JACOBS PUBLISHERS

OPEN ACCESS

Jacobs Journal of Nephrology and Urology

Research Article

Concurrent Chromosome 3p Mutations Are Associated With Worse Prognosis in Clear Cell Renal Carcinoma in an Institutional Cohort and the Cancer Genome Atlas

Christopher G. Keith[1], Fei Lian[1], Shilpa Sreedharan[1], Priscilla E. Davidson[1], Kenneth Ogan[1], Viraj A. Master[1], John G. Pattaras[1], Kenneth J. Carney[1], Peter T. Nieh[1], Megan Yu[1], John Gaspar[2], Jeremy Goecks[2], David L. Roberts[3], Michael R. Rossi[4,5], Rebecca S. Arnold[1,6], John A. Petros[1,5,6,7*]

[1]*Department of Urology, Emory University, Atlanta, Georgia*

[2]*Computational Biology Institute, George Washington University, Washington, DC, USA*

[3]*Division of General Medicine and Geriatrics, Department of Internal Medicine, Emory University, Atlanta, Georgia*

[4]*Department of Radiation Oncology, Division of Cancer Biology, Emory University, Atlanta, Georgia*

[5]*Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia*

[6]*Department of Urology, Atlanta Veterans Affairs Medical Center, Atlanta, Georgia*

[7]*Department of Hematology and Medical Oncology, Emory University, Atlanta, Georgia*

**Corresponding author:* *Dr. John A. Petros, Department of Urology, Emory University, Atlanta, Georgia,*

E-mail: jpetros@u-picardie.fr

Received: *09-19-2016*

Accepted: *09-25-2017*

Published: *10-15-2017*

Abstract

Purpose: Genes most frequently mutated in clear cell renal cell carcinoma (ccRCC), including *VHL*, *BAP1*, *SETD2*, and *PBRM1*, are present on the short arm of chromosome 3. While studies have analyzed mutations in each of these genes, little is known regarding the prognostic significance of co-existing chromosome 3p mutations.

Materials and Methods: We developed a high-throughput sequencing protocol for ccRCC specimens using Fluidigm Access Array with Illumina MiSeq sequencing. Our institutional cohort of 21 patients and The Cancer Genome Atlas (TCGA) ccRCC dataset were probed for co-occurring mutations in *VHL*, *BAP1*, *SETD2*, and *PBRM1*. Outcomes were evaluated using log-rank analysis.

Results: All patients in our institutional cohort who died (6/21) had mutations in two or more of the four chromosome 3p genes. In the TCGA cohort, after excluding cases with mutations only in *VHL* and *PBRM1* (n=72), the patients with concurrent chromosome 3p gene mutations (n=62) had significantly decreased overall (3.26 v. 9.74 years; p=0.0001) and recurrence-free survival (2.10 v. 6.42 years; p=0.0001) relative to the rest of the cohort (n=362). The difference in survival was independent of total mutational frequency and *BAP1* mutations. In addition, among those with *BAP1* mutations, those with additional chromosome 3p mutations (n=28) had decreased overall survival (p=0.045) compared to others with *BAP1* mutations alone (n=14).

Conclusions: Patients with mutations in 2 or more chromosome 3p genes, excluding those with mutations exclusively in *VHL* and *PBRM1*, have significantly decreased overall and recurrence-free survival. These results may be useful for risk stratification in patients with ccRCC

Keywords: Renal Cell Carcinoma; Kidney Neoplasms; Chromosome 3; High-Throughput Nucleotide Sequencing

Abbreviations:

BAP1 - BRCA1-Associated Protein 1;
Ccrcc – Clear Cell Renal Cell Carcinoma;
Cfdna – Cell-Free DNA;
Chr3p – Chromosome 3p *PBRM1* – Polybromo-1;
SETD2 - [Su (Var) 3–9, Enhancer Of Zeste [E(Z)] And Trithorax; (Trx) Domain–Containing Protein 2;
TCGA – The Cancer Genome Atlas;
VHL - The Von Hippel-Lindau Tumor Suppressor Gene

Introduction

In the United States, kidney cancer contributed to an estimated 63,920 new cases and 13,860 deaths in 2014, making it the 7th most deadly cancer in the country [1]. Around 90% of such cases are renal cell carcinoma (RCC) [2]. Clear cell RCC (ccRCC) accounts for 70-75% of all RCC and around 90% of metastatic RCC cases [3]. As such, the vast majorities of the cost, morbidity, and mortality associated with kidney cancer are due to ccRCC.

Loss of the short arm of chromosome 3 (chr3p) frequently contributes to sporadic ccRCC [4-6]. In addition, at least one of four genes present on chr3p, *VHL* (Von Hippel-Lindau Tumor Suppressor, E3 Ubiquitin Protein Ligase) (3p25.3), *BAP1* (BRCA associated protein 1) (3p21.1), *SETD2* (SET domain containing 2) (3p21.31), and *PBRM1* (polybromo-1) (3p21), are mutated in the majority of ccRCC cases [7]. While *VHL* defects are present in the majority of ccRCC cases, *VHL* mutations do not independently negatively impact prognosis. *SETD2* mutations correlate with a significantly higher relapse rate in some studies, but not decreased overall survival [8]. *BAP1* mutations and absent *BAP1* protein expression both are independent risk factors for poor overall and recurrence-free survival in patients with ccRCC [8-11]. *PBRM1* mutations have been associated, in some series, with worse patient outcome [9, 12-14]. However, in direct comparisons, cases with *BAP1* mutations have significantly worse survival than those with *PBRM1* mutations [15].

While co-occurring mutations of the chr3p genes are well described, little is known about the impact of co-existing somatic mutations in these genes. Some concurrent chr3p mutations, such as *VHL* and *PBRM1*, *VHL* and *SETD2*, and *PBRM1* and *SETD2*, have been reported to occur with a greater frequency than expected, suggesting a possible cooperative role in tumorigenesis [16]. In addition, the small population of cases with mutations in both *BAP1* and *PBRM1* were reported to have worse prognosis and aggressive tumor features [15, 16]. However, co-existing mutations in *BAP1* and *PBRM1* are negatively selected for, occurring with a significantly lower than expected frequency, in ccRCC [15, 17, 18].

As mutations in individual chr3p genes impact prognosis, and patients with co-existing *BAP1* and *PBRM1* mutations likely have worse outcome, we chose to specifically address if co-occurring mutations in 2 or more of the 4 chr3p genes associated with ccRCC impact survival. Using a small institutional cohort and The Cancer Genome Atlas (TCGA) dataset, we explored the predictive value of co-existing somatic mutations in *VHL*, *BAP1*, *SETD2*, and/or *PBRM1* as part of a diagnostic assessment of ccRCC.

Materials and Methods

Patient and sample collection

This single-site study was approved by the Emory University Institutional Review Board. Patients undergoing nephrectomy for renal mass at Emory University Hospital were eligible for participation. All provided written informed consent. Tumor tissue samples were stored in liquid nitrogen prior to DNA extraction. Specimens were confirmed by a pathologist to be ccRCC with tumor cellularity no less than 60%.

Isolation of DNA

Genomic DNA from frozen tissue was isolated using the Qiagen Gentra Puregene Tissue kit. Leukocyte control DNA was isolated using Qiagen DNA Mini and Blood Mini kit. DNA quantification was assessed by Qubit (Thermo Fisher Scientific Inc., Waltham, MA) fluorometer.

Gene Selection

A gene sequencing panel was developed based on published literature and mutational databases including the Catalogue of Somatic Mutations in Cancer (COSMIC) and TCGA. A total of 14 genes (*BAP1*, BRAF, CDKN2A, FGFR3, KDM5C, KIT, MET, MUC4, NFE2L2, *PBRM1*, PIK3CA, *SETD2*, TP53, *VHL*) known to be mutated in RCC were chosen for the panel. Custom primer sets for amplicons covering the known coding regions of the majority of these genes, including *BAP1*, *PBRM1*, *SETD2*, and *VHL*, were designed for the Fluidigm Access Array using D3 software. This manuscript focuses exclusively on the mutational results of chr3p genes: *BAP1*, *PBRM1*, *SETD2*, and *VHL*.

Library Prep and Next Generation Sequencing

A total of 22 ccRCC fresh frozen tumor specimens from our institutional cohort were available for sequencing. Briefly, 50 ng of tumor DNA per sample was used per specimen for amplicon preparation according to the Fluidigm Access Array standard protocol (DOI: http://dx.doi.org/10.1016/j.jmoldx.2015.04.008, DOI: http://dx.doi.org/10.1016/j.jmoldx.2015.04.008). Amplicon libraries were quantitated using Q-PCR and Agilent Bioanalyzer and run on the Illumina MiSeq instrument using the v2 kit. FASTQ data files for each

specimen were analyzed using a combination of the amplicon-aware tools, the GEMINI tool suite (DOI: 10.1371/journal.pcbi.1003153), and a local Galaxy server (DOI: 10.1093/nar/gkw343) at George Washington University. Read alignment and variant calling were done using the amplicon-aware tools, and GEMINI was used for variant annotation and filtering. A Galaxy workflow was created to run the entire analysis pipeline automatically.

TCGA data analysis

Data from the TCGA dataset Kidney Renal Clear Cell Carcinoma "sequenced tumors" (TCGA, Nature 2013) (DOI: 10.1038/nature12222) was analyzed using cBioportal (DOI: 10.1158/2159-8290, DOI: 10.1126/scisignal.2004088). The initial query included *VHL*, *PBRM1*, *SETD2* and *BAP1* to assess mutation exclusivity and co-occurrence. Overall and recurrence-free survival status was obtained based on clinical data downloaded from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp). Overall and recurrence-free survival data was compared using log-rank survival analysis of Kaplan-Meier survival curves in JMP 12 (JMP®, Version 12. SAS Institute Inc., Cary, NC) with median survival analyzed.

Analysis of mutational frequency in 100 most commonly mutated genes in ccRCC

To analyze whether prognostic information obtained by the "high-risk chr3p" delineation was due to overall increased mutational frequency, the 100 most significantly mutated genes in TCGA ccRCC, as published by TCGA Research Network (Supplementary Data file S3) were analyzed [7]. Comparisons of mutational frequency were conducted using the two-tailed T-test. Subsequently, survival analysis with matched mutational frequency was performed by stepwise removal of all cases with a given number of mutations in the 100 gene set, in ascending order, until there was no significant difference in mean number of mutations between groups. To further explore the impact of mutational frequency, we continued this stepwise removal for the "non-high-risk chr3p" group until it had a significantly higher degree of mutational frequency.

Multivariate analysis

Cox regression analysis was performed using IBM SPSS. For Cox regression, age was delineated by decade.

Results

Patient characteristics

Tumor samples and paired whole blood of 22 patients with ccRCC underwent deep sequencing. One patient (SS039) was lost to follow-up within a week of surgery. The remaining 21 patients had a mean post-operative follow-up of 52.5 months. Of the 21 patients, 4 were pathologically staged as metastatic at the time of surgery, and an additional 5 later developed metastatic disease (Table 1).

Impact of Chromosome 3p Mutations on Prognosis

In order to assess the impact of chr3p concurrent mutations, we divided the cohort into those with mutations in 2 or more of the chr3p genes compared to those with one or none. Representative amplicon coverage is shown in Figure 1. All 4 cases that were metastatic at time of surgery, 4 of the 5 patients subsequently diagnosed with metastatic spread, and all 6 patients who died within the follow-up period had mutations in 2 or more of these chr3p genes (Table 2). The difference in overall survival (p=0.027), but not grade (p=0.68), stage (p=0.43), or age (p=0.48), was significantly different between the two groups. Individual mutations are shown in Figure 2.

Mutational data in TCGA

To further explore patient outcomes with co-existing mutations in these four chr3p genes, we queried a published TCGA ccRCC dataset. Mutation results were available from 424 patients. At least one of the four genes was mutated in 72% of cases, with *VHL* in 51%, *PBRM1* in 36%, *SETD2* in 13%, and *BAP1* in 10% (Figure 3). *VHL* and *PBRM1* mutations had a tendency towards co-occurrence (p=0.003), *SETD2* and *PBRM1* had a tendency towards co-occurrence (p=0.013), and *BAP1* and *PBRM1* had a tendency towards mutual exclusivity (p=0.025).

Impact of concurrent chr3p mutations in TCGA

Of the TCGA cohort, 31.6% (n=134) had concurrent mutations in *VHL*, *PBRM1*, *SETD2*, and/or *BAP1*. The decrease in overall survival between those with mutations in 2 or more chr3p genes (n=134) compared to the rest of the ccRCC TCGA population (n=290) did not reach significance (median survival 7.02 v. 9.90 years; p=0.10). A similar trend was found for recurrence-free survival (4.73 v. 6.42 years; p=0.13). However, subgroup analysis revealed that cases with only *VHL* and *PBRM1* mutations (n=72) had significantly improved overall (3.26 v. 9.74 years; p=0.0012) and recurrence-free survival (2.10 v. 6.44 years; p=0.0007) compared to all other cases with mutations in two or more chr3p genes (n=62). After excluding those with only *VHL* and *PBRM1* mutations, the remaining group with mutations in 2 or more chr3p genes (n=62) had significantly decreased overall (3.26 v. 9.74 years; p=0.0001) and recurrence-free survival (2.10 v. 6.42 years; p=0.0001) relative to the rest of the TCGA cohort (n=362) (Figure 4).

Patient	Fuhrman Grade	Stage	Greatest tumor dimension(cm)	Age at Surgery	Last follow-up visit(months post-op)	Patient deceased (months post-op)	Metastatic spread
FL03	2	pT1N0MX	2.5	59.8	96.4		
SS044	2	pT1aNXMX	3	73.2	99.4		
FL14	2	pT1aNXMX	3.3	53.8	24		
SS018	2	pT1bNXMX	6	55.9	19.5		
SS039	2	pT1bNXMX	4.7	59.1	0.1		
SS045	2	pT1bNXMX	4.5	66.8	117.9		
FL06	2	pT1bNXMX	6.7	42.9	92.4		
FL05	4	pT1bNXMX	6.5	69.3	84.1		
FL12	2	pT2NXMX	7.2	81.3	71.1		
SS050	3	pT2NXMX	8.5	89.3	18.4		
FL17	3	pT3NXM1	13.4	64.3	3.1	5.8	yes*
FL13	2	pT3aNXMX	9.5	59.9	44.5		
SS031	3	pT3aNXMX	11.5	38.2	1.9	3.4	yes
FL16	3	pT3aN0M1	13	60.9	124.3		yes*
FL07	3	pT3aNXM1	12.5	72.2	48.2	48.2	yes*
SS026	3	pT3bN0MX	16	64.6	10.7		
SS033	3	pT3bN0MX	11.2	61.2	59		yes
SS034	3	pT3bN0MX	8	38.6	103.1		yes
FL04	3	pT3bNXMX	2.5	71	62.3		
SS035	3	pT3bNXM1	11.1	59.9	37.9	41.8	yes*
FL09	3	pT3cN0MX	18	75.3	11.5	26.3	yes
SS019	4	pT3cN1MX	11.5	69.1	1	6.5	yes

Clinical data for the 22 patient institutional cohort sorted by stage. Date of death was determined by analyzing the social security death index (SSDI) and electronic medical record for each patient. *indicates patient staged as metastatic (M1) at the time of surgery.

Table 1. Patient Characteristics

Patient	Gene	Position	Mutation type	AA change	Allele frequency
FL03	-	-	-	-	-
SS044	BAP1	52442574	nonsynonymous SNV	R57Q	9%
	PBRM1	52610581	frameshift deletion	T1198fs	42%
	VHL	10191558	nonsynonymous SNV	L184R	33%
FL14	PBRM1	52651424	frameshift deletion	Y558fs	8%
SS018	BAP1	52437676	nonsynonymous SNV	T495M	6%
	PBRM1	52643921	stopgain SNV	Q659X	24%
	VHL	10183795	nonsynonymous SNV	L87R	49%
SS039	-	-	-	-	-
SS045	BAP1	52439222	nonsynonymous SNV	G340D	7%
	BAP1	52439296	nonsynonymous SNV	A316T	11%
	SETD2	47084146	nonsynonymous SNV	P2381L	8%
	SETD2	47098402	nonsynonymous SNV	S2291F	11%
	SETD2	47164958	nonsynonymous SNV	V390I	8%
	SETD2	47165632	nonsynonymous SNV	S165P	8%
FL06	VHL	10191493	inframe deletion	Q164_V165del	36%
FL05	-	-	-	-	-
FL12	PBRM1	52584818	frameshift deletion	G1435fs	48%
	VHL	10188194	splice variant	splice	30%
SS050	VHL	10183776	nonsynonymous SNV	R82P	16%
FL17*	PBRM1	52696187	stopgain SNV	E164X	28%
	VHL	10188253	frameshift deletion	T133fs	12%
FL13	-	-	-	-	-
SS031*	PBRM1	52643380	frameshift deletion	L839fs	39%
	VHL	10191488	stopgain SNV	R161X	21%
	VHL	10183804	nonsynonymous SNV	D92N	9%
FL16*	SETD2	47162766	frameshift insertion	I1120fs	31%
	VHL	10188287	frameshift deletion	Q145fs	32%
FL07*	PBRM1	52584512	frameshift insertion	E1501fs	36%
	SETD2	47163569	stopgain SNV	K853X	55%
	VHL	10188197	nonsynonymous SNV	G114A	47%
SS026	-	-	-	-	-
SS033*	SETD2	47162531	stopgain SNV	Q1199X	12%
	VHL	10183664	nonsynonymous SNV	P45S	13%
	VHL	10183744	frameshift deletion	Q73fs	22%
SS034*	BAP1	52442591	nonsynonymous SNV	W52G	19%
FL04	-	-	-	-	-

	SETD2	47158174	stopgain SNV	R1509X	52%
	VHL	10188258	frameshift deletion	L135fs	45%
FL09*	BAP1	52442078	nonsynonymous SNV	C91G	26%
	PBRM1	52712514	splice variant	splice	35%
SS019*	BAP1	52439852	stopgain SNV	S287X	23%
	VHL	10183724	nonsynonymous SNV	S65P	42%

Table 2. Mutations detected in tumor samples of 22 patients.

Somatic mutations detected in tumor samples of the 22 patients with ccRCC. Patients are sorted by stage as in Table 1. Mutation rate expressed as percent of mutations relative to whole blood. *Patients with metastatic disease and/or death in follow-up period. - = no mutation present. SNV = single nucleotide variant. AA = amino acid

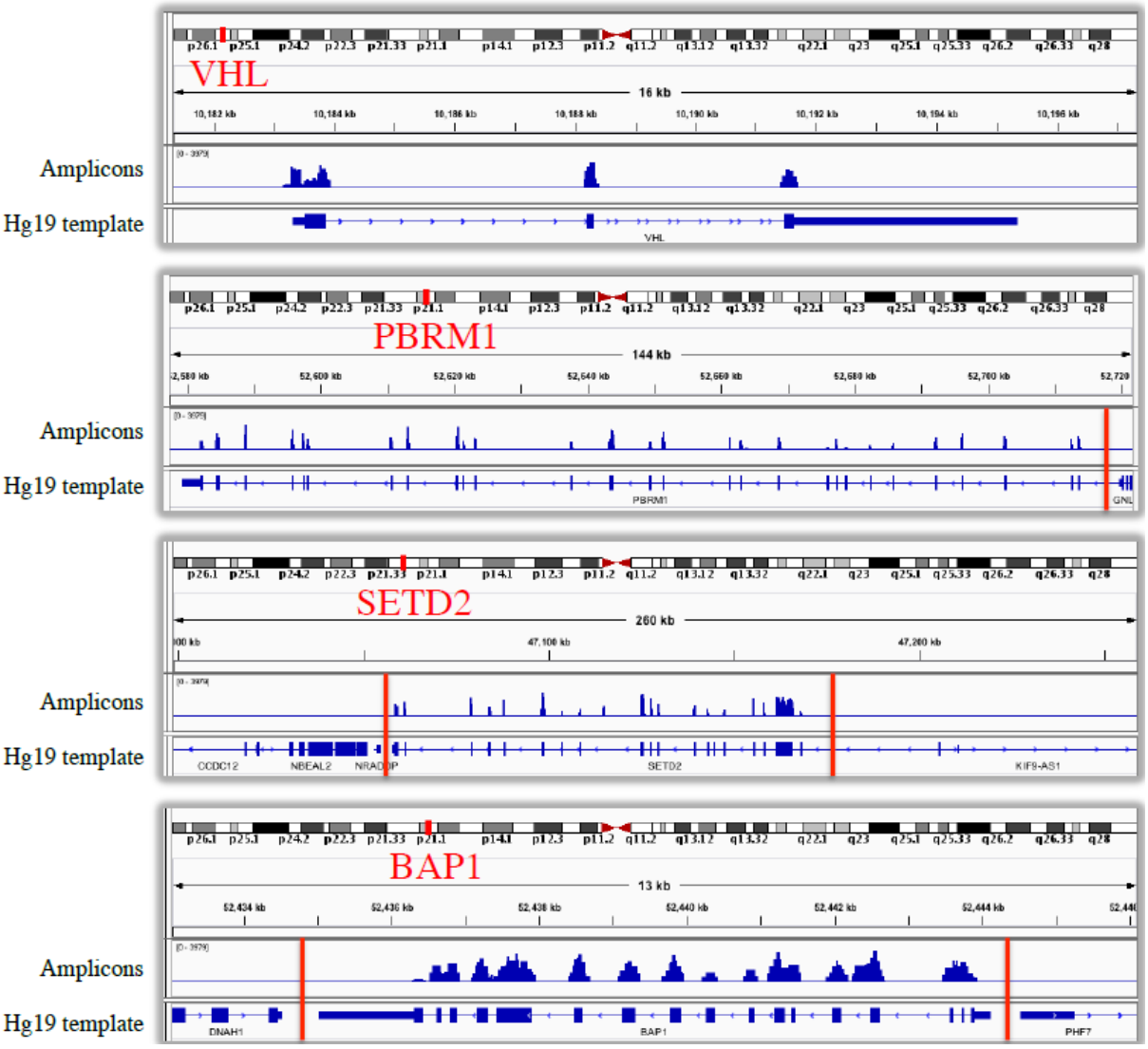
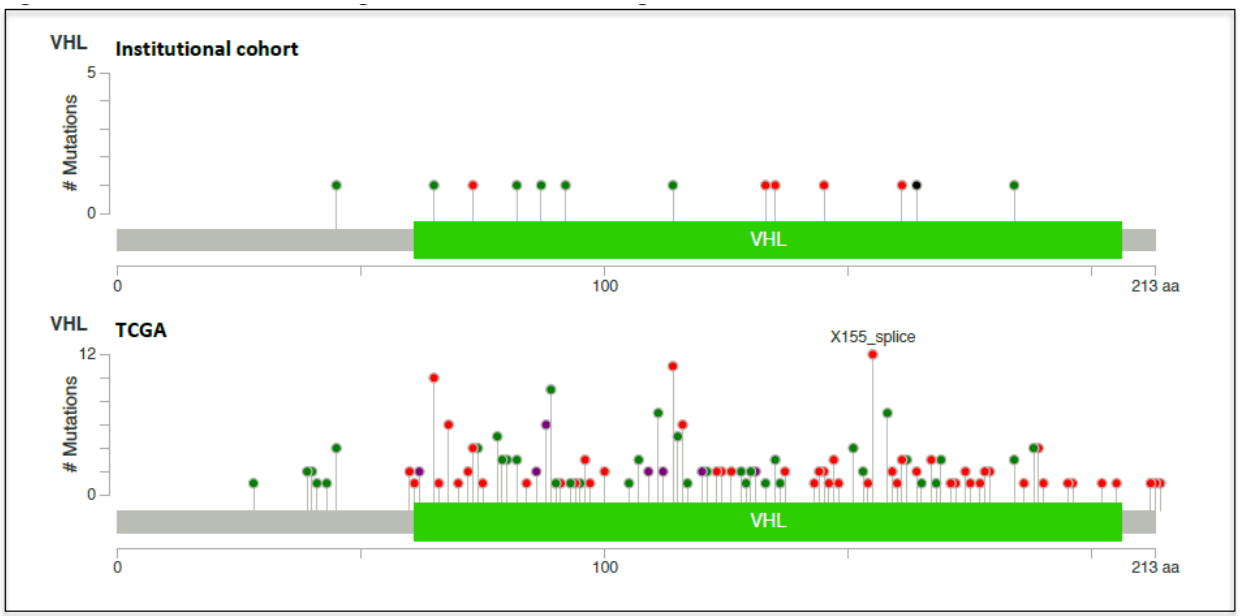
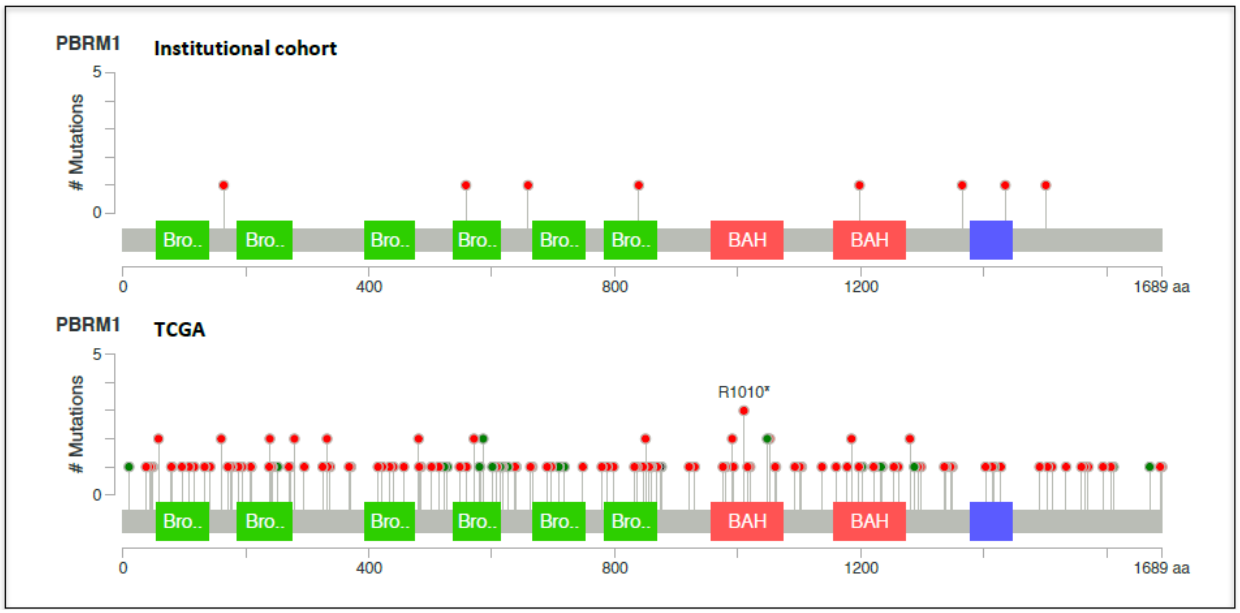
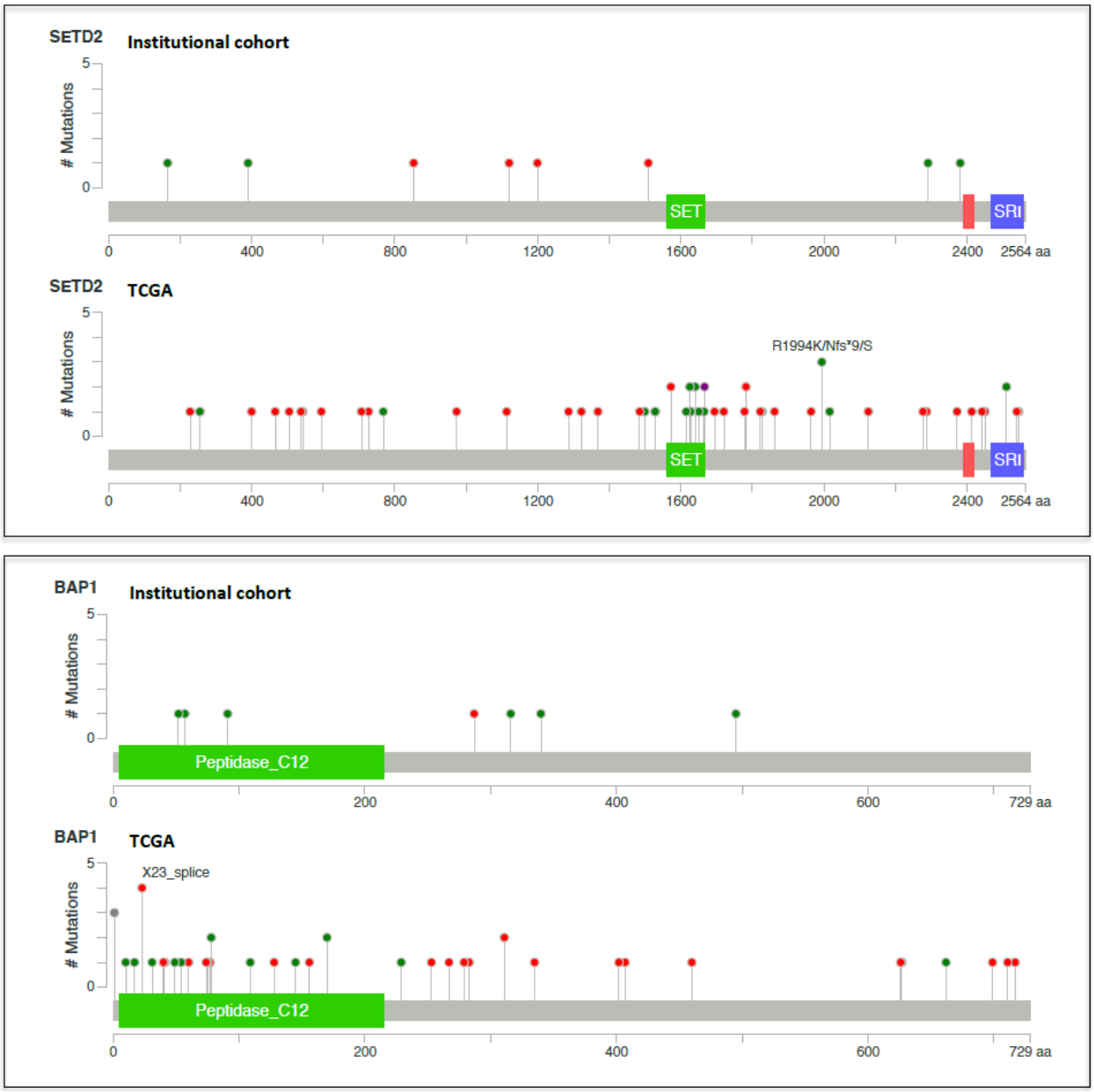


Figure 1. Representative primer coverage for chromosome 3p genes.

Representative exon coverage of the gene panel for the four chromosome 3p genes based on human genome 19 (hg19) in Integrated Genome Viewer (IGV). Primer amplicon read coverage is shown above chromosome 3p exome structure. When images contain multiple genes, edges of the specified genes are demarcated with red vertical lines.

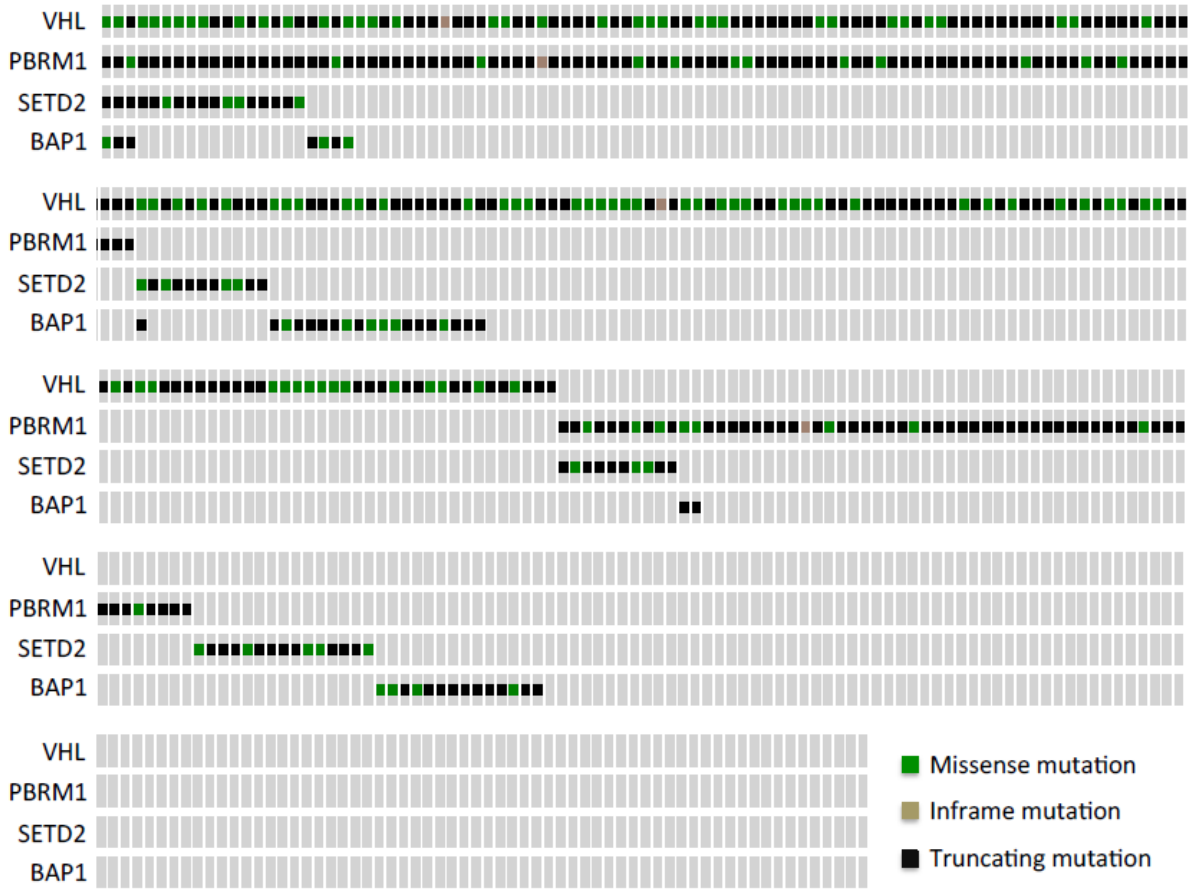

VHL
Institutional cohort
Mutations
5
0
VHL
0
100
213 aa
VHL
TCGA
X155_splice
Mutations
12
0
VHL
0
100
213 aa


PBRM1
Institutional cohort
Mutations
5
0
Bro..
Bro..
Bro..
Bro..
Bro..
Bro..
BAH
BAH
0
400
800
1200
1689 aa
PBRM1
TCGA
R1010*
Mutations
5
0
Bro..
Bro..
Bro..
Bro..
Bro..
Bro..
BAH
BAH
0
400
800
1200
1689 aa



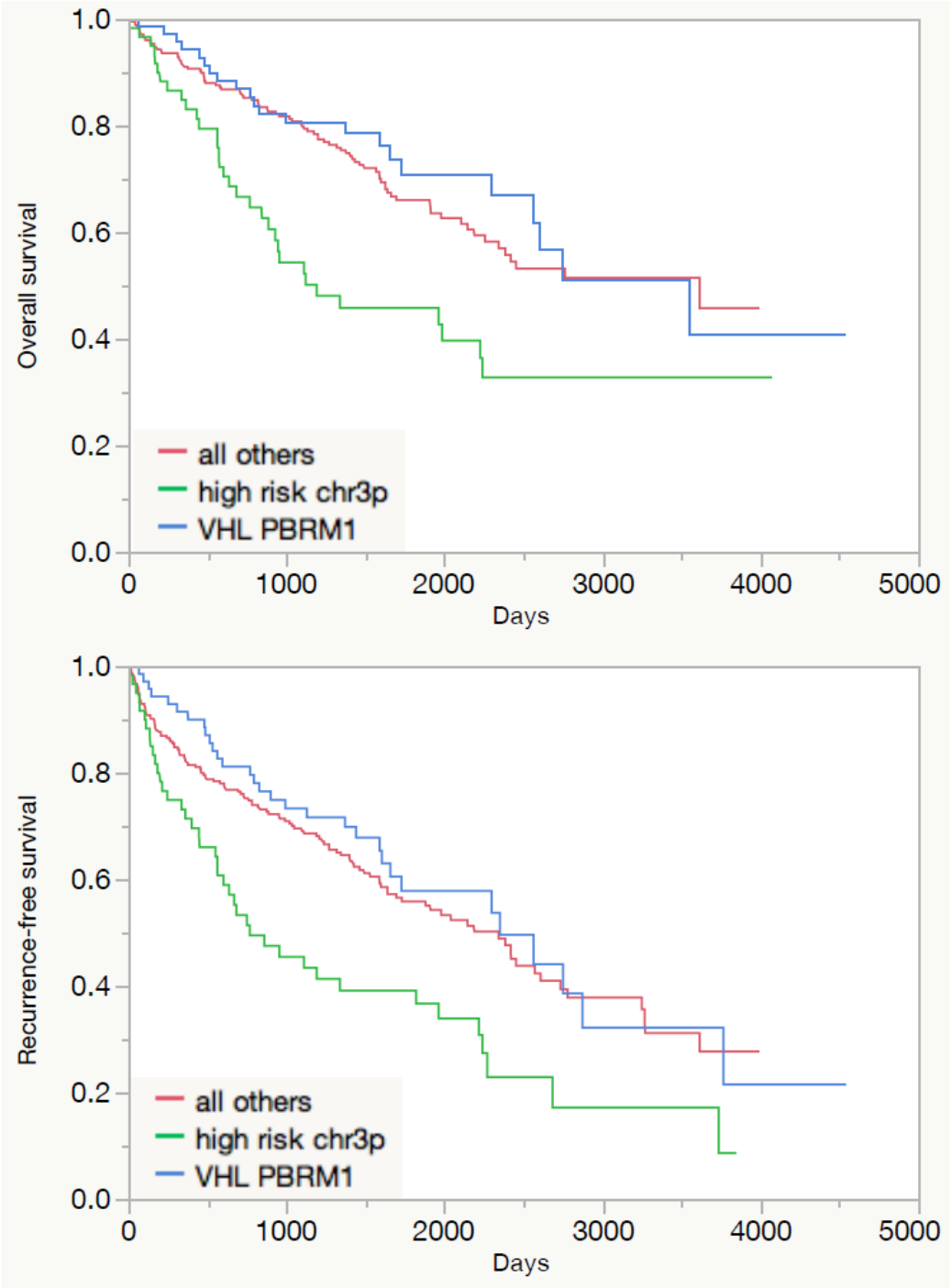
Mutational plot of chromosome 3p somatic mutations detected in tumor samples in the institutional cohort and TCGA. The x-axis corresponds to amino acid (aa) position, and the y-axis corresponds to total number of mutations. Green circles correspond to missense mutations, red to truncating mutations (including nonsense mutations and frameshift insertions/deletions), black to inframe insertions/deletions, and purple to sites affected by different mutation types at the same proportion. Protein domains are demonstrated by green, red, and purple boxes.

Figure 2. Somatic Chromosome 3p mutations in tumor samples.



Distribution of chromosome 3p gene mutations in The Cancer Genome Atlas produced using oncoprint available at <http://www.cbioportal.org/>. All individual cases are represented in the 5 rows. The first case depicted has mutations in all 4 genes, with missense mutations in VHL and BAP1 and truncating mutations in *PBRM1* and *SETD2*, and each case is reported sequentially based on mutation profile.

Figure 3. Chromosome 3p mutations in the Cancer Genome Atlas.



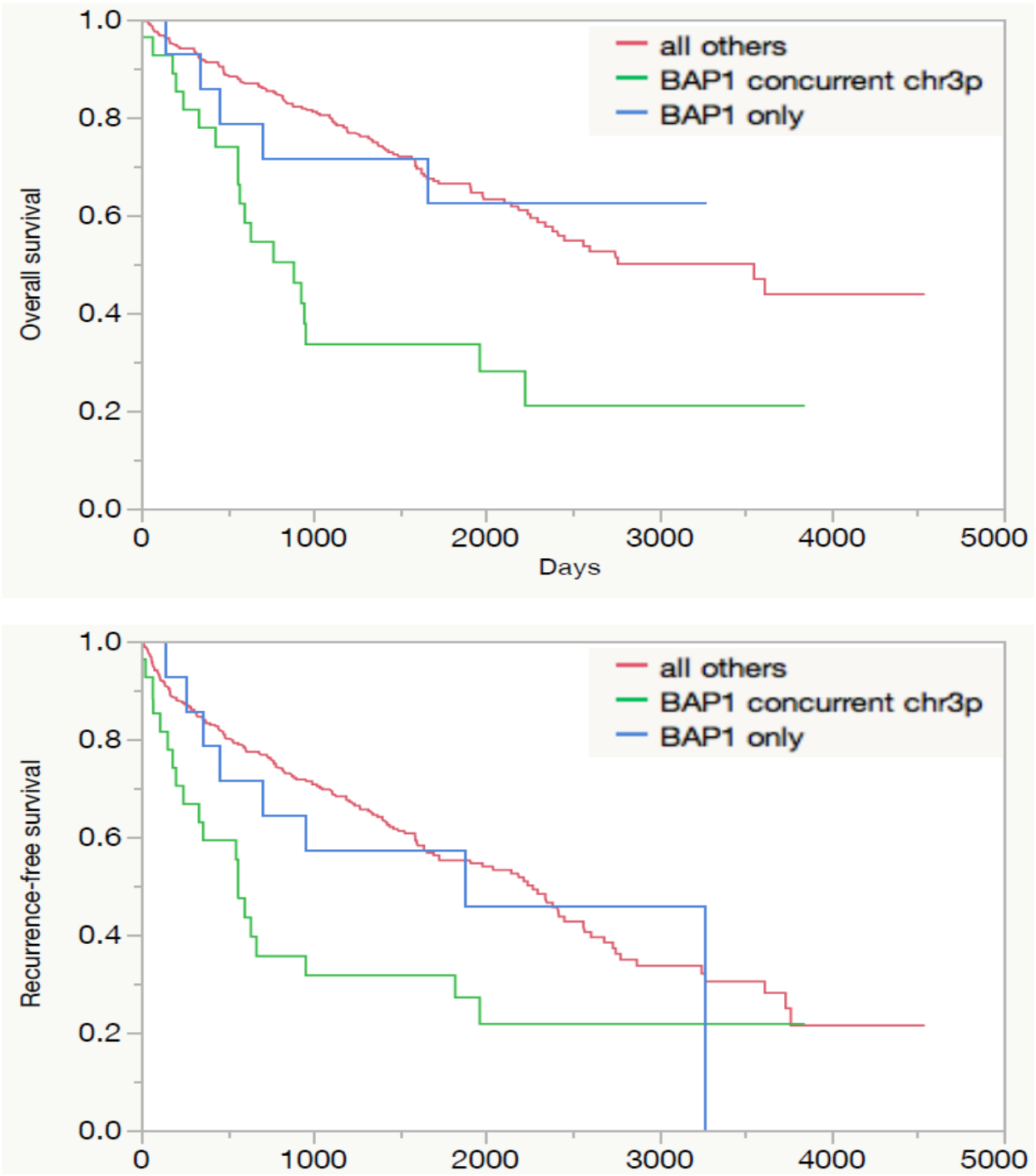
Survival curves comparing patients with only VHL and PBRM1 concurrent mutations (n=72), all other cases with 2 or more concurrent chromosome 3p mutations (n=62), and all other TCGA cases (n=290). The difference in overall and recurrence-free survival for the 2+ chr3p mutations (excluding *VHL* and *PBRM1*) compared to both other groups is significant ($p<0.01$). Markers for loss to follow-up are removed for better visualization of survival curves

Figure 4. Concurrent chromosome 3p mutations (excluding only *VHL* and *PBRM1* concurrent mutations) are associated with worse overall and recurrence-free survival in TCGA

Overall survival				
	Univariate analysis		Multivariate analysis	
Covariates	HR (95% CI)	p value	HR (95% CI)	p value
High-risk chr3p	2.11 (1.43-3.12)	<0.001*	1.91 (1.19-3.05)	0.007*
BAP1 mutation	2.13 (1.37-3.30)	0.001*	1.59 (0.98-2.59)	0.06
Mutational frequency	1.11 (1.01-1.23)	0.04*	0.98 (0.88-1.09)	0.73
Age	1.03 (1.02-1.05)	<0.001*	1.04 (1.02-1.05)	<0.001*
Recurrence-free survival				
	Univariate analysis		Multivariate analysis	
Covariates	HR (95% CI)	p value	HR (95% CI)	p value
High-risk chr3p	1.93 (1.37-2.73)	<0.001*	1.84 (1.21-2.80)	0.004*
BAP1 mutation	1.76(1.18-2.63)	0.006*	1.32 (0.84-2.06)	0.23
Mutational frequency	1.08 (0.99-1.18)	0.07	0.98 (0.89-1.07)	0.6
Age	1.02 (1.01-1.04)	<0.001*	1.03 (1.01-1.04)	<0.001*

Cox regression (proportional hazards) was used to assess the impact of high-risk chr3p status, *BAP1* mutations, mutational frequency, and age (by decade) on overall and recurrence-free survival. High-risk chr3p cases included those with 2 or more concurrent mutations in *VHL*, *PBRM1*, *SETD2*, or *BAP1*, with the exception of cases with only mutations in *VHL* and *PBRM1*. The BAP1 mutations category included any case with one or multiple BAP1 mutations. Mutational frequency was quantified by the total number of mutations in the 100 most significantly mutated ccRCC genes [7] in the ccRCC TCGA cohort. HR=hazard ratio, 95% CI=95% confidence interval. *p-value < 0.05

Table 3. Negative prognosis with high-risk concurrent chr3p mutations independent of BAP1 mutations, mutational burden, and age.



Overall and recurrence-free survival curves of TCGA ccRCC data for patients with BAP1 mutations with (n=28) or without (n=14) concurrent mutations in *VHL, BAP1* and/or *SETD2*. The difference is significant in overall (p=0.045) but not recurrence-free survival (p=0.15). Markers for loss to follow-up are removed for better visualization of survival curves.

Figure 5. Survival of patients with *BAP1* mutations with or without concurrent chr3p mutations.

Impact of BAP1 mutations on high-risk chromosome 3p group

As *BAP1* is the only chr3p gene in which mutations consistently have worse prognosis in ccRCC, we assessed whether poor prognosis in the concurrent chr3p group could be attributed to *BAP1* mutations. Corroborating the results of previous studies, patients with *BAP1* mutation (n=42) had decreased overall (2.59 v. 9.74 years; p=0.0006) and recurrence-free survival (1.83 v. 6.22 years; p=0.005) compared to the rest of the cohort (n=382). However, patients with only *BAP1* mutations (n=14) had significantly better overall survival (p=0.045) compared to those with mutations in *BAP1* and at least one additional chr3p gene (n=28) (Figure 5). The difference in recurrence-free survival was not significant (p=0.15). When analyzing those with only *BAP1* mutations (n=14) to the rest of the cohort (n=410), there was no difference in overall (p=0.88) or recurrence-free survival (p=0.78). This trend persisted after excluding those with *BAP1* and concurrent chr3p mutations. However, those with *BAP1* and concurrent chr3p mutations (n=28) had significantly reduced overall (p<0.0001) and recurrence-free survival (p=0.0007) compared to the rest of the TCGA population (n=396).

Impact of BAP1 and PBRM1 co-existing mutations

As concurrent mutations in *BAP1* and *PBRM1* are the only concurrent chr3p mutations that have been described in the literature to have poor prognosis, we assessed the impact of tumors with this mutational profile. Only 2 cases had mutations in *BAP1* and *PBRM1*, but not in *VHL*1 or *SETD2*. Both had high grade (Fuhrman 4 for both), high stage (3 and 4) and poor survival (overall 2 days and 572 days). An additional 4 cases had mutations in *VHL*, *PBRM1*, and *BAP1*, with high stage (3 and 4), but not necessarily grade (grades 2, 2, and 4 with one not reported), 3 of the 4 patients having recurrence (68 to 549 days) and 2 of 4 deaths (68 and 927 days). All four genes were mutated in 3 cases, with 1 of 3 having disease recurrence and death. Interestingly, these cases with somatic mutations in all four genes were not necessarily high stage (stage 1, 2, and 4) or Fuhrman grade (2 and 3). When these 9 cases with concurrent *PBRM1* and *BAP1* mutations were excluded from analysis, the difference in overall (p=0.0005) and recurrence-free survival (p=0.0003) between the remaining high-risk chr3p group (n=53) and the rest of the cohort (n=362) remained significant. Impact of total mutational frequency on high-risk chromosome 3p prognosis.

Increased tumor mutational burden has a negative impact on prognosis in a variety of tumor types, and recently was shown to be involved in ccRCC in sarcomatoid transformation, a poor prognostic indicator [19]. In analyzing cases with mutations in 2 or more chr3p genes, it is quite possible to be selecting for cases with increased total mutational burden. To further explore this possibility, we analyzed the 100 most significantly mutated genes in ccRCC [7] as a marker for increased mutational frequency. We found more mutations in the high-risk chr3p group compared to all other cases (mean 3.89 v. 2.28; p<0.0001). Therefore, to exclude the possibility that we were simply assessing the impact of higher mutational frequency on survival, we analyzed the impact of high-risk concurrent chr3p status against cases with matched mutational frequency in these 100 genes [7]. The significant difference in overall (3.26 v. 7.54 years; p=0.006) and recurrence-free survival (2.10 v. 6.30 years; p=0.005) persisted when comparing the high-risk concurrent chr3p group (n=62) to others with matched mutational frequency (n=150) (mean 3.89 v. 3.68 mutations; p=0.34). Compared to TCGA cases with significantly increased mutational frequency (n=65) (mean 4.57 v. 3.89 mutations; p=0.0035), the high-risk chr3p cohort continued to have significantly worse recurrence-free survival (p=0.037) and a trend toward worse overall survival (p=0.052).

High-Risk Chr3p Status Independently Associated With Decreased Overall and Recurrence-Free Survival

Compared to the rest of the TCGA cohort, the high-risk group with mutations in 2 or more chr3p genes, excluding cases with only *VHL* and *PBRM1* mutations, (n=62) was associated with higher Fuhrman grade (p=0.0091) and T-stage (p=0.0025). There was no significant difference in gender (p=0.20), N-stage (p=0.34), M-stage (p=0.092) or age (p=0.069). However, the difference in age reached significance with one-tailed T-test (mean 63.0 v. 60.3 for high-risk v. all others; p=0.034).

Multivariate testing revealed high-risk concurrent chr3p mutations were associated with decreased overall and recurrence-free survival independent of *BAP1* mutations, mutational frequency, or age (Table 3). In addition, concurrent chr3p mutations had significantly worse overall survival (HR 1.69 [1.13-2.52]; p=0.01), but not recurrence-free survival (HR 1.28 [0.89-1.85]; p=0.18), independent of Fuhrman grade, T stage, N stage, and M stage.

Discussion

In this study, we explored the association of concurrent chr3p mutations with prognosis in patients with ccRCC. Our findings suggest that co-existing somatic mutations in *VHL*, *PBRM1*, *SETD2*, and/or *BAP1*, with the exception of those with only mutations in *VHL* and *PBRM1*, are predictive of poor prognosis in ccRCC. These results were exclusive of *BAP1* mutations, total mutational frequency, or age.

Mutations in individual chromosome 3p genes are well-known to play a role in oncogenesis and, for *BAP1* and *PBRM1* mutations, prognosis in ccRCC. *VHL* is the most frequently mutated gene in ccRCC and normally inhibits hypoxia-inducible genes

by binding, destabilizing, and ubiquitinating hypoxia-inducibleα (HI factor 1 - 1α), which plays a role in angiogenesis and proliferation [20]. However, independent *VHL* mutations do not negatively impact survival of patients with ccRCC. *PBRM1* encodes an essential protein in a chromatin-remodeling complex (the PBAF SWI/SNF complex), which is implicated in replication, transcription, and DNA repair [21]. *PBRM1* mutations are associated with, in some ccRCC studies, worse survival [9, 12-14]. *SETD2* is a histone methyltransferase that plays a crucial role in homologous recombination repair [22]. As such, *SETD2* depletion results in significantly increased microsatellite instability [23, 24]. In large cohorts of ccRCC, *SETD2* mutations are associated with a significantly higher relapse rate but do not impact overall survival [8]. *BAP1* plays a critical role in binding host cell factor-1 (HCF-1), which subsequently binds to multiple transcription factors and recruits histone-modifying enzymes [17]. Both *BAP1* somatic mutations and lack of *BAP1* protein expression are associated with significantly worse prognosis in ccRCC [8-11].

Interestingly, a recent report suggests that *BAP1* mutations in ccRCC may only negatively impact survival in females [25]. In the TCGA cohort analyzed in our paper, 72% of females and 59% of males with *BAP1* mutations had concurrent chr3p mutations. While this difference in percentage was not significant (p=0.51), it could perhaps partially account for this finding of significantly worse survival with *BAP1* mutations in females but not males.

Previously, little was published about the impact of co-existing mutations in these chr3p genes. It was reported that some chr3p genes had higher frequency of co-existing mutations that would be expected by chance alone [16]. However, the only co-existing chr3p mutations reported to negatively impact prognosis, cases with concurrent *BAP1* and *PBRM1* mutations, have a negative selection bias. After excluding cases with concurrent *BAP1* and *PBRM1* mutations, we found the significantly reduced overall and recurrence-free survival associated with concurrent high-risk chr3p mutations persisted.

It is unclear why, in the TCGA cohort, the *VHL* and PBMR1 group has significantly improved prognosis relative to other cases with concurrent chr3p mutations. The *VHL* and *PBRM1* group had survival tracking the prognosis of other TCGA cases without co-occurring chr3p mutations, suggesting that the negative synergism associated with other chr3p mutational combinations does not exist with *VHL* and *PBRM1*. Further studies will be necessary to explore these findings.

Limitations

Our institutional cohort was limited by sample size (n=21) and an inability to compare specific chr3p mutation profiles or match for mutation burden. As such, our observation that of the 21 patients, those with 2 or more chr3p mutations had significantly worse outcome may have been confounded by increased total mutation burden. However, with the TCGA data, we found the decreased survival associated with concurrent chr3p mutations was independent of mutational frequency.

In addition, ccRCC is known to possess significant intratumor genetic heterogeneity [26-28]. Somatic mutations in our cohort and the TCGA may therefore not be indicative of the full genetic profile of ccRCC tumors. In order to better account for such heterogeneity, a future study involving at least 3 tumor biopsy sites would be ideal [28].

Future Directions

Studies demonstrate potential clinical utility of circulating tumor DNA in detecting a number of advanced cancers, including ccRCC [29]. Future studies of cfDNA somatic mutations for renal cancer, a so called "liquid biopsy," could theoretically provide access to prognostic genetic information without need for invasive biopsy.

Conclusions

Here, we described a novel approach to ccRCC risk stratification based on concurrent chromosome 3p mutations. Based on initial findings of worse overall survival with multiple chr3p gene mutations in a 21 patient ccRCC cohort, we probed TCGA data to further explore this association. Patients with co-existing chr3p somatic mutations, excluding those with only mutations in *VHL* and *PBRM1*, had significantly reduced overall and recurrence-free survival. This group comprised 14.6% of the patient population, and the survival difference was exclusive of total mutational frequency or *BAP1* mutations. Genotyping ccRCC tumors using the novel prognostic information demonstrated herein could be a valuable tool in risk-stratification and therapeutic planning for patients with ccRCC.

Acknowledgements

The authors would like to thank 1) the patients who participated in the TCGA and institutional urologic bank, for without them research of this nature would not be possible; 2) Mr. Larry Williams (The Breckenridge Group) for his support of this research; 3) Evans County Cares Foundation; 4) Emory's John and Mary Brock Diagnostic and Discovery Fund; 5) Emory Integrated Genomics Core (EIGC). The results here are in whole or part based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/ and using tools available at http://www.cbioportal.org.

References

1. Surveillance, Epidemiology, and End Results (SEER) Program (http://www.seer.cancer.gov) Research Data (1973-2011). April 2014: National Cancer Institute, DCCPS, Surveillance Research Program, Surveillance Systems Branch.

2. Ljungberg B, SC Campbell, HY Choi et al. The epidemiology of renal cell carcinoma. Eur Urol. 2011, 60(4): 615-621.

3. Shuch B, A Amin, AJ Armstrong et al. Understanding pathologic variants of renal cell carcinoma: distilling therapeutic opportunities from biologic complexity. Eur Urol. 2015, 67(1): 85-97.

4. Ogawa O, Y Kakehi, K Ogawa et al. Allelic loss at chromosome 3p characterizes clear cell phenotype of renal cell carcinoma. Cancer Res. 1991, 51(3): 949-953.

5. Kovacs G, R Erlandsson, F Boldog et al. Consistent chromosome 3p deletion and loss of heterozygosity in renal cell carcinoma. Proceedings of the National Academy of Sciences of the United States of America. 1988, 85(5): 1571-1575.

6. Brugarolas J. Journal of Clinical Oncology. 2014, 32(18): 1968-1976.

7. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature. 2013, 499(7456): 43-49.

8. Sato Y, T Yoshizato, Y. Shiraishi, et al. Integrated molecular analysis of clear-cell renal cell carcinoma. Nat Genet. 2013, 45(8): 860-867.

9. Hakimi AA, YB Chen, J Wren et al. Clinical and pathologic impact of select chromatin-modulating tumor suppressors in clear cell renal cell carcinoma. Eur Urol. 2013, 63(5): 848-854.

10. Joseph RW, P Kapur, DJ Serie, et al. Loss of *BAP1* protein expression is an independent marker of poor prognosis in patients with low-risk clear cell renal cell carcinoma. Cancer. 2014, 120(7): 1059-1067.

11. Kapur P, A Christie, JD Raman, et al. *BAP1* immunohistochemistry predicts outcomes in a multi-institutional cohort with clear cell renal cell carcinoma. J Urol. 2014, 191(3): 603-610.

12. Pawlowski R, SM Muhl, T Sulser, et al. Loss of *PBRM1* expression is associated with renal cell carcinoma progression. Int J Cancer. 2013, 132(2): 11-7.

13. da Costa WH, M Rezende, FC Carneiro, et al. Polybromo-1 (*PBRM1*), a SWI/SNF complex subunit is a prognostic marker in clear cell renal cell carcinoma. BJU Int, 2014. 113(5b): 157-163.

14. Nam SJ, C Lee, JH Park, et al. Decreased *PBRM1* expression predicts unfavorable prognosis in patients with clear cell renal cell carcinoma. Urol Oncol. 2015, 38(8): 340.e9–340.e16.

15. Kapur P, S Pena-Llopis, A Christie, et al. Effects on survival of *BAP1* and *PBRM1* mutations in sporadic clear-cell renal-cell carcinoma: a retrospective analysis with independent validation. Lancet Oncol. 2013, 14(2): 159-167.

16. Hakimi AA, I Ostrovnaya, B Reva, et al. Adverse Outcomes in Clear Cell Renal Cell Carcinoma with Mutations of 3p21 Epigenetic Regulators *BAP1* and *SETD2*: a Report by MSKCC and the KIRC TCGA Research Network. Clinical cancer research. 2013, 19(12): 3259-3267.

17. Pena-Llopis S, S Vega-Rubin-de-Celis, A Liao, et al. *BAP1* loss defines a new class of renal cell carcinoma. Nat Genet. 2012, 44(7): 751-759.

18. Pena-Llopis S. A Christie, XJ Xie, et al. Cooperation and antagonism among cancer genes: the renal cancer paradigm. Cancer Res. 2013, 73(14): 4173-4179.

19. Bi M, S Zhao, JW Said, et al. Genomic characterization of sarcomatoid transformation in clear cell renal cell carcinoma. Proc Natl Acad Sci U S A. 2016, 113(8): 2170-2175.

20. Cohen HT, FJ McGovern. Renal-Cell Carcinoma. New England Journal of Medicine. 2005, 353(23): 2477-2490.

21. Varela I, P Tarpey, K Raine, et al. Exome sequencing identifies frequent mutation of the SWI/SNF complex gene *PBRM1* in renal carcinoma. Nature. 2011, 469(7331): 539-542.

22. Edmunds JW, LC Mahadevan, AL Clayton. Dynamic histone H3 methylation during gene induction: HYPB/*SETD2* mediates all H3K36 trimethylation. Embo j. 2008, 27(2): 406-420.

23. Pfister SX, S Ahrabi, LP Zalmas, et al. *SETD2*-dependent histone H3K36 trimethylation is required for homologous recombination repair and genome stability. Cell Rep. 2014, 7(6): 2006-2018.

24. Li F, G Mao, D Tong, et al. The histone mark H3K36me3 regulates human DNA mismatch repair through its interaction with MutSalpha. Cell. 2013, 153(3): 590-600.

25. Ricketts CJ, LW M. Gender Specific Mutation Incidence and Survival Associations in Clear Cell Renal Cell Carcinoma (ccRCC). PLoS One. 2015, 10(10): e0140257.

26. Gerlinger M, S Horswell, J Larkin, et al. Genomic architecture and evolution of clear cell renal cell carcinomas defined by multiregion sequencing. Nat Genet. 2014, 46(3): 225-233.

27. Gerlinger M, AJ Rowan, S Horswell, et al. Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing. New England Journal of Medicine. 2012, 366(10): 883-892.

28. Sankin A, AA Hakimi, N Mikkilineni, et al. The impact of genetic heterogeneity on biomarker development in kidney cancer assessed by multiregional sampling. Cancer Med. 2014, 3(6): 1485-1492.

29. Goessl C, M Muller, B Straub, et al. DNA alterations in body fluids as molecular tumor markers for urological malignancies. Eur Urol. 2002, 41(6): 668-676.