Diverse *BRCA1* and *BRCA2* Reversion Mutations in Circulating Cell-Free DNA of Therapy-Resistant Breast or Ovarian Cancer

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ABSTRACT

Purpose: Resistance to platinum-based chemotherapy or PARP inhibition in germline *BRCA1* or *BRCA2* mutation carriers may occur through somatic reversion mutations or intragenic deletions that restore BRCA1 or BRCA2 function. We assessed whether *BRCA1/2* reversion mutations could be identified in circulating cell-free DNA (cfDNA) of ovarian or breast cancer patients previously treated with platinum and/or PARP inhibitors.

Experimental Design: cfDNA from 24 prospectively accrued *BRCA1*- or *BRCA2*-germline mutant patients, including 19 platinum-resistant/refractory ovarian cancer and five platinum and/or PARP inhibitor pre-treated metastatic breast cancer patients, was subjected to massively parallel sequencing targeting all exons of 141 genes and all exons and introns of *BRCA1* and *BRCA2*. Functional studies were performed to assess the impact of the putative *BRCA1/2* reversion mutations on BRCA1/2 function.

Results: Diverse and often polyclonal putative *BRCA1* or *BRCA2* reversion mutations were identified in cfDNA from four ovarian cancer patients (21%) and from two breast cancer patients (40%). *BRCA2* reversion mutations were detected in cfDNA prior to PARP inhibitor treatment in a breast cancer patient who did not respond to treatment, and were enriched in plasma samples after PARP inhibitor therapy. Foci formation and immunoprecipitation assays suggest that a subset of the putative reversion mutations restored BRCA1/2 function.

Conclusions: Putative *BRCA1/2* reversion mutations can be detected by cfDNA sequencing analysis in ovarian and breast cancer patients. Our findings warrant further investigation of cfDNA sequencing to identify putative *BRCA1/2* reversion mutations and to aid the selection of patients for PARP inhibition therapy.

TRANSLATIONAL RELEVANCE

Ovarian and breast cancers in women with germline *BRCA1* and *BRCA2* mutations are highly sensitive to platinum-based chemotherapy and PARP inhibitors. In this exploratory, hypothesis-generating study, we provide evidence that *BRCA1/2* reversion mutations, which based on preclinical studies would be anticipated to cause resistance to PARP inhibitors, are detectable in a subset of ovarian and breast cancer patients previously treated with platinum-based chemotherapy and/or PARP inhibitors. Given that these putative reversion mutations can be polyclonal within a patient and present frequently at low variant allele frequencies, very high sensitivity cfDNA assays are required to detect these reversion mutations, and may help determine which ovarian and breast patients are unlikely to benefit from PARP inhibition.

INTRODUCTION

BRCA1 and *BRCA2* play pivotal roles in homologous recombination (HR) DNA repair(1), and germline mutations affecting these genes result in an increased risk of early breast and ovarian cancer development(1). Complete loss of BRCA1 or BRCA2 function results in lack of HR repair of DNA double-strand breaks(1). Cancer cells arising in germline *BRCA1* and *BRCA2* carriers lose the wild-type allele, and, as a consequence, lose competent HR due to bi-allelic inactivation of *BRCA1* or *BRCA2*. In this context, DNA double-strand breaks are repaired by error prone mechanisms, such as non-homologous end-joining(2-4). Tumors harboring defective HR DNA repair have been shown to be sensitive to platinum-based chemotherapy and inhibitors of the Poly(ADP) ribose polymerase (PARP)(5,6), given that these agents induce double-strand breaks either directly or through the stalling and subsequent collapse of replication forks. *BRCA1* or *BRCA2* breast and ovarian cancers are reported to be sensitive to platinum-based chemotherapy and PARP inhibition(7-10), with platinum-based chemotherapy serving as the mainstay of treatment of ovarian cancer patients. Several PARP inhibitors have recently been approved for the treatment of advanced *BRCA1-* or *BRCA2*-mutant ovarian cancer(11), and are in phase III clinical trials for patients with *BRCA1-* or *BRCA2*-mutant breast cancer(12). Importantly, several mechanisms of resistance to these agents have been reported in preclinical models and in clinical studies(3,4). One mechanism of resistance to platinum-based chemotherapy and PARP inhibitors is in the form of reversion mutations or intragenic deletions that restore the open reading frame of the original germline *BRCA1* or *BRCA2* mutation, resulting in a functional protein with reacquisition of competent HR DNA repair(13-15).

Circulating tumor DNA (ctDNA) found in the plasma of cancer patients has been shown to constitute a source of tumor-derived DNA that can be employed for the analysis of sequencing-based biomarkers(16). Although ctDNA frequently comprises only a small fraction of total circulating cell-free (cf)DNA and varies according to disease burden and between cancer types(17), it is possible to detect much of the entire repertoire of somatic genetic alterations found in primary tumors or

metastatic disease in cfDNA samples if high-depth sequencing approaches are employed(18-20). In addition, multi-clonal *BRCA2* reversion mutations associated with resistance to PARP inhibitors have been identified in cfDNA from two metastatic prostate cancer patients with germline *BRCA2* mutations(21).

The aims of this exploratory, hypothesis-generating study were to define whether putative *BRCA1/2* reversion mutations can be detected in the cfDNA of *BRCA1* or *BRCA2* ovarian and breast cancer patients resistant or refractory to platinum-based chemotherapy or PARP inhibitors, to determine whether the putative *BRCA1/2* reversion mutations found in these patients could have an impact on BRCA1/2 function, and to develop analysis techniques that could potentially be employed in the implementation of biomarkers for future patient selection.

MATERIAL AND METHODS

Patient cohorts

Nineteen ovarian cancer and five breast cancer patients were prospectively accrued for this study. Inclusion criteria for the ovarian cancer patients encompassed proven *BRCA1* or *BRCA2* germline mutations, stage III or IV disease resistant or refractory to platinum-based chemotherapy, and availability of archived cancer tissue (Table 1). Patients with any other concurrent stage III/IV cancer were excluded. This study was approved by the Institutional Review Board (IRB) of Memorial Sloan Kettering Cancer Center (MSKCC)(IRB #13-128), and written informed consent was obtained from all participants. Radiologic recurrence within six months of last platinum administration was defined as resistant disease, whereas unresponsiveness to or progression during platinum therapy was defined as refractory disease(22). Peripheral blood samples (EDTA tubes) were collected at the time of scheduled chemotherapy following relapse or progression. Of the 19 ovarian cancer patients included, 18 had high-grade serous and one endometrioid ovarian cancer (Table 1). Inclusion criteria for the breast cancer patients entailed proven *BRCA1* or *BRCA2* germline mutations,

metastatic disease and prior treatment with platinum chemotherapy and/or PARP inhibitors. Samples were collected under studies approved by multicenter research ethics committees (ref. nos. 10/H0805/50 and 11/LO1595) in the United Kingdom. Peripheral blood samples (EDTA tubes) were collected upon disease progression, and serially after intervening therapy at subsequent disease progression (Table 2). This study is compliant with the Declaration of Helsinki.

Circulating cell-free (cfDNA) extraction from plasma

To avoid sample issues related to the stability of EDTA cfDNA, blood samples collected in EDTA tubes were processed within 2 hours of sample collection, centrifuged, and plasma samples were stored at −80°C until DNA extraction as previously described(20). DNA was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's instructions as previously described(16,20). DNA was quantified using the Qubit Fluorometer (Invitrogen, Thermo Fisher Scientific).

DNA extraction from peripheral blood leukocytes and tissue

Representative formalin-fixed paraffin-embedded (FFPE) tissue sections of all ovarian tumors and of core biopsies of metastases from the advanced breast cancer patients obtained at diagnosis (patient 1109 patient) and at recurrence (patient L031) were stained with nuclear fast red and microdissected with a sterile needle under a stereomicroscope (Olympus SZ61) to ensure >80% of tumor cell content, as previously described(23). In nine ovarian cancer cases, histologically distinct regions of the primary tumor or distinct anatomical sites including omental implants were available and microdissected (median of 1 (range 1-4) anatomically distinct regions per case). Genomic DNA was extracted from tumor samples and peripheral blood leukocytes using the DNeasy Blood and Tissue Kit (Qiagen) and quantified using the Qubit Fluorometer, as previously described(23,24).

Targeted capture massively parallel sequencing

Tumor DNA from ovarian cancer patients, cfDNA from ovarian and breast cancer patients and their respective germline DNA were subjected to targeted massively parallel sequencing in the MSKCC Integrated Genomics Operation (IGO) as previously described(24,25) using a custom panel of baits encompassing all exons and introns of *BRCA1* and *BRCA2*, and all exons of 141 additional genes reported to be involved in DNA repair, drug resistance, resistance to PARP-inhibitors/ platinum-salts, and genes recurrently mutated in ovarian cancer, including *TP53* (Supplementary Table S1)(26-28). In addition, baits tiling common single nucleotide polymorphisms (SNPs) were included to allow for copy number analysis(25). Serial plasma samples from breast cancer patients L031 and 1109 were subjected also to MSK-IMPACT sequencing targeting 410 key cancer genes, as previously described(25,29). Sequence data were analyzed as previously described (Supplementary Methods)(24,29), and in addition, variants in the cfDNAs and tumors were assessed using the SAMtools mpileup tool(30) and Varscan 2(31). Sequence data are available at the Sequence Read Archive (SRP100525).

Identification of putative *BRCA1/2* reversion mutations and intragenic deletions

Putative somatic reversion mutations or intragenic deletions were defined as somatic genetic alterations that would result in a restoration of the open reading frame of *BRCA1* or *BRCA2* in the cfDNA from a patient harboring a known germline mutation affecting *BRCA1* or *BRCA2*, respectively. To identify putative reversion mutations and intragenic deletions, we extracted all reads from *BRCA1* or *BRCA2* (i.e. the gene affected by the germline mutation in a given case). Among these reads, we used SAMtools mpileup tool(30) to search for 1) somatic small insertions and deletions (indels) that would restore the reading frame of BRCA1/2 in patients with germline indels, 2) somatic single nucleotide variants (SNVs) that restore the *BRCA1/2* reference allele in patients with germline point mutations, and 3) intragenic deletions that delete the *BRCA1/2* germline mutation and result in restoration of the open reading frame. For SNVs and indels, single reads

supporting a mutation were also examined owing to the limited fraction of ctDNA in total plasma cfDNA.

To account for potential large intragenic deletions (>40) that may not be aligned as single reads by BWA(32), we further included clipped reads aligning to multiple locations and spanning the germline mutation as putative intragenic deletions. All putative large intragenic deletions were visually inspected using the Integrated Genomics Viewer (IGV)(33).

All putative *BRCA1/2* reversion mutations and intragenic deletions were annotated using Oncotator(34), in conjunction with the respective *BRCA1/2* germline mutation. The cDNA changes predicted by Oncotator were applied consecutively to the BRCA1/2 cDNA transcripts and translated into amino acids. We further inferred the Levenshtein distance, which denotes the number of insertions, deletions and substitutions required to change one protein into the other(35). Each of the germline mutant and putative reversion mutant BRCA1/2 proteins were annotated with their respective Levenshtein distance to the wild-type BRCA1/2 protein. Any of the putative reversion mutations that differed in this metric compared to the germline mutation were flagged for manual review. Scripts to aid in the search of reversion mutations and compare their protein sequences are available online(36).

Quantification of tumor DNA in total plasma DNA

To ascertain the fraction of tumor ctDNA in the cfDNA obtained from plasma of ovarian cancer patients, we employed i) the *TP53* variant allele fractions (VAFs) of the ovarian tumors given that *TP53* mutations are present, clonal and truncal in >97% of high-grade serous ovarian cancers (HGSOCs)(37,38), ii) the tumor ploidy, local *TP53* copy number and tumor purity based on FACETS(39) and ABSOLUTE(40), and iii) the *TP53* VAF from plasma. If *TP53* mutations were not identified in cfDNA, a distinct clonal mutation was employed for analysis. The fraction of ctDNA in

cfDNA could not be defined in cases where only subclonal mutations from the ovarian tumor were detected in the respective cfDNA.

Given the lack of matched tumor tissue from the metastatic breast cancers subjected to targeted massively parallel sequencing, a different approach was employed to infer the fraction of ctDNA in total plasma DNA. The cfDNA was quantified on a Bio-Rad QX100 droplet (d)PCR using ribonuclease P (RNase P) as a reference, as previously described(20). At least two negative control wells without DNA were included in each run. The amount of amplifiable RNase P DNA and the number of RNase P copies were calculated using the Poisson distribution in QuantaSoft (Bio-Rad), and used together with the highest VAF identified by targeted massively parallel sequencing in the cfDNAs of the breast cancer patients to infer the fraction of ctDNA in cfDNA.

Droplet PCR (dPCR)

The putative c.85delG *BRCA1* reversion mutation identified by massively parallel sequencing in case OCT15 was validated using the Raindrop dPCR system (RainDance Technologies) as previously described(41). dPCR conditions were optimized as previously described(20), and assay sensitivity was tested using *BRCA1* wild-type DNA library spiked in with a *BRCA1* c.85delG mutant synthetic oligonucleotide. Massively parallel sequencing libraries from the ovarian tumor samples and plasma DNA samples of case OCT15 were loaded onto the RainDrop Source instrument for droplet generation for amplification (forward 5'-ACTTTGTGGAGACAGGTT-3', reverse 5'-TGAGCCTCATTTATTTTCTTTT-3' PCR primers), and loaded onto the Raindrop Sense instrument for droplet counting and fluorescence intensity readout as previously described(41). Libraries from germline DNA spiked in with 10, 100, and 1,000 c.85delG *BRCA1* synthetic oligonucleotide molecules were included in the run as controls and for gating purposes. Data were analyzed using the RainDrop Analyst data analysis software.

Targeted amplicon re-sequencing

The putative *BRCA2* reversion mutations and the somatic *SPEN* and *TGFBR1* mutations identified by massively parallel sequencing were validated in three plasma samples from case L031 using targeted amplicon re-sequencing. In case 1109 three somatic variants affecting *FAT3*, *ERCC4* and *KDM5C* were validated together with the putative *BRCA2* reversion mutations identified in the post-treatment plasma sample and in a tumor metastasis core biopsy affecting the liver obtained prior to treatment. At least 10ng of plasma DNA, microdissected tumor DNA and matching peripheral blood leukocyte-derived germline DNA were amplified using Taq Hifi polymerase (Ion AmpliSeq Library Kit 2.0, ThermoFisher Scientific), and libraries prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs). As controls, matched normal DNA from cases 1109 and L031 and plasma DNA from two unrelated advanced breast cancer patients not treated with PARP inhibitors or platinum-based chemotherapy and two tumor DNA samples obtained from unrelated breast cancer core biopsies were included. PCR conditions and primers are available on request. The quality and quantity of the amplification was tested using the Agilent 2100 Bioanalyzer and the KAPA Library Quantification Kit for Illumina (Kapa Biosystems), respectively. Amplicon re-sequencing of the putative *BRCA2* reversion mutations in the cfDNA samples of L031 and 1109 was performed twice independently, using an Illumina HiSeq2500 (first run) and an Illumina MiniSeq (Mid output kit; second run). Sequence data analyses are described in the Supplementary Methods. Only *BRCA2* reversion mutations present in plasma DNA with zero counts in the germline control and in the unrelated control samples were considered validated.

Cell lines

293T cells and U2OS cells were obtained from the American Type Culture Collection (ATCC) in 2008 and 2015, respectively. The identities of the cell lines were confirmed by short tandem repeat profiling after receipt as previously described(42), and tested for mycoplasma infection using a PCR-

based assay (ATCC) following the manufacturer's instructions (latest test in 2016). The cells were passaged no more than 20-25 times after thawing.

BRCA1 foci formation

The U2OS-double-strand break (DSB) reporter system was employed to define the ability of putative *BRCA1* somatic reversion mutations to recognize DSBs, as previously described(43-45) (Supplementary Methods).

BRCA2 interaction with PALB2 and RAD51

293T cells were transfected with pCDNA-HA-BRCA2 plasmids (i.e. wild-type HA-BRCA2, germline c.407delA HA-BRCA2del407 and putative reversion c.402_413delTCTAAATTCTTG HA-BRCA2REV) for 72 hrs, and lysed in lysis buffer (0.5% NP40, 25 mM Tris pH 7.5, 450 mM NaCl, 0.5 mM EDTA and proteinase inhibitors). The cell lysates were then incubated with anti-HA agarose beads (Sigma). After three washes with wash buffer (0.5% NP40, 25 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA), the beads were boiled in SDS sampling buffer, followed by western blotting with antibodies against HA (Santa Cruz), PALB2 (Novus Biologicals) and RAD51 (Santa Cruz).

RESULTS

BRCA1 reversion mutations in ctDNA from platinum resistant/refractory ovarian cancer patients

We developed a targeted capture sequencing assay comprising the coding regions of 141 genes and all intronic and exonic regions of *BRCA1* and *BRCA2* (Supplementary Table S1). We first established the potential of the assay for detecting ctDNA in patients with ovarian cancer, as prior studies suggested low rates of mutation detection in ctDNA of ovarian cancer patients(46). Massively parallel sequencing analysis of germline DNA from peripheral blood leukocytes, microdissected tumor and plasma DNA of 19 cases (*BRCA1*, n=12; *BRCA2*, n=7), using previously

validated methods(18,20), yielded median depths of coverage of 1,569x (range 852x-2,272x), 823x (range 272x-2,328x) and 1,978x (range 1,287x-4,157x), respectively (Supplementary Table S2). Given that somatic *TP53* mutations are present in >97% of all high-grade serous ovarian cancers (HGSOCs) and that the vast majority of these mutations are clonal (i.e. bioinformatically inferred to be present in virtually all cancer cells within a sample) and truncal (i.e. present as clonal mutations in all samples analyzed)(37,38), we reasoned that the *TP53* mutant allele fractions in cfDNA from patients with HGSOC could be employed to ascertain indirectly the fraction of tumor DNA in the total plasma DNA. In all 19 ovarian cancers sequenced, clonal *TP53* mutations were detected (Supplementary Table S2, Supplementary Fig. S1). In nine cases, multiple anatomically distinct areas of the ovarian cancer were microdissected and/or peritoneal implants and/or metastatic sites were available and sequenced separately; the *TP53* mutations in these nine cases with multi-region sequencing were found to be clonal and truncal (Supplementary Table S2, Supplementary Fig. S1). Other somatic mutations detected in the 19 ovarian cancers studied here included *NF1*, *ERCC4, RB1* and *CHEK2* (Supplementary Table S2, Supplementary Fig. S1).

Analysis of the cfDNA from these patients revealed the presence of the same somatic *TP53* mutation identified in the tumors from the respective ovarian cancer patients in 15 out of 19 cases (79%). *TP53* VAFs in the plasma DNA ranged from 0.06% to 32.7% (Supplementary Fig. S1, Supplementary Table S2), and only in 4 samples these *TP53* mutations were identified using our standard bioinformatics pipeline. In three cases (OCT1, OCT5, OCT12), none of the clonal *TP53* mutations present in the tumors were detected in the plasma, but other somatic mutations were identified, including *RB1*, *NF1* and *FAT3* mutations (Supplementary Fig. S1). In one case (OCT3) neither the clonal *TP53* nor the subclonal *NF1* somatic mutations present in the primary ovarian cancer were detected in the cfDNA (Supplementary Table S2, Supplementary Fig. S1). Overall, our assay identified ctDNA in plasma of 95% (18/19) patients with advanced ovarian cancer, and the median percentage of ctDNA in cfDNA was found to be 0.31% (range 0%-32.74%)(Table 1).

Having demonstrated the high sensitivity to detect ctDNA with our assay, we investigated whether putative *BRCA1/2* reversion mutations could be detected in cfDNA. Using a conservative bioinformatics strategy (see Methods), six putative somatic *BRCA1* reversion mutations in four patients OCT1, OCT5, OCT10 and OCT15 were identified with VAFs ranging from 0.0314% to 0.0850% (Fig. 1, Supplementary Fig. S2, Supplementary Table S3). Four of the six putative reversion mutations were flanked by microhomology sequences (Supplementary Table S3). Using dPCR(20) and spiked-in synthetic c.85delG oligonucleotides as controls, we detected the putative somatic c.85delC *BRCA1* reversion mutation in the cfDNA of patient OCT15 (Fig. 2A), which harbored a germline *BRCA1* c.68-69delAG mutation. By contrast, however, analysis of the pre-treatment primary tumors from the ovary and fallopian tube as well as a peritoneal implant of patient OCT15 showed no reliably detectable *BRCA1* reversion mutations (Fig. 2A). Validation of the reversion mutations in the other cases was not possible given that no or insufficient amounts of residual plasma DNA were available.

To ascertain whether these putative somatic *BRCA1* reversion mutations would restore the ability to recognize double-strand breaks, we employed the U2OS-DSB reporter system(43-45). Following 8 Gy of ionizing irradiation, we observed that three putative *BRCA1* somatic reversion mutations, all of which were flanked by microhomology sequences, namely c.108delC (OCT5), c.113delA and c.85delC (both OCT15), resulted in an induction of *BRCA1* foci to levels higher than those observed in U2OS cells expressing the respective germline *BRCA1* mutation (Fig. 2B). We therefore demonstrated that ctDNA sequencing can detect putative *BRCA1* reversion mutations, and that these mutations may restore BRCA1 function in *in vitro* assays.

***BRCA2* reversion mutations in ctDNA from breast cancer patients previously treated with platinum-salts and/or PARP inhibitors**

We next defined whether somatic reversion mutations would be detected in ctDNA of PARP inhibitor- and/or platinum-resistant advanced breast cancer patients harboring *BRCA1* (n=1) or *BRCA2* (n=4) germline mutations. Germline DNA extracted from peripheral blood leukocytes and a single (n=2) or two sequential (n=3) plasma DNA samples per patient were sequenced with the same custom targeted capture sequencing assay described above to a median depth of coverage of 537x (range 457x-630x) and 1,646x (range 1,163x-3,153x, respectively (Supplementary Table S2). In all cfDNA samples analyzed somatic mutations were identified (VAFs, 2.38%-54.54%), including somatic *TP53* mutations in two cases (Supplementary Table S2, Supplementary Fig. S3). Whilst the amount of cfDNA obtained per ml of plasma was similar between the breast and ovarian cancers studied here (breast median 7.8ng cfDNA/ml plasma (range 5.0ng-87ng) vs ovarian median 12.0ng cfDNA/ml plasma (range 4.8ng-32.4ng), p=0.2905, Mann-Whitney U test), the percentage of ctDNA was significantly higher in breast compared to ovarian cancer patients (breast median 12.7% (range 5.2-54.5%) vs ovarian 0.31% (range 0%-32.74%, p<0.0005, Mann-Whitney U test, Tables 1 and 2). It should be noted, however, that the methods for the quantification of ctDNA percentage in cfDNA applied to the breast and ovarian cancer samples differed, and these methodological differences may at least in part explain the distinct levels of ctDNA in cfDNA between the two groups. Despite this important caveat and consistent with the notion that the percentage of ctDNA in cfDNA was higher in the breast cancer patients than in the ovarian cancer patients studied here, SNVs and indels in the cfDNA of the metastatic breast cancer patients were identified using our standard bioinformatics approach, whereas these were detectable using the standard bioinformatics approach in the cfDNA of only 4/19 ovarian cancer patients.

Analysis of the sequencing data further revealed the presence of multiple putative somatic reversion mutations in two of the *BRCA2* germline mutation carriers (L031 and 1109) at VAFs ranging from 0.0549% to 0.2273% (Fig. 3, Supplementary Table S3). To validate the multiple polyclonal *BRCA2* mutations we developed an orthogonal amplicon sequencing strategy (see methods), which was

employed twice independently and confirmed the presence of all detected reversion mutations, and identified six additional putative *BRCA2* reversion mutations in the cfDNA of cases L031 and 1109 (Fig. 3, Supplementary Table S3), with no detected mutations in controls (method). In the plasma sample taken after carboplatin and PARP inhibitor treatment from patient 1109, harboring a c.750_753GACAdel germline mutation, up to nine distinct putative *BRCA2* reversion mutations were identified suggesting poly-clonality at resistance (Fig. 3A, Supplementary Table S3). Validated putative somatic reversion mutations were confirmed to restore the reading frame of *BRCA2* and were flanked by (micro) homology sequences (Supplementary Table S3). All of the reversion mutations preserved the BRC repeats, which have been shown to be essential for HR DNA repair of double-strand breaks(1,13).

We next sought to define whether the putative *BRCA2* reversion mutation identified in the cfDNA would be present in the matched tumor tissue. We obtained a tumor biopsy sample at initial diagnosis prior to carboplatin/PARP inhibitor treatment from case 1109 and a tumor sample at recurrence (i.e. synchronously with plasma sample 3) from case L031. The quality of DNA obtained from the L031 tumor biopsy was suboptimal and the targeted amplicon sequencing approach failed in this sample. Targeted amplicon sequencing of the initial diagnosis tumor sample from case 1109 confirmed the presence of the somatic *FAT3*, *ERCC4* and *KDM5C* mutations identified in the cfDNA, with VAFs ranging from 16%-55.6% (Supplementary Table S3); however, none of the putative *BRCA2* reversion mutations identified in cfDNA could be detected in the tumor tissue biopsy. This suggested that reversion mutations were selected by therapy, and were not detectable in the biopsied tumor prior to therapy.

Analysis of serial plasma DNA samples from one patient (L031) confirmed the presence of the putative *BRCA2* reversion mutations after carboplatin treatment and prior to treatment with the PARP inhibitor Talazoparib (Supplementary Table S3). The patient did not respond to Talazoparib,

with a differential response with some lesions unequivocally progressing. A decrease in the VAFs of the somatic putative *BRCA2* reversion mutation c.402_413delTCTAAATTCTTG immediately after treatment reflected lower levels of ctDNA in the cfDNA at that time point (with a decrease in tumor specific *SPEN* and *TGFBR1* mutations; Fig. 4A; Supplementary Fig. S4). After subsequent treatment, a novel c.389_406delTTTCCTGTCCACTTCTAA putative *BRCA2* reversion mutation, inferred to restore the open reading frame of the BRCA2 protein and initially detected at minimal levels, increased, suggesting a greater diversity in BRCA2 reversion mutations post-PARP inhibitor therapy (Fig. 4A; Supplementary Fig. 4). These results mirrored the progression of the disease and evidence of PARP inhibitor resistance in the patient.

To ascertain whether the putative somatic *BRCA2* reversion mutation identified in case L031 would show interaction with PALB2 and RAD51, we expressed full length BRCA2, the c.407delA patient-specific germline *BRCA2* mutation, and the c.402_413delTCTAAATTCTTG putative *BRCA2* reversion mutation in 293T cells. We observed that the *BRCA2* reversion mutation but not the *BRCA2* c.407delA germline mutation displayed an intact interaction with PALB2 and RAD51, which was at similar levels as those detected with the wild-type BRCA2 protein (Fig. 4B).

DISCUSSION

BRCA1 and *BRCA2* reversion mutations have been documented as potential mechanisms of resistance to platinum-based chemotherapy and PARP inhibitors in cell line models and patient samples(4,13,14,21,46). Here we report on the detection of putative *BRCA1* and *BRCA2* reversion somatic mutations in the cfDNA of platinum-based chemotherapy and/or PARP inhibitor resistant/ refractory ovarian and breast cancer patients harboring germline *BRCA1* or *BRCA2* germline mutations. We have observed these putative mutations in the cfDNA of 21% (4/19) of platinum resistant/refractory ovarian cancer patients, and 40% (2/5) of platinum and/or PARP inhibitors pre-treated breast cancer patients, suggesting that reversion mutations may not be uncommon in

patients following platinum-based chemotherapy and/or PARP inhibition. The putative reversion mutations in the form of small indels restored the reading frame before the aberrant stop codon produced by the germline mutation, and may have resulted in reacquisition of the DNA repair functions of BRCA1 or BRCA2 (Figs. 1 and 3). Consistent with this notion, *in vitro* studies revealed that three of the putative somatic *BRCA1* reversion mutations identified by targeted capture sequencing of cfDNA restored, at least in part, the ability of cells to elicit BRCA1 foci following ionizing ration treatment. In addition, one of the putative *BRCA2* mutations tested *in vitro* was found to restore the interaction with its partners PALB2 and RAD51. These putative reversion mutations could not be detected in the tumor tissue samples obtained at primary diagnosis, suggesting selection by therapy. No adequate tumor tissue was available contemporaneously with the cfDNA sample to define the frequency of these putative alterations in the resistant/refractory tumors.

Consistent with a recent report describing polyclonal reversion mutations in the cfDNA of two BRCA2 prostate cancer patients treated with PARP inhibitors(21), our findings suggest that polyclonal reversion mutations may also be found in cfDNA of BRCA1/2 ovarian and breast cancer patients treated with platinum-based therapy and/or PARP inhibitors, in particular in BRCA2 cancers. Importantly, however, all mutations were detected at very low allele frequencies in plasma, with more than one mutation present in 67% (4/6) of patients with reversion mutations. In two *BRCA2* breast cancer patients with disease progression after platinum-based chemotherapy and PARP inhibitor therapy, multiple concurrent somatic reversion mutations were detected by targeted capture sequencing and validated using orthogonal sequencing methods. Moreover, in one patient, the mutant allele fractions in plasma DNA increased after PARP inhibitor treatment and resistance development. These observations are consistent with the notion that resistance to targeted therapies may be polyclonal in a given cancer patient (e.g. polyclonal *ESR1* mutations as a mechanism of resistance to aromatase inhibition(47)), even in therapeutic strategies based on synthetically lethal approaches. It should be noted that the VAFs of the putative reversion mutations identified in the

cfDNA of subjects included here was low. Importantly, however, these VAFs were frequently similar to the allele fractions of *TP53* and/or other mutations (Supplementary Table S3) and consistent with the estimated ctDNA content (Table 1), suggesting that at least in a subset of patients these putative reversion mutations were clonally dominant at the time of sample collection. Moreover, it is most likely that similar to other targeted therapy resistance (e.g. EGFR inhibitors in *EGFR*-mutant non-small cell lung cancer), resistance to platinum-based therapy and PARP inhibition may be multifactorial in a single patient (e.g. polyclonal reversion mutations and/or other mechanisms of resistance to platinum-based chemotherapy or PARP inhibitors being present in distinct subclones within a tumor), or that these putative reversion mutations may cause resistance not only in a cell autonomous manner, but also through a bystander effect.

The presence of multiple reversion mutations within a given BRCA1 or BRCA2 patient may result from the strong selective pressures imposed by platinum-based or PARP inhibitor therapy and the type of DNA repair defects cancer cells with defective BRCA1 and BRCA2 display. Consistent with this hypothesis and the more frequent reporting of polyclonal reversion mutations in BRCA2 cancer (e.g. PARP-inhibitor resistant BRCA2-deficient pancreatic cancer cell line and in tumor tissue of BRCA2 ovarian cancer patients(13,48)), loss of BRCA2 function results in defective DNA repair of double strand breaks, as induced by platinum or PARP inhibition, and are repaired preferentially through single strand annealing and non-homologous end-joining(1,2). Given the selective pressure these agents provide in the context of BRCA2 deficient cells, multiple intra-genic deletions could eliminate the site of the germline *BRCA2* mutation and restore the open reading frame without the loss of domains essential for BRCA2 function. By contrast, non-homologous end-joining is the preferential mechanism of repair of DNA double strand breaks in BRCA1 breast cancers(1,2), which may be associated with a lower likelihood of multiple reversion events given the constraints of how a given germline mutation could be reversed and the maintenance of BRCA1 domains essential for its

function in HR DNA repair. Further studies on the polyclonality of *BRCA2* reversion mutations are required to clarify the mechanist basis of these observations.

Our exploratory, hypothesis-generating study has several limitations. Although we focused on BRCA1 and BRCA2 patients with advanced ovarian and breast cancer and performed high-depth sequencing of cfDNA, we did not detect somatic mutations in the cfDNA samples from one patient, and detected them in others at very low levels, which suggest that in a subset of platinum-based chemotherapy resistant/ refractory ovarian cancer patients, the levels of tumor DNA in plasma may be limited. In fact, recent studies have found that somatic mutations, including *BRCA1/2* reversion mutations, can be detected at higher allele frequencies in ascites of patients with ovarian cancer(46,49). Second, the bioinformatics approaches employed for the identification of somatic reversion mutations were able to nominate putative somatic reversion mutations, which were successfully validated using orthogonal methods. Owing to the nature of the sequencing performed (Illumina, 100bp reads), however, we would be unable to detect with a similar sensitivity large deletions that would result in reversion of the germline mutations. Therefore, our study may underestimate the frequency of somatic reversion mutations in the patient population analyzed. Third, we were unable to accrue tumor tissue synchronously collected with the cfDNA samples; therefore, we were unable to validate the presence of the putative reversion mutations in tumors. Fourth, the putative *BRCA1* and *BRCA2* reversion mutations identified in cfDNA were not tested by direct sequencing, given that the VAFs would be beyond the detection limits of Sanger sequencing. We did, however, validate these putative reversion mutations with orthogonal sequencing approaches and using distinct sequencing libraries, minimizing the likelihood of the putative reversion mutations described here constituting sequencing artifacts. Finally, owing to its small sample size, further studies are required to define the prevalence of *BRCA1/2* reversion mutations detected in cfDNA and to test whether they are causative of and predict the lack of therapeutic efficacy to platinum-based chemotherapy or PARP inhibitors, ideally in the context of a prospective

clinical trial or through the reanalysis of materials of a sufficiently powered prospective clinical trial, are required.

Despite these limitations, our study, in conjunction with recent studies(21,46), broadens the potential applications of cfDNA sequencing for the identification of somatic *BRCA1* and *BRCA2* reversion mutations. Further studies are warranted to define the prevalence of these reversion mutations in larger populations of BRCA1 and BRCA2 ovarian and breast cancer patients treated with PARP inhibitors and/or platinum-based chemotherapy, to define the chronology of the emergence of these mutations and the biological impact of their potential polyclonal nature, and to ascertain their role as predictive biomarkers for these therapeutic agents.

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TABLES

Table 1. Clinicopathologic characteristics of ovarian cancer patients included in this study.

ID	Tumor type	BRCA status	Date primary diagnosis	Stage primary disease	Neoadjuvant chemotherapy	Cycles platinum-based chemotherapy (n)	Date disease progression/ distant relapse	Primary refractory/ resistant disease	Secondary refractory/ resistant disease	Date blood draw	Disease location at blood draw	Time follow-up (years)	Follow-up	cfDNA (ng) per ml plasma	Fraction ctDNA in cfDNA (%)
OCT1	HGSOC	BRCA1	February 2008	Stage III	No	6	May 2010		Resistant	August 2013	Intra	8	DOD	27.9	NA
OCT2	HGSOC	BRCA2	February 2011	Stage III	No	6	May 2012		Refractory	August 2013	Intra and extra	3	DOD	20.4	32.74
OCT3	HGSOC	BRCA2	July 2010	Stage IV	No	6	July 2012		Resistant	August 2013	NED	6	AWD	16.3	0
OCT5	HGSOC	BRCA1	July 2012	Stage III	No	6	May 2013	Resistant	Refractory	September 2013	NED	4	AWD	12.0	NA
OCT6	HGSOC	BRCA1	May 2010	Stage IV	No	6	July 2011		Refractory	September 2013	Intra	4	DOD	7.6	2.49
OCT7	HGSOC	BRCA2	May 2008	Stage III	No	6	June 2009		Refractory	September 2013	Intra	8	DOD	6.2	0.32
OCT8	HGSOC	BRCA2	July 2006	Stage IV	No	6	July 2007		Refractory	September 2013	Intra and extra	8	DOD	5.9	1.60
OCT9	HGSOC	BRCA1	April 2011	Stage IV	No	6	August 2012		Refractory	October 2013	Intra and extra	3	DOD	12.0	0.08
OCT10	HGSOC	BRCA1	January 2012	Stage IV	Yes (4 cycles)	3	January 2013	Resistant	No	October 2013	Intra	4	NED	4.8	0.26
OCT11	HGSOC	BRCA2	June 2006	Stage IV	No	7	October 2008		Resistant	December 2013	Intra and extra	9	DOD	11.6	10.30
OCT12	HGSOC	BRCA2	June 2007	Stage III	No	6	July 2010		Refractory	June 2014	Intra	8	DOD	10.8	0.05
OCT13	HGSOC	BRCA1	December 2007	Stage II	No	6	January 2011		Refractory	June 2014	Intra	7	DOD	5.5	0.14
OCT14	HGSOC	BRCA1	January 2012	Stage III	No	6	June 2013		Resistant	June 2014	Intra	4	DOD	24.3	0.08
OCT15	HGSOC	BRCA1	August 2012	Stage IV	Yes (3 cycles)	3	October 2013		Resistant	August 2014	Intra	3	DOD	32.4	0.31
OCT17	HGSOC	BRCA1	August 2008	Stage IV	No	6	January 2010		Refractory	September 2014	Intra and extra	8	DOD	17.4	4.48
OCT18	HGSOC	BRCA2	May 1996	Stage II	No	6	December 2004		Resistant	October 2014	Intra	19	DOD	11.4	5.32
OCT19	HGSOC	BRCA1	September 2010	Stage III	No	6	December 2011		Refractory	December 2014	Intra	4	DOD	30	0.61
OCT20	HGSOC	BRCA1	June 2008	Stage III	No	6	February 2010		Refractory	December 2014	Intra	7	DOD	32.4	0.12
OCT21	Endometrioid (serous components)	BRCA1	June 2008	Stage III	No	14	December 2010		Refractory	March 2015	Intra and extra	7	DOD	15	0.24

AWD, alive with disease; DOD, dead of disease; HGSOC, high grade serous ovarian cancer; intra, Intra-abdominal; Intra and extra, intra- and extra-abdominal; MAF, mutant allele fraction; NA, not assessable due to the lack of a somatic mutation bioinformatically inferred as clonal in the tumor sample; hence, the fraction of ctDNA in cfDNA could not be calculated; NED, no evidence of disease; ND, not detectable.

Table 2. Clinicopathologic characteristics of breast cancer patients included in this study.

ID	Tumor type	BRCA status	Date primary diagnosis	Stage primary disease	Neoadjuvant chemotherapy	Cycles platinum-based chemotherapy (n)	Dates platinum-based chemotherapy	Dates PARP inhibitor therapy	Date disease progression/ distant relapse	Primary refractory/ resistant/ progressive disease	Date(s) blood draw	Disease location at blood draw	Time follow-up (years)	Follow-up	cfDNA (ng) per ml plasma	Fraction ctDNA in cfDNA (%)
L031	IDC	BRCA2	April 2003	Stage IIB	No	8	May-November 2014	April-June 2015	April 2013	PD post platinum	April 2015, June 2015, August 2015	LN, lung, bones	12	DOD	8.6 (P1), 8.1 (P2), 7.8 (P3)	9.8 (P1), 12.6 (P2)
1109	IDC	BRCA2	September 2005	NA	No	5	August - November 2010	March-October 2012	June 2010	PD>12 months post platinum (on maintenance endocrine)	October 2012, May 2013	liver, bone	8	DOD	28.1 (P1), 87.0 (P2)	15.0 (P1), 35.2 (P2)
1159	ILC	BRCA2	November 2010	Stage IIB	No	4	June-August 2014	March-April 2014	February 2011	PD on platinum	May 2014, October 2014	soft tissue, skin, LN, retroperitoneal	5	DOD	5.0 (P1), 7.0 (P2)	11.5 (P1), 12.8 (P2)
L046	IDC	BRCA1	May 2013	Stage IIB	Yes	5	June 2014		June 2015	PD on platinum	July 2015	chest wall	2	AWD	7.5	54.5
1211	IDC	BRCA2	May 2012	Stage IIIB	Yes	-	-	July - September 2015	May 2014	PD on platinum	June 2015	liver, lung, bone	4	DOD	7.6	5.2

AWD, alive with disease; DOD, dead of disease; IDC, invasive ductal carcinoma of no special type; ILC, invasive lobular carcinoma; LN, lymph node;

NA, primary disease staging information not available at the time of data freeze; P1, plasma sample 1; P2, plasma sample 2; P3, plasma sample 3;

PD, progressive disease.

FIGURE LEGENDS
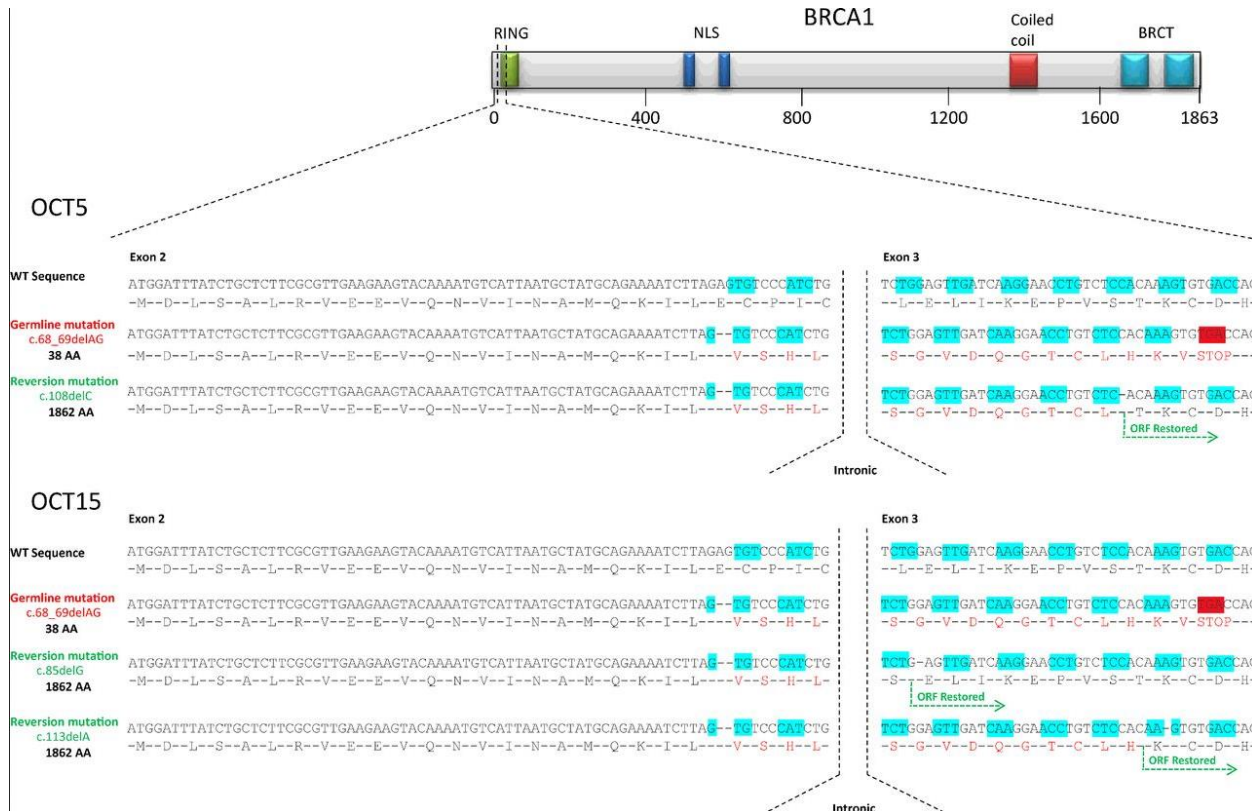
Figure 1: *BRCA1* open reading frame-restoring somatic mutations identified in cell-free DNA derived from ovarian cancer patients with *BRCA1* germline mutations resistant/ refractory to platinum-based chemotherapy.

Representation of the BRCA1 protein (top). Nucleotide and amino acid sequences for the affected genomic location shown are based on ENSEMBL transcript no. ENST00000357654.3. Representation of the predicted nucleotide and protein sequences for *BRCA1* wild-type (WT), germline mutation and putative reversion mutations from ovarian cancer patient OCT5 (top) and OCT15 (bottom). These three putative *BRCA1* reversion mutations were found to restore the BRCA1 open reading frame. Additional putative *BRCA1* reversion mutations are shown in Supplementary Fig. S2. Predicted protein lengths are shown in bold. The base triplets affected by a mutation are marked in light blue, and the aberrant amino acids produced by a given mutation are marked in red. Green arrows indicate the restored open reading frames. AA, amino acid; ORF, open reading frame; WT, wild type.
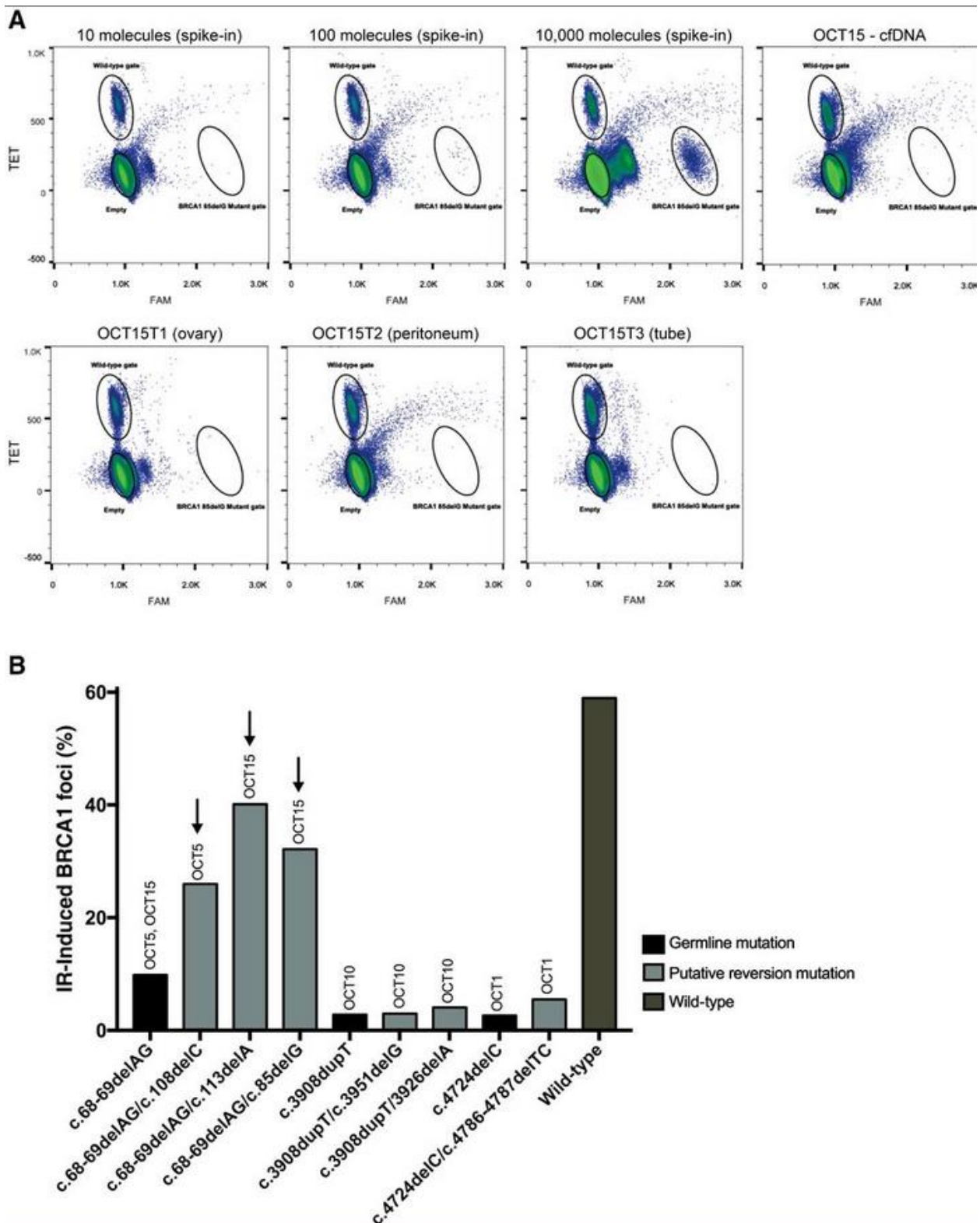
**Figure 2: Validation of putative *BRCA1* reversion mutation using dPCR and IR-induced BRCA1 foci formation.**

A, Validation of the putative *BRCA1* c.85delG reversion mutation in cfDNA and tissue samples from patient OCT15 harboring a *BRCA1* c.68-69delAG germline mutation using dPCR. Massively parallel sequencing libraries of germline DNA spiked in with 10, 100, and 1,000 *BRCA1* c.85delG synthetic oligonucleotide molecules were used as controls and for *BRCA1* c.85delG mutant gating (top). Massively parallel sequencing libraries from the plasma DNA (top right) and from three anatomically distinct ovarian tumor samples (i.e. ovary, peritoneum and fallopian tube; bottom) of case OCT15 were tested. The somatic *BRCA1* c.85delG mutation was confirmed in the cfDNA but was not detected in the pretreatment ovarian cancer tissues. **B**, U2OS cells were transfected with pcDNA-BRCA1(Δ510-1283) and *BRCA1* mutant plasmids (*BRCA1* germline and/or respective putative *BRCA1* reversion mutations of cases OCT1, OCT5, OCT10 and OCT15) or wild-type (WT) BRCA1 as control for 48 hrs (see Methods). Following 8Gy irradiation (IR), BRCA1 foci formation was assessed using immunofluorescence. Arrows indicate the *BRCA1* reversion mutations partially restoring BRCA1 foci formation.
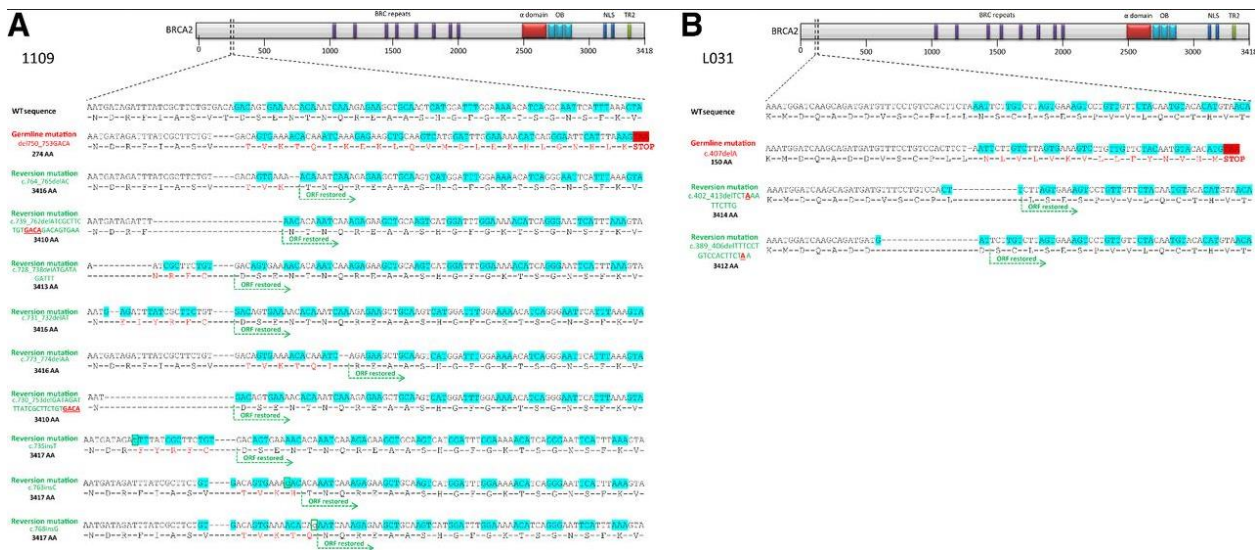
Figure 3: *BRCA2* open reading frame-restoring somatic mutations identified in cell-free DNA derived from breast cancer patients with *BRCA2* germline mutations after platinum-based chemotherapy.

Representation of the BRCA2 protein (top). Nucleotide and amino acid sequences for the affected genomic location shown are based on ENSEMBL transcript no. ENST00000380152.7. Representation of the predicted nucleotide and protein sequences for the *BRCA2* wild-type (WT), germline alteration and putative reversion mutations from patients **A**, 1109 and **B**, L031 are shown. The putative *BRCA2* reversion mutations presented in this figure were validated independently using targeted amplicon re-sequencing. Predicted protein lengths are shown in bold. The base triplets affected by a mutation are marked in light blue, and the aberrant amino acids produced by a given mutation are marked in red. Gaps represent the germline and somatic *BRCA2* reversion mutations identified. Four putative *BRCA2* reversion mutations were found to co-localize with the germline alteration, which is underlined in red in the reversion mutation sequences on the left. Insertions are highlighted by green squares. Green arrows indicate the restored open reading frames. AA, amino acid; ORF, open reading frame; WT, wild type.
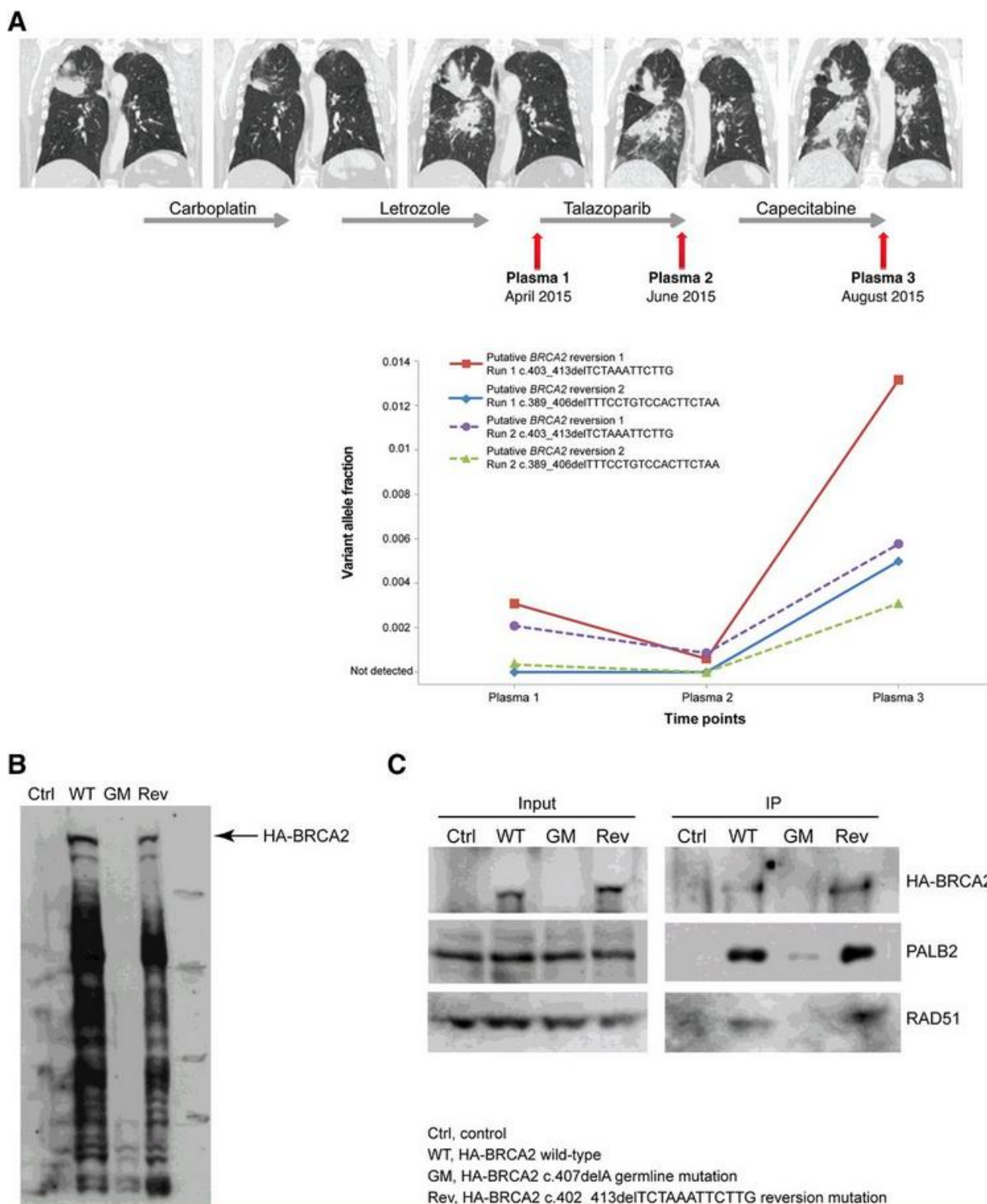
Figure 4. Serial analysis of putative *BRCA2* reversion mutations in cell-free DNA samples from breast cancer patient L031, and the interaction between reversion-mutant BRCA2, PALB2 and RAD51.

A, CT images during the course of therapy of breast cancer patient L031 demonstrating the initial response and subsequent progression of the lesions. Plasma samples were obtained before and after treatment with the PARP inhibitor Talazoparib and after Capecitabine therapy (top). Mutant allele frequencies of two somatic *BRCA2* reversion mutations identified by targeted massively parallel sequencing were assessed in two independent analyses in the plasma samples pre- and post PARP inhibitor treatment using targeted amplicon sequencing. **B**, 293T cells transfected with HA-BRCA2 wild-type (WT), HA-BRCA2 c.407delA germline mutant (GM) and HA-BRCA2 c.402_413delTCTAAATTCTTG somatic reversion-mutant plasmids Rev). Western blot performed using an anti-HA antibody revealed that the HA-BRCA2Rev was translated into mutant protein (predicted 3414AA) with a molecular weight similar to that of the wild-type protein (3418AA). The HA-BRCA2GM protein length is predicted to be 150AA. Immunoprecipitation of HA-BRCA2Rev and wild-type HA-BRCA2 revealed that HA-BRCA2Rev protein displays proficient interactions with PALB2 and RAD51 similar to that of the wild-type BRCA2 protein. AA, amino acid.