Aberrant promoter hypermethylation of PBRM1, BAP1, SETD2, KDM6A and other chromatin-modifying genes is absent or rare in clear cell RCC

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Keywords: PBRM1, BAP1, SETD2, KDM6A, KDM5C, MLL2, ARID1A, renal cell carcinoma, clear cell RCC, promoter methylation

Recent sequencing studies of clear cell (conventional) renal cell carcinoma (ccRCC) have identified inactivating point mutations in the chromatin-modifying genes PBRM1, KDM6A/UTX, KDM5C/JARID1C, SETD2, MLL2 and BAP1. To investigate whether aberrant hypermethylation is a mechanism of inactivation of these tumor suppressor genes in ccRCC, we sequenced the promoter region within a bona fide CpG island of PBRM1, KDM6A, SETD2 and BAP1 in bisulfite-modified DNA of a representative series of 50 primary ccRCC, 4 normal renal parenchyma specimens and 5 RCC cell lines. We also interrogated the promoter methylation status of KDM5C and ARID1A in the Cancer Genome Atlas (TCGA) ccRCC Infinium data set. PBRM1, KDM6A, SETD2 and BAP1 were unmethylated in all tumor and normal specimens. KDM5C and ARID1A were unmethylated in the TCGA 219 ccRCC and 119 adjacent normal specimens. Aberrant promoter hypermethylation of PBRM1, BAP1 and the other chromatin-modifying genes examined here is therefore absent or rare in ccRCC.

Introduction

Several tumor suppressor genes predisposing to inherited forms of renal cell carcinoma (RCC) have been identified but, until recently, with the exception of VHL, few classical tumor suppressor genes inactivated by point mutation had been identified in sporadic RCC, which is 96% of the disease. A large scale systematic re-sequencing study, three exome sequencing studies, as well as an exome sequencing study of chromosome 3p genes in clear cell (conventional) RCC (ccRCC) have identified several novel genes with inactivating point mutations, indicative of a tumor suppressor function. Many of these genes are involved in chromatin modification. The PBRM1 gene, which codes for the BAF180 subunit of the SWI/SNF chromatin remodeling complex, was reported to have point mutation in 41% of ccRCC and is the second most frequently mutated gene in ccRCC. The same study reported missense mutation of ARID1A, which codes for a different subunit of the SWI/SNF chromatin-remodeling complex, in two of seven RCC exomes sequenced. ARID1A is inactivated by a truncating point mutation in clear cell and endometrioid ovarian cancer, as well as in bladder and other cancers. The KDM6A and KDM5C genes, which encode enzymes that demethylate, and the SETD2 and MLL2 genes, which methylate important lysine residues of histone H3, each showed point mutations in 3% of ccRCCs. Point mutation of KDM6A and MLL2 has been reported in other cancer types. The BAP1 gene is a component of the ubiquitin-mediated proteolysis pathway (UMPP) and shows mainly inactivating point mutations in 8–14% of ccRCC. Somatic mutation of BAP1 is also present in melanoma and mesothelioma. Among other functions, BAP1 modifies chromatin by mediating deubiquitination of histone H2A, although this may not be the main mechanism of tumor suppression in RCC. Aberrant hypermethylation of the core promoter region within a CpG island is associated with loss of transcription of classical tumor suppressor genes in cancer. Hypermethylation is an alternative to point mutation or deletion for inactivation of one allele of the gene. Since several previously identified classical tumor suppressor genes, such as VHL, CDKN2A/p16INK4a, CDH1/E-cadherin and SDHB, are known to be hypermethylated in subsets of sporadic ccRCC, the recently identified tumor suppressor genes involved in chromatin modification might also be inactivated by aberrant promoter hypermethylation with the associated loss of mRNA expression in RCC. Immunohistochemical staining (IHC) studies have shown that most ccRCC negative by IHC for PBRM1 or BAP1 have an inactivating point mutation. There were no cases of point mutation or indels as the second inactivation event. The known high
frequency of LOH of 3p suggests the second hit in many tumors with point mutation but this has not been studied yet. aberrant promoter hypermethylation may therefore be the method of inactivation in the subset of 10–12% ccRCC negative by IHC for PBRM1 or BAP1 that show no evidence of point mutation and, also, for the inactivation of the second allele in some tumors with point mutation.

Knowledge of whether hypermethylation is a mechanism of inactivation of a particular tumor suppressor gene is important: (1) for an accurate assessment of the frequency of inactivation of the gene, which would indicate the relative importance of specific pathways and networks and, thereby, their biological significance in a specific disease; (2) for identification of molecular subtypes within a tumor type; (3) to determine the utility of a gene as a marker of diagnosis, prognosis or chemoresponse and (4) to understand the potential of the gene or protein as a therapeutic target, including for epigenetic drugs.35

The Cancer Genome Atlas (TCGA) uses the Illumina Infinium HumanMethylation27 probes for inactivating point mutation are included in the Infinium HumanMethylation27 BeadChip, the probe(s) for PBRM1, KDM6A or SETD2 are not located within a CpG island of either definition, presumably because of sequence-dependent features of the BeadChip chemistry.37 One of two Infinium probes for BAP1 was not located in the CpG island while the other probe was from the 5′ end of the island but relatively distant (1230bp) from the TSS (Table 1). We therefore designed primers for direct bisulfite sequencing or pyrosequencing of a region of the bona fide CpG island near the TSS using the Ensembl annotation of the TSS as nucleotide -1 of the 5′ UTR for PBRM1, KDM6A, SETD2 and BAP1 (Fig. 1). Methylation that surrounds the TSS is strongly linked with transcriptional silencing.20–21 The 1,000 bp of sequence centered on the TSS was generally unmethylated in a survey of human genes in 12 normal tissues.38 No Alu or other repetitive elements were detected in the amplicons to be sequenced.

The promoter CpG islands of PBRM1, KDM6A, SETD2 and BAP1 are unmethylated in ccRCC. The 50 ccRCC, 4 normal renal parenchyma and 5 RCC cell lines were unmethylated for PBRM1, KDM6A and SETD2 by direct bisulfite sequencing and unmethylated for BAP1 by pyrosequencing (Fig. 2). Because pyrosequencing provides a shorter sequence read-length we also examined a second area of the BAP1 promoter CpG island (Table 1). The 50:50 unmethylated:fully methylated DNA control showed approximately 50% methylation for PBRM1 and BAP1 (Fig. 2) and a bias toward the methylated template DNA for KDM6A and SETD2 (data not shown). Both KDM6A and KDM5C are located on the X chromosome and known to escape X-inactivation.39,40 Therefore, both alleles of KDM6A and KDM5C would be expected to be unmethylated in normal cells, as we observed (data not shown). There is little evidence for mutational inactivation of PBRM1 and the other chromatin-modifying genes in non-ccRCC. No point mutation of PBRM1 was found in 36 non-ccRCC and none of SETD2 and KDM5C in 65 non-ccRCC.3 Point mutation of KDM6A was reported in 1 of 5 papillary RCC and is found in types of cancer other than RCC.12 The BAP1 gene has not yet been examined for point mutation by sequencing in non-ccRCC. Consequently, we only examined the methylation status of these genes in a small number of non-ccRCC. We found PBRM1, KDM6A, SETD2 and BAP1 to be unmethylated in five papillary RCC and five chromophobe RCC.

The promoter CpG islands of KDM5C and ARID1A are unmethylated in the TCGA ccRCC data set. The Infinium probe for KDM5C is located within a bona fide CpG island and -49 bp upstream of the TSS. The two Infinium probes for ARID1A are both located within a bona fide CpG island 821 bp upstream and 863 bp downstream of the TSS. We examined the raw β-value
# Table 1. Information on the CpG loci interrogated for each gene

| Gene name | Chromosomal location | Bona fide CpG island | No. of Infinium probes and location relative to CpG island | Amplification and sequencing primers/Infinium Probes | CpGs read out of total number of CpGs in amplicon |
|-----------|---------------------|---------------------|---------------------------------------------------------|-----------------------------------------------------|-----------------------------------------------|
| PBRM1     | 3p21                | Yes                 | 1; outside                                              | F – TGGTGGTTGT AGTAAATTTT AGA R – GAGGGCTAAG GGAGGTTGAG  | 23/29                                         |
| KDM6A     | Xp11                | Yes                 | 1; outside                                              | F – GATAAGCTTG GTGTGTGTGT TT R – TAGTTTGA GTRAGAGAGA G  | 21/28                                         |
| SETD2     | 3p21                | Yes                 | 2; outside                                              | F – GTTTATGTGT TTYGGAGAGT TAT R – TGAGGCTAG AGGGAGAAGA  |                                               |
| BAP1      | 3p21                | Yes                 | 1; outside                                              | assay #ADS1756FS1, EpigenDX                           | 7/7                                           |
| KDM5C     | Xp11                | Yes                 | 1; within                                               | F - GATGAATAAG GGTGTTGGT GAT R – GTTGTGTGA TATCATTTT TTTTTTTGG | 3/6                                           |
| ARID1A    | 1p36                | Yes                 | 2; within                                               | cg11856093 _CAGGCCAGGG CTTTTGTGTC GGGCATGGTG TGGTGAGAGAG ACGCCGGCCG | 4                                              |
| MLL2      | 12q13               | No                  | 1                                                      | cg13007988 _ CGGGGAGAC TGTGGTGTC AAGAAGAGAGA TCTATGTTGCT TACTAAGGCT | 1                                              |

Gene name according to NCBI; chromosomal location according to NCBI; CpG island according to Takai and Jones criteria by CpG Island Searcher; number of probes in Infinium humanmethylation27 beadchip and position of probe relative to Takai and Jones CpG island; sequence of primers used and Infinium probes examined in our study, Y and R indicate degenerate T or C in forward and reverse primer respectively, primer sequences for the most 5’ area of BAP1 are proprietary and available as a commercial kit (Epigen DX); number of CpG loci read from total number of CpG loci in amplicon due to loss of sequence read at the 5’ end of the bisulfite sequencing amplicon or the 3’ end of the pyrosequencing amplicon.

![CpG island schematic of the genes studied](image)

**Figure 1.** CpG island schematic of the genes studied. Vertical red lines represent individual CpG loci in the island. The TSS is indicated by a vertical rectangle and the ATG by a hatched box. The horizontal black line indicates the area sequenced and the nucleotide position given is relative to the location of the TSS from Ensembl.
(methylation score) of the probes for these two genes in the available TCGA data set of 219 ccRCC compared with 119 matched adjacent normal renal tissue samples for evidence of aberrant hypermethylation in ccRCC. We considered a probe unmethylated if the \( \beta \)-value was \( \leq 0.15 \) and hypermethylated if the individual tumor had a \( \beta \)-value at least 0.2 higher than the \( \beta \)-value of the matched normal sample.\(^{37} \) By these criteria, there was no evidence of hypermethylation of the Infinium probe for \( KDM5C \) or the two \( ARID1A \) Infinium probes in the 219 TCGA ccRCC.

Considerations of the RCC specimen set and methylation assays. The 50 ccRCC screened for methylation status in our study are broadly representative of the disease.\(^{41} \) They comprise 25 low grade (I or II) of mainly 4 cm or under in size (stage I) and organ-confined (stage I or II) as well as 25, mainly high grade (III or IV), stage III or IV tumors (Table 2). A representative specimen set is important as the presence of an alteration may be associated with a particular pathologic subset as for example in a recent study that reported a significant correlation between \( BAPI \) point mutation and high grade ccRCC while \( PBRM1 \) point mutation was associated with low grade ccRCC.\(^{5} \)

That all 50 ccRCC specimens were determined by a pathologist to have \( \approx 70\% \) tumor cell content means that the sensitivity of detection of hypermethylation should not be overly diluted by unmethylated alleles from the normal cells that contaminate the tumor specimen. The TCGA ccRCC specimens are also assessed for adequate tumor cell content and 12% of 219 ccRCC showed \( VHL \) hypermethylation (\( \beta \)-value > 0.15), a frequency expected from prior studies.\(^{22-24} \) A conservative estimate of the typical minimal sensitivity of detection of methylation by the relevant assays might be 20% for direct bisulfite sequencing,\(^{42} \) 10% for pyrosequencing\(^{42} \) and 15% for Infinium analysis.\(^{37} \) While we observed evidence of amplification\(^{43} \) or sequencing bias, as assessed by inclusion of a 50% unmethylated normal DNA:50% fully methylated DNA control, for \( KDM6A \) and \( SETD2 \) this would not lead to underscoring of aberrant methylation. We prefer direct sequencing, as performed here, to subcloning of a mixed population of alleles in order to avoid potential cloning efficiency bias\(^{44} \) and artifact.\(^{45} \)

The 50 ccRCC studied here were obtained as a single biopsy from surgical resection, either radical or partial nephrectomy, performed pre-treatment. Since the entire tumor mass was not sampled, intratumor heterogeneity of an alteration could potentially result in an underestimate of methylation. However, in a recent study of intratumor heterogeneity, \( PBRM1 \) point mutation was considered ubiquitous, while point mutation of both \( SETD2 \) and \( KDM5C \) was shared, in multiple biopsies from the primary RCC in individuals with metastatic RCC (\( KDM6A, BAPI \) or \( ARID1A \) point mutation were not present in the 4 RCC in the study).\(^{46} \) Furthermore, point mutation of \( PBRM1 \) and the other chromatin-modifying genes was originally identified by sequencing of a single biopsy from each of several ccRCC.\(^{5-7} \) This suggests that if methylation of any of these genes was moderately frequent in ccRCC it would have likely been detected in the single biopsy from one or more of the 50 ccRCC examined by us or of the 219 ccRCC examined by TCGA.

The entire promoter CpG island of each gene was not assayed in this study. However, in our experience\(^{47,48} \) and that of others\(^{49,50} \) with bisulfite sequencing, the majority of individual CpG loci in the island, particularly within 500 bp of the TSS, are methylated in tumor suppressor genes that are aberrantly hypermethylated in cancer cells. In addition, in the human genome, there is evidence for significant correlation of co-methylation of CpG sites over distances shorter than or equal to 1,000 bp.\(^{51,52} \) Therefore, we believe the number of CpG loci interrogated for methylation status by sequencing near to the TSS in our study is sufficient to identify the presence of aberrant promoter hypermethylation. Similarly, it should be noted that the TCGA ccRCC Infinium BeadChip data for \( KDM5C \) and \( ARID1A \) are based on a more limited number of CpG loci, that the two \( ARID1A \) probes are located relatively distant (> 500 bp) to the TSS, and that we did not verify the Infinium probe methylation status by another technology, e.g., sequencing or quantitative methylation-specific PCR (qMSP). Taken together, the points considered above suggest that aberrant promoter hypermethylation of \( PBRM1, BAPI \) and the other chromatin-modifying genes examined here is absent or rare in ccRCC.

Why is hypermethylation of \( PBRM1 \) and other genes uncommon in ccRCC? The location of \( VHL, PBRM1, SETD2 \) and \( BAPI \) on chromosomal arm 3p means that a large deletion of 3p could result in simultaneous inactivation of one allele of all four genes in a single mutation event. In terms of conferring a growth advantage to a tumor cell, such a deletion event might be favored over hypermethylation of an allele of one of the four genes. A similar advantage has been postulated as a reason why homozygous deletion around \( CDKN2A \) at 9p21 that results in simultaneous inactivation of the \( INK4A \) and \( ARF \) tumor suppressors is more frequent than \( CDKN2A \) point mutation or hypermethylation in human cancer.\(^{51} \) However, the known hypermethylation of \( VHL \) in \( \approx 10-15\% \) of sporadic ccRCC\(^{22-24} \) argues against this idea, although the relative timing of inactivation of \( VHL \) to inactivation of \( PBRM1, SETD2 \) and \( BAPI \) in the initiation and development of ccRCC needs to be considered. A related point is that it has been noted\(^{54} \) and remains unclear why some classical tumor suppressor genes that contain a bona fide CpG island in the promoter region are susceptible to aberrant hypermethylation, i.e., \( BRCA1 \) and \( MLHI \), while others are not, i.e., \( BRCA2 \) and \( MSH2 \), since transcriptional silencing of any of these genes would be predicted to provide a growth advantage to a tumor cell. Lastly, it is possible that other mechanisms of epigenetic silencing, i.e., dysregulation of miRNA expression,\(^{55,56} \) aberrant methylation of an upstream regulatory gene,\(^{47} \) or histone modification in the absence of hypermethylation,\(^{57} \) act upon \( PBRM1 \) and the other tumor suppressor genes found to be unmethylated in our study.

Conclusions

Information on whether a tumor suppressor gene is hypermethylated is important to determine the relative contribution of the gene to the disease, to discover molecular subtypes and to assess its utility as a diagnostic, prognostic or chemoresponse marker as well as its potential as a therapeutic target. To our knowledge, this report is the first to examine the
Figure 2. For figure legend see page 491.
methylation status of the promoter of these genes identified by inactivating point mutation as important in the biology of RCC. We conclude that aberrant promoter hypermethylation of PBRM1, BAP1, SETD2, KDM6A and the other chromatin-modifying genes examined here is absent or rare in ccRCC.

Materials and Methods

Specimen preparation. The FCCC Institutional Review Board (IRB) approved the study and all patients provided written consent. Fifty fresh-frozen ccRCC and four normal renal parenchyma from patients with no history of RCC and of similar age (mean 66 y) to the average age of diagnosis of RCC (64 y) from 2005–2009 (http://seer.cancer.gov/statfacts/html/kidrp.html) were obtained from the Fox Chase Cancer Center (FCCC) Biospecimen Repository. A piece of each RCC embedded in optimal cutting temperature (OCT) compound was examined under a microscope with the assistance of a pathologist (ED) to identify an area with a tumor cell content ≥ 70% to be dissected out for DNA isolation by phenol/chloroform extraction and ethanol precipitation.58 The normal renal parenchyma specimens were similarly examined and determined to be non-neoplastic before DNA isolation. Clinicopathological data for the 50 ccRCC were obtained from the FCCC Kidney Keystone Database. The tumor set comprised 33 males and 17 females ranging from 33–85 y of age, with a median of 59 y, at diagnosis. The Fuhrman nuclear grade and clinical stage of the ccRCC are presented in Table 2. The ccRCC cell lines 786-0, 769-P, A498 and papillary RCC cell line ACHN were obtained from the American Type Culture Collection (ATCC). The ccRCC cell lines 786-0, 769-P, A498 and papillary RCC were obtained from the NCI-DCTD Tumor Cell Line Repository. The papillary RCC cell line ACHN were obtained from the American Type Culture Collection (ATCC). The ccRCC cell line Caki-1 was obtained from the Normal Renal Parenchyma Specimens were provided by Fox Chase Cancer Center via Institutional Support, and the other chromatin-modifying genes examined here is absent or rare in ccRCC.

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| ccRCC | Grade I | Stage I | Stage II | Stage III | Stage IV |
|-------|---------|---------|---------|-----------|---------|
|       | 3       |         |         |           |         |
|       | 20      | 2       |         |           |         |
|       | 4       | 1       | 3       | 8         |         |
|       | 9       |         |         |           |         |

Infinium HumanMethylation27 annotation and TCGA ccRCC data. The localization of the Infinium HumanMethylation27 probe sequence (available at ftp://ftp.illumina.com/Methylation/InfiniumMethylation/HumanMethylation27/) relative to the TSS and bona fide CpG island was examined for each gene in Ensembl (www.ensembl.org). Amplification and sequencing primers were designed to examine an area of sequence within 500 bp upstream or downstream of the TSS predicted by Ensembl in a CpG island that fulfilled the widely used definition criteria of Gardiner-Garden and Frommer56 as well as the modifications suggested by Takai and Jones65 using CpG Island Searcher (http://cpgislands.usc.edu/). The presence of Alu and other repetitive elements was examined by repeatmasker v3.3.0 (http://repeatmasker.org). Primer sequences are given in Table 1. We accessed the TCGA ccRCC raw data available at https://tcga-data.nci.nih.gov/tcga/tcgaCancerDetails.jsp?diseaseType=KIRC&diseaseName=Kidney%20Renal%20Clear%20cell%20carcinoma on September 12, 2012. The raw β-value from 219 ccRCC and 119 adjacent normal renal parenchyma run on the Infinium HumanMethylation27 BeadChip was examined to assess the methylation status for the relevant gene probes.

Bisulfite modification, PCR and sequencing of DNA. One microgram of specimen DNA was bisulfite modified using the EZ-DNA Methylation kit (Zymo Research Corporation) according to the manufacturer’s protocol. Approximately 100 ng of bisulfite-modified DNA was used as template for PCR amplification with the primers given in Table 1. The PCR product was run on a 1.5% agarose gel alongside a molecular weight marker, cut out and purified by the Qiaquick MinElute Gel Extraction Kit (Qiagen) then sequenced on an ABI 3130 sequencer. For BAP1, one primer was biotinylated before PCR amplification and the PCR product purified as above. Each amplicon was sequenced on a Pyrosequencing PSQ 96MA genetic analysis system using the Pyro Gold Reagent Kit according to the manufacturer’s instructions (Qiagen). A 50:50 mix of unmethylated DNA/M.SssI in vitro methylated DNA was run to control for amplification or sequencing bias for each gene analyzed.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Kirill Yudin for writing script to generate the schematic of CpG islands in Figure 1, Emmanuelle Nicolas PhD, Genetic Research Facility at FCCC, for design and optimization of BAP1 pyrosequencing, Suraj Peri PhD, Department of Biostatistics at FCCC for help with TSS data in Ensembl and Andrew Kossenkov PhD, Director of Bioinformatics at the Wistar Institute, Philadelphia for help with the interrogation of TCGA data. This publication was supported in part by grant number P30 CA006927 from the National Cancer Institute. Its contents are solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health. Additional funds were provided by Fox Chase Cancer Center via institutional support of the Kidney Keystone Program.
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