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ORIGINAL ARTICLE

Functional variant analyses (FVAs) predict pathogenicity in the BRCA1 DNA double-strand break repair pathway

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Abstract

Heritable mutations in the *BRCA1* and *BRCA2* and other genes in the DNA double-strand break (DSB) repair pathway disrupt binding of the encoded proteins, transport into the nucleus and initiation of homologous recombination, thereby increasing cancer risk [Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T. and Livingston, D.M. (1997) Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell*, 88, 265–275, Chen, J., Silver, D.P., Walpita, D., Cantor, S.B., Gazdar, A.F., Tomlinson, G., Couch, F.J., Weber, B.L., Ashley, T., Livingston, D.M. *et al.* (1998) Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. *Mol. Cell*, 2, 317–328]. To meet the challenge of correct classification, flow cytometry-based functional variant analyses (FVAs) were developed to determine whether variants in DSB repair genes disrupted the binding of BRCA1 to BARD1, PALB2, BRCA2 and FANCD2, phosphorylation of p53 or BRCA1 nuclear localization in response to DNA damage caused by diepoxybutane, mitomycin C and bleomycin. Lymphoblastoid cells from individuals with *BRCA1* pathogenic mutations, benign variants, and variants of uncertain significance or with known *BRCA2*, *FANCC* or *NBN* mutations were tested. Mutations in *BRCA1* decreased nuclear localization of BRCA1 in response to individual or combination drug treatment. Mutations in *BRCA1* reduced binding to co-factors, PALB2 and FANCD2 and decreased phosphorylation of p53. Mutations in *BRCA2*, *FANCC* and *NBN* decreased nuclear localization of BRCA1 in response to drug treatment, cofactors binding and p53 phosphorylation. Unsupervised cluster analysis of all and as few as two assays demonstrated two apparent clusters, high-risk *BRCA1* mutations and phenocopies and low-risk, fully sequenced controls and variants of uncertain significance (VUS). Thus, two FVA assays distinguish *BRCA1* mutations and phenocopies from benign variants and categorize most VUS as benign. Mutations in other DSB repair pathway genes produce molecular phenocopies. FVA assays may represent an adjunct to sequencing for categorizing VUS or may represent a stand-alone measure for assessing breast cancer risk.

Introduction

Breast cancer affects 11% of women, of whom 5–10% come from high-risk families with multiple affected individuals, some with ovarian cancer and oftentimes with earlier ages of onset. Among the women from high-risk families, 20% have a mutation in the genes, *BRCA1* and *BRCA2* (1). These mutations are stop-gain, small out-of-frame insertion or deletion alterations, splicing alterations and larger gene rearrangements that truncate the

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protein or remove important BRCA1 or BRCA2 protein domains (2). In addition, certain missense and in-frame indel variants disrupt protein function. In 5–10% of DNA sequencing tests (higher in some countries), missense and in-frame indel variants of uncertain significance (VUS) are identified (3). Some of these VUS may represent hypomorphic alleles, whose products retain partial activity and may be associated with only low-to-moderate risks of breast and ovarian cancer, whereas most tend to be reclassified as benign variants (2,4). The correct classification of variants represents a challenge for providing accurate genetic counseling.

Two general approaches, informatics analysis and functional analysis, have been used for curating newly identified genetic variants to determine whether they are pathogenic. One typical informatics approach, the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA), estimates the probability of pathogenicity for each variant using combined evolutionary sequence conservation (Align-GVGD—http://agvgd.iarc.fr/) with the likelihood of how the variant segregates with cancer in families, whether the variant is seen in combination with a known pathogenic mutation [which should be lethal for *BRCA1* or cause Fanconi Anemia (FA) for *BRCA2*, if the variant is pathogenic], the age of onset, the cancer type associated with the variant and the histology of the associated breast tumors (5,6). The accuracy of this method may be high for some variants, but may be overweighed for high-penetrance variants—hypomorphic alleles would show reduced penetrance for the cancer phenotype and would be non-lethal in the homozygous state (7). Alternatively, functional assays are performed in yeast or cultured cells—transcription activation, small colony phenotype, rescue of radiation resistance, ubiquitin ligase activity or cisplatin or PARB inhibitor sensitivity complementation assays (8,9). The accuracy of these methods has not been tested across a variety of variants and their adaption to the clinical environment has been limited.

DNA sequence analysis of *BRCA1* and *2* does not identify the risks for women who are members of high-risk families with mutations in other moderate-to-high-penetrance risk genes or with multiple predisposing risk single nucleotide polymorphisms (SNPs) that act in concert with one another or with the mutations in the moderate-to-high-risk genes (10). A key feature of these moderate-to-high-penetrance risk genes is that they are part of the FA-BRCA Molecular Pathway that plays a role in the repair of double-stranded breaks in DNA (11,12). In response to DNA damage, [ataxia telangiectasia mutated (ATM)] and [ataxia telangiectasia and Rad3-related (ATR)] kinases activate the FA core complex comprising FANCA, B, C, E, F, G, L and M, which then monoubiquinates FANCD2 and FANCI. This complex then interacts with other downstream proteins FANCD1 (BRCA2), FANCN (PALB2) and FANCJ (BRIP1) to incite DNA repair through homologous recombination (13). BRCA1 acts as an upstream regulator of the PALB2/BRCA2 complex, promoting its localization to DNA damage sites (14). BRCA1 exists mostly as a heterodimer with BARD1 forming a ubiquitin ligase (15).

Here, we extend the range of the flow cytometry-based functional variant analyses (FVAs) that we described previously to demonstrate how the transport of BRCA1 to the nucleus in response to DNA crosslinking and radiomimetic agents is disrupted by the presence of mutations in *BRCA1* or in other known breast cancer risk genes, creating phenocopies (16,17). Moreover, we demonstrate how known mutations in *BRCA1* and other risk genes modulate protein–protein interactions with BRCA1 and phosphorylation of p53. In turn, we demonstrate how these assays could be used as a basis for testing VUS and assessing mutations in other genes in the DSB repair pathway.

Results

Annotation of BRCA1 variant-bearing cell lines

Three sets of BRCA1 variants were assessed in these experiments, known pathogenic mutations, benign variants, and VUS (Fig. 1, Supplementary Material, Table S1). The mutations were either stopgain (p.Glu1250Ter, p.Arg1443Ter, p.Tyr1563Ter) (18–20), indel (c.66_67delAG) (21), or single nucleotide variants in the RING (p.Cys61Gly) (22), DNA binding (p.Ser1040Asn) (23), coiled-coil (p.Arg1443Gly) (19), or BCRT (p.Val1713Ala) domains (Supplementary Material, Table S1) (24,25). The variants with measurable frequencies of homozygotes for the minor allele (p.Asp693Asn, p.Ser784Leu, p.Pro871Leu, p.Glu1038Gly, p.Lys1183Arg, p.Ser1613Gly) were classified as benign and the remainders (p.Gln356Arg, p.Arg841Trp, p.Tyr856His, p.Glu1250Lys) were classified as VUS. For the cell lines from the 1000 Genomes Project with the benign variants or VUS, review of the genomic sequences demonstrated that one cell line, NA20412, had two VUS (p.Arg841Trp and p.S993N). The remainder of the cell lines did not have pathogenic mutations in known breast cancer genes (Supplementary Material, Table S1). Thus, with one possible exception, the cell lines with the benign variants truly represented controls. The assays reported here were shown to have small coefficients of variation (CVs) among the nine replications, indicating the quantitative and reproducible nature of each (see Supplementary Material, Figs S1–S3). Pairwise comparison between the CVs of controls and CVs of mutants, and phenocopies and VUS was made using the exact version of the Mann–Whitney test and yielded non-significant *P*-values among all comparisons.

Mutations in BRCA1 alter nuclear localization of BRCA1 in response to radiomimetic agents

BRCA1, BRCA2, PALB2, BARD1, FANCD2 and other members of this protein complex co-localize and form nuclear foci during S phase of the cell cycle (26,27). From their location along the synaptonemal complex in meiotic cells they play a role in recombination and genomic integrity. The process of forming nuclear foci is triggered by agents that promote DNA damage (28). To test the effect of such radiomimetic DNA damaging agents on BRCA1 nuclear localization, synchronized LCLs were treated with the DNA crosslinking drugs, mitomycin C (MMC) and diepoxybutane (DEB), and the DNA breakage drug, bleomycin (Bleo), both individually and in combination of the three, then total and nuclear BRCA1 were measured. The standardized (mean centered, standard deviation scaled per assay) amount of nuclear BRCA1 in LCLs with BRCA1 mutations was reduced compared with controls, both for the individual DEB (Mann–Whitney test $P = 0.001$), MMC ($P = 0.029$), Bleo ($P = 0.001$) or drug combination treatments ($P = 0.001$) (Fig. 2). Thus, these nuclear localization assays can readily discriminate known mutations from wild-type controls. The BRCA1 nuclear localization assays showed strong correlation with each other; the strongest correlations were observed for the DEB and Bleo treatments (see Supplementary Material, Fig. S4).

Mutations in BRCA1 alter binding to co-factors PALB2 and FANCD2

Mutations in *BRCA1* affect binding to cofactors—either by modifying the site at which the binding takes place, by modifying the folding of the BRCA1 protein and the protein binding sites, or by modifying the quantity of BRCA1 within the cells. BARD1 binds to the RING domain and PALB2 binds to the coiled-coiled domain. Other factors do not bind directly to BRCA1, but rather to the

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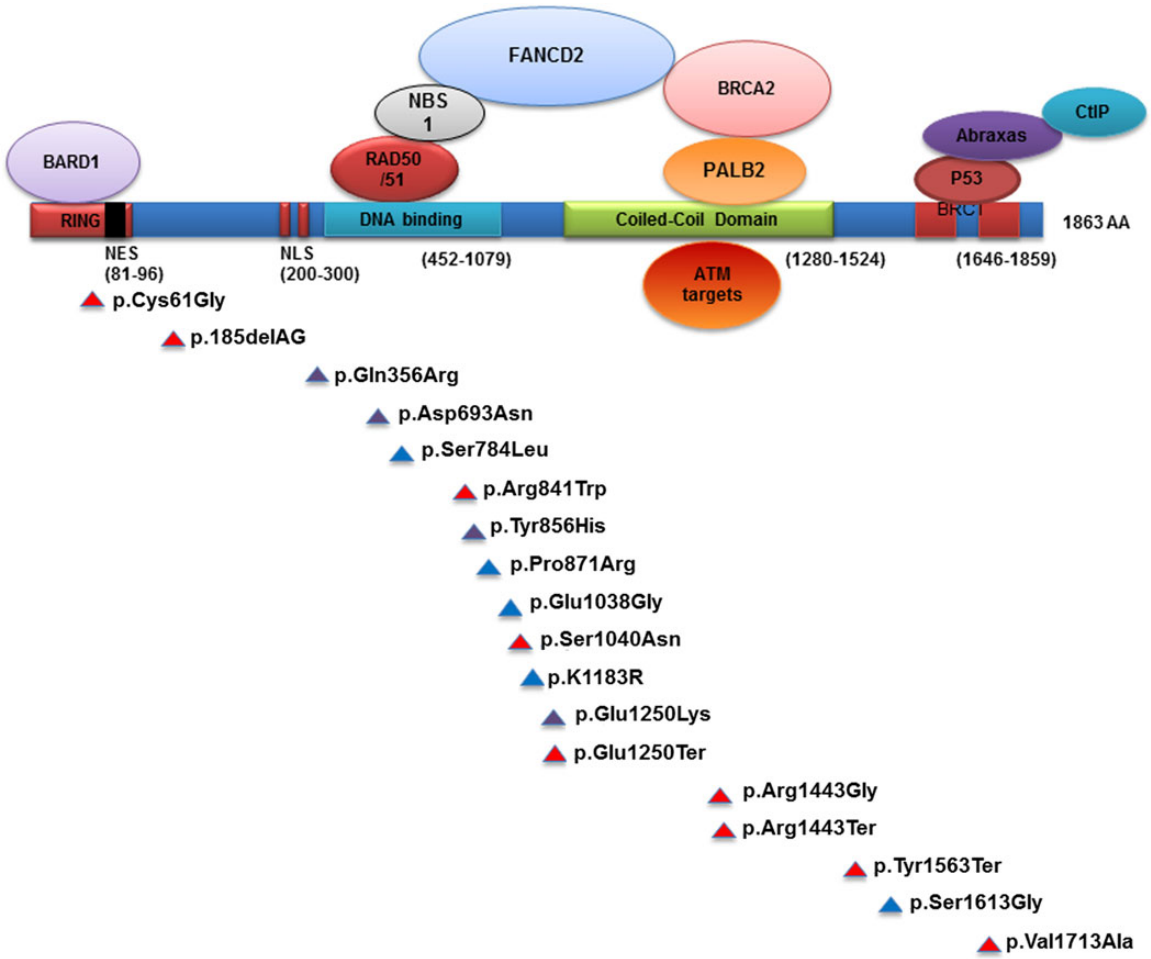

Figure 1. Map of BRCA1 protein demonstrating binding partners and binding sites. Cell lines included known pathogenic mutations (red), benign wild-type variants (blue) and VUS (purple).

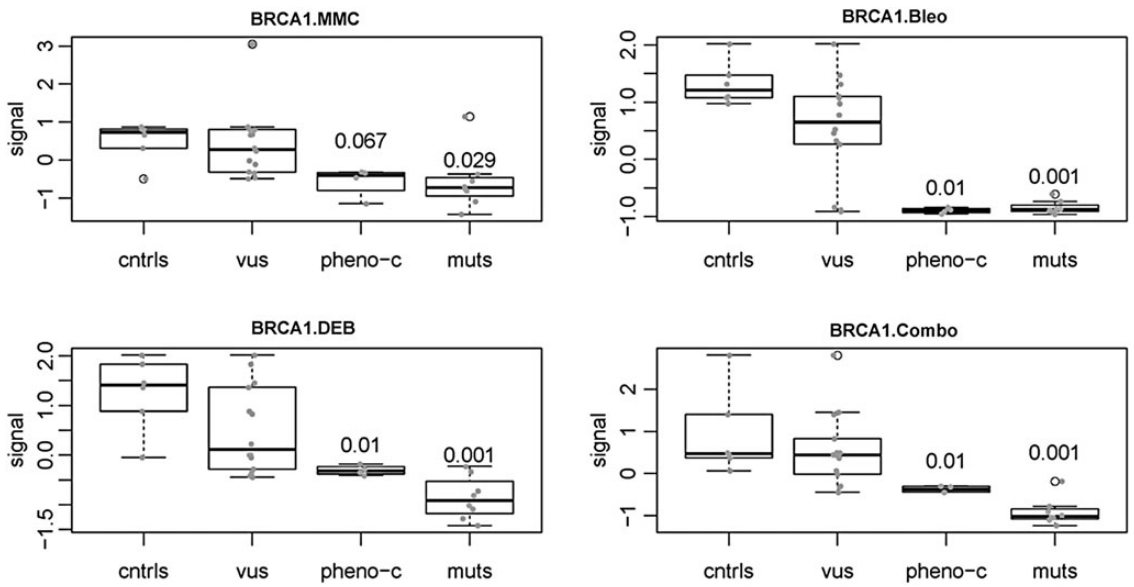

Figure 2. Cell culture in the presence of radiomimetic agents reduces nuclear localization in *BRCA1* mutation-bearing and phenocopy cell lines. Boxplots comparing the standardized localization of BRCA1 in response to MMC, Bleo, DEB or Combo treatment for control (cntrls), VUS, phenocopy (Pheno-C) or mutant (muts) LCLs. *P*-values of the pairwise comparisons of the various distributions of treatment groups relative to cntrls by the Mann–Whitney test are shown.

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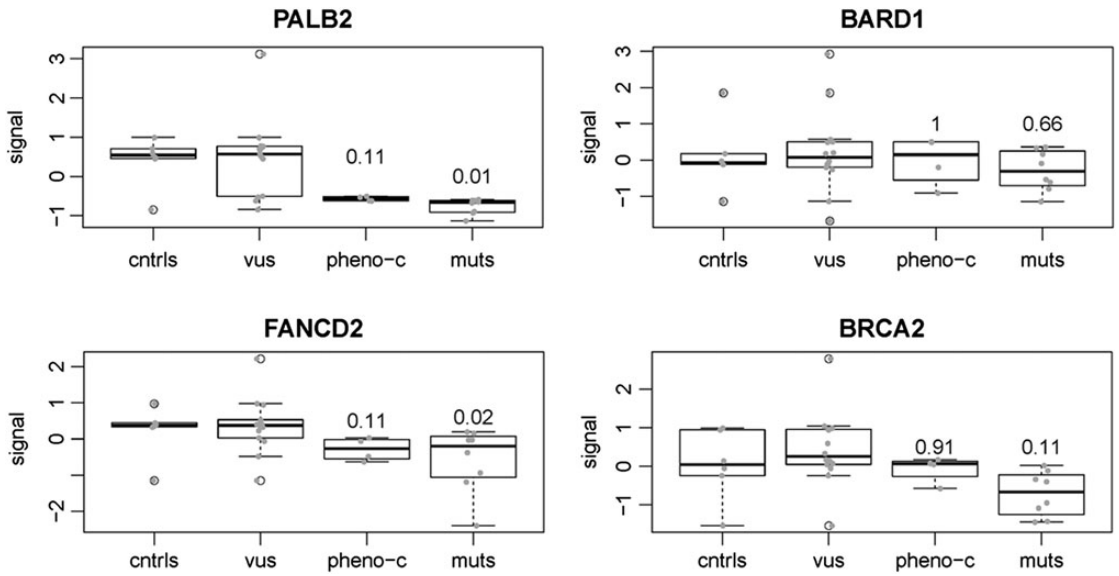

Figure 3. Binding of BRCA1 to interacting proteins is reduced in *BRCA1* mutation-bearing and phenocopy cell lines. Boxplots comparing the standardized binding of PALB2, BARD1, FANCD2 and BRCA2 to BRCA1 in cntrls, VUS, Pheno-C or muts LCLs. *P*-values of the pairwise comparisons of the various distributions of treatment groups relative to cntrls by the Mann–Whitney test are shown.

binding partners of BRCA1 forming a protein binding complex (Fig. 1). As observed in the protein co-immunoprecipitation assay, the binding of BRCA1 was markedly reduced to PALB2 (Mann–Whitney $P = 0.01$) and FANCD2 ($P = 0.02$), but not to BARD1 and BRCA2 in the mutant LCLs compared with control LCLs (Fig. 3). Thus, two of these binding assays discriminated high-risk mutations from benign variants. The binding of these proteins to BRCA1 was the same among the control and VUS cell lines. These PALB2, BRCA2 and FANCD2 binding assays showed strong correlation with each other, reflecting the known interaction of these factors with each other and their shared role in initiating homologous recombination (see Supplementary Material, Fig. S4) (13).

Mutations in BRCA1 decrease phosphorylation of p53

One of the functions of BRCA1 is to phosphorylate p53, an activity that may be altered by mutations in the *BRCA1* gene. For each of the cell lines, both total p53 and phospho-p53 were measured by the digital cell western (DCW) technique. Total p53 was the same among the control, *BRCA1* mutant and VUS LCLs (Fig. 4). Phospho-p53 and phospho-p53/total p53 ratio were reduced in the mutant LCLs compared with control LCLs (Mann–Whitney $P = 0.007$, $P = 0.005$, respectively); thus, measuring phospho-p53 may enhance the accuracy of identifying mutations. Phospho-p53 and phospho-p53/total p53 ratio were strongly correlated with each other and were also correlated with the nuclear localization assays, especially following treatment with DEB, Bleo or combination (Combo) (see Supplementary Material, Fig. S4).

A cluster of BRCA1 mutations that includes BRCA2, FANCC and NBN phenocopies

A heatmap of the unsupervised cluster analysis of all of these assays demonstrated two apparent clusters, suggestive of two categories, the mutants, which are interdigitated with each other, and the controls and VUS, which are clustered together (Fig. 5). The overlap of the VUS and controls is consistent with previous reports of >80% of VUS representing benign variants (4). Most of the FVAs correlated with each other. The pairwise correlation analysis suggests that one nuclear localization assay in response to a radiomimetic agent and the phospho-p53 assay would suffice for capturing the range of phenotypic effects in this data set (Supplementary Material, Table S2).

Two pathogenic mutations in *BRCA2* (p.Ser1982Argfs and p.Trp194Ter) as well as single pathogenic mutations in *FANCC* (c.456+4A>T) and *NBN* (c.657del5) in LCLs were assessed in all of these assays. These mutant cell lines showed reduction for the DEB (Mann–Whitney $P = 0.01$), Bleo ($P = 0.01$), and combo nuclear localization assays ($P = 0.01$), p53 phosphorylation assay ($P = 0.04$) and phospho-p53/total p53 ratio ($P = 0.038$), suggesting phenocopies for mutations in *BRCA1* (Figs 2, 4 and 5). The heatmap demonstrated that the *BRCA2*, *FANCC* and *NBN* LCLs were interdigitated with the BRCA1 mutations, supporting the idea that they represent phenocopies.

Discussion

FVAs using modified flow cytometry with fluorescently labeled antibodies were developed to address the need for high-throughput, quantitative immunoassays that assess the phenotypic effects of genetic variants on protein quantification, post-translational modification and interactions with other proteins. The selection of these assays assesses the role of BRCA1 and its binding partners in controlling the assembly and activity of macromolecular complexes that monitor double-strand breaks (DSBs) and DNA crosslinks, a role that has been previously termed, 'chromosomal custodian' (29). The FVAs comprise two principal methods. In DCW, fluorescent probes are annealed at room temperature to permealized fixed cells rapidly and assessed with modified flow cytometry to quantify proteins and their post-translational modifications. Based on individual signal intensity, each cell is measured independently for its protein expression level. For each experiment, 100 000 to a million data points are normalized and calculated as a digital value, hence DCW. Here,

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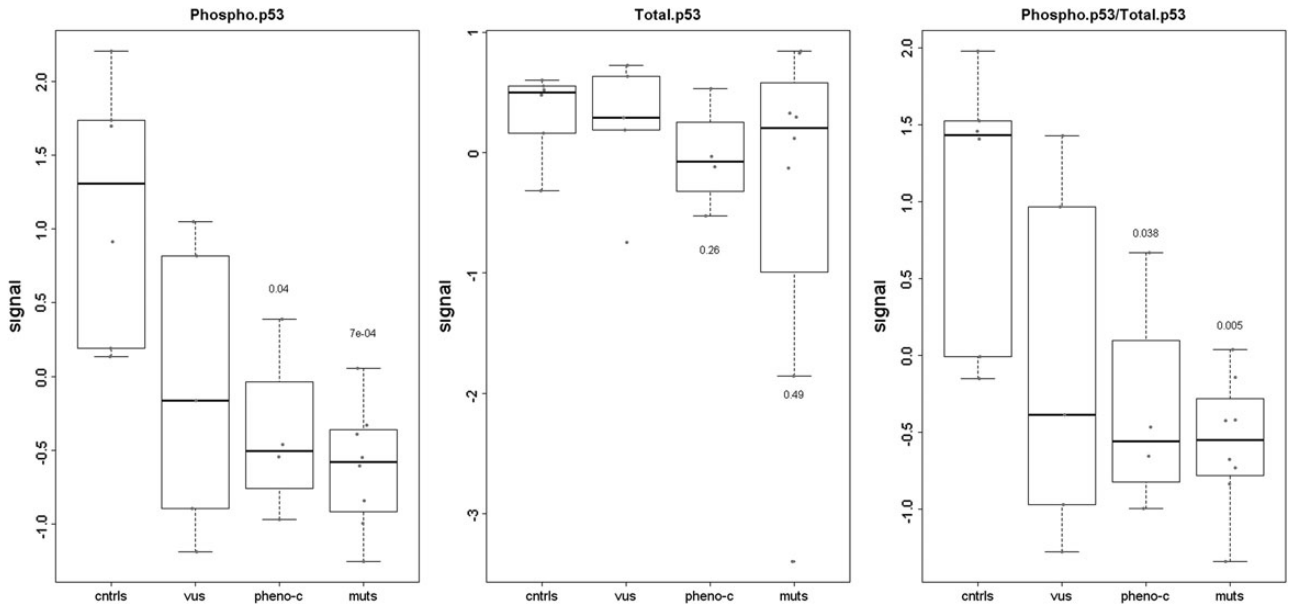

Figure 4. Phosphorylation of p53 is reduced in *BRCA1* mutation-bearing and phenocopy cell lines. Boxplots comparing standardized phospho-p53, total p53 and phospho-p53/total p53 ratio measured by DCW in cntrls, VUS, Pheno-C or muts LCLs. *P*-values of the pairwise comparisons of the various distributions of treatment groups relative to cntrls by the Mann–Whitney test are shown.

the technique was modified to include not only measurement of total and phospho-p53, but also measurement of BRCA1 in intact cells or the nuclear fraction. In turn, the nuclear localization of BRCA1 was augmented by exposing the cells to the radiomimetic drugs, MMC, DEB and Bleo. In the protein co-immunoprecipitation assay, a specific protein complex is bound to an antibody-coupled bead matrix, and then quantified for interactions with various binding partners. In the case of BRCA1, the bound proteins included PALB2, FANCD2, BRCA2 and BARD1. These FVAs are rapid, quantitative, low-cost and modular for multiple analytes. Thus, they can be readily adapted to the clinical laboratory unlike the previously described transcription activation, small colony phenotype, rescue of radiation resistance, ubiquitin ligase activity or cisplatin or PARB inhibitor sensitivity complementation assays that require transfection, use of non-host cells and extended periods of culture (8,9). For example, the cisplatin or PARB inhibitor sensitivity complementation assays in mouse ES cells takes 8 weeks from start to finish versus the 2 days for the FVAs (9). The aggregate large number of data points collected for each assay assures that the results are highly quantitative with narrow CVs and thus can measure small differences between different variants or treatment conditions. The assays can assess any variant in the *BRCA1* gene and are not confined to the BRCT domain as were the previously described proteolysis, phosphopeptide binding, and transcriptional activation assays (30).

When FVAs were applied to *MAP3K1* variants, three different classes were observed—*full gain-of-function mutations* whose molecular effects exceeded a threshold and produced a physical phenotype of 46, XY complete or partial gonadal dysgenesis, *partial gain-of-function alleles* whose molecular effects exceeded a threshold for some targets, but not others and did not produce a recognized physical phenotype, and *normal variants*, whose activities were constant for the various assays over the multiple controls (16,17). Conversely, when FVAs were applied to *BRCA1* variants, three different classes were observed—*full loss-of-function mutations* whose molecular effects exceeded a threshold and produced a physical phenotype of reduced localization, reduced binding and reduced phosphorylation, *partial loss-of-function or hypomorphic alleles* whose molecular effects were reduced for some assays, but not others, and did not produce a recognized physical phenotype (observed as blocks of red or dark orange in the heatmap (Fig. 5), and *normal variants*, whose activities were constant for the various assays over the multiple controls. Hypomorphic alleles have been observed previously for some *BRCA1* variants, such as p.Arg1699Gln in the BRCT domain of BRCA1 that abrogates the repression of microRNA-155 and is associated with a 2-fold increase in cancer risk (31).

Although most of the assays correlated with one another, sometimes discordant effects were observed (Supplementary Material, Table S2). However, when clustered, these assays blunted the hypomorphic effects of some alleles and resulted in two overall allelic classes—mutations and benign variants. As noted, this finding is in keeping with the observation that most VUS have been reclassified over time as benign variants, as additional data have accumulated (4). These observations are compatible with the previous suggestion that gene heterozygosity suffices for cancer predisposition, and may trigger a low, but quantitatively significant, level of genome instability that accumulates over many cell divisions (29).

Although the sample size was modest, mutations in other DSB repair genes produced phenocopies of the mutations in *BRCA1*. Reflecting the shared mechanisms, BRCA2 and FANCD2 bind to the BRCA1 complex, whereas NBN co-localizes with this complex at the site of the DSB. These observations suggest that this suite of FVAs may assess not only risks of VUS in *BRCA1*, but of any VUS in genes in the DSB pathway. Clearly to achieve clinical utility and validity, the assays will have to be adjusted to circulating B cells (or other circulating cells that express these proteins) and tested over a wider range of variants. As noted, only two assays may be required. Once validated, these assays could serve as an adjunct to sequencing when a VUS has been identified or might stand alone without sequencing for assessing breast and ovarian cancer risks.

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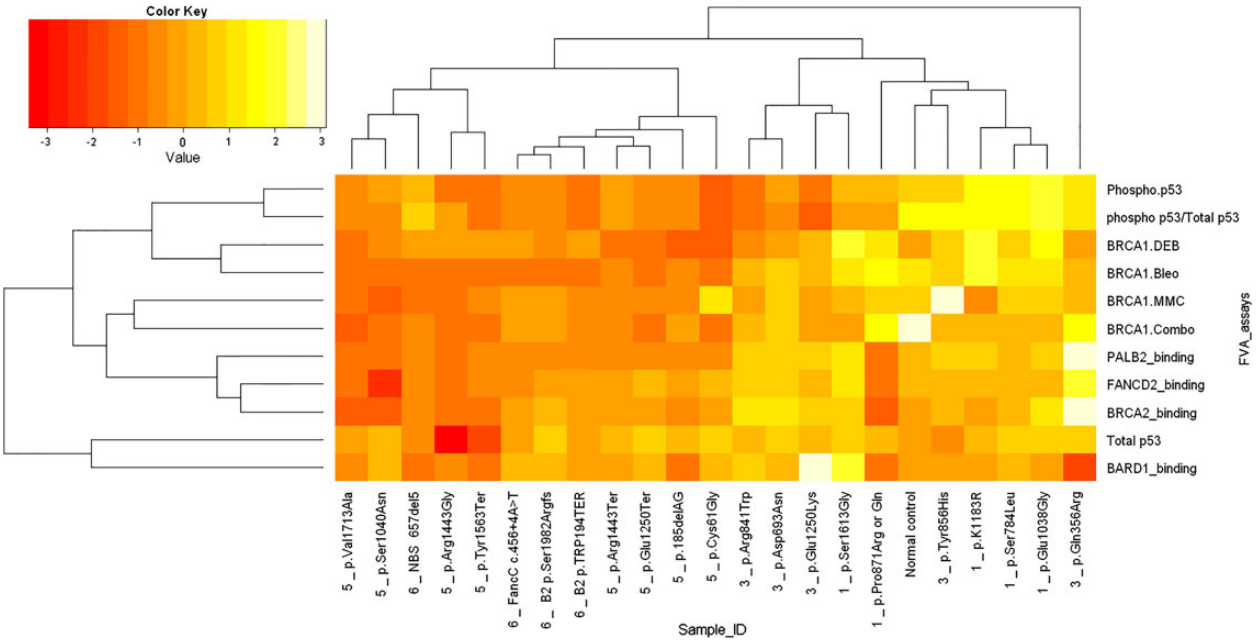

Figure 5. *BRCA1* mutation-bearing and phenocopy cell lines form a distinctive cluster. Heatmap of protein binding, p53 phosphorylation, and nuclear localization assays demonstrates two clusters—mutants and phenocopies (left) and controls (right). The mutants and phenocopies are interdigitated suggesting their similarity and the controls and VUS are interdigitated suggesting their similarity. B1, BRCA1;,B2, BRCA2; 1, control; 3, VUS; 5, mutant; 6, phenocopy.

Materials and Methods

Cell lines and mutations

Lymphoblastoid cell lines (LCLs) from subjects with sequence-identified variants in *BRCA1* (GM14097 p.Cys61Gly, GM14090 c.66_67delAG, GM20412 p.Arg841Trp, GM13711 p.Ser1040Asn, GM13713 p.Glu1250Ter, GM14637 p.Arg1443Ter, GM13710 p.Arg1443Gly, GM13708 p.Tyr1563Ter, GM14092 p.Val1713Ala), *BRCA2* (GM14805 p.Trp194Ter, GM14170 p.Ser1982Argfs), *FANCC* (GM20731 c.456+4A>T) and *NBN* (GM15788 c.657_661del5) were purchased from the Coriell Institute Human Genetic Cell Repository (Camden, NJ). LCLs with other sequence-identified variants in the *BRCA1* gene from the 1000 Genomes Project (GM12873 p.Gln356Arg, HG00099 p.Asp693Asn, GM 19740 p. Ser784Leu, GM18628 p.Tyr856His, GM11995 p.Pro871Arg, GM10850 p.Glu1038Gly, GM07056 p.Lys1183Arg, GM19084 p.Glu1250Lys, GM11894 p.Ser1613Gly) were also purchased from the Coriell Institute. The variants were classified as pathogenic mutations, benign variants or VUS (Supplementary Material, Table S1). Pathogenic variants were either truncating stopgain or indel, or SNVs with consistent reports of pathogenicity across databases and studies. Variants with observed homozygotes in the *BRCA1* gene for the minor allele were classified as benign, because homozygosity for deleterious mutations has been reported to be embryonic lethal (32,33). Variants with discordant reports were classified as VUS. The BRCA1-deficient (HCC1937), and heterozygous (HCC1937 BL) cell lines were purchased from the American Type Culture Collection, Manassas, VA., 20110 (34).

The sequences of the cell lines derived from the 1000 Genomes Project were analyzed for mutations in other genes reported as moderately to highly penetrant for breast cancer when mutated (AXIN2, BARD1, BMPR1A, BRCA1, BRCA2, FANCD2, GEN1, MLH1, PALB2, POLD1, POLE, PMS2, RAD51C, RAD51D, TP53, XRCC2). The variant annotation was performed by literature review as well as reports in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar, last accessed, 11 February 2015), the Sharing Clinical Reports Project (SCRP—http://www.clinicalgenome.org, last accessed, 11 February 2015), and the Breast Cancer Information Core Database (http://lgdfm3.ncifcrf.gov/bic/BIC.html, last accessed, 11 February 2015).

EBV immortalized B-LCLs were maintained in RPMI 1640 and DMEM supplemented with 10 and 15% fetal bovine serum, respectively, and cultured in CO_2 jacketed 37°C incubators according to the manufacturer's recommendations (GIBCO, Life Technologies, Grand Island, NY, USA). The cells were starved for 24 h for cell synchronization prior to all experiments. This study was approved by the institutional review board of the Albert Einstein College of Medicine.

Antibodies and FVA

Antibodies were obtained for native or phosphorylated forms of proteins. For protein co-immunoprecipitation assays, the following antibodies were used: BRCA1 (Origene TA310042), BRCA2 (Origene TA313520), PALB2 (Origene TA306814), BARD1 (Origene TA313499), FANCD2 (Origene TA307630). These antibodies were specific to their targets, as judged by western blot analysis that included the BRCA1-deficient (HCC1937) and heterozygous (HCC1937 BL) cell lines (Supplementary Material, Fig. S5) Total p53 (BD554294), and Phospho-p53 (BD560282). The BRCA1 antibody was covalently coupled to magnetic beads and the efficiency of binding was determined to be >90% (data not shown). All of the antibodies were conjugated with fluorochromes (Innova Bio lightning-link APC: 326-0010, FITC: 322-0010, PECy7: 762-0010, APCCy7: 765-0010, PECy5.5: 761-0010 and RPE: 703-0010).

Functional variant analyses

The general approach for the analyses included cell culture ± use of radiomimetic agents. The cell culture was followed by cell

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fixation, permealization and binding with labeled antibodies within cells or cell lysis with binding of BRCA1 protein complexes to beads and subsequent antibody binding to interacting proteins. For a typical experiment, 2 million isolated LCLs were cultured in suspension in each well of a six-well plate in RPMI medium (Invitrogen A2780), supplemented with 15% (v/v) FBS Defined Grade, 50 units/ml penicillin, and 50 g/ml streptomycin at 37°C with 5% CO_2. Three biological replicates and three technical replicates each were performed. The previously described methods were modified as follows (16,17).

For the BRCA1 nuclear localization assays, the cultured cells were serum starved for 24 h prior to treatment either by the vehicle or the radiomimetic drugs: 0.1 µg/ml Bleo, 0.5 µg/ml DEB and/or 1.5 µg/ml MMC or combination for 24 h. For nuclear extraction of cells, 200 000 cells cultured in 96-well plate were washed with PBS, then lysed using Cytolysis Buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$, 2 mM EDTA, 10% glycerol, 1% NP-40, 1% Triton X-100, 2.5 mM β glycerophosphate, 1 mM NaF, 1 mM dithiothreitol and complete protease inhibitors—Roche). This resulted in cytolysis of ~50% of cells. The nuclei and intact cells were collected by centrifugation, then resuspended in Staining Buffer (50 mM Tris–HCl pH 7.4, 100 mM NaCl, 5% FBS, 0.02% sodium azide). The whole prep was probed with fluorochrome-conjugated anti-BRCA1 and endoplasmic reticulum antibodies (whole-cell discrimination), stained with DAPI and analyzed by BD FACSAria II and quantified.

The binding of BRCA1-interacting proteins was measured by the FVA co-immunoprecipitation assays on Dynabeads. Using BRCA1 as bait, the binding of BRCA2, PALB2, BARD1 and FANCD2 were gated and quantified using fluorochrome-conjugated antibodies. The binding were compared for the mutant, VUS and wild-type LCLs. The relative abundance of total and phospho-p53 were measured by the FVA DCW analysis, as described previously to quantify ERK1/2 and p38 in cells using fluorochrome-conjugated anti-native and anti-phosphorylated protein antibodies (16,17). The quantified total p53, phospho-p53 and phospho-p53/total p53 ratio were compared for the mutant, VUS and wild-type LCLs.

Quantitative analysis

Flow cytometry was performed using BD FACSAria II equipped with Blue (488 nm), Green (532 nm), Yellow (561 nm), Red (638 nm) and Violet lasers (407 nm). Briefly, Side-scatter height was plotted against Side-scatter width to exclude doublets and to identify fixed intact cells and nuclei. Each target signal was normalized to total gated population of the single cell/bead measurements. All experiments were standardized with single color controls made using fluorophore labeled or bait target antibodies conjugated to Dynabeads using the methods described (17). To make results comparable across assays and cell types, the intensity signals were log transformed and standardized (mean centered, standard deviation scaled) on an assay basis. Unsupervised hierarchical cluster analysis was performed for all technical replicates from individual assays and presented as a dendrogram and heatmap. The mean of nine technical replicates was calculated for each individual cell line assay, and box plots and Mann–Whitney tests were performed to determine whether the differences in nuclear localization, protein binding and p53 phosphorylation were significant between cell lines from wild-type and other subgroups (phenocopies and mutants). The dependences among assays was assessed and presented using a Pearson correlation matrix (Supplementary Material, Fig. S4). Using all 11 assays, K-means clustering with $k = 2$ was used to classify the cohort of samples (i.e. cell lines) into $k = 2$ subgroups (wild-type versus mutant). K-means clustering ($k = 2$) was also conducted using each pair of assays and each of the 11 assays alone. The clustering results of each individual and paired assay were compared with the clustering of all 11 assays and reported as the ϕ coefficients (equivalent to the absolute values of Pearson's correlation coefficients) in Supplementary Material, Table S2.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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