr308 in a time-dependent manner (followed up to 48 h) (Fig. 2F). The strength of this BRAFi-induced p-AKT rebound level was weakest in *BRAF* mutant melanoma cell lines displaying the lowest basal level of p-AKT and WT PTEN expression such as M229. In contrast, BRAFi-induced strong phosphorylation of exogenous AKT1 Q79K in M229, to an extent similar to the endogenous p-AKT levels induced by BRAFi treatment in WM2664. Thus, consistent with our hypothesis, AKT1 Q79K amplified a weak BRAFi-induced signal upstream of AKT. Taken together, BRAFi treatment leads to rebound RTK upregulation, PIP_3 membrane accumulation, PHD membrane recruitment, and AKT activation. In addition, the AKT1 Q79K mutant, which can confer acquired BRAFi resistance (9), produces a signaling phenotype dependent on the cell context, which is related to the basal p-AKT level or PTEN status.

It is likely then that the AKT1 Q79K mutant would confer a BRAFi resistance phenotype in a cell context-dependent manner such that the greatest impact would be observed where the mutant AKT1 most robustly amplifies an adaptive response. To test this prediction, we treated multiple cell lines of varying *PTEN* genetic and protein expression status (Supplementary Fig. S4) with either DMSO or increasing vemurafenib concentrations for 3 days (Fig. 3A, left) or 10 days (Fig. 3A, right). From both short- and long-term drug treatment regimens, AKT1 Q79K expression robustly conferred vemurafenib resistance in M229 but weakly in M238 and WM2664 (as well as M263 and M249; Supplementary Fig. S8). In M229, where AKT1 Q79K and E17K expression conferred a two-log increase in BRAFi resistance, the expression of AKT1 WT led to no significant change in BRAFi sensitivity. PTEN knockdown in M229 increased the basal p-AKT (Thr308) level and further boosted the BRAFi-elicited p-AKT level rebound (Fig. 3B). PTEN knockdown in M229 conferred resistance to vemurafenib but rendered the AKT1 PHD mutants incapable of conferring further vemurfenib resistance (Fig 3C). Theses observations are consistent with the notion that WT PTEN activity limits the BRAFi-ellicited adaptive response and that

PHD, gain-of-function AKT1/3 mutants counteract this negative effect of WT PTEN to provide survival benefits under BRAF- or MAPK-inhibited conditions.

Given that AKT1 Q79K expression in M229 amplified BRAFi-induced p-AKT (Thr308) and conferred vemurafenib resistance, we tested whether AKT1 Q79K expression would render cell survival more dependent on AKT signaling in the presence of a BRAFi. First, we showed that vemurafenib-induced p-AKT (and the downstream substrate p-GSK3β) could be downregulated by co-treatment with the AKTi MK2206 in a dose-dependent manner (using 1h treatment to gauge on-target inhibition) (Fig. 3D). M229 expressing AKT1 Q79K exhibited reduced sensitivity to vemurafenib alone (as compared to M229 vector) but only slightly increased sensitivity to MK2206 alone (Supplementary Fig. S9). Importantly, the combination of BRAFi+AKTi was more effective than either agent alone in reducing the clonogenic growth of M229 expressing AKT1 Q79K (Fig. 3E), consistent with BRAF inhibition leading to an increase in MAPK-redundant, AKT-dependent survival. In additional PTEN-expressing, *BRAF* mutant melanoma cell lines (e.g., M238, M395; Fig. 4 and Supplementary Fig. S4 & S10) that are relatively insensitive to an AKTi alone (compared to a BRAFi or MEKi alone), the combination of BRAFi+AKTi (or MEKi +AKTi) was also more effective than either agent alone in reducing the clonogenic growth. Previous studies have suggested that the lack of PTEN expression correlated with cancer cell line sensitivity to AKT inhibition (18). Consistently, PTEN-non-expressing, *BRAF* mutant melanoma cell lines (e.g., WM2664, M249, M397) were exquisitely sensitive to AKT inhibition alone (vs. BRAF or MEK inhibition alone) (Fig. 4 and Supplementary Fig. S4 & S10). Thus, the intrinsic sensitivity of a subset of melanoma cell lines (those with no PTEN activity) to AKT inhibition and the BRAFi-induced sensitivity of another subset of melanoma cell lines (those with PTEN activity) to AKT inhibition rationalize the combination of PI3K-AKT and MAPK pathway inhibition to maximally suppress melanoma survival signaling.

We further tested the efficacy of combinatorial PI3K-AKT and MAPK pathway targeting using small molecule inhibitors (either in clinical use or in development) to suppress adaptive BRAFi resistance relative to the efficacies of single target/pathway targeting and dual targeting of the MAPK pathway (i.e., BRAFi+MEKi). We measured adaptive escape from drug-induced cell death by long-term clonogenic assays, visualizing and quantifying the growth of survival fractions after repeated drug treatments. For drug treatments, we selected doses sufficient to induce complete on-target inhibition (p-ERK for the MAPK pathway inhibitors; p-AKT for the PI3K inhibitor BKM120; p-AKT and p-GSK3β for the AKT inhibitors MK2206 and GSK2141795) at 1 h post-treatment (Supplementary S11 & S12; Fig. 3D; data not shown for the AKTi GSK795). The MAPK pathway inhibitors studied include the ATP competitive BRAFi vemurafenib (PLX4032) and the allosteric MEKi's AZD6244 and GSK1120212. In these long-term survival assays, BRAFi treatment alone (vs. DMSO) was used as the "reference" (Fig. 4). Dual-target MAPK pathway inhibition (BRAFi+MEKi) was more potent than single-target MAPK pathway inhibition, consistent with the clinical data demonstrating an improvement in response rate with the combination of dabrafenib and trametinib compared to dabrafenib alone (19). Notably, single-target MAPK pathway inhibition combined with PI3K or AKT inhibition (Fig. 4A to C; Supplementary Fig. S10) led to similarly efficacious or even more profound suppression of clonal growth escape than dual MAPK pathway inhibition. This is consistent with the notion that PI3K-AKT signaling provides either mutant BRAF-independent survival or BRAFi-induced compensatory survival. Additionally, triple treatment with BRAFi+MEKi +AKTi led to the most profound suppression of clonogenic growth regardless of PTEN expression status (Supplmentary Fig. S10). It is important to note that the drug treatment duration of these clonogenic assays most measured the slow growths of drug-tolerant persisters (Supplementary Fig. S5A).

In summary, we have shown that melanoma therapy based on MAPK pathway suppression can frequently unleash a rebound increase in PI3K-AKT pathway signaling in the tumors of treated patients. In cell lines, this signal crosstalk varied in strength, and one factor limiting the extent of a BRAFi-induced rebound in PI3K-AKT signaling was PTEN expression or activity. Interesting, a novel PHD mutant of AKT1 (Q79K), which was found to mediate acquired (late) BRAFi resistance, could counteract PTEN's negative effect, amplify this BRAFi-elicited rebound level of AKT activation (by enhancing its cell surface recruitment despite limiting PIP_3 levels), and confer BRAFi resistance (Fig. 4D). This BRAFi-induced, PI3K-AKT-dependent adaptive response, along with the intrinsic sensitivity of PTEN non-expressing, BRAF mutant melanoma cells to AKT inhibition, helped to explain the efficacy of combined PI3K-AKT and MAPK pathway targeting. Taken together, these findings provide key insights into the common clinical observation of partial initial tumor responses to BRAFi or BRAFi+MEKi therapy and a strong rationale for combinatorial, upfront targeting of both the PI3K-AKT and MAPK melanoma survival pathways.

Discussion

Strategies to deter acquired BRAFi resistance in melanoma have been based on the frequent occurrence of mechanisms which reactivate the MAPK pathway or the phenomenon of drug addiction displayed by the tumor cells with acquired resistance. These strategies have been either validated clinically or proposed to be tested clinically by the combination of BRAFi +MEKi or its intermittent dosing, respectively (19, 20). However, while the combination of BRAFi+MEKi likely achieves more profound pathway inhibition (compared with BRAFi or MEKi alone) and hence a higher rate of initial clinical responses, the prolonged durability of response is still cut short by the late acquisition of resistance, strongly suggesting that a strategy based solely on MAPK pathway inhibition would be missing another essential melanoma survival pathway. This is even more evident when patients who progressed on vemurafenib were then treated with the combination of BRAFi+MEKi; the secondary response rates were low and, if seen, the durability short (21). This work on adaptive BRAFi resistance and our recent study of the landscape of acquired BRAFi resistance mechanisms (9) strongly support upregulation of the PI3K-AKT pathway as a critical event during the early and late evolution of resistance to MAPK pathway inhibition in patients.

Early and late resistance could potentially be mechanistically linked. In other words, the effects of BRAF inhibition on melanoma signaling may influence and shape the evolutionary selective pressure on the residual tumor cells. We propose that, just as potent mutant BRAF inhibition provides strong selective pressure for MAPK reactivation, a potent BRAFi-induced AKT upregulation would attenuate selective pressure for gain-of-function lesions in the PI3K-AKT pathway. Based on our data, we surmise that BRAFi-induced AKT upregulation during the first month of treatment may be widespread as an adaptive response. In those tumors in which this adaptive response were attenuated (e.g., by wild type PTEN activity), genetic alterations up-regulating the PI3K-PTEN-AKT pathway would distinctly confer growth and/or survival advantage. In fact, in the setting of acquired or late BRAFi resistance, our data support the notion that BRAF inhibition can select for amplification of the early adaptive response driven by stable, genetic alterations, leading to dramatically enhanced survival dependency on the PI3K-PTEN-AKT pathway.

In this context, recent studies (22–24) and our own (unpublished data) place partial or complete genetic inactivation of PTEN at 10–30% of *BRAF* mutant melanoma tumors or short-term cultures. It is not yet clear how frequently loss of PTEN function occurs as a result of epigenetic (DNA methylation, small non-coding RNAs, etc.), transcriptional, and post-translational modification mechanisms in *BRAF* mutant melanomas. Presumably, the sub-group of *BRAF* mutant melanomas with wild type and functional PTEN expression (i.e.,

the low p-AKT cohort) would mount the weakest AKT-dependent adaptive response when treated with a MAPK pathway inhibitor. Interestingly, recent clinical data suggest that *BRAF* mutant, p-AKT low melanomas are more likely to regress in response to treatment with the MEKi selumetinib (AZD6244) than *BRAF* mutant, p-AKT high melanomas (25). The relative lack of responses of the *BRAF* mutant, p-AKT high melanomas may be explained by redundant survival pathways and/or a robust, AKT-dependent adaptive response. Another prediction from the current study is that the sub-group of *BRAF* mutant, wild type PTEN-expressing melanomas, when treated with a MAPK-targeted therapy, would be susceptible to the development of acquired resistance driven by genetic amplifiers of the PI3K-PTEN-AKT pathway.

The AKT-dependent adaptive melanoma response is likely an early event in a set or series of coordinated, stereotypic reprogramming of growth and survival signaling. Our data indicate that this AKT up-regulation is likely accompanied by a certain degree of MEK-ERK reactivation driven by CRAF, which is relatively much weaker compared to that driven by, for instance, *NRAS* mutations detected later during late or acquired resistance. This weaker form of MAPK reactivation occurring during the adaptive phase likely also constrains the initial efficacy of BRAF inhibitors and results from BRAFi-induced loss of ERK-dependent negative feedback which normally suppresses ligand/RTK-driven RAS/MAPK signaling via BRAFi-insensitive RAF dimers (11). Data presented here and our unpublished work show that certain RTKs (e.g., PDGFRβ, EGFR) are over-expressed and ligand-stimulated in a specific temporal order along the evolutionary temporal contiuum of adaptive resistance, spanning from an early period of maximal cell death induction, to an (overlapping) phase characterized by a surviving but slow-cycling subpopulation of drug-tolerant persisters (DTPs), and then another transition marked by renewed proliferative clonal escape (i.e., drug-tolerant proliferating persisters or DTPPs). These transitions could be marked by significant cellular morphologic and gene expression alterations (5). Subsequent to the DTPP stage, further clonal outgrowth can occur due to enhanced growth and proliferative fitness driven by specific genetic variants (e.g., *NRAS* mutations, mutant *BRAF* amplification, *AKT1/3* mutations) (9). Thus, late acquired resistance mechanisms not only mirror but also augment adaptive resistance mechanisms.

Melanoma therapeutics has entered the era of combinatorial approaches. It has become evident recently that about 20% *BRAF* mutant metastatic melanomas harbor readily screen-able genetic alterations which upregulate the PI3K-PTEN-AKT pathway. Our studies show that melanomas can adaptively up-regulate the PI3K-PTEN-AKT pathway early during MAPK-targeted therapy to compensate for MAPK pathway inhibition and, with further evolutionary selection, acquire genetic lesions to further enhance PI3K-AKT signaling for growth and survival. Thus, upfront, combinatorial targeting of both the PI3K-PTEN-AKT and MAPK pathway would be expected to curtail innate (lack of initial responses) (26), adaptive (limited initial responses), and acquired (cessation of responses) resistance to MAPK-targeted therapies.

Methods

Cell culture, infections and drug treatments

Cells were maintained in DMEM with 10% fetal bovine serum and glutamine. The M cell lines were established at UCLA with Institutional Review Board approval and routinely authenticated by mitochondrial DNA sequencing. A375, SK and WM cell lines were obtained from MSKCC and the Wistar Institute via Material Transfer Agreements and were not further authenticated except for verification of the *BRAF* mutant status. WT PHD, E17K PHD, and Q79K PHD of AKT1 fused to GFP as well as FLAG-tagged full-length AKT1, AKT1 E17K and AKT1 Q79K were sub-cloned into the doxycycline-repressible lentiviral

vector pLVX-Tight-Puro (Clontech, Inc.); viral supernatants generated by co-transfection with three packaging plasmids into HEK293T cells; and infections carried out with protamine sulfate. Stocks and concentrations of small molecule kinase inhibitors were made in DMSO. Cells were quantified using CellTiter-GLO Luminescence (Promega) or crystal violet staining followed by NIH Image J quantification.

Drug sensitivity, protein and PIP_3 detection

Cell proliferation experiments were performed in a 96 well format (five replicates) and drug treatments initiated at 24 h post-seeding for 72 h. Stocks and dilutions of PLX4032 (Plexxikon, Berkeley, CA), LY294002, MK2206, AZD6244, BKM120, GSK1120212, GSK2141795, GSK2118436 (Selleck Chemicals) were made in DMSO. In these short-term assays, surviving cells were quantified using CellTiter-GLO Luminescence (Promega) following the manufacturer's recommendations. Clonogenic assays were performed by plating cells at single cell density in 6-well plates and provided fresh media, doxycycline (if applicable) and vemurafinib (vs. DMSO) either every day or every other day. In these long-term assays, surviving clonogenic colonies were fixed by 4% paraformaldehyde and stained with 0.05% crystal violet. Cell lysates for Western blotting were made in RIPA buffer (Sigma) with protease (Roche) and phosphatase (Santa Cruz Biotechnology) inhibitor cocktails. Western blots were probed with antibodies against p-AKT (Ser473), p-AKT (Thr308), AKT, p-GSK3β (Ser9), GSK3β, p-ERK1/2 (T202/Y204), ERK1/2, PTEN, c-MYC, PDGFRβ, p-CRAF (Ser338), CRAF, EGFR, GFP, (Cell Signaling Technology) and FLAG, TUUBLIN (Sigma). Samples in the same figure sub-panels were run, transferred, blotted, and developed (with the same exposure time) together.

In immunohistochemistry (IHC) experiments, after deparaffinization and rehydration, all sections from tissue series with extensive microscopic or macroscopic melanin deposits in any tumor were first subjected to a bleaching step using 3% H_2O_2 for 2 hours at 55°C. Tissue sections were then antigen-retrieved with a vegetable steamer at 95° C for 30 min, and immunostaining with anti-p-AKT (Ser473) or anti-PDGFRβ (Cell Signaling Technology) was then performed followed by a standard streptavidin-biotin complex technique with horseradish peroxidase (HRP) and DAB chromogen (Vector labs). After mounting, stained slides were scanned in their entirety with Scan Scope CS section scanner (Aperio) at 40× magnification, and the images analysed by Tissue Studio 2.0 (Definiens). PIP_3 measurement was performed by a competitive PIP_3 Mass ELISA (Echelon K-2500). Briefly, the acidic lips of each sample were extracted by the TCA/chloroform/methanol method, and samples were normalized based on protein concentration. Cellular $PI(3,4,5)P_3$ quantities were calculated by comparing the values from the wells containing $PI(3,4,5)P_3$ extraction products to the values in the standard curve.

Fluorescent microscopy

Melanoma cell lines expressing GFP-AKT PHD or PHD mutants were cultured in 6-well plate over cover slides. Serum starved cells were treated with serum or a small molecule inhibitor for indicated durations. Cells were fixed with 4% paraformaldehyde and mounted by VECTASHIELD™ Mounting Media (Vector Lab). GFP signal was photographed with a Zeiss microscope (AXIO Imager A1) mounted with a CCD camera (Retiga EXi QImaging), and the images captured by Image-pro plus 6.0.

Apoptosis analysis

Cultures of indicated melanoma cell lines were treated with PLX4032 (at different time points) for increasing durations of time, fixed (at the same time point), permeablized, and treated with RNase (QIAGEN). Cells were then stained with Annexin V-V450 and anti-PDGFRβ FITC (BD Pharmingen) for 15 min at room temperature, and mixed with 7-AAD

before sample loading (LSR II Flow Cytometry, BD Bioscience). Flow cytometry data were analyzed by the FACS Express V2 software.

Data quantification

Clonogenic assays were stained with 0.05% crystal violet and photographed, and colonies were quantified by NIH Image J (U.S. National Institutes of Health, Bethesda, MD, USA). Stained immunohistochemical slides were scanned with Scan Scope CS section scanner (Aperio) with 40× magnification, and analysed by Tissue Studio 2.0 image analysis software (Definiens). Briefly, tumor-rich regions were selected as region of interest (ROI), and the software was trained to recognize tumor cells by hematoxylin and DAB intensity, nuclear size, and nuclear morphology. Cell morphology was simulated per parameter 5.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

This study provides a mechanistic link between early, adaptive and late, acquired BRAF inhibitor resistance in melanoma, with early BRAFi-induced signaling alterations shaping the subsequent evolutionary selective pressure. These findings argue for upfront, combined targeting of the mutant *BRAF* genotype and a pervasive drug-adaptive, AKT-dependent tumor response.

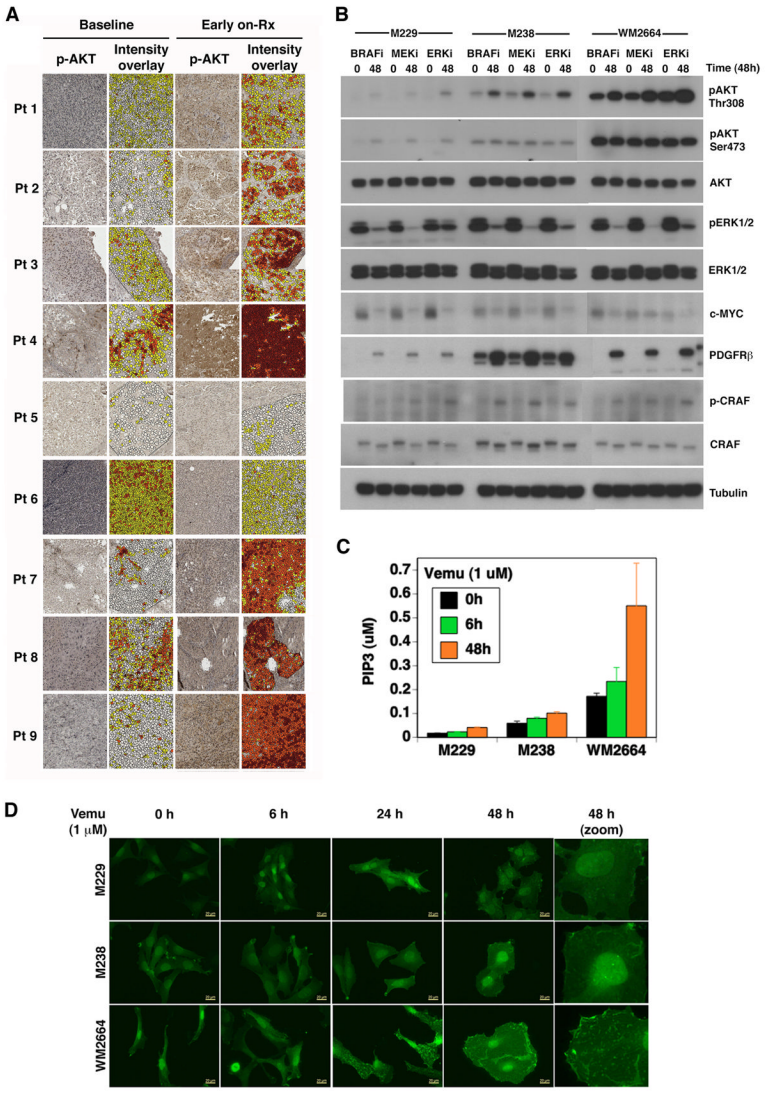

Figure 1.

MAPK pathway inhibition leads to rebound upregulation of AKT signaling in melanoma tumors and cell lines. **A,** BRAFi (vemurafenib or dabrafenib) or BRAFi+MEKi treatment led to increased p-AKT Ser473 levels in early on-treatment tumor biopsies (days #4 to 25) relative to the patient-matched, baseline (pre-treatment) biopsies. p-AKT IHC of melanin-bleached tumor sections (400X; quantification shown as heat intensity). **B,** *BRAF* mutant melanoma cell lines were treated with the indicated MAPK inhibitor (1 μM) or DMSO for 48 h. Phospho- and total protein levels were then probed by Western blotting. Tubulin, loading control. **C,** Lipids were extracted from the indicated cell lines (n = 3 per group) treated with vemurafenib (1 μM) for increasing durations (h), and the levels of PIP_3 levels were detected by ELISA (average of biologic triplicates; error bar, standard deviation). **D,** Localization of WT-PHD-GFP cells treated with vemurafenib (1 μM) for increasing durations (h) (scale bar, 20 micron). Note the cellular morphologic response to vemurafenib treatment in the surviving sub-populations. Photomicrographs representative of two independent experiments.

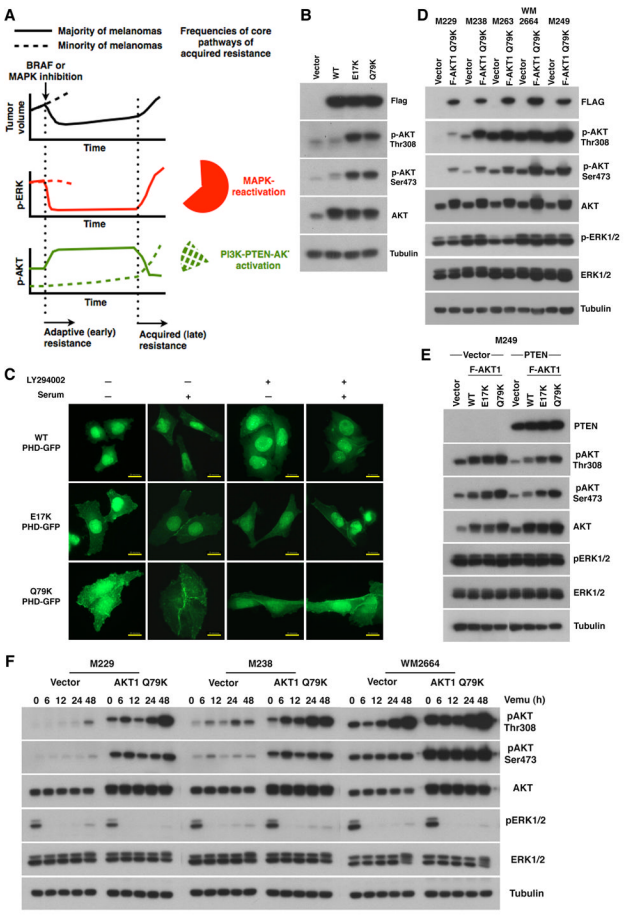

Figure 2.

AKT1 Q79K mutant amplifies BRAFi-induced PI3K-AKT signaling in the presence of PTEN. **A,** A hypothetical mechanistic link between early, adaptive and late, acquired resistance to BRAF or MAPK pathway inhibition in *BRAF* mutant melanomas. Model depicting temporal response patterns of tumor volume and pathway status (p-ERK and p-AKT) to BRAF or MAPK targeting as distinct subsets (majority vs. minority). The frequencies of ERK and AKT activation status early on treatment is reversely correlated with (and hence may influence) the relative frequencies of acquired resistance mechanisms in the two core pathways (shown as pie charts). **B,** AKT1 E17K and Q79K displayed up-regulation of activation-associated phosphorylation. Indicated constructs were transfected into human HEK293T, and levels of phospho- and total proteins were probed by Western blotting. Tubulin, loading control. **C,** AKT1 PHD containing the E17K or Q79K mutations localizes to the cell surface independently of serum stimulation (10% FBS, 1h), and serum-induced cell-surface localization of mutant PHD (vs. WT PHD) is less sensitive to a PI3K inhibitor (20 μM LY294002, 1h). PHD of AKT1 was fused to GFP, expressed stably in M229 and visualized (scale bar, 20 micron). **D,** Melanoma cell lines stably expressing vector or doxycycline-repressible FLAG-tagged AKT Q79K (doxycycline withdrawal, 48 h) were probed for endogenous and exogenous p-AKT and indicated total protein levels. **E,** Stable expression of PTEN WT and FLAG-AKT1 WT and mutants (vs. vector) in M249 revealed stronger PIP_3 signal-amplifying effects of AKT1 PHD mutants with PTEN WT re-expression. **F,** Indicated stable melanoma cell lines were treated with vemurafenib (1 μM) for increasing durations (h) without change of media. Protein lysates were probed for levels

of endogenous p-AKT (vector) vs. exogenous p-AKT (AKT1 Q79K) and indicated phospho- and total protein levels.

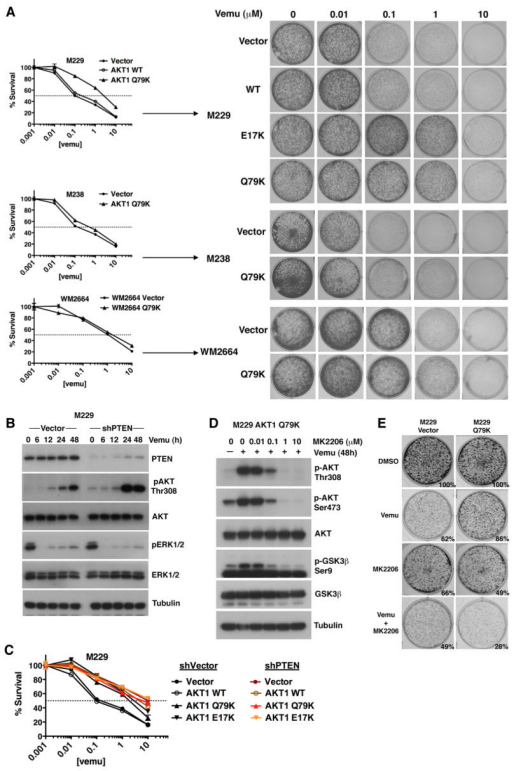

Figure 3.

AKT PHD mutants confer vemurafenib resistance in a PTEN context-dependent manner. **A,** Indicated stable melanoma cell lines were withdrawn from doxycycline (48 h), treated with indicated concentrations of vemurafenib (vemu) for 72 h, and survival (relative to DMSO-treated controls; mean ± SEM, n = 5) measured by the MTT (left; dashed line, 50% inhibition) or clonogenic assays (right). Media and vemurafenib at indicated concentrations were replenished every two days for ten days, starting two days after doxycycline withdrawal. **B,** The levels of vemurafenib-indued p-AKT in M229 without or with PTEN knockdown as shown by Western blotting. **C,** The effects of AKT1 PHD mutant expression on vemurafenib sensitivity (MTT) in the contexts of PTEN WT expression or its knockdown. **D,** M229 stably expressing AKT1 Q79K was treated with DMSO or vemurafenib (1 μM, 48 h) without and with increasing concentrations of the AKT1/2/3 inhibitor MK2206 (1 h), and the lysates probed for the indicated phospho- and total protein levels. **E,** M229 stably expressing vector or AKT1 Q79K was withdrawn from doxycycline (48 h), seeded, treated with at vemurafenib (1 μM) and/or MK2206 (5 μM) every two days (four treatments, 9 d drug exposure), fixed/stained, and quantified (% survival relative to DMSO).

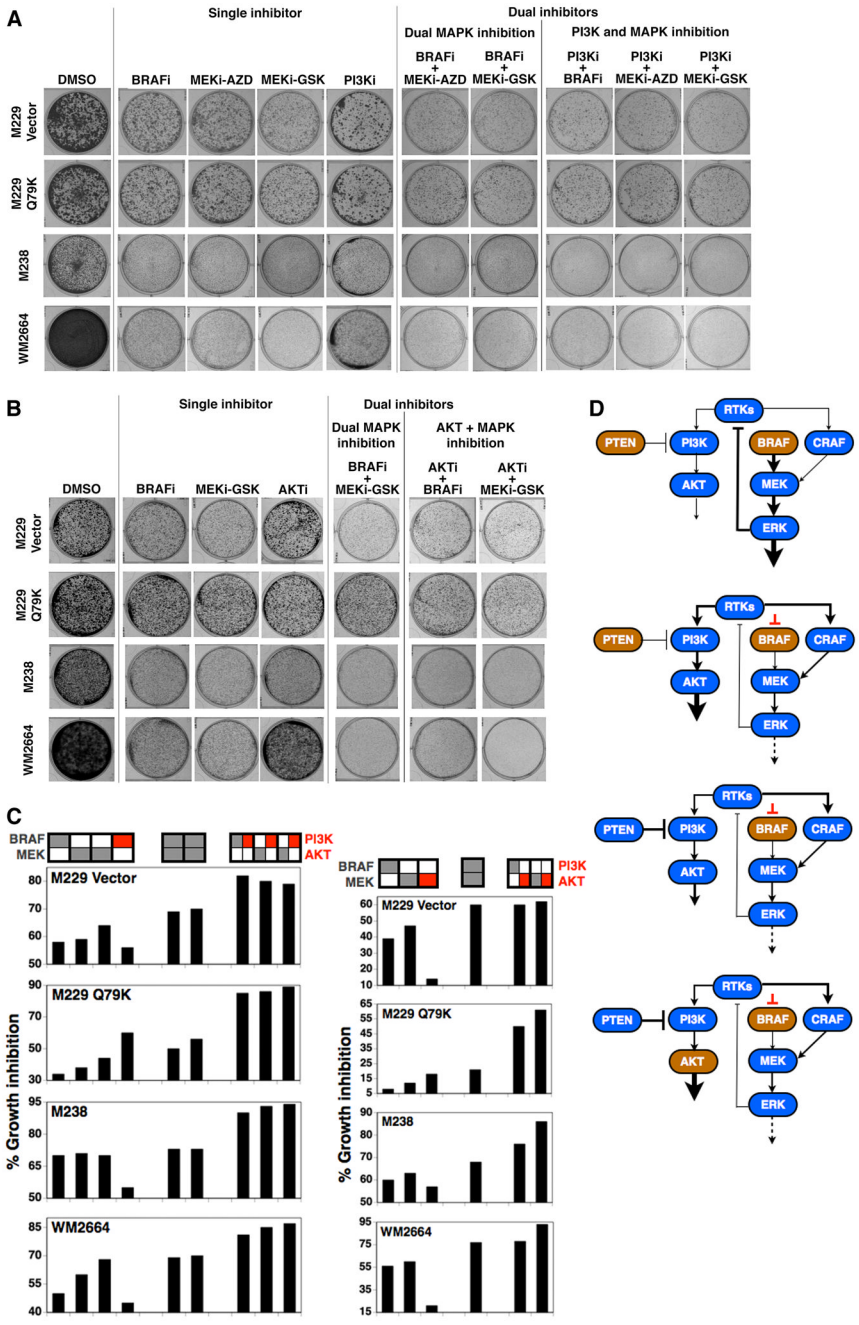

Figure 4.

Clonogenic suppression of melanomas via co-targeting of the PI3K-AKT and MAPK pathways regardless of PTEN status. **A,** M229 vector or M229 AKT1 Q79K stable cell lines (2 days after doxycycline washout), M238, and WM2664 were treated with DMSO or indicated inhibitor(s) every day. BRAFi (vemurafenib), MEKi-AZD6244, and PI3Ki were used at 1 μM (except for WM2664, 0.4 μM). MEKi-GSK was used at 0.01 μM (except for WM2664, 0.004 μM). All cultures were fixed and stained with crysal violet (results shown representative of two additional experiments). MAPK inhibition (BRAFi, vemurafenib; MEKi, AZD6244 or GSK1120212) indicated in grey; PI3K inhibition (PI3Ki, BKM120) indicated in red. **B,** BRAFi (vemurafenib), MEKi-GSK (GSK1120212), and AKTi

(MK2206) were used at 1, 0.01 and 5 μM for M229 vector and M229 AKT1 Q79K and at 0.5, 0.005 and 2.5 μM for M238, and WM2664. **C,** Quantification of clonogenic growths in A and B expressed as percentage growth inhibition (relative to DMSO). MAPK inhibition (at BRAF or MEK) indicated in grey; PI3K or AKT inhibition in red. **D,** Model showing that BRAFi treatment of *BRAF* mutant melanoma can lead to a context-dependent, adaptive RTK-PI3K-AKT up-regulation. BRAFi (or MAPKi) treatment de-represses RTK upregulation, resulting in activation of PI3K/AKT and CRAF. With WT PTEN activity, this BRAFi-induced, rebound AKT up-regulation is weak. In this context, AKT activating mutants can counteract the effect of WT PTEN and amplify PIP_3 signaling. Brown, mutated; blue, wild type.