METHODS

Human Mutation

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

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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

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., 2004], 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

Additional Supporting Information may be found in the online version of this article.

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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 first or second-degree relatives; 2) early onset of the disease (<50 years of age); 3) occurrence of bilateral breast cancer; or 4) occurrence of breast and ovarian cancer in the same individual.

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 YCp*GAL*::*BRCA1* which contains the human *BRCA1* gene under the galactose-inducible promoter *GAL1*p 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 QuikChange 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: *MAT***a***/MATα ura3-52/ura3-52 leu2-3,112/leu2-Δ98 trp5-27/TRP5 ade2-40/ade 2-101 ilv1-92/ilv1-92 arg4-3/ARG4 his3Δ5′-pRS6-his3Δ3′/his3-Δ200 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 $MgCl_2$, 0.1 M EDTA, 25 mM HEPES, 5 mM dithiothreitol, 0.3 M $(NH_4)_2SO_4$, and 10% glycerol; pH 7.4) plus 10 µl of protease inhibitor solution (4.4 mg phenylmethylsulphonyl fluoride, 62 mg pepstatin, 50 mg chemostatin, and 725 ml DMSO in 1 ml H_2O). 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), diluted 1:5,000, is used as secondary antibody. As loading control, we determined the level of the 3-phosphoglycerate kinase (PGK) with the anti-&agr3PGK 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 Interchromosomal 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 *ade2-40* and *ade2-101*, 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 *BRCA1*wt and the controls, aliquots containing 10^7 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 5×10^7 cells/ml were inoculated in a total volume of 5 ml (10^7 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.N132 K, p.Y179C, p.N550 H, p.S1164I, p.S1512I, p.I1766S, p.A1789 T, were identified. Among those, two variants, the 3610G>T (c.3491G>T, p.S1164I) and the 5484G>A (c.5365G>A, p.A1789 T), were studied for the first time in this analysis; the variant 5416T>G (c.5295T>G, p.I1766S) has been classified as deleterious mutation by other studies [Carvalho et al., 2007]. Three have been previously reported as probably neutral: the 655A>G (c.536A>G, p.Y179C) has been reported by Judkins et al. [2005]; the 1767A>C (c.1648A>C, p.N550 H) was classified as probably neutral by Tavtigian et al. [2006]; and the 515C>A (c.396C>A, p.N132 K) 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.S1512I) 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); 1118A>G (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.M1652I). Two of them, p.F486L and p.P1010S, were selected as negative controls; p.F486L was previously reported as neutral variant [Judkins et al., 2005], whereas p.P1010S has never been reported before. Summary information on the 13 *BRCA1* missense variants is reported in Table 1.

Finally, three known missense variants (p.C61G, p.A1708E, and p.M1775R) previously reported as deleterious mutations were chosen as positive (i.e., loss of function) controls; the first one maps in the RING FINGER domain and the last two in the BRCT

Table 1. Description of the BRCA1 Missense Variants Analyzed

BRCA1 variant[a]	Systematic nomenclature[b]	Number of families harboring the variant	Sift[c]	Polyphen[d]	BIC[e]	Pathogenicity	Previous classification
477G>A, p.D120N	c.358G>A	1	0.25	0.45	0	Null	Not classified
515C>A, p.N132K	c.396C>A	1	0	1.924	1	Damaging	Neutral [Easton et al., 2007]
655A>G, p.Y179C[f]	c.536A>G	2	0.03	2.41	35	Damaging	Probably neutral [Judkins et al., 2005]
1118A>G, p.T333A	c.999A>G	1	0.19	1.151	0	Null	Not classified
1575T>C, p.F486L[f]	c.1456T>C	2	0.71	0.815	37	Null	Neutral [Judkins et al., 2005]
1767A>C, p.N550H[f]	c.1648A>C	2	0.13	1.570	35	Probably deleterious	Probably neutral [Judkins et al., 2005]
2576T>C, p.S819P	c.2457T>C	1	0.08	0.08	0	Null	Not classified
3147C>T, p.P1010S	c.3028C>T	1	0.9	0.618	0	Null	Not classified
3610G>T, p.S1164I	c.3491G>T	1	0.01	2.158	0	Damaging	Not classified
4654G>T, p.S1512I	c.4535G>T	2	0.03	1.77	50	Damaging	Neutral [Tavtigian et al., 2006]
5075G>A, p.M1652I	c.4956G>A	4	0.00	1.713	39	Probably neutral	Neutral Tavtigian et al., 2006]
5416T>G, p.I1766S	c.5295T>G	1	0.01	1.94	5	Damaging	Deleterious [Carvalho et al., 2007]
5484G>A, p.A1789T	c.5365G>A	1	0.02	1.23	0	Probably deleterious	Not classified

[a]DNA mutation nomenclature is as used in the BIC database (http://research.nhgri.nih.gov/bic) according to GenBank accession number U14680.1 with numbering starting at the first residue.
[b]Mutation nomenclature listed with a "c." uses GenBank cDNA reference sequence U14860.1 with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, following the journal guidelines (www.hgvs.org/mutnomen).
[c]$P < 0.05$, deleterious substitution.
[d]Score: 1.5–2, probably damaging substitution; and >2, damaging substitution.
[e]Number of times that the variant has been described in Breast Cancer Information Core (http://research.nhgri.nih.gov/bic/) revised in January 2006.
[f]Both patients with the p.Y179C variant also carried the p.F486L and the p.N550H variants.

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 it 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.N550 H, 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.N550 H 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 a cousin, were affected by breast cancer. The affected cousin was found negative for the variants (Supplementary Figs. S1 and S2).

The variant p.S1164I was detected in Family PI397, also the carrier of the p.E1172X *BRCA1* deleterious mutation. The proband was unaffected, but her mother and three of her mother's sisters were affected by ovarian cancer; moreover, four third-degree relatives were affected by breast and ovarian cancer. We showed the presence of a variant allele, in association with the deleterious mutation, in a unaffected cousin and an unaffected aunt of the proband, and their absence in three unaffected cousins of the proband (Supplementary Fig. S1).

The variant p.A1789 T was detected in Family PI258. The proband was affected by breast cancer at 32 years of age. The proband's mother, affected by breast and ovarian cancer diagnosed at 46 and 50 years of age, respectively, was found to be the carrier of the variant (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.N550 H.

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

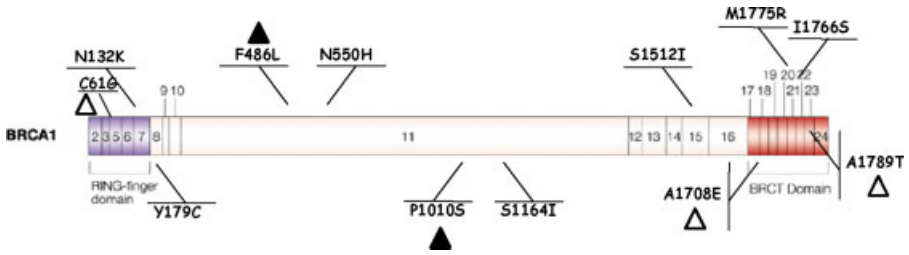


Figure 1. Location of variants in BRCA1. Location of variants and negative (black triangle) and positive (open triangle) controls. SQ, serine and threonine cluster domain; NLS, nuclear localization signal; BRCT, BRCA1 C-terminus domain; RING, RING FINGER domain. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 2. Histopathological Features of Tumors

Sample	Variants	Tumor	Age at diagnosis	Histopathology type	Grade[a]	Lymph node metastasis	ER/PR
PI432	N132K	Breast	47	DCI	3	–	+/+
PI340	Y179C/F486L/N550H	Breast	48	DCI	3	–	+/+
PI403	Y179C/F486L/N550H	Breast	42	DCI	n.a.	n.a.	n.a.
PI98	S1512I	Breast	39	DCI	3	–	–/–
PI226	S1512I	Breast	39	n.a.	n.a.	n.a.	n.a.
PI222	I1766S	Ovary	52	n.a.	n.a.	n.a.	n.a.
PI335	P1010S	Breast	29	DCI	3	–	n.a.
PI258	A1789T	Breast	32	DCI	3	–	n.a.

[a]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.

mutation, will lead to death during embryogenesis. The method has the pitfall that the clearly deleterious mutation and the sequence variant of interest must be in *trans* for the analysis to be valid. We evaluated the co-occurrence in our sample of 276 families. Only the variant p.S1164I was found to co-occur in a family (Family PI397) in which a deleterious mutation was found. This variant co-occurs with the stop mutation p.E1172X in all six individuals we tested in the family, so we supposed that it is localized in the same allele (in *cis*), with respect to the deleterious mutation.

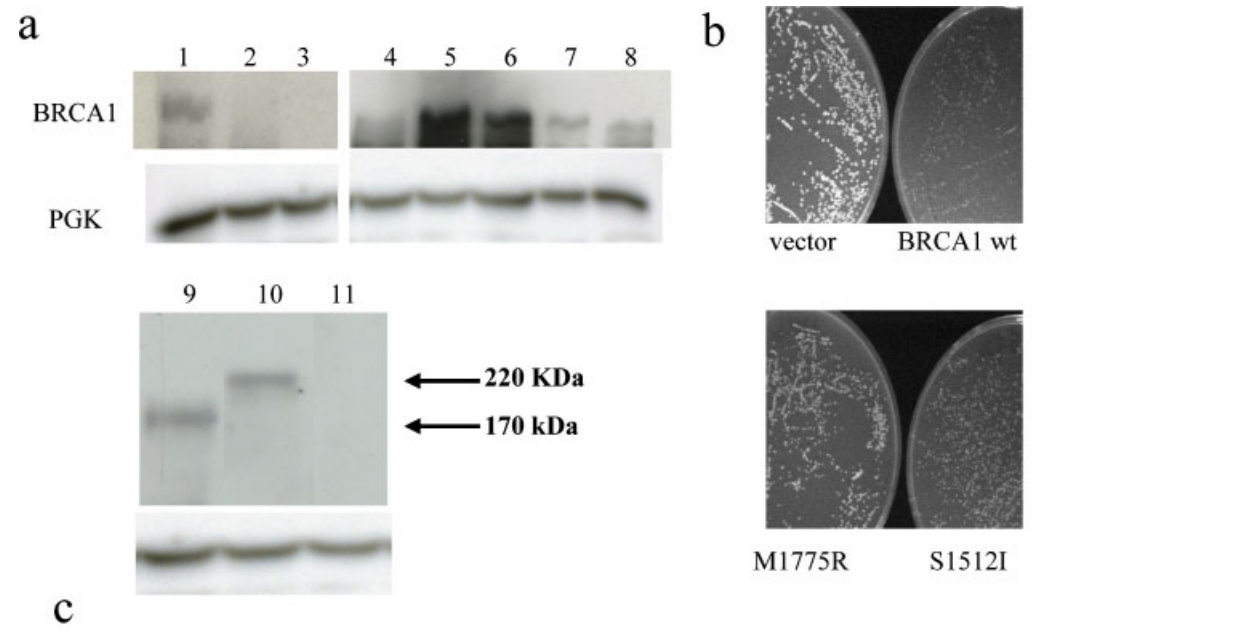
The BRCT Missense Variants Inhibit the Growth Suppression Phenotype

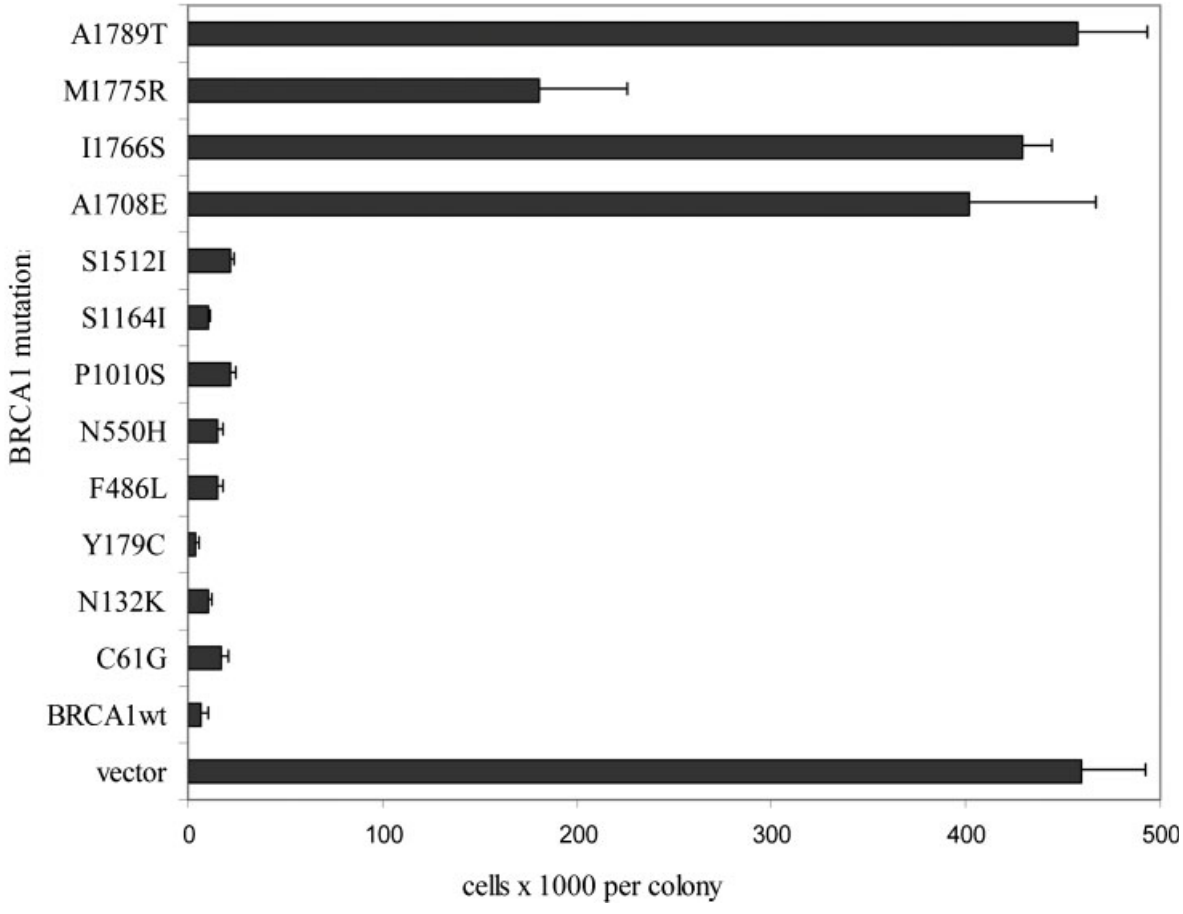
The expression of the wild-type *BRCA1* gene inhibits growth in *S. cerevisiae* [Humphrey et al., 1997]. Taking advantage of this phenotype, the SCP assay has been proposed to distinguish cancer predisposing missense mutations from harmless polymorphisms [Coyne et al., 2004]. In this study, we constructed 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 downstream 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.A1708E; 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 *BRCA1*wt led to formation of colonies much smaller than those formed by the strain carrying the empty vector, confirming that *BRCA1*wt 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 missense variants p.C61G, p.N132 K, p.Y179C, p.F486L, p.N550 H, p.P1010S, p.S1164I, and p.S1512I did not complement the growth 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., 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

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 *BRCA1*wt or the missense variants under the galactose-inducible promoter were pregrown in glucose medium. As reported in Materials and Methods, 1×10^7 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 vital 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.N132 K 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.N550 H, 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.A1789 T. 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 *BRCA1*wt and five missense variants; 30 &mgrg of total proteins were loaded on each lane: lane 1, extract from RS112 expressing *BRCA1*wt (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.A1789 T, 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 *BRCA1*wt 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 *BRCA1*wt 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.]

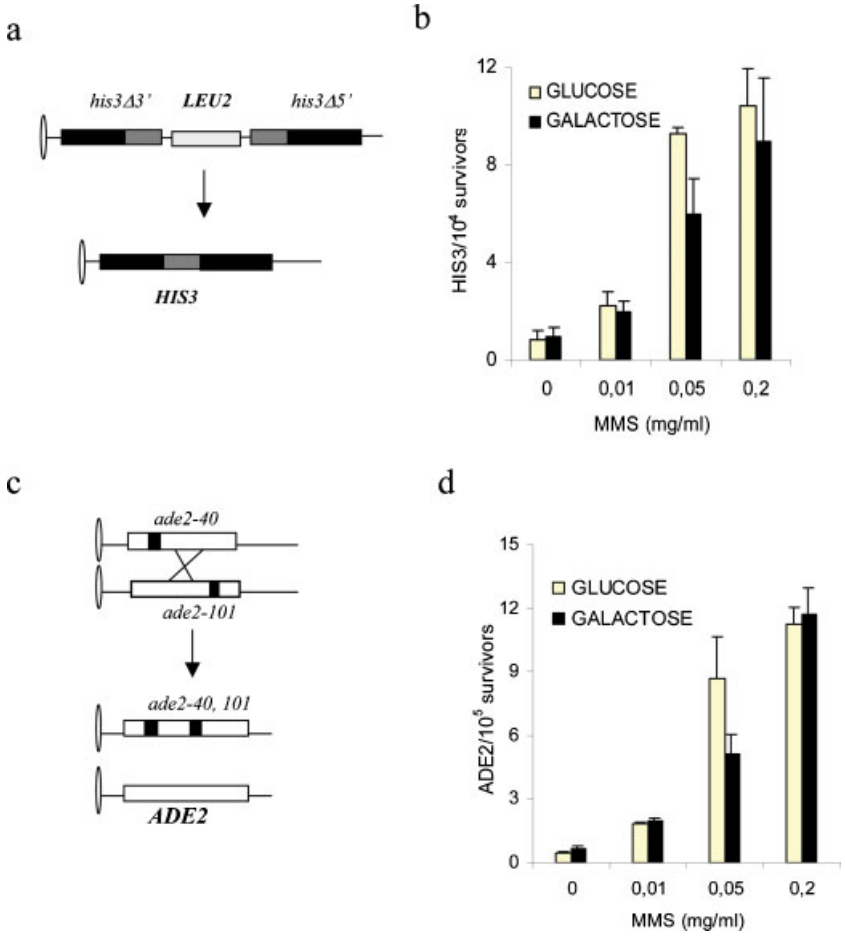


Figure 3. HR is induced by MMS in glucose and in galactose. The RS112 strain of *S. cerevisiae* contains two HR systems. **A:** The intrachromosomal recombination event occurs between the two duplicated and differentially deleted *his3* alleles, leading to the *HIS3* reversion and the loss of *LEU2* [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.]

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.A1789 T 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 interchromosomal recombination in yeast, while the neutral p.F486L and p.P1010S did not. Moreover, the *BRCA1*wt 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.N132 K, p.Y179C, p.S1164I, and p.I1766S should be assessed as potentially deleterious, and the p.N550 H and p.S1512I variant as neutral. The p.A1789 T 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.

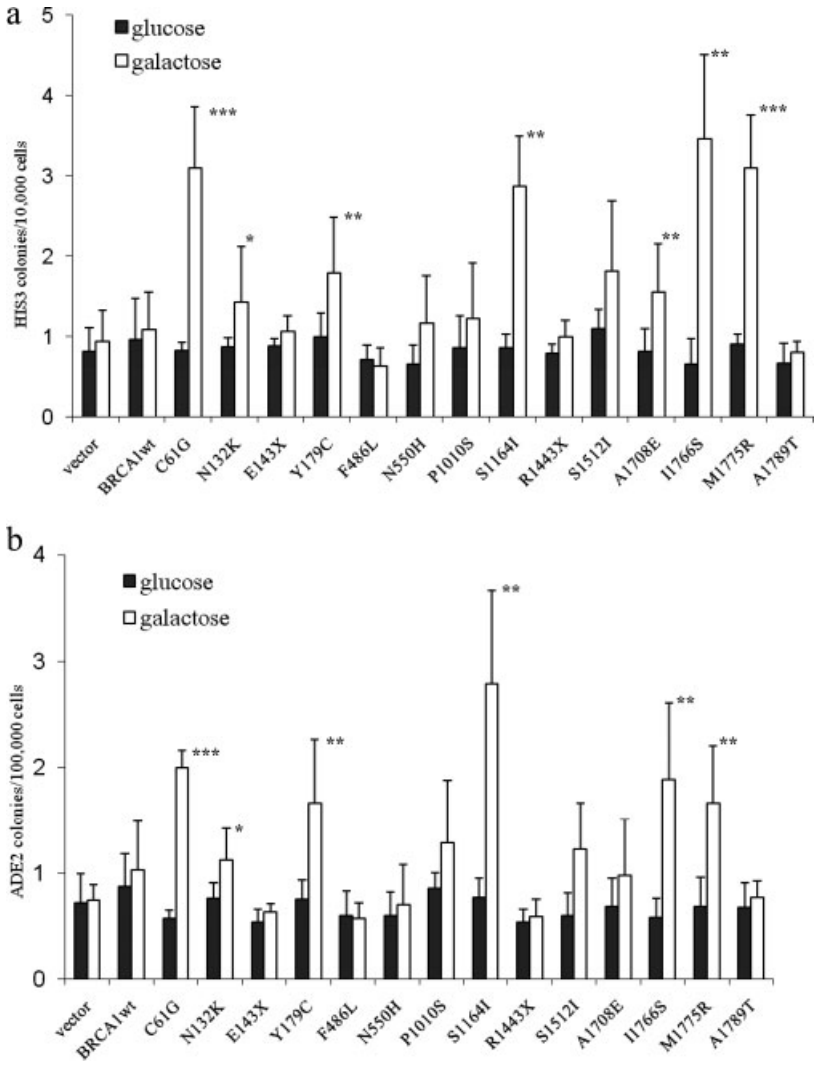


Figure 4. Effect of BRCA1 missense variant expression on yeast HR. Single colonies of the RS112 strain containing the plasmid expressing the *BRCA1*wt or missense variants were first pregrown in glucose. Then, 10^7 cells were inoculated in glucose and galactose medium for 24 hr at 30°C. As described 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. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

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.

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 *BRCA1*wt. 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 *BRCA1*wt-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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