EGFRvIII and *Pten* Deletion Mutations Influence Genotype-Dependent Kinome Activation in Immortalized Murine Astrocyte Models of Glioblastoma

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Abstract

Glioblastoma (GBM) is the most common malignant primary brain tumor. Receptor tyrosine kinase (RTK) pathways are frequently mutated in GBM, including alterations in the RTK Epidermal Growth Factor Receptor (EGFR). Moreover, EGFR variant III (EGFRvIII) is the most common activating mutation in GBM. Given its frequency, specificity, and role in promoting gliomagenesis, EGFR dysfunction is a prime target for drug treatment. However, multiple resistance mechanisms, such as co-occurrence of EGFRvIII and deletion of the tumor suppressor PTEN, prevent effective treatment via activation of alternate kinase pathways. To observe the differential kinase activation involved in EGFRvIII- and PTEN loss- driven gliomagenesis and identify potential kinase targets for dual therapy, we examined immortalized murine astrocytes derived from non-germline genetically engineered mouse models expressing four core genotypes: wild-type *EGFR* + wild-type *Pten* (C), wild-type *EGFR* + *Pten* deletion (CP), *EGFRvIII* + wild-type *Pten* (CEv3), and *EGFRvIII* + *Pten* deletion (CEv3P). We used RNA sequencing and multiplexed inhibitor bead affinity chromatography and mass spectrometry (MIB-MS) to determine the baseline transcriptomes and kinomes of each genotype. We determined that cell lines exhibited genotype-dependent baseline RNA expression and kinase activity as a result of EGFRvIII and/or *Pten* deletion relative to C astrocytes and across genotypes. We observed several differentially-activated kinases that represent potential targets for dual treatment with EGFR TKI. We are currently examining changes in the kinome profile of each cell line after both acute and chronic treatment with tyrosine kinase inhibitors (TKI). These results will help define the kinase networks involved in tumor development and resistance and may aid in the identification of potential personalized combination therapy with EGFR TKI for GBM patients.

Introduction

Glioblastoma (GBM), a grade IV astrocytoma, is the most common malignant primary brain tumor[10]. Current treatment includes surgical resection of the tumor and adjuvant radiation therapy plus the chemotherapeutic temozolomide[13]. However, survival rates for GBM remain extremely low with a five-year survival rate less than 10% and a recurrence rate of >90%[10]. Although GBM is diagnosed histologically and treated uniformly, it is genetically heterogeneous. The genes most frequently mutated in glioblastoma are involved primarily in three intracellular pathways: Retinoblastoma (RB), Receptor Tyrosine Kinase (RTK), and TP53[1]. RTKs are regulators of intracellular signaling pathways that control multiple cellular functions, such as proliferation, metabolism, and survival[6]. Moreover, overexpression and mutation of RTKs has been shown to promote tumorigenesis across multiple cancer types[8]. Furthermore, these RTK amplifications and mutations are the most common targetable alterations in GBM, identified in 66% of primary tumors[6].

Alterations in Epidermal Growth Factor Receptor (EGFR) are the primary RTK lesions in GBM[6]. Activation of EGFR by Epidermal Growth Factor (EGF) results in dimerization and autophosphorylation, inducing various downstream signaling cascades that regulate multiple cellular functions, including proliferation, apoptosis, migration, and survival[8]. Mutations, rearrangements, and amplifications of EGFR are the most common genetic alteration in GBM, presenting in over 50% of tumors[6]. The most common EGFR mutation, EGFR variant III (EGFRvIII), is the most common activating mutation in glioblastoma and occurs in 25 – 33% of all tumors[14]. EGFRvIII lacks exons 2 – 7, resulting in a truncated extracellular domain, which prevents the receptor from binding to the ligand[6]. This causes constitutive activation of downstream signaling, commonly activating the phosphatidylinositol 3-kinase (PI3K) pathway, resulting in ligand-independent increases in proliferation and survival and promotion of gliomagenesis[4,14].

Given the frequency of occurrence and role in promoting GBM tumorigenesis, EGFR mutations are a prime target for drug treatment with Tyrosine Kinase Inhibitors (TKI)[5]. However, despite the number of available EGFR inhibitors, multiple resistance mechanisms prevent effective single-agent treatment, with only 10 – 20% of patients exhibiting response to EGFR TKI[8]. One such mechanism involves the co-existence of mutations in downstream effectors or alternate activation of other RTK pathways; moreover, there is a frequent co-occurrence of RTK mutation and PTEN deletion in GBM[6]. PTEN, the phosphatase and tensin homolog deleted on chromosome 10, is a tumor suppressor that negatively regulates PI3K signaling by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP_3), and also performs multiple other tumor suppressor functions[5]. *PTEN* is frequently lost or mutated in GBM at a rate of 40%; this deletion maintains signaling, which in effect promotes tumorigenesis[5,6]. Though presence of wild-type PTEN is associated with sensitivity to EGFR TKI in cell lines harboring EGFRvIII, PTEN deletion confers resistance when expressed with EGFRvIII as a result of constitutive activation of downstream pathways[5,8]. The kinase networks involved in EGFRvIII- and PTEN loss- driven pathogenesis and resistance have not yet been determined.

We examined murine astrocytes immortalized via deletion of cyclin-dependent kinase inhibitor 2A (*Cdkn2a*), which occurs in approximately 60% of GBM and is also frequently mutated alongside PTEN deletion and EGFRvIII[6]. These astrocytes were derived from non-germline genetically engineered mouse models expressing four core genotypes: wild-type *EGFR* + wild-type *Pten* (C), wild-type *EGFR* + *Pten* deletion (CP), *EGFRvIII* + wild-type *Pten* (CEv3), and *EGFRvIII* + *Pten* deletion (CEv3P). To investigate EGFRvIII- and PTEN loss- driven kinase activation and identify potential targets for drug treatment, we used RNA sequencing (RNA-seq) and multiplexed inhibitor beads/mass spectrometry (MIB-MS) to determine the baseline kinase expression profile (transcriptome) and activity profile (kinome) of each genotype. We determined that *EGFR* and *Pten* mutation status influenced the transcriptomes and kinomes of Cdkn2a

(Ink4a/Arf)-null cell lines. These core genotypes exhibited differential RNA expression and kinase activity levels relative to astrocytes expressing wild-type *EGFR* and wild-type *Pten*, identifying several potentially attractive target kinases for dual therapy with EGFR TKI.

Methods

***Astrocyte culture*.** Ink4a/Arf-null murine astrocytes were derived from Cdkn2a-null genetically engineered mice. Non-germline genetically engineered mouse (nGEM) models were generated by expressing either wild-type *EGFR* + wild-type *PTEN*, wild-type *EGFR* + PTEN deletion, *EGFRvIII* + wild-type *PTEN*, or *EGFRvIII* + *PTEN* deletion in these astrocytes. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) and maintained at 37°C and 5% CO_2.

***RNA-sequencing*.** Total RNA from samples of each cell line was purified using the RNeasy Plus Mini Kit (Qiagen). RNA-sequencing was performed as previously described[3,12]. Briefly, poly(A) RNA was captured from 2 μg total isolated RNA with magnetic oligo-dT mRNA capture beads and beads were resuspended in 1X Fragment, Prime and Elute Buffer. In a thermocycler, mRNA was fragmented for 7 minutes at 94°C for a desired mean library fragment size of ~275 bp. The fragmented RNA was eluted and incubated with primers to synthesize first strand cDNA via reverse transcriptase. Double-stranded cDNA was synthesized, and A-tailing reaction was then performed. Adapters were ligated to dscDNA fragments, and library was amplified by PCR. Sample quality and fragment size were checked using Agilent 2200 TapeStation. Library was sequenced with an Illumina NextSeq-500, and analysis was performed as previously described[3,12].

***Multiplexed inhibitor bead affinity chromatography/mass spectrometry*.** Cells were harvested for baseline MIB-MS analysis. Cells were lysed in MIB lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 2.5 mM $NaVO_4$,

protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany), 500 µL phosphatase inhibitor cocktail 2 (Sigma-Aldrich, St. Louis, MO), 500 µL phosphatase inhibitor 3 (Sigma-Aldrich, St. Louis, MO)] and incubated on ice for 10 minutes. Lysates were sonicated 3 x 15 seconds and centrifuged at maximum speed (19,090 rpm) for 10 minutes at 4°C. Supernatant was filtered through 0.2 µm filter. BCA assay was conducted to equalize the input protein to 5 mg across samples. Lysates were brought to 1 M NaCl using Ambion 5 M NaCl and multiplexed inhibitor (PP58-, Purvalanol B-, UNC-21474-, Buparlisib-, and VI-16832-coated) beads were mixed in equal volumes and loaded into columns. MIB columns were washed with 2 mL high-salt MIB wash buffer [50 mM HEPES (pH 7.5), 1 M NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA], and lysates were passed through the columns. MIB columns were washed with 5 mL high-salt MIB wash buffer, 5 mL low-salt buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA], and then 500 µL 0.1% SDS in low-salt wash buffer. Twice, 500 µL MIB elution buffer [0.5% SDS, 1% β-mercarptoethanol, 0.1 M Tris-HCl (pH 6.8), LC-MS grade water] was added and eluate was collected after boiling columns for 15 minutes. Samples were treated with dithiothreitol (DTT) and iodoacetamide to reduce and alkylate the kinases and were then concentrated by filtration. Proteins were precipitated after methanol/chloroform extraction and incubated in 5 µL trypsin overnight at 37°C to digest the proteins. Samples were then extracted using ethyl acetate and cleaned using C-18 PepClean spin columns (Pierce Biotechnology, Rockford, IL). Mass spectrometry analysis was performed as previously described[3,12].

Results

EGFRvIII and *PTEN* loss have been implicated in GBM tumorigenesis, but effects of these mutations on kinome activation that promotes this process have not yet been determined. We investigated this issue *in vitro* by using RNA-seq and MIB-MS to examine the baseline transcriptomes and kinomes of immortalized cell lines harboring either EGFRvIII, *Pten* deletion, or both, in comparison to wild-type EGFR and wild-type *Pten* astrocytes.

RNA-seq was performed to define the baseline kinase expression profile and multiplexed inhibitor bead affinity chromatography/mass spectrometry (MIB-MS) was used to determine the baseline kinase activity profile for each cell line: C, CP, CEv3, and CEv3P. To assess the similarities and differences between the transcriptomes and kinomes of each cell line, principal component analysis (PCA) was performed. PC1 and PC2 were plotted to analyze common and unique variants between cell lines and between replicates for each line.

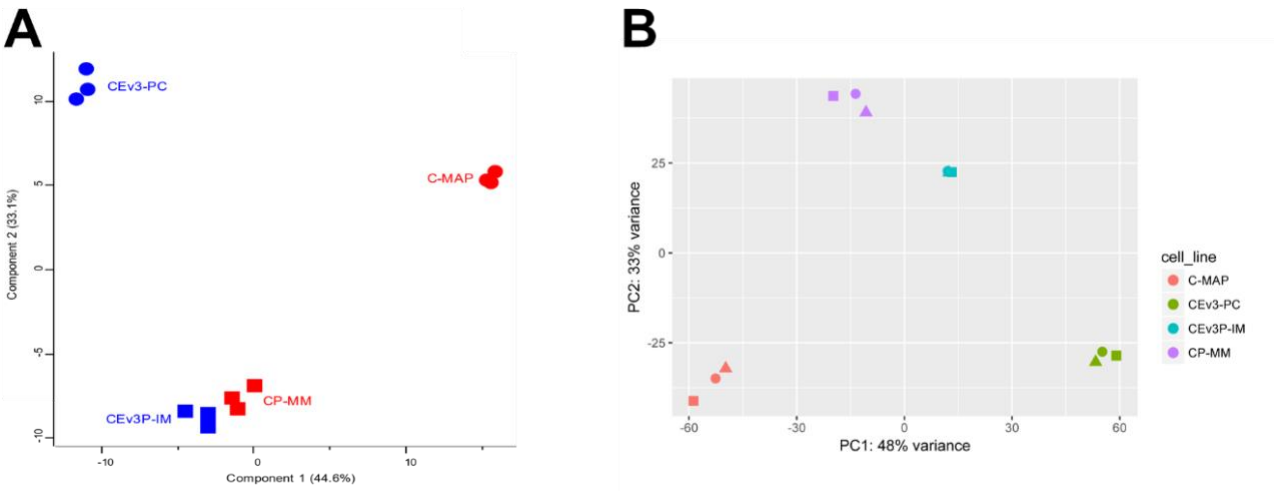


Figure 1. EGFRvIII and Pten deletion mutations cluster according to kinomes and transcriptomes. Principal Component Analysis was performed for kinome (A) and transcriptome (B) analysis, where Principal Component 1 (PC1) clusters by EGFR status and Principal Component 2 (PC2) clusters by Pten status.

Based on principal component analysis, samples appear to cluster based on EGFRvIII status (PC1) and *Pten* deletion status (PC2). Replicates for each cell line cluster together according to cell line, validating the consistency between replicates. Loss of Pten blunts the effect of EGFR versus EGFRvIII, as shown by the decrease in variation of PC1 between CEv3P and CP compared to the difference between CEv3 and C. Additionally, the relative relationships between samples are similar between kinome analysis (**Figure 1A**) and transcriptome analysis (**Figure 1B)**; thus, the results are consistent between MIB-MS and RNA-seq. Overall, PCA

indicates that EGFRvIII and *Pten* deletion mutation statuses influence kinase expression and activity levels of these cell lines.

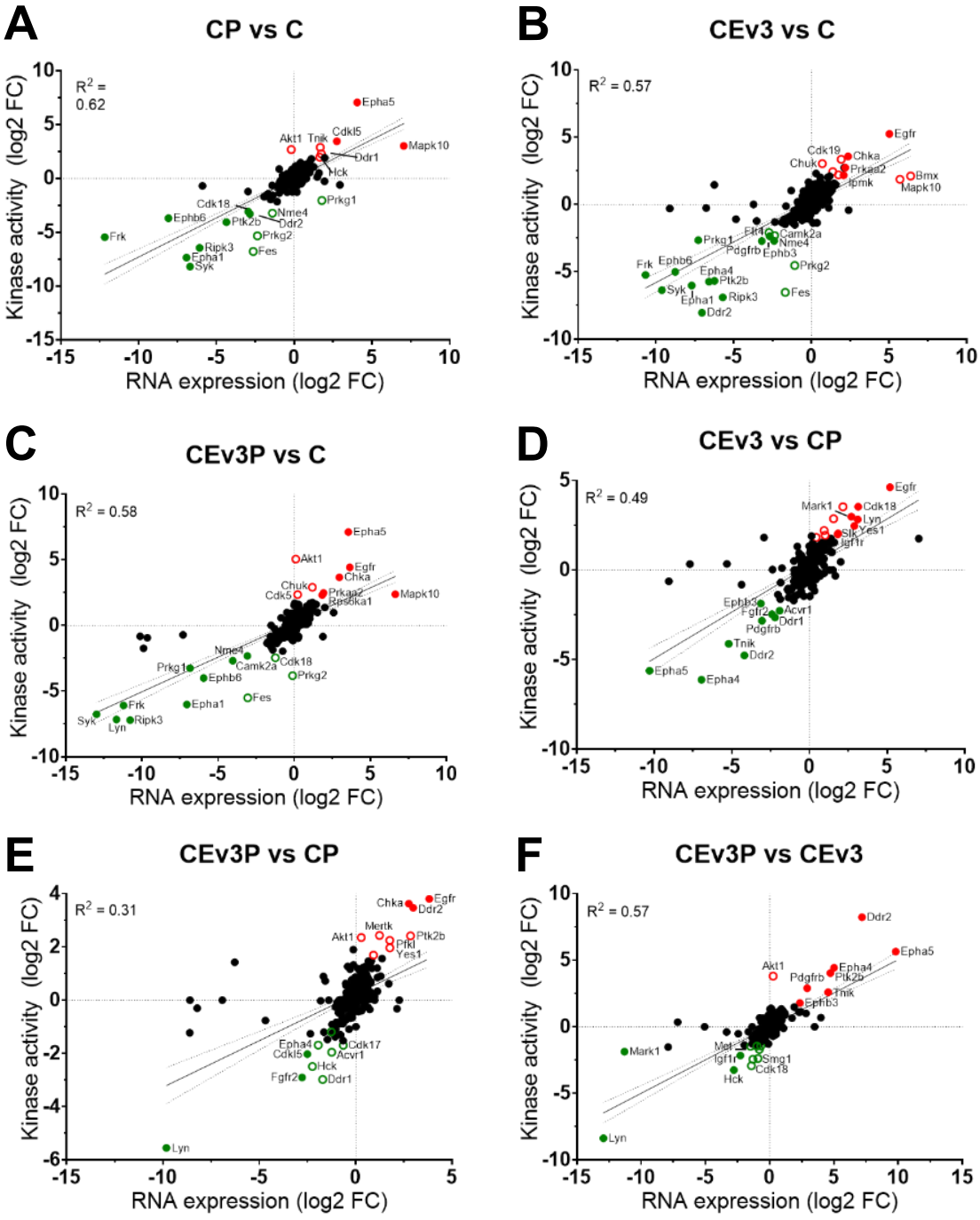
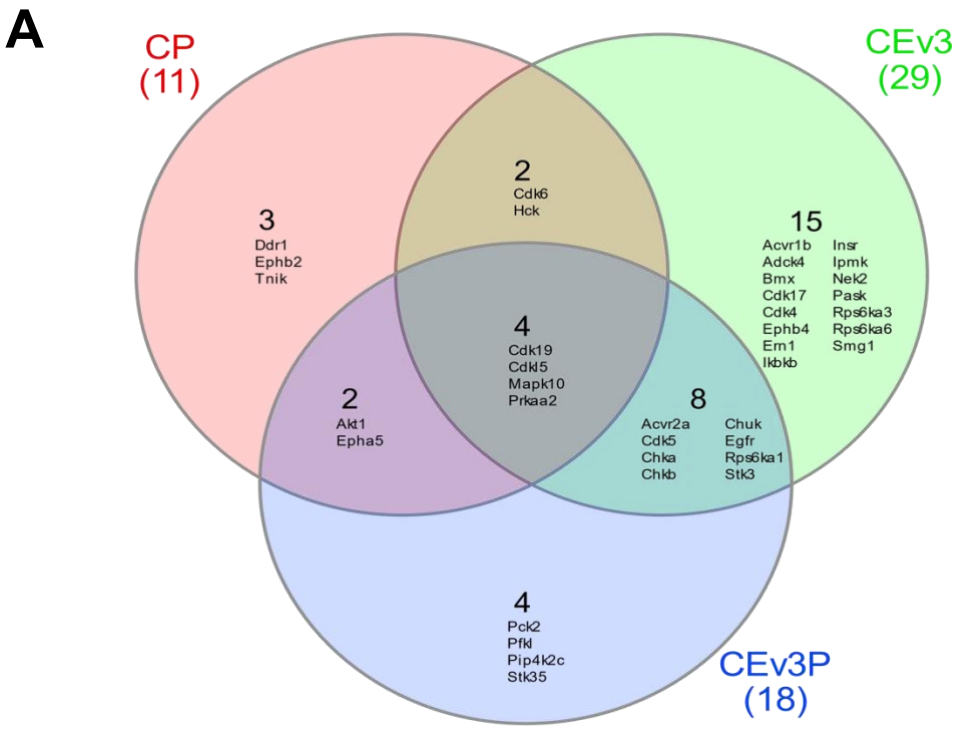


Figure 2. The four core genotypes result in differential kinase activity and RNA expression. Results from RNA-seq and MIB-MS analysis were plotted for each cell line relative to wild-type (**A-C**) and relative to the alternate genotypes (**D-F**). Differential RNA expression and kinase activity (closed circles) and differential kinase activity (open circles) shown between cell lines in terms of fold change (FDR Q<0.001).

Cell lines were harvested and analyzed by RNA sequencing and MIB-MS to determine the transcriptome and kinome profiles for all cell lines. RNA-seq determines the level of gene expression, and MIB-binding represents a function of kinase activation – increased binding indicates increased kinase activation, whereas decreased binding indicates decreased kinase activity level. RNA expression and kinase activity levels were plotted to compare the profile of each cell line to wild-type (C), and additionally graphed relative to each of the other genotypes separately. Differential core genotypes expressing EGFRvIII and/or *Pten* deletion resulted in varying fold-changes in kinase activity and RNA expression levels between cell lines (**Figure 2**).



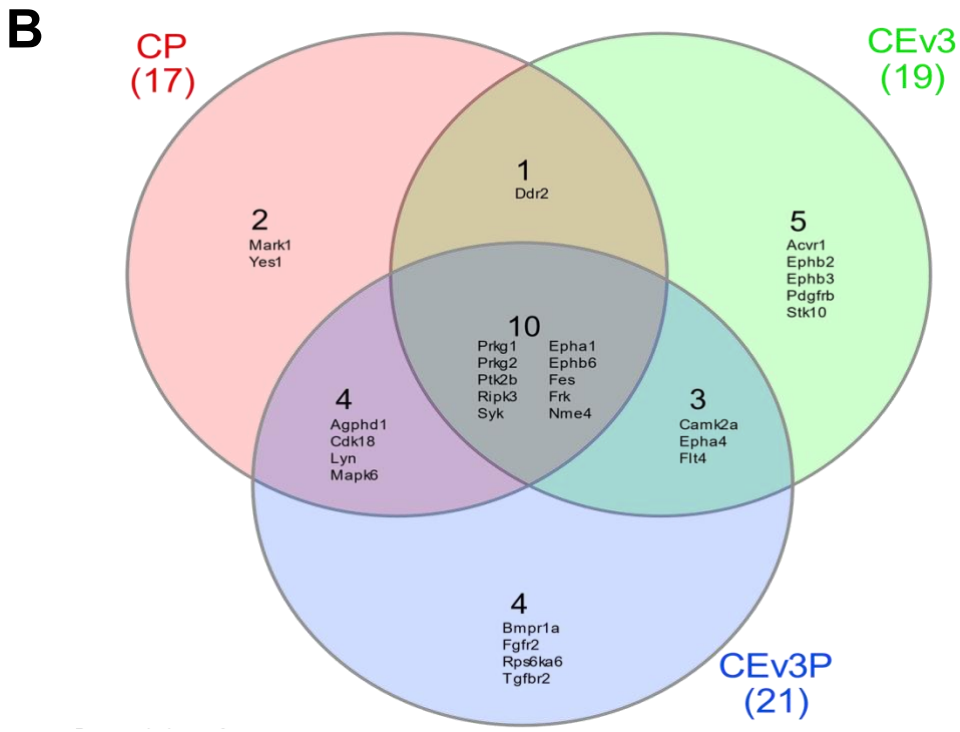


Figure 3. Cell lines exhibit differential kinase activity. Kinases with upregulated activity (**A**) or downregulated activity (**B**) compared to activity level in C astrocytes.

There was increased activity of a total of 38 kinases and decreased activity of a total of 29 kinases relative to Ink4a/Arf-null astrocytes expressing wild-type *EGFR* and wild-type *Pten* (C) (**Figure 3A, 3B**). The kinases with upregulated activity across all three cell lines were Cdk19, Cdk15, Mapk10, and Prkaa2 compared to astrocytes harboring both wild-type EGFR and wild-type *Pten*. The kinases with downregulated activity across all three cell lines relative to C astrocytes were Prkg1, Prkg2, Ptk2b, Ripk3, Syk, Epha1, Ephb6, Fes, Frk, and Nme4.

There was an increase in activity of Ddr1, Ephb2, and Tnik solely in CP relative to C astrocytes, presumably as a result of *Pten* loss in CP astrocytes. Also solely in CP, there was decreased activity of kinases Mark1 and Yes1, suggesting the loss of *Pten* influenced downregulation of these kinases. The kinases upregulated in both *Pten*-mutant cell lines, CP and

CEv3P, relative to C astrocytes were Akt1 and Epha5. There was a decrease in activity of kinases Agphd1, Cdk18, Lyn, and Mapk6 in these cell lines.

Relative to C astrocytes, CEv3 singularly exhibited increased activity of Acvr1b, Adck4, Bmx, Cdk17, Cdk4, Ephb4, Em1, Ikbkb, Insr, Ipmk, Nek2, Pask, Rps6ka3, Rps6ka6, and Smg1, which suggests these kinases were upregulated as a result of EGFRvIII expression. CEv3 also specifically exhibited decreased activity of Acvr1, Ephb2, Ephb3, Pdgfrb, and Stk10 compared to C, suggesting EGFRvIII influenced the reduction in activity of these kinases.

CEv3P exclusively exhibited increased kinase activity of Pck2, Pfkl, Pip4k2c, and Stk35, and decreased activity of Bmpr1a, Fgfr2, Rps6ka6, and Tgfbr2, suggesting that the combination of EGFRvIII and *Pten* deletion possibly cooperates to influence the activation of these kinases that were not differentially-activated in cell lines harboring either only EGFRvIII or *Pten* loss. Additionally, notably fewer kinases were activated in CEv3P compared to CEv3, indicating that deletion of *Pten* blunts the effect of CEv3, consistent with principal component analysis.

In both EGFR-mutant cell lines, CEv3P and CEv3, there was increased activity of Acvr2a, Cdk5, Chka, Chkb, Chuk, Egfr, Rps6ka1, and Stk3 relative to C. Both CEv3P and CEv3, but not CP, exhibited downregulated activity of Camk2a, Epha4, and Flt4, suggesting the presence of EGFRvIII affected the decrease in activity of these kinases.

These results show that EGFRvIII and *Pten* deletion mutations influence genotype-dependent kinase expression and activity levels when compared to astrocytes harboring wild-type EGFR and wild-type *Pten*.

Discussion

Genomic sequencing of GBM identifies EGFRvIII and PTEN deletion in a large percentage of tumors, and these mutations have been shown to promote tumorigenesis *in vivo*[5,6,14]. Though EGFR mutation has emerged as an attractive target for drug treatment, multiple resistance

mechanisms have been implicated in affecting the responses of EGFR-mutant cell lines to single-agent EGFR tyrosine kinase inhibitor treatment in GBM[8]. Consequently, determining the profiles of these cell lines will aid in the identification of potential kinase targets for dual treatment with EGFR TKI for a more effective precision therapy approach. Herein, we investigated the baseline transcriptome and kinome states of four core genotypes expressing EGFRvIII, *Pten* deletion, or both, in order to aid in determining the differential kinase expression and activation involved in EGFRvIII- and *Pten* loss- driven gliomagenesis. Principal component analysis showed that EGFRvIII and *Pten* deletion influenced the transcriptome and kinome profiles of each cell line. RNA-seq and MIB-MS showed that EGFRvIII and/or *Pten* deletion influences differential baseline RNA expression and baseline kinase activity of the four core genotypes relative to wild-type and across genotypes.

There was a general increase in activity of kinases involved in the regulation of cell proliferation, survival, signal transduction, and metabolism. Cyclin dependent kinases Cdk19 and Cdk15 were upregulated across all cell lines except C, consistent with their role in proliferation. In addition, there was increased activity of Mapk10 across all cell lines relative to C, as well as Rps6ka3 in CEv3, indicating a possible increase in the activity of MAPK/JNK and MAPK/ERK pathways, which also promote increased growth and migration of cells. Other kinases involved in the regulation of cell metabolism showed increased activity, including Prkaa2 in all cell lines except C and Pfkl in CEv3P. Additionally, there was upregulated activity of multiple other RTKs, including Ddr1, Ephb2, Insr, Ephb4, and Epha5 in various cell lines. In EGFR-mutant cell lines, there was upregulation of EGFR activity, which is consistent with the effects of EGFRvIII, and in *Pten*-mutant cell lines, there was increased activity of Akt1, indicating increased stimulation of the PI3K pathway, which is consistent with the effects of *Pten* loss.

There was a general decrease in activity of kinases with proposed tumor suppressor functions, including Ripk3, Syk, Frk, and Nme4, across all cell lines except C. Other kinases with

roles in growth inhibition, including Tgfbr2 in CEv3P and Lyn in CP and CEv3P, were downregulated as well. Decreased activity of these kinases indicates a decrease in negative regulation of proliferation in EGFR- and/or *Pten*-mutant cell lines compared to wild-type. Since MIB-binding represents a function of kinase activation, we will perform immunoblots for phospho and non-phospho targets to measure downstream pathway signaling for the notable differentially-activated kinases.

Though tumor suppressor proteins are difficult to target in cancer therapy, the observed overactive kinases may serve as attractive targets for combination drug treatment with EGFR TKI. Specifically, Ephb4 (upregulated in CEv3) and Epha5 (upregulated in both CP and CEv3P) are Ephrin (Eph) receptors of the RTK family. Eph receptor tyrosine kinases have been implicated in cancer growth and Ephb4 and Epha5 are altered in 1.4% and 1.1% of GBM, respectively, which makes these kinases potentially attractive for targeted treatment of EGFR-mutant GBM with and without *PTEN*[2,7,9,11]. Also upregulated were Rps6ka3 in CEv3 and Rps6ka1 in both CEv3 and CEv3P, which phosphorylate targets in the MAPK/ERK pathway[9,11]. Since this pathway stimulates processes such as cell proliferation and survival, targeting these two kinases could be beneficial in treating EGFRvIII-expressing GBM with or without *PTEN*. Ikbkb and Chuk were hyperactive in CEv3 and both CEv3 and CEv3P, respectively; these kinases are involved in the activation of NF-kappa-B, which leads to cell growth and prevention of apoptosis[9,11]. Thus, these kinases could also be potential targets for treatment of patients with tumors harboring EGFRvIII with and without *PTEN*. A potential target in cell lines expressing EGFRvIII and wild-type *Pten* is Nek2, a cell-cycle regulator which has been associated with tumorigenesis and is altered in 1.1% of GBM[2,7,9,11,]. Finally, Akt1 of the PI3K pathway was upregulated in both CP and CEv3P and is also altered in 1.4% of GBM[2,7]. Since PI3K signaling promotes cell growth, targeting Akt1 could be effective for *Pten*-mutant tumors that lack negative regulation of this pathway. Overall, these results from kinome profiling identify the genotype-dependent kinase activation involved in processes behind

EGFRvIII- and *Pten* loss- driven tumorigenesis, and identify several preliminary kinase targets for future combination therapy with EGFR TKI.

Though baseline kinome profiling identifies potential kinase targets for dual treatment, resistance may still occur in response to EGFR inhibition as a result of dynamic reprogramming of the kinome. Subsequently, observing the kinases that are differentially-activated in direct response to drug treatment may identify targets that more accurately predict effective dual therapy; targeting these kinases would ideally inhibit the activation of additional pathways that contribute to EGFR TKI resistance. In order to investigate the role of EGFRvIII and *Pten* deletion in adaptive kinome reprogramming and resistance, we will perform RNA-seq and MIB-MS for these cell lines after one-time treatment with 3 μM afatinib, an EGFR TKI. Compared to the baseline kinome state, we would expect significant changes in MIB-binding, and thus kinase activity, of alternate kinase pathways across all cell lines in response to drug treatment, which is indicative of kinome response to TKI. We are also culturing these astrocytes in media with either of two EGFR TKI, gefitinib or erlotinib, in order to investigate the response of these resistant cell lines after chronic treatment. Whereas determining the response of cell lines after immediate treatment shows adaptive resistance to EGFR inhibition, we will perform RNA-seq and MIB-MS for cell lines grown in long-term culture with low-dose gefitinib or erlotinib to identify the kinase networks involved in acquired resistance. These results may be more applicable to patient care, as acquired resistance could arise during chronic drug treatment. Dynamic kinome profiling will aid in determining the kinase networks involved in both acute and acquired resistance mechanisms, which will be relevant to developing personalized combination treatments for genome-sequenced GBM patients.

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