A Yeast Recombination Assay to Characterize Human 
BRCA1 Missense Variants of Unknown Pathological 
Significance

Maria Adelaide Caligo,1 Fabrizia Bonatti,1 Lucia Guidugli,1 Paolo Aretini,1 and Alvaro Galli2*

1Sezione Genetica Oncologica Divisione di Patologia Dipartimento di Oncologia Università di Pisa, Pisa, Italy
2Laboratorio di Terapia Genica e Molecolare, Istituto di Fisiologia Clinica, Italian National Research Council (CNR), Pisa, Italy

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Abstract: The BRCA1 tumor suppressor gene is found mutated in familial breast cancer. Although many of the mutations are clearly pathological because they give rise to truncated proteins, several missense variants of uncertain pathological consequences have been identified. A novel functional assay to screen for BRCA1 missense variants in a simple genetic system could be very useful for the identification of potentially deleterious mutations. By using two prediction computer programs, Sorting Intolerant from Tolerant (SIFT) and Polymorphism Phenotyping (PolyPhen), seven nonsynonymous missense BRCA1 variants likely disrupting the gene function were selected as potentially deleterious. The budding yeast Saccharomyces cerevisiae (S. cerevisiae) was used to test these cancer-related missense mutations for their ability to affect cell growth and homologous recombination (HR) at the HIS3 and ADE2 loci. The variants localized in the BRCA1 C-Terminus (BRCT) domain did not show any growth inhibition when overexpressed in agreement with previous results. Overexpression of either wild-type BRCA1 or two neutral missense variants did not increase yeast HR but when cancer-related variants were overexpressed a significant increase in recombination was observed. Results clearly showed that this genetic system can be useful to discriminate between neutral and deleterious BRCA1 missense variants.

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Key words: BRCA1 missense variants; Saccharomyces cerevisiae; homologous recombination; yeast small colony phenotype assay

METHODS

Introduction

In the Western world, one of the most common neoplasia of women is breast cancer, which affects approximately 6 out of 100 women before the age of 74 years. About 80% of breast cancers is sporadic and is diagnosed in women without any familial aggregation. However, the remaining 20% of cases are inheritable and about 40% of those are caused by mutations in one of the two tumor suppressor genes, BRCA1 (MIM# 113705) and BRCA2 (MIM# 600185) [Miki et al., 1994; Wooster et al., 1994].

The tumor suppressor BRCA1 gene encodes a nuclear phosphoprotein that is involved in many cellular processes including homologous recombination (HR) and DNA repair [Narod and Foulkes, 2004]. The gene is highly polymorphic, with many common single-base changes. Many of the germ-line mutations found in BRCA1 give rise to truncated nonfunctional proteins that can predispose to breast and ovarian cancer. However, the pathological consequence of many missense mutations found in breast and/or ovarian cancer families remains to be ascertained.

Several predictive methods have been proposed to distinguish cancer-related variants from neutral polymorphisms, including the pattern of cosegregation of the variant with disease in affected carrier families [Goldgar et al., 2004], the nature and the position of amino acid substitution [Grantham, 1974; Mirkovic et al., 2004], the degree of conservation among species [Abkevich et al., 1993], and the inactivation of the wild-type allele either by loss of heterozygosity (LOH) or promoter hypermethylation in the tumor of the carrier[Blackwood and Weber, 1998]. Recently, a combined method that integrates the molecular biology data with the familial and clinical history has been useful classify the missense mutations [Osorio et al., 2007]. Several functional assays to identify missense mutations of BRCA1 are also available [Carvalho et al., 2007]; some methods take advantage of the ability of the BRCT domain to activate the transcription of a reporter gene both in mammalian cells and in Saccharomyces cerevisiae (S. cerevisiae) [Phelan et al., 2005]. Other functional assays rely on the ability of BRCA1 mutations to rescue the radiation resistance of the BRCA1 defective HCC1937 human cell line or are based on the determination of the ubiquitin ligase activity, mediated by the interaction of the RING domain of BRCA1 with the BARD1 gene [Carvalho et al., 2007; Scully et al., 1999].

The expression of human wild-type BRCA1 in the budding yeast S. cerevisiae inhibits growth and this peculiar phenotype has been exploited to characterize several missense mutations [Coyne
et al., 2004; Humphrey et al., 1997]. A functional assay named “yeast small colony phenotype assay” (SCP) has been proposed to evaluate the pathogenicity of BRCA1 missense mutations. In general, the SCP assay can only identify mutations localized within the BRCT and this is in agreement with predictions based on structure modeling [Coyne et al., 2004].

In this study, we identified several BRCA1 missense variants by the mutational analysis of 276 breast and/or ovarian cancer families. By in silico analysis, seven missense variants were identified as potentially not functional. Then, we tested them for the identification of potentially deleterious mutations by SCP assay. Moreover, as BRCA1 is involved in HR and the yeast is an excellent genetic model system to investigate factors affecting HR, we determined the effect of BRCA1 missense variant expression on yeast HR at two distinct chromosomal loci (HIS3 for intrachromosomal and ADE2 for interchromosomal recombination). Results clearly indicate that the yeast S. cerevisiae can be a very helpful tool to classify BRCA1 missense variants.

Materials and Methods

Samples

DNA samples from 276 individuals belonging to 276 breast and/or ovarian cancer families were analyzed for BRCA1 and BRCA2 germline mutations. All patients were from the University Hospital of Pisa. The selection criteria were as follows: 1) occurrence of two or more cases of breast and/or ovarian cancer in the same individual. 124 HUMAN MUTATION, Vol. 30, No. 1, 123–133, 2009

Mutation Screening

The screening of mutations in the BRCA1 and BRCA2 genes was performed by direct sequencing. DNA sequencing was carried out using BigDye terminator v 3.1 mix (Applera-Applied Biosystems, Foster City, CA) and different primers (available upon request). Capillary gel electrophoresis and data collection was performed on an automated DNA sequencer (ABI 3100; Applera-Applied Biosystems). Sequences analysis were performed using SeqScape Software (Applera-Applied Biosystems).

Variant Selection

To identify nonsynonymous amino acid changes likely to disrupt BRCA1 gene function we used two comparative evolutionary bioinformatic programs: Sorting Intolerant From Tolerant (SIFT; http://blocks.fhcrc.org/sift/SIFT.html) and Polymorphism Phenotyping (PolyPhen; http://tux.embl-heidelberg.de/ramensky/polyphen.cgi).

SIFT is a multiple sequence alignment tool based on the premise that important amino acids will be conserved among species in a protein family, so that changes of amino acids conserved in the family should affect protein function [Ng and Henikoff, 2003]. PolyPhen is an automatic tool for prediction of possible impact of an amino acid substitution on the structure and function of a human protein [Ramensky et al., 2002].

The DNA mutation numbering is based on the cDNA sequence of BRCA1 (GenBank: U14680.1), with a “c” symbol before the number. For the numbering, +1 corresponds to the A of the ATG translation initiation codon in the reference sequence, following the journal guidelines (www.hgvs.org/mutnomen). Otherwise, the DNA mutation nomenclature is as in the Breast Cancer Information Core (BIC) database according to reference sequence GenBank U14680.1, where +1 corresponds to the first base of exon 1.

LOH Analysis

LOH analysis was carried out on tumor tissue excised from the index individual case of each family. Neoplastic and normal cells were collected separately by manual microdissection from 7-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, Hilden, Germany). Amplification was carried out by PCR using primers located in the exon in which the missense variant was identified and the resulting products were analyzed by sequencing on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences analysis were performed using SeqScape Software. If one of the two bases identified in the electropherogram at the same position, corresponding to the variant position, disappeared, the sample was scored as positive for LOH.

BRCA1 Promoter Methylation Profile

DNA was extracted from formalin-fixed paraffin-embedded tumoral sections after manual microdissection. The methylation profile of BRCA1 gene promoter was evaluated by methylation specific PCR (MSP) using the EZ DNA methylation kit (Zymo Research, Orange, CA). The method is based on the conversion of unmethylated cytosines to uracil by sodium bisulfite treatment. By using specific primers that distinguish methylated cytosines from unmethylated cytosines uracil-transformed in the same promoter region, it is possible to PCR amplify differentially methylated DNA. We considered a promoter as methylated if a PCR product is obtained by using primers specific for methyl-CpG.

Plasmids and DNA Manipulation

The plasmid YCpGAL::BRCA1 which contains the human BRCA1 gene under the galactose-inducible promoter GAL1p was obtained from Craig Bennett (Duke University, Durham, NC) [Westmoreland et al., 2003]. The missense variants were constructed by site-directed mutagenesis with specific oligonucleotides using QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primer sequence is available upon request.

Plasmid DNA preparation was carried out using the Wizard miniprep kit (Promega, Madison, WI). The mutations were checked by DNA sequence analysis.

Yeast Strain

The diploid strain RS112 of S. cerevisiae, obtained from Robert Schiestl (University of California, Los Angeles [UCLA], Los Angeles, CA) has the following genotype: MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-A98 trp5-27/TRP5 ade2-40/ade 2-101 ile1-92/ile1-92 arg4-3/ARG4 his3A5–>pRS6-his3A3–/his3-A200 lys2/lys2-801. Media preparation and yeast culturing was carried out according to standard techniques. Yeast was transformed with plasmid DNA by using the lithium acetate method with single-strand DNA as carrier, following the procedure described in [Gietz et al., 1995]. Transformants were selected in solid medium lacking uracil (SC–URA). Colonies were grown for 4 days at 30°C and analyzed further.
Protein Extract Preparation and Western Blotting

The level of BRCA1 expression was determined in yeast cells transformed with the BRCA1 expression vector after 24 hr induction in galactose medium. Single clones were pregrown in 10–20 ml of SC–URA glucose medium for 24 hr at 30°C. Then, cell pellets were washed in water and split in two aliquots: one was inoculated in 20 ml of SC–URA glucose and the other one in 20 ml of SC–URA galactose. The cultures were incubated at 30°C for 24 hr under constant shaking. Thereafter, pellets were washed twice in ice cold water and resuspended in 0.5 ml of suspension buffer (50 mM KCl, 5 mM MgCl₂, 0.1 M EDTA, 25 mM HEPES, 5 mM dithiothreitol, 0.3 M (NH₄)₂SO₄, and 10% glycerol; pH 7.4) plus 10 μl of protease inhibitor solution (4.4 mg phenylmethylsulfonyl fluoride, 62 mg pepstatin, 50 mg chomestatin, and 725 ml DMSO in 1 ml H₂O). Total protein extracts were prepared according to the method of Kimmerly et al. [1988]. Cell lysis was performed by vortexing five times for 30 s with acid-washed glass beads [Del Carratore et al., 2004]. A total of 30 μg of protein yeast extract was electrophoresed on a 6% SDS-polyacrylamide gel and transferred overnight in a cold room on a polyvinylidene fluoride membrane. BRCA1p is analyzed using anti-BRCA1 monoclonal antibody Ab4 diluted 1:100 (clone SD118; Calbiochem, Gibbstown, NJ), which recognizes the exon 11 of the BRCA1 protein. Anti-mouse horseradish peroxidase-linked (Amersham Biosciences, Piscataway, NJ), which recognizes the exon 11 of the BRCA1 protein. As loading control, we determined the level of the 3-phosphoglycerate kinase (PGK) with the anti-β-actin3PGK antibody from Molecular Probes (Invitrogen, Carlsbad, CA).

SCP Assay

For each missense variants three independent transformants were analyzed. Single colonies were picked up from –URA glucose plates and inoculated in 5 ml liquid –URA medium containing 2% glucose. Under these growth conditions the synthesis of BRCA1 is repressed. Cultures were then incubated at 30°C for 48 hr under constant shaking. Thereafter, cells were counted, diluted in sterile distilled water, and plated in SC–URA plates containing 5% galactose. Usually, 200–250 cells per dish were plated. Under these conditions, the GAL1 promoter confers a high level of protein expression [Galli and Schiestl, 1998]. Plates were incubated at 30°C for 4–6 days or until colonies reached the largest size. Then, three colonies were picked up from each plate, resuspended in 1 ml sterile water, and counted with a hemocytometer.

Intra- and Intercromosomal Recombination Assay

The RS112 strain was constructed from the haploid RSY6 and, consequently, carries the same intrachromosomal recombination substrate as RSY6 [Schiestl et al., 1988, 1989]. This substrate consists of two his3 alleles, one with a deletion at the 3’ end and the other with a deletion at the 5’ end, which share 400 bp of homology. These two alleles are separated by the LEU2 marker and by the plasmid DNA sequence. An intrachromosomal recombination event leads to HIS3 reversion and loss of LEU2 [Schiestl et al., 1988]. The diploid RS112 strain also contains the two alleles ade-2-40 and ade-2-101, located in two homologous chromosomes that allow the measurement of interchromosomal recombination events [Schiestl, 1989]. To determine the frequency of intrachromosomal and interchromosomal recombination, single colonies were inoculated into 5 ml of SC–URA–LEU medium and incubated at 30°C for 24 hr. Thereafter, cultures were washed twice in sterile distilled water and counted. For each BRCA1 variant as well as the BRCA1wt and the controls, aliquots containing 10⁷ cells were inoculated in 5 ml of SC–URA–LEU medium containing 5% galactose. In parallel, the same number of cells was inoculated in 5 ml of SC–URA–LEU glucose-containing medium. Both glucose and galactose cultures were incubated at 30°C for 24–30 hr under constant shaking. Thereafter, cells were washed twice, counted, and appropriate numbers were plated onto complete medium to determine the number of vital cells, and onto solid medium lacking histidine or adenine to determine the frequency of intrachromosomal and interchromosomal recombination, respectively. The RS112 strain carrying the empty vector was exposed to methyl methanesulfonate (MMS) as follows: aliquots containing 5 × 10⁷ cells/ml were inoculated in a total volume of 5 ml (10⁷ cells/ml) of SC–URA–LEU glucose or galactose with different doses of MMS. Then, cultures were incubated at 30°C for 4 hr under shaking, washed, counted, and plated as described [Schiestl, 1989; Schiestl et al., 1989].

Data Comparison and Statistical Analysis

The frequency of recombination obtained after growth in galactose medium was compared to that in glucose medium. For each BRCA1 protein, at least six independent experiments were carried out. Results were statistically analyzed using the Student’s t-test.

Results

SIFT and PolyPhen Prediction Programs Identify Probably Deleterious Nonsynonymous Missense Variants

The mutational analysis of 276 hereditary breast and ovarian cancer families, performed by automatic direct sequencing of all coding regions and intron–exon boundaries, has revealed several novel as well as previously described variants of BRCA1 gene. By using two prediction software programs, SIFT and PolyPhen, seven nonsynonymous variants likely disrupting the gene function, p.N132K, p.Y179C, p.N550H, p.S1164I, p.S1312L, p.I1766S, p.A1789T, were identified. Among those, two variants, the 3610G>T (c.3491G>T, p.S1164I) and the 5484G>A (c.5365G>A, p.A1789T), were studied for the first time in this analysis; the variant 5416T>C (c.5295T>C, p.I1766S) has been classified as deleterious mutation by other studies [Carvalho et al., 2005; Deffenbaugh et al. [2002] and confirmed by Tavtigian et al. [2006]. Finally, the variant 4654G>T (c.4535G>T, p.S1521D) was reported as neutral by Deffenbaugh et al. [2002] and confirmed by Tavtigian et al. [2006].

Out of the 13, 6 were predicted as neutral by both programs: 477G>A (c.358G>A, p.D120N); 1118G>4 (c.999A>G, p.T333A); 1575T>C (c.1575T>C, p.F486L); 2576T>C (c.2457T>C, p.S819P); 3147C>T (c.3028C>T, p.P1010S); and 5057G>A (c.4956G>A, p.N132K) has been reported as likely to be of no or little clinical significance by Easton et al. [2007]. Finally, the variant 4654G>T (c.4535G>T, p.S1521D) was reported as neutral by Deffenbaugh et al. [2002] and confirmed by Tavtigian et al. [2006].

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Finally, three known missense variants (p.C61G, p.A1708E, and p.M1775R) previously reported as deleterious mutations were selected as negative controls; p.C61G was previously reported as deleterious mutation by other studies [Easton et al., 2007]. Finally, the variant 1767A>C (c.1648A>C, p.N550H) was classified as probably neutral by Tavtigian et al. [2006]; and the 515C>A (c.396C>A, p.N132K) has been reported as likely to be of no or little clinical significance by Easton et al. [2007]. Finally, the variant 4654G>T (c.4535G>T, p.S1521D) was reported as neutral by Deffenbaugh et al. [2002] and confirmed by Tavtigian et al. [2006].

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Finally, three known missense variants (p.C61G, p.A1708E, and p.M1775R) previously reported as deleterious mutations were selected as negative controls; the first one maps in the RING FINGER domain and the last two in the BRCT
domains [Ruffner et al., 2001; Carvalho et al., 2007]. The mutation p.C61G is due to a G to T transversion at nt 181 of the BRCA1 cDNA. p.A1708E is due to a C>A transversion at nt 5123 of the BRCA1 cDNA, and p.M1775R is due to a T>G transversion at nt 5324 of the BRCA1 cDNA. The location of selected missense variants and negative and positive controls is depicted in Figure 1.

Each selected variant was found in a single family with the exception of the three variants, p.Y179C, p.F486L, and p.N550H, which were found to be associated in two unrelated families.

The control variants p.C61G, p.A1708E, and p.M1775R were not found in the breast and ovarian cancer family group we studied. The histopathological features of the proband’s tumor are shown in Table 2.

Segregation Analysis

Familial history information was available for all carriers of the variants considered and the pedigrees are included in the Supplementary Figs. S1 and S2 (available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat). The segregation analysis of the variant allele with the disease was performed only in Families PI403, PI397, PI258, and PI222, for which DNA samples of unaffected or affected relatives were available.

The variants p.Y179C and p.N550H were both detected in two unrelated families, Families PI403 and PI340, in which the neutral variant p.F486L has also been found. The proband from Family PI403 was affected by breast cancer at 42 years of age. Two second-degree relatives in the paternal branch, the proband’s grandmother and her aunt, were affected by breast cancer. The affected cousin was negative for the variants (Supplementary Fig. S1).

The variant p.I1766S was found in Family PI222. The proband had ovarian carcinoma diagnosed at 42 years of age. A DNA sample was available from an unaffected sister of the proband; she tested negative for the mutation (Supplementary Fig. S1).

Inactivation of the BRCA1wt Allele by LOH or Promoter Methylation

The inactivation of the wild-type allele in the tumor tissue of the patient carrying the variant allele is considered to be indicative of the pathogenicity of such a variant. Inactivation may occur through loss of part of a chromosome, detected by LOH analysis, or by gene expression silencing due to promoter hypermethylation. LOH analysis of four cases and a methylation profile of two cases was performed.

LOH was not observed in the tumor DNA of the p.N132 K carrier in Family PI432. The wild-type allele was lost in the tumor DNA of the proband of Family PI340 carrying the three variants p.Y179C, p.F486L, and p.N550H.

The tumor DNA of both patients carrying the variant p.A1789 T showed no LOH of the wild-type allele but the methylation profile of BRCA1 gene promoter of both samples showed hypermethylation.

Co-Occurrence

Co-occurrence is really a test for embryonic lethality due to inheritance of a compound heterozygous null genotype. The underlying assumption is that inheritance of a genuine high-risk missense substitution in BRCA1, along with a clearly deleterious
the family, so we supposed that it is localized in the same allele (in
with the stop mutation p.E1172X in all six individuals we tested in
in which a deleterious mutation was found. This variant co-occurs
variant p.S1164I was found to co-occur in a family (Family PI397)
evaluated the co-occurrence in our sample of 276 families. Only the
expression of these proteins assessed by Western blot indicated
and p.A1708E; the neutral p.P1010S; and the p.Y179C. The
missense variants: the three BRCT mutants p.A1789 T, p.I1766S,
recombination in Yeast
Variants But Not the
BRCA1
Recombination in Yeast
Variants But Not the
BRCA1
The Expression of Known Cancer-Related Missense
BRCA1 Variants But Not the BRCA1 Wild-Type Increased
Recombination in Yeast

The BRCT Missense Variants Inhibit the Growth
Suppression Phenotype

The expression of the wild-type BRCA1 gene inhibits growth in
S. cerevisiae [Humphrey et al., 1997]. Taking advantage of this
phenotype, theSCP assay has been proposed to distinguish cancer
predisposing missense mutations from harmless polymorphisms
[Coyne et al., 2004]. In this study, we constructed 12 missense
mutations localized throughout the whole sequence of the BRCA1
gene. To further validate the SCP assay, we constructed one
mutant within the RING FINGER domain, two mutants down-
stream from this domain, four mutants in the DNA binding
domain, one in the serine and threonine (SQ) cluster domain, and
four in the BRCT domains (Fig. 1). The vector allows a high level
of protein expression when galactose is present in the culture
medium [Humphrey et al., 1997]. To determine if the BRCA1 was
expressed in the RS112 strain, we carried out Western blot analysis
of the total protein extract from yeast culture in galactose. The
BRCA1wt protein was expressed after 24 hr in galactose (Fig. 2A,
lane 1). No protein was seen when the same culture was grown in
glucose (Fig. 2A, lane 2) or in the extracts from yeast carrying the
vector (Fig. 2A, lane 3). We also checked the expression of five
missense variants: the three BRCT mutants p.A1789 T, p.I1766S,
and p.A1778E; the neutral p.P1010S; and the p.Y179C. The
expression of these proteins assessed by Western blot indicated
different levels of expression (Fig. 2A, lanes 4–8), suggesting that the
stability of the proteins may be affected by the mutations.
However, in another yeast strain this difference has not been seen,
indicating that the different protein level might be dependent on
the genetic background of the strain used [Bennett et al., 2008].
When the same amount of cells from the same culture were plated
onto galactose, the expression of BRCA1wt led to formation of
colonies much smaller than those formed by the strain carrying
the empty vector, confirming that BRCA1wt inhibited yeast
growth (Fig. 2B) [Humphrey et al., 1997; Coyne et al., 2004].
We quantified this effect by counting the number of cells per colony
by picking up three colonies per plate and counting the cells as
previously reported [Coyne et al., 2004]. The colonies from the
BRCA1-expressing strain contained an average of 7,000 cells per
colony; on the other hand, colonies carrying the empty vector
contained an average of 459,000 cells per colony (Fig. 2C). The
suppression phenotype, in fact the average number of cells per
colony ranged from 4,000 to 22,000 (Fig. 2C), but they could not
be classified as neutral because they map outside the BRCT
domains. The three BRCT mutants p.A1708E, p.I1766S, and
p.A1789 T fully inhibited the growth suppression, as the number
of cells per colony ranged from 402,000 to 457,500. The expression
of the p.M1775R variants that gave colonies containing about
180,500 cells per colony only partially complemented the SCP, as
reported by others (Fig. 2C) [Coyne et al., 2004; Humphrey et al.,
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1997].

The Expression of Known Cancer-Related Missense
BRCA1 Variants But Not the BRCA1 Wild-Type Increased
Recombination in Yeast

The SCP assay can evaluate missense variants that are located
only within the BRCT domains of the BRCA1 protein [Billack and

Table 2. Histopathological Features of Tumors

<table>
<thead>
<tr>
<th>Sample</th>
<th>Variants</th>
<th>Tumor</th>
<th>Age at diagnosis</th>
<th>Histopathology type</th>
<th>Grade</th>
<th>Lymph node metastasis</th>
<th>ER/PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI1432</td>
<td>N132K</td>
<td>Breast</td>
<td>47</td>
<td>DCI</td>
<td>3</td>
<td>–</td>
<td>+/+</td>
</tr>
<tr>
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<td>DCI</td>
<td>3</td>
<td>–</td>
<td>+/+</td>
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<td>n.a.</td>
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</tr>
<tr>
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<td>3</td>
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<td>n.a.</td>
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<tr>
<td>P1222</td>
<td>I1766S</td>
<td>Ovary</td>
<td>52</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
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<tr>
<td>P1335</td>
<td>P1010S</td>
<td>Breast</td>
<td>29</td>
<td>DCI</td>
<td>3</td>
<td>–</td>
<td>n.a.</td>
</tr>
<tr>
<td>P1258</td>
<td>A1789T</td>
<td>Breast</td>
<td>32</td>
<td>DCI</td>
<td>3</td>
<td>–</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

*According to the Bloom-Richardson grading system for breast cancer (www.ccrcal.org/Vol_1/BloomRichardsonGradeForBreastCancer_CA.htm).
ER, estrogen receptor; PR, progesterone receptor; DCI, ductal carcinoma infiltrating; n.a., data not available; –, absence; +, presence.
Monteiro, 2004; Coyne et al., 2004; Humphrey et al., 1997]. Therefore, novel assays to investigate the pathogenic impact of BRCA1 missense mutations located throughout all of the BRCA1 domains are needed. As the biological functions of BRCA1 appear to be related to DNA repair and recombination, we tested the effect of the expression of the wild-type and 12 missense variants of the BRCA1 protein on yeast HR [Narod and Foulkes, 2004; Scully et al., 2004; Zhang and Powell, 2005]. For a more complete
evaluation we used the diploid yeast strain RS112 that contains two distinct HR systems and, therefore, allows the simultaneous measurement of intrachromosomal and interchromosomal recombination events at the HIS3 and ADE2 loci, respectively (Fig. 3A and D) [Schiestl, 1989; Schiestl et al., 1989]. The mechanisms by which HR occurs in this strain have been extensively studied and we previously reported that DNA double strand breaks formed during DNA replication are able to stimulate HR [Galli and Schiestl, 1995, 1998, 1999; Schiestl et al., 1988]. Although this yeast strain is widely used to study HR, there are few data on induced recombination in galactose media. Recently, we used this strain to screen a human cDNA library and we found that many human cDNAs increase yeast HR by two- to six-fold in galactose [Collavoli et al., 2008]. Here, we further characterized the HR induction by exposing the cells to different MMS doses in galactose or glucose SC–URA–LEU medium. Each MMS dose induced a significant increase of intra- and interchromosomal recombination as compared to the control. Particularly, at the lowest MMS dose the HR was stimulated at both loci by two- to four-fold in glucose and in galactose. The increase of HR is not affected by the medium because both intra- and interchromosomal recombination were induced to the same extent in glucose and in galactose (Fig. 3B and D).

To determine whether BRCA1 expression affects HR, yeast cells containing the vector with the BRCA1wt or the missense variants under the galactose-inducible promoter were pregrown in glucose medium. As reported in Materials and Methods, 1 × 10^6 cells were inoculated in 5 ml of galactose medium and, for comparison, in glucose medium. Cultures were incubated at 30°C for 24 hr. Thereafter, cells were counted and plated in complete and selective media to score for viable cells and recombinants, and incubated at 30°C until colonies formed. The expression of full-length BRCA1 did not affect either HIS3 or ADE2 recombination (Fig. 3A and B). The expression of the three variants, p.C61G (located in the RING FINGER domain) and p.N132K and p.Y179C (located downstream from the RING FINGER domain) induced a significant increase of HIS3 and ADE2 recombination (Fig. 4A and B). The expression of missense variants carrying mutation within the DNA binding and BRCT domain gave quite different results. The expression of the p.F486L, p.N550H, and p.P1010S mutants did not increase recombination, while the expression of the p.S1164I variant showed a significant increase of both intra- and interchromosomal recombination (Fig. 3A and B). The p.S1512I variant did not have any influence on yeast recombination, nor did the BRCT variant p.A1789T. The expression of the known cancer-related missense variant p.A1708E induced a significant increase of HIS3 recombination but not of ADE2 recombination. However, the other known deleterious variant p.M1775R significantly increased both recombination events (Fig. 4A and B) [Mirkovic et al., 2004; Phelan et al., 2005]. Moreover, the expression of the I1766S variant showed a significant increase of intra- and interchromosomal recombination (Fig. 4A and B). We also constructed two new BRCA1 mutants that expressed two deleted proteins: the p.R1443X and the p.E143X. The p.R1443X deletion mutant was constructed by the insertion of a stop codon at nucleotide 4446 in exon 13 encoding a protein lacking the BRCT domains (Fig. 1), with a putative molecular weight of 170 kDa. The p.E143X deletion mutant was constructed by the insertion of a stop codon at nucleotide 546 in exon 7. The molecular weight of this truncated protein is expected to be 17 kDa. As shown in Figure 2A, the p.R1443X indeed is smaller than the BRCA1wt (Fig. 2A, lanes 9 and 10). When the total protein extract from yeast cells producing the p.E143X was analyzed by western blot, no band was detectable at high molecular weight; this demonstrated that the BRCA1wt is not produced. On the other hand, the early truncated protein (17 kDa) is not recognized by the antibody used (see Materials and Methods). The expression of these BRCA1-truncated proteins did not affect HR in yeast (Fig. 4A and B).

Discussion

Several assays have been proposed to distinguish between cancer-related mutations and neutral polymorphisms of the tumor suppressor gene BRCA1 [Mirkovic et al., 2004; Phelan et al., 2002]. Prediction programs, such as SIFT and PolyPhen, are very useful as a starting point, but often can produce misclassification of the variants and need to be supported by functional assays [Easton et al., 2007; Rajasekaran et al., 2007]. Transcriptional activation assays using a heterologous DNA binding domain have been recognized as very helpful systems to correlate the BRCA1 mutations to cancer [Monteiro and Humphrey, 1998; Vallon-Christersson et al., 2001; Monteiro et al., 1997]. In the present study, we chose seven missense variants, identified among 267 breast and/or ovarian cancer families; two nonpathogenic variants; and three cancer-related missense variants. As novel functional assays are necessary to classify BRCA1 missense variants as pathogenetic, we proposed to use the yeast S. cerevisiae as genetic tool. This organism is very easy to manipulate, simple to cultivate, and provides a large collection of viable mutants that could be very helpful to identify new genetic factors involved in the BRCA1 pathway [Westmoreland et al., 2003]. The expression of full-length BRCA1, achieved by using a galactose-inducible promoter, has been previously reported to inhibit yeast cell growth in a haploid yeast strain [Humphrey et al., 1997]. Here, we demonstrated that the GAL1 promoter-driven expression of full-length BRCA1 is also able to inhibit cell growth in a diploid strain. As previously reported, only

Figure 2. Expression and effect of BRCA1 missense variant on colony size in yeast. The strain RS112 of S. cerevisiae was transformed with plasmids carrying BRCA1wt or the selected missense variants. Single URA3+ transformants were grown in –URA glucose for 24 hr at 30°C and then plated in –URA galactose. A: Western blot analysis of yeast cells expressing BRCA1wt and five missense variants; 30 μg of total proteins were loaded on each lane: lane 1, extract from RS112 expressing BRCA1wt (galactose); lane 2 and 3, extracts from noninduced RS112 (glucose) and RS112 carrying the empty vector. In lane 4 to 8, extracts from RS112 expressing the variants were loaded as follows: p.A1789T, p.I1766S, p.A1708E, p.P1010S, and p.Y179C. In lanes 9 and 10, extracts from yeast expressing the truncated p.R1443X protein and BRCA1wt were loaded; in lane 11, extracts from yeast expressing the truncated p.E143X were loaded. The level of the 3-PGK was determined as loading control. B: The expression of BRCA1wt or the missense variants affect the colony size in the RS112 strain. Here, 200–250 cells were plated in SC–URA galactose and incubated for 4–5 days. The expression of BRCA1wt and p.S1512I gave smaller colonies than those derived from the expression of the p.M1775R variants or from the RS112 containing the empty vector. C: The effects on colony size were quantified by counting the number of cells per colony. Then, single colonies each plates were resuspended in 1 ml water and the number of cells was counted by a hemocytometer. Three colonies on each plate were checked for cell number. Results are reported as mean of four independent experiments ± standard deviation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
the BRCA1 variants carrying mutations in the BRCT domain are capable of suppressing the growth-inhibition phenotype [Coyne et al., 2004]. The expression of BRCT missense variants also inhibited the growth suppression in the RS112 yeast strain, confirming that the SCP assay could help to classify some BRCA1 missense variants as deleterious. Western blot analysis showed that the level of the p.A1708E and p.I1766S, which suppressed the growth inhibition phenotype, was higher than in the wild-type and the other variants. On the other hand, the level of the variant p.A1789T is lower than the level of the other two BRCT variants, indicating that the growth suppression phenotype is not due to changes in the expression and/or stability of the BRCA1 variants.

As the SCP assay is BRCT domain–specific, we set up a novel test named “yeast HR–based assay,” which could help to distinguish the deleterious variants from the neutral polymorphisms. The assay measures the properties of BRCA1 variants to affect spontaneous HR in the model organism S. cerevisiae. We showed that the expression of the cancer-related mutations p.C61G, p.A1708E, and p.M1775R significantly affected intrachromosomal and/or intrachromosomal recombination in yeast, while the neutral p.F486L and p.P1010S did not. Moreover, the BRCA1wt did not affect yeast HR, suggesting that missense variants that do not alter HR frequency should be considered as potentially neutral. These results strongly suggest that the yeast S. cerevisiae could be a useful genetic tool to classify BRCA1 missense variants as deleterious. Particularly, our results indicated that the variants p.N132K, p.Y179C, p.S1164I, and p.I1766S should be assessed as potentially deleterious, and the p.N550H and p.S1512I variant as neutral. The p.A1789T variant might also be deleterious, although it did not affect HR as it showed a clear effect on cell growth inhibition and clinical data showed cosegregation with disease in the family and wild-type allele inactivation in the tumor sample. The HR results are not dependent on the expression level of the proteins because the five BRCA1 variants that are expressed at different levels have different effects on HR.

Figure 3. HR is induced by MMS in glucose and in galactose. The RS112 strain of S. cerevisiae contains two HR systems. A: The intrachromosomal recombination event occurs between the two duplicated and differentially deleted his3 alleles, leading to the HIS3 reversion and the loss of LEU2 [Schiestl et al., 1988]. B: The inducibility of intrachromosomal recombination was assayed in glucose and galactose media after exposing yeast cells to different MMS doses. The frequency of intrachromosomal recombination is expressed as the number of HIS3 colonies/10,000 cells. Data are reported as mean of four independent experiments ± standard deviation. Results were statistically analyzed using the Student’s t-test. The P value was < 0.001 at any MMS dose compared to the control. C: The RS112 strain is also heteroallelic for ade2-40 and ade2-101 so that interchromosomal recombination events between homologs leading to ADE2 can be measured [Schiestl et al., 1989]. D: The inducibility of interchromosomal recombination was determined in glucose and in galactose after exposing yeast cells to different MMS doses. The frequency is expressed as the number of ADE2 colonies/100,000 cells. Data are reported as the mean of four independent experiments ± standard deviation. Results were statistically analyzed using the Student’s t-test. The P value was < 0.001 at any MMS dose compared to the control. Roughly, by comparing results from glucose vs. galactose, intra- and interchromosomal recombination were increased to the same extent at both loci. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
In general, this novel yeast recombination assay has provided data that are in agreement with the predictive analysis carried out by the computer programs (see Table 1). In fact, any predicted deleterious mutation increased recombination, at least at one locus (see Fig. 4). Although this novel functional assay is not directly applicable in clinical practice because it requires expressing the missense variant in yeast, it could be a very useful aid in the evaluation of the pathological significance of BRCA1 missense variants. However, results from this assay have to be compared and evaluated with all data obtained from other assays to give a more complete characterization of the missense variants. Importantly, our assay allows the characterization of mutations located within a specific domain. Indeed, while all four BRCT mutants were positive in the SCP assay, 3 out of 4 BRCT mutants affected HR.

DNA damage such as double strand breaks induces HR in mitotically growing cells of S. cerevisiae in order to repair the DNA [Galli and Schiestl, 1995, 1998; Kupiec, 2000]. Conversely in mammalian cells, DNA double strand breaks are primarily repaired by nonhomologous end joining. In addition, HR has been shown to provide a very faithful and efficient DNA repair pathway mainly in mammalian replicating cells where it is critical to maintain genome integrity [West, 2003]. The main goal of this study is to develop a new assay to characterize BRCA1 missense variants and not to deeply investigate the role of BRCA1 in HR. However, we could hypothesize that those BRCA1 variants affecting recombination could have some impact on yeast DNA repair or interact with proteins that lead to an elevated level of endogenous DNA damage. Recently, we reported that BRCA1 interaction with the carboxy-terminal domain of RNA polymerase II is mediated by the BRCT domain of BRCA1 [Bennett et al., 2008]. To address whether the BRCT domain was important for HR, we measured the HR in the strain expressing the BRCT deleted p.R1443X variant. As this protein did not affect recombination, we conclude that this domain is involved in HR. As expected, the early-truncated p.R143X did not affect HR again indicating that protein-protein interactions are important.

Figure 4. Effect of BRCA1 missense variant expression on yeast HR. Single colonies of the RS112 strain containing the plasmid expressing the BRCA1wt or missense variants were first pregrown in glucose. Then, 10⁷ cells were inoculated in glucose and galactose medium for 24 hr at 30°C. As described in Materials and Methods, cells were counted and plated to score for cell surviving fraction, the frequency of HIS3 intrachromosomal recombination, and ADE2 interchromosomal recombination. A: Effect of BRCA1 protein expression on HIS3 intrachromosomal recombination. B: Effect of BRCA1 protein expression on ADE2 interchromosomal recombination. Data are reported as mean of six to nine independent experiments ± standard deviation. Results were statistically analyzed using the Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001.
Some yeast mutants affecting DNA replication have a hyperrecombination phenotype, indicating that HR is also stimulated when the DNA replication is impaired [Galli et al., 2003]. Although there is no direct evidence that BRCA1 is directly involved in DNA replication, it interacts with several proteins that function in cell cycle checkpoint activation and the genome surveillance complex [Wang et al., 2000; Durant and Nickoloff, 2005]. Many BRCA1 interacting proteins have a yeast counterpart; therefore, the expression of such variants could affect the activity of these proteins leading to an increase of HR. It seems somehow contradictory that some BRCA1 variants negative in the SCP assay and, therefore, growth suppressors, were able to increase HR as much as the BRCA1wt. This is probably due to the profound difference between the two assays. In the SCP assay, yeast cells are plated onto galactose and incubated for several days (4–6 days). During that time, the BRCA1wt protein can accumulate and, consequently, inhibit the “colony” growth. We basically measured the colony growing efficiency of a single cell that has been “forced” to form a colony. In the recombination assay, yeast cells were grown for 24 hr in galactose or glucose, washed, and then plated in glucose media to score for recombinants and survivors. Therefore, this assay measures the HR events occurring during the 24-hr induction in liquid medium. To determine whether the growth suppressor variants inhibited cell replication during the 24-hr in galactose, the colony-forming efficiency of the BRCA1wt-expressing strain was measured after 24 hr in galactose by plating 200 cells per plate in glucose medium. The results showed that the colony-forming efficiency was as high as 80% and, under 24-hr galactose induction, the culture underwent one replication cycle. This indicates that DNA replication has occurred and could be related to HR.

As mentioned before, BRCA1 is involved in many aspects of DNA damage response and affects DNA double strand break repair, cell cycle checkpoint, and HR; therefore, this novel functional assay could be also exploited to investigate the biological activity of BRCA1 and the mechanisms underlying BRCA1 tumorigenesis [Deng, 2006; Narod and Foulkes, 2004; Durant and Nickoloff, 2005].

The HR assay clearly has potential predictive value and can be added to the other previously used modalities to help understanding the pathogenic role of specific BRCA1 variants. However, the characteristics of the assay are not well defined and, like SIFT and PolyPhen, the yeast-based HR assay is not yet applicable in the clinical setting with a high level of confidence.

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References


