Complex MSH2 and MSH6 mutations in hypermutated microsatellite unstable advanced prostate cancer

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A hypermutated subtype of advanced prostate cancer was recently described, but prevalence and mechanisms have not been well-characterized. Here we find that 12% (7 of 60) of advanced prostate cancers are hypermutated, and that all hypermutated cancers have mismatch repair gene mutations and microsatellite instability (MSI). Mutations are frequently complex MSH2 or MSH6 structural rearrangements rather than MLH1 epigenetic silencing. Our findings identify parallels and differences in the mechanisms of hypermutation in prostate cancer compared with other MSI-associated cancers.
Recently, exome sequencing of metastatic prostate cancers revealed that a subset of patients harboured tumors with markedly elevated single-nucleotide mutation rates, defining a new hypermutated subtype. This phenotype was subsequently observed in primary prostate cancer in a tumour that harboured an MSH6 mutation. However, mechanisms that lead to hypermutation and the prevalence of this distinct subtype have not been completely defined. Comprehensive cancer genomics efforts recently published by The Cancer Genome Atlas Research Network (TCGA) reported that 16% of colon cancers and up to 35% of endometrial cancers exhibit hypermutation. For both colon and endometrial cancers, about three quarters of hypermutated tumors were associated with phenotypic microsatellite instability (MSI) and loss-of-function DNA mismatch repair genes via mutation or epigenetic silencing. Therefore, we hypothesized that hypermutated prostate cancer may also be associated with DNA mismatch repair (MMR) gene defects and MSI.

In this study, we identified hypermutation in 7 of 60 patients with advanced prostate cancer. Using a targeted deep sequencing approach, we find that all hypermutated tumors have somatic mutations in MMR genes and associated MSI. In four of seven hypermutated cases, MMR mutations were complex structural rearrangements in MSH2 and MSH6. We conclude that somatic rearrangements in MSH2 and MSH6 are an important mechanism leading to hypermutation and MSI in advanced prostate cancer.

**Results**

**Prevalence of hypermutation.** We identified hypermutated cases in exome sequencing data sets of advanced prostate cancer samples from two sources: a panel of patient-derived xenografts (PDX) and metastatic specimens obtained through a rapid autopsy programme (Supplementary Table 1). Exome data for PDX tumors was from Kumar et al., where hypermutation was previously characterized. In the autopsy samples where hypermutation status had not been previously established, we defined hypermutation as >300 somatic protein altering mutations based on the distribution of total mutation burden in metastatic tumors, which had matched normal tissue available (Supplementary Fig. 1; Supplementary Table 1). We identified hypermutation in 3 of 15 PDX tumors (Table 1), and in metastatic tumors from 5 of 50 autopsy patients (Table 2). There was partial overlap between the two patient groups: five of the PDX tumors were derived from autopsy patients, including one with a hypermutated genome (LuCaP 147). Therefore, there were a total of 7/60 unique patients with hypermutated tumors, for an overall prevalence of 11.6%. Hypermutation status was 100% concordant at different metastatic sites, and was also concordant between primary tumour and metastasis in two patients where primary prostate tumors were available (Table 2).

**Identification of MSH2 and MSH6 rearrangements.** Because exome sequencing has limitations in detecting structural rearrangements and larger insertion/deletion (indel) mutations, we investigated alterations in DNA MMR pathway genes in hypermutated and non-hypermutated cases using a targeted deep sequencing approach (BROCA assay) that included capture of intronic and flanking DNA sequences (Supplementary Table 2). We developed a bioinformatics pipeline to accurately detect structural variation, copy number variation and indel mutations of all sizes.

All three PDX hypermutated tumors had complex structural rearrangements in MSH2, MSH6 or both genes (Table 1; Fig. 1a; Supplementary Figs 2–4), while only 1 of 20 non-hypermutated xenografts had mutations in these genes (LuCaP 145, derived from a patient with neuroendocrine prostate cancer, Supplementary Fig. 5). A second loss-of-function mutation in MSH2 or MSH6 was detected in the three hypermutated PDX tumors, but not in LuCaP 145, supporting a requirement for bi-allelic gene inactivation underlying the hypermutated genome.

We detected mutations with predicted loss-of-function in MSH2, MSH6 or both genes in four of five rapid autopsy patients

| Table 1 | MMR gene mutations in prostate cancer PDX. |
|-----------------|-----------------|-----------------|-----------------|
| | PDX tumour* | Patient-derived from | Hypermutated?1 | MSI | MMR gene mutation(s)2 |
| LuCaP 58 | Yes | Yes | (1) MSH6 del exon 8 to 3’UTR |
| LuCaP 73 | Yes | Yes | (2) MSH2 frameshift (c.3799_3800del) |
| LuCaP 147, 147CR | 05–165 | Yes | Yes | (1) MSH2 and MSH6 copy loss (del 3Mb) |
| | | | | (2) MSH2-FBXO71 inversion |
| | | | | (1) MSH2-C2orf61 343 kb inversion |
| | | | | (2) MSH2-KCNK12 74 kb inversion |
| | | | | (3) MSH2-KCNK12 40 kb inversion |
| | | | | |

MMR, mismatch repair; MSI, microsatellite instability; PDX, patient-derived xenografts.

*Matched pairs of androgen-sensitive and castration-resistant sublines (for example, LuCaP 35 and LuCaP 35CR) and tumour lines derived from the same patient are listed numerically and grouped in the same row.

1Hypermutation status was previously determined in these samples in Kumar et al.1

2Mosaic MSH6 frameshift mutations observed in a poly G tract in exon 5 (c.3261dup/del) and poly A tract in exon 7 (c.3573del) were detected in several hypermutated samples and are not included in the table because they are presumed to be due to MSI.
Hypermutated tumours have phenotypic MSI. MSH2 and MSH6 are mismatch DNA repair genes that act together as a heterodimer, and bi-allelic inactivating mutations of either gene are predicted to result in MSI. PCR of microsatellite loci revealed MSI in all hypermutated tumours, from both PDX and autopsy patients (Fig. 1b; Supplementary Data 1). Phenotypic MSI was also detected directly from targeted next-generation data for all hypermutated tumours, from both PDX and autopsy patients (Fig. 1b; Supplementary Data 1). Immunohistochemistry (IHC) for DNA MMR proteins in hypermutated tumours demonstrated complete loss of MSH2 and/or MSH6 in a pattern consistent with the inactivating mutations detected by sequencing (Fig. 1c; Supplementary Fig. 11). Non-hypermutated tumours were microsatellite stable (Tables 1 and 2; Supplementary Data 1) and had intact MSH2 and MSH6 protein expression by IHC in MSI-positive prostate cancers, but very few MMR mutations have been identified. We speculate that technical limitations have led to an underestimation of MMR gene mutations in prostate cancer.

Our findings support the conclusion that the hypermutated subtype of prostate cancer is chiefly due to loss-of-function mutations in MSH2 and MSH6 that result in MSI. Mutations were predicted to be bi-allelic in all cases except 00–010, which may harbour a second undetected mutation. Most interestingly, four of seven hypermutated cases had complex structural rearrangements in MSH2 and MSH6 that were not detected by exome sequencing in the same samples, and would also not be expected to be detected by traditional exon-based Sanger sequencing methods. Several previous studies have reported MMR protein loss and MSI in both primary and advanced prostate cancers, but very few MMR mutations have been identified. We speculate that technical limitations have led to an underestimation of MMR gene mutations in prostate cancer.

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Our finding of predominantly MSH2 and MSH6 mutations is in contrast to colon and endometrial cancer, where MSI is most often due to MLH1 epigenetic silencing. This supports an alternate mechanism by which MSI is acquired in prostate cancer. A recent study demonstrated that DNA translocations and deletions in advanced prostate cancer occur in a highly hypermutated context, and that these alterations are frequently found in MSI tumours. This suggests that the acquisition of MSI may involve the accumulation of multiple genomic alterations, rather than a single driver event.

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Methods

**Patients and specimens.** The LuCaP series of prostate cancer xenografts were obtained from the University of Washington Prostate Cancer Biorepository. Human primary and metastatic prostate cancer tissues were obtained as part of the University of Washington Prostate Cancer Donor Rapid Autopsy Programme.

In summary, we have shown that complex structural rearrangements in MSH2 and MSH6 or both genes. Shown is a representative complex MSH2 rearrangement present in hypermutated cases LuCaP 147 and 05S-165 (LuCaP 147 was derived from autopsy patient 05S-165). Breakpoints were confirmed by Sanger sequencing. Genomic coordinates are hg19. Detail on additional structural rearrangements and other mismatch repair gene mutations is provided in Tables 1 and 2 and Supplementary Figs 2–9.

(b) Hypermutated tumors exhibited microsatellite instability by PCR. Shown is representative data for LuCaP 58, which is positive for MSI in 3/5 mononucleotide marker systems (MONO-27, BAT-25 and NR-24, arrows). All hypermutated tumors tested were MSI-PCR positive in at least 2/5 loci (Supplementary Data 1). (c) Hypermutated tumors LuCaP 58, 73 and 147 have loss of MSH2 and MSH6 proteins by IHC. Similar results were observed in hypermutated tumors from rapid autopsy patients (Supplementary Fig. 11). A representative non-hypermutated tumour (LuCaP 23.1) has intact expression. LuCaP 145 had mono-allelic mutations in MSH2 and MSH6 but was not hypermutated. IHC shows loss of MSH6 protein expression in some tumour cells. Scale bars, 0.1 mm.
and stable diaminobenzidine (Invitrogen Corp.). All sections were light-ly counterstained with haematoxylin and mounted with Cytoseal XYL (Richard Allan Scientific). Mouse or rabbit immunoglobulin-G was used at the same concentration as the primary antibody for negative controls. Antibodies and dilutions used for IHC are given in Supplementary Table 4.

Immunostaining was assessed using a quasi-continuous score system, created by multiplying each intensity level (0 for no brown colour, 1 for faint and fine brown chromogen deposition and 2 for clear and coarse granular chromogen clumps) with the corresponding percentage of cells expressing the particular intensity, and then summing all values to get a final score for each sample (scores ranging from 0 to 200). Only nuclear staining was evaluated. Samples with damaged tissue core, missing tissue core or poor quality of tissue were excluded from final analysis.

Microsatellite instability PCR. MSI-PCR testing was performed by the University of Washington (UW) clinical genetics and solid tumors laboratory using the Promega MSI analysis kit (Promega, Madison, WI, USA) following the manufacturer’s instructions. Specimens demonstrating instability within two or more of the mononucleotide markers included in this panel were considered ‘MSI positive’, others were considered ‘MSI negative’. The microsatellite loci tested in the Promega MSI analysis kit were NR-21, BAT-26, BAT-25, NR-24 and MONO-27 (Ganbahn Accession # XM_033393, U41210, L04143, X60152, AC007684, respectively).

**MLH1 methylation analysis.** Two to four hundred nanograms of DNA from each sample was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) and eluted in 20 μl volume, according to manufacturer’s protocol.

SYBR Green qPCR to detect methylated and unmethylated MLH1 was performed using a CFX 96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with a final reaction volume of 20 μl, consisting of 500 nM each primer, 9 μM probe, 2X SYBR Green Universal SYBR Green Supermix at the following conditions: 95°C for 3.5 min, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. The unique primer sequences for methylated MLH1 were 5'-CGGATACGGTATTTAAGG-3’ (forward) and 5’-CTCTCCATCCCTCTCCATAACA-3’ (reverse) (ref. 19). The four primers each also included a 20 bp GC-rich tail (5’-GGGTTCGAAGGGGTCTAGT-3’) at their 5’ end. Repetitive Alu sequence (AguC4) was used to normalize for the amount of input DNA. The absolute quantitation of methylated and unmethylated MLH1 in each sample was determined by using the Epitect human methylated- and unmethylated DNA (Qagen, Germantown, MD, USA) to create a standard curve. The SYBR Green assay results are expressed as ratios between methyl-MLH1 or unmethyl-MLH1 values and the ALUC4 control values. The error bars represent the s.e.m.

**Exome sequencing.** Exome sequencing for autopsy samples was performed using the Nimblegen EZ SeqCap kit (Roche, Basel, Switzerland). The libraries were hybridized and captured using the EZSeqCap V1 or V2 solution-based probe, amplified and sequenced on either the Illumina multiplexed sequencing, which is available for free download.

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**Confirmation of MSH2 and MSH6 structural rearrangements.** To validate structural rearrangement calls, we designed primers against regions flanking putative breakpoints using either PrimerBLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) or Primer3 (http://bioinfo.ut.einet.primer3-0.4.0/input.htm). We used the iProof High-Fidelity PCR kit (Bio-Rad) to perform PCR under the following conditions: 98°C for 35 s followed by 30–40 cycles of 55–69°C for 30 s, 72°C for 30 s and 72°C for 10 min. Primers are listed in Supplementary Table 5. We submitted resulting PCR products to Genewiz for Sanger sequencing and aligned fragments to the human genome reference sequence (hg19) using BLAT from the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway).

Copy number changes were confirmed by genomic microarray. One microgram of high molecular weight genomic DNA from each sample was labelled by random priming using the Agilent Genomic DNA Enzymatic Labelling Kit (Cy3- dye). A pool of reference normal DNA (Promega) was labelled with Cy5-dUTP. Cy3 and Cy5 probes were combined and hybridized to Agilent 2 × 40K SurePrint G3 CGH Microarrays and washed following the manufacturer’s specifications. Fluorescence array images were collected using the Agilent DNA microarray scanner G2505C and Agilent Feature Extraction software. Data analysis was performed with the Robust Multi-Copy Number Software, the RASST2 segmentation algorithm and default Adjacent settings for significance, gain and loss thresholds, with at least six probes per segment used to identify regions of CNV for each sample. Results of copy number analysis by genomic microarray are given in Supplementary Fig. 14.

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Author contributions

C.C.P. conceived and designed the study, coordinated sample acquisition and processing and performed primary data analyses. C.M., A.K. and P.S.N. assisted with the study design. T.W., J.S., R.L.V., E.C. and J.F.T. assisted with the study design and reviewed the manuscript. R.L.V., C.M. and E.C. were involved in metastasis and PDX tissues collection and selection. A.K., J.M. and S.J.S. performed confirmatory Sanger sequencing studies. C.M. and X.Z. performed and analyzed the IHC studies. C.S., I.C. and A.K. assisted with the genomic sequencing. I.C., S.I.S., C.C.P. and A.K. coordinated informatics analyses. W.M.G. and M.Y. performed MLH1 methylation studies. C.C.P., P.S.N., R.L.V., T.W. and J.S. directed the research. C.C.P. wrote the manuscript, with contributions from P.S.N., A.K. and C.M.

Additional information

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