

ORIGINAL ARTICLE

RAD51 foci as a functional biomarker of homologous recombination repair and PARP inhibitor resistance in germline BRCA-mutated breast cancer

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Background: *BRCA1* and *BRCA2* (*BRCA1/2*)-deficient tumors display impaired homologous recombination repair (HRR) and enhanced sensitivity to DNA damaging agents or to poly(ADP-ribose) polymerase (PARP) inhibitors (PARPi). Their efficacy in germline *BRCA1/2* (g*BRCA1/2*)-mutated metastatic breast cancers has been recently confirmed in clinical trials. Numerous mechanisms of PARPi resistance have been described, whose clinical relevance in g*BRCA*-mutated breast cancer is unknown. This highlights the need to identify functional biomarkers to better predict PARPi sensitivity.

Patients and methods: We investigated the *in vivo* mechanisms of PARPi resistance in g*BRCA1* patient-derived tumor xenografts (PDXs) exhibiting differential response to PARPi. Analysis included exome sequencing and immunostaining of DNA damage response proteins to functionally evaluate HRR. Findings were validated in a retrospective sample set from g*BRCA1/2*-cancer patients treated with PARPi.

Results: RAD51 nuclear foci, a surrogate marker of HRR functionality, were the only common feature in PDX and patient samples with primary or acquired PARPi resistance. Consistently, low RAD51 was associated with objective response to PARPi. Evaluation of the RAD51 biomarker in untreated tumors was feasible due to endogenous DNA damage. In PARPi-resistant g*BRCA1* PDXs, genetic analysis found no in-frame secondary mutations, but *BRCA1* hypomorphic proteins in 60% of the models, *TP53BP1*-loss in 20% and *RAD51*-amplification in one sample, none mutually exclusive. Conversely, one of three PARPi-resistant

gBRCA2 tumors displayed *BRCA2* restoration by exome sequencing. In PDXs, PARPi resistance could be reverted upon combination of a PARPi with an ataxia-telangiectasia mutated (ATM) inhibitor.

Conclusion: Detection of RAD51 foci in gBRCA tumors correlates with PARPi resistance regardless of the underlying mechanism restoring HRR function. This is a promising biomarker to be used in the clinic to better select patients for PARPi therapy. Our study also supports the clinical development of PARPi combinations such as those with ATM inhibitors.

Key words: germline BRCA, PARP inhibitor resistance, homologous recombination, RAD51, TP53BP1, ATM

Introduction

BRCA1 and *BRCA2* encode essential proteins for DNA homologous recombination repair (HRR) [1]. Loss of function of either gene impairs this high-fidelity DNA repair pathway and results in genetic instability and an increased risk of breast or ovarian cancer in germline *BRCA1/2* (gBRCA) mutation carriers [2, 3]. Defective HRR increases sensitivity of gBRCA-mutated tumors to DNA damaging agents including anthracyclines, platinum salts, or to novel agents that block parallel DNA repair pathways, including poly(ADP-ribose) polymerase inhibitors (PARPi) [4–6]. PARP inhibition blocks the repair of DNA single-strand breaks and results in stalling of replication fork progression by trapping PARP on the DNA break [7]. Both contribute to the accumulation of DNA double-strand breaks (DSBs) that HRR-deficient cells cannot repair efficiently.

PARPi are well-tolerated agents and elicit anticancer efficacy in metastatic gBRCA tumors. Their use has been approved for advanced ovarian cancer [olaparib (Lynparza[®]), rucaparib (Rubraca[®]) and niraparib (Zejula[®])] and for gBRCA breast cancer (BC) [8–10]. Final results from other phase III clinical trials are awaited, both in the early and advanced BC setting (NCT01905592, NCT01945775, NCT02032823).

Primary resistance to PARPi in a subset of gBRCA patients limits the potential of gBRCA status as the only biomarker of response to that of an enrichment strategy [11]. In addition, acquired resistance in monotherapy responders is a challenge. Previous studies using *in vitro* models, transgenic mice and human tumor samples have delineated two types of resistance mechanisms to PARPi in gBRCA cells: (i) independent of HRR (cellular extrusion of the PARPi, PARP1 loss, FANCD2 overexpression, SLFN11 inactivation or CHD4 loss) and (ii) dependent on HRR recovery, either by BRCA-independent mechanisms (loss of 53BP1, REV7/MAD2L2, PAXIP1/PTIP, Artemis) or by BRCA-dependent mechanisms [12–22]. The latter include secondary *BRCA1/2* mutations that restore the reading frame and the expression of partially functional hypomorphic BRCA1 proteins (BRCA1-11q alternative splice isoform, the RING-less BRCA1 generated by downstream translation initiation, or HSP90-mediated stabilization of BRCA1 C-terminal mutants). Most work in gBRCA clinical samples has focused on ovarian cancer and has established that HRR recovery through secondary *BRCA1/2* mutations may act as a resistance mechanism to platinum salts and PARPi. Conversely, little is known about *in vivo* PARPi resistance mechanisms in gBRCA BC [22].

Patterns of DNA aberrations in the tumors (genomic scars) resulting from HRR deficiency may aid in distinguishing HRR-deficient from HRR-proficient tumors [23–25]. However, genomic scars in gBRCA tumors may persist after restoration of

HRR function [26]. In order to improve patient selection for PARPi monotherapy among gBRCA mutation carriers, especially in the metastatic setting, there is a clear need for a functional biomarker of HRR status to be used in the clinic. Previous work by others showed that induction of nuclear foci of the HRR protein RAD51 after neoadjuvant chemotherapy is a measure of HRR functionality in BC biopsies and predicts treatment response [27]. Here, we sought to investigate RAD51 foci as an indicator of functional HRR and its correlation with PARPi resistance in the gBRCA setting. We further explored potential treatment strategies for PARPi-resistant BC.

Methods

Study design

A collection of patient-derived tumor xenograft (PDX) models was generated by implanting tumor samples from patients with a germline *BRCA1/2* mutation and breast or ovarian cancer. Their sensitivity to PARPi was evaluated, and the functionality of the HRR pathway was analyzed and compared between the PARPi-sensitive versus the PARPi-resistant PDX samples to find a functional test correlating with response. An exploratory analysis in a set of 20 tumor samples including patients treated with PARPi at our institution was employed to confirm the findings and the potential clinical interest of the functional test. A new therapeutic PARPi combination was tested *in vivo* in PARPi-resistant PDX models.

See further methods in [supplementary material](#), available at *Annals of Oncology* online.

Results

gBRCA PDX panel

Fresh tumor samples prospectively collected for implantation into nude mice yielded a total of 12 PDX models (11 gBRCA1 and 1 gBRCA2) ([supplementary Table S1](#), available at *Annals of Oncology* online). Five models were derived from patients with metastatic disease who had been treated with PARPi, three of which prior to olaparib treatment and two at progression after a sustained partial response (PR) ([supplementary Table S1](#), available at *Annals of Oncology* online). Persistence of the gBRCA mutations was confirmed in all models but PDX274, and they were associated with loss of heterozygosity, i.e. loss of the wild type allele ([supplementary Figure S1](#), available at *Annals of Oncology* online).

Olaparib treatment in the gBRCA PDX collection distinguished a subset of PARPi-resistant tumors (Figure 1A) [assessed by modified Response Evaluation In Solid Tumors (mRECIST), see [supplementary methods](#), available at *Annals of Oncology*

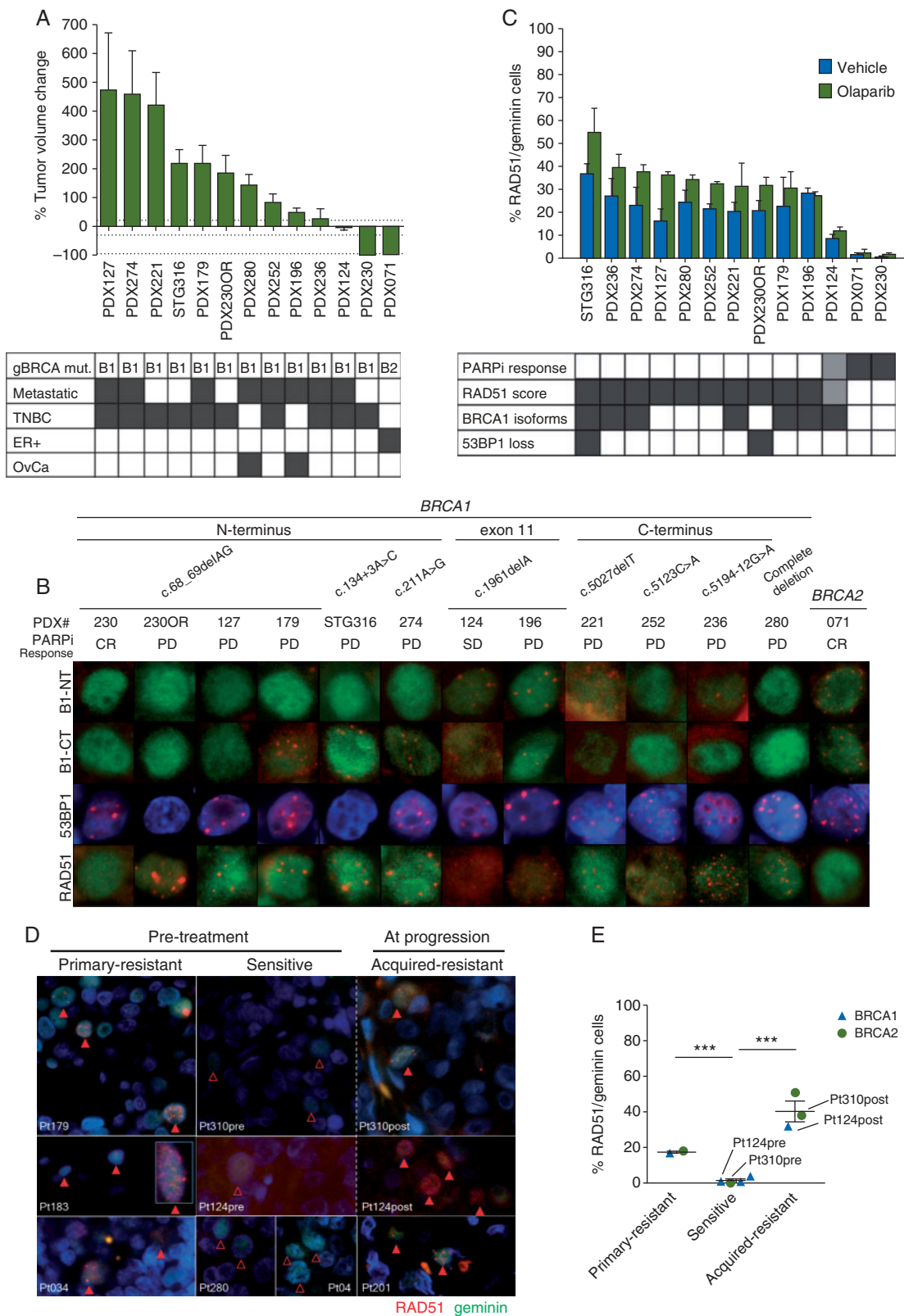


Figure 1. Homologous recombination repair markers and PARPi response. (A) Antitumor activity of olaparib in gBRCA patient-derived tumor xenografts (PDXs). Best response to olaparib is plotted as the percentage of tumor volume change after at least 21 days of treatment. +20%, -30% and -95% are marked by dashed lines to indicate the range of CR (complete response), PR (partial response), SD (stable disease) and PD (progressive disease). Mut, mutation; B1, mutation in *BRCA1*; B2, mutation in *BRCA2*; Metastatic, PDX derived from a metastatic lesion (otherwise, derived from a primary tumor); TNBC, triple negative BC; ER+, estrogen receptor positive BC; OvCa, ovarian cancer. (B) Immunofluorescence staining of BRCA1, 53BP1 and RAD51 across the PARPi-sensitive and PARPi-resistant gBRCA PDX models. Detection of

online]. Treatment with olaparib exhibited antitumor activity in three gBRCA models: two complete responses (CR) and one stable disease (SD). The remaining nine PDX models were olaparib-resistant (PD). An additional PDX model with acquired resistance (PDX230OR) was generated from its PARPi-sensitive counterpart (PDX230) after >100 days exposure to olaparib (supplementary Figure S2, available at *Annals of Oncology* online), totaling 13 gBRCA1/2 models. Among the four PDX derived from gBRCA primary tumors, 50% showed CR. The sensitivity to PARPi-treatment in the PDXs from metastatic patients previously treated with olaparib mirrored the patients' clinical response to olaparib (supplementary Table S1 and Figure S3, available at *Annals of Oncology* online).

BRCA1/2 sequencing, BRCA1 expression and nuclear foci formation in gBRCA PDX samples

No frameshift-correction or genetic reversion of the inherited mutation—the so-called secondary BRCA mutations—occurred in the gBRCA1 PARPi-resistant PDXs. *BRCA1* mRNA expression was variable across models and absent in PDX280, a model with a large deletion encompassing the complete *BRCA1* gene (supplementary Figure S4, available at *Annals of Oncology* online). To investigate the potential expression of hypomorphic BRCA1 isoforms and their recruitment to DNA damage sites, we set up immunostaining assays for the DNA damage response (DDR) proteins: BRCA1, RAD51 (as functional HRR marker) and γ H2AX (as DNA damage marker), with geminin (as S/G2-cell cycle marker) (supplementary Figure S5, available at *Annals of Oncology* online).

PDX124 and PDX196 harbor a *c.1961delA* mutation in *BRCA1* exon 11 and express the BRCA1- Δ 11q splice isoform (p.Ser264_Gly1366del) [20] which forms nuclear foci detected with both B1-NT and B1-CT antibodies, as expected (Figure 1B and supplementary Figure S6, available at *Annals of Oncology* online). BRCA1 nuclear foci were also detected in five additional gBRCA PDX models: PDX179, STG316, PDX274, PDX221 and PDX236 (supplementary Table S1, available at *Annals of Oncology* online and Figure 1B). Western blot confirmed the expression of BRCA1 isoforms at the respective predicted sizes [Δ 11q, RING-less, and C-terminal truncated mutant proteins (supplementary Figure S7, available at *Annals of Oncology*

online)]. In summary, hypomorphic BRCA1 isoforms were detected by immunofluorescence (IF) to form nuclear foci in seven PDX models, six with primary or acquired resistance to PARPi and one model showing disease stabilization (PDX124).

Analysis of 53BP1 loss and exome sequencing in gBRCA PDX samples

The assessment of 53BP1 nuclear foci by IF in olaparib-treated PDX samples identified 53BP1 loss in two PARPi-resistant models: PDX230OR and STG316 (Figure 1B). Exome sequencing unveiled somatic mutations in *TP53BP1* in both models (supplementary Table S1, available at *Annals of Oncology* online). The PD model PDX280 harbors a non-previously reported missense mutation in the PARPi resistance gene *SLFN11* p.H661D. The SD model PDX124 displays a focal *RAD51* amplification and high protein expression (supplementary Figure S8A and B, available at *Annals of Oncology* online). Mutations in other known PARPi resistance genes (*PARP1*, *REV7/MAD2L2*, *PAXIP1/PTIP*, *Artemis*, *CHD4*) were not identified.

Nuclear foci formation of the HRR protein RAD51

The observed recruitment of hypomorphic BRCA1 isoforms to DNA damage sites and/or 53BP1 loss in PARPi-resistant PDXs may help restore their ability to accomplish HRR. As a functional surrogate of HRR, we sought to detect RAD51 nuclear foci in geminin-positive cells and nuclear co-localization with BRCA1. RAD51 nuclear foci were detected in 11 PDXs in olaparib-treated samples, including all models expressing hypomorphic BRCA1 isoforms and/or lacking 53BP1 (Figure 1B). RAD51 foci co-localized with BRCA1 foci in all PDX models expressing hypomorphic BRCA1 isoforms (supplementary Figure S9, available at *Annals of Oncology* online). The three PARPi-resistant models that lacked hypomorphic BRCA1 isoform expression or 53BP1 loss (PDX127, PDX252 and PDX280) exhibited RAD51 foci suggesting that recovery of HRR occurs via BRCA1-independent mechanisms in these models (Figure 1B). Olaparib-treated samples from PARPi-resistant PDXs showed higher percentage of RAD51-positive cells versus those from PARPi-sensitive models ($36 \pm 2\%$ in PARPi-resistant versus $5 \pm 3\%$ in PARPi-sensitive, $P = 0.0017$) (Figure 1C). Analysis of γ H2AX foci ruled out PARPi pharmacodynamic differences as the reason for this differential response

BRCA1 [with an antibody towards the N-terminus of BRCA1 (B1-NT) or C-terminus (B1-CT)], 53BP1 and RAD51 nuclear foci in olaparib-treated PDX models. CR, PD and SD are indicated. For *BRCA1*, the location of the mutation within the gene is indicated. DAPI staining is shown in blue. Green nuclei indicate geminin-positive cells (S/G2 phase of the cell cycle). (C) RAD51 nuclear foci formation discriminates PARPi-resistant tumors. Quantification of geminin positive cells with RAD51 nuclear foci detected by immunofluorescence in FFPE samples from tumors treated with vehicle (black bars) or olaparib (green bars). The graph displays mean \pm SEM from three independent tumors. The association with PARPi-response is shown in the supplementary Table S1, available at *Annals of Oncology* online. Dark grey, CR; light gray, SD; white, PD. For RAD51, dark gray means high RAD51 score; light gray, intermediate RAD51; white, low RAD51. Expression of hypomorphic BRCA1 isoforms and loss of 53BP1 is depicted in dark gray. (D) RAD51 in patients' tumors is associated with PARPi clinical response. IF of RAD51 and geminin in the pretreatment setting using a pretreatment tumor sample (or the most recent metastatic sample). Samples from three PARPi-resistant patients (Pt179, skin metastasis of TNBC; Pt183, dermal lymphatic carcinomatosis of ovarian cancer; Pt034, lymph node metastasis of ER+ BC) and four PARPi-sensitive patients (Pt310pre, liver metastasis of ER+ BC; Pt124pre, primary TNBC; Pt280, peritoneal implant of ovarian cancer; Pt04, lymph node metastasis TNBC) are shown. For acquired resistance, samples obtained from three patients at PARPi progression (Pt310post, liver metastasis; Pt124post, skin metastasis; Pt201, skin metastasis of ER+ BC) are shown. Empty arrowheads show geminin-positive cells devoid of RAD51 nuclear foci. Solid arrowheads indicate RAD51/geminin-positive cells. DAPI staining is shown in blue. (E) Quantification of RAD51/geminin-positive cells from tumor samples shown in panel D. Pt183 was not scored as the tumor did not contain 100 geminin-positive cells. Unpaired *t* test: * $P < 0.05$; ** $P < 0.01$.

(supplementary Figure S10, available at *Annals of Oncology* online). The unexpected evidence of endogenous DNA damage and repair markers in untreated samples (supplementary Figure S5 and S10, available at *Annals of Oncology* online) prompted us to also score RAD51 foci in untreated tumors (Figure 1C). Importantly, untreated samples of PARPi-resistant tumors showed a significantly higher baseline percentage of RAD51-positive cells compared with PARPi-sensitive tumors ($24 \pm 2\%$ versus $3 \pm 2\%$, $P = 0.0025$). Thus, HRR functionality was frequent and associated with PARPi resistance in this gBRCA PDX panel.

RAD51/geminin score and response to PARPi in patients' samples

We confirmed the feasibility of detecting RAD51 and γ H2AX foci in FFPE tumor samples from patients, by firstly staining for RAD51 and geminin in 20 patients' tumor samples including some matched with the gBRCA PDX models ($n = 7$) (supplementary Figure S11A–C, available at *Annals of Oncology* online). These results prompted us to further assess the potential clinical utility of the RAD51/geminin score as a functional biomarker of PARPi treatment in patients treated with various PARPi at our institution ($n = 10$ tumors), including two paired pre-/post-PARPi samples. This cohort included eight patients with germline mutations in *BRCA1/2* (*BRCA1*, $n = 5$; *BRCA2*, $n = 3$; diagnosis of BC, $n = 6$; ovarian cancer, $n = 2$). The samples had been collected prior to ($n = 7$) or at progression to treatment with a PARPi ($n = 3$). We stained and scored for RAD51 (Figure 1D and E). Importantly, PARPi-resistant tumor samples showed an inverse relationship between the RAD51 score and clinical efficacy of the PARPi. Exome sequencing identified a *BRCA2*-secondary mutation in one tumor with acquired resistance and RAD51 foci (supplementary Figure S12, available at *Annals of Oncology* online).

Platinum salts in olaparib-resistant tumors

HRR recovery/retention that limits PARPi efficacy may not imply resistance to platinum-based treatments in gBRCA cancers. A previous study in gBRCA ovarian cancer showed a 40% response rate to platinum chemotherapy in the setting of resistance to olaparib [28]. We next assessed the efficacy of cisplatin in the two HRR proficient, RAD51-positive ovarian cancer PDX models (PDX196 and PDX280) (Figure 2A). Response to cisplatin was confirmed in both PDX models and in the clinic for Pt280 (data not available for Pt196 due to carboplatin hypersensitivity). Next, we assessed the activity of platinum-based chemotherapy in advanced/metastatic BC in the context of PARPi resistance. We previously reported that PDX127 showed resistance to PARPi but response to platinum, in agreement with the clinical response of the patient [29]. Similarly, the PARPi-resistant models, RAD51-positive model PDX252 exhibited significant tumor regression when treated with cisplatin (Figure 2A). In the PARPi-resistant PDX236 and PDX274, cisplatin-only slowed tumor growth as compared with vehicle, while its combination with olaparib achieved PR and SD, respectively (Figure 2B). These results highlight that platinum-based therapies can be active in PARPi-resistant metastatic BC and suggests that RAD51 foci formation does not predict resistance to platinum in this setting.

Ataxia-telangiectasia mutated blockade plus PARP inhibition in olaparib-resistant tumors

We further explored the potential of DDR inhibitors to enhance PARPi antitumor activity. The ataxia-telangiectasia mutated (ATM) kinase is activated in response to DNA DSBs, signals to cell cycle checkpoints and DNA repair pathways, and is reciprocally synthetic lethal with PARP [30]. As previously suggested, we hypothesized that ATM inhibition is a treatment option for PARPi-resistant *BRCA1*-deficient tumors that restore HRR through loss of *TP53BP1* or *REV7/MAD2L2* by enabling ATM-dependent end resection [17, 18]. We tested this hypothesis in three ATM-expressing PDXs (supplementary Figure S13A, available at *Annals of Oncology* online): STG316, a model that lacks 53BP1, and in PDX127 and PDX280, which are devoid of hypomorphic *BRCA1* isoforms, and presumably achieve PARPi resistance by a 'loss of 53BP1'-like mechanism. In fact, PDX127 harbors a missense mutation in PRCC p.P55T, within the interaction domain with *REV7/MAD2L2* (supplementary Figure S13B, available at *Annals of Oncology* online). The best antitumor activity of the olaparib combination with the ATM inhibitor AZD0156 was achieved in PDX127 (SD) (Figure 2C). We investigated whether ATM inhibition resulted in restoration of HRR deficiency by impairing RAD51 foci formation [17, 18] (supplementary Figure S13C, available at *Annals of Oncology* online). Unexpectedly, RAD51 foci formation was marginally reduced in combination-treated tumors, arguing that ATM inhibition may exert a broader effect in signaling the olaparib-induced DDR beyond its effects on HRR (supplementary Figure S13C, available at *Annals of Oncology* online). Quantification of DNA damage by γ H2AX staining (pan-nuclear, Figure 2D and foci formation, supplementary Figure S13D, available at *Annals of Oncology* online) showed a significant increase of pan- γ H2AX-positive cells upon ATM plus PARPi as compared with olaparib monotherapy in combination-responders PDX127 and STG316. These results suggest an induction of replication stress in these combination-sensitive models. These results are of interest since an international phase I clinical trial testing the tolerability of olaparib in combination with AZD0156 in solid tumors is currently ongoing (NCT 02588105).

Discussion

There is a need to refine the determinants of PARPi efficacy beyond gBRCA mutations, especially in the metastatic setting. Our analysis of RAD51 foci in a total of 20 BC patient samples, 10 gBRCA1 and 10 gBRCA2, provides new evidence in favor of restoration of HRR functionality as a frequent mechanism of PARPi resistance, and demonstrates the potential of functional biomarkers to discriminate tumors that will fail PARPi monotherapy. The RAD51 foci assay may capture the dynamic changes in DNA repair that occur throughout tumor evolution and may, therefore, more effectively identify the HRR-deficient *BRCA1/2*-mutated tumors. Unexpectedly, while previous studies reported low levels of baseline DNA damage as a potential limitation to evaluate HRR [27, 31], we were able to detect it and score for RAD51 in untreated samples, which correlated with PARPi response. This highlights that an IF assay for RAD51 staining is feasible in FFPE samples and suggests that testing for RAD51 may

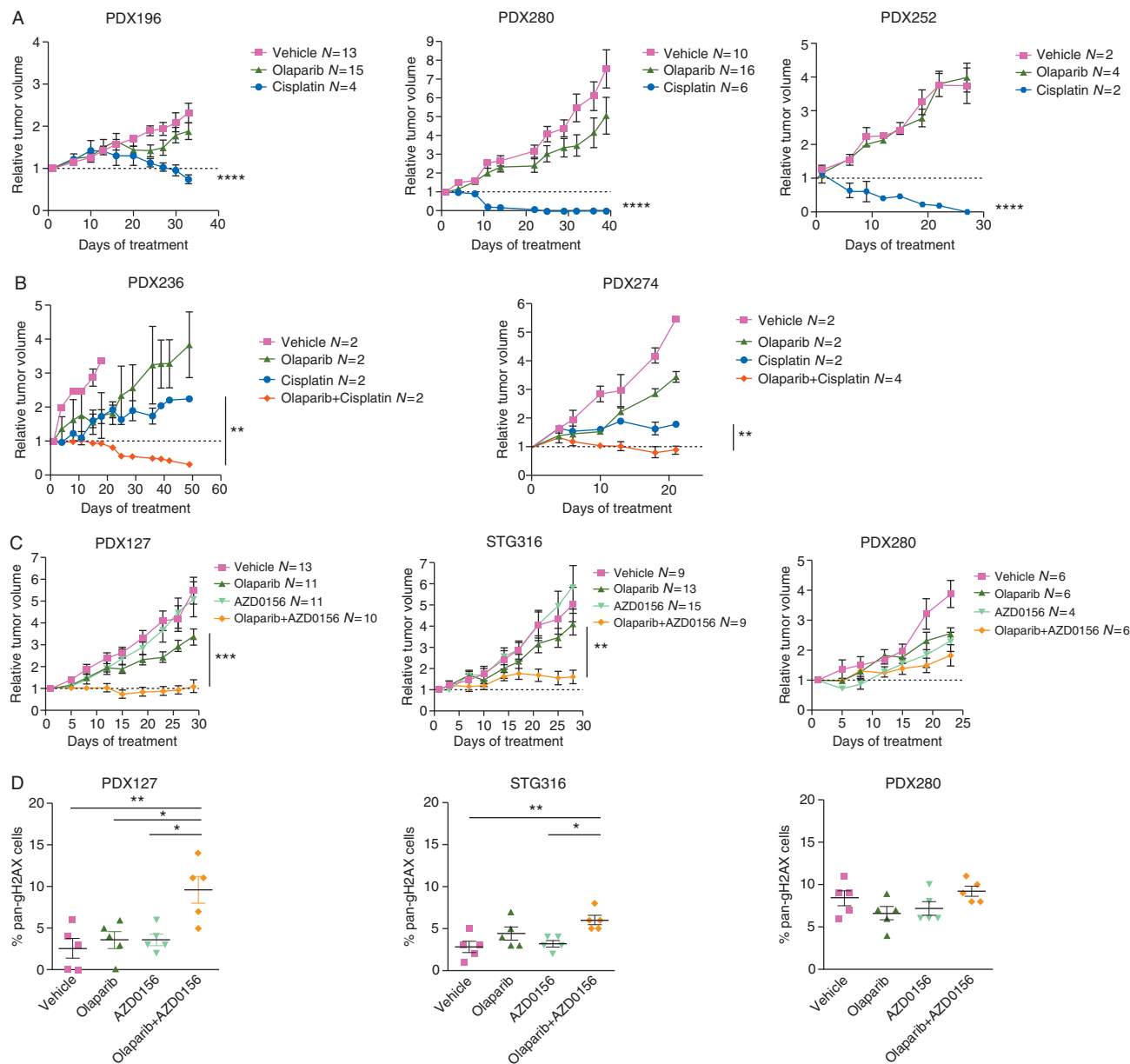


Figure 2. Cisplatin or ATM inhibition overcomes PARPi resistance. (A) Relative tumor volume (RTV) of vehicle, cisplatin or olaparib in PDX196, PDX280 and PDX252. Cisplatin was administered 6 mg/kg weekly unless RTV < 0.5. Olaparib was administered daily at 50 mg/kg (5 doses/week). Number of tumors per arm is indicated. (B) RTV of vehicle, cisplatin, olaparib or its thereof combination in PDX236 and PDX274. Cisplatin and olaparib were administered as in panel A. (C) RTV of vehicle, olaparib, AZD0156 or the combination of treatments in PDX127, STG316 and PDX280. Olaparib was administered daily at 50 mg/kg (5 doses/week) and AZD0156 was administered three times per week at 2 or 2.5 mg/kg. (D) Quantification of pan-nuclear γ H2AX-positive cells in PDX127, STG316 and PDX280 treated with vehicle, olaparib, AZD0156 or the combination of drugs at the end point of experiments shown in panel C. All figures show mean and SEM. Statistical *P*-values are shown when relevant: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001 (two-way ANOVA).

be directly transferrable to the clinical setting when a larger study confirms our findings.

Restoration of HRR can be achieved by secondary BRCA mutations and may be captured by sequencing techniques [32, 33]. We identified a *BRCA2* secondary mutation in one BC patient with acquired resistance to olaparib, whereas we did not detect in-frame secondary mutations in any gBRCA1 PDX model. Our data suggest that hypomorphic BRCA1 isoforms contribute to HRR restoration in gBRCA1 BC [20, 21]. Importantly, high RAD51 score predicted poor response to PARPi monotherapy independently of the

underlying mechanism of HRR restoration. Further research is needed to establish the RAD51 score cut-off that differentiates responders from nonresponders to PARPi monotherapy and to evaluate the potential impact of RAD51-independent resistance mechanisms that involve replication fork stabilization [14, 16, 34, 35]. A high RAD51 foci score may encourage the use of combination therapies with PARPi, such as those that inhibit HRR [29, 36], or that enhance DNA damage [9, 37, 38]. Here, we propose that a subset of PARPi-resistant gBRCA tumors benefit from combined PARP plus ATM blockade [17, 30, 39].

Our study unveils coexistence of various mechanisms of PARPi resistance in each individual tumor, such as hypomorphic BRCA1 isoforms together with *RAD51* amplification or 53BP1 [40] loss. In conclusion, this emphasizes the need of comprehensive functional tests for measuring HRR activity such as the *RAD51* assay to better select patients who will benefit most from PARPi monotherapy and those who may benefit from a combination therapy.

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Disclosure

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are employees of AstraZeneca. All remaining authors have declared no conflicts of interest.

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