RAD51 foci as a biomarker predictive of platinum chemotherapy response in ovarian cancer

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RAD51 Foci as a Biomarker Predictive of Platinum Chemotherapy Response in Ovarian Cancer
Amanda J. Compadre1, Lillian N. van Biljon1, Mark C. Valentine1, Alba Llop-Guevara2, Emily Graham1, Bisiayo Fashem1, Andrea Herencia-Ropero2,3, Emiliee N. Kotnik1, Isaac Cooper1, Shariska P. Harrington1, Lindsay M. Kurok1, Carolyn K. McCourt1, Andrea R. Hagemann1, Premal H. Thaker1, David G. Mutch1, Matthew A. Powell1, Lulu Sun5, Nima Mosammaparast5, Violeta Serra2, Peinan Zhao6, Elena Lomonosova1, Dineo Khabele1, and Mary M. Mullen1

ABSTRACT

Purpose: To determine the ability of RAD51 foci to predict platinum chemotherapy response in high-grade serous ovarian cancer (HGSOC) patient-derived samples.

Experimental Design: RAD51 and γH2AX nuclear foci were evaluated by immunofluorescence in HGSOC patient-derived cell lines (n = 5), organoids (n = 11), and formalin-embedded tumor samples (discovery n = 31, validation n = 148). Samples were defined as RAD51-High if >10% of geminin-positive cells had ≥ 5 RAD51 foci. Associations between RAD51 scores, platinum chemotherapy response, and survival were evaluated.

Results: RAD51 scores correlated with in vitro response to platinum chemotherapy in established and primary ovarian cancer cell lines (Pearson r = 0.96, P = 0.01). Organoids from platinum-nonresponsive tumors had significantly higher RAD51 scores than those from platinum-responsive tumors (P < 0.001). In a discovery cohort, RAD51-Low tumors were more likely to have a pathologic complete response (RR, 5.28; P < 0.001) and to be platinum-sensitive (RR, 0.31; P = 0.05). The RAD51 score was predictive of chemotherapy response score [AUC, 0.90; 95% confidence interval (CI), 0.78–1.0; P < 0.001]. A novel automatic quantification system accurately reflected the manual assay (92%).

Conclusions: RAD51 foci are a robust marker of platinum chemotherapy response and survival in ovarian cancer. The utility of RAD51 foci as a predictive biomarker for HGSOC should be tested in clinical trials.

Introduction

Most patients with high-grade serous ovarian cancer (HGSOC) are diagnosed with advanced-stage disease for which the standard treatment includes platinum chemotherapy, and up to 80% of patients are candidates to receive maintenance therapy with PARP inhibitors (PARPis; refs. 1, 2). Carboplatin and PARPi sensitivity. To minimize unnecessary toxicity and rationaly triage patients to therapy, we need an assay that reflects current tumor response to DNA-damaging chemotherapeutic agents.

Here, we assessed the utility of an assay that reflects the current ability of tumors to perform HR. The DNA repair protein RAD51 is an optimal biomarker for such an assay for several reasons. First, upon a double-strand break (DSB) in DNA, the kinase ATM rapidly phosphorylates histone H2AX (γH2AX). This activates a signaling cascade that generates single-stranded DNA with 3’ overhangs. These overhangs are bound by replication protein A, which is ultimately replaced by RAD51 via mediator proteins, including BRCA1 and BRCA2. Finally, sister chromatid strand invasion and DNA synthesis lead to faithfully repaired DNA. Because many upstream events must occur for RAD51 to bind to DNA, and RAD51 is necessary to complete HR,
RAD51 provides a comprehensive readout for many independent steps of HR. Second, upon binding, RAD51 forms a nucleoprotein filament that can be visualized microscopically as foci, and the inability to form RAD51 foci has been used as a functional marker of HR deficiency (6–8). Third, RAD51 foci assays predicted response to platinum chemotherapy and to PARPis in patient-derived breast and ovarian cancer xenografts (9–11). Similarly, quantification of RAD51 foci in breast cancer patient samples predicted response to platinum chemotherapy (12, 13). However, whether RAD51 foci can accurately predict response to platinum chemotherapy in readily available HGSOC formalin-fixed paraffin-embedded (FFPE) patient tumor samples is unknown. Furthermore, RAD51 foci in FFPE are currently quantified manually, limiting translation into clinical care. Here, we rigorously test this biomarker in patient-derived cell lines, organoids, and FFPE samples. Furthermore, we describe a novel automated quantification method and show its accuracy in both a discovery cohort and a validation cohort. We demonstrate that automated quantification of RAD51 foci is a robust and reliable method to accurately predict HGSOC patient response to platinum chemotherapy and survival in clinically available samples.

**Materials and Methods**

**Patient population**

Tissues for primary ovarian cancer cell lines and organoid generation were collected prospectively as part of our Gynecologic Oncology biorepository (IRB No. 201105400 and IRB No. 201706151) after obtaining patient consent. Tissues for formalin fixation and paraffin embedding were collected prospectively (IRB No. 201407156) as part of a National Cancer Institute-funded project studying patients with advanced-staged HGSOC undergoing neoadjuvant chemotherapy. Patients were included if they had stage III–IV HGSOC and underwent neoadjuvant chemotherapy and interval cytoreductive surgery. Tissue specimens were collected before neoadjuvant chemotherapy. Chemotherapy response was assessed at the time of interval cytoreductive surgery and defined according to a validated histopathologic scoring system (14–19). A chemotherapy response score of 1 was considered little to no response and 3 was considered a pathologic complete response (pCR). Progression-free survival (PFS) and overall survival (OS) were calculated from the time of interval cytoreductive surgery.

FFPE ovarian cancer tissue microarrays were obtained from the University of Kansas and the Anatomic and Molecular Pathology Core Laboratories at Washington University. The microarrays contained normal, primary, and metastatic tumors from patients with HGSOC. Samples were obtained at primary or interval cytoreductive surgery after platinum chemotherapy. BRCA mutation status was reported, but status of the mutation (benign vs. pathogenic) was unknown. Platinum chemotherapy resistance was defined as recurrence within 6 months of completing platinum chemotherapy.

This study was conducted in accordance with guidelines set forth by the Belmont Report. All patients provided written informed consent.

**DNA/RNA sequencing, BRCA status, LOH, HRDI, DNA repair genes**

All tumors were examined by a pathologist to determine tumor cellularity and necrosis, and only samples of 60% tumor cellularity or higher with <20% necrosis were sequenced. DNA and RNA were extracted from tumors embedded in optimal cutting temperature compound tissues using Qiagen’s DNaseasy Blood and Tissue Kit (cat No. 69504) according to the manufacturer’s protocol. Samples underwent whole-genome sequencing (WGS) to an average depth of 30X. Reads were aligned with bwa (version 0.7.8) and duplicates were removed with picard-tools (version 2.21.7). Base quality recalibration was performed per GATK best practices. Somatic variants were called using MuTect-2 with Gnomad variants as a germline resource and filtered using GATK (version 4.1.9.0). LOH, telomeric allelic imbalance, and large-scale transitions, were calculated with ascatNGS (version 2.1.1) as previously described (20), and these values are added to generate the HRDi score as a proxy for the commercially available Myriad myChoice assay (21). A high HRDi score was considered any value ≥42 and a high LOH score ≥16 as previously described (22–25). Germline and somatic DNA data regarding BRCA status were obtained from next-generation sequencing (NGS) gene panel tests obtained as standard-of-care.

RNA sequencing of primary tumor samples was performed using the Illumina TruSeq stranded Total RNA library kit following the recommended protocol, with paired-end Illumina sequencing of 151 bp read length, with an average of approximately 125 million paired reads per sample and an average of approximately 134 million reads mapped per sample. Transcript quantification was performed using kallisto (26). Downstream analysis was then performed in R.

**Cell lines**

ES2 (cultured in McCoy’s plus 10% FBS and 1% penicillin and streptomycin) and OVCAR8 cells (cultured in RPMI plus 10% FBS and 1% penicillin and streptomycin) were obtained from the National Cancer Institute. COV362 and TykNu cells (Sigma-Aldrich) were cultured in DMEM plus 10% FBS, 1% l-Glutamine, and 1% penicillin and streptomycin. PEO1 and PEO4 cells (Sigma-Aldrich) were cultured in RPMI plus 10% FBS. UWB1 and UWB1-B1 cell lines were generously provided by Lee Zhou (Massachusetts General Hospital Cancer Center, Harvard Medical School), and cultured in a 1:1 ratio of RPMI and MEGM BulletKit (Lonza) supplemented with 3% FBS and 1% penicillin and streptomycin. All cells were maintained at 37°C in a 5% CO2 incubator. Cell lines were confirmed mycoplasma negative with the MycoAlert Mycoplasma Detection Kit (Lonza) before experiments. Cells were used for 2 to 3 months then discarded.
Development of primary cell lines
To develop patient-derived ovarian cancer cells, ovarian cancer patient ascites were collected from patients with advanced-stage HGSOC, mixed in a 1:1 ratio with RPMI supplemented with 20% FBS and 1% penicillin and streptomycin, and cultured in flasks. After 7 to 14 days, the attached and proliferating cells were passaged and used for experiments. Primary cells were used for 1 to 2 passages then discarded.

Cell line immunofluorescence
Cells (40,000 per well) were plated in an 8-well chamber slide. Cells were then treated with 10 Gy of ionizing radiation at 37°C. After treatment, the cells were washed with cold PBS, fixed with 2% paraformaldehyde for 10 minutes, permeabilized with 0.2% Triton X-100 in PBS for 20 minutes, and then washed and blocked (30 minutes) with staining buffer (PBS, 0.5% BSA, 0.15% glycine, and 0.1% Triton X-100). Cells were then incubated overnight at 4°C with primary antibodies (Supplementary Table S1) in staining buffer. Cells were then stained with secondary antibodies (Supplementary Table S1) and DAPI (Sigma) and imaged using a Leica TCS SPE inverted confocal microscope. Raw images were exported, and foci were counted with JCountPro (27, 28). At least 100 cells were analyzed for each treatment group in duplicate.

Western blot analysis
Cultured cells were lysed in 9 mol/L urea, 0.075 mol/L Tris, pH 7.6, and proteins were quantified by the Bradford assay. Protein lysates (60–100 μg) were subjected to reducing SDS-PAGE by standard methods and transblotted. Each membrane was probed with corresponding horseradish peroxidase-conjugated secondary antibodies (Supplementary Table S1) and chemiluminescence was measured on a ChemiDoc (Bio-Rad Laboratories).

M200 Pro plate reader (Tecan, Inc.). IC50 values were determined in GraphPad Prism (GraphPad Software, Inc.).

Organoid generation
Tumor biopsies and/or malignant ascites were collected from patients with HGSOC. For solid tumors, samples were manually minced then chemically and mechanically digested with the gentle-MACS Octo Dissociator with Heaters (Miltenyi Biotec; No. 130096427), 2 mg/mL Type II Collagenase (Life Technologies; No. 17101015), and DNase (NEB; No. M0303S). Both the homogenate and the ascites cell pellet were filtered (Laboratory Source; No. T50476) and treated with DNase and red blood cell lysis buffer (BioLegend; No. 420301). Single-cell suspensions of tumor or ascites were resuspended in 75% Cultrex (R&D Systems; No. 353300502) and 25% organoid base media [Advanced DMEM/F12 (Thermo Fisher Scientific; No. 12634028)] supplemented with 1% penicillin-streptomycin (Millipore Sigma; No. P0781), 1× Glutamax (Life Technologies; No. 35050061), and 1% HEPES (Life Technologies; No. 15630080). The cell suspension was plated onto a 6-well plate, in approximately 35-μL droplets, and placed into a 37°C incubator to solidify. The organoids were cultured in the base media plus 50 ng/mL EGF (PeproTech; No. 10026), 10 ng/mL FGF-10 (PeproTech; No. 10026), 10 ng/mL FGF2 (PeproTech; No. 100–18B), 1× B27 (Life Technologies; No. 17504044), 10 mmol/L nicotinamide (Sigma-Aldrich; No. N0636), 1.25 mmol/L N-acetylcysteine (Sigma-Aldrich; No. A9165), 1 μmol/L prostaglandin E2 (R&D Systems; No. 2296), 10 μmol/L SB202190 (Sigma-Aldrich; No. 57076), 500 nmol/L A83–01 (Sigma-Aldrich; No. SML0788), and 10 μmol/L ROCK inhibitor (R&D Systems, No. Y27632; ref. 29).

Organoids were classified as platinum-responsive or platinum-nonresponsive according to the parent tumor’s CRS score (1–2: platinum-nonresponsive; 3: platinum-responsive) or their platinum-free interval (<6 months: platinum-nonresponsive; ≥6 months: platinum-responsive). In vitro carboplatin sensitivity was used when clinical data were not available (n = 1).

Organoid platinum sensitivity assays
For organoid carboplatin sensitivity assay, 20,000 cells per 10 μL of 75% Cultrex were plated into the wells of a black walled 96-well plate. On day 2, media containing 1, 5, 10, 25, 50, and 75 μmol/L of carboplatin (Teva; No. 00703424601) were added to the organoids. On day 7, an equal volume of CellTiter-Glo 3D Cell Viability Assay (Promega; No. G9681) was added to each well, and the luminescence was read using a Tecan plate reader. The percentage of cell viability was calculated and graphed using Microsoft Excel and GraphPad Prism.

Organoid immunofluorescence
Organoids were plated in Cultrex and cultured in an 8-chamber dish for 3 to 5 days. The organoids were treated with 10 Gy of ionizing radiation at 37°C and fixed with 2% paraformaldehyde for 10 minutes. They were then washed with staining buffer, permeabilized (PBS with calcium and magnesium, 0.2% Triton X-100) for 20 minutes, washed with staining buffer, and then blocked with staining buffer for 30 minutes. Organoids were incubated overnight at 4°C with primary antibodies (Supplementary Table S1), washed with staining buffer, stained with secondary antibodies (Supplementary Table S1) and DAPI (Sigma), and mounted with Prolong Gold Antifade Mountant (Sigma). Organoids were imaged on a Leica TCS SPE inverted confocal microscope. Raw images were exported, and foci were counted with JCountPro (27, 28). At least 100 cells were analyzed for each treatment group in duplicate.
FFPE immunofluorescence

Hematoxylin and eosin–stained slides of FFPE samples were screened for diagnosis, cellularity, and necrosis by a board-certified pathologist. Staining methods were adapted from Llop-Guevara and colleagues (13). FFPE blocks were cut into 4-μm sections and deparaffinized in organic solvents. Slides were dehydrated, submerged in DAKO Antigen Retrieval Buffer (pH 9.0), and incubated at 110°C in a steam rice cooker for 30 minutes. The slides were then cooled on ice for 30 minutes, followed by 5 minutes in distilled water. Samples were permeabilized with DAKO Wash Buffer for 5 minutes, then blocked with blocking buffer (DAKO Wash buffer; 1% BSA) for 5 minutes. The primary antibodies (Supplementary Table S1) were diluted in DAKO Antibody Diluant and incubated for 1 hour. Then, samples were washed, blocked for 5 minutes, and incubated for 30 minutes in blocking buffer with secondary antibodies (Supplementary Table S1). DAPI (Sigma) was added, and the slides were dehydrated with increasing concentrations of ethanol. Samples were then mounted with prolong gold anti-fade reagent and stored at −20°C. Stained samples were imaged on a Leica Thunder Imager Microscope.

The amount of DNA damage was quantified by scoring the percentage of geminin-positive cells with 2 or more γH2AX foci. Only samples with a γH2AX score of >25% were evaluated for RAD51 to avoid false negatives as a result of inadequate dsDNA breaks to mount an HR response. RAD51 foci were quantified by scoring the percentage of geminin-positive cells with 5 or more RAD51 foci of approximately 3-μm diameter. At least 100 geminin-positive cells per sample were assessed. All samples were scored by 2 independent, masked reviewers. Tumors were defined as RAD51-Low if the RAD51 score was <10% and RAD51-High if the RAD51 score was >10%.

Automated foci analysis

Microscopy images from the RAD51 foci assay were imported into R environment. After denoising, smoothing, and thresholding, a 2-dimensional convolution was applied to segment all the foci in the image. Then, the cells were segmented by using a denoising and adaptive thresholding method, the foci-positive cells were counted, and the ratio to all cells was calculated. With multiple images from each patient, a median ratio was computed to estimate the RAD51 score. Using the linear coefficient of linear regression modeling, an automated cutoff value of 6% was determined to identify RAD51-High tumors.

Statistical analysis

Descriptive statistics were used to characterize baseline differences between patients. Missing data were excluded from the analysis. Categorical factors were compared between groups by using the χ² or Fisher exact test, as appropriate. Independent Student t and Mann–Whitney U tests were used to compare normally and nonnormally distributed continuous variables, respectively. One-way ANOVA was used as appropriate. Pearson rank correlation coefficient was used for correlation analyses. Sensitivity analysis was performed. Poisson regression was used to report relative risks (RRs). Areas under the receiver-operating characteristic curves were used to compare the predictive performance of RAD51 scores and genomic scars. DFS and OS were calculated as the time from surgery to physical or radiographic evidence of disease recurrence, date of death, or date of last contact if no recurrence occurred. Patients alive or without recurrence were censored at the date of last contact. Platinum resistance was defined as progression-free interval ≥6 months (30). The Kaplan–Meier method was used to estimate survival times, and distributions were compared using the log-rank test. Univariate and multivariate Cox proportional hazards regression analysis were used as indicated. Two-tailed 95% CIs and P values were calculated, and P < 0.05 was considered significant. GraphPad Prism 9 software and SPSS version 27 were used for statistical analysis and IC50 value calculations.

Sample size

On the basis of previously published research, we anticipated that at least 30% of tumors would be scored as RAD51-Low (11, 31, 32). Furthermore, from our institution’s previous clinical trial evaluating neoadjuvant chemotherapy in ovarian cancer, we projected that 35% to 40% of patients would have a pCR. Therefore, the necessary sample size to detect a significant RR of having a pCR for the RAD51-Low patients was calculated (12, 32). If the expected RR of RAD51-Low was higher than 3.5, then 26 participants would provide the study with 80% power, at a 2-sided significance level of 0.05, including 11 (30%) patients with a RAD51-Low score and 24 (70%) patients with a RAD51-High score (33). A validation cohort of 148 patients was used to confirm the automated quantification methods developed in the discovery cohort.

Data availability

The data generated or used in this study will be made available upon request. Please contact the corresponding author for requests.

Results

RAD51 foci are associated with in vitro platinum chemotherapy response in ovarian cancer cell lines and patient-derived ovarian cancer cells

Upon binding to sites of DNA DSBs, RAD51 forms a nucleoprotein filament that can be visualized microscopically as foci, and the ability to form RAD51 foci has been suggested as a functional read-out for HR proficiency (6, 7). To determine whether RAD51 foci would predict platinum response independent of genomic HR status, we treated 8 established HGSOC cell lines—2 BRCA1/2 wild-type (WT), 2 BRCA1 mutated, 1 BRCA2 mutated, 1 BRCA1 methylated, 1 BRCA1 WT restored, and 1 with a BRCA2 reversion mutation—with 10 Gy of radiation to cause DNA damage. We then used immunofluorescence to assess for the G2/S phase cell-cycle marker geminin, γH2AX foci (34), and RAD51 foci. We only analyzed samples in which >25% of geminin-positive cells had ≥5 γH2AX foci, indicating sufficient dsDNA breaks to mount an HR response. Therefore, only irradiated cells were analyzed. We assigned each cell line a RAD51 score—defined as the percentage of geminin-positive cells with ≥5 RAD51 foci—and found that RAD51 score correlated significantly with carboplatin IC50 value (Pearson r = 0.92, P = 0.001; Fig. 1A and B). In contrast, the γH2AX score did not correlate with carboplatin IC50 value (P > 0.05; Supplementary Fig. S2A). We performed the same evaluation in 5 patient-derived ovarian cancer cell cultures—3 platinum-resistant (progression-free interval ≥6 months) and 2 platinum-sensitive (progression-free interval ≥6 months; Supplementary Table S2; Supplementary Fig. S1). One patient had a BRCA1 mutation. RAD51 foci, but not γH2AX foci, significantly correlated with carboplatin sensitivity in vitro (Pearson r = 0.96, P = 0.01; Fig. 1C and D; Supplementary Fig. S2B). Cells obtained from patients with platinum-resistant disease had higher RAD51 scores than those from patients with platinum-sensitive disease (87.4% vs. 69.9%). These data indicate that RAD51 foci might be a reliable marker of chemotherapy response in HGSOC.

Unlike RAD51 foci, we suspected that RAD51 total expression would not be associated with platinum chemotherapy response. We used the cancer cell line encyclopedia (35) to determine RAD51
RAD51 foci in patient-derived ovarian cancer organoids are predictive of clinical platinum chemotherapy response

We next examined whether RAD51 foci in patient-derived ovarian cancer organoids would predict patients' clinical responses to carboplatin (Supplementary Fig. S1). Tumor organoids are 3-dimensional models that recapitulate tumor cell clonal heterogeneity, the tumor microenvironment, and cell–cell and cell–matrix interactions. Thus, they are more clinically relevant than cell lines for evaluating drug sensitivity, functional biomarkers, and processes such as DNA damage response (29). We generated 11 patient-derived ovarian cancer organoids (Fig. 1E) from patients with stage IIC or IV HGSOC and no prior therapy (Supplementary Table S3). One parent tumor had a pathogenic BRCA mutation. Organoids were classified as platinum-responsive (n = 6, 55%) or platinum-nonresponsive (n = 5, 45%) according to the corresponding patient’s chemotherapy response score (3 = platinum-resistant, 2 = platinum-nonresistant, progression-free interval (PFI) ≥ 24 months = platinum-responsive, < 24 months = platinum-nonresponsive). There was high correlation between the 2 samples (r = 0.81, Pearson r = 0.81, P < 0.001; Supplementary Table S2). Organoids were categorized according to the corresponding patient’s chemotherapy response score, with 6 organs classified as platinum-responsive (n = 6, 100%; Fig. 1F and G) and 5 organs classified as platinum-nonresponsive (Fig. 1H). 

The RAD51 foci assay is feasible and reliable in FFPE samples

Given the cost and time involved in generating primary ovarian cancer cell lines and organoids, we next assessed the possibility of measuring RAD51 foci in FFPE tumor samples. Fig. 2A shows our workflow and scoring criteria, which were adapted from Llop-Guevara and colleagues (13). First, all FFPE samples must have a γH2AX score of > 25%, indicating sufficient dsDNA breaks to mount RAD51 binding. Cells were then screened for geminin and RAD51 (RAD51 score). Per previously validated cutoff values established in patient-derived xenografts and patient breast cancer samples (9, 13), tumors were defined as RAD51-High if > 10% of geminin-positive cells had ≥ 5 RAD51 foci and RAD51-Low if ≤ 10% of geminin-positive cells had ≥ 5 RAD51 foci (Fig. 2A). To assess the specificity of this assay, we knocked down RAD51 with two separate siRNAs in 2 HGSOC cell lines, 1 BRCA WT (ES2), and 1 BRCA1 mutant (COV362; 37), embedded them in agarose, cut FFPE sections, and tested our methods and RAD51 antibody. We observed significantly fewer RAD51 foci in cells in which RAD51 was knocked down, both with and without irradiation (Fig. 2B and C). To assess the sensitivity of our assay, we irradiated RAD51-High HGSOC cell lines, embedded them in agarose, and tested our methods. With increasing doses of irradiation, the percentage of cells with RAD51 foci increased, demonstrating a broad dynamic range (Fig. 2D).

Having confirmed the sensitivity and specificity of our assay, we evaluated RAD51 foci in 31 HGSOC tumor biopsies obtained from primary tumor sites before the patients received neoadjuvant carboplatin and paclitaxel (Fig. 2E). All patients had advanced-stage disease (stage IIC or IV) and at least mixed high-grade serous histology (Table 1). All patients had contributive testing results. We found discrete quantifiable foci that classified tumors as RAD51-High and RAD51-Low that allowed for formal analysis. In addition, using a subset of patients (n = 17), we compared 2 tumor samples obtained from different locations at the time same (Fig. 2F). There was high correlation between the 2 samples (n = 17, Pearson r = 0.83, P < 0.001, Cohen’s Kappa coefficient 0.81, P < 0.003), indicating the results of this assay may not entirely depend on the biopsy location.

RAD51 foci in FFPE patient samples predict platinum chemotherapy response and survival

To investigate the accuracy of the RAD51 foci assay in predicting platinum chemotherapy response in patient-derived FFPE tumor samples, we evaluated the association between RAD51 scores and platinum chemotherapy response in pretreatment HGSOC tumor biopsies. The association between platinum chemotherapy response and RAD51 score was significant (Fig. 3A). Somatic and germline mutations in BRCA1 and BRCA2 were evaluated by standard-of-care NGS-based gene panel tests. Chemotherapy response was assessed at the time of interval cytoreductive surgery and defined according to a validated histopathologic scoring system (14–19). Thirteen out of 31 patients (41.9%) had a pCR (Fig. 3A). All FFPE patient tumors had a γH2AX score of > 25%, indicating sufficient dsDNA breaks to cause RAD51 foci formation. Twelve (38.7%) tumors were RAD51-Low and 19 (61.3%) were RAD51-High.
Table 1. Patient characteristics.

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<th>Validation Cohort* (n = 149)</th>
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<td>67.4 ± 10.9</td>
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<td>No</td>
<td>24 (77.4)</td>
<td>118 (79.2)</td>
</tr>
<tr>
<td>Missing</td>
<td>–</td>
<td>4 (2.7)</td>
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<td>Median follow-up (mo)</td>
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<tr>
<td>41.9 ± 22.7</td>
<td>51.2 ± 47.2</td>
<td>(1.1 – 88.7)</td>
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<td></td>
<td>100% (68%)</td>
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Note: Data are n (%) unless stated otherwise; ± denotes standard deviation. Abbreviation: PFI, progression-free interval.

*BRCA mutation status determined by germline testing or whole-genome sequencing. Limited information regarding pathogenicity of mutations.

RAD51-High (Supplementary Table S4). Pretreatment RAD51-Low tumors were more likely to have a pCR than RAD51-High tumors (RR, 5.28; 95% CI, 1.8–15.37; P < 0.001; Fig. 3B). In addition, RAD51-Low tumors were more likely to be platinum-sensitive than RAD51-High tumors (100% vs. 68%; P = 0.03; RR, 0.99; P < 0.001; Fig. 3C). The assay predicted platinum sensitivity with 100% specificity, 100% positive predictive value, and 48% sensitivity. Overall, RAD51 score was associated with survival (Fig. 3D) and predicted chemotherapy response (AUC: 0.90; 95% CI, 0.78–1.0; P < 0.001; Fig. 3E).

Figure 2. Validation of RAD51 immunofluorescence assay in formalin-fixed paraffin-embedded (FFPE) samples. A, RAD51 immunofluorescence assay in high-grade serous ovarian cancer (HGSOC) FFPE tumor samples. B, RAD51 scores in 2 HR-proficient HGSOC cell lines after transfection with 2 separate short-interfering RNA (siRNA) targeting RAD51 (siRAD51) or a noncoding region (siControl) and exposed to γ-irradiation. Cells were treated, fixed, embedded, and cut into 4-µm sections for evaluation. All foci were counted in 4 technical replicates with ≥100 geminin-positive cells per experiment. C, Western blot of the HGSOC cell lines after treatment with two siRNA (siRAD51-1: 124a01; siRAD51-2: s53193d0) targeting RAD51. D, Dynamic range of RAD51 scores in 2 FFPE HR-proficient HGSOC cell lines after treatment with 0, 5, and 10 Gy of γ-irradiation. E, Representative images of geminin, RAD51, and colocalization of geminin/RAD51 at ≥10 with ≥63 insets in a patient-derived FFPE HGSOC sample. F, Correlation in HGSOC FFPE tumor samples between RAD51 scores obtained from 2 separate samples from the same patient.
Figure 3.
RAD51 score predicts platinum chemotherapy response and survival in ovarian cancer in a discovery cohort. A, RAD51 score and pathologic complete response (pCR) in patients with high-grade serous ovarian cancer before neoadjuvant chemotherapy. Dotted black line indicates manual quantification 10% cutoff value, which delineates RAD51-High and RAD51-Low tumors. All foci were counted in n > 100 geminin-positive cells per experiment. Technical replicates were performed for 30% of samples. B, Proportion of pretreatment RAD51-Low (n = 12) and RAD51-High (n = 19) tumors that had a pCR versus chemotherapy response score of 1–2 (RR 5.28; 95% CI 1.8–15.37; P < 0.001). C, Proportion of pretreatment RAD51-Low (n = 12) and RAD51-High (n = 19) tumors that were platinum-sensitive versus resistant (RR ∞, P = 0.05). *, P < 0.05 and ****, P < 0.0001 by the Student two-tailed t test. D, Kaplan–Meier curves evaluating progression-free survival (left) and overall survival (right) in patients (n = 31) stratified by RAD51 scores. E, Receiver operating characteristic curve evaluating the predictive performance of RAD51 score and pathologic complete response. F, Correlation between manual quantification and novel automated quantification in patient-derived FFPE tumor samples.
internal control, we calculated γH2AX and RAD51 scores for all benign tissue. γH2AX scores were noted to be <25%, and RAD51 scores were consistently less than 10 (mean 4.5 ± 4.6). γH2AX scores were >25% for 148/149 (99.3%) tumors analyzed. Primary tumor samples were used for analysis when available (n = 141). Metastatic tumors (n = 7) were used when primary samples were unavailable. A broad range of RAD51 scores was noted (0.8–56.9%; Fig. 4A). Thirty-four (22.9%) tumors were RAD51-Low and 114 (77.1%) were RAD51-High (Supplementary Table S5). RAD51-Low tumors were more likely to be platinum-sensitive than RAD51-High tumors (100% vs. 73.1%, P = 0.005; RR = 100%, P < 0.001; Fig. 4B). On multivariate analysis, patients with RAD51-Low tumors had significantly longer PFS (20.0 vs. 10.0 months; HR, 0.53; 95% CI, 0.33–0.85; P = 0.008; Fig. 4C) and OS (123.2 vs. 70.8 months; HR, 0.43; 95% CI, 0.25–0.75; P = 0.003; Fig. 4D) than patients with RAD51-High tumors when controlling for stage and residual disease at time of cytoreductive surgery. Overall, RAD51 scores in metastatic tumors showed fair correlation with RAD51 scores in primary tumors (Cohen’s Kappa coefficient 0.3, P < 0.001). On univariate analysis, RAD51-Low score in primary tumors was more strongly associated with survival than RAD51-Low score in metastatic tumors (HR, 0.47; 95% CI, 0.29–0.76; P = 0.002 vs. HR, 1.2; 95% CI, 0.77–1.88; P = 0.4;
Fig. 5A and B). RAD51-Low scores in tumor samples obtained from both primary and interval cytoreductive surgery were associated with survival (Fig. 5A and B).

Overall, a RAD51-Low score predicted platinum sensitivity with 30% to 48% sensitivity, 100% specificity, and 100% positive predictive value in both discovery and validation cohorts (Fig. 5C).

Discussion

Current biomarkers of platinum chemotherapy response in HGSOC have limitations. We provide several lines of novel evidence that RAD51 foci are clinically valid and can accurately predict response to platinum chemotherapy in ovarian cancer samples with great precision. First, we show that a RAD51 foci assay can accurately...
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predict platinum chemotherapy response in established and patient-derived ovarian cancer cell lines and patient-derived ovarian cancer organoids. In line with other studies evaluating RAD51 foci in patient-derived xenografts and cells, we found that in total approximately 25% of HGSOCC tumors are RAD51-Low (9, 31). Second, we show that our methods can reproducibly provide a RAD51 score in HGSOCC FFPE tissue. Third, we establish that RAD51 foci are predictive of platinum chemotherapy response using novel automated quantification in a collection of 31 FFPE pretreatment ovarian tumor samples. Finally, we validate these findings in a cohort of 148 tumor samples. We demonstrate that RAD51 foci can predict platinum sensitivity with 100% specificity and 100% positive predictive value.

We interpret our findings in HGSOCC cells, organoids, and patient FFPE samples as follows: RAD51 foci formation reflects proficiency in many upstream HR events and thus is a well-established readout for HR proficiency (39, 40). Because platinum chemotherapy causes dsDNA breaks in the S-phase due to interstrand lesions as well as intrastrand lesions undergoing nucleotide excision repair, cells that cannot form RAD51 foci cannot use HR to repair DNA and thus die in response to chemotherapy (41, 42). As a result, the RAD51-Low cells and organoids are platinum-responsive, and patients with RAD51-Low tumors are more likely than those with RAD51-High tumors to show a good response to platinum chemotherapy. In conclusion, although platinum sensitivity is multifactorial and the result of diverse cellular processes, including drug uptake, DNA damage signaling, nucleotide excision repair, cell-cycle checkpoints, and cell death pathways (43), our results support RAD51 as a biomarker predictive of response to platinum chemotherapy in ovarian cancer.

RAD51 foci offer an extremely valuable tool for clinical decision making. As almost every patient with ovarian cancer is treated with platinum chemotherapy, it is important to identify patients who will certainly benefit from standard-of-care chemotherapy to avoid unnecessary toxicity of ineffective drugs and to maximize the benefit received from specific therapies. Our results show that the probability of a patient with a RAD51-Low tumor demonstrating platinum sensitivity is 100%. These results are unprecedented for a biomarker predictive of platinum chemotherapy response in ovarian cancer and offer great promise for the precise treatment of this disease.

Our findings are consistent with other reports that BRCA mutation status alone does not unequivocally determine platinum chemotherapy response. Similar to Gorodnova and colleagues (44), we found that patients with BRCA mutations had longer survival and about 40% of these patients achieved a pCR (44–46). Within the discovery cohort, 44% (n = 4) of tumors with BRCA mutations were scored as RAD51-High, and these patients all had poor responses to platinum chemotherapy and decreased survival. Given limited data regarding BRCA mutation pathogenicity in our validation cohort, it is difficult to draw further conclusions regarding the use of this assay in a cohort of patients with BRCA mutations. Nonetheless, data from the discovery cohort confirm findings from prospective clinical trials such as SOLO1 and PRIMA that there is a subset of BRCA-mutated patients who do not respond as expected to DNA damaging therapy (45, 46). Possible explanations for differences in chemotherapy response in BRCA-mutated tumors include restoration of BRCA function by, for example, hypomorphic protein expression, stabilization of the BRCA1 C-terminal domain, alternative splicing or alternative translation initiation of BRCA genes, reversion mutations, or by dysregulation of DSB end resection, including loss of 53BP1 (47–51). Exploratory analysis of our samples suggest increased BRCA mRNA and increased BRCA foci in at least a portion of our RAD51-High BRCA mutated samples. Further work is necessary to understand the mechanisms behind differential responses in these cells, but our results support further study of the incorporation of RAD51 foci within the current standard-of-care genomic biomarkers.

In this work, we overcome several potential limitations of a RAD51-based assay for assessing ovarian cancer samples. FFPE samples are the most accessible source for patients’ tumors, as formalin fixation is a standard procedure performed routinely in pathology laboratories. However, formalin fixation affects tissue antigens through the formation of methylene bridges that modify protein conformation and epitopes, resulting in poor antibody reactivity. To ensure the immuno-reactivity of the fixed antigens (γH2AX, Geminin, RAD51), in our assays, we first carefully validated antigen retrieval steps (antigen retrieval buffer, temperature, and time) and specificity of antibodies in FFPE cell lines. Second, this assay is technically challenging. However, we demonstrate reproducible, optimized methods adapted from investigations in other cancer types and patient-derived xenograft models (13, 52). We validated our assay in prospectively obtained HGSOCC samples (53). We observed 2 factors that affected RAD51 staining quality. Samples that were 3 to 4 μm thick provided the best image quality. In addition, biopsies that were of the primary tumor and immediately placed in fixative had higher quality results than samples from large tissue collections such as a cytoreduction surgery obtained from metastatic sites. A third potential limitation of the RAD51 foci assay was that previous studies suggested significant tumor heterogeneity in RAD51 scores (31). Although we observed this in our validation cohort, this was not noted in the discovery cohort. In our discovery cohort, we evaluated 4 quadrants of an entire tumor slice as opposed to small tumor biopsies on a microarray in the validation cohort. Therefore, we hypothesize that tumor heterogeneity can be overcome by evaluating larger tumor samples. Nonetheless, on the basis of our findings, the primary tumor should be evaluated for RAD51 score when possible. Finally, manual counting of RAD51 foci can be subjective and time consuming. Thus, we developed an automated image processing method to quantify RAD51 foci and observed a strong correlation with the manual quantification. This automated scoring system both removes bias from the assay and once validated, will allow this assay to be widely implemented in histopathology laboratories.

We note 3 important limitations of our study. First, a proportion of platinum-sensitive tumors were RAD51-High. Therefore, further investigation is necessary to more accurately stratify RAD51-High tumors. Second, tumor samples were only obtained at cytoreductive surgery, and so we are unable to make conclusions regarding the use of this biomarker throughout a patient’s treatment course to determine the utility of a repeat challenge with platinum chemotherapy. In addition, our study focused on the correlation between RAD51 foci and platinum response. Therefore, given the importance of HR status in PARPi response, future work should assess the ability of the RAD51 foci assay to predict PARPi response.

In conclusion, we demonstrate that RAD51 is a robust and reliable biomarker predictive of response to platinum chemotherapy. Further studies are needed to prospectively evaluate this biomarker for translation into clinical care.

Authors’ Disclosures

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Authors’ Contributions

A.J. Compadre: Conceptualization, data curation, software, formal analysis, validation, investigation, visualization, methodology, writing—original draft, writing—review and editing. L.N. van Billoon: Conceptualization, data curation, software, formal analysis, validation, investigation, visualization, methodology, writing—original draft, writing—review and editing. M.C. Valentine: Data curation, software, formal analysis, investigation, writing—original draft, writing—review and editing. A. Llop-Guevara: Conceptualization, methodology, writing—review and editing. E. Graham: Data curation, software, validation, investigation, visualization, methodology, writing—review and editing. B. Fokhemi: Methodology, writing—original draft, writing—review and editing. A. Herencia-Ropero: Conceptualization, investigation, methodology, writing—review and editing. E.N. Kotnik: Data curation, methodology, writing—review and editing. I. Cooper: Validation, methodology, writing—review and editing. S.P. Harrington: Resources. L.M. Kuroki: Resources, data curation, methodology, writing—review and editing. C.K. McCourt: Resources, data curation, methodology, writing—review and editing. A.R. Hagemann: Resources, data curation, methodology, writing—review and editing. P.H. Thaker: Resources, data curation, methodology, writing—review and editing. D.G. Match: Resources, data curation, funding acquisition, methodology, project administration, writing—review and editing. M.A. Powell: Conceptualization, resources, funding acquisition, methodology. L. Sun: Validation, methodology, writing—review and editing. N. Mosammamparast: Conceptualization, resources, methodology, writing—review and editing. V. Serra: Conceptualization, supervision. P. Zhao: Software, formal analysis, writing—review and editing. E. Lomonosova: Conceptualization, resources, data curation, software, formal analysis, funding acquisition, methodology, writing—review and editing. D. Khabele: Conceptualization, resources, methodology, writing—review and editing. M.M. Mullen: Conceptualization, resources, data curation, software, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, project administration.

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Note

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References

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