Analysis of circulating cell-free DNA identifies multi-clonal heterogeneity of BRCA2 reversion mutations associated with resistance to PARP inhibitors

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Running Title
cfDNA finds PARPi resistance mutations in prostate cancer

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AA and CJL are named inventors on patents describing the use of PARP inhibitors and as such stand to gain financially as part of the ICR “Rewards to Inventors” Scheme.
ABSTRACT
Approximately 20% of metastatic prostate cancers harbor mutations in genes required for DNA repair by homologous recombination (HRR) such as BRCA2. HRR defects confer synthetic lethality to PARP inhibitors (PARPi) such as olaparib and talazoparib. In ovarian or breast cancers, olaparib resistance has been associated with HRR restoration, including by BRCA2 mutation reversion. Whether similar mechanisms operate in prostate cancer, and could be detected in liquid biopsies, is unclear. Here, we identify BRCA2 reversion mutations associated with olaparib and talazoparib resistance in prostate cancer patients. Analysis of circulating cell-free DNA reveals reversion mutation heterogeneity not discernable from a single solid tumor biopsy and potentially allows monitoring for the emergence of PARPi resistance.

STATEMENT OF SIGNIFICANCE
The mechanisms of clinical resistance to PARPi in DNA-repair deficient prostate cancer have not been described. Here we show BRCA2 reversion mutations in prostate cancer patients with metastatic disease who developed resistance to talazoparib and olaparib. Furthermore, we show PARPi resistance is highly multiclonal, and that cfDNA allows monitoring for PARPi resistance.

INTRODUCTION
Men who carry heterozygous deleterious germline mutations in BRCA2 are predisposed to aggressive prostate cancer (1-4). Metastatic prostate tumors that arise in these patients frequently lose their remaining functional BRCA2 allele (4,5). Lack of functional wild type BRCA2 is associated with defects in the repair of double-strand DNA breaks using HRR and with a characteristic mutational spectrum (6,7). Cells lacking HRR must repair double-strand DNA breaks through more error-prone forms of DNA repair such as non-homologous end joining. HRR-deficient tumors show increased sensitivity to drugs such as platinum salts and inhibitors of the DNA repair protein PARP1 (PARPi) that induce double-strand breaks by stalling replication forks and causing replication fork collapse (8,9).
PARPi agents, including olaparib and talazoparib, inhibit PARP enzymatic function and trap PARP1 protein on damaged DNA (10). Multiple mechanisms of resistance to PARPi have been proposed (reviewed in [11]). Amongst these, secondary reversion mutations in the BRCA1/2 genes have been associated with clinical resistance to platinum or olaparib (12-14). However, the mechanism of resistance in prostate cancer, and to PARPi other than olaparib, is unknown.

RESULTS
We studied a patient (patient 1) who harbored a germline BRCA2 mutation (p.K1872X, chr13:32,914,106 A→T). This mutation introduces a premature stop codon in BRCA2 exon 11 by converting a lysine codon (AAA) to a stop codon (TAA), truncating the encoded protein at residue p.1872. Patient 1 was initially diagnosed with a Gleason 6 localized prostate cancer, and was treated with radiation therapy in 2009. His disease subsequently recurred and progressed despite a series of androgen-directed therapies, including nilutamide, enzalutamide, and abiraterone. In June 2014, he underwent biopsy of a bone metastasis as part of a research protocol, and was then treated with the PARPi talazoparib, to which his disease initially responded. The Prostate-Specific Antigen (PSA) of Patient 1 decreased from 16.52 at talazoparib initiation to a nadir of 0.66 after three months on treatment (Figure 1a). However, after seven and a half months of treatment, radiographic and PSA testing showed disease progression. A second metastatic biopsy, this time from the liver, was obtained at this time.

First, we performed mutation and DNA copy number analysis on DNA extracted from the pre-talazoparib and resistant solid tumor biopsies. Tumor content was estimated at greater than 60% by histopathological review of tumor sections (Figure 1b) and variant allele frequency analysis. We identified extensive genome-wide loss of heterozygosity (LOH) and the presence of the BRCA-associated mutation signature three (6), both signature hallmarks of HRR deficiency (15) (Supplementary Figure S1-S3). The pathogenic p.K1872X BRCA2 allele frequency
in this sample was 65%, compared to 50% in the germline DNA sample, and only a single copy of the \textit{BRCA2} locus was present in pre-talazoparib DNA, suggesting loss of the wild type allele (\textit{Supplementary Figure S2, Figure 1c}). Loss of wild type \textit{BRCA2} would be expected to sensitize the tumor to talazoparib, consistent with the observed decrease in PSA after treatment initiation (\textit{Figure 1a}). The resistant biopsy bore two secondary mutant \textit{BRCA2} alleles that carried deletions of 177 and 66 nucleotides respectively. Each allele eliminated the pathogenic p.K1872X mutation and removed 59 or 22 amino acids from the predicted encoded protein, while restoring the open reading frame to encode the critical C terminal region of \textit{BRCA2} (\textit{Figure 1c}, labeled \textit{D1} and \textit{D2}, \textit{Supplementary Tables S1, S2}). Alleles \textit{D1} and \textit{D2} were predicted to produce proteins 3359 and 3396 amino acids in length that carried an altered BRC repeat domain 7 (\textit{Figure 1d}). Previous observations suggest these altered proteins would highly likely restore HRR function and be resistant to talazoparib (12). Reversion deletions in \textit{BRCA2} that eliminate BRC repeats 5-8 have been shown in cell line models to produce \textit{BRCA2} proteins with sufficient HRR function to confer cisplatin and PARPi resistance (12,13). We confirmed that alleles \textit{D1} and \textit{D2} were exclusively present in the resistant tumor biopsy and not the pre-talazoparib or germline DNA by PCR amplification (\textit{Figure 1e}). These observations suggest that suggesting these alleles were not present in these samples until after talazoparib therapy was initiated.

Targeted sequencing of circulating cell-free DNA (cfDNA) was performed on plasma that was collected from patient 1 when the resistant liver biopsy was performed. At a local read depth of 200x, 48 DNA reads (24%) bore the p.K1872X allele while 38 reads (19%) bore the wild-type allele. We identified 7 DNA fragments corresponding to deletion \textit{D1}, 37 fragments corresponding to \textit{D2}, and five additional deletion alleles not observed in the solid tumor (\textit{Figure 1c}, labeled \textit{D3} through \textit{D7}, \textit{Supplementary Tables S1, S2}). A total of 84 reads, when aligned to the genome, contained deletion alleles \textit{D1} through \textit{D7}, accounting for the valley in read depth visible at this locus (\textit{Figure 1c}). All deletion alleles eliminated the pathogenic p.K1872X mutation and restored the open reading frame of \textit{BRCA2}, producing
predicted proteins of residue length 3409 to 3212 compared to the 3418 amino acid full length BRCA2 protein. Notably, deletion D4 was observed in cfDNA more than four times as frequently as deletion D1, but D4 was observed zero times in the solid biopsy. Targeted sequencing of a paraffin section isolated from a physically distinct portion of the resistant liver biopsy using an orthogonal sequencing technology (Ion Torrent) at 1000x coverage depth identified deletion D1 but not deletion D4 (Supplementary Tables S1, S2). Applying this approach to a paraffin-embedded biopsy of patient 1’s metastatic lesion that was obtained before talazoparib therapy did not identify any of the deletions in Figure 1C. These observations raised the possibility that deletions other than D1 and D2 originated from a distinct metastasis that was not biopsied, or from a physically separate region of the same metastasis.

We next analyzed cfDNA isolated from a second prostate cancer case. Patient 2 was diagnosed with widespread metastatic prostate cancer in 2013 and progressed on numerous systemic therapies, including leuprolide, bicalutamide, enzalutamide, abiraterone, and eventually docetaxel. In late 2015, germline genetic testing demonstrated that he carried a germline heterozygous deletion of two nucleotides in BRCA2 (p.R259fs) that introduced a stop-gain prematurely truncating the protein at residue 274. The PSA of patient 2 decreased on olaparib treatment from 355 to 42 after four months of treatment, with a nadir of 40.63 (Figure 2a). Scans at four months of treatment showed a radiographic response (decrease in size of bony lesions, visceral lesions, and lymphadenopathy) and functional improvement (ECOG score improvement from 2 to 1) (Figure 2b, 2c). However, by seven months of treatment, the patient’s PSA had reached 96.10, and a repeat bone scan at that time demonstrated numerous new bone and hepatic metastases (Figure 2c).

DNA copy number analysis of cfDNA of patient 2 identified single copy loss at the BRCA2 locus (Supplementary Figure S4). In cfDNA isolated from plasma before treatment resistance, at a local read depth of 950x, 258 reads (27%) bore the wild type allele while 711 reads (73%) bore the pathogenic p.R259fs allele (Supplementary Figure S5). In cfDNA isolated from plasma after treatment
resistance, at a local read depth of 968x, 459 reads (44%) bore the wild-type allele. The remaining 509 reads bore the pathogenic p.R259fs allele. Any combination of somatic insertions or deletions (indels) in the p.R259fs allele that restored the reading frame before the stop-gain at residue 274, and did not produce a new stop-gain, would produce an almost full length BRCA2 protein. We identified 105 distinct reads bearing somatic alterations beginning upstream of the germline stop-gain mutation, with 82 of these in exons 9 and 10 immediately preceding the stop gain (Figure 2d). Indel mutations were most frequent immediately upstream of the germline stop-gain mutation, the region a priori most likely to harbor reversion mutations. Each of the 34 distinct alleles resulting from indels in this region was predicted to restore the open reading frame of BRCA2 (Figure 2e). Predicted protein products from these indels are listed in Supplementary Figure S6. The number of distinct reads bearing each type of reversion allele ranged from 22 to 1, with deletion sizes ranging from 1 to 42 bp. Reversion indels on exon 9 were confirmed to be in cis with p.R259fs, as the tumor bore only one copy of BRCA2 (Supplementary Figure S4) and the same DNA read contained both the germline p.R259fs frameshift and the somatic indel.

A total of 82 of the 968 reads in this region (8%) bore an indel. We performed two statistical analyses to support the genetic evidence that indels at this locus, including those observed at low frequency in cfDNA, were likely to represent bona fide reversion alleles. To produce a reversion at this locus an indel of any size must, when combined with the pathogenic germline deletion of two bp, produce a nucleotide change divisible by three (add 2 bp, lose 1 bp, lose 4 bp, etc.). The probability that 82 randomly generated indels would all be of one of these lengths is \((\frac{1}{3})^{82}\), or \(7.5 \times 10^{-40}\). We also compared the length of reversion indels to length of all 10,652 indels observed in sequenced cfDNA across 73 genes. Indels that produced a predicted reversion were significantly longer than the overall distribution (mean length 10.2 bp altered, s.d. 12.6 vs. mean length 1.8 bp altered, s.d. 2.7, \(P < 3 \times 10^{-16}\), Wilcoxon rank sum test, Supplementary Figure S7). This analysis also held for reversion indels supported by a single read (mean length 15.5, s.d. 15.9, \(P =\))
DISCUSSION

Here we report the first mechanistic description of talazoparib resistance, the first \textit{BRCA2} reversion mutations identified in prostate cancer, and the first cases of multi-clonal \textit{BRCA2} reversion mutations as a mechanism of PARPi resistance. The multi-clonal nature resistance in metastatic disease, in the context of a single evolutionary stimulus, was striking. Based on extrapolation from the ovarian and breast cancer literature, these reversion mutations are likely to confer resistance to platinum-based therapies. The broader impact of this finding for this disease is that it provides a relatively non-invasive readout of resistance to PARP inhibitors, and likely to platinum-based agents, and will allow for personalization of therapy based on this readout. Specifically, in patients with BRCA alterations, the emergence of these reversions at very low PSA levels may allow for switching to non-PARPi and non-platinum based therapies earlier in the course of disease progression. In addition, for these same patients, significant decreases in cfDNA levels of these reversion mutations on subsequent therapies may signify the emergence of clones that may again be sensitive to PARP inhibition.

These findings highlight the profound selection pressure exerted by PARP inhibitors on BRCA-deficient tumors to restore BRCA function. The two patients described in this study were selected for analysis because they responded and then later relapsed on PARPi therapy, and because pre- and post-treatment samples were available. No other patients with BRCA mutations were treated with PARPi and had appropriate biopsies or cfDNA samples available for inclusion in this study. Analysis of larger cohorts will provide information about the frequency and kinetics of PARPi reversion mutations in this patient population. Our work further suggests that in order to optimize use of PARPi, strategies will need to be developed to mitigate such mechanisms of resistance. We find that the emergence of somatic genomic alterations conferring treatment resistance can be more comprehensively detected with non-invasive cfDNA approaches capable of capturing genomic
alterations from multiple lesions simultaneously. Overall, this study supports the use of cfDNA to identify emergence of reversion mutations conferring resistance to PARPi in the mCRPC setting.

METHODS

Exome sequencing
The studies undertaken were part of an IRB-approved protocol. All patients provided written informed consent for the studies performed. Biopsies from bone or liver were fresh-frozen in OCT and preserved in liquid nitrogen. 7µm tissue sections were H&E stained and assessed to be at least 50% tumor. DNA was isolated from five 40µM tissue sections using the QIAamp Fast DNA Tissue kit (QIAGEN), with tissue disruption performed by ceramic beads (MP BioMedicals). DNA was quantified by Qubit fluorimeter and verified by a BioAnalyzer DNA 12000 (Agilent). DNA libraries were prepared using 500 ng of DNA as input to the Hyper kit (Kapa), barcoded using TruSeq (Illumina), with three cycles of PCR. Whole exome capture of gene coding regions and a small shoulder region of intron-exon boundaries was performed using SeqCap EZ Exome V3 (64.1 Mb, Nimblegen). Library fragment size was verified by BioAnalyzer High Sensitivity DNA assay (Agilent). Libraries were sequenced on an Illumina HiSeq 4000 instrument. Patient 1’s germline, pretalazoparib, and resistant biopsies were sequenced to a mean depth of 33x, 88x, and 95x respectively.

FFPE sequencing
Tumor samples were macrodissected from unstained 5µm FFPE sections by comparison with a hematoxylin and eosin (H&E)-stained slide. Genomic DNA was extracted using a Macherey-Nagel NucleoSpin Tissue Kit (Clontech, Mountain View, CA). Amplicon libraries were generated from a custom Ion AmpliSeq (Ion Torrent, Carlsbad, CA) panel. 20 ng of DNA derived from FFPE was amplified by PCR using AmpliSeq primers and HiFi master mix (Ion AmpliSeq v. 2.0). Amplicons were treated with FuPa (Ion Torrent) to partially digest sequences and phosphorylate
amplicons. Library concentration was measured with the Ion Library Quantitation Kit. Libraries were amplified for 20 cycles using emulsion PCR on Ion Sphere particles (ISPs) at a 1:2 ratio of library molecules/ISPs (280 × 10^6 molecules/reaction) (Ion Xpress Template kit version 2.0; Ion Torrent). Positive templated ISPs were biotinylated and enriched with MyOne streptavidin C1 dynabeads (Life Technologies/Thermo Fisher, Carlsbad, CA) and sequenced on an Ion Torrent PGM using the Ion PGM 200 sequencing kit 2.0, according to manufacturer's instructions.

cfDNA sequencing

Whole blood was collected in EDTA, centrifuged at 1600 rcf, and chilled at 4°C within two hours of collection. Plasma and buffy coat were stored at -80°C. Germline DNA was extracted from buffy coat using the DNeasy Blood and Tissue kit (QIAGEN, manufacturer's instructions) and quantified with a NanoDrop spectrophotometer (Thermo Scientific). Circulating cfDNA was extracted from 6mL of plasma using the Circulating Nucleic Acids kit (QIAGEN, manufacturer's instructions) and quantified with a Qubit 2.0 Fluorometer and a Qubit dsDNA HS Assay Kit (Life Technologies). 10 to 100 ng of DNA was used for targeted DNA capture; gDNA was sheared to 180 nt fragments by ultrasonication (Covaris); cfDNA samples do not require shearing. A-tailing, end repair, Illumina-compatible adapter ligation and between 12 to 17 cycles of PCR amplification were performed. Products were quantified by Nanodrop. Samples were hybridized to a custom NimbleGen SeqCap panel targeting the exonic regions of 73 genes (Supplementary Table S3) for a minimum of 16 hours at 47°C following the SeqCap EZ system protocols. Libraries were purified with AMPure beads (Agencourt) and quantitated by Qubit. Sequencing was performed on an Illumina MiSeq (V3 600 cycle kit). Patient 1’s pre-olaparib and resistant cfDNA samples were sequenced to a mean depth of 220x. Patient 2’s cfDNA samples were sequenced to a mean depth of 400x (germline) and 1,400x (pre-olaparib and resistant).

Bioinformatics analysis
FASTQ files from Illumina instruments were aligned against HG19 (genome.ucsc.edu) using BWA version 0.7.12 (16), with duplicates marked using Picard MarkDuplicates version 2.7.1. FASTQ files from Ion Torrent were aligned against HG19 using TMAP version 3.4.1. BAM/SAM file manipulation was performed with samtools version 1.3.1 (17). Genotyping was performed using the GATK version 3.3-0-g37228af (18). Functional annotation was performed in ANNOVAR version 2016Feb01 (19). Copy number calling of solid tumor exomes was performed using CNVkit version 0.8.1 (20). Genome coverage was calculated using bedtools version 2.26 (21). The bounds and frequency of reversion alleles in Patient 1's post-talazoparib DNA were defined by DNA reads that included sequence from both sides of the deleted region. Reversion alleles present in Patient 1 and Patient 2's DNA were identified by visual inspection and Pindel version 0.2.5b9 (Supplementary Methods) (22). Mutation signature analysis was performed in R (23) with the deconstructSigs (24) package. Sequencing data are available at synapse.org, doi:10.7303/syn9652227.

**PCR primers and conditions**

PCR amplification was performed on genomic DNA using the primer pair forward: 5' CTTGATTCTGGTATTGAGCCAGT 3', reverse: 5' TGAGCTGGTCTGAATGTTCG 3', amplified for 35 cycles at an annealing temperature of 62°C with Q5 polymerase (NEB) using manufacturer's standard conditions. This primer pair was designed to produce a 775 nt product on HG19 reference DNA.

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REFERENCES


**FIGURE LEGENDS**

**Figure 1:** PARPi resistance by multiple large deletions. 1a: Timeline of patient 1’s serum PSA level before and during treatment with talazoparib. 1b: H&E stains of the solid tumor biopsy pre- and post-talazoparib were consistent with approximately 60% tumor purity. 1c: DNA read coverage of patient 1’s germline, pre- and post-talazoparib solid tumor, and post-talazoparib liquid biopsy.
Horizontal white lines indicate the span of deletions, numbered D1 through D7, with deletion frequency indicated at the right side. The location and frequency of the p.K1872X/wild type allele is noted in gold/blue. **1d:** Schematic protein models for BRCA2 WT, p.K1872X, and reversion isoforms. **1e:** PCR amplification of the region around deletions D1 and D2 in patient 1’s germline, pre-talazoparib, and post-talazoparib solid biopsy confirms the presence of deletions D1 and D2 in post-talazoparib DNA.
Figure 2: Olaparib resistance in Patient 2 was associated with reversion mutations. 2a: Timeline of patient 2’s serum PSA level before and during treatment with olaparib, showing a drop in PSA from 355 to a nadir of 40, followed by increase associated with drug resistance. 2b: CT scans from patient 2 pre-olaparib, on treatment, and after resistance show a liver metastasis at 17 mm on 2/8/16, decreased to 6 mm on 6/16/16 after olaparib treatment began, and at 18 mm on 9/13/16 respectively. 2c: Bone scans of patient 2 at times matching 2b, showing widespread metastasis, treatment response, and recurrence of metastatic lesions. 2d: Counts of indels observed exclusively in resistant cfDNA, binned into 50 bp intervals across BRCA2. Exon bounds shown at top. The pathogenic stop gain in p.R259fs allele is at nucleotide 1050, noted with red arrow. 2e: Diagram of BRCA2 reversion mutation alleles observed in patient 2. Wild type BRCA2 nucleotide and protein sequence, p.R259fs nucleotide and protein sequence, and BRCA2 reversion alleles present in patient 2’s post-olaparib DNA in all of exon 9 and the first 48 nucleotides of exon 10. Black vs. grey bars indicates observed vs. inferred nucleotide sequence. Deleted nucleotides shown as a thin black line with tics for each deleted nucleotide, insertions highlighted in blue, mutations highlighted in orange, alternate splice acceptor highlighted in green. For clarity, nucleotides are draw on reversion alleles where insertions are present; otherwise nucleotide sequence corresponds to WT. Count of each allele is shown at right.
Figure 1

**a**

PSA

- **6/9/14** first biopsy
- **1/22/15** second biopsy

**b**

- pre-talazoparib
- post-talazoparib

**c**

- Coverage for germline
- Coverage for pre-talazoparib solid biopsy
- Coverage for post-talazoparib solid biopsy
- Coverage for post-talazoparib liquid biopsy

**d**

- WT
  - BRC Repeats (1-8)
  - DNA-Binding domain
  - TR2/NLS

- **p.K1872X**
  - BRC Repeats (1-6)

- **revertant**
  - BRC Repeats (1-6, 8)
  - DNA-Binding domain
  - TR2/NLS

**e**

- germline
- pre-talazoparib solid biopsy
- post-talazoparib solid biopsy
Figure 2

(a) Graph showing PSA levels over time.

(b) CT scans showing tumor progression:
- 2/8/16: 17 mm tumor
- 6/16/16: 6 mm tumor
- 9/13/16: 18 mm tumor

(c) Lateral views of CT scans:
- 2/8/16: Initial scan
- 6/16/16: Post-treatment scan
- 9/13/16: Follow-up scan

(d) Mutation analysis of BRCA2:
- Exon 9: p.R259fs stop gain
- Exon 10: p.R259fs stop gain

(e) Allele analysis showing the presence of revertant alleles.
Analysis of Circulating Cell-free DNA Identifies Multi-clonal Heterogeneity of BRCA2 Reversion Mutations Associated with Resistance to PARP Inhibitors


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