The breast and ovarian cancer susceptibility genes \textit{BRCA1} and \textit{BRCA2} (\textit{BRCA1/2}) are key components of the Fanconi anemia (FA)/homologous recombination (HR) pathway of DNA repair. Previous work had shown that cancer cells with deleterious FA/HR pathway mutations are hypersensitive to poly(ADP-ribose) polymerase (PARP) inhibitors. Importantly, however, only about half of the cancer patients with germline FA/HR pathway mutations respond to PARP inhibitors, raising the question of why a substantial fraction of HR-deficient cancers are resistant to these agents in the clinic. Based on previous work in the Swisher and Kaufmann laboratories, we proposed to test the \textit{hypothesis} that two different conditions must be met for ovarian cancer to be hypersensitive to platinum and PARP inhibitors: The FA/HR pathway must remain disabled and NHEJ must remain intact and functional. Although we proposed two aims, the aim in previously banked specimens was removed before the present grant was awarded, leaving us with the following aim: \textit{Correlate biomarkers of HR deficiency and NHEJ pathway integrity in pre-treatment biopsies with response to a PARPi in a prospective single-agent PARPi phase 2 clinical trial in sporadic ovarian carcinoma.} Over the past 11 months we have i) completed IRB and HRPO review of our project, ii) developed and performed rigorous validation of our IHC assays, and, as of a few weeks ago, iii) begun accessioning samples from the phase 2 rucaparib trial (Ariel 2. ClinicalTrials.gov identifier NCT01891344).
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INTRODUCTION

Poly(ADP-ribose) polymerase (PARP) is an abundant nuclear enzyme that regulates five different DNA repair pathways (1, 2). Building on preclinical observations that defects in homologous recombination (HR) repair, which are found in 30-50% of ovarian cancers, sensitize cells to killing by PARP inhibitors (3-5), five separate phase 3 trials involving PARP inhibitors have opened or are about to open in ovarian cancer (2). Nonetheless, in a recent decision the Food and Drug Administration has declined to approve the PARP inhibitor olaparib for ovarian cancer, citing (in part) the need for additional information that will permit better identification of patients most likely to respond to this agent. In collaboration with Elizabeth Swisher (University of Washington), the present synergistic translational leverage project is assessing multiple aspects of DNA repair pathway integrity in pretreatment biopsies from a large multi-institution phase 2 study of the PARP inhibitor rucaparib. In particular, the Kaufmann laboratory is using immunohistochemistry to assess expression of proteins in the nonhomologous end-joining (NHEJ) pathway (53BP1, Ku70, Ku80, DNA-PKcs, XRCC4, DNA ligase IV) as well as PARP1. This group of proteins was chosen based on our preclinical studies showing that PARP inhibitors activate the error-prone NHEJ repair pathway in homologous recombination-deficient ovarian cancer cells (6, 7) and that loss of any of the proteins in this pathway will simultaneously impair NHEJ and PARP inhibitor-induced killing (op. cit. and additional unpublished observations).

KEYWORDS

Key words: ovarian cancer, drug resistance, rucaparib, phase 2, DNA repair, homologous recombination, nonhomologous end-joining (NHEJ), immunohistochemistry, poly(ADP-ribose) polymerase, Ku70, Ku80, PARP1, 53BP1, DNA-PK, Artemis, DNA ligase IV, XRCC4.
OVERALL PROJECT SUMMARY

Consistent with our Statement of Work, we prepared paperwork for the IRB and HRPO regarding the analysis of deidentified samples from the phase 2 rucaparib trial. Both determined that the research was exempt.

Simultaneously, we started the process of validating the proposed IHC assays. For each of these assays, our strategy is purchase a commercial antibody that has been reported by the supplier to give a robust signal in IHC. Once conditions are identified that permit staining of tissues reported on the supplier website to stain positive with the antibody, we assess the staining in a small group of ovarian cancers to make sure that formalin fixed, paraffin embedded (FFPE) samples of the cancer of interest will stain. We then endeavor to confirm that the staining is specific for the antigen of interest. In particular, we fix and embed cells with different amounts of the antigen of interest (as a consequence of siRNA or gene targeting) and assess the impact on staining. Results to date can be summarized as follows:

**DNA-PKcs:** Using a polyclonal antibody from Bethyl Labs, we have identified conditions that yield nuclear staining of varied intensity in different ovarian cancer specimens (Fig. 1). To assess specificity of the staining, we examined M059J and M059K cells, a glioblastoma line that lacks DNA-PKcs (8) and a line from the same patient that has DNA-PKcs restored. Staining was negative in formalin fixed, paraffin embedded M059J cells and was restored when DNA-PKcs was restored (Fig. 2), leading to the conclusion that the staining is specific.

**Ku70:** We identified a commercially available anti-Ku70 antibody that stained FFPE ovarian cancers to varying extents (Fig. 3). Control experiments using Ovcar8 cells, chosen for these studies because of their ease of transfection and clear-cut ovarian cancer origin, confirmed that the signal is markedly diminished when Ku70 is absent (Fig. 4), demonstrating specificity of the staining.

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**Figure 1.** Examples of DNA-PKcs staining in ovarian cancer.

**Figure 2.** DNA-PKcs IHC validation. DNA-PKcs blotting (right) or staining of DNA-PKcs-deficient M059J and -proficient M059K after formalin fixation and paraffin embedding (left).

**Figure 3.** Examples of Ku70 staining in ovarian cancer.

**Figure 4.** Ovcar8 cells were transfected with control siRNA or K70 siRNA were subjected to SDS-PAGE followed by immunoblotting (left) or staining with anti-Ku70 antibody (right).
**Ku80:** Using a commercially available anti-Ku80 antibody from Abcam, we have established conditions that yield nuclear staining of variable intensity across a series of ovarian cancers (Fig. 5). Initial experiments examining staining in Ovcar8 cells lacking Ku80 due to siRNA treatment failed to show any change in staining despite a dramatic knockdown in antigen (not shown). We are currently repeating this experiment and will begin working with a different anti-Ku80 antibody if the Abcam antibody lacks the required specificity.

**XRCC4:** Staining with the initial antibody from Santa Cruz Biotechnology yielded weak staining across all ovarian cancer examined. Use of an alternative antibody from Becton Dickinson has demonstrated good staining in Ovcar8 cells and markedly diminished staining when XRCC4 was downregulated with siRNA (Fig. 6). We are staining a series of test ovarian cancer specimens at present.

**53BP1.** The original antibody chosen for these studies and obtained commercially (from Abcam) gave varied staining in a series of ovarian cancers (Fig. 7). Using Ovcar8 cells, we were able to knock down 53BP1 efficiently (Fig. 8). The nuclear staining with this antibody was markedly diminished, although a cytoplasmic staining pattern persisted. We have subsequently tested an additional rabbit polyclonal antibody, which yielded exclusively nuclear staining but did not give a strong a differential between control and 53BP1 knockdown cells. We are currently acquiring a third antibody (murine monoclonal) to test.
**PARP1:** We have identified assay conditions that provide nuclear staining of varied intensity in different ovarian cancer specimens (Fig. 9). To confirm that the staining is due to PARP1 and not cross-reactivity with a different antigen, we have investigated two types of controls. First, we obtained human HCT116 PARP1\(^{-}\) cells engineered in the laboratory of Eric Hendrickson, an expert in somatic cell gene targeting. Staining of parental HCT116 and putative PARP1\(^{-}\) cells was strong and indistinguishable, but our immunoblotting demonstrated the continued presence of PARP1 in the supposed gene targeted cells. Accordingly, we next tried to knock down PARP1 using several two different siRNAs, but knockdown was limited and we observed only a limited difference in signal between the control and siRNA-treated cells as the primary antibody was titered out (not shown). We have subsequently obtained two additional clones of PARP1-targeted HCT116 clones from the Hendrickson lab. As of August 15 these have been expanded; and samples have been sent for embedding in paraffin and simultaneously saved for immunoblotting.

**DNA ligase IV:** The initial antibody obtained from Abcam failed to stain any of the ovarian cancer specimens examined. We have obtained anti-DNA ligase IV from Santa Cruz Biotechnology and are currently staining parental Ovcar8 cells and Ovcar8 cells with DNA ligase IV knocked down by siRNA.

**Artemis:** Our initial commercial antibody from Abcam stained cytoplasm rather than nucleus, which is the presumed site of this repair protein. Accordingly, we obtained HCT116 parental and DCLRE1C gene targeted cells (“Artemis\(^{-}\)” from the Hendrickson laboratory and confirmed the absence of Artemis protein (Fig. 10). Because cytoplasmic staining was also observed in the Artemis\(^{-}\) cells with this first antibody, we obtained a second commercial antibody (from Fisher Thermo) but could not identify conditions that distinguished between parental and Artemis\(^{-}\) cells. In studies beyond the scope of this grant (supported by Institutional funds), during the course of this year we purified human recombinant Artemis and attempted to raise a mouse monoclonal antibody in the Mayo Clinic Hybridoma Core Facility. Although we obtained some ELISA positive wells during primary screening, none of the resulting clones would selectively react with Artemis in western blots. We will screen additional commercial antibodies for specific staining of Artemis.

In summary, we have validated assays working for DNA-PKcs, Ku70 and 53BP1 (although we are trying to improve the 53BP1 assay). We close to having assays for PARP1, Ku80 and XRCC4. We are continuing to develop the assays for DNA ligase IV and Artemis.

**Sample acquisition:** The phase 2 clinical trial that is providing samples for the correlative assays in the Kaufmann and Swisher laboratories (ClinicalTrials.gov identifier NCT01891344) opened during the reporting period. Deidentified specimens from 6 patients were obtained through August 15, 2014, and are being stored for staining in batches.
KEY RESEARCH ACCOMPLISHMENTS

Nothing to report (per instructions that hitting project milestones are not key research accomplishments)

CONCLUSION

A majority of the proposed assays are up and running, with the remainder quickly falling into place. After initial delays, the samples are now being acquisitioned. Thus, we are on track to acquisition, stain and score all samples before the end of the funding period in two years.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS


INVENTIONS, PATENTS AND LICENSES

None

REPORTABLE OUTCOMES

None

OTHER ACHIEVEMENTS

None

REFERENCES

2. Scott CL, Swisher EM, Kaufmann SH. PARP Inhibitors: Recent Advances and Future Development. 2014;submitted:


APPENDICES

None