Title: Deconvoluting mechanisms of acquired resistance to RAF inhibitors in BRAF V600E mutant human glioma

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Statement of Translational Relevance

Targeted therapy is increasingly used in the clinical setting. In the treatment of gliomas, the focus has been on the use of RAF pathway inhibitors, but relatively little is known about mechanisms of resistance to these drugs in glioma. Identifying and characterizing mechanisms of resistance to RAFl/ MEKi is a critical step to the effective use of molecularly targeted therapies in patients with gliomas. With this cohort of paired glioma specimens, we have identified a range of putative genomic and adaptive mechanisms of resistance. More importantly, it is possible to use this information to identify a different combination of targeted therapy that could have clinical efficacy at the time of progression. These observations underscore the importance of comprehensive genomic analysis of gliomas as well as prioritizing tissue collection following progression on targeted therapy, despite the relative risks of craniotomy, as a means to identify targetable mechanisms of resistance.
Abstract

Purpose:

Selective RAF-targeted therapy is effective in some patients with BRAF V600E mutated glioma, though emergent and adaptive resistance occur through ill-defined mechanisms.

Experimental Design:

Paired pre-/ post- RAF inhibitor (RAFi)-treated glioma samples (N=15) were obtained and queried for treatment-emergent genomic alterations using DNA and RNA sequencing. Functional validation of putative resistance mechanisms was performed using established and patient-derived BRAF V600E-mutant glioma cell lines.

Results:

Analysis of 15 tissue sample pairs identified thirteen alterations conferring putative resistance were identified among nine paired samples (including mutations involving ERRFI1, BAP1, ANKHD1, and MAP2K1). We performed functional validation of mechanisms of resistance, including loss of NF1, PTEN, or CBL, in BRAF V600E mutant glioma lines, and demonstrate they are capable of conferring resistance in vitro. Knockdown of CBL resulted in increased EGFR expression and phosphorylation, a possible mechanism for maintaining ERK signaling within the cell. Combination therapy with a MEKi or EGFR inhibitor was able to overcome resistance to BRAFi, in NF1 knockdown and CBL knockdown, respectively. Restoration of wild-type PTEN in B76 cells (PTEN-/-) restored sensitivity to BRAFi. We identified and validated CRAF upregulation as a mechanism of resistance in one resistant sample. RNAseq analysis identified two emergent expression patterns in resistant samples, consistent with expression patterns of known glioma subtypes.

Conclusions:

Resistance mechanisms to BRAFi in glioma are varied and may be predict effective precision combinations of targeted therapy, highlighting the importance of a personalized approach.
Introduction

Extracellular signal-regulated kinase (ERK) signaling pathway alterations are common in pediatric low- and high-grade gliomas (pLGG and pHGG), and also occur in adult LGG and HGG (1,2). The most common oncogenic BRAF mutation, p.V600E, renders the BRAF kinase constitutively active and able to signal in its monomeric form, uncoupled from upstream regulation (3,4). Initially identified in melanoma, colon and lung cancers, among others, BRAF\textsuperscript{V600E} represents a targetable mutation and selective mutant RAF-inhibitors (RAFi) have successfully demonstrated activity in some cancers (5,6). Treatment-emergent resistance has been well-characterized in melanoma, and its appearance over time prompted the combination of RAFi with MEK inhibitors (MEKi), leading to improved durability of responses (7,8). Given the dismal prognosis of adult and childhood HGG, establishing the role of RAF targeted therapy in this patient population is critical (9). Early clinical trial results and individual reports demonstrate efficacy for RAFi and / or MEKi in some patients with gliomas, particularly in LGG (10-12). Many patients’ tumors, however, do not respond or respond only transiently.

Mechanisms of resistance to RAFi are well-described, but vary considerably between and within cancer types. Many mechanisms involve alterations or expression changes that maintain ERK signaling such as aberrantly-spliced BRAF (13), mutations in RAS (14), loss of NF1 (15), and CRAF switching (16). Other cancers such as colorectal and thyroid cancers, rely on increased HER kinase activation through loss of negative feedback for resistance (17,18). It is unclear whether gliomas develop treatment-emergent resistance via the same mechanisms described in other cancers, or whether resistance is acquired in unique, lineage-specific patterns (19). Identifying and characterizing mechanisms of resistance to RAFi/ MEKi in gliomas is a critical step to the effective use of molecularly targeted therapies in patients with gliomas. Work by our group and others has identified some mechanisms of resistance in glioma that are shared with other cancers, such as activation of receptor tyrosine kinases (RTK) or autophagy (20-22). We have also identified a unique mechanism of resistance to RAFi in glioma, an \textit{in-cis} mutation that promotes BRAF dimerization as a means to evade inhibition (19). A major barrier to studying treatment emergent resistance in gliomas is the scarcity of paired pre- and post-treatment specimens, as post-treatment biopsies are rarely clinically indicated. Through a multi-institutional collaboration, we have gathered a cohort of fifteen paired samples from patients with LGG and HGG, before and after treatment with RAFi or RAFi/ MEKi. Using genomic and expression data, we identify and validate putative mechanisms of resistance from many of these samples in BRAF\textsuperscript{V600E} glioma models.

Methods / Materials

Study approval
Primary patient samples were obtained from Children’s Hospital Colorado (COMIRB 95-500) in accordance with local and federal human research protection guidelines and institutional review board (IRB) regulations. Patient specimens were also obtained under clinical trial NCT03593993 in accordance with IRB regulation at the Johns
Hopkins Hospital (IRB00158788). Non-clinical genetic analysis on UCSF specimens was performed under the UCSF Brain Tumor Research Center Tissue Bank Study and approved by the UCSF IRB (IRB: 10-01318). All tumor specimens were given a sequential unique identifier to ensure complete deidentification.

**Cell culture and cell line creation**

Cells were maintained in a humidified incubator at 37°C and 5% CO₂. The B76 cell line was generated from a cultured tumor sample obtained during surgery, and grown in OptiMEM Reduced Serum media supplemented with 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) (21). The DBTRG-5MG cell line (RRID:CVCL_1169) was obtained from the American Type Culture Collection (ATCC) and grown in RPMI-1640 supplemented with 10% FBS and 1% P/S. The AM38 cell line (RRID:CVCL_1070) was purchased from the Japan Health Sciences Foundation Health Science Research Resources Bank (Osaka, Japan), and grown in MEM supplemented with 20% FBS and 1% P/S. MAF-794 and MAF-905_3 cell lines were generated from tumor surgically removed from patients at Children’s Hospital Colorado, and grown in OptiMEM Reduced Serum media supplemented with 15% FBS and 1% P/S (21). The BT40 cell line was obtained from the Brain Tumor Research Center (BTRC) Tissue Bank at the University of California, San Francisco (UCSF) and grown in DMEM supplemented with 10% FBS and 1% P/S. Vemurafenib-resistant MAF-749 and AM38 cell lines were previously established and characterized (22).

pLentiCRISPR-E was a gift from the laboratory of Phillip Abbosh (Addgene #78852, RRID:Addgene_78852). CRISPR constructs against three distinct regions of the NF1 gene were obtained from ABM (pLenti-U6-NF1sgRNA-SFFV-CAS9-2A-Pur). Lentivirus was produced in 293T cells and DBTRG-5MG cells were infected with the filtered supernatant. Cell lines were stably selected in puromycin prior to use. pBABE-PTEN-puro was a gift from the laboratory of William Sellers (Addgene plasmid #10785, RRID:Addgene_10785). TTIGFP-MLUEX plasmids encoding MEK1 and MEK1 p.E203K were a gift from the laboratory of Neal Rosen. Cells were first infected with pMSCF-rTa3-PGK-Hygro and selected in hygromycin. Stable cultures were then infected with TTIGFP construct and selected in puromycin. GFP was induced by exposure to doxycycline for 24 hours and cells were GFP-sorted to create a stable, doubly-infected population, as previously described (23). Cultures were maintained in media containing TET-free FBS. shRNA targeting CRAF1 (TRCN0000001068, GAGACATGAAATCCAACAATA) was purchased from the Functional Genomics Facility (Denver, Colorado) which is supported by the Cancer Center Support Grant (P30CA046934). For CBL knockdown, Silencer Select Cbl-siRNA (sense: CCAUCUGGUAGAUCCGUUt, antisense: AACGGAUCUACCACGAUGGgt, cat#4390824, assay ID s2478) and Silencer Select negative control No.1 siRNA (cat# 4390843) purchased from ThermoFisher Scientific were transfected in AM38 or BT40 cells using Lipofectamine RNAiMAX reagent (cat# 13778030) according to manufacturer’s protocol. Twenty-five pmol of siRNA were used to transfect cells in a 6-well plate.
Cell lines were authenticated by short tandem repeat (STR) profiling upon receipt and every 6-12 months subsequently. Cell lines were used for up to 60 months or 30 passages (whichever was less) after thawing from frozen stocks. All lines were routinely verified free of mycoplasma infection (at receipt and every 6-12 months) by the MycoAlert Detection Kit (Lonza) or the MycoHunter Mycoplasma Detection kit (GenHunter).

**Antibodies and reagents**
Antibodies against EGFR (#2232, RRID:AB_331707), AXL (#8661, RRID:AB_11217435), phospho-ERK (#9101, RRID:AB_331646), ERK (#9102, RRID:AB_10694066), β-actin (#4967, RRID:AB_330288), GAPDH (#2118, RRID:AB_561053), and vinculin, were obtained from Cell Signaling Technologies. Anti-CBL (#05-440, RRID:AB_2290887) was obtained from MilliporeSigma. Anti-NF1 (A300-140A; RRID:AB_2149790) was obtained from Bethyl Antibodies. Vemurafenib, cobimetinib, neratinib, dabrafenib, trametinib, ZM336372, LY3009120, and belvarafenib were obtained from SelleckChem. Drugs for *in vitro* studies were dissolved in DMSO to create 10 or 100mM stock solutions and stored at -20°C.

**Immunoblot**
Protein samples were collected in stringent RIPA buffer, 1% NP-40 lysis buffer, or 1% NETN lysis buffer and resolved by SDS-PAGE gels. Visualization was accomplished using a Cell Signaling secondary HRP-conjugated anti-rabbit (#7074, RRID:AB_2099233) or anti-mouse (#7076, RRID:AB_330924) and G:Box imager (Syngene) or LI-COR Odyssey Fc Imaging system. Human phospho-RTK array was performed according to the manufacturer’s protocol (Proteome Profiler Human phospho-RTK array kit, #ARY001B, R&D systems) with 200 μg of cell lysate as previously described (19).

**Cell Proliferation Assays**
Cells were seeded in 96-well plates at a density of 1,500-2,000 cells per well and treated with the appropriate drugs. Cell growth was quantified using Cell Counting Kit-8 (CCK-8, Dojindo) or WST1 reagent (Roche). Alternatively, cells were plated in 96-well plates and placed in an IncuCyte Zoom (Sartorius). Phase images were collected every 4 hours for 96 hours. In some experiments using the Zoom plate reader, cells were incubated with 62.5 nM Cytotox Green reagent (Sartorius) over 96 hours to quantify cell death.

**Clonogenic Assays**
Cells were seeded at 1000 cells/well in 12-well plates, and treated with the appropriate drugs for 14 days, drug being washed and re-administered every 3-4 days. After 14 days, cells were fixed and stained in 3:1 2% crystal violet:methanol for imaging. Cell growth was quantified by dissolution of crystal violet in 33% glacial acetic acid, and reading the absorbance of the solution at 590nm.

**Nucleic acid purification**
RNA was isolated using the Quick-RNA Miniprep Plus kit (Zymo Research) for fresh or frozen tissue specimens and the PinPoint Slide RNA Isolation System II kit (Zymo Research) for FFPE-preserved specimens. DNA was isolated from frozen tissue using the DNeasy Blood and Tissue Kit (Qiagen).

**RNA sequencing analysis**

RNA was extracted from paired specimens as described above. mRNA quality was assessed for library preparation using DNA Analysis ScreenTape (Agilent Technologies). Libraries for cDNA were generated using either TrueSeq (for frozen samples) or TrueSeq RNA Access (for FFPE-preserved samples) library kits (Illumina). Libraries were sequenced using either the Illumina HiSeq4000 platform, or NovaSEQ 6000 platform, both with paired-end reads (2 × 150). Forty million reads/sample were collected on average. Resulting sequences were filtered and trimmed, removing low-quality bases (Phred score <15), and analyzed using a custom computational pipeline consisting of open-source trimmomatic (RRID:SCR_011848), gSNAP (RRID:SCR_005483), and Cufflinks (RRID:SCR_014597). R was used for differential gene expression alignment and discovery. Data was then analyzed using either GSEA 3.0 (The Broad Institute, RRID:SCR_005724) or IPA (Qiagen, RRID:SCR_008653).

**DNA sequencing analysis**

See supplemental methods for analysis details. In brief DNA from pre- and post-treatment specimens was extracted from formalin-fixed, paraffin embedded tissue using QIAamp DNA FFPE Tissue Kit (Qiagen). For some samples, capture-based next-generation sequencing was performed using an assay that targets all coding exons of 479 cancer-related genes (UCSF500 Cancer Panel) (24). For other samples whole genome sequencing was performed. For all specimens, candidate mutations were compared against known published genomic alterations conferring resistance or likely pathogenic mutations (COSMIC Catalogue of Somatic Mutations in Cancer, RRID:SCR_002260).

**Statistical analysis**

All statistics are presented as the mean ± the standard error of the mean (SEM). Student’s t-test, Dunnett’s multiple comparisons test, and ANOVA were performed using GraphPad Prism software version 7 or 8 (RRID:SCR_002798), using data collected from at least three biological replicates per experiment as appropriate. Significance was determined at p≤0.05. Where used, *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

**Results**

**Patient characteristics**

Paired tissue samples were obtained from fifteen patients with BRAF<sup>V600E</sup> mutated LGG or HGG, before and after treatment with RAF-targeted therapy (Figure 1A). Median age at diagnosis was sixteen years (range 2-46), with one patient over age 30 (46 years) at diagnosis. 33% were female (5/15). Seven patients had a diagnosis of glioblastoma (47%), while four had anaplastic pleomorphic xanthoastrocytoma (PXA; 27%), three LGG (20%), and one anaplastic astrocytoma (7%). Eleven patients (73%) received RAF
inhibitor (RAFi) monotherapy, the remaining four (27%) received combined RAFi/MEKi. All but one patient responded to treatment with a complete response (N = 5), partial response (N = 4), or stable disease (N = 5; Figure 1B). Median duration of therapy was twelve months (range 2-40 months).

Clinical genomic sequencing of pre-treatment tissue revealed homozygous loss of CDKN2A/B in twelve patients (80%), which commonly co-occurs with BRAF alterations and is associated with a worse prognosis. Other accompanying alterations included TERT promoter mutation or gene amplification in three (20%), missense mutations in TP53 in two (13%), and a missense mutation in ATRX in one (Figure 1B). We assembled twelve of the most frequently mutated genes in glioblastoma and found no additional mutations in these genes in any of our samples (see Supplemental methods and Figure S1). Median overall survival was 44 months (range 5-104; Figure 1C). Median progression free survival in all patients was 11 months (range 2-40; Figure 1D).

In our small cohort, there was no difference in progression free survival between patients on RAFi monotherapy (12 months) as compared with those on combination RAFi/MEKi (13.5 months; 95% CI 0.27-2.95). This may be due in part to more patients with HGG receiving RAFi/MEKi.

**Novel post-treatment mutations provide rationale for tumor progression**

Next generation sequencing (NGS) was performed on pre- and post- RAFi-treated tissue to identify putative genomic mechanisms of resistance. Post-RAFi NGS was compared to pre-RAFi, and shared somatic variants were removed. Manual comparison of remaining alterations revealed putative etiologies for RAFi resistance in nine (60%) of the patient-derived paired tumor samples (Figure 2). All were present in sub-clonal variant allele frequencies (VAF). Alterations in each of these genes has been identified in association with BRAF alterations (Figure S2). These included alterations in genes that modulate RTK activity such as a missense alteration in CBL^{C384R}. Alterations in RAS/RAF signaling were also identified, with two resistant samples harboring NF1 missense mutations conferring loss of tumor suppressor function (NF1^{W1831*, NF1^{D6996*}}). One sample harbored an activating alteration in BRAF (L514V, in cis with V600E) which we previously reported, and confirmed that the second site mutation functions to enhance dimerization (19). In another sample we identified an activating alteration in MAP2K1 (E203K) that has been documented in melanoma (23). Emergent mutations in PTEN (R130G) and PIK3C2G (R722H) were identified as potential drivers of PI3K/mTOR signaling, thereby mediating resistance (25). No likely pathogenic driver alteration was identified in the remaining six paired samples (40%). Several of these candidate alterations were selected for in vitro validation, and the remainder are described below.

**PIK3C2G** encodes for the catalytic subunit type 2 gamma of phosphatidylinositol-4-phosphate 3-kinase (commonly known as PI3-kinase, or PI3K). As a member of the PI3K family of proteins, its role seems to be primarily related to a range of cell functions including proliferation and survival. While mutations in the PI3K family member PIK3CA (encoding the alpha catalytic subunit) are found commonly in cancer, mutations in genes for other subunits, including PIK3C2G, are not as well studied. Alterations in
PIK3C2G are often amplifications (26), and missense mutations are often considered to be of uncertain functional significance (27). The alteration identified in our post-treatment sample (patient #15), PIK3C2G<sup>R722H</sup> occurs in the PI3K accessory domain (PI3Ka) and has been documented as a somatic mutation in nasopharyngeal carcinoma, though its function is unknown (28).

Two sample pairs had emergent alterations affecting cell cycle signaling, BAP1 or ANKHD1. BAP1 is a deubiquitinase involved in chromatin modification that functions as a tumor suppressor gene and is frequently lost in uveal and cutaneous melanoma (29), among other cancers. Loss of BAP1 in combination with BRAF<sup>V600E</sup> is associated with metastatic uveal melanoma and inferior outcomes (29). Its loss is thought to be an early driver mutation in some melanoma, and germline loss of BAP1 is associated with melanomas containing BRAFV600E and bi-allelic BAP1 loss (30). In our cohort, BAP1 loss was only identified in one patient’s post-treatment sample. The role of BAP1 in resistance to RAFi, however, is unconfirmed at this time. A second paired sample demonstrated alterations in a gene associated with chromatin modification, loss of ARID1A via a focal deletion. ARID1A is a member of the SWI/SNF family, which is thought to regulate gene transcription by altering chromatin structure. ARID1A loss is common in several cancers, including serous ovarian and gastric carcinomas. Its loss is associated with activation of PI3K signaling, among other mechanisms, and has been implicated in resistance to HER2-inhibitors in breast cancer (31).

Two cell lines derived from patients with BRAF<sup>V600E</sup> mutant glioma were evaluated for treatment-emergent resistance as clinically relevant resistance models (AM38 and MAF-794). Patient-derived cultures were treated with low dose vemurafenib in cell culture until resistant clones emerged (approximately 12 weeks). NGS from sensitive and resistant clones was compared for emergent mutations. SNVs conferring functional alterations in ERRFI1<sup>S251*</sup> and TET2<sup>V1199E</sup> were observed in resistant clones as possible mechanisms of resistance in vitro (Figure 2). The ERBB receptor feedback inhibitor 1 (ERRFI1) modulates RTK signaling and is downregulated or mutated as a mechanism of resistance to RTK inhibitors (32). The alteration that emerged in our sample of RAFi-resistance AM38 cells, ERRFI1<sup>S251*</sup>, is also associated with loss of function and a putative resistance mechanism, supported by the finding that p-EGFR is upregulated in resistant cells compared to parental (Figure S3). Tet methylcytosine dioxygenase 2 (TET2) plays an important role in epigenetic modification of DNA by catalyzing the conversion of methylcytosine to 5-hydroxymethylcytosine. The mutation that emerged in resistant MAF-794 cells, TET2<sup>V1199E</sup>, is located within the catalytic domain, adjacent to a zinc-binding motif and likely disrupts proper protein folding (33). While this particular alteration has been documented in several solid tumors (34), TET2 loss of function is most common in myeloid cancers, where it is sufficient to promote oncogenesis (35).

**Loss of NF1 confers resistance to RAF-targeted therapy**

We undertook validation of selected putative mechanisms of resistance to RAFi or RAFi/ MEKi combination therapy. Loss of NF1 is commonly associated with resistance to RAFi (15). To validate that loss of NF1 can confer adaptive resistance in glioma, stable NF1 knockdown lines were created using two different CRISPR sgRNA
constructs. Decreased NF1 protein expression was verified on immunoblot in BRAF\(^{V600E}\) glioma cell lines NMCG1 and DBTRG (Figure 3A). NF1 knockdown decreased the sensitivity glioma cells to RAFi by shortening the duration of ERK inhibition in response to RAFi (Figure 3B). Additionally, downstream RAS effectors such as phospho-S6 were less sensitive to RAFi inhibition following NF1 knockdown (Figure 3C).

We evaluated the effect of NF1 knockdown on sensitivity to RAFi in vitro. Consistent with biochemical findings, we observed loss of NF1 expression conferred a survival advantage, with less growth inhibition in both NMCG1 and DBTRG in response to RAFi (dabrafenib; Figure 3D,E) and less apoptosis (Figure 3F,G). When the cells were treated with a combination of RAFi+MEKi, resistance was partially overcome, though not completely, suggesting a dose-dependent effect (Figure 3H). Interestingly, two paired samples demonstrated loss of NF1: one patient had received combination RAFi/MEKi prior to developing the mutation, while the other had received RAFi alone. The recurrent nature of this finding suggests that the small change in sensitivity to RAFi or RAFi/MEKi can be clinically significant.

**Loss of CBL confers resistance to RAF-targeted therapy**

CBL is an E3 ubiquitin-protein ligase that functions as a negative regulator of RTK such as EGFR and AXL (36). The SNV identified in our cohort, p.C384R, is located within the ring-finger domain and is associated with loss of CBL function and increased RTK activation (36,37). It has been identified previously as a somatic mutation in cancer, but its function in resistance to targeted therapy is unknown (27). We hypothesized that loss of CBL activity would lead to upregulation of upstream RTK (including AXL and EGFR) and emergent resistance to RAFi. We validated the effect of CBL loss on RAFi sensitivity in glioma using two glioma cell lines: AM38 and BT40. siRNA effectively knocked down CBL expression for 4-6 days following treatment (Figure 4A,B). We observed that both EGFR and AXL expression were upregulated on immunoblot following CBL knockdown. We performed a phospho-RTK array to identify other changes in protein phosphorylation and observed that EGFR phosphorylation increased significantly following CBL knockdown (Figure 4C).

Given the dependence of many HGG on EGFR-driven RTK activation, we hypothesized that CBL knockdown would alter sensitivity to RAFi treatment. Indeed, loss of CBL prevented growth inhibition by a RAFi (vemurafenib) over time (Figure 4D) and at a range of doses in both AM38 and BT40 (Figure 4E,F). To test the efficacy of combination therapy, we evaluated sensitivity to RAFi (vemurafenib), MEKi (cobimetinib), and EGFRi (neratinib), alone or in combination. In both glioma lines, loss of CBL conferred relative resistance to single drug and combination therapy with most combinations. Sensitivity to combination therapy with RAFi+MEKi or RAFi+EGFRi was relatively good despite CBL knockdown (Figure 4G,H). Of note, the patient in our cohort who developed this alteration was only treated with RAFi monotherapy. It is unknown whether they would have been sensitive to combination treatment following the development of resistance.

**PTEN loss as a mechanism of resistance**
**PTEN** is a tumor suppressor gene with many critical functions including its role as a protein tyrosine phosphatase (PTP) and negative regulator of PI3K/AKT/mTOR signaling (38). Its loss is quite common in human cancers and a known mechanism of intrinsic and acquired resistance to RAFi in melanoma (39), where it acts to augment PI3K/AKT/mTOR signaling and decrease apoptosis in response to RAFi (25). The missense alteration that occurred in patient #12 in our cohort (Figure 2) was within the phosphatase domain at p.R130G, a common alteration described previously in glioma (34).

Several BRAF\textsuperscript{V600E} mutant glioma cell lines are known to have PTEN loss, including B76. We evaluated the role of PTEN loss in B76, which also demonstrates reduced sensitivity to RAFi at baseline. We stably expressed PTEN in B76 cells using retroviral infection with puromycin selection (Figure 5A). Restoration of PTEN mildly increased the sensitivity of B76 to vemurafenib, shifting the IC50 to vemurafenib from 18.8 μM to 10.4 μM (p < 0.0001, 2-way ANOVA; Figure 5B). Of note, we have previously demonstrated that inhibition of autophagy can also overcome resistance to vemurafenib in B76 cells (21).

**CRAF activation is a mechanism of resistance to RAFi**

RNA sequencing was performed on 12 sample pairs, and using the Ingenuity Pathway Analysis (IPA) to interrogate our data, we observed strong predictions of alterations in cAMP signaling at resistance. In melanoma, this phenomenon is associated with a switch from BRAF to CRAF (RAFI) dependency (16,40). In the RAFi-resistant patient-derived cell line MAF-905_3, we hypothesized that intrinsic resistance to RAFi was due to enhanced activity of CRAF mediated by a transition from BRAF to CRAF-dominated signaling. Accordingly, we knocked down RAF1 using shRNA in MAF-905_3 cells and observed increased sensitivity to RAFi (p < 0.0001 by ANOVA; Figure 5C). MAF-905_3 cells with RAF1 knockdown also demonstrated inhibition of ERK phosphorylation in response to vemurafenib as compared with the control (Figure 5D). We next evaluated the effect of treatment with the BRAF\textsuperscript{V600E} inhibitor, vemurafenib, the pan-RAF inhibitor LY3009120, or the combination, on ERK activity. Both drugs inhibited ERK phosphorylation (Figure 5E).

We hypothesized that a pan-RAF inhibitor would be more effective at inhibiting glioma growth than the V600E-specific RAF inhibitors. MAF-905_3 cells were treated with increasing doses of vemurafenib or two different pan-RAF inhibitors, LY3009120 (41,42) or belvarafenib (43), which is currently in clinical trial testing (NCT02405065). Both pan-RAF inhibitors were superior to vemurafenib monotherapy (p < 0.001 by ANOVA; Figure 5F). Similar results were seen with a clonogenic assay, where vemurafenib monotherapy was unable to effectively inhibit cell growth, while LY3009120 was quite potent (p < 0.0001; Figure 5G).

We next evaluated the effect of inhibiting signaling through BRAF\textsuperscript{V600E} mutant monomers as well as through RAF-dimerization by combining vemurafenib with
LY3009120. We observed enhanced cell growth inhibition with the combination compared to either drug alone at a range of concentrations (Figure 5H). Similarly, we evaluated the effect of a different CRAF inhibitor, ZM336372 (44), alone or in combination with vemurafenib. We observed little effect of ZM336372 alone, but significant growth inhibition upon combination with vemurafenib (p < 0.05; Figure 5I). These data suggest that ERK signaling in BRAFV600E mutant glioma can occur through V600E-independent RAF dimerization upon development of resistance (19).

RNA expression changes as a mechanism of resistance
While we identified putative mechanisms of resistance in two-thirds of the patient samples and were able to validate several of these, a clear genomic mechanism was not identified in the remaining paired samples (N = 6). We hypothesized that resistance might be mediated on a gene expression level in these tumors. To test this hypothesis, we performed bulk RNA sequencing on nine paired patient samples and three patient-derived cell line RAFi-sensitive/resistant pairs. Patient samples and derived cell lines were clustered by predicted upstream regulators using IPA, sorting by those regulators that are predicted to be significantly activated or inactivated after treatment with RAFi to the level of p<10^-10 in one or more samples, and at least p<10^-3 in all others (Table S1). From this, unsupervised hierarchical clustering identified two groups characterized by either high or low TGFB1 activity (Figure 6A). We identified from this analysis a number of putative regulators known to be of clinical relevance in brain cancers (Figure 6B), which showed a modest correlation between cluster affiliation and activation/deactivation score (r^2=0.45). To determine whether the observed expression patterns were features of all recurrent glioblastoma, we added 4 BRAF wild type paired samples to the analysis and observed that they did not demonstrate increased TGFB1 expression. Moreover, BRAF-mutant samples demonstrated enrichment of gene sets related to ERK signaling pathways at recurrence, consistent with preservation of elevated ERK signaling (Figure S4).

We used GSEA to identify differential enrichment in gene sets between the TGFB1-high and TGFB1-low groups identified by IPA. Many gene sets associated with cancer progression and chemoresistance were differentially enriched between the groups (Figure 6C). Of note, upon comparing the post-treatment samples to each other using GSEA we also observed clear and significant enrichment of the mesenchymal (TGFB1-high) and proneural (TGFB1-low) genotypes (Figure 6D,E). Other transcription factors and epigenetic modulators were differentially enriched between TGFB high/low groups, including EGFR signaling, YAP1, and KRAS (Figure 6D,E; Table S2). These data suggest BRAF-mutant glioma escape targeted therapy along conserved pathways expressed in a subset of GBM.

We evaluated differential gene set enrichment upon emergent resistance in tumors with no identified genomic mutation (n = 4), those with RTK mutations (n = 3), those with cell cycle mutations (n = 2), and those with RAS-ERK pathway mutations (n = 2). We did not...
identify any clear pattern in expression change (Table S3), likely due to the very small sample size.

**Discussion**

The role of BRAF\textsuperscript{V600E} mutations in glioma pathogenesis and prognosis are well described in pediatric glioma, and increasingly studied in adult glioma (45,46). Multiple case reports and early phase clinical trials support RAF-targeted therapy in patients with BRAF\textsuperscript{V600E} alterations, though resistance is common (10,47). While RAF targeted therapy is increasingly used in the clinical setting, relatively little is known about mechanisms of resistance to RAFi in glioma (19,22). This knowledge gap is due to the relative rarity of BRAF-mutant glioma and the difficulty associated with obtaining clinical specimens at time of progression. Given the range of resistant mechanisms identified within and between cancer types, understanding resistance in glioma is critical to advance the use of targeted therapies. With this cohort of 15 paired glioma specimens, the largest dataset of its type to date, we have identified a range of putative genomic and adaptive mechanisms of resistance and performed functional validation using patient-derived glioma lines with BRAF\textsuperscript{V600E} mutations.

Our data indicate that resistance mechanisms among gliomas are various, but largely follow the patterns recognized in other cancers. We identified genomic alterations that were putative mechanisms of resistance in 60% of paired samples. These fell into several broad categories, with some affecting RTK activity, RAS/ERK signaling, PI3K/mTOR signaling, or cell cycle signaling. Most of the alterations we found have been previously observed as somatic mutations in cancer, though the pathogenicity of some had not been explored previously (34). Moreover, putative resistance alterations were present at variant allele frequencies consistent with a sub-clonal population within the tumor, suggesting intra-tumoral heterogeneity upon resistance. Notably, the effect of some of these alterations may be quantitatively subtle, but sufficient for clinical resistance. For example, the alteration in MAP2K1 (MEK1\textsuperscript{E203K}) identified in our sample pair occurs commonly in melanoma but likely only decreases sensitivity to MEKi by a factor of 10 and often co-occurs with BRAF or NRAS hotspot mutations (23). Given the poor blood brain barrier penetration of RAFi, this small change in sensitivity may be sufficient to confer a survival advantage in vivo.

Some of the resistance alterations we identified are common in BRAF wild type glioma. Specifically, loss of NF1 is present in 35% of pLGG and 10% of GBM, while PTEN loss occurs in 40% of GBM (48). Here, we demonstrated that loss of either of these tumor suppressors can confer relative resistance to targeted therapy, consistent with findings in other cancers (15,39). While these alterations were only detected following selective pressure from RAFi in our paired samples, co-occurrence of BRAF\textsuperscript{V600E} and NF1 or PTEN alterations occur in 0% or 12%, respectively, of adults with BRAF\textsuperscript{V600E}-mutant glioma (Schreck et al., manuscript in preparation). This co-mutation pattern may be one
reason HGG are overall less sensitive to RAF-targeted therapy (47) and should be considered in analysis of ongoing clinical trials.

A genetic mechanism of resistance was not identified in 40% of our patient samples, suggesting the potential for adaptive, rather than genomically acquired, mechanisms. RNAseq analysis on all available specimens revealed two primary patterns of adaptive resistance to RAFi, one driven by increased TGFB1 with an expression pattern most consistent with the mesenchymal subtype of glioblastoma previously classified. The other showed decreased TGFB1 and was most similar to the proneural glioblastoma subtype. These data may suggest that adaptive resistance emerges along conserved pathways already exploited in glioblastoma and other HGG. Another adaptive pattern of resistance identified using IPA was CRAF switching, which results in increased expression of CRAF as the dominant RAF isoform and permits ongoing ERK signaling despite selective pressure from a Class 1 RAFi (16,40). Targeting dimer disruption using either a pan-RAF inhibitor LY3009120 (41,42) or belvarafenib (43), or shRNA knockdown of RAF1 proved effective at disrupting adaptation in our model system, which suggests that combined therapy with a dimer-disrupter and MEKi may be efficacious in patients with BRAFV600E mutant glioma who have progressed on the currently FDA-approved RAFi. Clinical trials using the dimer disrupters DAY-101 (NCT03429803), BGB-283 (NCT03905148), and BGB-3245 (NCG04249843) are currently ongoing in patients with pediatric glioma and other cancers, either alone or in combination with MEKi, and interim result reporting suggest promising outcomes.

Although this is the largest cohort to date of paired pre-/post-RAFi glioma samples, the relatively small sample size remains a limitation given the rarity of paired tumor samples from patients with glioma. Additionally, we primarily evaluated genomic mechanisms of resistance. One additional means of resistance to targeted therapy in glioma is autophagy (21,22), but it is unclear whether autophagy contributed to adaptive resistance in our cohort. Despite these limitations, we have clearly demonstrated that a broad range of resistance mechanisms arise through both genomic and adaptive mechanisms. Moreover, in several of these instances, a different combination of targeted therapy could have had clinical efficacy at the time of progression. These observations underscore the importance of comprehensive genomic analysis of both LGG and HGG as well as prioritizing tissue collection following progression on targeted therapy, despite the relative risks of craniotomy, as a means to identify targetable mechanisms of resistance.

In sum, we have demonstrated that a number of putative and functionally validated genomic and adaptive mechanisms of resistance emerge in response to RAFi in glioma. We have also identified combination therapies able to overcome adaptive resistance. These results confirm the need for a more comprehensive approach to targeting critical conserved resistance pathways in individual cases.
References


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Figures and Figure Legend

**Figure 1**: Clinical and pathological characteristics of 15 patients with BRAF<sup>V600E</sup> mutant glioma at time of diagnosis. A) Relative prevalence of histopathologic diagnosis, sex, age, and best response in cohort. B) Clinical features and genomic alterations in pre-treatment tumor tissue from each patient. C) Progression free survival and D) overall survival in all patients. LGG = low grade glioma, aPXA = anaplastic pleomorphic xanthoastrocytoma, AA = anaplastic astrocytoma, GBM = glioblastoma, SD = stable disease, PR = partial response, CR = complete response, PD = progressive disease, SNV = single nucleotide variant, Ampl = amplification. * Samples with whole exome sequencing.

**Figure 2**: Putative resistance drivers in the resistant sample from 15 paired treatment samples and 2 patient-derived cell lines. Colored boxes indicate presence of an alteration. Numbers within colored boxes represent the variant allele frequency (VAF). fs = frameshift, del = deletion, XRT = radiation therapy.

**Figure 3**: Loss of NF1 confers resistance to RAF-targeted therapy. (A) Immunoblot of NF1 expression in DBTRG and NMCB1 parental lines (P), and stably-infected NF1 knockdown using two different sgRNAs (T1 or T3). (B) Immunoblot of DBTRG treated with 30nM dabrafenib and samples collected at a range of time points after treatment. (C) Immunoblot of DBTRG cells treated with a range of dabrafenib doses for 24 hours. (D) Growth of NMCB1 or (E) DBTRG parental or NF1 knockdown lines in response to increasing doses of dabrafenib at 72hrs. Data are representative of 4 experiments. (F) Cytotox Green expression as a marker of apoptosis upon treatment with a range of dabrafenib concentrations in NMCB1 at 72hrs (N = 3) and (G) DBTRG at 64 hours (2 independent experiments with triplicate at each time point). (H) Growth assay in DBTRG at 48 hours after treatment with RAFi (dabrafenib, 10 nM), MEKi (trametinib, 10 nM) or the combination. Dab = dabrafenib, Tram = trametinib.

**Figure 4**: Loss of CBL expression confers resistance to RAF-targeted therapy. Immunoblot confirms knockdown of CBL by siRNA in (A) AM38 and (B) BT40 cells as compared with control siRNA ‘C’ or untransfected cells ‘U’ at the specified number of days post-transfection. (C) Phospho-RTK array was performed three days after siRNA transfection, and phospho-EGFR protein levels were quantified. (D) Relative cell viability in response to 5 uM vemurafenib over time in AM38, or as measured after three days of vemurafenib treatment at a range of doses in (E) AM38 and (F) BT40. (G) AM38 and (H) BT40 were treated for 72 hours with RAFi (vemurafenib, 5 or 0.5 µM, respectively), MEKi (cobimetinib, 1 or 0.5 µM, respectively), EGFRi (neratinib 1 µM), or their combinations 24 hours after transfection with control or CBL siRNA. For all experiments, relative cell viability is quantified in relation to DMSO-treated control.

**Figure 5**: CRAF switching and PTEN loss are mechanisms of resistance to RAFi. (A) Immunoblot showing successful re-introduction of PTEN into B76 cells. (B) Cell viability normalized to DMSO controls after five days treatment with B76 cells transduced with functional PTEN (2-way ANOVA, p < 0.0001) or (C) with control plasmid or RAF1-
shRNA (2-way ANOVA, p < 0.0001). (D) Immunoblot of MAF-905_3 cells transfected with control or CRAF siRNA and treated with RAFi (2 µM vemurafenib) for 0-24 hrs. (E) Immunoblot of MAF-905_3 cells treated with BRAFV600E-inhibitor Vem (2 µM), pan-RAF inhibitor LY (5 µM), or the combination for 1 or 4 hours. (F) Cell viability normalized to DMSO controls in MAF-905_3 cells after 5 days of treatment with Vem, LY, or Bel at a range of concentrations (p < 0.0001). (G) Soft agar colony-forming assay with MAF-905_3 treated with Vem (2 µM) or LY (5 µM), and quantified measuring 590nM light absorbance after crystal violet treatment. (H) Normalized cell viability in MAF-905_3 cells after five days of treatment with Vem (2 µM), LY (concentration as noted), and the combination, (I) or with CRAF inhibitor ZM336372 (25 µM), Vem (10 µM), or the combination. Bel = belvarafenib, LY = LY3009120, Vem = vemurafenib.

**Figure 6:** RNA sequencing reveals transcriptional changes accompany the development of resistance. (A) Heat map showing the top 79 upstream regulators consistently significantly predicted by IPA to be significantly activated or deactivated in 12 paired patient samples and tumor-derived cell lines. (B) Mean z-scores of the upstream regulators identified by IPA plotted as TGFB1-high vs -low. (C) Grouped similar gene sets found by GSEA to be enriched pre/post treatment in the TGFB-high and TGFB-low phenotypes. (D-E) Highlighted gene sets identified by GSEA as enriched post-RAFi in either the TGFB1-high (D) or TGFB1-low (E) clusters.
Figure 1

A. Pie charts showing the distribution of diagnoses (GBM, LGG, aPXA, AA), sex (male, female), age at diagnosis (11-20, 21-30, ≤10, >30), and best response (CR, PR, PD, SD).

B. Heatmap depicting the patient ID, histopathological diagnosis, sex, age at diagnosis, exposure time, best response, and alterations in various genes (BRAF V600E, CDKN2A/B, TERT, TP53, ATRX, EGFR, ERBB2, IDH1, NF1, PDGFRA, PIK3R1, PIK3CA, PTEN, RB1).

C. Kaplan-Meier curve showing progression-free survival over months.

D. Kaplan-Meier curve showing overall survival over months.
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