Whole-genome analysis of papillary kidney cancer finds significant non-coding alterations

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One Sentence Summary: The first detailed whole genome analysis of papillary kidney cancer and identifies several novel non-coding variants.

Abstract: Papillary renal cell carcinoma (pRCC) constitutes 10-15% of kidney tumors. Recent advances in DNA sequencing have significantly deepened our understanding of the molecular genetics of this disease. However, much of this work has been limited to coding alterations in traditional cancer driver genes. Moreover, despite identifying specific sub-groups of alterations, researchers cannot find a clear molecular etiology for a significant proportion of the tumors. To address this, we carry out the first whole genome analysis of pRCC. We take a comprehensive approach, trying to explain the cases lacking classic drivers. Traditionally, research in pRCC has focused on alterations in the *MET* gene. First, we elaborate on these results, validating a germline SNP in *MET* (rs11762213), predicting prognosis. Next, we analyze mutations in the non-coding regions throughout the entire genome. We discover several potentially impactful hotspots, including some in *MET* non-coding regions and inside a long non-coding RNA, *NEAT1*. The *NEAT1* mutations, moreover, are associated with increased expression and worse cancer-specific survival. Finally, we analyze genome-wide mutational patterns. We notice that these are dictated mostly by the prevalence of methylation-associated C-to-T transitions. Also, we observe significantly more mutations in open chromatin regions in tumors with chromatin modifier alterations.

Introduction

Renal cell carcinoma (RCC) makes up over 90% of kidney cancers and currently is the most lethal genitourinary malignancy (*1*). Papillary RCC (pRCC) accounts for 10%-15% of the total RCC cases (*2*). Unfortunately pRCC has been understudied and there are no current forms of effective systemic therapy for this disease. For many years, the only prominent oncogene in pRCC (specifically type 1) that physicians were able to identify was *MET*, a tyrosine kinase receptor for hepatic growth factor. An amino acid substitution that leads to constitutive activation and/or overexpression are two mechanisms of dysfunction of *MET* in tumorgenesis. Recently, the Cancer Genome Atlas (TCGA) published its first result on pRCC (*3*), which improves our understanding of the genomic basis of this disease. Several more genes and specific sub-clusters were identified to be significantly mutated in pRCC. Nevertheless, a significant portion of pRCC cases still remains "driver-unknown". Therefore we think it is the right time to explore the rest

98% of the genome with whole genome sequencing (WGS). Non-coding regions, previously overlooked in cancer, has been showed to be involved in tumorigenesis (*4-6*). Mutations in non-coding regions may cause disruptive changes in both cis- and trans-regulatory elements. Understanding non-coding mutations helps fill the missing "dark matter" in cancer research.

Looking at the mutations at a higher level, multiple endogenous and environmental mutation processes shape the somatic mutational landscape observed in cancers (*7*). Analyses of the associated genomic alterations give information of cancer development, shed light on mutational disparity between cancer subtypes and even indicates potential new treatment strategies (*8*). Additionally, genomic features such as replication time and chromatin environment govern mutation rate along the genome, contributing to spatial mutational heterogeneity. While identifying mutation signatures is possible using data from whole exome sequencing (WXS), whole genome sequencing (WGS) gives richer information on mutation landscape and minimizes the potential confounding effect of exome capture process and driver selection.

In this study, we comprehensively analyzed 32 pRCC WGS data along with an extensive set of WXS data in multiple levels. We went from microscopic examination of driver genes to analyses of whole genome sequencing variants and finally to investigation of high-order mutational features. First, we focused on *MET*, a proto-oncogene which play a central role in pRCC, especially in Type 1. For the first time we validated rs11762213, a germline exonic single nucleotide polymorphism inside *MET*, as a predictive SNP for cancer-specific survival (CSS) in pRCC. We also found several potentially impactful non-coding alternations around *MET* promoter and first two exons. Then we went onto cases not as easily explained as those with MET alterations. We analyzed nearly 150,000 non-coding mutations and found several potentially high-impact mutations in non-coding regions. Further zooming out, we discovered pRCC exhibits mutational heterogeneity in both nucleotide context and genome location, indicating underlying vibrant mutational processes interplay. Methylation is the leading factor influencing mutation landscape. Methylation status drives the intra-sample mutation variation by giving rise to more C>T mutations in the CpG context. APOBEC activity, although infrequently observed, leaves an unequivocal mutation signature in a pRCC genome but not in ccRCC.

Results

1.   Probing an exonic SNP in MET, rs11762213, in pRCC prognosis.

We begin with *MET* coding variants. Although many MET somatic mutations are believed to play a central role in pRCC, a germline SNP, rs11762213, has been discovered to predict recurrence and survival in a RCC cohort. ccRCC predominated the initial discovery RCC cohort (*9*). This conclusion was later validated in ccRCC cohort but never in pRCC (*10*). We evaluated whether this SNP has a prognostic effect in pRCC. Using an extensive WXS set of 207 patients (see Methods), we found 12 patients carry one risk allele of rs11762213 (G/A, Table 1). No homozygous A/A was observed. The cancer-specific survival is statistically significantly worse in patients with the risk allele (p < 0.037, Peto & Peto modification of the Gehan-Wilcoxon test; p < 0.044, log-rank test, Fig 1).

Genotype (rs11762213)	G/A (n = 12)	G/G (n = 195)
Sex, No. (%)		
Male (%)	7 (58)	142 (73)
Female (%)	5 (42)	53 (27)
Age, median (IQR), y	53 (46-59.5)	60 (53-69)
Race, No. (%)		
White	9 (75)	130 (67)
Black or African American	3 (25)	47 (24)
American Indian or Alaska native	0	2 (1)
Asian	0	5 (3)
NA	0	11 (6)
Tumor type, No. (%)		
Type 1	4 (33)	86 (44)
Type2	7 (58)	66 (34)
Unclassified	1 (8)	25 (13)
Not centrally reviewed	0	18 (9)
T stage, No. (%)		
T1	7 (58)	132 (68)
T2	2 (17)	18 (9)
T3	3 (25)	43 (22)
T4	0	2 (1)
N stage, No. (%)		
N0	5 (54)	32 (16)
N1	0	18 (9)
N2	1 (8)	2 (1)
NX	6 (50)	143 (74)
M stage, No. (%)		
M0	5 (42)	74 (38)
M1	1 (8)	6 (3)
MX/NA	6 (50)	115 (59)
AJCC stage, No. (%)		
I	7 (58)	125 (64)
II	1 (8)	11 (6)
III	2 (17)	39 (20)
IV	2 (17)	10 (5)
NA	0	10 (5)
Median follow-up for surviving patients, days (IQR)	266 (118-643)	493 (167-884)

AJCC: American Joint Committee on Cancer; IQR: interquartile range
Due to rounding, the percentages may not add up to one

Table 1. Clinical characteristics of subjects in pRCC The Cancer Genome Atlas Cohort

2. *Mutation hotspots in non-coding region*

Despite the fact *MET* is the most common driver alteration, about 20% presumably *MET*-driven yet *MET* wild-type pRCC samples were left unexplained (3). Therefore, we scanned the *MET* non-coding regions. We observed one mutation in *MET* promoter region in a type 1 pRCC sample (Fig 2A). This sample has no evidence of a nonsynonymous mutation in *MET* gene but it has copy number gain of *MET*. Additionally, we observed 6/32 (18.8%) samples carry mutations in the first or the second introns of *MET* (Fig 2A). Notably, RNA splicing variants involving exon 1-3 were found in approximately 5% pRCC samples and thought to be a cancer-driving event. However, we were not able to find statistically significant association between splicing events and intronic mutations.

We further expanded our scope and ran FunSeq2 (5) to identify potentially high-impact, non-coding variants in pRCC. First, we identified a high-impact mutation hotspot on chromosome 1. 6/32 (18.8%) samples have mutations within this 6.5kb region (Fig 2B). This hotspot locates at the upstream of *ERRFI1* (ERBB Receptor Feedback Inhibitor 1) and overlaps

with the predicted promoter region. ERRFI1 is a negative regulator of EGFR family members, including EGFR, HER2 and HER3. Due to a very limited sample size here, our test power was inevitably low. We didn't observe statistically significant changes among mutated samples in mRNA expression level, protein level and phosphorylation level of EGFR, HER2 and HER3 (S1-S3).

Another potentially impactful mutation hotspot is in *NEAT1*. We saw mutations inside this nuclear long non-coding RNA in 5/32(15.6%) samples (FIG 2C). Several studies indicated *NEAT1* is associated in many other cancers (*11, 12*). It promotes cell proliferation in hypoxia (13) and alters the epigenetic landscape, increasing transcription of target genes (*14*).

All the mutations we found fell into a speculated promoter region of *NEAT1*. We noticed *NEAT1* mutations were associated with higher *NEAT1* expression (Fig 2D, $p < 0.044$, two-sided rank sum test). We also found *NEAT1* mutations were associated with worse prognosis (Fig 2E, $p < 0.022$, log-rank test).

We used DELLY2 (REF) to perform structural variants (SVs) calling from WGS reads information (Supplements). The SV discovery approach has high sensitivity and resolution than array-based method, which was employed in the TCGA analysis. In the end we found about 500 SV events, includes deletions, duplications, inversions and translocations. We confirmed three cases carrying deletions affecting *CKDN2A*. One sample, TCGA-B9-4116, which had extensive amplification of *MET*, showed multiple SVs of various classes hitting *MET* regions. However, we didn't find SVs affecting *MET* except this one example.

3.   Mutation spectra of pRCC

To further get a high-order overview of the mutation landscape, we summarized the mutation spectra of 32 whole genome sequenced pRCC samples (Fig 3A). C>T in CpGs showed the highest mutation rates, which were roughly ten to twenty-fold higher than mutation rates in other nucleotide context.

We used principle components analysis (PCA) to reveal factors that explain the most inter-sample variation. The loadings on the first principle component (which explains 12.5% of the variation) demonstrated C>T in CpGs contributes the most to inter-sample variation (Fig 3B). C>T in CpGs is highly associated with methylation. It reflects the spontaneous deamination of cytosines in CpGs, which is much more frequent in 5-methyl-cytosines. So we further explored the association between C>T in CpGs and tumor methylation status. We confirmed this by showing samples from methylation cluster 1 (hypermethylated group, Supplement 4-5) had higher PC1 scores as well as higher C>T mutation counts and rates in CpGs (Fig 3C). This trend was further confirmed using WXS as well (S6). Especially the most hypermethylated group, CpG island methylation phenotype (CIMP), showed the greatest C>T in CpGs. Therefore, methylation status was the most prominent factor that shapes the mutation spectra across patients.

Using a LASSO-based approach (see Methods) to identify mutation signatures in both WGS and WXS samples, we found one Type II pRCC case out of 155 somatic WXS sequenced samples exhibited APOBEC-associated signature 2 and 13. APOBEC mutation pattern enrichment analysis (see Method) further confirmed the presence of APOBEC activity in pRCC (Fig 3D). It was statistically enriched of APOBEC mutations (adjusted p-value < 0.0003).

This Type II pRCC case with APOBEC activities was centrally reviewed by six pathologists in the original study. Thus this tumor is likely a special case of Type 2 with genomic alterations share some similarities with urothelial cancer (UC), which often carries APOBEC mutation signatures. Indeed it had non-silent mutations in *ARID1A* and *MLL2* and a synonymous mutation in *RXRA*, all are identified as significantly mutated genes in UC. Potential pRCC driver events, for example low expression of *CDKN2A* or non-synonymous alternations in significantly mutated genes of pRCC, were absent in this sample.

Prominent APOBEC activities were also incidentally detected in three upper track UC samples sequenced and processed in the same pipeline with pRCC samples. This result is consistent with TCGA bladder urothelial cancer study (*15*). Noticeably, along with the one Type II pRCC case, all four samples showed significantly higher *APOBEC3A* and *APOBEC3B* mRNA expression level (p < 0.0022 and p < 0.0039 respectively, one-side rank sum test, S7).

Consistent with previous studies (16), we could not detect statistically significant APOBEC activities in an extensive WXS dataset consisting of 418 clear cell RCC (ccRCC) samples, even after resampling to avoid p-value adjustment eroding the power. Accordingly, very low level of APOBEC signatures (<15%) was found in less than 1%(4/418) samples. With a much larger sample size, this result was unlikely to be confounded by detecting power.

4.   Defects in chromatin remodeling affects mutation landscape

Chromatin remodeling genes are frequently mutated in pRCC and many other cancers including ccRCC. We postulate defects in chromatin remodeling cause dysregulation of chromatin environment. This further alters the mutation landscape, specifically increases mutation rate in previously open chromatin. To test this hypothesis, we tallied the number of mutations inside DNase I hypersensitive sites (DHS) in HEK293, a cell line is derived from human embryonic kidney cells, the closest match we could find in ENCODE DHS database. 12/32 samples with non-silent mutations in eleven chromatin remodeling, cancer associated genes show higher genome-wide mutation counts (p < 0.032, one-side rank-sum test), partially driven by an even higher mutation counts in DHS region (p < 0.003, one-side rank-sum test). The median number of mutations in DHS region considerably increases by about 50% (75.5 versus 112). The effect is still significant after normalizing against the total mutation counts (p < 0.015, one-side rank-sum test, Fig 3E).

Replication time is known to correlate greatly with mutation rate. Early replicated regions have lower mutation rate but the difference dissipates when DNA mismatch repair becomes defective (*17*). We found a trend of shifting to earlier replication in the chromatin remodeling genes mutated group (S8). However, a non-parametric permutation Kolmogorov–Smirnov test (see Methods) failed to detect a statistical significance (p > 0.05). Given there is no significant association with replication time, the mutation rate rise we saw in open-chromatin region is likely to be a direct result of chromatin environment change rather than an indirect effect via replication time shift.

Discussion

We comprehensively analyzed both WGS and an extensive set of WXS of pRCC, finely scrutinizing local high-impact events as well as giving a macro overlook of the mutation

landscape. Our work further completed the genomic alteration landscape of pRCC (Fig 4). Beyond traditionally driver events, we suggested several novel noncoding alterations that could potentially drive tumorgenesis

First, we validated an exonic SNP in MET, rs11762213, as a prognostic germline variance in pRCC for the first time. The original discovery was made in a mixed RCC samples, predominated by ccRCC, and later confirmed in a large ccRCC cohort. It was unclear whether rs11762213 only predicts the outcome in ccRCC. In this study, we concluded that the alternative allele of rs11762213 also forecasts unfavorable outcome in pRCC patients. The mechanism of this exonic germline SNP remains unsettled. Remarkably, pRCC has two subtypes. We noticed cancer-specific deaths in our cohort concentrate in type 2 patients. Thus we hypothesized rs11762213 potentially has different prognostic power in two subtypes. A larger pRCC dataset is required to test our hypothesis. Nevertheless, this finding is potentially very meaningful in clinical management of pRCC patients. rs11762213 genotyping could become a reliable, low-cost risk stratification tool for patients.

Interestingly, MAF of rs11762213 among African American patients is 3.0%, higher than MAFs observed in general African populations in both 1000 Genome phase 3 dataset (0.2%) and the ExAC dataset (1.27%). This implies a possible effect of rs11762213 on pRCC incidence among African Americans that is worth further investigation. Perhaps this variant could play a role in the significant racial disparities are known to exist in the overall incidence, histologic distribution, and survival of African Americans with kidney cancer.

Besides, in MET non-coding regions, we also discovered mutations associated with MET promoter and first two introns. Although the implication is unknown, our analysis suggests there is a mutation hotspot in MET that calls for further research.

Expand our scope from coding to non-coding, we found several potentially significant non-coding mutations relevant to tumorigenesis. A mutation hotspot was found upstream of ERRFI1, an important regulator of the EGFR pathway, which may serve as a potential tumor suppressor. EGFR inhibitors have been used in papillary kidney cancer with an 11% response rate observed (REF). These mutations potentially disrupt regulatory elements of ERRFI1 and thus play a role in tumorigenesis. However, likely limited by a small sample size, we were not able to detect statistically significant functional changes in ERRFI1 and related pathways. Another non-coding hotpot is in NEAT1, a long non-coding RNA that has been speculated to involved in cancer. Patients carrying mutations in NEAT1 have higher NEAT1 expression and worse prognosis. NEAT1 has been shown to be hypermutated in other cancers and some studies also linked high NEAT1 association with unfavorable prognosis in several other tumors (18, 19).

Last, focusing on the high-level landscape of mutations in pRCC, we identified mutation rate dispersion of C>T in the CpG motif contributes to the largest proportion of inter-sample variations. We further pinned down the cause of dispersion by showing the hypermethylated cluster, identified in the previous TCGA study (3), has higher C>T rate in CpGs. This hypermethylated cluster is associated with later stage, type 2 pRCC, SETD2 mutation and poorer prognosis. Although increased C>T in CpG is likely the results of hypermethylation, we cannot rule out the possibility the change of mutation landscape plays a role in cancer development. For example, C>T in methylated CpG causes loss of methylation, which could have effects on trans-elements recruitment.

Significant APOBEC activities and consequential mutation signatures were observed in one Type II pRCC case. APOBEC activities were known to be prevalent in UCs (15, 16). We also successfully detected prominent APOBEC signatures in all three UC samples processed in the same pipeline as pRCCs. Intriguingly, despite being considered to have the same cellular origin with pRCC, we were not able to detect significant APOBEC activities in ccRCC. This is in agreement with previous studies (16). Interestingly, APOBEC mutation signature was also found in a small percentage of chromophobe renal cell carcinoma (20), which is believed to have a different cellular origin. APOBEC activities have been linked with genetic predisposition and viral infection (21). Although we could not rule out sample processing contamination, given a statistically robust signal in our conservative algorithm, it is plausible that a small fraction of otherwise driver mutation absent Type II pRCCs might be etiologically and genomically similar to UC. Since standard treatment for UC involves cytotoxic chemotherapy and radiation, this finding could have a meaningful clinical impact.

Chromatin remodeling pathway is highly mutated in pRCC (3). Several chromatin remodelers, for example *SETD2*, *BAP1* and *PBRM1*, have been identified as cancer drivers in pRCC. We demonstrated pRCC with defects in chromatin remodeling genes show higher mutation rate in general, driving by an even higher mutation rate in open chromatin regions. By adapting a defective chromatin remodeling pathway, tumor alters its mutation rate and landscape, which could further provide advantage in cancer evolution. However, excessive mutation in functional important open chromatin regions would also lead to disastrous mutational meltdown.

Materials and Methods

Data acquisition

We downloaded pRCC and ccRCC WXS SNV calls and pRCC WGS variation calls from TCGA Data Portal (https://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp) and TCGA Jamboree. pRCC samples that failed the histopathological review were excluded. pRCC RNAseq, RPPA and methylation data were downloaded from TCGA Data Portal as well. Repli-seq and DHS data were obtained from ENCODE (https://www.encodeproject.org/).

Testing rs11762213 on prognosis

We downloaded pRCC clinical outcomes from TCGA Data Portal (https://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp). Excluding criteria are "Follow-up days" not available and identified as non-pRCC by histopathological review (3). In total, we included 207 patients in our analyses. The majority of samples, 158 out of 207, were supported by high-quality, curated SNV callings from two centers. 100% genotype concordance rate was observed in samples harbor the minor allele (A, 10 samples) in germline as well as samples with homozygous reference allele (GG, 148 samples). Also, these curated rs11762213 genotypes were in agreement with automated callsets. With proved high confidence in accuracy of genotyping rs11762213 in germline, we recruited additional 49 samples from single-center, automated calls to form an extensive patients set.

Cancer-specific survival was defined using similar method as described in a ccRCC study (10). Deaths were considered as cancer-specific if the "Personal Neoplasm Cancer Status" is "With Tumor". If "Tumor Status" is not available, then the deceased patients were classified as

cancer-specific death if they had metastasis (M1) or lymp node involvement (>= N1) or died within two years of diagnosis. An R package, "survival", was used for the survival analysis.

Mutation spectra study

WGS Mutations were extracted from with flaking 5' and 3' nucleotide context. Then the raw mutation counts were normalized based on trinucleotide frequency in the whole genome.

To identify signatures in the mutation spectra, we used a robust, objective LASSO-based method. First, 30 known signatures were downloaded from COSMIC (http://cancer.sanger.ac.uk/cosmic/signatures). Then we solve a positive, zero-intercept linear regression problem with L1 regularizer to obtain signatures and corresponding weights for each genome. The penalty parameter lambda was determined empirically using 10-fold cross-validation individually for every sample. Last, we discharged signatures that composite less than 5% of the total detectable signatures.

Methylation association analysis

In total, we collected HumanMethylation450 BeadChip array data for 139 samples that are either methylation cluster 1 or 2. We used an R package "IMA" to facilitate analysis (*22*). After discharging sites with missing values or on sex chromosomes, we obtained beta-values on 366,158 CpG sites in total. Then we test beta-values of each site by Wilcoxon rank sum test between two methylation clusters. After adjusting p-value using Benjamini-Hochberg procedure, we called 9,324(2.55%) hypermethylation sites. These sites must have an adjusted p-value of less than 0.05 and mean beta-values in methylation cluster 1 are 0.2 or higher than the ones in methylation cluster 2.

APOBEC enrichment analysis

We used the method described by Roberts et al. (*16*). For every C>{T,G} and G>{A,C} mutation we obtained 20bp sequence both upstream and downstream. Then enrichment fold was defined as:

$$Enrichment\ Fold = \frac{Mutation_{TCW/WGA} \times Context_{C/G}}{Mutation_{C/G} \times Context_{TCW/WGA}}$$

Here TCW/WGA stands for T[C>{T,G}]W and W[G>{A,C}]A. W stands for A or T. p-value for enrichment were calculated using one-side Fisher-exact test. To adjust for multiple hypothesis testing, p-values were corrected using Benjamini-Hochberg procedure.

Replication time association

In order to avoid cell type redundancy, we only kept Gm12878 as the representative of all lymphoblastoid cell lines. Wave smoothed replication time signal is averaged in a +/- 10kb region from every mutation. To avoid potential selection effects, we removed mutations in exome and flanking 2bp. Regions overlap with reference genome gaps and DAC blacklist (https://genome.ucsc.edu/) were removed. Last, we picked the median number from 11 cell types at each mutation position for further analysis.

To test the significance of replication time of non-coding mutations between two groups, we adapted a conservative non-parametric Kolmogorov–Smirnov test (K-S test) using empirical p-value. We assigned all the mutation with its percentile among all mutations replication time shifted +/- 100kb from the origin (represents the background replication time). Then we calculate the K-S test statistics of mutation counts of 100 bins in two groups and compare. To obtain the empirical p-value, we randomly permutated the chromatin remodeling genes mutation labels for 1,000 times to estimate the test statistics distribution.

Supplementary Materials

Supplement figure 1. Mutations on *ERRFI1* promoter region has no effect on *ERRFI1* RNA expression.

Supplement figure 2. Mutations on *ERRFI1* promoter region has no effect on *ERR* family protein levels.

Supplement figure 3. Mutations on *ERRFI1* promoter region has no effect on *ERR* family Phosphorylation.

Supplement figure 4. Volcano plot of rank sum test of all CpG probe sites between methylation cluster 1 and 2.

Supplement figure 5. Volcano plot of rank sum test of all CpG probe sites between methylation cluster 1 and 2 after grouped by functional regions.

Supplement figure 6. Comparison of C>T in CpGs mutation counts and fractions in pRCC WXS set among three different methylation clusters.

Supplement figure 7. The expression levels of *APOBEC3A* and *APOBEC3B* are significantly higher in samples carrying APOBEC signatures

Supplement figure 8. Replication time distribution shifts among samples having chromatin remodeling genes mutations.

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