Deficiency in BRCA-dependent DNA interstrand crosslink (ICL) repair is intimately connected to breast cancer susceptibility and to the rare developmental syndrome Fanconi anemia. Bona fide Fanconi anemia proteins, BRCA2 (FANCD1), PALB2 (FANCN), and BRIP1 (FANCJ), interact with BRCA1 during ICL repair. However, the lack of detailed phenotypic and cellular characterization of a patient with biallelic BRCA1 mutations has precluded assignment of BRCA1 as a definitive Fanconi anemia susceptibility gene. Here, we report the presence of biallelic BRCA1 mutations in a woman with multiple congenital anomalies consistent with a Fanconi anemia–like disorder and breast cancer at age 23. Patient cells exhibited deficiency in BRCA1 and RAD51 localization to DNA-damage sites, combined with radial chromosome formation and hypersensitivity to ICL-inducing agents. Restoration of these functions was achieved by ectopic introduction of a BRCA1 transgene. These observations provide evidence in support of BRCA1 as a new Fanconi anemia gene (FANCS).

**SIGNIFICANCE:** We establish that biallelic BRCA1 mutations cause a distinct FA-S, which has implications for risk counselling in families where both parents harbor BRCA1 mutations. The genetic basis of hereditary cancer susceptibility syndromes provides diagnostic information, insights into treatment strategies, and more accurate recurrence risk counseling to families.

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INTRODUCTION

The identification of mutations within 16 different Fanconi anemia genes has been particularly instructive regarding how specific aspects of DNA crosslink repair affect human disease phenotypes (1–4). Interstrand crosslink (ICL) repair is initiated by a core Fanconi anemia protein complex, which requires monoubiquitination of the FANCD2 and FANCI proteins for localization to DNA crosslinks. ICL recognition by the canonical ubiquitinated D2-I complex is thought to direct subsequent DNA replication–dependent processing of ICLs in S-phase to DNA double-strand break (DSB) intermediates that require BRCA1 and BRCA2 for homology-directed DNA repair (5, 6). Recent evidence also implicates the BRCA proteins and RAD51 at earlier stages of ICL repair (5, 7, 8). The putative multifactorial involvement of individual Fanconi anemia genes in different aspects of ICL repair may account for the diverse spectrum of phenotypes exhibited in this syndrome. Notably, biallelic carriers of BRCA2 (FANCD1) and PALB2 (FANCN) display a severe Fanconi anemia phenotype and solid tumor development (9–11), whereas this is not observed in patients with mutations in canonical Fanconi anemia genes.

Given the many connections between BRCA1 and both the D2-1 and BRCA2–PALB2 arms of ICL repair (2, 3), it is somewhat paradoxical that biallelic BRCA1 mutations have not been previously identified as a cause of Fanconi anemia. Despite high carrier frequencies in Ashkenazi Jewish populations, no patient with biallelic mutations in BRCA1 had been reported until recently (12), presumably because most combinations of deleterious BRCA1 mutations would result in embryonic lethality. This individual harbored a hypomorphic BRCA1 BRCT (BRCA1 C-terminal) repeat missense allele in trans to a frameshift mutation within BRCA1 exon 11. She presented with short stature, developmental delay, microcephaly, and ovarian cancer at age 28, with hypersensitivity to carboplatin-based chemotherapy. Although this case report established that biallelic deleterious BRCA1 mutations allow viability in humans, the absence of a cell line from this patient and the lack of extensive phenotypic examination precluded a definitive assessment of whether biallelic BRCA1 mutations cause Fanconi anemia. We report here on a second patient harboring biallelic BRCA1 mutations, early-onset breast cancer, and multiple developmental and cellular anomalies consistent with a new Fanconi anemia subtype (FA-S).

RESULTS

The proband presented at birth with microsomia and dysmorphic features (Fig. 1A). Growth parameters were less than the 0.4 percentile at term [birth weight, 1,990 g; height, 40.5 cm; head circumference (HC), 27 cm], and subsequent catch-up growth was not evident at 25 years of age (weight, 40 kg, −3.03 SD; 135 cm tall, −4.35 SD; HC, 48.5 cm, approx. −4 to −5 SD). Additional congenital abnormalities included sparse hair, upslanted palpebral fissures, blepharophimosis, a narrow palate, dental malocclusion, a high-pitched and hoarse voice, hyper- and hypopigmented skin lesions, duodenal stenosis, and a slightly enlarged left kidney. She has proximally inserted thumbs (Fig. 1A), second digit camptodactyly, 2–3 toe syndactyly, and hyperextensible knees, as well as a history of hip dislocation. Conductive hearing loss was diagnosed at 4 years of age. Bone age at 2 years 3 months was delayed (1 year and 6 months; −2 SD), but had normalized by 9 years. The patient also has mild intellectual disability with significantly delayed speech. At 23 years of age, she was diagnosed with ducal breast carcinoma that was estrogen and progesterone receptor–positive and HER2 negative. Mastectomy was performed, followed by treatment with docetaxel, fluorouracil–epirubicin–cyclophosphamide and radiotherapy. A prophylactic mastectomy was performed on the contralateral breast at age 25. The patient did not experience unusual treatment-associated toxicity and has not been diagnosed with bone marrow failure to date.

To identify the molecular basis for her syndromic presentation, the patient was originally ascertained as part of an international effort to identify gene(s) for Dubowitz syndrome.

Figure 1. An individual with early-onset breast cancer, clinical features of Fanconi anemia, and biallelic BRCA1 mutations. A, the affected individual at 3 and 23 years old showing hypertelorism, epicanthal folds, ptosis, strabismus, blepharophimosis, broad nasal bridge and nasal tip, and proximally inserted thumbs. B, sequencing of BRCA1 in family members demonstrated that both BRCA1 alleles in the proband were inherited from the heterozygous parents. III: 2, proband; III: 3, brother; II: 3, father; II: 4, mother.
(OMIM 223370) through the FORGE (Finding of Rare Disease Genes) Canada consortium. Dubowitz syndrome is a disorder characterized by microcephaly, cognitive and growth delay, and increased risk of malignancy and immunodeficiency, and is suspected to be genetically heterogeneous. Whole-exome sequencing (WES) was performed on the patient’s genomic DNA as part of this analysis. Sequencing results revealed biallelic compound heterozygous variants in the BRCA1 gene. A 4-bp deletion at the beginning of exon 10 (NM_007294:c.594_597del) was found on one of the alleles, which predicts a truncated protein with the N-terminal 198 amino acids of BRCA1 followed by an out-of-frame stretch of 35 amino acids within exon 11 not related to the canonical BRCA1 protein (p.Ser198Argfs*35). The second allele showed a point mutation in exon 18 (NM_007294:c.5095C>T), resulting in a single amino acid substitution (p.Arg1699Trp) within the BRCT repeats (Fig. 1B). This missense mutation has previously been reported as a pathogenic mutation, predisposing carriers to breast and ovarian cancers (13–15). There were no other compelling variants that explained her developmental phenotype, and none that were shared with other patients diagnosed with Dubowitz syndrome.

As BRCA1 is a major gene related to hereditary breast cancer, we performed further pedigree and genetic analysis of the family (Figs. 1B and 2A). The proband is the second of three children born to nonconsanguineous Finnish parents. Segregation analysis in the family did not identify any other siblings with biallelic mutations, or any with the syndromic features seen in the proband. Sibling III: 3 was found to be heterozygous at the c.5095 position and has no history of cancer. The BRCA1:c.594_597del and c.5095C>T missense mutations were confirmed to be maternally and paternally derived, respectively, which is consistent with autosomal recessive inheritance. The proband’s mother (II: 4) carries the BRCA1:c.594_597del and presented with bilateral low-grade ovarian serous adenocarcinoma (estrogen receptor or progesterone receptor–positive) at the age of 50. The maternal aunt (II: 5) also presented with ovarian cancer at age 50 (immunohistochemistry and mutation status is unknown). Given that the proband inherited the BRCA1:c.594_597del from her mother, the high incidence of breast and ovarian cancers in the family strongly implicates the truncating BRCA1:c.594_597del as a deleterious mutation. To further understand the potential pathogenicity of each allele, loss of heterozygosity (LOH) analysis was performed on genomic DNA extracted from the tumors of the proband and her mother (Fig. 2B). Ovarian cancer tumor blocks from the mother (II: 4) demonstrated loss of the wild-type...
(WT) BRCA1 allele with retention of the BRCA1:c.594_597del allele, consistent with BRCA1:c.594_597del as a deleterious mutation. Interestingly, in the proband, who carries both BRCA1:c.594_597del and c.5095C>T alterations, the breast tumor did not display LOH at either BRCA1 allele, suggesting that both mutant genes were dysfunctional, and therefore no selective pressure existed in the tumor to lose either allele.

To further investigate the extent of BRCA1 deficiency in the proband, we performed an immunoblot for BRCA1 on primary skin fibroblast lysates from the proband (KFB14-1 cells) and sibling III: 3 (KFB14-2 cells). KFB14-1 cells showed significantly reduced expression of full-length BRCA1 (Fig. 3A), in comparison with KFB14-2 cells, in agreement with reported in vitro experiments documenting that p.Arg1699Trp leads to BRCT misfolding and reduced proteolytic stability (16). RT-PCR and sequencing of the cDNA from the KFB14-1 cells demonstrated that most of the carboxy-terminal expressed BRCA1 mRNA in the proband carried the p.Arg1699Trp mutation (Fig. 3B), consistent with a nonsense-mediated decay process reducing levels of the BRCA1:c.594_597del mRNA. Given the known involvement of BRCA1 in ICL repair and the clinical similarities to Fanconi anemia, chromosomal breakage tests were performed on peripheral blood lymphocyte and proband skin fibroblast cultures. Upon treatment with diepoxybutane (DEB), proband lymphocytes showed increased chromosomal breakage and radial chromosome formation (30% of cells displayed radial chromosomes at 0.2 μg/mL DEB), which are well within the range established for a diagnosis of Fanconi anemia (Fig. 3C and D; ref 17). Mitomycin C (MMC)–treated skin fibroblast cultures from the proband also exhibited increased radial chromosomes in comparison with heterozygous sibling control fibroblasts, although quantification was not possible due to a failure to obtain sufficient numbers of proband fibroblast metaphases (Fig. 3C). These cellular data, in concert with the clinical presentations of growth failure, microcephaly, dysmorphic faces, and other congenital anomalies, are supportive of a diagnosis of Fanconi anemia–like disorder in the proband.

**Figure 3.** Characterization of BRCA1 function in the proband-derived cells. A, immunoblot for BRCA1 from fibroblast cell lysates shows reduced BRCA1 expression in the proband compared with the heterozygous sibling. KFB14-1, fibroblast cells derived from the proband III: 2. KFB14-2, fibroblast cells derived from the sibling III: 3. B, RT-PCR followed by cDNA sequencing showed that approximately 80% of BRCA1 mRNA in KFB14-1 cells contains the p.Arg1699Trp mutation, whereas approximately 50% of BRCA1 mRNA in KFB14-2 cells is WT. C, examples of metaphase chromosomes from peripheral blood lymphocytes [treated with 0.2 μg/mL diepoxybutane (DEB)] and fibroblast KFB14-1 cells [treated with 10 ng/mL MMC or 0.1 μg/mL DEB]. Arrows, radial chromosomes or chromatid breaks. D, quantification of abnormal chromosomes in chromosome breakage tests from peripheral blood samples of the proband and sibling.
cells (Fig. 4A and B). We next treated fibroblast cultures with MMC to test the integrity of Fanconi anemia–BRCA pathway function in proband cells in ICL repair. Similar to the condition of IR, MMC-treated KFB14-1 cells showed less BRCA1 and RAD51 foci (30.56% and 28.89%, respectively) compared with KFB14-2 cells (66.26% and 62.5%, respectively; Fig. 3B; Supplementary Fig. S1). To further ascertain whether mutant BRCA1 exhibited deficiency in DSB recognition, we examined localization of an epitope-tagged carboxy-terminal region of BRCA1 containing the BRCT repeats to nuclease-induced

**Figure 4.** Defective BRCA1 DNA repair function in proband-derived cells. **A,** immunofluorescence showing reduced BRCA1 and RAD51 foci formation at IR-induced damage sites. Complementation of KFB14-1 cells with BRCA1Δ312-1283 (KFB14-1/BRCA1Δ) restored BRCA1 and RAD51 foci formation. **B,** quantification of the percentage of fibroblasts with BRCA1 and γH2AX colocalization and/or percentage of cyclin A–positive cells with RAD51 foci. **C** and **D,** cell-survival assay showing the survival curve of fibroblasts treated with the PARP inhibitor olaparib (**C**) or MMC (**D**). KFB14-1, KFB14-2, and KFB14-1/BRCA1Δ cells were treated with olaparib or MMC at the indicated concentrations for 72 hours. BRCA1Δ312-1283 successfully restored resistance to olaparib or MMC in KFB14-1 cells. Error bars, SEM from three independent experiments.
breaks present within a single genomic location in U2OS cells (Supplementary Fig. S2). The BRCA1 fragment containing p.Arg1699Trp was observed at 22.11% of DSBs, similar to another known cancer-related BRCT mutant, p.Pro1749Arg (20.11% of DSBs; P = 0.059), but significantly less than the WT BRCT fragment (73.58%, P = 0.003). Interestingly, the p.Val1736Ala mutation found in the other biallelic carrier yielded an intermediate deficit in DSB localization, consistent with her less severe clinical presentation of developmental anomalies (12) and the less direct interaction of Val1736 with BRCT-binding phospho proteins (Supplementary Fig. S2).

BRCA-mutant and other HR-deficient cells are exquisitely sensitive to PARP (poly-ADP ribose polymerase 1 and 2) inhibition (18, 19). KFB14-1 cells were hypersensitive to the PARP inhibitor olaparib (LC50, 2.66 μmol/L) in comparison with KFB14-2 cells [LC50, 25.1 μmol/L (Fig. 4C)]. Moreover, olaparib treatment for 36 hours revealed persistent γH2AX foci in proband-derived KFB14-1 cells, in comparison with sibling control KFB14-2 cells (Supplementary Fig. S3). These results strongly suggest defective BRCA1-dependent DNA repair in KFB14-1 cells. To verify that the pathogenic phenotype is due to the deficiency of BRCA1, we complemented the KFB14-1 cells by retroviral transduction of a cDNA expressing BRCA1 with WT BRCT repeats.

Because of the difficulty of stable reconstitution with full-length BRCA1 in primary cells, we used a BRCA1Δ512-1283 transgene, which has been reported to restore radiation resistance in BRCA1 BRCT mutant breast cancer cells (20). When complemented with BRCA1Δ512-1283, KFB14-1 cells recovered RAD51 foci formation after IR or MMC exposure (Fig. 4A and B). Furthermore, BRCA1Δ512-1283 reconstitution restored resistance in KFB14-1 cells to olaparib and to MMC (Fig. 4C and D).

**DISCUSSION**

This study demonstrates that biallelic BRCA1 mutations cause a Fanconi anemia–like cellular and clinical phenotype in a human patient. The only other known patient with biallelic BRCA1 mutations also had a missense mutation in the BRCT repeats and presented similarly to the proband in this study with microcephaly, short stature, and developmental delays. Although the chromosomal breakage test was not available, the other patient experienced significant toxicity from carboplatin, suggestive of hypersensitivity to ICL reagents. In both cases, the patients had either breast or ovarian cancer, but did not develop bone marrow failure, which is similar to the FA-O (RADS1C) complementation group (21–23). It would appear prudent, however, to follow hematologic indices in patients with biallelic BRCA1 (FANCS) mutations until more data are available.

The viability of biallelic BRCA1 mutations also has important implications for genetic counseling. These two patients, together, indicate that there is a risk for couples in which each partner harbors a pathogenic BRCA1 mutation of having a child with FA-S, particularly when one of the alleles is a missense change to the BRCT repeats. On a more general note, this study demonstrates the importance of looking at all genes in a WES study, and highlights the overlap between incidental and diagnostic findings.

**METHODS**

**Samples and Cell Lines**

The patient provided written informed consent in accordance with the Declaration of Helsinki before enrollment in this study and gave additional specific approval for the publication of full facial images. The Institutional Review Board and Ethical Committee approved this study.

Skin fibroblast cells KFB14-1 and KFB14-2 were derived from proband III:2 and sibling III:3, respectively. Cells were maintained in DMEM:F12 mix (Invitrogen) with 10% fetal bovine serum and penicillin/streptomycin. After three to four passages, Sanger sequencing was used to confirm the originally assigned BRCA1 gene sequences in the proband and sibling-derived cell lines. The U2OS–DSB reporter cell line was cultured in DMEM (Invitrogen) with 10% calf serum and penicillin/streptomycin. Stable expression of a BRCA1Δ512-1283 fibroblast cell line (KFB14-1/BRCA1Δ) was generated by retroviral transduction.

**Antibodies and Chemicals**

Antibodies used for protein blots and immunofluorescence are as follows: mouse monoclonal anti-BRCA1 antibody D9 (Santa Cruz Biotechnology), anti-γH2AX (Millipore), anti-RAD51 (Santa Cruz Biotechnology), anti-RPA (Novus), and anti-Cyclin A (Santa Cruz Biotechnology). Drugs used are olaparib (ChemieTek) and MMC (Sigma).

**WES and Data Analysis**

Patient DNA was extracted from whole blood using standard protocols. DNA samples were processed by the University of Washington Center for Mendelian Genomics (UW-CMG). The initial quality control entailed DNA quantification, sex typing, and molecular “fingerprinting” using a 96-plex genotyping assay. Library construction (using 1 μg of genomic DNA) and exome capture were automated (PerkinElmer Janus II) in 96-well plate format. Libraries underwent exome capture using the approximately 37-Mb target from Roche/
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NimblegenSeqCap EZ v2.0. Before sequencing, library concentration was determined by triplicate qPCR and molecular weight distributions were verified on Agilent Bioanalyzer 2100. Individual exome multiplex-compatible libraries were pooled into sets of 24 to 96 samples using liquid handling robotics. Sequencing-by-synthesis was carried out on the IlluminaHiSeq platform (2 × 50 bp reads). Current throughput was sufficient to complete 3 to 4 multiplexed exomes per lane at high coverage (60–80x mean coverage).

Sequencing data processing involved the following: (i) base calling (Illumina RTA 1.13), (ii) demultiplexing and conversion into unaligned BAM files by lane and index sequence (IlluminaBasecall10so-

mand Picard ExtractIlluminaBarcodes); (iii) alignment to the human reference (hg19/GRC37) using BWA (24). Read pairs not mapping within ±2 SDs of the average library size (~125 ± 15 bp for exomes) were removed. Following QC, individual-level data were merged and subjected to the following steps: (i) base quality recalibration using GATK TableRecalibration; (ii) reads with duplicate start positions were removed; (iii) indel realignment using GATK IndelRealigner; and (iv) variant calling using multiple samples with the UnifiedGeno-
typer routine of GATK 1.6.11 (25).

Variants observed in more than 10% of other UW-CMG samples prepared using the same capture probe set were removed and the remaining variants were filtered for QUAL ≥ 250, GQ ≥ 50, QD ≥ 5, MapQ ≥ 0.99, AB < 0.8|NA, absence of SnpCluster or HRunFilter flags, no InDels ± 5 bp, maximum 1000G/ESP frequency of 0.001 for de novo/domi-

nant variants and 0.01 for recessive variants. Variants were annotated using Ensembl Variant Effect Predictor v2.6 (26), and the analysis was limited to events with the following predicted consequences: FRAME_ SHIFT, INFRAME, NONCODING_CHANGE, NON_SYNONYMOUS, CANONICAL_SPLICE, STOP_GAINED, STOP_LOST, UNKNOWN. In case of overlapping annotations or multiple isoforms, one annotation was picked based on whether the annotation was in CCDS, the avail-

ability and value of SIFT/PolyPhen scores, and the splice-site distance.

Chromosome Breakage Studies

The lymphocyte cultures were established from whole blood as routinely applied to make chromosomal preparations for karyotype analysis. DEB was used at a final concentration of 0.1–0.22 μg/ml for chromosome fragility tests in lymphocytes and 0.1 μg/ml in fibroblasts.

LOH Analysis

Genomic DNA was extracted from paraffin-embedded tumor samples following laser-capture microdissection (LCM; Skin Disease Research Center cores of University of Pennsylvania). Primers 5′-AGGA GAGAGACGCTTTCAC-3′, 5′-CTGATTTACCTCCGTTC-3′ and 5′-CAGCCCTCGATTTGTCAC-3′, 5′-TCTCCGGGACTTCTAG-3′ were used to amplify exon 10 and exon 18, respectively. Sanger sequencing was performed on ABI3730 (University of Pennsylvania Genomics Facility).

Cell Survival Assay

Cell survival assay was performed using Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay kits following the manufacturer’s instructions (Promega). Experiments were repeated three times, and data represented as the mean of six replicate wells ± SEM.

Immunofluorescence

Cells grown on coverslips were fixed using 3% paraformalde-
hyde/2% sucrose solution. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 5 minutes at 4°C. Following incubation with primary antibody for 20 minutes at 37°C in a humidified chamber, cells were washed with PBST and then incubated with secondary antibody for 20 minutes at 37°C in the humidified chamber. After extensive washing with PBST, coverslips were mounted onto glass slides using VECTASHIELD mounting medium containing 4’,6-diamidino-2-phenylindole (Vector Laboratories).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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