Helicase-inactivating BRIP1 mutation yields Fanconi anemia with microcephaly and other congenital abnormalities

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Abstract

Fanconi anemia is a genetically and phenotypically heterogeneous disorder characterized by congenital anomalies, bone marrow failure, cancer, and sensitivity of chromosomes to DNA cross-linking agents. One of the 22 genes responsible for Fanconi anemia is BRIP1, in which biallelic truncating mutations lead to Fanconi anemia group J and monoallelic truncating mutations predispose to certain cancers. However, of the more than 1000 reported missense mutations in BRIP1, very few have been functionally characterized. We evaluated the functional consequence of BRIP1 p.R848H (c.2543G > A), which was homozygous in two cousins with low birth weight, microcephaly, upper limb abnormalities, and imperforate anus and for whom chromosome breakage analysis of patient cells revealed increased mitomycin C sensitivity. BRIP1 p.R848H alters a highly conserved residue in the catalytic DNA helicase domain. We show that BRIP1 p.R848H leads to a defect in helicase activity. Heterozygosity at this missense has been reported in multiple cancer patients but, in the absence of functional studies, classified as of unknown significance. Our results support that this mutation is pathogenic for Fanconi anemia in homozygotes and for increased cancer susceptibility in heterozygous carriers.

[Supplemental material is available for this article.]

INTRODUCTION

Fanconi anemia is a genetically heterogeneous multisystemic disorder characterized by congenital anomalies, progressive bone marrow failure, cancer susceptibility, and cellular hypersensitivity to DNA cross-linking agents. At least 22 genes harbor mutations causing Fanconi anemia phenotypes (Mamrak et al. 2017; Niraj et al. 2019). Genes responsible for Fanconi anemia encode proteins in the BRCA/Fanconi DNA damage response pathway. Different protein complexes in the pathway collaborate in DNA damage repair, especially the removal
and resolution of DNA interstrand cross-links to protect DNA replication fork stability (Kottemann and Smogorzewska 2013; Niraj et al. 2019). Proteins in the upstream core complex participate in DNA damage detection and signaling, culminating in the monoubiquitylation of the FANCD2/FANCI complex and its recruitment to interstrand cross-links (Niraj et al. 2019). Interstrand cross-links are then excised and bypassed in a process that generates double-stranded breaks. Resolution of the double-stranded breaks requires homologous recombination orchestrated by the downstream Fanconi anemia proteins, which include BRCA2 (FANCD1), BRIP1 (FANCJ), PALB2 (FANCN), RAD51C (FANCO), and BRCA1 (FANCS). Biallelic mutations in genes encoding proteins involved in homologous recombination cause Fanconi anemia and monoallelic mutations in these genes predispose to breast, ovarian, and other cancers (Niraj et al. 2019).

Because of the diversity of symptoms that can overlap with other genetic disorders, Fanconi anemia can be challenging to diagnose. Although bone marrow failure and cancer are hallmarks of Fanconi anemia, they may not develop until adulthood and some patients never develop these complications (Alter 2014). If Fanconi anemia is clinically diagnosed, the large number of causative genes and limited genotype–phenotype correlation make it difficult to predict which gene is responsible. We present here the identification of a BRIP1 variant and diagnosis of Fanconi anemia in a consanguineous family with two affected cousins presenting with multiple congenital abnormalities.

RESULTS

Clinical Presentation
We evaluated a 5-yr-old female for a history of microcephaly and congenital malformations (HO3; Fig. 1A). She was the child of consanguineous parents of Palestinian ancestry. She presented at birth with low weight (1.5 kg), microcephaly, bilateral upper limb malformation, including four fingers and clenched hands, and imperforate anus. At age 5.5 yr she is of normal height, although with poor weight gain, and can speak, walk, and run and attends preschool. Macrocytosis present soon after birth resolved by 5 mo; other complete blood count (CBC) values were near or within normal ranges (Supplemental Table 1). A brain computed tomography (CT) scan at age 5 mo and chromosomal microarray at age 2.5 mo were normal. Her brother, parents, and grandparents have none of the above manifestations.

A male cousin (HO7) of the proband presented with low birth weight, microcephaly, polydactyly, imperforate anus, and malformation of the gastrointestinal (GI) tract. At age 22 yr he has finished school and works with his father. He is reportedly healthy with normal blood counts. He had three deceased siblings with similar phenotypes. A sister, born with a small head, imperforate anus, and intestinal torsion, died at age 18 yr; the cause of death was reported as red blood cell hemolysis and septicemia. A brother, born with kidney problems and imperforate anus, died at 1 yr of age. A second brother, born with imperforate anus, died at 10 d of age. His parents and grandparents have none of the above manifestations. The two cousins are related to each other through both parents. A great grandfather died of a hematological cancer; there is no other known history of cancer in the family.

Gene Discovery
Genomic DNA samples from the two affected cousins were evaluated by whole-exome sequencing (Table 1). Given the consanguinity of the family, data was filtered for shared homozygous rare variants predicted by in silico tools to be damaging. Three variants met these criteria: BRIP1 c.2543G > A, p.R848H (NM_032043.2) at Chr 17:59770823C > T, with one gnomAD entry; ENO4 c.1589A > T, p.D530V (NM_001242699.1) at Chr 10:118638853A > T,
with 34 gnomAD entries; and MED13 c.563T > C, p.L188P (NM_005121.2) at Chr 17:60112877A > G, with 93 gnomAD entries. The variants in ENO4, encoding an enolase, and MED13, encoding a component of the mediator complex, were considered unlikely to explain the phenotype and were not investigated further. Damaging variants in BRIP1, encoding a DNA helicase, are known to cause Fanconi anemia, which can include the features present in the affected cousins (Levitus et al. 2005; Levran et al. 2005; Litman et al. 2005;
Sanger sequencing of peripheral blood-derived genomic DNA confirmed BRIP1 c.2543G > A, p.R848H to be homozygous in the affected cousins with no indication of somatic mosaicism (Table 2; Fig. 1A,B; Supplemental Fig. S1). BRIP1 c.2543G > A was confirmed to be heterozygous in each parent of the affected cousins and heterozygous or not present in all other unaffected family members evaluated. Female relatives (HO2 and HO6) known to be heterozygous for BRIP1 c.2543G > A are currently ages 29 and 48. BRIP1 c.2543G > A was not present among exomes from approximately 1200 individuals (approximately 2400 alleles) in our in-house Palestinian databases. Nor does BRIP1 c.2543G > A appear among approximately 10,000 cancer-free women (~20,000 alleles) older than age 70 yr (https://whi.color.com).

BRIP1 (FANCJ) encodes a member of the DEAH family of ATP-dependent DNA helicases involved in interstrand cross-link repair. BRIP1 contains an amino-terminal helicase catalytic domain and a carboxy-terminal domain that includes a BRCA1 binding domain (Fig. 1C; Cantor et al. 2001, 2004). R848H affects a residue located at the carboxy-terminal end of the catalytic domain and is predicted to be damaging by PolyPhen-2 (score = 1.00) and SIFT (P = 0.00). Arginine at residue 848 is conserved in all sequenced species and in other members of the DEAH helicase family (Fig. 1D).

Chromosome Breakage Analysis
The identification of a homozygous BRIP1 mutation suggested the possibility of Fanconi anemia. Consistent with a diagnosis of Fanconi anemia, peripheral blood lymphocytes from the proband HO3 showed increased chromosomal breakage after exposure to increasing concentrations of mitomycin C (MMC), compared to peripheral blood lymphocytes from her unaffected brother HO4 (Fig. 2). Proportions of cells with at least one radial chromosome after treatment with 150 nM MMC were 24 of 30 cells from the proband and 0 of 30 cells from her unaffected brother, Fisher (two-tailed) P = 3.29 × 10^{-11}. Proportions of cells with at least one acentric chromosome after treatment with 150 nM MMC were seven of 30 cells from the proband and 0 of 30 cells from the unaffected brother, Fisher (two-tailed) P = 0.011.

Helicase Activity
BRIP1 is known to unwind double-stranded DNA in the 5'→3' direction (Cantor et al. 2004). To determine whether the mutation identified in our patients alters this activity, we evaluated BRIP1 activity in vitro, using recombinant enzyme and a forked duplex substrate in which a

| Table 2. BRIP1 variant identified by whole-exome sequencing |
|------------------------------------------------------------|
| **Gene** | Chromosome | HGVS DNA reference | HGVS protein reference | Variant type | Predicted effect | dbsNP/ dbVar ID | Genotype | ClinVar ID | gnomAD allele frequency |
|----------|-------------|---------------------|------------------------|--------------|-----------------|----------------|-----------|------------|------------------------|
| BRIP1    | Chr17: 59770823C > T (hg19) | NM_032043.2: c.2543G > A | p.Arg848His | Substitution | Missense | rs374334794 | Homozygous | SCV001429629 | 0.0000319 |

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DNA duplex is flanked by single-stranded noncomplementary arms (Cantor et al. 2004; Gupta et al. 2005). Recombinant FLAG-tagged nonmutant and mutant BRIP1 was transiently expressed in HEK293T cells and isolated by immunoprecipitation. Increasing amounts of nonmutant BRIP1 exhibited increasing unwinding of a 26-bp forked duplex substrate, as indicated by the appearance of a band corresponding to the labeled single-stranded portion of the substrate (Fig. 3A). As a negative control, BRIP1 p.P47A, a variant known to lack catalytic activity (Cantor et al. 2004), exhibited no unwinding. BRIP1 p.R848H also showed no unwinding of the substrate, indicating that the helicase activity of this variant is compromised (Fig. 3A,C). The presence of all proteins was confirmed by western blot (Fig. 3B).

DISCUSSION

The limb, GI, kidney, and growth abnormalities seen in this family have all been described in patients with Fanconi anemia group J (Shimamura and Alter 2010; Fiesco-Roa et al. 2019; Niraj et al. 2019). This constellation of congenital abnormalities in combination with cellular hypersensitivity to DNA cross-linking support a diagnosis of Fanconi anemia. The known role for BRIP1 mutations in Fanconi anemia, segregation with the phenotype in the family, and compromised helicase activity support our conclusion that BRIP1 p.R848H is the causal allele for the phenotype of this family. BRIP1 p.R848H has been reported in another patient with clinically diagnosed Fanconi anemia but functional significance of the mutation was not established (Steinberg-Shemer et al. 2019). BRIP1 p.R848H has been reported from six different sources on ClinVar and in breast and prostate cancer studies (Kim et al. 2016; Momozawa et al. 2020), all designating the variant as "uncertain significance." Here we confirm the association of BRIP1 p.R848H with Fanconi anemia and demonstrate that the mutation impairs BRIP1 protein function.

BRIP1 variants causing Fanconi anemia include both protein-truncating and missense variants. Like R848H, all other BRIP1 missense mutations causing Fanconi anemia target residues in the helicase domain. In addition, all other missense variants that have been functionally evaluated show deficits in helicase activity (Wu et al. 2010; Guo et al. 2014; Bharti et al.
Patient-derived FA-J cells, which lack BRIP1, are sensitive to interstrand cross-linking agents (Litman et al. 2005). Cellular resistance to interstrand cross-linking can be restored by the reintroduction of BRIP1 (Peng et al. 2007). Restoration of resistance requires BRIP1 helicase activity but not BRIP1–BRCA1 interaction, demonstrating the importance of the helicase domain and suggesting an explanation for why missense mutations particularly targeting the helicase domain may be causal for Fanconi anemia.

Neither of the surviving affected individuals in our family have thus far been diagnosed with bone marrow failure. The similar congenital abnormalities in the three deceased siblings of HO7 suggest that they were also homozygous for BRIP1 p.R848H, but their hematological phenotypes are not known. Hematological abnormalities are a hallmark feature of Fanconi anemia but are not present in all patients. The cumulative incidence of bone marrow failure in Fanconi anemia generally is 50% by age 40, with annual risk peaking by age 7 yr (Alter 2014). Homozygosity or compound heterozygosity for BRIP1 truncating alleles was associated with bone marrow failure in early childhood in 12 of 14 cases, generally by the age of 6 yr.
All BRIP1 truncating alleles yield loss of the helicase domain and are likely to lead to loss of protein (Levitus et al. 2004; Levitus et al. 2005; Levran et al. 2005; Litman et al. 2005; Steinberg-Shemer et al. 2019). Missense alleles, which may retain at least partial protein expression and function (Levitus et al. 2005; Bharti et al. 2018; Moyer et al. 2020), may cause a less severe phenotype. A recent analysis of genotype–phenotype associations in Fanconi anemia found an increased frequency of congenital abnormalities in patients with null genotypes compared to those with hypomorphic genotypes, suggesting that phenotypic severity may be influenced by the type of mutation (Fiesco-Roa et al. 2019), although other studies have found conflicting results (Faire et al. 2000; Castella et al. 2011; Steinberg-Shemer et al. 2019). Most reports of homozygous or compound heterozygous BRIP1 missense mutations have not included phenotypic details (Levitus et al. 2005; Chandrasekharappa et al. 2013; Steinberg-Shemer et al. 2019). Additional phenotypic information from individuals with BRIP1 missense genotypes will be needed to determine whether the class of BRIP1 mutation influences phenotype severity. Protection from bone marrow failure may result from hematopoietic somatic mosaicism caused by the correction of one mutant allele in a somatic reversion event (Shimamura and Alter 2010). Somatic mosaicism is ruled out in our patients by detection of only the BRIP1 c.2543G > A allele in peripheral blood.

Cancer risks for individuals with Fanconi anemia caused by biallelic BRIP1 mutations have not been documented. However, patients with biallelic mutations in certain other Fanconi anemia genes have cumulative risks of 20% for leukemia and 30% for solid tumors by age 40 (Alter 2014). Biallelic mutations in BRCA2/FANCD1 or BRCA1/FANCS are generally embryonic lethal; surviving patients have very high cancer risks and BRCA2/FANCD1 patients have extremely elevated rates of spontaneous and induced chromosome breakage (Gowen et al. 1996; Hakem et al. 1996; Ludwig et al. 1997; Hirsch et al. 2004; Alter et al. 2007; Myers et al. 2012; Domench et al. 2013; Shaheen et al. 2014; Sawyer et al. 2015; Loizidou et al. 2016; Seo et al. 2018, Weinberg-Shukron et al. 2018). It is likely that the patients in our study who are homozygous for BRIP1 p.R848H will also be at increased risk for these malignancies and will benefit from ongoing surveillance.

Heterozygous BRIP1 loss-of-function mutations predispose to ovarian cancer (Rafnar et al. 2011; Ramus et al. 2015; Norquist et al. 2016; Weber-Lassalle et al. 2018), and current National Comprehensive Cancer Network guidelines indicate that risk-reducing salpingo-oophorectomy should be considered between the ages of 45–50 for women with these mutations (https://www.nccn.org/professionals/physician_gls/pdf/genetics_screening.pdf). These loss-of-function mutations include rare BRIP1 missense mutations that have been shown experimentally to have no or greatly reduced protective activity against DNA-damaging agents (Moyer et al. 2020). BRIP1 p.R848H, with defective helicase activity, may also increase the risk of ovarian cancer. Early studies also implicated BRIP1 as a breast cancer susceptibility gene (Seal et al. 2006; De Nicolo et al. 2008), although there has been conflicting evidence (Easton et al. 2016; Couch et al. 2017). A recent study suggests that BRIP1 may be associated specifically with triple-negative breast cancer (Shimelis et al. 2018). The family of this study is not informative for cancer risk among female carriers, because the female relatives known to be heterozygous for the missense are presently ages 29 and 48.

Of more than 1000 different BRIP1 missense mutations reported in ClinVar, very few have been functionally evaluated. The clinical significance of the vast majority is listed as uncertain with respect to both cancer predisposition and Fanconi anemia group J. Six missense mutations previously identified in patients with Fanconi anemia (R251C, Q255H, A349P, H396D, W647C, R707C) have been shown to be deficient for helicase activity (Levitus et al. 2005; Levran et al. 2005; Wu et al. 2010; Chandrasekharappa et al. 2013; Guo et al. 2014; Bharti et al. 2018). BRIP1 p.P47A, originally identified in a breast cancer patient and used as a negative control in our study, is also helicase-deficient (Cantor et al. 2004). A recent study
evaluated 18 additional BRIP1 missense mutations identified in ovarian and early-onset breast cancer patients for protein stability and effects on cell growth, cell cycle progression, and chromosome breakage in the presence of DNA damaging agents (Moyer et al. 2020). Thirteen of these mutations were null or depleted in one or more of these functions, whereas five had fully normal function. Our finding that BRIP1 p.R848H lacks helicase activity adds to the small but growing number of BRIP1 missense mutations with demonstrated functional impact.

The similar congenital abnormalities in the cousins in our study suggested a genetic disorder. Although their features are consistent with Fanconi anemia, the physical features of Fanconi anemia overlap with those of other multisystem genetic disorders. Fanconi anemia commonly presents with blood pancytopenia (Shimamura and Alter 2010), but CBC results for the proband were not significant enough to prompt further evaluation of bone marrow or chromosome breakage analysis. The phenotypic and genetic heterogeneity of Fanconi anemia can make it challenging to reach a clinical diagnosis and to predict the genetic cause. In this case, exome sequencing provided a genetic diagnosis that led to a clinical diagnosis of Fanconi anemia and the opportunity for surveillance appropriate to the ongoing risks for cancer and bone marrow abnormalities.

METHODS

Exome Sequencing
Genomic DNA was extracted from peripheral blood samples, quantified using Qubit v.3 and quality checked by gel electrophoresis. Library preparation was carried out using TruSeq Capture Exome Kit (Illumina). This kit provides coverage of 45 Mb of exonic content. After sequencing on NextSeq 500, data was uploaded onto our server and reads were aligned to the reference human genome (hg19) using BWA aligner. Prior to variant calling by GATK (Genome Analysis Toolkit), mapped reads (BAM format) went through preprocessing steps by removing PCR duplicates, realigning around indels, and recalibrating base quality. The final list of variants was annotated by ANNOVAR (http://annovar.openbioinformatics.org/) (Wang et al. 2010) using several databases of minor allele frequency such as gnomAD (https://gnomad.broadinstitute.org) and PopFreqMax as well as variant effect predictors such as SIFT (Sorting Intolerant From Tolerant; http://sift.bii.a-star.edu.sg), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and REVEL (Ioannidis et al. 2016). Variants with low coverage, which were synonymous or predicted benign (SIFT, PolyPhen-2, REVEL), or with frequency >0.001 on gnomAD, PopFreqMax, or in our Palestinian in-house database were excluded. Candidate variants were validated by Sanger sequencing and tested for cosegregation with the phenotype in additional family members. Primers used for validation of BRIP1 c.2543G > A were 5′-CCAGCTGAGATCTTACCAGA-3′ and 5′-CAACATTTTGATGCAAGTT-3′.

Chromosome Breakage Analysis
Chromosome breakage analysis was performed essentially as previously described (Tenenbaum-Rakover et al. 2015). Peripheral blood lymphocytes were cultured for 72 h with 0, 150, or 300 nM mitomycin C. At least 30 metaphase spreads were evaluated by microscopy for chromosomal aberrations.

BRIP1 Isolation by Immunoprecipitation
Constructs in which nonmutant or mutant BRIP1 with a carboxy-terminal FLAG tag was expressed from the pCMV-3Tag-3 vector (Stratagene) were generated using the Gibson assembly method (Gibson et al. 2009). HEK293T cells in two 15-cm dishes were transfected
with 60 µg of DNA and 150 µL of Lipofectamine 2000 (ThermoFisher) per dish, according to manufacturer’s instructions, and protein was isolated essentially as described (Wu et al. 2010). Cells were lysed 48 h after transfection in buffer containing 10 mM Tris-HCl, pH 7.4, 130 mM NaCl, 1% Triton X-100, 10 mM sodium fluoride, 1 mM sodium orthovanadate and a protease inhibitor cocktail (ThermoFisher). BRIP1 was immunoprecipitated from clarified cell lysate with anti-DYKDDDDK magnetic agarose beads (ThermoFisher), using 7.6 µL bead slurry per mg protein. Immune precipitates were washed twice in 50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.5% Nonidet P-40 and twice in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40. Beads were stored as a 50% slurry in storage buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 0.1% Tween-20, 0.5 mM TCEP (tris(2-carboxyethyl) phosphine)) in aliquots at −80°C.

Helicase Assays
DNA helicase assays were performed on BRIP1-containing immune precipitates (Moser et al. 2000; Kamath-Loeb et al. 2004), and activity was detected by the displacement of an oligonucleotide labeled with IRDye700 from a partial duplex substrate. Based on previously published substrates (Gupta et al. 2005; Rudolf et al. 2006), the forked substrate consisted of the labeled oligonucleotide X50 (5′-IRD700-GCTCGAGTCTAGACTGCAGTTGAGAGCTTGC TAGGACGGATCCCTCGAGG) and an unlabeled oligonucleotide (5′-CCTCGAGGGATCGTCCTAGCAAGAGGATTTTTTTTTTTTTTTTTT) (IDT), annealed to form a 24-bp duplex flanked by 26-nt single-stranded DNA tails. Immune precipitates containing BRIP1 were diluted in storage buffer, and helicase activity was routinely assayed at dilutions of 1:128 to 1:32; activity was linear in this range. Helicase reactions (20 µL) contained 40 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM MgCl2, 2% glycerol, 100 ng/mL bovine serum albumin, 2 mM dithiothreitol, 2 mM adenosine triphosphate, 0.5 nM partial duplex DNA substrate, and BRIP1 immune precipitate (Cantor et al. 2004). Reactions were incubated for 30 min at 30°C, with mixing, and stopped with a mixture of 2.2 µL 10× loading dye (LI-COR Biosciences) and 0.8 µL 125 mM unlabeled X50 oligonucleotide. Reaction products were resolved on 10% polyacrylamide TBE gels (ThermoFisher), visualized on an Odyssey infrared imaging system (LI-COR Biosciences), and quantified using Image Studio Lite (LI-COR Biosciences).

Western Blots
In parallel to each helicase assay, equivalent amounts of BRIP1 immune precipitate 1:32 dilutions were separated by SDS/PAGE, transferred to Immobilon-FL membrane (Millipore), and immunoblotted with mouse anti-FLAG M2 (Sigma-Aldrich, F1804, 1:1000). Proteins were detected with IRDye680RD-conjugated secondary antibody, followed by analysis with an Odyssey infrared imaging system (LI-COR Biosciences).

ADDITIONAL INFORMATION

Data Deposition and Access
Our patient consent does not permit us to make patient sequence data publicly available. The BRIP1 variant identified in this study was submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and can be found under the accession number SCV001429629.

Ethics Statement
This study was approved by the institutional review boards of Bethlehem University and the University of Washington, Seattle. Written informed consent was provided by adults and by parents of minor children.
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Author Contributions
M.N.K., M.C.K., and E.L.L. designed the study. M.N.K. supervised data collection. L.K. obtained, analyzed, and interpreted data. S.B.P. designed and performed biochemical assays. C.C., A.A.R., T.J., S.L., and G.R. obtained data. O.L. performed chromosome breakage testing. F.Z. analyzed data. S.B.P. wrote the manuscript and prepared figures. M.N.K., M.C.K., E.L.L., and B.M.N. contributed to manuscript revisions.

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Competing Interest Statement
The authors have declared no competing interest.
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