Philip H. Abbosh MD-PhD
Molecular Therapeutics Program
Institute for Cancer Research
Fox Chase Cancer Center
Philadelphia, PA 19111
215-214-4212
philip.abbosh@fccc.edu

Kidney Cancer Association
P.O. Box 803338 #38269
Chicago, IL 60680-3338

Re: Young Investigator Award final progress report

1 Jun 2018

Dear Kidney Cancer Association

I am grateful to have received the Young Investigator Award and my lab and I have been working very hard towards the goals set out in my proposal. As you will recall, the hypotheses that were proposed were not supported by our data in the last update. However, we have made significant progress in exploring the role of *PBRM1* in kidney cancer and have made several interesting findings.

Foremostly key to the successes we have achieved in the last year was our ability to finally re-express BAF180 in *PBRM1* -/- ccRCC cell lines (**Figure 1**). This allowed us to test the hypothesis that *PBRM1*-null cell lines would be more sensitive to EZH2-mediated Polycomb inhibition. We tested two EZH2 inhibitors, UNC1999 and EPZ011989, in these 6 paired cell lines and found that there was no difference in their sensitivities (not shown), suggesting that in ccRCC, SWI-SNF loss does not confer the same vulnerability to Polycomb inhibitors that has been seen in other malignancies which harbor loss of SWI-SNF components. We then tested other typical drugs that are commonly used in metastatic ccRCC including sunitinib and everolimus in the paired cell lines and again found no differences in sensitivities with respect to PBRM1-status. These disappointing findings led us to ask how *PBRM1* loss / reintroduction changed gene expression.

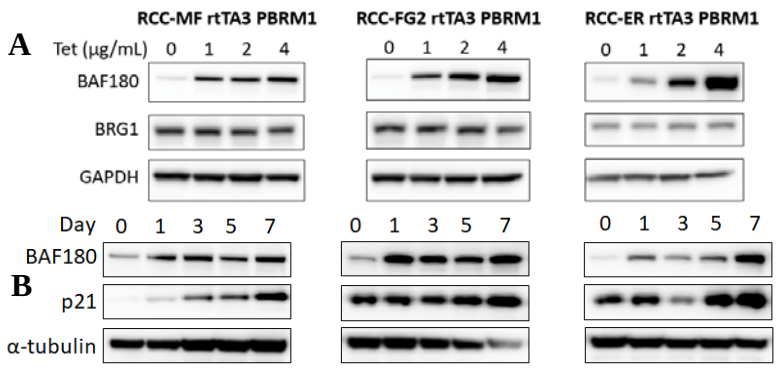

Figure 1: PBRM1 cDNA was stably introduced into cells expressing the reverse-tetracycline transcriptional activator (rtTA3) under the control of a tetracycline-inducible promoter. Cells were treated with increasing doses of tetracycline for 3 days (in **A**) or with 4 µg/mL tetracycline on a time course (in **B**). Then, Western blot was performed on cell lysates to show that BAF180 was re-expressed in a dose and time-dependent manner, that its target gene p21 is also induced, and that a SWI-SNF component (BRG1) is not altered on BAF180 induction.

PBRM1 loss in ccRCC cell lines activates type II Interferon signaling. Because SWI/SNF is intimately involved in chromatin modification and expression of target genes, we performed RNA-seq on sham and tetracycline-treated cells (7d treatment) to evaluate signaling pathways and gene expression changes that might be altered after its reintroduction. We hypothesized that Polycomb targets would be upregulated. To our surprise, Polycomb target genes were not altered. We then performed Hallmark gene set enrichment

analysis (GSEA; Liberzon 2015) to check for alterations in other pathways. To prioritize candidate pathways, we looked for gene sets that were enriched in all three cell lines after 7 days of tetracycline (4 μg/mL) with p<0.05 and q<0.25. Re-introduction of inducible BAF180 into each of the 3 *PBRM1* -/- cell lines led to a strong decrease in the 'Interferon-γ response' gene set (normalized enrichment score <-2.06, p<0.001, q≤0.031 for all cell lines; **Figure 2**). This was the only commonly altered gene set, although 'TNFα signaling via NFκB,' 'Oxidative phosphorylation,' 'G2M checkpoint,' and 'E2F targets' were enriched positively or negatively in 2 of 3 isogenic pairs. G2M checkpoint and E2F targets (lower in induced cells) may be reflective of overall slower growth in these cells, which we and others have observed.

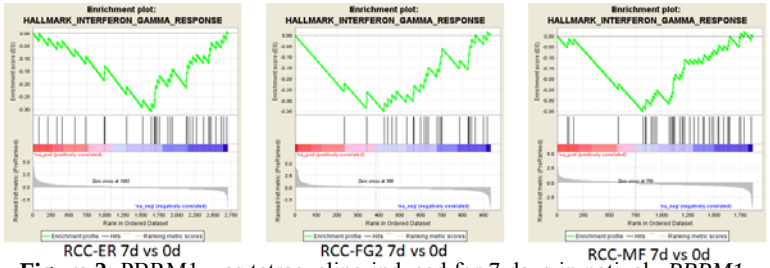

Figure 2: PBRM1 was tetracycline-induced for 7 days in natively *PBRM1* null ccRCC cell lines followed by RNAseq and GSEA. This revealed that Interferon-γ pathway is enriched in PBRM1 null cells. Genes to the right are ranked more highly after induction, and genes to the left are ranked more highly prior to induction.

These findings may have clinical implications, as an active Interferon-γ pathway appears to be a biomarker for response to PD1 blockade (Ayers 2017). Interferon-γ signaling in tumor infiltrating Tcells also is required for response to combination ICB (Patel 2017). Additionally, Interferon-γ directly regulates PD-L1 and PD-L2 transcription at their respective promoters though IRF1 binding and transactivation (Garcia-Diaz 2017). On the other hand, chronic Interferon-γ signaling may actually mediate anti-CTLA-4 therapeutic resistance (Gao 2017). These previous models make our findings highly relevant but their meanings ambiguous. We therefore sought to confirm our findings in human tumors and search for additional information to clarify whether the role of Interferon-γ signaling might be associated with ICB response or resistance.

Loss of PBRM1 and SETD2 in human cancers also activated type II Interferon signaling. We also analyzed TCGA ccRCC cases to determine if this relationship between somatic *PBRM1* loss and immune signaling is relevant in human ccRCC tumors. Using GSEA, we saw that *PBRM1*-mutant ccRCCs carried upregulation of genes in the 'TNFα signaling via NF-κB' gene set (normalized enrichment score=1.75, p<0.001; q=0.019; **Figure 3, top**). 'Interferon-γ response' was slightly enriched but not statistically significant. The Hallmark gene set 'Hypoxia' was also enriched in *PBRM1*-mutant tumors (p=0.002; q=0.076), congruently with prior published studies showing that *PBRM1* augments the hypoxic response in ccRCC.

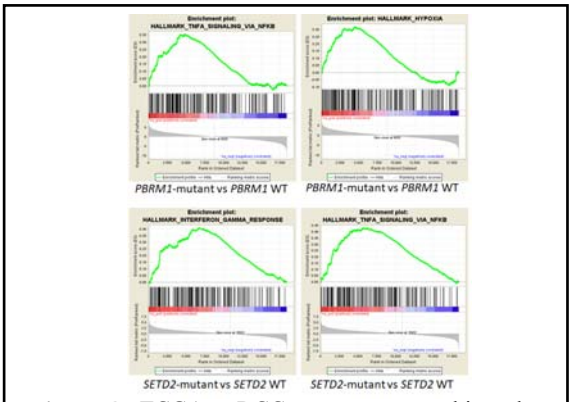

Figure 3: TCGA ccRCC tumors were subjected to Hallmark GSEA as grouped by *PBRM1*, *SETD2*, or *BAP1* mutation status. Several inflammatory pathways were enriched in *PBRM1* and *SETD2*-mutant tumors.

We repeated this analysis by grouping tumors by *SETD2* mutation status and saw that several immune signaling pathways were activated including 'Interferon γ response,' 'TNFα signaling via NF-κB,' 'Inflammatory response,' 'Allograft rejection,' 'IL2-STAT5 signaling,' and 'IL6 JAK STAT3 signaling' (normalized enrichment score≥1.57, p<0.001, q≤0.011 for all gene sets; **Figure 3, bottom**). In contrast, *BAP1*-mutant ccRCC tumors did not have any Hallmark immune or inflammatory pathways which were enriched with p<0.05.

We followed up the interesting finding that *PBRM1* loss increased TNFα signaling through NF-κB by probing

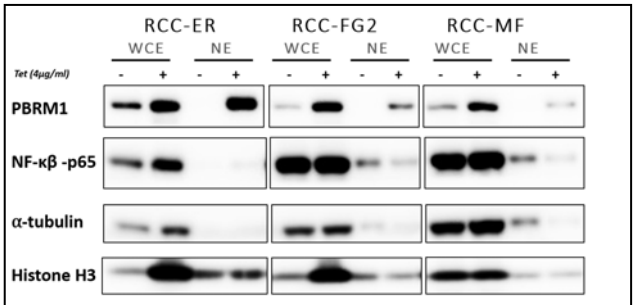

Figure 4: Whole cell or nuclear extracts (WCE or NE, respectively) were probed for the specific anitgens. RCC-FG2 and RCC-MF cells had higher nuclear p65 subunit levels when BAF180 was absent compared to when BAF180 was present.

nuclear vs whole cell extracts for the NF-κB p65 subunit. Because NF-κB localizes to the nucleus when activated, higher NF-κB signaling should result in higher NF-κB protein detectable in the nucleus. Indeed, we found that there was increased nuclear presence of the NF-κB p65 subunit in the *PBRM1*-null state compared to induction with tetracycline for 7 days in RCC-MF and RCC-FG2 cells (**Figure 4**).

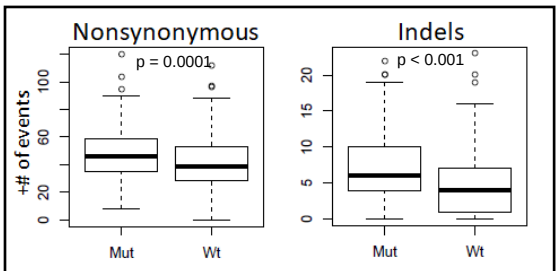

Figure 5: Tumors were grouped according to their *PBRM1* status and the burden on SNVs (left) and indels (right) were compared. *PBRM1*-mutant (mut) tumors had higher burden of both types of variants.

Loss of PBRM1 and SETD2 in ccRCC results in a higher mutation burden and more neoantigens. BAF180 has been shown to mark sites for non-homologous end joining and cancer-associated *PBRM1* mutants are unable to rescue this DNA repair defect. SETD2 has been shown to methylate histone H3-K36 at the sites of DNA damage, marking these sites for repair by either mismatch repair or homologous repair. We therefore hypothesized that *PBRM1* or *SETD2* loss in ccRCC would lead to a higher number of mutations per tumor genome due to decreased DNA repair capacity. We used the mutation calls from TCGA ccRCC cohort to compare the mutation load in either *PBRM1* or *SETD2* mutant vs WT tumors. We found that indeed, *PBRM1*-mutant tumors carried a higher mutation load (median 54.5 versus 44 $p<6x10^{-5}$, Mann-Whitney U test; **Figure 5**). Although *SETD2*-mutant tumors had a higher mutation load too, this was not statistically significant (p=0.06). We also performed this comparison on BAP1-mutant tumors and found that mutation load did not differ (p=0.4). On average, 50% of nonsynonymous mutations result in ≥1 neoantigen. Therefore, higher neoantigen burden is expected in *PBRM1*-mutant tumors due to their higher mutation load. Of note, *PBRM1*-loss is associated with statistically significantly higher *nonsynonymous variant* burden *and* higher *indel* burden (median 6 vs 4 indels/tumor, $p=4x10^{-6}$). Indels result in 9-fold more neoantigens than single nucleotide variants (Turajiic, 2017). Because neoantigens and neoantigen burden have been shown to be a marker for (and possibly central to the response to) ICB and other immunotherapeutics, this finding supports the idea that mutant tumors are more antigenic and may be predisposed to ICB response.

CD8 infiltrate is altered in SETD2 and PBRM1-mutant tumors. We characterized the CD8 infiltrate in TCGA ccRCC tumors with two methods. Firstly, CIBERSORT was performed on RNAseq data from TCGA ccRCC tumors. CIBERSORT assigns a fractional abundance of 22 different immune cell types based on expression of genes which are specific to each cell type (Gentles, 2015). Tumors were grouped by *PBRM1* and *SETD2* mutation status. We found that *PBRM1*-mutant tumors had slightly reduced CD8 infiltrates compared to *PBRM1*-WT tumors (median 0.11 vs 0.14, p=0.004 for *PBRM1*-mutant vs WT, p=0.013), but neither *SETD2* mutation (median 0.19 vs 0.16, mutant vs WT respectively, p=0.12) nor *BAP1* mutation (0.16 vs 0.13, mutant vs WT, respectively, p=0.09) associated with CIBERSORT CD8 score (**Figure 6**). Similar results were found when CD8 scores were calculated using single sample GSEA to score Tcell infiltrate (0.86 vs 1.22, p=0.003 for *PBRM1*-mutant vs WT; 1.27 vs 1.11, p=0.66 for *SETD2*-mutant vs WT; 1.36 vs 1.08, *BAP1*-mutant vs WT, p=0.11). Analyzing tumors based on their combined mutation status (*PBRM1* or *SETD2* vs WT *PBRM1* and *SETD2*) did not change this pattern, most likely because many *SETD2*-mutant tumors also harbor *PBRM1* mutations.

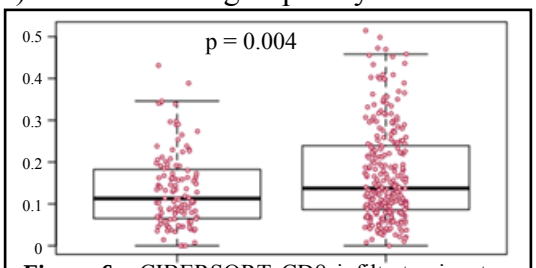

Figure 6: CIBERSORT CD8 infiltrate signature score was compared between PBRM1 and SETD2 WT and mutant (mut) tumors.

Lastly, we also used the Rooney et al cytolytic score (calculated as geometric mean of GZMA and PRF1 expression) and found that *PBRM1*-mutant tumors had less inflammation than *PBRM1*-WT tumors (median 27.8 vs 30.9 , p=0.018 for *PBRM1*-mutant vs WT), but again neither *SETD2* mutation (median 31.2 vs 29.1, p=0.8 mutant vs WT) nor *BAP1* mutation (median 29.6 vs 28.6 p=0.09, mutant vs WT) were associated with tumor inflammation.

These data suggest that tumors with mutation in either *PBRM1* or *SETD2* have active Interferon-γ and NF-κB/TNFα signaling, higher mutation loads, and (by correlation) higher neoantigens. Paradoxically, the

Tcell infiltrate and cytolytic activity within these tumors is lower. It is not possible to determine if these driver mutations predispose to ICB response because none of these patients were treated with ICB. Given the previously established importance of the Interferon-γ pathway in immune checkpoint response, we hypothesize that mutations predispose to response by activating type II Interferon signals in tumor cells. Interestingly, *PBRM1*-mutant ccRCCs tend to have a better overall survival than *BAP1*-mutant ccRCCs. It is possible that activated Interferon-γ signaling 'keeps them in check' until they break equilibrium and metastasize, when even then it is possible that the immune system may still control their growth.

KCA funding was the primary support for these final analyses. Although the project evolved from what we initially proposed, we have made some significant findings which are congruent with recent work by the work of Eliezer Van Allen, a former KCA Young Investigator, and colleagues which suggest that *PBRM1* mutation is a marker for successful immune checkpoint inhibitor us in patients with metastatic kidney cancer. Similarly, Pan et al showed that *PBRM1* loss makes tumor cells susceptible to immune checkpoint blockade in a a mouse model of cancer, further corroborating these ideas. My group has now submitted proposals to the Department of Defense (Kidney Cancer Research Program) and the AACR Kure It Kidney Cancer Immunotherapy award based on this work. Although neither project was funded, both received favorable reviews and I anticipate resubmitting these in the coming funding cycle.

I sincerely appreciate the opportunity to be a steward of the KCA's support and hope to continue to this successful research in the future.

Thank you,

Philip Abbosh MD-PhD