BRCA Reversion Mutations in Circulating Tumor DNA Predict Primary and Acquired Resistance to the PARP Inhibitor Rucaparib in High-Grade Ovarian Carcinoma

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Conflicts of interest

K.K. Lin, C. Say, L.-T. Vo, E. Mann, J.D. Isaacson, L. Maloney, H. Giordano, and T.C. Harding are employees of Clovis Oncology and may own stock or have stock options in that company. M.I. Harrell has nothing to disclose. A.M. Oza has served on steering committees for AstraZeneca, Clovis Oncology, and Tesaro. A. Oaknin has served on advisory boards for Roche, AstraZeneca, PharmaMar, Clovis Oncology, and Tesaro and received support for travel or accommodation from Roche, AstraZeneca, and PharmaMar. I. Ray-Coquard has served on an advisory board for AstraZeneca, Pharmammar, and Roche. A.V. Tinker has served on an advisory board for and received grants from AstraZeneca. E. Helman is an employee of Guardant Health and may own stock or have stock options in that company. M.R. Radke has nothing to disclose. D.M. O'Malley has served on an advisory board for Clovis Oncology, AstraZeneca, Janssen, Gynecologic Oncology Group, Myriad, and Tesaro; has served on steering committees for Clovis Oncology, Amgen, and Immunogen; has served as a consultant to AbbVie, AstraZeneca, Tesaro, Health Analytics, and Ambry; and his institution has received research support from Agenus, Ajinomoto, Array BioPharma, AstraZeneca, Bristol-Myers Squibb, Clovis Oncology, ERGOMED Clinical Research, Exelisis, Genentech, GlaxoSmithKline, Gynecologic Oncology Group, ImmunoGen, INC Research, inVentiv Health Clinical, Janssen Research and Development, Ludwig Institute for Cancer
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ABSTRACT

A key resistance mechanism to platinum-based chemotherapies and PARP inhibitors in BRCA-mutant cancers is the acquisition of BRCA reversion mutations that restore protein function. To estimate the prevalence of BRCA reversion mutations in high-grade ovarian carcinoma (HGOC), we performed targeted next-generation sequencing of circulating cell-free DNA (cfDNA) extracted from pretreatment and postprogression plasma in patients with deleterious germline or somatic BRCA mutations treated with the PARP inhibitor rucaparib. BRCA reversion mutations were identified in pretreatment cfDNA from 18% (2/11) of platinum-refractory and 13% (5/38) of platinum-resistant cancers, compared to 2% (1/48) of platinum-sensitive cancers ($P = 0.049$). Patients without BRCA reversion mutations detected in pretreatment cfDNA had significantly longer rucaparib progression-free survival than those with reversion mutations (median, 9.0 vs. 1.8 months; HR, 0.12; $P < 0.0001$). To study acquired resistance, we sequenced 78 postprogression cfDNA, identifying eight additional patients with BRCA reversion mutations not found in pretreatment cfDNA.

SIGNIFICANCE: BRCA reversion mutations are detected in cfDNA from platinum-resistant or -refractory HGOC and are associated with decreased clinical benefit from rucaparib treatment. Sequencing of cfDNA can detect multiple BRCA reversion mutations, highlighting the ability to capture multiclonal heterogeneity.
Cancers with defective homologous recombination repair (HRR) have been shown to be exquisitely sensitive to platinum-based chemotherapies and PARP inhibitors (1,2). The best characterized mechanism for HRR deficiency is the presence of deleterious mutations in \textit{BRCA1} or \textit{BRCA2} (BRCA), which are predominantly frameshift, nonsense mutations that result in truncated BRCA proteins. PARP inhibitors demonstrate synthetic lethality in cancer cells with HRR deficiency and clinical efficacy for cancers harboring deleterious germline or somatic \textit{BRCA} mutations, including ovarian, breast, prostate, and pancreatic carcinomas (3-8).

Multiple potential mechanisms of acquired resistance to platinum-based chemotherapies and PARP inhibitors have been described, although few have been identified in clinical samples (9). A key mechanism of clinical resistance is the acquisition of reversion mutations, which are somatic base substitutions or insertions/deletions (indels) that are typically close to the primary protein-truncating mutation and restore the open reading frame (ORF) of the gene and functional protein, switching the neoplastic cell from HRR deficient to proficient (10-12). The restoration of HRR function within the cancer promotes drug resistance by enabling DNA damage repair induced by PARP inhibitor and/or platinum-based chemotherapy, undermining the basis of synthetic lethality and ultimately promoting cell survival. Reversion mutations in multiple HRR pathway genes, including \textit{BRCA1}, \textit{BRCA2}, \textit{RAD51C}, \textit{RAD51D}, and \textit{PALB2}, have been reported in ovarian, prostate, and breast carcinomas as a mechanism of acquired resistance to platinum-based chemotherapies and PARP inhibitors (13-18).
Recent studies have identified somatic *BRCA* reversion mutations in circulating cell-free DNA (cfDNA) in germline *BRCA* mutation carriers with ovarian, fallopian tube or primary peritoneal carcinoma (collectively termed high-grade ovarian carcinoma, HGOC) (19,20). However, to date there is no reported study in a large prospective cohort of patients with HGOC to determine the prevalence of *BRCA* reversion mutations after prior platinum-based chemotherapies and assess their relationship with clinical efficacy of PARP inhibitor treatment.

Therefore, to study primary and acquired resistance to the PARP inhibitor rucaparib, we sequenced cfDNA from blood plasma samples collected before and after rucaparib treatment in patients (*n* = 112) with *BRCA*-mutant carcinomas enrolled in the phase II study of rucaparib in relapsed HGOC, ARIEL2 (NCT01891344).

**RESULTS**

**Detection of *BRCA* Reversion Mutations in Pretreatment Samples**

To study primary resistance to rucaparib, we first performed targeted next-generation sequencing (NGS) of cfDNA extracted from plasma samples collected just prior to rucaparib treatment (pretreatment) in 112 patients with germline or somatic *BRCA*-mutant HGOC in ARIEL2 (Supplementary Fig. S1) (21,22). The median cfDNA extracted from the 2–3 mL plasma samples was 19.1 ng (range, 4.8–1428 ng), and the median unique molecule coverage was 1,971× (range, 586×–6,029×).

Since *TP53* is ubiquitously mutated in HGOC, the presence of *TP53* mutations in cfDNA can be used as an indicator of the presence of neoplastic DNA in the bloodstream (23).
Somatic TP53 mutations were detected in 96% (107/112) of the sequenced pretreatment cfDNA samples, indicating that shedding of neoplastic DNA was frequent in patients with relapsed HGOC. Of these cases, primary deleterious BRCA mutations (germline or somatic) were detected in 97 cfDNA samples, which was required to determine whether a secondary mutation restores the ORF (i.e., reversion). A significantly lower level of the serum marker CA-125 was found at study enrollment in patients with no TP53 or BRCA mutations detected in the cfDNA ($P = 0.025$; Supplementary Fig. S2). The median somatic TP53 mutation allele frequency (MAF) detected in the cfDNA was 3.4% (range, 0.13%–80.4%; Supplementary Table S1). Primary somatic BRCA mutations accounted for 31% (30/97) of patients, and germline BRCA mutations accounted for the remainder (Table 1); the median somatic BRCA MAF detected in the cfDNA was 5.7% (range, 0.18%–77.4%). The observed MAFs of the primary somatic BRCA and TP53 mutations were significantly correlated ($R = 0.93$; $P < 0.0001$), supporting the notion that these are clonal driver mutations of HGOC (Supplementary Fig. S3).

The 97 patients with sequenced cfDNA samples containing the primary deleterious BRCA mutations had a median of three prior chemotherapy regimens (range, 1–4 regimens) (Table 1). Of these 97 patients, 48 were classified as platinum-sensitive, 38 as platinum-resistant, and 11 as platinum refractory at study entry based on time to relapse following the most recent platinum-based chemotherapy. Interestingly, we found BRCA reversion mutations in cfDNA from 18% (2/11) of platinum-refractory and 13% (5/38) of platinum-resistant cancers, compared to 2% (1/48) of platinum-sensitive cancers ($P = 0.049$) (Fig. 1). Apart from platinum sensitivity, no other baseline
characteristics were significantly associated with the presence of BRCA reversion mutations (Supplementary Table S2).

Sequencing of cfDNA identified BRCA reversion mutations in both BRCA1 (n = 4) and BRCA2 (n = 4) (Fig. 1). All four cases with BRCA1 reversion mutations in pretreatment cfDNA were found within the large exon 11 of BRCA1, whereas the BRCA2 reversion mutations were found within and outside of exon 11 of BRCA2 (Supplementary Fig. S4). Although the BRCA reversion (secondary) mutations were somatic events, the primary deleterious BRCA mutations preceding those events were either germline (n = 5) or somatic (n = 3) in origin. The eight patients with BRCA reversion mutations detected in cfDNA samples had received a median of four prior chemotherapy regimens (range, 3–4 regimens) and had a median platinum-free interval of 0.7 months (range, 0–10.9 months). Three of the five platinum-resistant cases had a platinum-free interval of <1 month, suggesting that their disease was at the borderline of platinum resistant and platinum refractory.

To confirm the mutations detected in cfDNA, pretreatment plasma samples were available for six of the eight patients with BRCA reversion mutations for sequencing by an alternative targeted NGS-based cfDNA assay (see Methods). This independent NGS-based cfDNA assay also identified BRCA reversion mutations in all six patients (Supplementary Table S1). Overall, a highly significant correlation of MAF for the primary deleterious BRCA mutations was found between the two independent NGS-based cfDNA assays (Supplementary Fig. S5).

In addition to cfDNA analysis, tumor tissues (archival and/or pretreatment biopsies) for NGS were available from 95 of 97 patients. The same primary deleterious BRCA
mutations were detected by NGS of cfDNA and tumor tissues in all cases (Supplementary Table S1). The zygosity of the primary \textit{BRCA} mutations was evaluable for 85% (82/97) of tumors. In all evaluable cases (82/82), the primary \textit{BRCA} mutations were homozygous, suggesting loss of heterozygosity of the wild-type allele and biallelic inactivation (Fig. 1). This finding is consistent with the previous report of ubiquitous biallelic inactivation of \textit{BRCA} in HGOC (24).

Of the 97 patients with sequenced cfDNA samples containing primary \textit{BRCA} mutations, 48 had matched pretreatment tumor biopsies (collected within 28 days prior to the first dose of rucaparib) for NGS, including four of the eight patients with \textit{BRCA} reversion mutations detected in cfDNA (Fig. 1). A \textit{BRCA} reversion mutation was also identified by NGS in each of the four available pretreatment tumor biopsies. In addition, sequencing of pretreatment tumor biopsies identified a large \textit{BRCA1} deletion event (366 base pairs [bp]) that restored the ORF from a patient with platinum-refractory cancer (Fig. 1). Due to the size of cfDNA fragments (approximately 170 bp) and short paired-end sequence reads (150–175 bp), this large deletion was not initially detected by the NGS-based cfDNA assays, but a post-hoc bioinformatics analysis of the cfDNA sequencing data did identify the 366 bp deletion of \textit{BRCA1}.

Overall, a significant association was found between detection of \textit{BRCA} reversion mutations in matched pretreatment tumor biopsies and cfDNA ($P < 0.0001$). In contrast, \textit{BRCA} reversion mutations were not detected in sequenced archival tumor specimens ($n = 97$) collected at the time of primary diagnosis prior to any platinum-based treatment for ovarian cancer (10% [5/48] vs. 0% [0/97]; $P = 0.0034$).
Compared to NGS of pretreatment tumor biopsies, we found sequencing of cfDNA detected additional $BRCA$ reversion mutations at baseline that also restored the ORF, highlighting the ability of the NGS-based cfDNA assay to capture multiclonal heterogeneity (Fig. 1, Supplementary Table S1). For instance, a patient with platinum-resistant cancer and a primary somatic $BRCA1$ mutation (c.1045G>T; p.E349*) had four unique $BRCA1$ reversion mutations detected in cfDNA, including base substitutions that resulted in a single amino acid change and deletions that restored the ORF (Fig. 2). In comparison, in the pretreatment tumor biopsy, only one of the four $BRCA1$ reversion mutations was identified.

Another patient with platinum-resistant cancer and a primary somatic $BRCA1$ mutation (c.2679delG; p.K894fs) had eight unique $BRCA1$ reversion mutations detected in cfDNA, consisting of different lengths of deletions (ranging from deletions of 2 to 29 bp) (Supplementary Table S1). Of the eight $BRCA1$ reversion mutations in cfDNA, only one was detected in the contemporaneous tumor biopsy. Similarly, a germline $BRCA1$ mutation carrier (c.3770_3771delAG; p.E1257fs) with platinum-refractory cancer had five unique $BRCA1$ reversion mutations in cfDNA that were deletions ranging from one to 31 bp. A paired tumor biopsy was not available for this patient.

Sufficient tumor biopsy specimens were available from 8 of the 41 platinum-resistant or -refractory cases without secondary $BRCA$ reversion mutations for laser-captured microdissection of neoplastic cells to determine whether reversion to the wild-type $BRCA$ sequence was present. However, we did not observe reversion to the wild-type $BRCA$ sequence in any of the cases tested (data not shown).
We hypothesized that patients without BRCA reversion mutations would be more sensitive to rucaparib therapy than those with reversion mutations. Consistent with our hypothesis, patients with BRCA-mutant cancers but no BRCA reversion mutations detected in pretreatment cfDNA had significantly longer progression-free survival (PFS) after rucaparib treatment than those with reversion mutations, with a median PFS of 9.0 and 1.8 months, respectively (HR, 0.12; 95% CI, 0.05–0.26; \( P < 0.0001 \)) (Fig. 3A). Furthermore, within the platinum-resistant or -refractory BRCA-mutant subgroup, patients without BRCA reversion mutations had significantly longer PFS than those with reversion mutations, with a median PFS of 7.3 and 1.7 months, respectively (HR, 0.16; 95% CI, 0.07–0.42; \( P < 0.0001 \)) (Fig. 3B). In fact, five of the seven platinum-resistant or -refractory cases with pretreatment BRCA reversion mutations had progressive disease as defined by Response Evaluation Criteria In Solid Tumors version 1.1 (RECIST) at the first scheduled response assessment (8 weeks following rucaparib treatment) (Supplementary Fig. S6). In addition, all seven of these platinum-resistant or -refractory cases with BRCA reversion mutations had an increase from baseline in sum of target lesions as defined by RECIST (Fig. 3C). On the other hand, most patients without BRCA reversion mutations derived clinical benefit from rucaparib even though they had been resistant to their last platinum-based chemotherapy. We next explored whether the MAF of the BRCA reversion mutations detected in pretreatment plasma was associated with rucaparib PFS. Linear regression analysis
showed a nonlinear association between BRCA reversion MAF and PFS ($P = 0.69$) (Supplementary Fig. S7).

**Detection of BRCA Reversion Mutations in Postprogression Samples**

To study acquired resistance to rucaparib, we performed NGS of cfDNA extracted from plasma samples collected immediately following progression while on rucaparib treatment in 78 patients who had BRCA-mutant carcinomas (Supplementary Fig. S1).

Similar to pretreatment plasma samples, somatic TP53 and BRCA mutations were detected in 81% (63/78) of postprogression plasma samples (Supplementary Table S1). The median TP53 MAF detected in postprogression cfDNA was 5.7% (range, 0.17%–68.8%; Supplementary Table S1).

Postprogression plasma samples were available from seven of the eight patients with BRCA reversion mutations detected in pretreatment plasma samples. As expected, BRCA reversion mutations detected before treatment were also detected after progression (Supplementary Table S3). For patients with multiple BRCA reversion mutations, changes were observed in the relative MAFs of the different reversion mutations (normalized to TP53 MAF to control for variation in the neoplastic component of cfDNA), suggesting potential expansion of certain clonal fractions. For example, in the patient with platinum-resistant cancer and a primary somatic BRCA1 mutation (c.2679delG; p.K894fs), the reversion mutation c.2740_2750del11 increased from a relative MAF of 7.2% before treatment to 25.9% after progression, and seven additional BRCA reversion mutations were detected after progression (Supplementary Fig. S8). Furthermore, sequencing postprogression plasma samples identified eight additional patients with BRCA reversion mutations not found in pretreatment plasma samples.
Most of these patients derived clinical benefit on rucaparib treatment, with a median PFS of 9.0 months, before eventually progressing. These BRCA reversion mutations were found in all three platinum status groups: platinum refractory (n = 1), resistant (n = 5), and sensitive (n = 2). The acquired BRCA1 reversion mutations were found within and outside of exon 11 of BRCA1, whereas the BRCA2 reversion mutations were all outside of exon 11 of BRCA2 (Supplementary Fig. S4).

The primary BRCA mutations for all eight of these cases were germline in origin (Supplementary Table S1).

To study the kinetics of acquisition of BRCA reversion mutations, we profiled cfDNA collected at multiple time points between pretreatment and postprogression. Interestingly, in four of the eight patients with acquired BRCA reversion mutations, the reversion mutations were detected in plasma samples collected prior to progression, at a median of 3.4 months (range, 0.7–8.3 months) before progression. By contrast, the remaining four patients had BRCA reversion mutations detected in plasma samples only at the time of radiological/clinical progression.

**DISCUSSION**

In this study, we sequenced cfDNA from a large cohort of patients with recurrent, BRCA-mutant HGOC and found that BRCA reversion mutations were more commonly detected in pretreatment cfDNA from patients with platinum-resistant or -refractory than platinum-sensitive disease at study entry. Previous studies examining BRCA reversion mutations in cfDNA were restricted to germline BRCA mutation carriers with HGOC (19,20). We found that BRCA reversion mutations were also detected in cfDNA from
patients with deleterious somatic BRCA mutations. Furthermore, we showed that patients without BRCA reversion mutations in cfDNA prior to starting therapy had significantly longer PFS when treated with the PARP inhibitor rucaparib than patients with reversion mutations. Patients without reversion mutations at baseline appeared to benefit from rucaparib treatment, suggesting that resistance to platinum does not always predict resistance to rucaparib.

A critical question is how the detection of BRCA reversion mutations should impact clinical decision-making in patients on PARP inhibitor therapy. The answer may depend on whether the reversion mutation is detected in a tumor biopsy or in cfDNA. In the case of a tumor biopsy, we previously observed a RAD51D reversion mutation in one progressing lesion, while the rest of metastatic sites were stable (17). Continuing PARP inhibitor therapy in this setting seems reasonable, potentially combined with local treatment of the progressing site (i.e., radiation or surgical resection). On the other hand, the finding of reversions in cfDNA reflects a more global tumor assessment. We had four patients in whom reversion mutations were detected in plasma samples prior to radiologic progression according to RECIST criteria, a median of 3.4 months prior to progression (range, 0.7-8.3 months) and four patients in whom reversion mutations were detected at the time of progression. In no cases were reversion mutations identified in cfDNA that did not associate with concurrent or imminent progression, suggesting that these findings warrant a change in therapy.

Reversion mutation in an HRR gene such as BRCA is an acquired resistance mechanism observed for the different clinically tested PARP inhibitors, including olaparib, talazoparib, and rucaparib (15-18). The utility of changing from one PARP
inhibitor to another is uncertain. Given the impact of reversion mutations in restoring DNA homologous recombination-directed repair, a cancer with a BRCA reversion mutation that has just progressed on one PARP inhibitor should not be treated with another single-agent PARP inhibitor. The next generation of clinical trials are evaluating a variety of therapies post PARP inhibitor progression, and we suggest that these trials should stratify patients at trial entry for the presence of reversion mutations in tumor tissue and/or cfDNA.

The association between BRCA reversion mutations and platinum resistance found in this study is consistent with previous studies that sequenced ovarian carcinomas (13,14,20). In addition, the frequency of pretreatment BRCA reversion mutations in platinum-resistant or -refractory cancers observed in this study (14%) was similar to the frequency found in a recent study of cfDNA from a smaller cohort of platinum-resistant or -refractory ovarian cancer patients (21%; 4/19) (20).

Since equal volumes of plasma samples from the same blood draw were used to perform two independent NGS-based cfDNA assays in this study, the low starting volume of 2–3 mL of plasma and resulting cfDNA extraction yield may not be optimal for detecting low allele frequency indels in cfDNA. Therefore, to estimate the prevalence of BRCA reversion mutations, we required the sequenced cfDNA samples to harbor the primary somatic BRCA and TP53 mutations, indicating shedding of neoplastic DNA with detectable key driver mutations. From a typical 10 mL whole blood draw, an average yield of approximately 5.5 mL of plasma would likely produce higher cfDNA extraction yield, potentially resulting in even higher probe coverage and sensitivity.
BRCA reversion mutations were identified in pretreatment and postprogression cfDNA from a minority of patients with platinum-resistant or -refractory HGOC in this study, suggesting the existence of other primary and acquired resistance mechanisms. In addition to reversion mutations, other mechanisms of resistance to platinum agents and PARP inhibitors have been reported by others (9). A potential mechanism of resistance for cancers with BRCA1 mutations in exon 11 is increased expression of a naturally occurring alternative splice isoform that lacks exon 11 but still has residual BRCA1 activity (25). If this resistance mechanism was commonly at play, we would expect a lower rate of BRCA1 reversion events in carcinomas with exon 11 mutations than in those with mutations outside of exon 11, which was not the case in this series. Another potential mechanism of chemo resistance recently found in ovarian carcinomas is promoter fusion that results in overexpression of the drug efflux pump MDR1 (14). Unfortunately, the NGS-based cfDNA assays that were tested in this study do not sequence the regions around the ABCB1 gene, which encodes MDR1, to detect such fusion events. Future studies may incorporate assays that can assess the frequency of ABCB1 fusion events and other resistance mechanisms to PARP inhibitors.

In summary, this study found BRCA reversion mutations were detected in cfDNA of platinum-resistant or -refractory HGOC and are associated with decreased clinical benefit from PARP inhibitor therapy. For patients who relapsed from prior chemotherapies and need to urgently find the next treatment options, this type of minimally invasive assay can efficiently detect BRCA reversion mutations that predict resistance to PARP inhibitors and may provide information on tumor heterogeneity.
METHODS

Patient Cohort and Sample Collection

Archival tumor and pretreatment and postprogression tumor biopsies were collected from patients who enrolled in ARIEL2, an international, multicenter, two-part, phase II open-label (21,22). Part 1 of ARIEL2 enrolled patients who received at least one prior platinum-based regimen and had platinum-sensitive disease. Part 2 enrolled patients who had received at least three, but no more than four, prior chemotherapy regimens. Only patients in whom a germline or somatic BRCA mutation was identified were included in this analysis. Blood plasma samples were prospectively collected before treatment (during the 28-day screening period and just prior to day 1 of the first cycle of rucaparib treatment), during treatment (day 1 of subsequent treatment cycles), and after progression.

PFS was defined as the time from the first dose of rucaparib to investigator-assessed disease progression or death from any cause. Tumor response was assessed by the investigators using RECIST, with computed tomography scans at screening and every 8 weeks during treatment. Serum CA-125 measurements were taken at screening (before rucaparib), day 1 of each cycle, the end of treatment, and when clinically indicated. The cutoff date for the clinical efficacy data was December 31, 2017.

Patients whose disease progressed during their most recent platinum-based treatment were classified as platinum refractory. Patients whose disease relapsed within 6 months after the last platinum agent was administered were classified as platinum resistant,
those whose disease relapsed more than 6 months were classified as platinum sensitive.

ARIEL2 was approved by the institutional review board at each study site and was done in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines of the International Conference on Harmonisation. Patients provided written informed consent before participation.

**Plasma Sample Preparation and NGS of cfDNA**

Whole blood was collected in the S-Monovette 9 mL hematology EDTA tubes. Within 30 minutes of blood collection, the EDTA tubes were centrifuged at 800 × g for 10 minutes. After transferring plasma into 1 mL aliquots, the plasma samples were centrifuged a second time at 14,000 rpm for 10 minutes to remove any remaining cellular debris and stored at -70°C or below prior to cfDNA extraction.

Blinded plasma samples of equal volume (2–3 mL) were sent to Guardant Health (Redwood City, CA) and Foundation Medicine (Cambridge, MA) for targeted NGS using the Guardant360 (cfDNA assay 1) (26) and FoundationACT (cfDNA assay 2) (27), respectively. Details of the bioinformatics methods for read processing and variant calling are described in the analytical validation studies of the two NGS-based cfDNA assays (26,27). Both cfDNA assays are based on the Illumina HiSeq sequencing platform and sequence a panel of cancer-related genes, including all exons of *BRCA1*, *BRCA2*, and *TP53*. The Guardant360 assay was used to sequence 112 pretreatment and 78 matched postprogression plasma samples. For confirmation of mutations detected by the Guardant360 assay, the FoundationACT assay was utilized to sequence 28 pretreatment plasma samples.
BRCA reversion mutations included the following: (i) a base substitution that changed a nonsense mutation to a missense mutation and (ii) an insertion/deletion that restored the ORF. For an insertion/deletion to restore the ORF and be classified as a reversion mutation, the combined effect of the primary deleterious mutation and secondary mutation should produce a nucleotide change that is divisible by three. For instance, if a primary deleterious mutation is a deletion of 2 bp and the secondary mutation is an upstream or downstream deletion of 4 bp, the two mutations result in a net deletion of 6 bp (divisible by three). A larger intragenic deletion that deletes the primary deleterious mutation can also result in restoration of the ORF. Large intragenic deletions that can restore the ORF were visually confirmed using the Integrated Genomics Viewer (28).

**NGS of Tumor Specimens**

Sequencing of formalin-fixed paraffin-embedded (FFPE) tumor specimens was performed using the Foundation Medicine T5 NGS-based assay (29), which sequences the exons of 287 cancer-related genes, including BRCA1, BRCA2, and TP53. Of the 112 patients with cfDNA sequenced, 97 had archival tumors and 59 had pretreatment tumor biopsies for NGS.

Classification of deleterious BRCA mutations was previously described (21). We defined primary BRCA mutation as a deleterious BRCA mutation that was detected in the tumor tissue. Due to differences in variant annotation between the different NGS assays, we verified that the cfDNA change of the primary BRCA mutations detected in the plasma sample was the same as that of the mutations detected in the tumor tissue.

To identify reversion to the wild-type BRCA sequence, DNA was extracted from tumor cells that were laser-captured, microdissected, and sequenced for specific BRCA
mutations by Sanger sequencing (University of Washington, Seattle, WA) as previously described (13,30). The BROCA-HR assay was used to sequence DNA extracted from whole blood to identify germline BRCA mutations (30).

**Statistical Methods**

Comparisons of CA-125 levels in patients with and those without BRCA or TP53 mutations detected in pretreatment cfDNA were analyzed using the Mann-Whitney test. Pearson correlation coefficient was calculated for the MAF of primary somatic BRCA and TP53 mutations.

Comparisons of BRCA reversion mutation frequencies in different patient subgroups (e.g., platinum status) were analyzed using Fisher’s exact test. Assessment of cfDNA as a molecular marker of efficacy was a prospectively planned, exploratory objective of ARIEL2. PFS was analyzed using the Kaplan-Meier method and a Cox proportional hazard model, and the log-rank test was used to compare the PFS of patients with and those without BRCA reversion mutations.

**Authors’ Contributions**

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Figure 1. Detection of BRCA reversion mutations in pretreatment cfDNA and tumor biopsy. The platinum-free interval and platinum status is based on patient’s most recent platinum-based treatment. The primary deleterious BRCA mutations are categorized based on their germline or somatic origin. All tumors with evaluable zygosity of the BRCA mutation were found to be homozygous; tumors with indeterminate zygosity are indicated with hatching. The number of BRCA reversion mutations detected by each assay is indicated; an empty cell denotes no reversion mutation was detected. A dash within a cell denotes a pretreatment tumor biopsy sample was not available for sequencing.

Figure 2. Multiple BRCA1 reversion mutations detected in pretreatment cfDNA from one patient. Detected allele frequencies of the primary somatic BRCA1 mutation (c.1045G>T) and the four unique reversion mutations by NGS of cfDNA extracted from a pretreatment plasma sample. The nucleotide and corresponding protein sequence changes of the primary and reversion mutations are shown in red.

Figure 3. Patients without BRCA reversion mutations detected in pretreatment cfDNA have significantly longer rucaparib PFS in (A) all BRCA-mutant cases and (B) in platinum-resistant or -refractory BRCA-mutant cases. (C) Best percentage change from baseline in sum of longest diameter of target lesions per RECIST as assessed by the investigators for patients with both baseline and postbaseline measurements.
Primary somatic \textit{BRCA1} mutation \texttt{c.1045G>T (p.E349*)}

Reversion mutation \#1
\texttt{c.1046A>G (p.E349G)}

Reversion mutation \#2
\texttt{c.1047G>T (p.E349D)}

Reversion mutation \#3
\texttt{c.1039_1077del39(p.L347_P359del)}

Reversion mutation \#4
\texttt{c.1035_1055del21 (p.D345_K351del)}
Figure 3

A

No reversion ($n = 89$)

- Median (months): 9.0
- 95% CI: 7.3–9.4
- HR, 0.12
- 95% CI, 0.05–0.26
- $P < 0.0001$

Reversion ($n = 8$)

- Median (months): 1.8
- 95% CI: 1.6–5.4
- HR, 0.16
- 95% CI, 0.07–0.42
- $P < 0.0001$

B

No reversion ($n = 42$)

- Median (months): 7.3
- 95% CI: 5.3–9.0
- HR, 0.16
- 95% CI, 0.07–0.42
- $P < 0.0001$

Reversion ($n = 7$)

- Median (months): 1.7
- 95% CI: 1.6–3.2
- HR, 0.16
- 95% CI, 0.07–0.42
- $P < 0.0001$

C

Percent change from baseline

- Refractory
- Resistant
- Sensitive
- BRCA reversion
- Ongoing

*BRCA reversion

++

+++
Table 1. Baseline characteristics of patients with primary *BRCA* mutations detected in pretreatment cfDNA samples from ARIEL2

<table>
<thead>
<tr>
<th></th>
<th>Patients with cfDNA sequenced (n = 112)</th>
<th>Patients with primary <em>BRCA</em> mutations detected in cfDNA (n = 97)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range), y</td>
<td>60.5 (33–82)</td>
<td>60.0 (33–82)</td>
</tr>
<tr>
<td>Histological classification, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>108 (96.4)</td>
<td>93 (95.9)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>2 (1.8)</td>
<td>2 (2.1)</td>
</tr>
<tr>
<td>Mixed</td>
<td>2 (1.8)</td>
<td>2 (2.1)</td>
</tr>
<tr>
<td><em>BRCA</em> mutation type, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germline</td>
<td>71 (63.4)</td>
<td>67 (69.1)</td>
</tr>
<tr>
<td>Somatic</td>
<td>41 (36.6)</td>
<td>30 (30.9)</td>
</tr>
<tr>
<td><em>BRCA</em> gene with deleterious mutation, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>BRCA1</em></td>
<td>69 (61.6)</td>
<td>60 (61.9)</td>
</tr>
<tr>
<td><em>BRCA2</em></td>
<td>43 (38.4)</td>
<td>37 (38.1)</td>
</tr>
<tr>
<td>Number of prior chemotherapy regimens, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12 (10.7)</td>
<td>8 (8.2)</td>
</tr>
<tr>
<td>2</td>
<td>11 (9.8)</td>
<td>10 (10.3)</td>
</tr>
<tr>
<td>3</td>
<td>52 (46.4)</td>
<td>47 (48.5)</td>
</tr>
<tr>
<td>4</td>
<td>37 (33.0)</td>
<td>32 (33.0)</td>
</tr>
<tr>
<td>Platinum response to last therapy, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitive</td>
<td>56 (50.0)</td>
<td>48 (49.5)</td>
</tr>
<tr>
<td>Resistant</td>
<td>44 (39.3)</td>
<td>38 (39.2)</td>
</tr>
<tr>
<td>Refractory</td>
<td>12 (10.7)</td>
<td>11 (11.3)</td>
</tr>
<tr>
<td>Median time since cancer diagnosis (range), mo</td>
<td>49.4 (12–186.8)</td>
<td>49.6 (12–186.8)</td>
</tr>
</tbody>
</table>