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Clinical and Pathologic Impact of Select Chromatin Modulating Tumor Suppressors in Clear Cell Renal Cell Carcinoma

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Abstract

Background—Historically, *VHL* was the only frequently mutated gene in clear cell renal cell carcinoma (ccRCC), with conflicting clinical relevance. Excitingly, recent sequencing efforts identified several novel, frequent mutations of histone modifying and chromatin remodeling genes in ccRCC, including *PBRM1*, *SETD2*, *BAP1* and *KDM5C*. Intriguingly, *PBRM1*, *SETD2* and *BAP1* are located in close proximity to *VHL* within a commonly lost (~90%) 3p locus. To date the clinical and pathologic significance of mutations in these novel candidate tumor suppressors is unknown.

Objective—To determine the frequency of and render the first clinical and pathologic outcome associated with mutations of these novel candidate tumor suppressors in ccRCC.

Design, Setting, and Participants—Targeted sequencing was performed in 185 ccRCC and matched normal tissues from a single institute. Pathologic features, baseline patient characteristics and follow-up data were recorded.

Statistical Analysis—The linkage between mutations and clinical and pathologic outcomes was interrogated with Fisher's exact test (for stage and Fuhrman nuclear grade) and the permutation log-rank test (for cancer specific survival).

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Conflict of Interest:
None to report for any author

Results and Limitations—*PBRM1*, *BAP1*, *SETD2* and *KDM5C* are mutated at 29%, 6%, 8% and 8%, respectively. Tumors with mutations in *PBRM1* or any of *BAP1*, *SETD2* or *KDM5C* (19%) are more likely to present with stage 3+ diseases, p=0.01 and p=0.001, respectively. Small tumors (<4cm) with *PBRM1* mutations are more likely to exhibit stage 3 pathologic features (OR 6.4, p=0.001). *BAP1* mutations tend to occur in Fuhrman Grade 3–4 tumors (p=0.052) and associate with worse cancer specific survival (p=0.01). Clinical outcome data is limited by the number of events.

Conclusion—Most mutations of chromatin modulators discovered in ccRCC are loss-of-function, which associate with advanced stage, grade, and possibly worsened cancer specific survival. Further studies validating the clinical impact of these novel mutations and future development of therapeutics remedying these tumor suppressors are warranted.

Keywords

Chromatin; Histone; Mutation; Outcome; Renal Cell Carcinoma

Introduction

The mutational landscape of clear cell renal cell carcinoma (ccRCC), the most common and aggressive form of renal cell carcinoma, has been radically transformed in the last two years due to several kidney cancer genomics projects. Long considered to be a disease dominated by the mutation of a single gene, *VHL*, recent studies identified several frequently mutated genes, including *PBRM1* (1), *SETD2*, *KDM5C* (2, 3) and *BAP1*(4, 5).

Dalgliesh et al. performed selected exon sequencing of candidate cancer genes on 101 ccRCCs and discovered several novel recurrent mutations in chromatin remodeling genes, including *SETD2* and *KDM5C* (2). In a follow-up study, these authors identified truncating mutations of *PBRM1* in 41% of ccRCCs, nominating *PBRM1* as the second most common mutated gene behind *VHL* (1). A subsequent report by Guo et al. confirmed the frequent mutations of *VHL* (27%), *PBRM1* (21%), *KDM5C* (9%) and *SETD2* (4%) in ccRCC, and identified *BAP1* mutations in 8% cases (4). Interestingly, a recent study suggested a strong association between BAP1 mutations and advanced ccRCC tumor grade (5). Although whether and how mutations of these chromatin modulating genes contribute to the pathogenesis of ccRCC is unknown, disruption of chromatin biology has become an emerging theme constituting a new pathobiology underlying oncogenesis.

These findings are particularly intriguing for several reasons. First, these four genes all function in chromatin biology. Although the significance of this chromatin connection is currently unclear, histones and chromatin are essential building blocks of genomic architecture and dysregulation leads to transcriptional disruption of gene expression (6, 7). Second, *PBRM1*, *SETD2* and *BAP1* are located in close proximity at the 3p21 locus, right next to the 3p25 locus where *VHL* resides (Supplemental figure 1). Hence, the signature single copy loss of 3p (>90%) in ccRCC (8) would simultaneously impair four tumor suppressors that might be functionally linked. Third, *PBRM1*, *BAP1* and *SETD2* have recently been implicated in the pathogenesis of cancers other than ccRCC (9–13).

The clinical relevance of the *VHL* mutation in ccRCC has thus far been conflicting in both mutation frequency and its relationship to adverse tumor features and clinical outcomes. (14) Plausible causes underlying the wide range of *VHL* loss include tumor purity, tumor and patient heterogeneity, promoter methylation, etc. Nevertheless, many contemporary series estimate that *VHL* is impaired through mutations and promoter silencing in upwards of 90% of ccRCC (14, 15). Hence, the mutation status of *VHL* alone is quite unlikely to be useful as a biomarker for disease aggressiveness. On the other hand, a recent phylogenetic assessment

of tumor heterogeneity in ccRCC suggested that mutations of chromatin modulators are secondary events and contribute to invasive and metastatic phenotypes (16). Hence, the mutation status of chromatin modulators in ccRCC might offer important prognostic insights and thus render novel therapeutic strategies. Accordingly, we set out to investigate the prevalence and the pathologic and clinical significance of these mutations in ccRCC patients from a single institute.

Methods

Patient Samples

Tissue samples from 185 treatment naive patients, undergoing either radical or partial nephrectomy for sporadic ccRCC from December of 2001 to December of 2011 were collected based on tissue availability and quality of samples with 85% of tumors collected after 2007 All patients had signed informed consents for tissue utilization and the study had been approval from our Institutional Review Board. All tumor staging was based on the AJCC/UICC TNM 7th Edition. All tumors were reviewed by a group of dedicated uropathologists to confirm the histopathologic diagnosis. Fresh frozen tumors and paired normal tissue blocks were identified and macro-dissected from areas marked by a uropathologist for maximal tumor density. DNA was extracted from tissue samples using DNEasy (Qiagen) and was quantified using a Nanodrop spectrophotometer (Invitrogen).

Integrated Mutation Analysis

Mutation analysis of entire coding regions of *VHL*, *PBRM1*, *SETD2*, *BAP1* and *KMD5C* was performed using PCR amplification and bidirectional Sanger sequencing. Details of mutation analysis can be seen in Supplemental Methods.

Statistical Analysis

The association between the clinical and pathologic outcome and individual mutations was evaluated using Fisher's exact test with two-tailed p values (for stage and Fuhrman nuclear grade) or log-rank permutation test (for cancer specific survival) (17). Odds ratios were used to estimate the strength of association for co-expression or mutual exclusivity among mutations as well as between mutations and pathologic features with 95% confidence intervals estimated using a logarithmic transformation. Multiplicity adjustment is not considered and, as a result, our conclusions are presented in suggestive rather than confirmatory language.

Results

Patients

The demographics, clinical, and pathologic characteristics of the 185 ccRCC patients are presented in Table 1. Patients were grouped into low risk (AJCC 1–2) and high risk (AJCC 3–4) for an analysis of association with gene mutations. Pathologic Fuhrman nuclear grades were grouped into low grade (1–2) and high grade (3–4) for mutation associations. Eighteen percent of patients developed metastasis: 21 patients presented with metastatic disease (cytoreductive nephrectomy) and 12 patients developed de novo distant metastasis. Seventeen patients (9%) died at last follow-up with 10 (5.4%) dying from ccRCC.

Frequencies and Types of Mutations

Overall, 119 of the 185 (65%) tumors harbored genetic mutations in at least one of the five target genes. *VHL* mutations were found in 49.2% of tumors, *PBRM1* in 29.2%, *SETD2* in 7.6%, *KDM5C* in 7.6%, and *BAP1* in 5.9% (Figure 1 heat map). Supplemental Table 2 lists

clinical and pathologic information of these 119 patients. In total, truncating mutations, i.e., frameshift and nonsense, represent 67%, 82.5%, 78.5%, 35.5% and 36% of mutations in *VHL*, *PBRM1*, *SETD2*, *KDM5C* and *BAP1*, respectively (Supplemental Tables 3 and 4, and Supplemental Figures 2 and 3). Individual gene maps with notations of mutation types and their positional relationships to the indicated functional domains are depicted in Figure 2.

Mutations and Clinical Outcomes

There is a tendency of mutual exclusivity between *PBRM1* and *BAP1* mutations, which is supported by a recent report (5), but it did not reach statistical significance (p=0.18) (Figure 1). Interestingly, tumors with *PBRM1* mutations (n=54) were more likely to present with advanced stages (p=0.01) (Figure 3). Although there was no association between tumors with mutations in *BAP1*, *SETD2* or *KDM5C* alone and advanced stages at presentation, there was a statistically significant connection (p=0.001) between those with any of the three mutations (n=35) and advanced stages, likely reflecting the relatively low prevalence of individual mutations (<10%). A subset analysis of tumor by mutation type was not suggestive of any clinical or pathologic trends with the exception of *SETD2* mutations. All *SETD2* mutations were frameshift (n=11) with the exception of 3 missense mutations that score as either neutral or low by functional impact (18). When excluding missense mutations, *SETD2* mutations alone were associated with advanced tumor stages (p=0.02). Furthermore, tumors with *BAP1* mutations were more likely to have higher Fuhrman nuclear grades (3–4), but this was not quite statistically significant (p=0.052), whereas none of the remaining gene mutations was associated with Fuhrman grade (Figure 3). There were 12 tumors that underwent sarcomatoid de-differentiation; however, no associations between specific individual mutations with sarcomatoid changes were detected. (data not shown).

Mutation Impacts on Small Tumors

In terms of tumor sizes, there was no statistically significant association between mutation and size (Data not shown). Since small kidney tumors (≤4cm), incidentally found by imaging studies, are generally considered as less aggressive and might be reasonably managed with close monitoring, we wished to determine if there is a connection between underlying genetic abnormalities and pathologic stages in such tumors. Primary kidney tumors, less than 4cm, are classified as pathologic T1a when no aggressive pathologic features are observed. On the other hand, pathologic T3a tumors are those that grossly extend into the renal vein or muscle containing branches, or invade into sinus or perirenal fat. Overall, 69 tumors were smaller than 4cm, comprising 48 (pT1a) and 21 (pT3a) tumors. Remarkably, we discovered that small tumors with *PBRM1* mutations alone (n=12) were greater than 6 times more likely to be at pathologic T3a than those (n=9) with wild type *PBRM1* (OR 6.44 [1.8–24.7], p=0.001). Similarly, small tumors with mutations in *BAP1*, *SETD2* and/or *KDM5C* also exhibited a higher propensity to be at pT3a (OR 5.3 [1.2–28.8], p=0.03). In total, tumors with any of the *PBRM1*, *BAP1*, *SETD2* and/or *KDM5C* mutations were likely to be at higher stage (OR 10.3 [2.8–44.7], p<0.001), supporting the notion that chromatin dysfunction participates in disease progression.

Mutations and Metastasis

Based on our cohort and targeted genetic studies, there was no statistically significant association between specific mutations of chromatin modulators and the presence or the subsequent development of metastasis. Nevertheless, 27% and 29% of patients with either *BAP1* or *SETD2* mutations presented with or developed metastasis, compared to 18% of metastasis in the entire cohort (p value not statistically significant, Supplemental Table 5).

Mutations and Survival

Survival analysis was limited by the number of events at the time of analysis. However, *BAP1* mutations were significantly associated with worse survival outcomes on Kaplan Meier analysis (Fig 4; p=0.013 permutation log rank). On the contrary, there was no statistically significant association between other mutations and survival.

Discussion

Although inactivation of the *VHL* gene is the most prevalent (upwards of 90%) genetic alteration based on certain reports and likely represents the primary initiating event for the pathogenesis of ccRCC, numerous lines of evidence indicate that the loss of *VHL* alone is insufficient to cause ccRCC and provides neither prognostic nor therapeutic prediction values (19, 20). Consistent with these notions, our study identified *VHL* mutations in nearly half of our cohort and detected no prognostic significance. Genetic loss of heterozygosity studies of ccRCC have implicated the chromosome 3p21 locus as a region harboring additional tumor suppressors besides *VHL* (21). Three large-scale genomic studies have identified several recurrently mutated genes in ccRCC, including *PBRM1*, *BAP1*, *SETD2* and *KDM5C*. Remarkably, all have important roles in the epigenetic control of gene expression, and three of them (*PBRM1*, *BAP1* and *SETD2*) are closely situated in the frequently lost 3p21 locus. Hence, our current study focused on addressing the clinical and/or pathologic significance of mutations in this newly identified class of candidate tumor suppressors.

In agreement with the recent reports, our data validate prevalent mutations of *PBRM1*, positioning it as the second most commonly mutated gene in ccRCC, right behind *VHL*. The *PBRM1* gene encodes the Polybromo 1 (BAF180) protein, the chromatin targeting subunit of the Polybromo SWI/SNF complex (PBAF) (22). SWI/SNF complexes are large ATP-dependent chromatin-remodeling machines that mobilize nucleosomes along the DNA (23). Genetic data on *PBRM1* from both ours and others' indicate loss-of-function as the leading feature of tumor-derived *PBRM1* mutations. However, how dysfunction of these *SWI/SNF* genes contributes to tumorigenesis is largely unknown. Nevertheless, our clinical and pathologic interrogation provides an invaluable hint. We found that small tumors with PBRM1 mutations are 6 fold more likely to be categorized as pT3a instead of pT1a, suggesting a role of PBRM1 in bridling cell invasiveness. SETD2 is a histone H3 lysine 36 (H3K36) methyltransferase that regulates mRNA splicing and transcription elongation (24, 25); BAP1 interacts with and deubiquitinates host cell factor-1 (HCF-1), a transcription co-activator, that regulates cell proliferation (26); and KDM5C a histone 3 trimethyl-lysine 4 (H3K4Me3) demethylase that erase active transcription marks (27). Mutation rates of these three genes occur in 5–10% of ccRCC and most mutations are loss-of-function, supporting their roles as tumor suppressors. Despite the relatively low mutation incidences and short clinical follow up, we were able to deduce certain clinical/pathologic features associated with individual mutations. In terms of *SETD2*, we identified 11 loss-of-function and 3 low impact missense mutations(18). Among the 11 tumors bearing *SETD2* loss-of-function mutations, 10 occurred in stage 3+, of which 2 presented with and 2 subsequently developed metastatic diseases. In total, the overall metastatic rate of patients with *SETD2* mutated primary tumors is 36% (4/11), implicating a functional connection between *SETD2* and cancer metastasis. With respect to *BAP1* mutations, we demonstrated associations with high Fuhrman nuclear grades and worse cancer specific survivals, underscoring its tumor suppressor role in both mesothelioma and uveal melanoma. In regard to the *KDM5C* mutation alone, we were unable to detect significant associations with specific clinical/pathologic states, indicating that a larger cohort and a longer follow up may be required to unveil its role in the pathogenesis of ccRCC.

Limitations of this study include the lack of a significant number of cancer specific outcomes and gene expression. Nevertheless, the majority of mutations in *VHL*, *PBRM1* and *SETD2* are nonsense/frameshift truncating mutants and invariably cause loss of the protein function/product, as do the essential splice site mutations we identified in *BAP1*. While there is a fair amount of missense mutations in *BAP1* and *KMD5C*, we use the validated MUTATIONASSESSOR computational algorithm to predict the functional impact of these mutations (Supplemental table 2). This analytic filter suggests that the mutations are functionally deleterious.. Furthermore, we were unable to address the issue of epigenetic gene silencing through mechanisms such as methylation or polyadenylation. However, publically available, unpublished data from the Cancer Genome Atlas project (TCGA) does not implicate any of these genes as frequently methylated other than *VHL*.

Rationales underlying mechanism-based therapeutics in advanced and metastatic ccRCC have evolved predominantly around the VHL/HIF angiogenic axis. The subsequently proven clinical benefit of administering anti-angiogenic agents to ccRCC patients has led to the approved first-line use of such agents, including Sunitinib and Pazopanib. However, despite these mark strides against this deadly disease, most advanced-stage patients eventually succumb to their illness, urging for the development of new targeting strategies. Data thus far demonstrated that the involvement of this new class of tumor suppressors, i.e. chromatin modulators, in cancers is clearly beyond ccRCC. Thus, further clinical, pathologic and mechanistic interrogations likely yield novel therapeutic insights that impact diverse cancers.

Conclusions

To our knowledge, our data presents the first clinical/pathologic assessment of a novel class of tumor suppressors, consisting of chromatin modulating factors, in ccRCC. Our study indicates that mutations of *PBRM1*, *SETD2*, *BAP1* and/or *KDM5C* in kidney cancers are associated with advanced stage, grade and tumor invasiveness. Remarkably, small (<4cm) tumors with *PBRM1* mutations, the second most commonly mutated gene in ccRCC, are significantly linked to a higher tumor stage. Furthermore, *BAP1* mutations appear to associate with worse cancer specific survival. Further studies to validate clinical impacts and future therapies to target this new class of cancer contributory chromatin-modulating genes in ccRCC are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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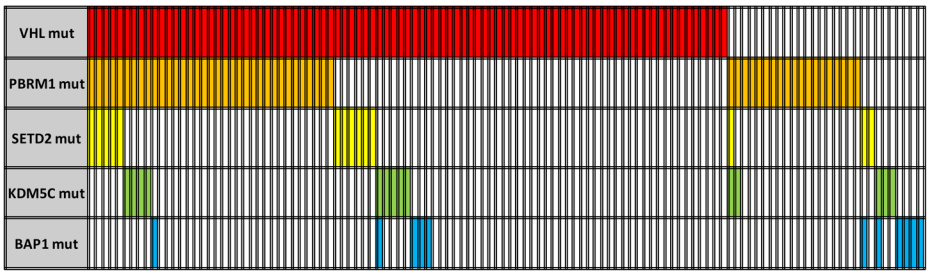


Figure 1.
Heat map of mutations in affected samples (65% – 119/185).

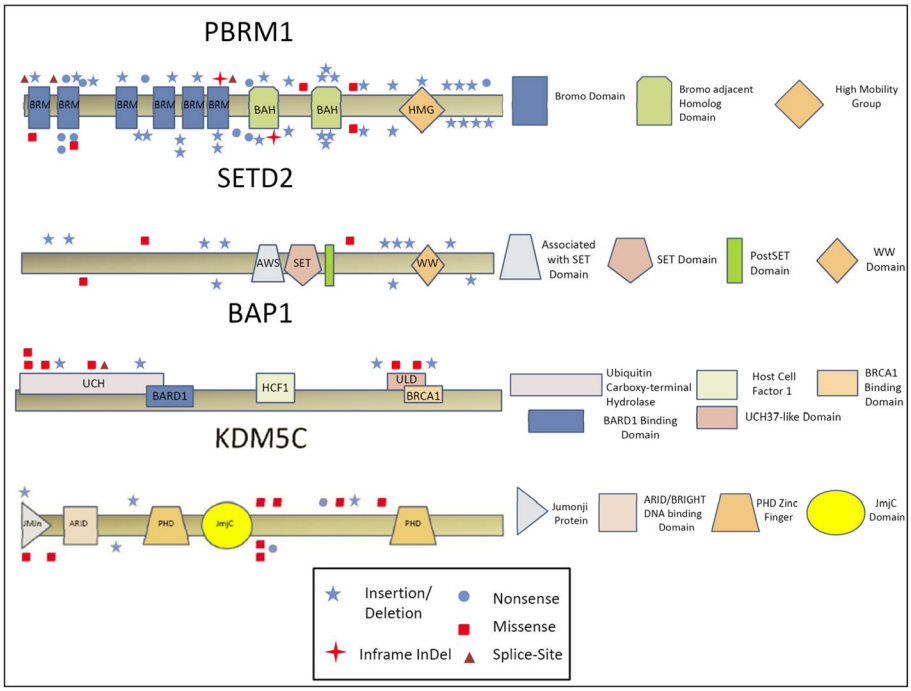
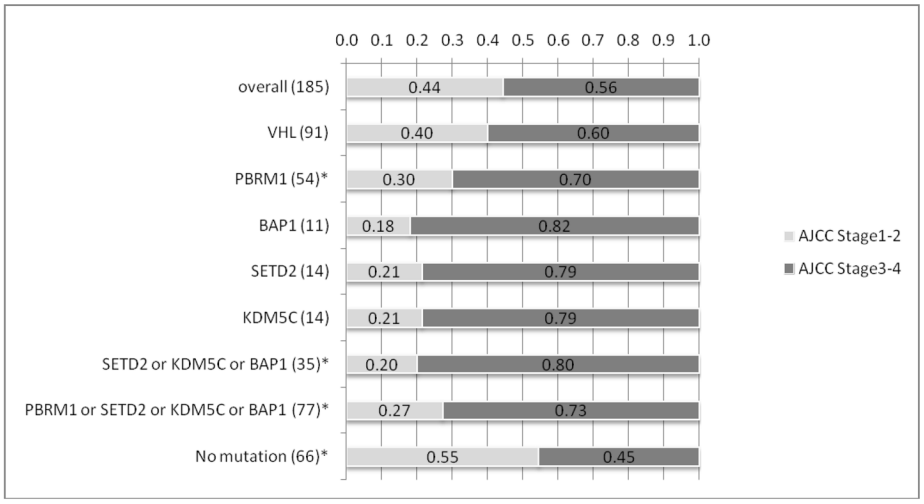


Figure 2.
Gene maps with mutation types, locations, and gene domains.

(A)



(B)

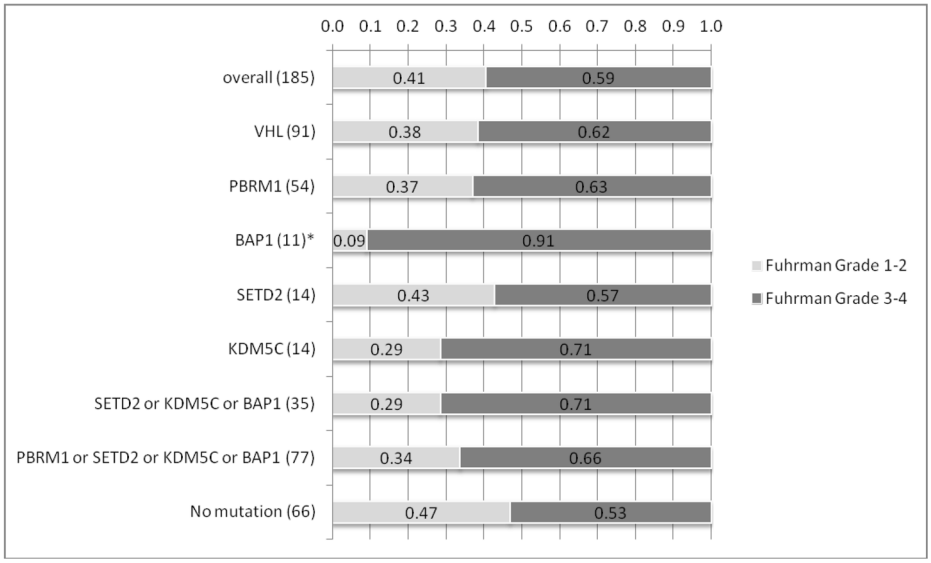
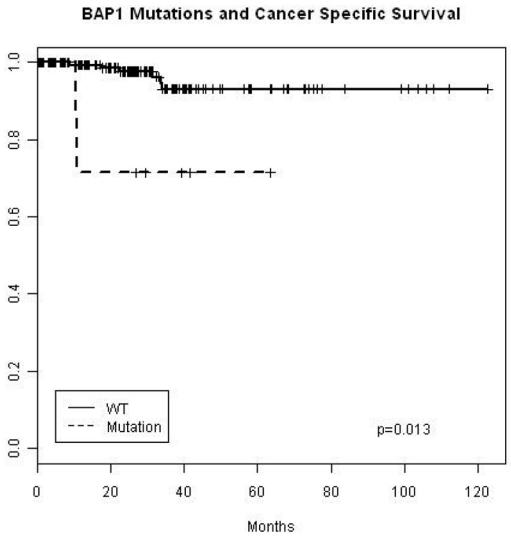


Figure 3.

(A) The associations of between individual mutations and mutation combinations and AJCC stages. (B) The associations of individual mutations and mutation combinations with Furhman nuclear grades. * Indicates statistical significance ($p<0.05$; Fischer's exact test).

(A)



(B)

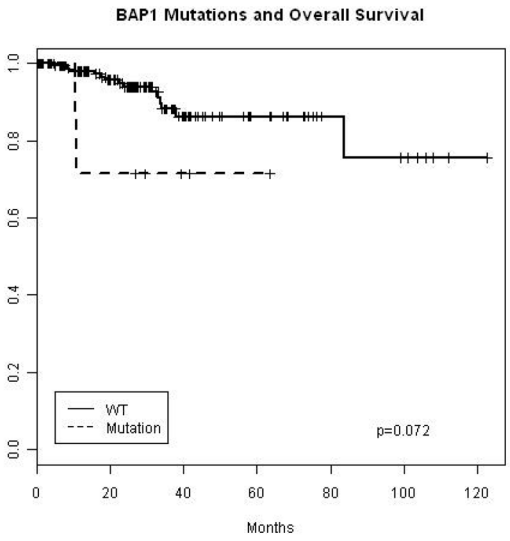


Figure 4.
Kaplan Meier survival plot for *BAP1* mutation and (A) cancer specific and (B) overall survival.

Table 1

Clinical and pathologic characteristics of the cohort.

Number of Patients	185
Age (Median, IQR)	61.4 (53.4,68.4)
Gender	Female–29% Male–71%
Race	White–90% Black–4% Other–6%
BMI	29.5 (25.6,34.1)
Labs	Hb–13.8 (12.5,14.7) Ca –9.3 (9,9.6)
Presentation	Incidental–77% Local–18% Systemic–5%
Pathologic Stage	T1a–25.9% T1b–13.5% T2a/b–7% T3–51.4% T4–2.2%
AJCC Clinical Stage	1–38.4% 2–5.9% 3–43.8% 4–11.9%
Fuhrman Nuclear Grade	1–0.5% 2–40% 3–48% 4–11.5%
Tumor Size (cm) (median, IQR)	5 (3.3,8.2)
Metastasis Metastasis at Presentation De Novo Metastasis Contralateral Recurrence	18% (33) 11.5% (21) 6.5%(12) 1%(2)
Followup Survivors (months) – average	31 (1–123)
Death Death from RCC	17 (9%) 10 (5.4%)

Table 2

Pathologic stages (pT1a to pT3a) in tumors ≤4cm. Statistically significant findings are bolded (p<0.05; Fischer's exact test).

Mutation	*VHL*	*PBRM1*	*BAP1*	*SETD2*	*KDM5C*	*BAP1* or *SETD2* or *KDM5C*	*PBRM1* or *BAP1* or *SETD2* or *KDM5C*
Overall Frequency (n=185)	49%	29%	6%	8%	8%	19%	42%
Frequency in Tumors ≤4cm (n=69)	49%	29%	3%	9%	6%	16%	39%
Pathologic T1a (n=48)	21 (44%)	8 (17%)	1 (2%)	3 (6%)	1 (2%)	4 (8%)	11 (23%)
Pathologic T3a(n=21)	13 (62%)	12 (57%)	1 (5%)	3 (15%)	3 (15%)	7 (33%)	16 (76%)
Odds Ratio (CI)	2.1 (0.7 –6.9)	6.4 (1.8 –24.7)	2.3 (0.03 –188)	2.5 (0.3 –20.2)	7.6 (0.6 –418.6)	5.3 (1.2 –28.8)	10.3 (2.83–44.7)
	p=0.20	**p=0.001**	p=0.52	p=0.36	p=0.08	**p=0.03**	**p<0.001**