Rare *BRIP1* Missense Alleles Confer Risk for Ovarian and Breast Cancer

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ABSTRACT

Germline loss-of-function mutations in BRCA1 interacting protein C-terminal helicase 1 (BRIP1) are associated with ovarian carcinoma and may also contribute to breast cancer risk, particularly among patients who develop disease at an early age. Normal BRIP1 activity is required for DNA interstrand cross-link (ICL) repair and is thus central to the maintenance of genome stability. Although pathogenic mutations have been identified in *BRIP1*, genetic testing more often reveals missense variants, for which the impact on molecular function and subsequent roles in cancer risk are uncertain. Next-generation sequencing of germline DNA in 2,160 early-onset breast cancer and 1,199 patients with ovarian cancer revealed nearly 2% of patients carry a very rare missense variant (minor allele frequency < 0.0001) in *BRIP1*. This is 3-fold higher than the frequency of all rare BRIP1 missense alleles reported in more than 60,000 individuals of the general population ($P < 0.0001$, χ^2 test). Using CRISPR-Cas9 gene editing technology and rescue assays, we functionally characterized 20 of these missense variants, focusing on the altered protein's ability to repair ICL damage. A total of 75% of the characterized variants rendered the protein hypomorph or null. In a clinical cohort of >117,000 patients with breast and ovarian cancer who underwent panel testing, the combined OR associated with BRIP1 hypomorph or null missense carriers compared with the general population was 2.30 (95% confidence interval, 1.60–3.30; $P < 0.0001$). These findings suggest that novel missense variants within the helicase domain of BRIP1 may confer risk for both breast and ovarian cancer and highlight the importance of functional testing for additional variants.

Significance: Functional characterization of rare variants of uncertain significance in *BRIP1* revealed that 75% demonstrate loss-of-function activity, suggesting rare missense alleles in *BRIP1* confer risk for both breast and ovarian cancer.

Introduction

BRCA1 interacting protein C-terminal helicase 1 (BRIP1) is a member of the Fanconi anemia (FA) pathway of proteins with an established role in DNA interstrand cross-link (ICL) repair (1, 2). The N-terminal domain is comprised of seven highly conserved DEAH helicase motifs that function in the unwinding of DNA, preferentially at forked duplex substrates and D-loops (3, 4), and in the resolution of guanine quadruplexes (G4-DNA; refs. 5–7). The N-terminal region also includes an iron–sulfur (Fe-S) binding domain that distinguishes BRIP1 from other DEAH helicase family members. A single missense mutation in this domain (A349P) abrogates BRIP1's ability to unwind DNA strands and displace proteins bound to DNA (8, 9). The C-terminal domain contains an essential phosphorylation site, S990, required for the binding of BRIP1 to the tumor suppressor, BRCA1 (10). Cells lacking BRIP1 are unable to repair ICL damage, resulting in cell death (2, 11) and homozygosity or compound heterozygosity for missense, nonsense, or frameshift mutations leads to Fanconi anemia (12, 13).

BRIP1 was reported to be the third most common ovarian cancer susceptibility gene with nearly 0.9%–2.5% of all patients with ovarian cancer carrying a splice, stop, or frameshift defect (14–16). Rare missense variants are also associated with an increased risk for ovarian cancer, but the function of these variants is unknown (14). Mutations in *BRCA1* and *BRCA2*, also members of the Fanconi anemia pathway, confer high risk for the development of ovarian and breast cancers (17–19). Given that *BRIP1* is a member of the Fanconi anemia pathway, it was logically assumed that *BRIP1* mutations might similarly confer risk for both ovarian and breast cancers. Family studies initially pointed to truncating mutations in *BRIP1* (including the hotspot Fanconi anemia allele, R798X) as low-penetrance breast cancer susceptibility alleles (20, 21). Recent studies, however, have brought into question the role that pathogenic *BRIP1* mutations play in risk for breast cancer (16, 22, 23).

Next-generation sequencing (NGS) for cancer susceptibility alleles frequently includes analysis of *BRIP1*. *BRIP1* missense variants are identified more frequently than nonsense or frameshift mutations. The vast majority of these missense alleles are reported as variants of uncertain significance (VUS). As of May 2019, 950 missense variants in *BRIP1* have been reported to ClinVar with 933 clinically classified as a VUS. Most of the *BRIP1* variants in ClinVar are linked to the conditions "familial cancer of the breast" and "neoplasm of the ovary" (24). To better understand how *BRIP1* VUSs contribute to cancer risk, we functionally characterized rare and novel missense variants identified in patients with ovarian and early-onset breast cancer by assessing ICL DNA repair activity. The missense variants that we classified as deleterious (nulls and hypomorphs) were significantly enriched in a cohort of >117,000 patients with breast and ovarian cancer who underwent clinical testing. Our findings highlight

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the importance of functional testing for *BRIP1* VUSs and the need for further studies to determine the role that missense variants play in cancer risk.

Patients and Methods

Cohorts and patient materials

Early-onset breast cancer cohort

Breast cancer subjects were recruited to The Young Women's Breast Cancer Program (YWBCP) at Washington University (St. Louis, MO) during the period 2005–2012 (IRB-approved protocols 04-1009 and 2012C0097). The YWBCP enrolled women from all 50 states. The majority of subjects were Caucasian. African Americans made up 3.7% of the cohort, and there were smaller numbers of Native American and Asians. All of the 2,160 subjects investigated in this study had invasive breast cancer diagnosed at age $\leq$40 years. For those subjects for whom *BRCA1* and/or *BRCA2* testing data were available, 15.7% carried mutations. Germline DNA was prepared form white blood cells for the vast majority of subjects. All coding exons in *BRIP1* (GenBank: NM_032043) were sequenced to an average of >500 reads using the TruSeq Custom Amplicon Kit v1.5 and a MiSeq instrument with Reagent Kit v2 (Illumina). Variants were identified using Miseq Reporter software (v2.5.1).

Ovarian cancer cohort

A total of 1,199 ovarian cancer cases were identified from the University of Washington Gynecologic Oncology Tissue Bank, the Mayo Clinic Ovarian Spore Tissue Bank, or from the ARIEL2 part 1 clinical trial. Sequencing of germline DNA was completed using BROCA as described previously (25). Clearly damaging mutations for the University of Washington and ARIEL2 cases have been reported previously (15).

Clinical diagnostic laboratory breast and ovarian cancer cohorts

Data in the clinical laboratory cohort included 117,346 women referred for clinical genetic testing with a multigene panel inclusive of *BRIP1* in the period (Jul 2012–Sep 2018). Multigene panel testing on genomic DNA isolated from blood or saliva was carried out by NGS with deletion/duplication analysis. Targeted coding exons and adjacent intronic nucleotides of the *BRIP1* gene were enriched for sequencing. Additional follow-up Sanger sequencing was performed for any regions missing or with insufficient read depth coverage for reliable heterozygous variant detection (26). ICD10 codes were used to filter the deidentified data for applicable cases.

Exome Aggregation Consortium and FLOSSIES

Two publically available, online sequencing datasets were utilized to estimate population frequencies of *BRIP1* variants. Germline variants from 60,327 unrelated individuals of European, African, Latino, South Asian, East Asian, and Middle Eastern descent were analyzed from Exome Aggregation Consortium (ExAC; ref. 27). In addition, germline variants from 9,884 cancer-free women, over the age of 70, of European and African descent from the Fabulous Ladies Over 70 project (FLOSSIES, https//whi.color.com) were analyzed. We chose to include all the FLOSSIES controls to better sample *BRIP1* genetic diversity among women of African descent.

CRISPR-Cas9

pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid #48138). CRISPR-Cas9 target sites near candidate missense variants were identified using CRISPOR (28). Guide sequences with *in silico* specificity scores > 50 and zero exonic, off-target effects were selected for exons 3, 8, 15, and 16. Guide RNAs were cloned into the pSpCas9(BB)-2A-GFP construct as described previously (29). HeLa and HEK293TN cells were cotransfected with CRISPR constructs and DNA template oligos containing missense variants using Lipofectamine 2000 (Thermo Fisher Scientific). After 48 hours, live GFP-positive cells were single-cell plated using a FACSAria III (BD Biosciences) flow cytometer. DNA ligase IV inhibitor SCR7 (1.0 μmol/L; APExBIO) was added to the media and colonies were maintained in 96-well plates. DNA was prepared from individual HeLa and HEK293TN clones using Quick-DNA Miniprep Kit (Zymo Research) and incorporation of missense variants was detected using PCR and Sanger sequencing (primers list in Supplementary Materials and Methods). Clones that failed to incorporate missense variants but contained homozygous or compound heterozygous frameshift mutations were further screened by Western blot analysis for loss of protein expression and chosen as null controls.

Cell culture

HeLa (obtained from J. Parvin laboratory) and HEK293TN (System Bio, LV900A-1) cells were cultured in DMEM (Sigma Aldrich) with 10% FBS at 37°C in a 5% CO_2 containing atmosphere. Cell lines were confirmed *Mycoplasma* negative using the MycoAlert Assay (Lonza). The estimated number of passages between authentication of cancer cell lines and completion of experiments is 20. Additional details regarding authentication are provided in the Supplementary Materials and Methods.

Karyotypes

Cells were incubated for 14 hours at 37°C in media containing mitomycin C (MMC; 60 nmol/L). KaryoMAX Colcemid Solution (Thermo Fisher Scientific) was added dropwise and cells were incubated for an additional 45 minutes at 37°C. Cells were collected, resuspended in 0.56% KCl, and placed in a 37°C water bath for 10 minutes to bloat. After incubation, cells were immediately spun down, removed from KCl solution, and fixed in 3:1 methanol to acetic acid. Cells were dropped onto slides in 55°C humid chamber and stained with KaryoMAX Giemsa Stain Solution (Thermo Fisher Scientific). Metaphase spreads were imaged on BIOREVO BZ-9000 (Keyence) at 100× magnification and images were captured and processed with BZ-II Viewer.

Clonogenic growth assays

Cells (5,000) were plated in each well of a 6-well dish and allowed to adhere. Cells were incubated for 24 hours at 37°C in media containing MMC (Sigma Aldrich), cisplatin (Sigma Aldrich), hydroxyurea (Sigma Aldrich), or TMPyP4 (Abcam) then washed 2 times with PBS before fresh media was added. After 7 days, cells were washed with PBS, fixed in 7:1 methanol to acetic acid for 10 minutes, and incubated with crystal violet (0.5% in 25% methanol) for 30 minutes at room temperature with gentle shaking. Crystal violet was removed and cells were washed 3 times with water and allowed to dry. Cells were destained with methanol for 30 minutes at room temperature with gentle shaking. Equal volumes of solution were removed from the destained cells and plated in optically clear 96-well plates. Optical density of crystal violet was measured at 580 nm using the Synergy 2 Microplate Reader with Gen5 Software v1 (BioTek). Colony survival values plotted are relative to an untreated control.

Cell-cycle analysis

Cells were incubated for 16 hours at 37°C in normal media or media containing MMC (Sigma Aldrich). Cells were trypsinized, collected as

a single-cell suspension, and fixed in ice-cold 70% ethanol for at least 10 minutes on ice. Fixed cells were RNaseA treated for 30 minutes at room temperature. Propidium iodide was added and cells were incubated for an additional 15 minutes before analysis using a FACSCalibur Flow Cytometer (BD Biosciences).

Cycloheximide chase assay

HeLa *BRIP1*$^{-/-}$ clone D2 was transfected with pCDH-BRIP1 wild-type (WT) and mutant constructs using Lipofectamine 2000 (Thermo Fisher Scientific). At 48 hours posttransfection, equal number of transfected cells were plated into 12-well plates and allowed to adhere. Cycloheximide (30 μg/mL) was added at time 0 and cells were collected at 0, 1, 2, 4, 6, and 8 hours. Protein was quantified by Western blot analysis and values were normalized to time 0. Analysis of protein degradation was performed from three independent experiments for each construct.

Statistical analysis

Statistical tests were performed using GraphPad Prism 5 for Windows (GraphPad Software, Inc). Analysis of G_2–M accumulation was performed from three independent experiments. *P* values were calculated using one-way ANOVA with Tukey *post hoc* test for pairwise comparisons. Western blot quantification of CRISPR clones was performed from three independent experiments. *P* values were calculated using one-way ANOVA with Dunnett multiple comparison test to *BRIP1*$^{+/+}$ clone. Protein half-life was determined using a nonlinear regression one-phase decay. *P* values by functional grouping were calculated using one-way ANOVA with Tukey multiple comparison test. Results were considered statistically significant when $P < 0.05$.

Results

Increased number of *BRIP1* missense allele carriers in ovarian and early-onset breast cancer cohorts

Sequencing germline DNA from 1,199 patients with ovarian and 2,160 patients with early-onset breast cancer revealed 6 patients with ovarian (0.50%) and 8 patients with breast cancer (0.37%) carried a frameshift or nonsense mutation in *BRIP1* (Supplementary Fig. S1). The hotspot R798X mutation was seen in 1 patient with ovarian and 3 patients with breast cancer along with four additional nonsense and four frameshifts. The frequency of truncating mutations in both cohorts was higher than reported by ExAC (0.19%) and FLOSSIES (0.19%). Consistent with previous articles (16), the enrichment of truncating mutations reached statistical significance in the ovarian cancer cohort ($P < 0.05$; Fisher exact test) but not the breast cancer cohort.

Rare missense alleles (MAF $\leq$ 0.0005) were more common than nonsense and frameshift mutations. Thirty-six patients with ovarian (3.0%) and 43 patients with breast cancer (2.0%) carried a rare or novel missense allele (**Fig. 1**; refs. 30, 31). The frequency of rare alleles was significantly higher ($P < 0.001$ both cohorts, Pearson χ^2 test) than all rare alleles reported by the ExAC general population (1.6%) and FLOSSIES (1.4%), with the greatest enrichment observed for very rare variants (MAF < 0.0001; $P < 0.0001$). The majority of rare alleles (54/64) clustered within the highly conserved helicase domain (1–888 amino acids) and are predicted *in silico* to be deleterious to protein function (Supplementary Table S1; ref. 32). The P47A mutation, known to have reduced ATPase activity and helicase deficiency (3) was observed in both the breast and ovarian cancer cohorts. Four additional rare alleles in the C-terminal region of the helicase domain were seen in both cohorts (Q540L, I691L, Q740H, and A745T).

Several of the missense alleles we identified have been described as mutations. The A349P and R707C variants associated with Fanconi anemia (12, 33) were each seen once in the ovarian cohort. Substitution of proline for alanine at amino acid 349 results in helicase deficiency and the mutant protein acts as a dominant negative *in vitro* (9). The novel K52R allele was identified in one individual with ovarian cancer. This mutation, like A349P perturbs WT helicase function in a dominant-negative manner (1). A subset of the missense variants identified fall within the seven highly conserved motifs of a DEAH helicase, but most lie outside of these domains (Supplementary Table S1). None of the missense alleles involve the amino acids known to be essential for BRCA1 binding (S990, P991, and F993; ref. 10).

Of the 64 different rare variants identified in patients with breast or ovarian cancer, 57 appear in ClinVar. Most are classified as VUSs ($n = 46$) or having conflicting interpretations of pathogenicity ($n = 10$) in association with familial cancer of breast, neoplasm of ovary,

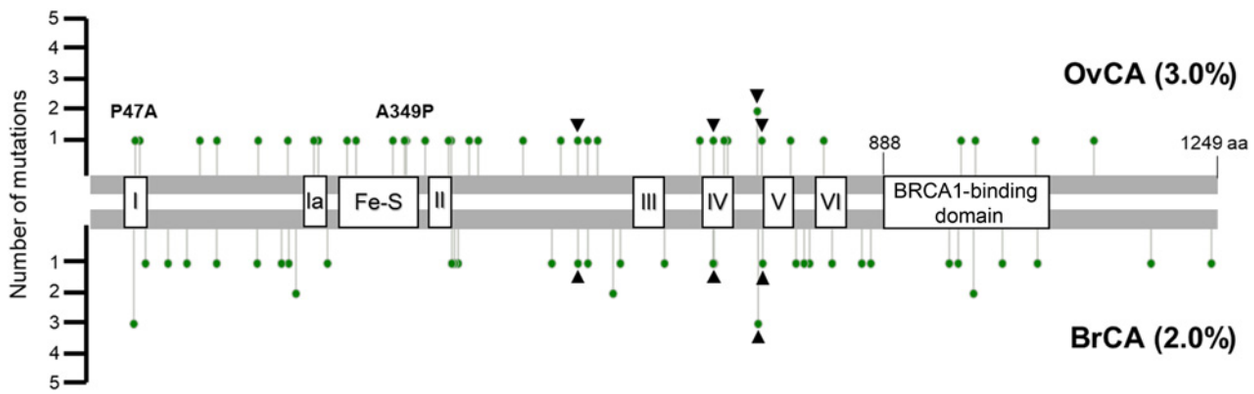

Figure 1.

Graphical summary of *BRIP1* rare (MAF < 0.0005) and novel missense variants identified in patients with ovarian and early-onset breast cancer. The ATPase reduced, helicase-deficient P47A (helicase domain I) was seen in both cohorts. Helicase-deficient, dominant negative–mutant A349P (Fe-S domain, 276–362 aa) was identified in a single patient with ovarian cancer. Rare alleles in the C-terminal region of the helicase domain (Q540L, I691L, Q740H, and A745T) identified in both cohorts are indicated with black arrowheads. Mutation plot was generated using cBioPortal MutationMapper tool. Helicase domain motifs: I (39–57); Ia (245–258); II (385–398); III (610–624); IV (689–710); V (748–775); and VI (819–836). BRCA1-binding domain: 888–1063 aa.

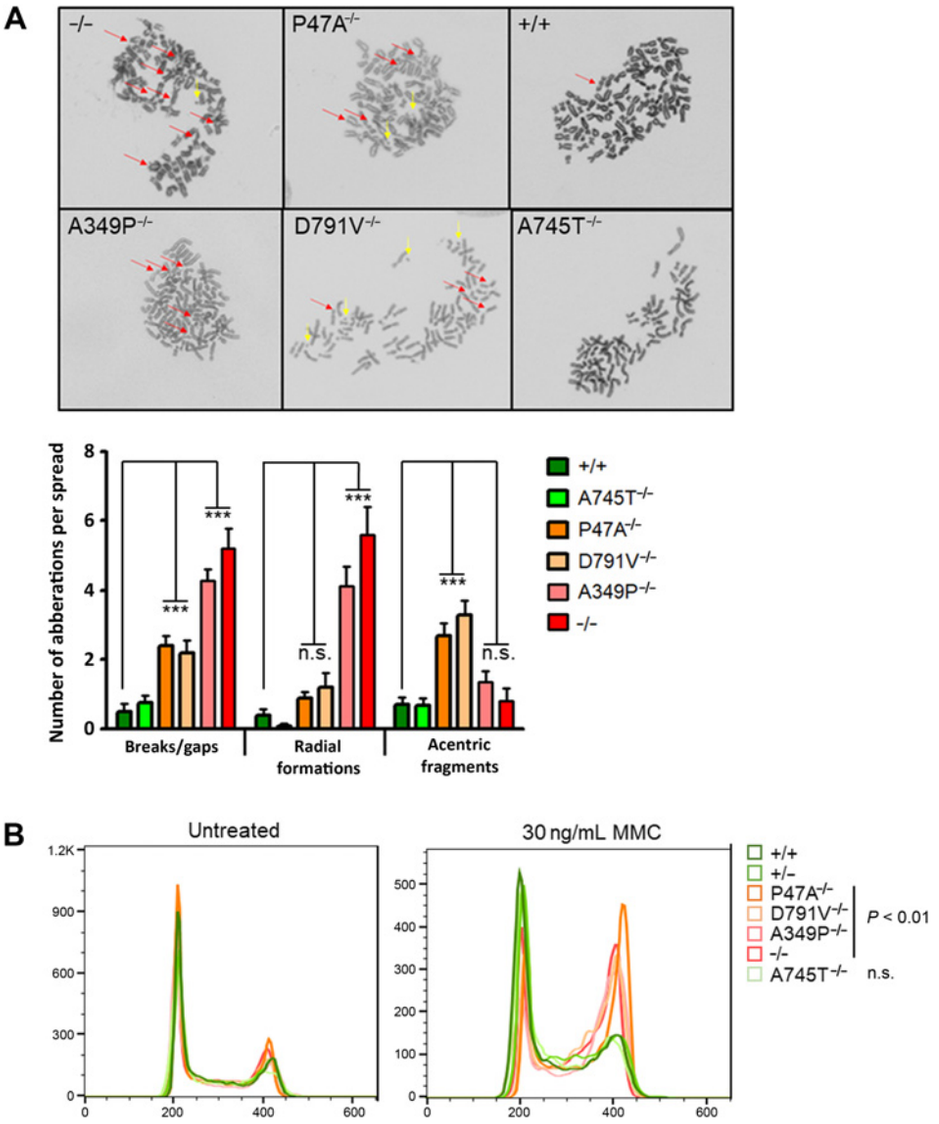

Figure 2.

Missense alleles in *BRIP1* increase sensitivity to ICL damage. **A,** Representative karyotypes of independent HeLa clones after exposure to MMC (60 nmol/L). Chromosomal breaks, gaps, radials (red arrows), and acentric fragments (yellow arrows) were observed in −/−, A349P$^{-/-}$, P47A$^{-/-}$, and D791V$^{-/-}$. The average number of breaks/gaps, radial formations, and acentric fragments per metaphase spread were calculated for each genotype ($n = 15$ spreads per cell line). Statistical significance determined by two-way ANOVA with a Bonferroni posttest to *BRIP1*$^{+/+}$. ***, $P < 0.0001$. **B,** Overlay of representative cell cycles from independent HeLa clones with and without MMC treatment. Statistical significance calculated by averaging percentage of cells in G_2–M-phase from three replicates (one-way ANOVA with Tukey multiple comparison test). n.s., not significant.

Fanconi anemia complementation group J (FA-J), and/or hereditary cancer-predisposing syndrome (review dates 2016–2018). Only A349P is described as likely pathogenic (Supplementary Table S2).

Of the 36 patients with ovarian cancer and 43 patients with breast cancer carrying a rare *BRIP1* missense variant, 6 (7.6%) also carried a pathogenic mutation in *BRCA1* or *BRCA2* (Supplementary Table S1). The cooccurrence of *BRCA1/2* mutations was not observed in any of the *BRIP1* frameshift or nonsense mutations carriers.

Missense alleles in *BRIP1* confer sensitivity to ICL damage

To assess the function of missense variants in the C-terminal helicase region, we utilized CRISPR-Cas9 gene editing to create isogenic HeLa cell lines lacking BRIP1 protein and/or expressing candidate missense variants identified in our cohorts. The helicase-deficient P47A and A349P mutations were generated to serve as positive controls for loss-of-function missense mutations (Supplementary Fig. S2). We generated HeLa cell lines expressing two representative candidate VUSs identified in our cohorts, A745T and D791V. A745T was identified in both cohorts and D791V was seen in a patient with early-onset breast cancer with a strong family history of breast and other cancers. HeLa clones expressing missense mutations carry the mutation of interest on one homologue and a frameshift mutation, predicted to undergo nonsense-mediated decay, on the other. RT-PCR sequencing confirmed that the missense allele was expressed with trace or no expression of the frameshift transcript consistent with compound heterozygosity (Supplementary Fig. S2A). qRT-PCR demonstrated that *BRIP1* transcript levels were similar in clones expressing the missense alleles to those in *BRIP1*$^{+/+}$ and *BRIP1*$^{+/-}$ clones (a positive control for hemizygosity). As expected, qRT-PCR showed significantly reduced *BRIP1* transcript levels in −/− clones (Supplementary Fig. S2B).

Cells lacking BRIP1 protein were sensitive to ICL damage and exhibit characteristic chromosomal aberrations when treated with ICL agents. These clastogenic effects were seen in patients with FA-J (34). MMC treatment of *BRIP1*$^{-/-}$ and *BRIP1* A349P$^{-/-}$ HeLa clones resulted in characteristic chromosomal breaks, gaps, and radial formations (**Fig. 2A**). Karyotypes of *BRIP1* P47A$^{-/-}$ cells treated with MMC showed modest numbers of chromosomal breaks and gaps but

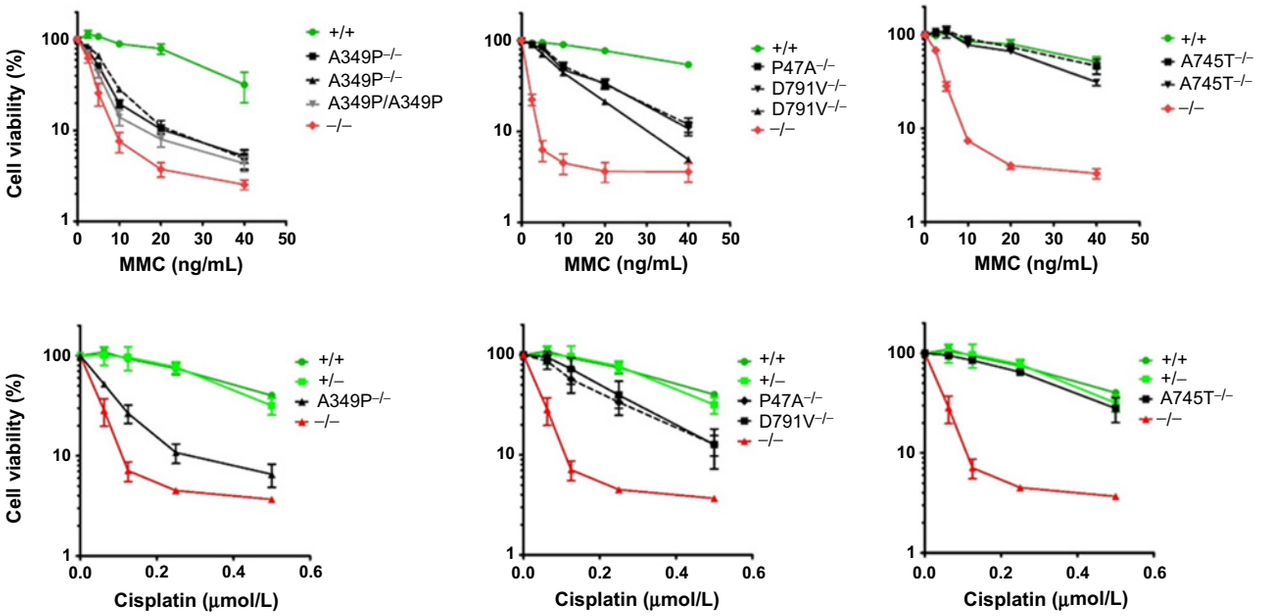

Figure 3.

Missense alleles classified as null, hypomorphic, or WT based on ICL damage–induced cell killing. Clonogenic growth assays of independent HeLa clones after exposure to increasing concentrations of MMC and cisplatin. Data presented are the average of three replicates, with error bars indicating one SD. Cisplatin experiments were performed simultaneously with a single *BRIP1*$^{+/+}$, $^{+/-}$, and $^{-/-}$ clone for control.

were characterized by many acentric fragments that were seen much more frequently than in the WT and null clones (**Fig. 2A**). *BRIP1* D791V$^{-/-}$ clones had karyotypes similar to *BRIP1* P47A$^{-/-}$ cells with increased acentric fragments and chromosomal breaks. *BRIP1* A745T$^{-/-}$ clones, on the other hand, had normal karyotypes, similar to WT HeLa cells (**Fig. 2A**).

There were no differences in proliferation between the clones (ATP cell viability assay) and no differences in cell cycle when cells were grown in normal media (**Fig. 2B**). Treatment with MMC, however, was associated with a statistically significant increase in G_2–M for the clones expressing P47A, D791V, A349P, and null alleles compared with *BRIP1*$^{+/+}$, $^{+/-}$, and A745T$^{-/-}$ clones (**Fig. 2B**). Accumulation of cells in G_2–M-phase and increased rates of apoptosis are phenotypes observed in FA-J patient cells (2).

To further assess the sensitivity of mutants to ICL, we performed clonogenic growth assays with clones treated with increasing concentrations of MMC. As expected, null and A349P-mutant–expressing cells were extremely sensitive, showing reduced cell viability at concentrations as low as 2.5 ng/mL. The estimated IC_{50}s were between 4 and 7 ng/mL for null and A349P cells and strikingly different than the IC_{50} for WT cells (>30 ng/mL; **Fig. 3**). Interestingly, clones expressing the helicase-deficient P47A allele and D791V allele showed intermediate sensitivity to MMC (**Fig. 3**). They were largely resistant to MMC at low doses but more much more sensitive than WT cells at high doses (IC_{50} between 5 and 15 ng/mL), consistent with a hypomorphic phenotype. In contrast, clones expressing the A745T allele were resistant to MMC damage, much like WT clones.

Treatment with a second ICL agent, cisplatin, paralleled the findings with MMC (**Fig. 3**). *BRIP1*$^{-/-}$ and *BRIP1* A349P$^{-/-}$ clones were much more sensitive to the cytotoxic effects (IC_{50} ~0.1 μmol/L) than clones expressing hypomorphic alleles, P47A and D791V (IC_{50} ~0.2 μmol/L) and homozygous or hemizygous WT alleles (IC_{50} ~0.4 μmol/L).

BRIP1 localizes to sites of stalled replication forks and may be necessary for replication fork restart and the timely progression through cell cycle (35, 36). To test whether BRIP1 activity was necessary for release of stalled replication forks in our cell lines, we performed the same clonogenic growth assays for cells treated with hydroxyurea, an antimetabolite that stalls replication forks. Although there was some variability in the sensitivity among the HeLa clones investigated, it was not associated with *BRIP1* genotype (Supplementary Fig. S3).

Several studies have shown that BRIP1 preferentially binds and can unwind G4-DNA *in vitro* and thus BRIP1 may be essential for the resolution of G4 structures *in vivo* (5, 6, 9, 37, 38). We performed clonogenic growth assays using the G4 stabilizing agent, TMPyP4 and did not observe any differences in cell viability between clones (Supplementary Fig. S3). However, we noticed that even WT HeLa cell lines were exquisitely sensitive to this particular drug, which is known to have many off-target effects, and thus may not reflect the effects of G4 stabilization alone.

CRISPR-Cas9 gene editing of HEK293TN cells validated the findings with HeLa clones. As was seen with HeLa cells, no differences in cell viability were observed in HEK293TN treated with hydroxyurea (Supplementary Fig. S4A). Genetically modified HEK293TN cells confirmed *BRIP1* D791V$^{-/-}$ and *BRIP1*$^{-/-}$ clones were sensitive to MMC and cisplatin, and showed G_2–M-phase accumulation similar to that observed in the genetically modified HeLa clones (Supplementary Fig. S4B).

Unstable proteins linked to hypomorphic variants

Hypomorphic alleles can reflect deleterious changes in transcription, RNA processing or stability, and/or changes in the protein that affect its function and/or stability. It has been shown that the substitution of a proline for an alanine at position 47 results in an unstable protein (1). Western blot analysis of BRIP1 in our HeLa and

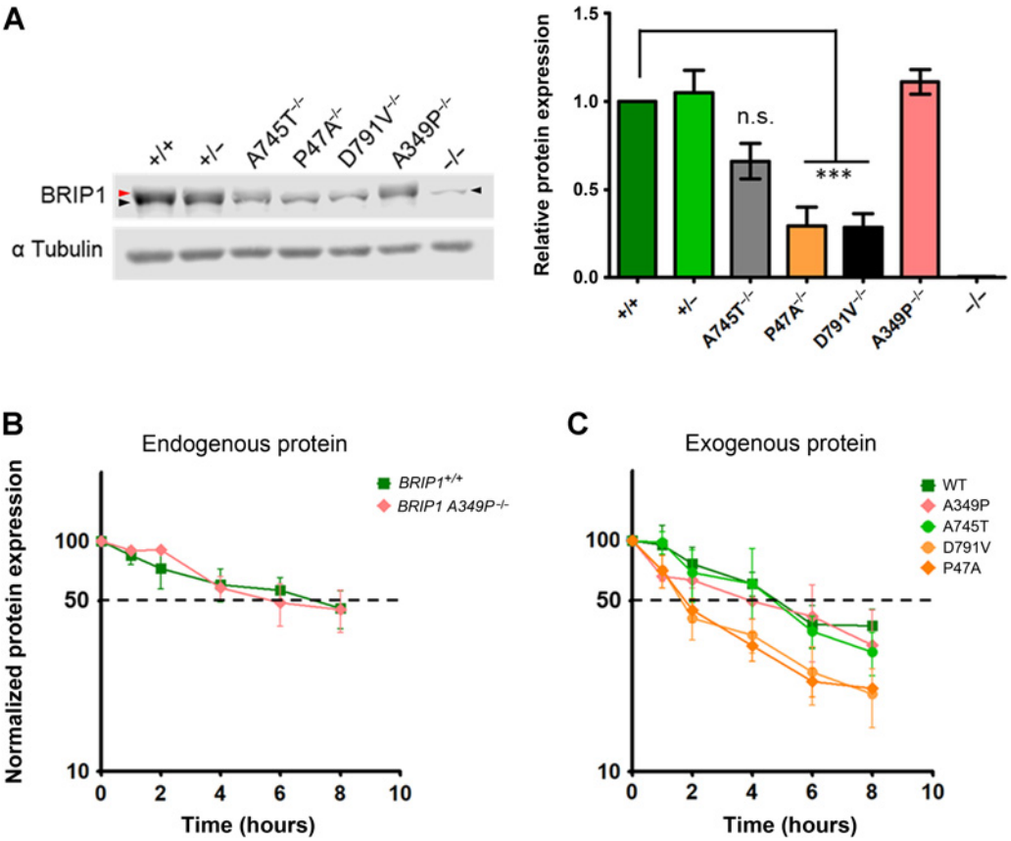

Figure 4.

BRIP1 hypomorphs exhibit protein instability. **A,** Representative Western blot showing variable protein expression in HeLa clones. Red arrowhead, BRIP1 protein; black arrowhead, nonspecific band of lower mass. Quantification of relative BRIP1 levels (right). Data presented are the average of three replicates, with error bars indicating one SD. Statistical significance determined by one-way ANOVA with a Dunnett multiple comparison test to $BRIP1^{+/+}$. ***, $P < 0.0001$. **B,** Cycloheximide chase analysis of endogenous WT and A349P protein. Dotted black line, 50% protein expression. Data presented are the average of three replicates, with error bars indicating one SD. **C,** Cycloheximide chase analysis of exogenously expressed WT, A349P, A745T, D791V, and P47A protein. Dotted black line, half-life. Data presented are the average of three replicates, with error bars indicating one SD. n.s., not significant.

HEK293TN CRISPR clones showed that *BRIP1* P47A$^{-/-}$ and *BRIP1* D791V$^{-/-}$ clones had significantly lower levels of endogenous BRIP1 than WT (+/+), +/−, A745T$^{-/-}$, and A349P$^{-/-}$ clones (**Fig. 4A**). Using a cycloheximide chase assay, we determined that endogenous A349P and WT proteins had similar BRIP1 half-lives at 5–6 hours (**Fig. 4B**). Because BRIP1 levels in the P47A and D791V clones were so low at baseline, it was not possible to accurately determine the half-lives of the endogenous proteins.

Overexpression of the mutant proteins in a HeLa *BRIP1*$^{-/-}$ cell line combined with cycloheximide chase assays showed that exogenous WT (+/+), A349P, and A745T proteins have half-lives of 4–5 hours. The P47A and D791V hypomorphs showed decreased protein stability with a half-life of less than 2 hours (**Fig. 4C**), confirming that P47A is an unstable protein and pointing to protein stability as a factor contributing to ICL sensitivity in D791V cells.

Rescue experiments classify additional missense alleles as WT, hypomorphic, or null

In an effort to functionally characterize additional rare and novel alleles identified in patients with breast and ovarian cancer, we undertook rescue experiments using our *BRIP1*$^{-/-}$ HeLa cell lines. Expression constructs for 20 missense variants were evaluated. These included the previously characterized P47A and A349P as controls plus 18 variants of unknown function, located in the C-terminal helicase region (15 from our discovery cohorts and three very rare variants previously reported in ovarian cases; ref. 14). HeLa clones stably expressing Y822H, E636K, E511G, G690E, and the hypomorph D791V were readily established. Clonogenic growth assays and cell-cycle analysis revealed that none of the alleles fully rescued the ICL effects despite having BRIP1 protein levels comparable with WT clones (Supplementary Fig. S5A and S5B). Y822H, E636K, E511G, and D791V all showed partial rescue in cell viability assays, similar to the endogenous hypomorph P47A and D791V alleles (CRISPR-Cas9 clones; **Fig. 3**). Cell-cycle analysis showed that when treated with MMC, the HeLa cells stably expressing those four missense alleles were all characterized by accumulation of cells in G_2–M-phase.

Of the five variants investigated, G690E was the only example in which there was no evidence for rescue of MMC cytotoxicity in the clonogenic growth assay. The dose-response curve for the G690E rescue was nearly identical to that seen with the null clone and similar to the endogenous A349P allele (CRISPR-Cas9 clone; **Fig. 3**). The substitution of a glutamic acid for a glutamine at position 690 was thus considered to result in a null protein. Given that A349P protein can increase sensitivity of cells to ICL damage in the presence of WT

protein and is thus classified as a dominant negative (9), we created a stable clone expressing G690E in *BRIP1*$^{+/+}$ HeLa cells and tested cell viability after exposure to MMC. The presence of G690E exogenous protein did not increase the sensitivity of WT cells to ICL damage (Supplementary Fig. S5C), indicating that it is unlikely to act as a dominant negative. Analysis of protein stability showed that the G690E protein has a reduced half-life (~2 hours), comparable with D791V. The G690E variant, although null, was thus different than the A349P dominant negative (a stable protein). Reduced protein stability might explain in part why G690E it is a loss-of-function mutation (Supplementary Fig. S5D) but other biochemical defects cannot be ruled out.

Despite multiple attempts (five independent transfections), we were unable to establish stable clones expressing WT or the WT-like A745T protein in HeLa *BRIP1*$^{-/-}$ cells. The inability to stably express other members of the Fanconi anemia pathway has been reported previously (39). We determined that stable transfection of *BRIP1*$^{-/-}$ HeLa cells to express exogenous *BRIP1* variants was possible only with hypomorphic or null alleles.

We devised a transient rescue assay that allowed us to characterize all missense variants (WT, hypomorph, and null alleles). *BRIP1*$^{-/-}$ HeLa cells were transfected with pCDH-Puro-*BRIP1* constructs, grown 48 hours, and then put under puromycin selection and challenged with MMC. Cells were allowed to grow 10–15 days and subjected to a second MMC challenge. Fifteen to 20 days later total numbers of clones were determined (Supplementary Fig. S6A and S6B). Survival after two rounds of MMC treatment was dependent on exogenous protein expression (Supplementary Fig. S6C). The MMC doses used for these transient rescue assays were determined on the basis of effects observed with known positive and negative controls. The high dose (30 ng/mL) kills or severely impedes growth of all *BRIP1*$^{-/-}$ cells and cells expressing null or hypomorph alleles of *BRIP1*. The lower dose (15 ng/mL) kills or severely impedes *BRIP1*$^{-/-}$ cells and cells expressing null alleles, but has a minor or no effect on cells expressing hypomorphic alleles (detailed methods describing the transient rescue assay can be found in Supplementary Materials and Methods with representative images shown in Supplementary Fig. S6B).

Growth of clones that survived two rounds of MMC selection was used to classify variants. If greater than 50% of the transfected, twice challenged cells grew out at both low and high doses of MMC (compared with the unchallenged puromycin-only selected population), then the reexpressed allele was deemed WT in function. If less than 50% of challenged cells grew out at either low or high dose of MMC, reexpressed alleles were classified as hypomorphs, having partial rescue ability but not to WT levels. If none of the challenged cells grew out at either dose of MMC, alleles were deemed null in function. All transient rescue experiments were replicated (2–4 replicates). Alleles with vagaries in protein expression and/or phenotype across replicates were excluded. In addition to testing alleles for ICL damage sensitivity, we assessed protein stability of each allele by transient exogenous overexpression of mutant proteins in HeLa null cell lines (Supplementary Fig. S7).

Twenty missense alleles, located in the helicase domain, were successfully screened (**Table 1**). Of these, five alleles (25%) were classified as WT, including A745T and Q740H alleles that were identified in both of our cohorts. Nine variants (45%) fell into the category of hypomorphs, including both the P47A and D791V alleles characterized in our CRISPR model, as well as the three additional hypomorph alleles characterized by stable rescue. The remaining six alleles (30%) were classified as null, including the A349P and G690E allele. Most of the alleles classified as hypomorph and null-created proteins with significantly shorter half-lives than WT protein, with null proteins trending to be more unstable than hypomorphs (**Table 1**;

Table 1. Key features of functionally characterized missense alleles.

Functional class	Expression construct	Conserved motif	Clone growth			Protein half-life (95% CI)	CONDEL
			Puro only	MMC ng/mL 15	MMC ng/mL 30		
WT	WT		+++	++	++	5.7 (3.9–8.5)	NA
	S407G		+++	++	++	8.2 (4.2–10.8)	Damaging
	H587L		+++	++	++	4.0 (3.0–6.3)	Neutral
	Q740H		+++	++	++	8.1 (5.5–11.2)	Damaging
	A745T		+++	++	++	4.9 (3.2–6.11)	Damaging
	L844I		+++	++	++	ND	Neutral
Hypomorph	P47A[a]	H-I	+++	++	+	2.9 (2.3–4.0)	Damaging
	E511G		+++	++	+	4.4 (93.5–6.0)	Damaging
	E636K		+++	+	−−	3.4 (2.6–4.6)	Damaging
	I691L	H-IV	+++	++	+	2.7 (2.0–4.1)	Damaging
	G649S		+++	++	+	3.6 (2.3–4.8)	Damaging
	I782V		+++	++	+	ND	Neutral
	D791V		+++	+	−−	3.0 (2.3–4.2)	Damaging
	K797R		+++	+	−−	2.5 (1.7–3.7)	Damaging
	Y822H	H-VI	+++	++	+	3.8 (3.3–4.5)	Damaging
Null	A349P[a]	Fe-S	+++	−−	−−	5.6 (3.8–8.6)	Damaging
	V676E		+++	−−	−−	2.6 (2.1–3.3)	Damaging
	G690E	H-IV	+++	−−	−−	2.3 (1.5–3.3)	Damaging
	R777C		+++	−−	−−	2.2 (1.5–3.7)	Damaging
	C832Y	H-VI	+++	−−	−−	2.9 (2.3–3.9)	Damaging
	R865W		+++	−−	−−	1.8 (1.4–2.4)	Damaging
	EV		+++	−−	−−	NA	NA

Abbreviation: NA, not applicable; ND, not determined.
[a]Previously characterized as deleterious missense variant.

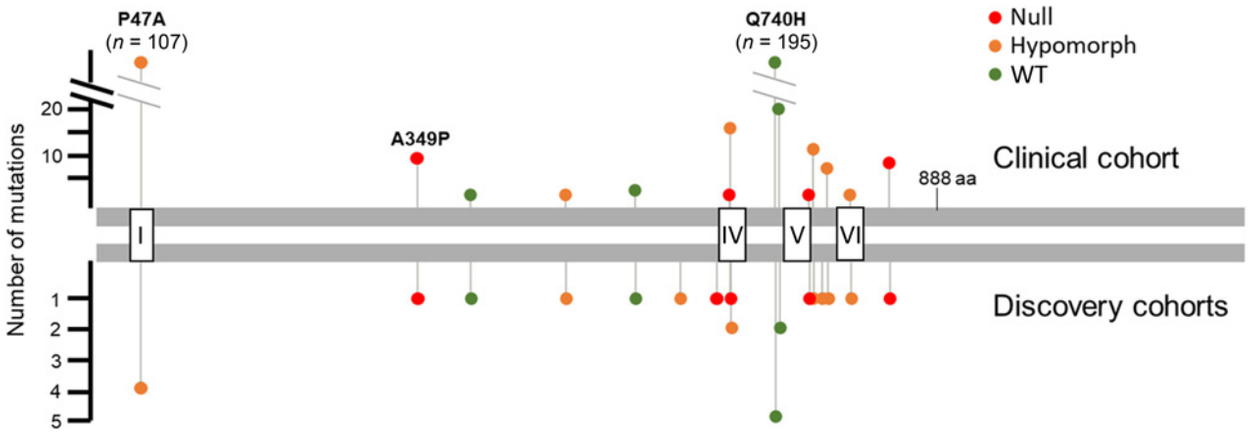

Figure 5.
Occurrence of functionally characterized variants in clinical testing. Q740H and A745T account for a majority of the WT alleles (green) identified. P47A was the most common hypomorph allele (orange) detected. Null alleles (red) were seen at lower frequencies. Helicase domain motifs: IV (689–710); V (748–775); and VI (819–836). I, helicase domain I.

Supplementary Fig. S7). The exception to this observation is the stable, dominant-negative, null A349P protein. We noted that some of the WT-like alleles created proteins with much greater half-lives than WT protein and may in fact result in an increased stability of BRIP1 protein.

BRIP1 null and hypomorphs are seen in women with breast and ovarian cancer who have undergone clinical cancer genetic testing

BRIP1 is included in many cancer susceptibility NGS multigene panel tests (MGPT) and is frequently evaluated in patients with breast and ovarian cancer. Clinical testing data for 101,759 patients with breast cancer and 15,587 patients with ovarian cancer, who had NGS MGPT in the period 2012–2018, identified 14 of the 20 missense alleles that we functionally classified as WT, hypomorphic, or null. Of the six alleles not detected (E636K, G649S, V676E, D791V, C832Y, and L844I), two were reported in ClinVar as having been identified in patients with familial cancer of the breast.

Of the 14 alleles identified in the clinical cohort and evaluated in this functional study, 323 patients with breast (0.32%) and 56 patients with ovarian (0.36%) cancer carried one of the alleles tested. The WT Q740H and A745T were the most frequently observed rare variants (**Fig. 5**; **Table 2**). However, 161 patients (0.14%) carried an allele deemed null or hypomorphic in our functional assays. Strikingly, the

Table 2. Occurrence of functionally characterized missense alleles in patient and control populations.

			Clinical cohort			
		Discovery cohorts (n = 3,359)	Breast cancer (n = 101,759)	Ovarian cancer (n = 15,587)	EXAC (n = 60,327)	FLOSSIES (n = 9,884)
WTs	S407G	1	1	—	—	—
	H587L	1	2	—	—	—
	Q740H	5	163	32	56	8
	A745T	2	18	2	8	1
	L844I[a]	—	—	—	—	—
Hypomorphs	P47A[b]	4	96	11	29	5
	E511G	1	1	—	—	—
	E636K	1	—	—	—	—
	I691L	2	13	2	—	—
	G649S[a]	—	—	—	—	—
	I782V	1	10	1	2	1
	D791V	1	—	—	—	—
	K797R	1	3	4	—	1
	Y822H	1	1	—	1	—
Nulls	A349P[b]	1	7	2	3	—
	V676E	1	—	—	—	—
	G690E	1	1	—	—	—
	R777C	1	1	—	—	—
	C832Y[a]	—	—	—	—	—
	R865W	1	6	2	1	2

[a]Rare missense variants previously reported in ovarian cancer cases (14).
[b]Previously characterized as deleterious missense variant.

I691L hypomorph allele was identified in 15 additional patients with breast and ovarian cancer but was not observed in FLOSSIES or ExAC controls. Clinical testing confirms P47A as a recurrent *BRIP1* risk allele and the frequency of our newly characterized hypomorph and null alleles is comparable with the frequency observed for A349P.

Combining the discovery and clinical cohorts, 178 patients with ovarian and breast cancer carried a hypomorph or null missense variant (0.15%), which is significantly greater than the expected frequency in the general population (ExAC 0.06%; OR = 2.47; 95% confidence interval = 1.73–3.54; $P < 0.0001$).

Discussion

Our study shows that greater than 2% of patients with ovarian and early-onset breast cancer carry a rare missense variant in *BRIP1* DNA helicase. At present, the effect of these missense variants on protein function and the cancer risk they confer is largely unknown. Our functional characterization of 20 rare missense variants in the BRIP1 helicase domain (18 of which had not previously been studied) revealed that most (15/20, 75%) significantly impaired ICL repair and are thus loss-of-function defects. A majority was hypomorphs (9/15) and six were considered to be nulls.

Patients with breast and ovarian cancer will frequently undergo genetic testing for mutations in cancer susceptibility genes. Testing has been widely adopted because of the recognized benefit to patients and their family members. Positive or negative findings help guide cancer surveillance for the patient and her family members. Identification of a germline mutation in *BRCA1* or *BRCA2* is central to both primary prevention (surgical prophylaxis) and for those women who have breast or ovarian cancers, to decisions regarding best approaches for adjuvant and/or second-line treatment. Risk reducing oophorectomy and mastectomy in patients with *BRCA1/2* mutations is associated with lower rates of ovarian and breast cancer with a decrease in all-cause mortality (40). Currently, germline testing is most often for a large number of cancer susceptibly genes. The role that mutations play in some of the genes tested is not fully understood and frequently they are low or moderate penetrance mutations. A complexity of panel testing is that rare and/or previously uncharacterized VUSs will be identified more frequently than pathogenic mutations. Rare variants, particularly those identified in moderate to low penetrance susceptibility genes, pose a challenge to health care providers. The risk associated with these variants is usually unknown and family-based studies (penetrance and expressivity analyses as well as tumor studies) to classify variants are not feasible. Ambiguity surrounding the potential function of rare variants can create unwanted anxiety in patients and family members and may lead to unnecessary medical interventions (41–43). Functional studies are needed to better interpret cancer risk and guide disease management strategies.

Reduced protein stability is a frequent feature of hypomorphic alleles. We found that most of the hypomorph and null alleles we evaluated have reduced half-lives and low steady state levels of BRIP1 protein. Although lower protein levels could explain the reduction in ICL repair activity we observed, it is likely that some of the missense changes directly affect the helicase activity of the mutant protein. We showed that *BRIP1*$^{+/-}$ HeLa and *BRIP1*$^{+/-}$ HEK293TN cells do not show an increase in sensitivity to MMC or cisplatin (**Figs. 2B** and **3**; Supplementary Fig. S4A and S4B), and from that we conclude haploinsufficiency does not confer increase sensitivity to ICL damage. One of the controls we investigated, the unstable P47A protein, is ATPase deficient and lacks helicase activity (3). Biochemical characterization of helicase activity of the unstable alleles could further determine the functional effects of missense alleles that are associated with reduced ICL repair. A second control allele we investigated (A349P) is a null based on assays that measure effects of MMC on chromosome fragmentation, cell viability, and cell cycle, but has normal protein stability (**Figs. 2**, **3**, and **4B** and **C**). Other BRIP1 functions that could be affected by single amino acid substitutions include DNA binding, nuclear localization, processivity of unwinding, displacement of proteins, binding to other proteins, dimerization, and recruitment to sites of damage (4, 9, 44–47). Ideally, all of these functions could be considered as part of missense variant analysis. The currently available reagents present challenges to fully characterize the function of BRIP1 protein variants. The lack of specific antibodies for G4-DNA made it impossible for us to assess the effects of variants on G4-DNA resolution. Additional reagents will be critical for *in vivo* functional assays.

Creating BRIP1-null cell lines in HeLa and HEK293 was relatively simple as both cell lines seem to thrive without BRIP1 protein. In contrast, rescuing the null phenotype with reexpression of WT proteins proved to be unexpectedly difficult. Stable clones rescued with WT BRIP1 protein could only be created after challenging the cells with ICL-damaging agents to force survival dependency on the exogenously expressed protein. A doxycycline inducible, controlled expression system could provide a way to overcome this obstacle and should be explored in future efforts to characterize additional variants.

In our initial discovery efforts, we noticed that three very rare alleles (R579C, K979E, and Q227E) were seen twice in our breast cancer cohort but were not observed in the ovarian cancer cohort. In addition, a cluster of five different missense alleles, located in the highly conserved Fe-S domain, were identified in our ovarian cancer cohort but none were observed in this domain in the breast cancer cohort (**Fig. 1**). These idiosyncrasies may allude to functional regions of the protein that are cancer type–specific and could explain the difference in occurrence of *BRIP1* mutations between ovarian and breast cancer.

A745T and Q740H account for a majority of the WT alleles identified in patients with ovarian and breast cancer, who underwent clinical genetic testing and carry one of the rare missense alleles functionally tested in our study. Of the remaining individuals who carry one of the other 18 alleles tested, most harbor a putative hypomorph or null variant. Remarkably, 15 patients carry the hypomorph I691L allele, which was not observed in either ExAC or FLOSSIES control populations. This suggests that some rare alleles in *BRIP1* are more penetrant than others.

Although *BRIP1* mutations have been described as the third or fourth most frequent causes of inherited ovarian cancer (14–16, 48), the analyses have been largely restricted to nonsense or frameshift alleles. Family-based studies to analyze penetrance of very rare alleles are impractical, making it difficult to assess cancer risk associated with rare missense alleles identified in *BRIP1*. The clinical utility for understanding the functional impact of missense variants in susceptibility genes has been made clear by the extensive research on *BRIP1*-binding partner, *BRCA1*. Classification of VUSs identified in patients with ovarian and breast cancer can contribute to early detection and screening of disease and improve treatment options by personalizing care with targeted therapies. This study highlights the importance of uncovering the functions of BRIP1 in tumor development and the role of *BRIP1* missense variants in protein function.

Disclosure of Potential Conflicts of Interest

M.E. Richardson is a variant assessment scientist (paid consultant) for Ambry Genetics. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: C.L. Moyer, P.J. Goodfellow
Development of methodology: C.L. Moyer, J.L. Gillespie
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.L. Moyer, J.L. Gillespie, R. Doberstein, E.M. Swisher, P.J. Goodfellow
Writing, review, and/or revision of the manuscript: C.L. Moyer, J. Ivanovich, M.E. Richardson, S.H. Kaufmann, E.M. Swisher, P.J. Goodfellow
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.L. Moyer, J.L. Gillespie
Study supervision: C.L. Moyer, P.J. Goodfellow

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