A Novel Breast Cancer–Associated *BRIP1* (*FANCJ*/*BACH1*) Germline Mutation Impairs Protein Stability and Function

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Abstract

Purpose: *BRCA1-interacting protein 1* (*BRIP1*; *FANCJ*/*BACH1*), which encodes a DNA helicase that interacts with BRCA1, has been suggested to be a low-penetrance breast cancer predisposing gene. We aimed to assess whether *BRIP1* mutations contribute to breast cancer susceptibility in our population and, if so, to investigate the effect of such mutation(s) on BRIP1 function.

Experimental Design: A series of 49 breast/ovarian cancer families, devoid of a *BRCA1*/*BRCA2* mutation, were screened for *BRIP1* mutations. Functional analyses, including coimmunoprecipitation and stability assays, were employed to further characterize a previously unreported variant.

Results: Five sequence alterations were identified, of which four had been already described. Herein, we report a novel *BRIP1* germ-line mutation identified in a woman with early-onset breast cancer. The mutation consists of a 4-nucleotide deletion (c.2992-2995delAAGA) in *BRIP1* exon 20 that causes a shift in the reading frame, disrupts the BRCA1-binding domain of BRIP1, and creates a premature stop codon. Functional analysis of the recombinant mutant protein in transfected cells showed that the truncation interferes with the stability of the protein and with its ability to interact with BRCA1. Loss of the wild-type *BRIP1* allele with retention of the mutated one was observed in the patient's breast tumor tissue.

Conclusions: These results, by showing that the newly identified *BRIP1* c.2992-2995delAAGA mutation is associated with instability and functional impairment of the encoded protein, provide further evidence of a breast cancer–related role for BRIP1.

It is estimated that ~5% to 10% of all breast cancer cases result from an inherited predisposition to the disease (1). Thus far, two major high-penetrance susceptibility genes have been identified: *BRCA1* and *BRCA2*. Inheritance of a defective allele of either gene is sufficient to confer breast cancer predisposition with an estimated lifetime risk of ~35% to 80% (refs. 2–5 and reviewed in ref. 6). However, mutations in the *BRCA* genes fail to explain occurrence of the neoplasia in all breast cancer–prone families (7). Other well-established cancer susceptibility genes, such as *TP53*, *PTEN*, and *LKB1*, contribute only to a small proportion of breast cancer in the general population, as their germ-line mutations are very rare. The remainder breast cancer risk is, as yet, unexplained and likely due to cooperative defects in a larger number of low-penetrance genes rather than to mutations in highly penetrant ones (7). In keeping with this notion, suitable candidates have been analyzed in search for functionally deficient variants that segregate with the cancer phenotype. Genes whose products are known to interact with BRCA1 and/or BRCA2 and to be involved in DNA repair or in the regulation of cell cycle progression are usually prioritized for such investigation (8). As a result, mutations of *ATM*, *CHEK2*, *RAD50*, *Nibrin*, *PALB2*, and *BRCA1-interacting protein 1* (*BRIP1*; *FANCJ*/*BACH1*) have been found to associate with a modest (~2-fold) increase of breast cancer risk (9–14).

BRIP1 was first identified as a 1,249–amino acid protein that interacts with the BRCA1 BRCT *in vivo* (15). BRIP1 is

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A. De Nicolo and M. Tancredi contributed equally to this work.

Electronic Database Information: The URLs and accession numbers for data presented herein are as follows: GenBank (http://ncbi.nlm.nih.gov/GenBank/) for *BRIP1* genomic (accession no. NT.010783) and cDNA (accession no. NM.032043) sequences and Primer3 Software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.www.cgi).

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ubiquitously expressed, colocalizes with BRCA1 at sites of DNA damage, and contributes to its DNA repair function (15). Specifically, BRIP1-BRCA1 interaction, which has been shown to depend on the cell cycle–regulated phosphorylation of BRIP1 at Ser^{990} (16–18), is required for timely repair of DNA double-strand breaks and for DNA damage–induced checkpoint control during the G_2-M phase of the cell cycle (17).

Interestingly, *BRIP1* maps to chromosome 17q22 near the *BRCA1* locus (15). The frequent documentation of breast carcinomas that are wild-type for the *BRCA* genes but exhibit allelic losses encompassing the 17q21-q22 region suggests that this chromosomal region may harbor an additional breast cancer susceptibility gene (19). Thus, both its functionally relevant interaction with BRCA1 and its chromosomal location render *BRIP1* a candidate tumor suppressor gene.

In addition, the BRIP1 NH_2 terminus shares substantial sequence homology to the catalytic and nucleotide-binding domains of known members of the DEAH helicase family. Indeed, BRIP1 was shown to be a *bona fide* ATP-dependent, 5′-3′ DNA helicase (20). Mutations in helicases (such as *XPB*, *XPD*, *WRN*, *BLM*, and *RecQL4*) have been described in association with human genetic disorders (xeroderma pigmentosum, Werner syndrome, Bloom syndrome, and Rothmund-Thomson syndrome, respectively), which are characterized by genome instability and predisposition to cancer (reviewed in refs. 21–24).

The demonstration that certain *BRIP1* mutations, detected in women with early-onset breast cancer, result in the synthesis of enzymatically defective proteins (20) provides biochemical support for the aforementioned notion that *BRIP1* is a breast cancer gene. However, thus far, studies have failed to find any highly penetrant *BRIP1* mutations, and most of the newly identified sequence alterations have not been resolved in terms of their biological significance (14, 25–28).

More recently, *BRIP1* was shown to be biallelically inactivated in patients with Fanconi anemia (FA), a genetic disease (which can be autosomal recessive or X-linked) characterized by multiple congenital abnormalities, bone marrow failure, cellular hypersensitivity to interstrand DNA cross-linking agents, and susceptibility to cancer (29). Specifically, *BRIP1* mutations were found in patients with FA belonging to the complementation group J (30–33). Similarly, a biallelic inactivation of the breast cancer gene *BRCA2* has been described previously in patients with FA complementation group D1 (34). More recently, biallelic defects in *PALB2* have been shown to cause FA subtype N (35, 36).

The aim of our study was to evaluate the contribution of alterations at the *BRIP1* locus to breast cancer susceptibility in a cohort of 49 breast or breast/ovarian families in which no *BRCA1/BRCA2* mutations were detected.

Materials and Methods

Family recruitment. A total of 49 breast or breast/ovarian cancer patients belonging to as many families mainly originating from central-northern Italy and referred to the University Hospital in Pisa (37) were defined as probands (index individuals) and were analyzed for *BRIP1* germ-line mutations. All probands displayed no detectable deleterious mutations on full screening of *BRCA1* and *BRCA2* genes. In addition, when tested for the *CHEK2* 1100delC disease-linked mutation (11), they all resulted negative. Of the 49 families, 40 (80%) were associated with breast cancer and 9 (20%) with both breast and ovarian cancer (Supplementary Table S1). Most of the families included ≥3 breast/ovarian cancer cases. Thirty-five pedigrees showed also cases of other types of neoplasia. The average age at diagnosis of breast cancer among patients was 43.8 years, ranging between 21 and 73 years (Supplementary Table S1). Family histories were obtained through detailed interviews and pedigrees were traced as far backward and laterally as possible. Families were classified as high-risk or moderate-risk cancer families depending on the criteria they met. Inclusion criteria for the 38 high-risk families were as follows: (*a*) ≥3 cases of breast and/or ovarian cancer in first/second-degree relatives or (*b*) ≥2 cases of breast and/or ovarian cancer in first/second-degree relatives, of which at least one with bilateral or early-onset (≤40 years) breast cancer or with breast and ovarian cancer. Each of the 4 moderate-risk families displayed 2 cases of breast/ovarian cancer in first/second-degree relatives. In addition, 7 families with single cases of bilateral or early-onset breast cancer or breast/ovarian cancer along with multiple (≥2) cases of other kinds of tumors in first/second-degree relatives were included in the study. Written informed consent to collect pedigree data and a blood specimen for a study on cancer susceptibility was obtained from all probands. As controls, 50 individuals, from the same geographic region as the cancer cases, were anonymously recruited. They were all cancer-free at the time of blood donation and their age ranged between 21 and 87 years (average, 48.5 years).

Mutation screening. A DNA sample of the index individual of each cancer family was analyzed. Genomic DNA was extracted from peripheral blood lymphocytes using a QIAamp DNA Mini kit (Qiagen). Twenty-two fragments of the *BRIP1* gene, including both coding exons and flanking intronic sequences, were amplified by PCR using Taq Gold polymerase (Applied Biosystems). The size of PCR products ranged between 214 and 380 bp. Screening for *BRIP1* mutations was carried out by single-strand conformation polymorphism. Genomic DNA from cases showing an altered migration pattern was amplified and analyzed by bidirectional sequencing using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems) on an ABI model 3100 capillary sequencer. Oligonucleotides for single-strand conformation polymorphism and sequencing were designed using the Primer3 Software based on the sequence information obtained from the GenBank databases. Primer sequences and PCR conditions are available on request.

Loss of heterozygosity analysis. Loss of heterozygosity analysis was carried out on tumor tissue excised from the index individual of the Pisa88 family. Laser-capture microdissection was done to isolate pure populations of neoplastic and normal breast cells from 5 μm sections of the formalin-fixed, paraffin-embedded breast carcinoma tissue block. The DNA was extracted from the dissected tumor and normal cells using a QIAamp DNA Mini Kit (Qiagen). Amplification was carried out by PCR using primers located in exon 20, and the resulting products were analyzed by sequencing.

Plasmids. The full-length BRIP1 cDNA-containing pENTR/D-TOPO (wtBRIP1 pENTR/D-TOPO) vector (15) served as template to generate a 4-nucleotide deletion (Δ) at the relevant site using the QuickChange mutagenesis kit (Stratagene). After sequencing to confirm the mutation, the ΔBRIP1-pENTR/D-TOPO vector was modified to insert an in-frame Flag-encoding COOH-terminal sequence (ΔBRIP1Flag-pENTR/D-TOPO). Next, both ΔBRIP1 and ΔBRIP1 Flag-encoding cDNA were transferred, by LR recombinase-mediated reaction (Invitrogen), into a pcDNA-DEST47 destination vector (Invitrogen) for expression in mammalian cells. The ΔBRIP1-pcDNA-DEST47 vector was further used as template for PCR amplification. The PCR product was *Not*I/*Apa*I digested and subcloned into a pcDNA3.1 vector (Invitrogen) encoding a COOH-terminal Myc-6xHys tag (ΔBRIP1-pcDNA3.1/MycHis). All cloned cDNA constructs were fully sequenced before use. The mammalian expression vector pcDNA3.1 Myc-6xHis containing full-length wtBRIP1 cDNA (wtBRIP1-pcDNA3.1/MycHis) was described previously (15). pEGFP-C2 (Clontech) and pRL-TK (Promega) plasmids were used as controls to ensure efficiency of transfection.

Table 1. *BRIP1* sequence alterations

Exon/intron	Nucleotide change*	Effect on protein	Allele frequency † (%)
Intron 5	IVS5-31C>G	Unknown	1/98 (1)
Exon 19	2637G>A	$Glu^{879}Glu$	32/98 (33)
Exon 19	2755C>T	$Pro^{919}Ser$	48/98 (49)
Exon 20	2992-2995delAAGA	$Glu^{998}fsX1057$	1/98 (1)
Exon 20	3411C>T	$Tyr^{1137}Tyr$	81/98 (83)

*Numbering based on RefSeq NT_010783.13 (for the intronic variant) and NM_032043 (for all the variants within the coding region). For exonic variants, numbering starts at codon 1.
† Frequency of second allele listed.

In vitro ***transcription and translation.*** Expression plasmids, generated as described above, and encoding the wild-type and the ΔBRIP1 protein products, were *in vitro* transcribed and translated using a TNT kit (Promega) according to the manufacturer's specifications.

Establishment of a lymphoblastoid cell line. A lymphoblastoid cell line (LCL) was established by EBV infection of the Pisa88 proband peripheral blood lymphocytes using standard protocols and served as a source for DNA, RNA, and proteins (detailed below).

DNA extraction and PCR amplification and analysis. Genomic DNA was extracted from the Pisa88 LCL using a QIAamp DNA Mini Kit (Qiagen) and amplified with appropriate primer pairs depending on the applications. Analysis of PCR products by bidirectional sequencing was carried out as described above. For electrophoretic analysis, PCR products were resolved on 4% low melting point agarose (Life Technologies) or 8% polyacrylamide denaturing gels and stained with ethidium bromide and SYBR Gold (Invitrogen), 1:10,000 dilution, respectively. The 4-bp length difference caused by the mutation allowed direct discrimination of the mutant and wild-type fragments (amplified with a pair of primers specifically designed to give rise to a small product encompassing the mutation site). Bands were visualized under UV light. The Image J software was used for quantitative analysis of the intensity of the bands.

Cell culture and transfection. HeLa, U2OS, and 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum (Gemini) and antibiotics in a humidified incubator at 37°C with an atmosphere containing 10% CO_2. Grown to 50% confluence, cells were transfected with 5 to 10 μg of wtBRIP1 or ΔBRIP1-pcDNA3.1/MycHis constructs using FuGene 6 transfection reagent (Roche Diagnostic).

RNA extraction, cDNA synthesis, and reverse transcription-PCR amplification and analysis. RNA was isolated from the Pisa88 LCL or from green fluorescent protein (GFP)–transfected, Myc-wtBRIP1-transfected, or Myc-ΔBRIP1-transfected 293T cells using the RNeasy mini kit (Qiagen). cDNA synthesis was carried out using Superscript II RT RNase H^- reverse transcriptase (Invitrogen) and random hexamers (Invitrogen) according to the manufacturer's instructions. Reverse transcription-PCR to analyze the expression levels of endogenous transcripts in the Pisa88 LCL was carried out with the same primer pairs used for DNA, and the amplified fragments were discriminated on agarose and polyacrylamide gels as detailed previously. Reverse transcription-PCR to compare mutant versus wild-type transcripts in transfected cells was carried out using specific primer pairs that allowed selective amplification of the exogenously expressed constructs. In both cases, mock reverse transcription-PCR (from mRNA reverse transcribed with no addition of reverse transcriptase) served as controls and confirmed the absence of DNA contamination.

Immunoprecipitation, immunoblotting, and protein stability analysis. Forty-eight h after transfection, cells were collected, washed with PBS (pH 7.4), and lysed in NETN buffer [0.5% NP-40, 20 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, and 1 mmol/L EDTA] supplemented with 1 tablet/50 mL of complete protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation (16,000 × *g* for 10 min) and incubated on ice for 1 h with 1 to 2 μg of the relevant antibody. Next, 20 μL of slurry protein A/G Sepharose beads (Amersham Biosciences) was added to each lysate, and incubation continued with rotation for 40 min at 4°C. Beads were retrieved by centrifugation and washed three times in NETN buffer. Proteins bound to the beads were eluted by boiling in Laemmli sample buffer, separated by SDS-PAGE, and electrotransferred to nitrocellulose membranes (Invitrogen). After blocking in 5% milk, Western blot assays were carried out with the indicated primary antibodies for 1 h at room temperature followed by incubation with horseradish peroxidase–conjugated secondary antibodies for 1 h at room temperature. Aliquots of lysates were also run and blotted for tubulin as a control for protein loading. Equal transfection efficiency among the samples was ensured by Western blot detection of the cotransfected pEGFP-C2 plasmid. All blots were developed using Enhanced Chemiluminescence Plus Western blotting detection system (Amersham Biosciences) and Biomax BLUE XB-1, MR, or XAR films (Kodak). Cycloheximide-chase experiments were done as described (38). The Image J software was used for densitometric analysis of the films.

To obtain whole-cell lysates from EBV-immortalized lymphocytes, the cells were harvested, washed once in PBS, and lysed in NETN buffer as described previously. Whole-cell lysates were separated by SDS-PAGE and subjected to Western blotting as detailed above.

Antibodies. Two different rabbit polyclonal antibodies, I-104 and I-82, were generated against BRIP1 in New Zealand White rabbits. I-104 was produced against a COOH-terminal peptide of BRIP1 (N-KTHEIEIKNFKPSPSKNKGM-C). I-82 was generated against a recombinant full-length flag-tagged wild-type BRIP1 (rBRIP1) produced in High Five insect cells. I-82 was affinity purified using rBRIP1 coupled to a solid support. The anti-BRCA1 rabbit antiserum was purchased from Upstate Biotechnology. The anti-BARD1 affinity-purified polyclonal antibody was purchased from Bethyl Laboratories. The following monoclonal antibodies were used: anti-BRCA1 MS110 (39), anti-Myc clones 4A6 (Upstate Biotechnology) and 9B11 (Cell Signaling Technology), anti-α-tubulin clone DM1A (Sigma), and anti-GFP clone C163 (Zymed Laboratories). Secondary antibodies used for immunodetection were horseradish peroxidase–conjugated sheep anti-mouse IgG and donkey anti-rabbit immunoglobulin (Amersham Biosciences; 1:5,000 dilution).

Results

Mutational analysis of the *BRIP1* gene done in our cohort of 49 index individuals led to the identification of four germ-line sequence alterations within the *BRIP1* coding region together with one intronic variant (Table 1). Three sequence changes (c.2637G>A, c.2755C>T, and c.3411C>T) had already been reported with comparable allelic frequencies (15, 25, 26, 28) and all were classified as neutral polymorphisms. The intronic variant IVS5-31 C>G in the region located between exons 5 and

6 was reported previously as well (27). Neither of the two heterozygous missense mutations (c.139C>G and c.897G>A) described by Cantor et al. were found in our set of patients.

Interestingly, we identified a novel heterozygous sequence change in the last exon (exon 20) of *BRIP1*: a 4-nucleotide deletion starting at 2,992 (c.2992-2995delAAGA) that causes a slippage of the open reading frame, leading to a premature stop at codon 1057 (Fig. 1A and B). The sequence from the deletion to the stop bears no homology to wtBRIP1. Importantly, the amino acid change (amino acids 998-1,057) caused by the newly identified mutation partially encompasses the BRCA1-binding domain (amino acids 979-1,006) of BRIP1 (20) and might be predicted to affect the interaction between BRIP1 and BRCA1.

The c.2992-2995delAAGA mutation was detected in a 49-year-old woman that had been diagnosed with breast cancer at the age of 40 years and exhibited a family history of multiple cancers (Fig. 1C). No breast cancer cases other than the proband and her mother (who was diagnosed at the age of 76 years) were known to have occurred in the family. However, the pedigree displayed two cases of colorectal cancer in the proband's paternal aunt and grandmother. Moreover, both brothers of the paternal grandmother had been diagnosed with a neoplastic disease (lymphoma and lung cancer). Unfortunately, because DNA samples from any other suitable family member(s) were not available, cosegregation of the mutated allele with the cancer phenotype through the family could not be tested. Nevertheless, when 50 matched healthy control individuals were analyzed, they all tested negative for the c.2992-2995delAAGA mutation.

BRIP1 would conform to the classic paradigm for tumor suppressor genes if loss of the wild-type allele could be detected in tumor cells isolated from the affected individual. To address this possibility, we searched for loss of heterozygosity at the *BRIP1* locus in breast carcinoma tissue from the patient carrier of the novel mutation. Remarkably, sequence analysis of the DNA extracted from the laser-capture microdissected specimen revealed that loss of the wild-type allele had occurred in the tumor cells, with retention of the mutant allele (Fig. 2). This finding underscores the notion that *BRIP1* has tumor-suppressive function and might participate in breast tumorigenesis.

We then sought to elucidate the possible biological consequences of the mutant *BRIP1* allele by investigating whether the deletion had any effect on the function of the corresponding protein. Because the frameshift caused by the mutation was predicted to give rise to a premature translational stop, we decided to characterize the truncated protein product. First, we generated several BRIP1 constructs bearing the relevant mutation (ΔBRIP1) with or without a COOH-terminal tag (Flag or Myc). As a control, we used the wtBRIP1-pcDNA3.1/MycHis described previously (15). *In vitro* transcription and translation reactions were programmed with the wild-type and mutant plasmids. The obtained products were resolved by SDS-PAGE and analyzed by Western blotting using antibodies against BRIP1 or the Myc tag. As predicted, all the ΔBRIP1 constructs generated electrophoretically distinguishable protein products

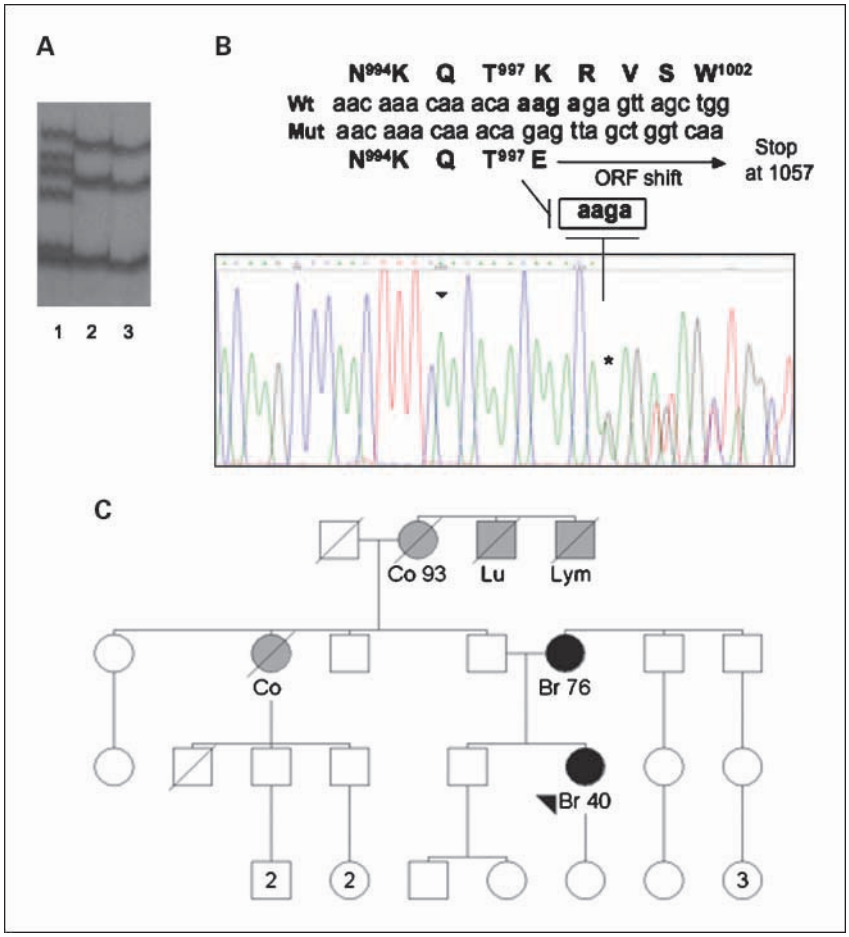

Fig. 1. A novel *BRIP1* sequence alteration identified in the family Pisa88. *A,* identification of a novel *BRIP1* sequence variant. Single-strand conformation polymorphism analysis after PCR amplification using primers located in *BRIP1* exon 20 reveals an altered electrophoretic pattern in the sample corresponding to the proband of the family Pisa88 (*lane 1*). *Lanes 2* and *3,* families Pisa61 and Pisa137, respectively, show a normal migration pattern as ascertained in healthy control individuals (data not shown). *B,* characterization by sequence analysis of the *BRIP1* gene alteration detected in the index case of the family Pisa88. The chromatogram displays a 4-nucleotide deletion, which starts at position 2,992 (GenBank database sequence reference NM.032043, nucleotide numbering starting at codon 1; *asterisk*), and causes a shift of the open reading frame (*ORF*). The sequence of the wild-type and mutated alleles appear overlapped. The wild-type and mutated cDNA sequences are detailed (starting point marked by an *arrowhead* in the chromatogram). The corresponding amino acid sequences are reported as well. The open reading frame shift (starting at codon 998) caused by the deletion results in a premature stop codon at position 1,057. *C,* pedigree of the family Pisa88, exhibiting the c.2992-2995delAAGA mutation. *Black-filled symbols,* individuals diagnosed with breast cancer (*Br,* breast cancer); *gray-filled symbols,* individuals diagnosed with cancer other than breast (*Co,* colon cancer; *Lu,* lung cancer; *Lym,* lymphoma). *Arrowhead,* index case.

that migrated ~20 kDa faster than the wtBRIP1 one, consistent with the expected size for ΔBRIP1 being ~200 amino acids shorter than the wild-type protein (Fig. 3A, *lanes 3-5* versus *lane 2*). In addition, we ascertained that the mutant BRIP1 was not detected by an antibody generated against the extreme COOH terminus of the protein, whereas, under the same conditions, the full-length wtBRIP1 was easily recognized (Fig. 3B). These results show that the *BRIP1* c.2992-2995delAAGA mutation leads to the synthesis of an identifiable truncated protein that lacks the COOH terminus.

Next, we aimed to determine the expression of the ΔBRIP1 protein *in vivo*. For this purpose, a LCL was established, by EBV infection, from the peripheral blood lymphocytes of the heterozygous patient, and analysis of the endogenous BRIP1 protein was done by Western blotting. Only a wtBRIP1 product was observed when either a polyclonal antibody (Fig. 3C, *lane 5*) or a pool of monoclonal antibodies (data not shown) were used. This observation indicates that ΔBRIP1, if expressed, is significantly less abundant than wtBRIP1 in EBV-immortalized lymphocytes.

Because mRNA bearing a premature termination codon are known to be targeted for degradation via a surveillance mechanism named nonsense-mediated mRNA decay (reviewed in refs. 40, 41), we searched for an imbalanced expression of the transcripts (mutant versus wild-type) that could explain the absence of a detectable mutant protein. As a result, direct sequencing and PAGE analysis of reverse transcription-PCR products revealed the presence of both mutant and wild-type alleles in the Pisa88 LCL (Supplementary Fig. S1A and B). Furthermore, the expression levels of both transcripts appeared comparable as confirmed after quantitation of the band intensities (Supplementary Fig. S1B and C; data not shown). This finding is consistent with the notion that premature termination codons residing within the last exon do not trigger nonsense-mediated mRNA decay (42–44) and argues against preferential allelic degradation as an explanation for the inability to detect the mutant protein.

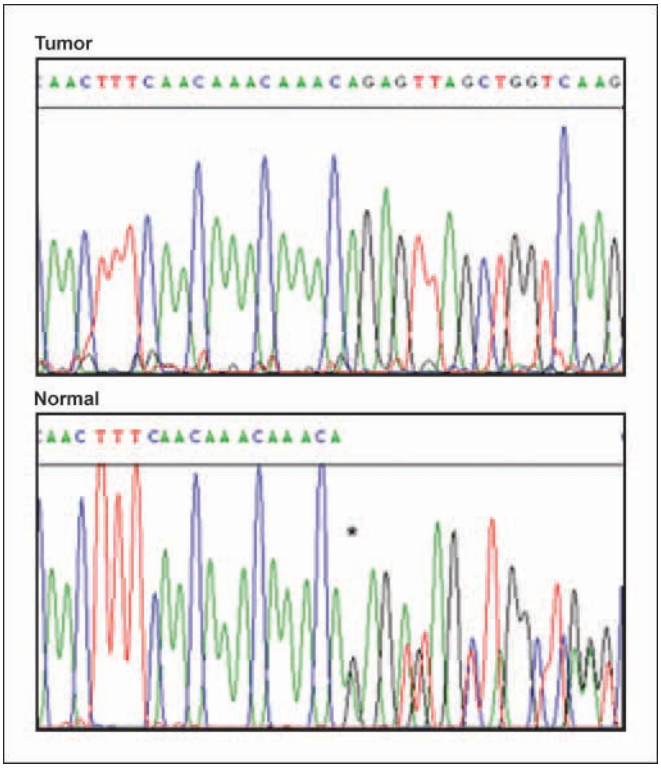

Fig. 2. Breast tumor tissue from Pisa88 proband displays loss of heterozygosity at the *BRIP1* locus. Chromatograms of tumor and normal breast tissue DNA from Pisa88 index individual. Loss of the wild-type allele with retention of the mutant one is evident in the tumor sequence (*top*). Normal tissue (*bottom*) shows both mutated and wild-type alleles. *Asterisk,* beginning of the overlapping sequences.

We therefore reasoned that the lack of expression of ΔBRIP1 in the patient's immortalized lymphocytes could be due to the inherent instability of the mutant protein. To test this possibility, we set out to characterize an exogenously overexpressed ΔBRIP1 protein in cultured cells. HeLa cells were transiently transfected with Myc-wtBRIP1 or Myc-ΔBRIP1 encoding constructs. Using anti-Myc or anti-BRIP1 antibodies, we were unable to detect a mutant protein product of the predicted size (data not shown). A low expression of the truncated protein was observed after transfection of the relevant constructs in U2OS cells (data not shown). We then exploited the 293T cell line, which expresses SV40 large T antigen, to allow T antigen-dependent amplification of the SV40 origin-containing plasmids. Even in this setting, the expression of the ΔBRIP1 protein was lower than the expression of the wild-type counterpart (Fig. 4A). Cotransfection with GFP- or luciferase-encoding plasmids allowed us to confirm equal efficiency of the transfection for both wild-type and ΔBRIP1 constructs (Fig. 4A; data not shown). Furthermore, we ruled out that the mutation could affect the protein by rendering ΔBRIP1 insoluble; indeed, the mutated protein was easily extractable in cell lysis buffer from transfected cells with no significant residual amount detected in the pellet (data not shown). Moreover, a fairly equal level of expression for both Myc-wtBRIP1 and Myc-ΔBRIP1 transcripts was shown by reverse transcription-PCR carried out with primers specifically designed to amplify the exogenous mRNA (Supplementary Fig. S2). These observations suggest that the lower level of ΔBRIP1 detected after transfection might be due to instability of the truncated protein caused by the mutation.

We sought to address this possibility by using a cycloheximide-chase method (38) to determine the half-lives of both mutant and wild-type proteins. Transient transfection of 293T cells using equal amounts of the aforementioned Myc-wtBRIP1-encoding and Myc-ΔBRIP1-encoding constructs was done followed by cycloheximide treatment to block new protein synthesis. When cell lysates obtained at different time points were blotted with an anti-Myc-specific antibody, ΔBRIP1 appeared to be, indeed, significantly less stable than wtBRIP1 (Fig. 4B). An estimated half-life of ~1 h was observed for the mutant protein, whereas wtBRIP1 was shown to be significantly more stable, having a half-life of ~5 h (Fig. 4C), consistent with previous findings (15). These results indicate that the c.2992-2995delAAGA mutation impairs the stability of the corresponding truncated BRIP1 protein.

Although these studies show that the mutant BRIP1 protein is unstable, we were intrigued by the fact that the c.2992-2995delAAGA mutation disrupts the previously described BRCA1-binding domain (15, 20) and wondered whether ΔBRIP1, even in low concentration, could still interact with BRCA1. To this end, we attempted to assess, by coimmunoprecipitation analysis, the potential effect of the novel *BRIP1*

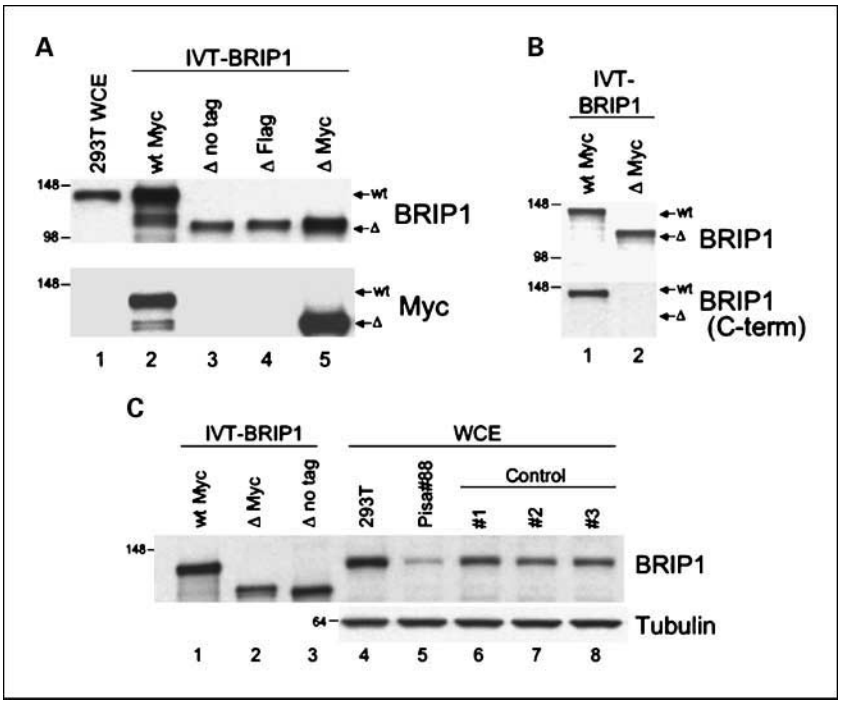

Fig. 3. *BRIP1* 2992-2995delAAGA gives rise to a truncated protein that is not detectable *in vivo* by Western blot analysis. *A,* electrophoretic mobility of endogenous (*lane 1*) and *in vitro* transcribed and translated BRIP1 (IVT-BRIP1) proteins (*lanes 2-5*). IVT-ΔBRIP1 proteins (*lanes 3-5*) exhibited faster electrophoretic mobility compared with the IVT-wtBRIP1 (*lane 2*), consistent with the smaller size of the mutated protein products. Flag- and Myc-tagged IVT-ΔBRIP1 proteins (*lanes 4* and *5*) migrated slightly slower than IVT-ΔBRIP1 with no tag (*lane 3*). *B,* I82 antibody, generated against full-length rBRIP1, recognized both IVT-wtBRIP1 and IVT-ΔBRIP1 proteins (*top blot*). The I104 antibody, raised against BRIP1 extreme COOH terminus, failed to recognize ΔBRIP1 while still being able to react with wtBRIP1 (*bottom blot, lane 2* versus *lane 1*). *C,* Western blot analysis of EBV-immortalized lymphocytes from the Pisa88 proband revealed absence of a truncated BRIP1 protein (*lane 5*). IVT-wt/IVT-ΔBRIP1 proteins (*lanes 1-3*), 293T cell extract (*lane 4*), and EBV-immortalized lymphocyte lysates from unrelated individuals (*lanes 6-8*) were run as controls. *Left,* molecular mass (in kDa).

sequence change on the BRIP1-BRCA1 interaction *in vivo*. 293T cells were transfected with GFP and either Myc-wtBRIP1-encoding or Myc-ΔBRIP1-encoding constructs, and immunoprecipitation with an anti-Myc monospecific antibody was carried out followed by Western blotting for BRCA1. BRCA1 was always nearly absent in the immunoprecipitation from ΔBRIP1-transfected cells, whereas a clearly detectable BRCA1 signal was observed even when serial dilutions of the Myc-IP from wtBRIP1-transfected cells were loaded onto the gel (Fig. 5). These results indicate that ΔBRIP1, which bears a significantly altered BRCA1-binding domain, is impaired in its ability to interact with BRCA1. In addition, because BRIP1 and BARD1 have been reported to indirectly interact with one another, BRCA1 being the mediator of such interaction (15), we asked whether the defective binding of ΔBRIP1 to BRCA1 translated into a defective interaction with BARD1 as well. Indeed, in Myc-ΔBRIP1 complexes, the amount of coimmunoprecipitated BARD1 was significantly decreased compared with what was observed in Myc-wtBRIP1 complexes (Fig. 5). Furthermore, results from reciprocal immunoprecipitation experiments (BRCA1 immunoprecipitation followed by Western blotting for Myc) consistently showed a significantly impaired interaction between BRCA1 and the mutant BRIP1 protein (Supplementary Fig. S3).

Taken together, these results indicate that the c.2992-2995delAAGA mutation affects BRIP1 function and impairs its ability to interact with BRCA1.

Discussion

Our goal was to evaluate whether and to what extent defects in the *BRIP1* gene contribute to familial breast cancer in selected *BRCA1/BRCA2*-negative breast/ovarian cancer families. The mutational screening we carried out led to the identification of five *BRIP1* sequence changes in a series of 49 affected individuals. Four alterations (IVS5-31C>G, c.2637G>A, c.2755C>T, and c.3411C>T) had already been reported (15, 25–27), of which only one (c.2755C>T) causes a nonsynonymous amino acid substitution and introduces a serine ($Pro^{919}Ser$) in the BRCA1-binding domain, thus generating a potential phosphorylation site. An association between such polymorphism and an increased breast cancer risk by the age of 50 years has been suggested (45), but it is, as yet, not confirmed (28, 46).

The fifth sequence variant we characterized (c.2992-2995delAAGA) is a unique mutation that causes a change of 9 of 28 amino acids within the BRCA1-binding domain (979-1,006) of BRIP1 and introduces a premature stop codon. The mutation was identified in a woman diagnosed with early-onset breast cancer who lacked a strong family history for the disease. Importantly, we found loss of heterozygosity at the *BRIP1* locus in breast cancer tissue from the same patient.

To our knowledge, this is the first report of a heterozygous germ-line mutation in the *BRIP1* gene, which gives rise to a truncated protein product, predicted to be impaired in its biological role and is associated with loss of the wt*BRIP1* allele in the corresponding breast tumor tissue. The latter result is consistent with the classic Knudson's two hit hypothesis for loss of tumor suppressor function (47) and supports the speculation that the novel mutation we describe herein might have contributed to the development of breast cancer in the patient. Notably, although most of the *BRIP1* sequence alterations described thus far associated with FA have a truncating effect (31, 32), the c.2992-2995delAAGA mutation was not reported previously in FA-J patients.

Although analysis of BRIP1 transcripts evidenced a fairly equal expression of both wild-type and mutant alleles [thus suggesting that the c.2992-2995delAAGA mutation, which falls in *BRIP1* last exon, is not subject to nonsense-mediated mRNA decay (43)], Western blotting on lysate from Pisa88 LCL

revealed undetectable levels of endogenous mutant protein. Consistent with this observation, the recombinant ΔBRIP1 protein was inefficiently expressed in transfected cells because it is unstable. Indeed, instability of mutated BRIP1 proteins has already been reported in studies where cells from the heterozygous carriers could be directly tested (15). Furthermore, truncated BRIP1 species have not been detected, thus far, in FA-J cells (31–33).

Given the instability of the truncated protein, the interaction with BRCA1 being preserved or disrupted would likely represent an irrelevant point *in vivo*. However, one cannot rule out the possibility that ΔBRIP1 is still expressed at low levels in tissues other than lymphoid. When coimmunoprecipitation experiments were done in transfected cells, we observed that the described c.2992-2995delAAGA mutation affects BRIP1 interaction with BRCA1. In fact, although both Ser^{990} and Phe^{993} (recently suggested to play a major role in anchoring BRIP1 binding to the first and second BRCT, respectively; ref. 16) are preserved in ΔBRIP1, it is conceivable that the altered amino acid sequence starting at 998 might have an effect on the interaction.

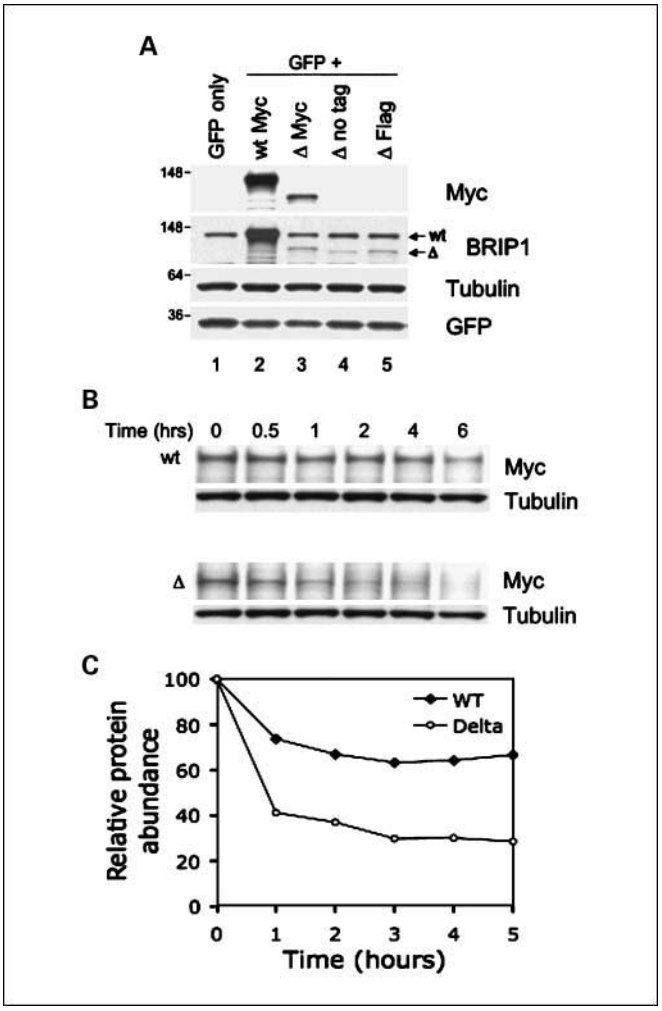

Fig. 4. Recombinant ΔBRIP1 is unstable when expressed in 293T cells. *A,* lysates from 293T cells transfected with a GFP-encoding plasmid alone (*lane 1*) or in combination with either Myc-wtBRIP1-encoding (*lane 2*) or Myc-ΔBRIP1-encoding (*lanes 3-5*) constructs were resolved by SDS-PAGE and probed with the indicated antibodies. A significantly reduced abundance of ΔBRIP1 compared with wtBRIP1 was evidenced with both anti-Myc (*lane 3* versus *lane 2*) and anti-BRIP1 (*lanes 3-5* versus *lane 2*) antibodies. *B,* cycloheximide-chase analysis of 293T cells expressing Myc-tagged wild-type or ΔBRIP1 proteins. Equal amounts of lysates, prepared at the indicated time points, were analyzed by Western blot with anti-Myc and anti-tubulin antibodies. A significant decrease of the ΔBRIP1 signal intensity was detectable already after 1 h as opposed to the steady level of wtBRIP1 signal. The experiment was repeated three times with consistent results. *C,* densitometric analysis of a representative cycloheximide-chase experiment. Films were scanned and analyzed using the Image J software. The intensity of each band, after normalization to tubulin, which served as loading control, was plotted to determine the half-lives of the wild-type and mutant BRIP1 proteins.

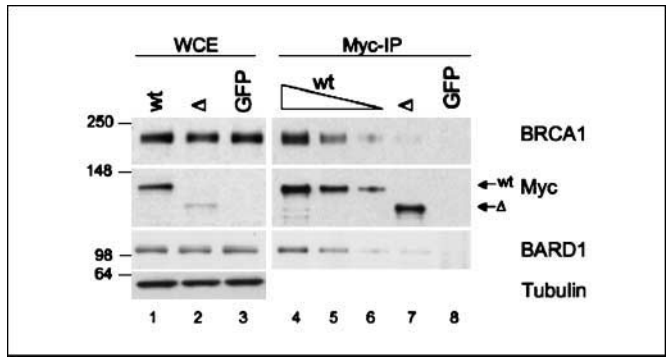

Fig. 5. ΔBRIP1 exhibits defective ability to interact with BRCA1. 293T cells were transfected with Myc-wtBRIP1-encoding, Myc-ΔBRIP1-encoding, or GFP-encoding plasmids. Lysates (*lanes 1-3*) and corresponding Myc-IP (*lanes 4-8*) were analyzed by Western blotting with the indicated antibodies. BRCA1 and BARD1 were detected in Myc-wtBRIP1 complexes even when the immunoprecipitate was diluted up to 10-fold (*lanes 4-6*), whereas they were both barely detectable in undiluted Myc-ΔBRIP1 complexes (*lane 7*). Immunoprecipitate from GFP-transfected cells (*lane 8*) served as control.

Interestingly, disease-linked mutations occurring in the BRCT domains are sufficient to abrogate BRCA1-BRIP1 interaction *in vivo* (48). Furthermore, mutations within the helicase domains of BRIP1 were shown to interfere with double-strand break repair in a BRCA1 binding-dependent manner (20). One may, therefore, hypothesize that impairment or loss of such interaction, due to *BRIP1* mutations that perturb the stability of the protein and/or its ability to bind to the partner, may be a critical cancer-predisposing event. In addition, more recent studies have reported BRCA1-independent functions of BRIP1 (49), and our observations by protein chromatography[5] suggest that the majority of BRIP1 exists in a native complex without BRCA1. In such a scenario, one can imagine that, in the patient's breast tumor tissue, which, as shown here, has lost the wild-type allele, the instability of ΔBRIP1 combined with possible functional defects of the residual mutant protein might render the tumor cells virtually BRIP1 deficient, thus having a dramatic effect on biological processes in which BRIP1 is involved.

Taken together, our data suggest a causative role for *BRIP1* in breast cancer disease reinforcing the possibility that *BRIP1* mutations may account for a small percentage of inherited breast cancer cases. A recent model of breast cancer susceptibility suggests that a sizeable fraction of the still unaccounted familial aggregation (50) may be best explained by a large number of low-penetrance genes whereby their individual small effects combine multiplicatively (7, 51). Of note, Seal et al. have recently reported that *BRIP1* truncating mutations act as low-penetrance breast cancer alleles in the British population (14). Interestingly, *BRIP1* truncating mutations were found to be

[5] R. Drapkin, unpublished data.

associated with a high relative risk for carriers ages <50 years as opposed to *BRIP1* missense variants, which were described, instead, to associate with a much lower risk (14).

In conclusion, the screening we carried out revealed a previously unreported constitutive, functionally deleterious *BRIP1* mutation in a woman with early-onset breast cancer and, importantly, loss of heterozygosity at the *BRIP1* locus in the corresponding breast tumor tissue. The scarce number of breast cancer cases in the Pisa88 pedigree is consistent with the low penetrance suggested for *BRIP1* mutations whereby some of the unaffected individuals might be potential carriers. Unfortunately, the inability to check for segregation of the mutation in other individuals of the same family did not allow us to explore the possible link between *BRIP1* mutations and neoplasia in tissues other than breast.

It would be interesting to know whether heterozygous *BRIP1* mutation carriers in FA families have an excess risk of developing breast cancer or other malignancies. Thus far, unlike *BRCA2*, whose monoallelic mutations confer high risk of breast cancer and whose biallelic mutations predispose to childhood solid and hematologic malignancies (52), *BRIP1* seems to be linked only to a modest risk of breast cancer. Nevertheless, recent studies suggest that *BRIP1* may also be inactivated via an epigenetic mechanism in certain head and neck cancers (53). Future investigation will certainly help to gain insights into the role of BRIP1 in the FA pathway and to shed light on how that relates to its tumor suppression function.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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