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## Exome sequencing identifies frequent mutation of the SWI/SNF complex gene *PBRM1* in renal carcinoma

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### Summary

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#### Author contribution

IV and PT performed the main analytical aspects of the study. PS, HD, GLD, M-LL, GB, CH, LM, SM performed the follow-up sequencing and analyses. KR, DJ, JT, AB, CG, DG, MJ, CL, JM, AM, LS contributed to the data processing, mapping and variant calling informatics. CG and KWL performed statistical analyses. SR, RJK, JA contributed samples and data for the clinical series. DJA, AR, DAL, LFAW, DAT, PAP-M performed the transposon screening and analyses. DH, CKO, WC, CS performed the siRNA and functional work. VM, AF performed the missense mutation analysis. KD, KF and JC performed the expression analyses. PJC, BTT, MRS, PAF directed the study and wrote the manuscript, which all authors have approved.

#### Author information

Exome sequence data have been deposited at the European Genome-Phenome Archive (<http://www.ebi.ac.uk/ega/>) hosted by the European Bioinformatics Institute under accession EGAS00001000006 and expression data has been deposited with Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession GEO22316.

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The genetics of renal cancer is dominated by inactivation of the *VHL* tumour suppressor gene in clear cell carcinoma (ccRCC), the commonest histological subtype. A recent large-scale screen of ~3500 genes by PCR-based exon re-sequencing identified several new cancer genes in ccRCC including *UTX* (*KDM6A*)<sup>1</sup>, *JARID1C* (*KDM5C*) and *SETD2*<sup>2</sup>. These genes encode enzymes that demethylate (*UTX*, *JARID1C*) or methylate (*SETD2*) key lysine residues of histone H3. Modification of the methylation state of these lysine residues of histone H3 regulates chromatin structure and is implicated in transcriptional control<sup>3</sup>. However, together these mutations are present in fewer than 15% of ccRCC, suggesting the existence of additional, currently unidentified cancer genes. Here, we have sequenced the protein coding exome in a series of primary ccRCC and report the identification of the SWI/SNF chromatin remodeling complex gene *PBRM1*<sup>4</sup> as a second major ccRCC cancer gene, with truncating mutations in 41% (92/227) of cases. These data further elucidate the somatic genetic architecture of ccRCC and emphasize the marked contribution of aberrant chromatin biology.

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Exome sequencing based on a solution phase capture approach<sup>5</sup> was performed on seven cases of ccRCC, three of which carry *VHL* mutations, and matching normal DNAs (See Supplementary information and Supplementary Table 1). Captured material was sequenced using 76 basepair paired-end reads on the Illumina GAIIx platform. After read alignment, variant calling was performed using a naïve Bayesian classifier algorithm for substitutions and a split-read mapping approach (PinDel<sup>6</sup> with substantial cancer-aware output filtering) for insertion/deletions (See Supplementary Material for details). These algorithms aim to identify somatically acquired coding and splice-site variants (i.e. present in the tumour but not in the matching normal), and all mutations reported here were confirmed by PCR-based capillary sequencing. 156 somatic mutations were identified, of which 92 were missense, 9 nonsense, 1 canonical splice site, 1 stop codon read-through, 11 frameshift and 42 synonymous (Supplementary Table 2).

In four cases truncating mutations were identified in *PBRM1*. *PBRM1* maps to chromosome 3p21 and encodes the BAF180 protein, the chromatin targeting subunit of the PBAF SWI/SNF chromatin remodelling complex<sup>7</sup>. The gene is comprised of 6 bromodomains involved in binding acetylated lysine residues on histone tails, 2 bromo-adjacent homology domains important in protein-protein interaction and an HMG DNA binding domain<sup>4</sup>. PBAF complex-mediated chromatin remodelling is implicated in replication, transcription, DNA repair and control of cell proliferation/differentiation<sup>4,7</sup>. The *SMARCB1* and *BRG1* components of this complex have inactivating mutations in rhabdoid tumours<sup>8,9</sup> and *BRG1* mutations have been reported in multiple tumour types<sup>10</sup>. The *PBRM1* mutations included three frame-shifting insertions and a nonsense mutation; all judged to be homozygous from SNP array and mutant allele read count data. *PBRM1* was not included in our previous PCR-based sequencing screen<sup>2</sup> and was the only gene, apart from *VHL*, with recurrent truncating mutations in the seven cases screened.

We next sequenced *PBRM1* in a further 257 RCC cases, including 36 cases of papillary, chromophobe and other non-ccRCC cancers. Truncating mutations were identified in a remarkable 88/257 (34%) (Figure 1) of cases, all diagnosed as ccRCC (for full data see Supplementary Tables 3, 4). *PBRM1* mutations were all found in the context of chromosome 3p loss of heterozygosity (38/38) where SNP array data was available (<http://www.sanger.ac.uk/cgi-bin/genetics/CGP/cghviewer/CghHome.cgi>). Two in-frame deletion mutations were identified – a predicted 6 amino-acid deletion (p.M1209\_E1214delIMFYKKE) in the second BAH (bromo-adjacent homology) domain likely involved in protein-protein interactions within the SWI/SNF complex<sup>4</sup> and deletion of an isoleucine codon (Ile57) in the first bromodomain (Figure 1). Both deletions remove amino acids conserved to *C. elegans* and both were in cases with 3p LOH. The ratio of nine

missense to zero silent mutations suggests that a proportion of the missense mutations are likely to be pathogenic. Six of nine missense mutations occur in bromodomains and one in the second BAH domain (Figure 1). The bromodomains of PBRM1 have been shown to have preferential binding to different acetylated lysine configurations of histone tails, suggesting they may contribute to “reading” of the histone code<sup>11</sup>. The likelihood of the missense mutations having functional impact was assessed using a scoring system calibrated with protein domain alignments from Pfam (see Supplementary Methods). Three missense mutations (p.T232P, p.A597D and p.H1204P) could be scored with these alignments. This set of mutations was predicted to be deleterious, having a significantly lower mean score than a typical null set of *in silico* generated random missense mutations falling onto the scorable parts of the gene (p-value 0.01 Figure 2), making these mutations interesting candidates for functional studies.

Four *PBRM1* truncating mutations have been previously described in breast cancer<sup>12</sup>. Although there is frequent 3p21 LOH in small-cell lung cancer, no evidence for *PBRM1* inactivation was found<sup>13</sup>. To further evaluate the contribution of *PBRM1* mutation in human cancer, copy number was evaluated and the coding exons were sequenced through a series of 727 cancer cell lines of various histologies. SNP array copy number analysis (<http://www.sanger.ac.uk/cgi-bin/genetics/CGP/cghviewer/CghHome.cgi>) identified one homozygous deletion in the HCC-1143 breast cancer cell line, previously described<sup>12</sup>. Sequencing analysis identified five homozygous truncating mutations (Supplementary Table 5). Frame-shifting deletions were identified in the *VHL*-mutant A704 renal cancer, NCI-H2196 small-cell lung cancer and TGBC24TKB gall bladder cancer lines. Nonsense mutations were identified in the NCI-H226 squamous-cell lung cancer and PANC-10-05 pancreatic adenocarcinoma lines. Interestingly, a *PBRM1* truncating mutation has been reported in a comprehensive pancreatic cancer mutational screen<sup>14</sup>.

To obtain further support that *PBRM1* can act as a cancer gene, we examined data from several insertional mutagenesis screens in mice. Analyses of transposon insertion sites from a forward genetic screen performed using a conditional Sleeping Beauty transposon system<sup>15</sup> in a mouse pancreatic cancer model<sup>16</sup> revealed a significant enrichment of insertion events in *Pbrm1* amongst all genes hit using Monte Carlo simulation analyses as previously described<sup>17</sup>. Insertions were found in pancreatic dysplasia, intraductal (panIN) and high grade invasive tumours suggesting *Pbrm1* inactivation is an early event in this model. The mixed forward and reverse pattern of insertions is indicative of inactivation, as demonstrated by RT-PCR showing premature termination of the *Pbrm1* cDNA via splicing into the inserted transposon (Figure 3). These data suggest that loss of *Pbrm1* cooperates with *Kras* in driving pancreatic tumour development in this model. Intriguingly, *Setd2*, previously implicated human ccRCC, was also found to rank significantly in frequency among all insertion sites and two tumours had both *Setd2* and *Pbrm1* insertions. These comparative oncogenomic data provide independent support for *PBRM1* as a cancer gene and suggest further investigation of the role of *PBRM1* (and *SETD2*) in human pancreatic cancer is warranted.

Abrogation of *PBRM1* expression via siRNA knockdown in ccRCC cell lines was investigated to assess possible consequences of *PBRM1* loss. Greater than 60% knockdown of *PBRM1* RNA and protein resulted in a significant increase in proliferation 4/5 RCC lines (Figure 4A, B and Supplemental Information). No effect was seen, however, in A704 which carries a homozygous truncating *PBRM1* mutation and expresses no PBRM1, confirming the specificity of the assay. Further, knockdown of *PBRM1* resulted in significantly increased colony formation in soft-agar and increased cell migration (Figure 4C, D), indicative of an increase in transformed phenotype. Taken together, these data support PBRM1 having a tumour suppressor role in ccRCC.

Transcriptional profiling before and after *PBRM1* knockdown was performed using gene expression microarrays. Gene set enrichment analysis following *PBRM1* knockdown showed that PBRM1 activity regulates pathways associated with chromosomal instability and cellular proliferation (Figure 4E, Supplementary Table 6), the latter being consistent with previous studies identifying PBRM1 as critical transcriptional regulator of p21/*CDKN1A* in breast cancer cell lines<sup>12</sup> and work showing that PBRM1/Baf180 is implicated in regulating TP53 mediated replicative senescence<sup>18</sup>. The PBAF complex has been shown to localise at kinetochores during mitosis<sup>19</sup> and SMARCB1 has been implicated in spindle checkpoint control<sup>20</sup>, which would support the loss of PBRM1 giving rise to a chromosomal instability/spindle checkpoint expression phenotype. It may be of interest to further explore spindle checkpoint control in *PBRM1* mutated ccRCC as a potential therapeutic opportunity.

Previous work has demonstrated that *VHL* loss alone is insufficient for ccRCC tumourigenesis arguing the need for additional genetic events<sup>21,22</sup> (Teh, unpublished) and has further suggested the existence of a 3p21 “gatekeeper” ccRCC mutation based on LOH studies<sup>23</sup>. The data presented here strongly suggest that inactivation of *PBRM1* comprises this second major mutation in ccRCC development. Nearly all (36/38) *PBRM1* mutant cases fall into the hypoxia signature group as described previously<sup>2</sup>, including 13/14 cases without demonstrable *VHL* point mutations where expression data is available – further indicating the importance of *PBRM1* in typical ccRCC development. The SWI/SNF complex has been implicated in the normal cellular response to hypoxia, with impairment of the complex rendering cells resistant to hypoxia-induced cell cycle arrest<sup>24</sup>, which would be consistent with selection for frequent loss of *PBRM1* in ccRCC. Multiple cancers have apparently concomitant *VHL*, *PBRM1* and *SETD2* mutations, with all three genes mapping to chromosome 3p, suggesting that the mutations are non-redundant functionally. Half (55/107) of cases in this series with a demonstrable *VHL* mutation<sup>2</sup> have a *PBRM1* mutation. Strikingly, all 9 cases with a *SETD2* mutation have a mutation in either *PBRM1* or *VHL*, with 6 of 9 cases having mutations in all three genes. Physical linkage of these three ccRCC cancer genes together with their potential interaction may be the key driver for the large scale 3p LOH seen in most cases of ccRCC – being particularly parsimonious in requiring only four genetic events to unmask three tumour suppressor genes as opposed to six if the genes were on different chromosomes.

Several other mutated genes of potential interest were identified. In particular, *ARID1A* encoding the BAF250A subunit of the SWI/SNF complex was found to have two heterozygous missense mutations - p.R1020K,c.3059G>A and p.L1872P,c.5615T>C. Both cases (PD2126, PD2127) have a *PBRM1* truncating mutation. Two homozygous *ARID1A* deletions were found in SNP 6.0 data (<http://www.sanger.ac.uk/cgi-bin/genetics/CGP/conan/search.cgi>) in the LB1047-RCC ccRCC and NCI-SNU-5 gastric carcinoma cell lines and loss of *ARID1A* expression has been reported in RCC<sup>25</sup>. Frequent truncating *ARID1A* truncating mutations have recently been reported in clear cell ovarian carcinoma<sup>26,27</sup>. These data all point to *ARID1A* being a cancer gene, likely operative in ccRCC. PD2127 was also found to have a heterozygous truncating mutation in *ARID5B*, related to *ARID1A* and recently implicated in childhood acute lymphoblastic leukaemia susceptibility<sup>28</sup>. The extent to which the other mutated genes identified here contribute to ccRCC will await large-scale follow-up screens. Similarly, exome and whole genome sequencing on a large number of cases is likely to yield further insights.

The identification of a second major cancer gene in ccRCC further defines the genetic and molecular architecture of this tumour type. It is remarkable that *PBRM1*, like the majority of the other non-*VHL* mutated cancer genes identified in ccRCC, is involved in chromatin regulation – again at least in part at the level of histone H3 modification and recognition. Understanding the contribution of *PBRM1* mutation to clinical disease progression and

outcome as well the potential for exploiting SWI/SNF complex abrogation therapeutically are important future areas of renal cancer research.

## Methods Summary

DNA samples from ccRCC patients tumour and matching normal were all obtained under local IRB and LREC approvals for this study and processed as previously described<sup>2</sup>. DNA fragmentation, library preparation and solution phase hybrid capture were according to manufacturer instructions (Agilent Technologies, US) and modified from previously published protocols<sup>5</sup>. Capillary-based Sanger sequencing for confirmations and PBRM1 followup were done as previously described<sup>2</sup> with manual inspection of all sequencing traces. mRNA was extracted from snap-frozen mouse pancreatic lesions and subjected to RT-PCR using a nested PCR approach utilising primers of mouse Pbrm1 exon 23/24 and the Carp- $\beta$ -Actin Splice acceptor sequence of the T2Onc transposon cassette. Resulting bands were gel-purified and subjected to capillary-based Sanger sequencing. PBRM1 or scrambled control siRNAs (Santa Cruz, CA) were transfected into ccRCC cell lines using Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's conditions. Real-time PCR and western blotting were all done utilising standard protocols essentially as described<sup>1</sup>. Expression analyses were carried out as previously described<sup>2</sup>.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

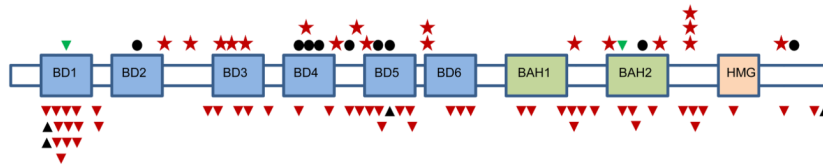
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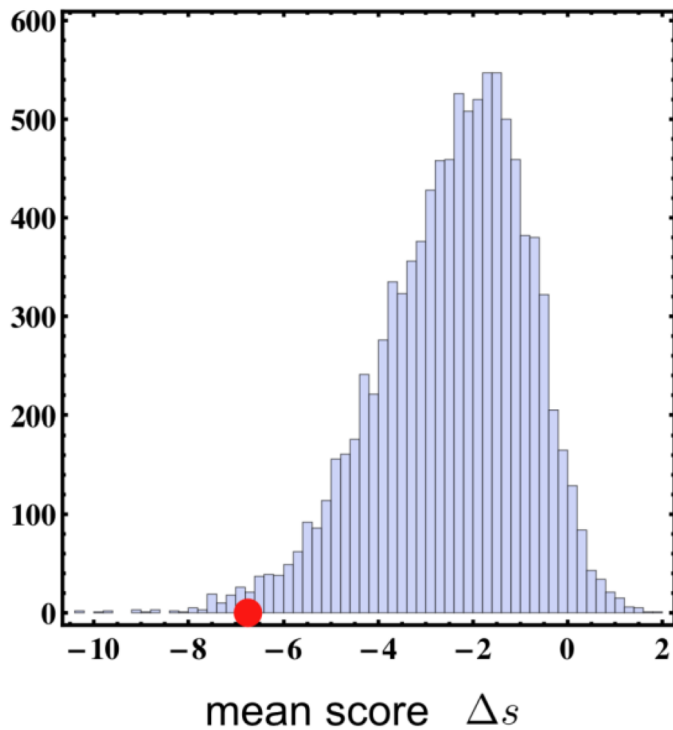
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**Figure 1. PBRM1 somatic mutations**

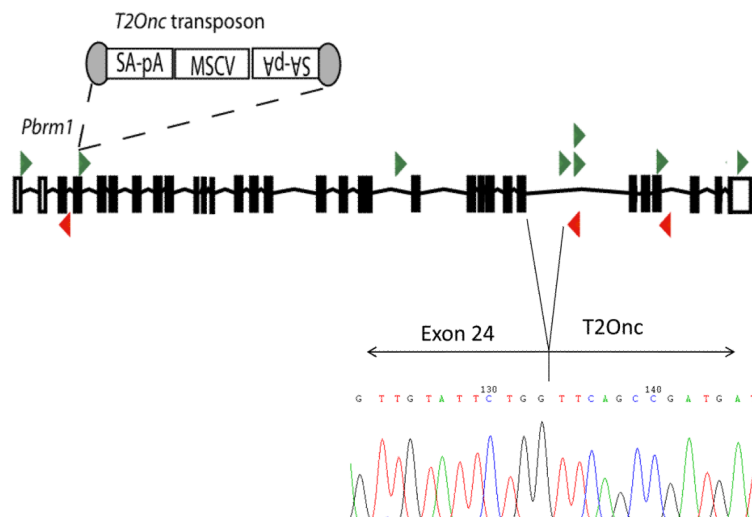
Representation of PBRM1 transcript with boxes BR1-BR6, BAH1-2 and HMG indicating the positions of the bromodomains 1-6, bromo-adjacent homology domains and high-mobility group domain, respectively. Relative positions of mutations are indicated by symbols. Stars – nonsense, dots – missense, red triangles – frameshift deletions, black triangles – frameshift insertions and green triangles – in-frame deletions. Splice-site mutations are not depicted.



**Figure 2. Analysis of PBRM1 missense mutations**

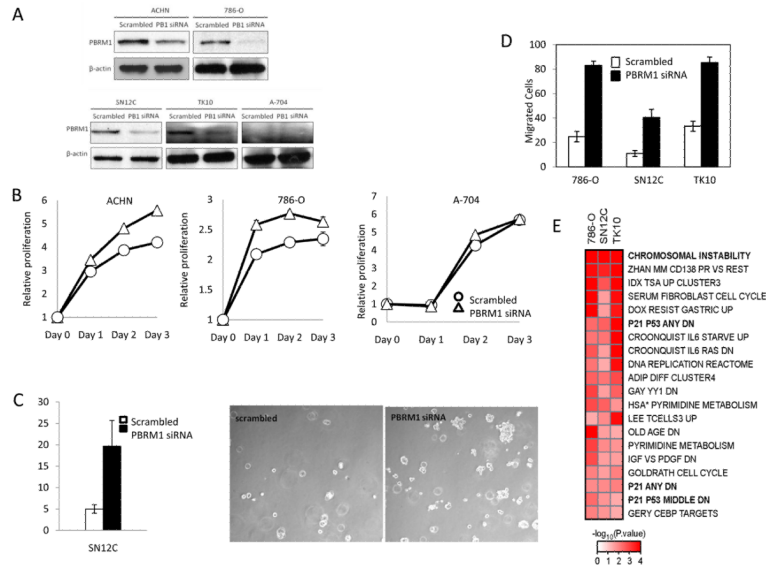
Bars represent histogram of the mean score of *in silico* generated random missense mutations (10,000 sets of three mutations that can be scored) and the red circle denotes the mean score of the somatic mutations that could be scored (T232P  $\Delta s = -7.78$ , A597D  $\Delta s = -9.69$ , H1204P  $\Delta s = -2.76$ ). The somatic set is significantly different from the null set (p-value 0.01). They have a higher negative mean score and are thus predicted to be more deleterious on average.





**Figure 3. *Pbrm1* is frequently mutated in a mouse model of pancreatic cancer**

To identify genes that co-operate with *K-Ras* in the formation of pancreatic cancer a conditional allele of *K-Ras*<sup>G12D</sup> and *Pdx1*-Cre were combined with a conditional *Sleeping Beauty* transposase driver and the *T2Onc*<sup>tg</sup> transposon donor allele<sup>29</sup>. Expression of Cre results in expression of *K-Ras*<sup>G12D</sup> and transposon mobilization within the epithelial compartment of the pancreas. Isolation of the transposon insertion sites from a panel of 153 pancreatic cancers and pre-neoplastic lesions generated from this model revealed a common insertion site in *Pbrm1* suggesting that loss of *Pbrm1* co-operates with *K-Ras*<sup>G12D</sup> in pancreatic cancer development. Statistical analysis was performed as previously described<sup>30</sup>. Transposon insertions in the forward strand of *Pbrm1* are shown in green. Insertions in the reverse orientation are shown in red. A chromatogram from sequencing of RT-PCR products from one tumour is shown demonstrating splicing of exon 24 of *Pbrm1* into the inserted transposon, thus truncating the transcript.



#### Figure 4. Knockdown of *PBRM1* expression in RCC cell lines

(A) Verification of *PBRM1* knockdown by western blotting. (B) Silencing *PBRM1* increased the proliferation of ACHN and 786-O with wild type *PBRM1*, but not A704 with a homozygous *PBRM1* truncating mutation. Data represent means of triplicate experiments with standard deviation,  $p < 0.01$ . (C) Knockdown of *PBRM1* enhanced colony formation in SN12C cells. Data represent means of triplicate experiments with standard deviation,  $p < 0.01$ . (D) Knockdown of *PBRM1* enhanced cell migration in 786-O, SN12C and TK10 cells. Data represent means of triplicate experiments with standard deviation,  $p < 0.01$ . (E) Gene sets that are most significantly deregulated following *PBRM1* knockdown in three RCC cell lines using curated gene sets obtained from MSigDB (<http://www.broadinstitute.org/gsea/msigdb/>) and additional curated gene sets obtained from the *PGSEA* package (see Supplemental Material for details).