Effect of the expression of \textit{BRCA2} on spontaneous homologous recombination and DNA damage-induced nuclear foci in \textit{Saccharomyces cerevisiae}

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The tumour-suppressor gene \textit{BRCA2} has been demonstrated to be involved in maintenance of genome integrity by affecting DNA double-strand break repair and homologous recombination. Protein-truncating mutations in \textit{BRCA2} predispose women to early onset breast and ovarian cancers and account for 15–30% of familial breast cancer risk. In contrast, the human cancer risk due to missense mutations, intronic variants, and in-frame deletions and insertions in the \textit{BRCA2} gene, called unclassified variants, has not been determined. Here, we want to define if the yeast \textit{Saccharomyces cerevisiae} is a good model to study the role of \textit{BRCA2} in DNA recombination and repair and to characterise the unclassified \textit{BRCA2} missense variants. Therefore, we expressed the wild-type \textit{BRCA2} in yeast and determined the effect of \textit{BRCA2} on yeast homologous recombination, methyl methanesulphonate (MMS)-induced \textit{Rad51} and \textit{Rad52} foci and MMS sensitivity. The expression of \textit{BRCA2} induces a high increase in both intra- and inter-recombination events and confers a higher MMS resistance as compared with the negative control. This may suggest that \textit{BRCA2} gets involved in DNA repair pathways in yeast. Moreover, the expression of \textit{BRCA2} did not affect the number of cells carrying \textit{Rad51} or \textit{Rad52} nuclear foci. Finally, we aimed to investigate if yeast could be reliable system to set up a functional assay to distinguish a mutated protein from a neutral polymorphism. Therefore, we have expressed two neutral (M1915T and A2951T) and one pathogenic variant (G2748D) in yeast and checked the effect on recombination. The neutral M1915T variant increased intra-chromosomal recombination by almost 2-fold and the other neutral A2975T variant increased intra-chromosomal recombination 2.5-fold as compared with the control. On the other end, the pathogenic variant G2748D did not increase intra- and inter-chromosomal recombination in yeast and, consequently, confers a phenotype very different from the wild-type \textit{BRCA2}. Moreover, we have also evaluated whether the expression of the selected unclassified variants affects homologous recombination in yeast. Results indicated that the expression of the selected \textit{BRCA2} variants differentially affects yeast recombination suggesting that yeast could be a very promising genetic system to characterise \textit{BRCA2} missense variants.

Introduction

Breast cancer is the most common malignant neoplasia of women in the western world affecting approximately 6 out of 100 women before the age of 74. About 80% of breast cancers are sporadic and are diagnosed in women without any familial aggregation. However, the remaining cases are inheritable and about 40% of those are caused by mutations in one of the two tumour-suppressor genes, \textit{BRCA1} (Breast Cancer 1) and \textit{BRCA2} (Breast Cancer 2) (1, 2). Inherited mutations in \textit{BRCA2} have been associated with an increased lifetime risk of breast cancer. There are hundreds of deleterious germline mutations found in \textit{BRCA2} that give rise to truncated non-functional protein. However, the pathological consequence of many missense mutations, also called ‘unknown classified variants’ (UCVs), found in breast and/or ovarian cancer families, remains to be ascertained, posing a problem for patients and their health care providers. UCVs account for over 35% of all reported mutations in \textit{BRCA1} and approximately 50% of \textit{BRCA2} (Breast Cancer Information Core Database: http://research.nhgrl.nih.gov/bic/).

The \textit{BRCA2} protein provides an important function in promoting homologous recombination (HR) repair of damaged DNA in cells, presumably through its interaction with the \textit{RAD51} recombinase, which may underlay its role in tumour suppression (3–6). Human \textit{BRCA2} protein contains several functional domains and although the role of the N-terminal domain of the protein is uncertain, the central part (about 1000 amino acids) of \textit{BRCA2} contains eight \textit{BRCA2} repeats that bind \textit{RAD51} (5, 7) (Figure 1). Structural analysis revealed five distinct domains in the \textit{BRCA2} C-termini. The first domain comprises 190 amino acids consisting of mainly \(\alpha\)-helices, termed the helical domain. This is followed by the three structurally related domains of approximately 110 amino acids (OB1, OB2 and OB3; (aa 2620–3184) that exhibit homology and structural similarity to the OB (oligonucleotide/oligosaccharide-binding)-fold that is present in most prokaryotic and eukaryotic ssDNA-bonding proteins, including SSB (ssDNA-binding protein) and RPA (replication protein A) (Figure 1). Consistent with the presence of three OB-fold domains, the \textit{BRCA2} C-terminal fragment was shown to bind with high affinity to single- and double-stranded DNA (8, 9).

The functional assays available for UCV testing on \textit{BRCA2} are based on mammalian cell cultures transfected with plasmid vectors expressing the mutated \textit{BRCA2} to evaluate the efficiency of DNA double-strand breaks repair by HR or the survival to genotoxic agents as compared with the wild-type protein (10–12). All those assays are labour intensive and time consuming; the recently proposed computational classification of UCVs can significantly reduce the time by identifying the UCVs most likely to be deleterious, but still need to be supported by functional studies (13).

The budding yeast \textit{Saccharomyces cerevisiae} represents a powerful genetic model: this organism is easy to grow and genetically manipulate. The expression of human wild-type \textit{BRCA1} in the yeast \textit{S. cerevisiae} inhibits growth, conferring a
small-colony phenotype has been exploited to characterise several UCVs (14). Moreover, the expression of mutated BRCA1 protein in the yeast S. cerevisiae appears to increase HR (15). Therefore, yeast can be used as model organism to distinguish the deleterious from neutral mutations.

In this study, we expressed the wild-type BRCA2 in yeast and analysed the effect on HR. Moreover, we have studied the effect of BRCA2 on rad51 and rad52 nuclear foci induced by MMS to test whether BRCA2 could affect DNA damage repair in yeast. Finally, we expressed several missense BRCA2 variants and investigated whether yeast could be a promising system to develop as functional assay to characterise the UCVs.

Fig. 1. BRCA2 functional domains and location of selected variants. Schematic diagram of the BRCA2 protein. From the N-terminus to the C-terminus: a transactivation core sequence, eight BRC motifs, the DNA-binding region (helical domain and OB-folds), the single Rad51-binding site (TR2 region) regulated by Cdk phosphorylation and the NLS. The arrows represent the location of the selected missense variants: the neutral variants are underlined, the pathogenic variant is in bold.

Of 505 breast and/or ovarian cancer families, we have selected 343 patients negative for BRCA1/2 mutation carriers of 83 novel and previously described variants of BRCA2. Out of these 83, we have selected 40 missense UCVs, excluding synonymous and intronic variants. Among these 40 missense UCVs, we have chosen eight variants that are not previously described or classified in other papers (S286P, M927V,T1011R, L1019V, N1878K, S2006R, R2108C and V3091I). Moreover, we have chosen four variants as controls: we have selected the G2748D as pathogenic control (Table I, (19, 20)); the variants M1915T and A2951T have been chosen as neutral controls (Table I, (10, 20, 21)). The S286P variant maps near the N-terminus; the M927V variant is located before the BRCA repeat domain (Figure 1); the variants T1011R, L1019V, N1878K and S2006R are localised in the BRCA repeat domain (Figure 1); the R2108C variant is located right after the BRCA repeats in the BRAF35 interaction domain (Figure 1); the V3091I variant is located in the OB-fold ssDNA binding domain (Figure 1). Moreover, we have constructed specific vectors to express the BRCA2 neutral variants M1915T and A2951T (Figure 1 underlined, Table I) and the deleterious G2748D (Figure 1 in bold, Table I).

In order to identify whether non-synonymous amino acid changes are likely to disrupt BRCA2 gene functions, we used three comparative evolutionary bioinformatics programs: Sorting Intolerant From Tolerant (SIFT; http://blocks.fhcrc.org/sift/SIFT.html, (22)), Polyorphism Phenotyping (PolyPhen; http://tux.embl-heidelberg.de/ramensky/polyphen.cgi, (23)) and Align-GVGD (http://agvgd.iarc.fr/agvgd_input.php, (24)). SIFT is a multiple sequence alignment tool based on the premise that important amino acids will be conserved amongst species in a protein family in such a way that changes of amino acids conserved in the family should affect protein function (22). PolyPhen is an automatic tool for the prediction of the possible impact an amino acid substitution could have on the structure and function of a human protein (23). Align-GVGD, which compares different sequences by simple alignment, is a mathematically simple missense substitution analysis algorithm, based on the Grantham difference, which measures the biochemical differences between pairs of amino acids (25). This method predicts variants in the query sequence based on a combination of Grantham Variation (GV), which measures the amount of observed biochemical evolutionary variation at a particular position in the alignment, and Grantham Deviation (GD), which measures the biochemical difference between the reference and amino acid encoded by the variant (24, 26). The missense variants are described in Table I.

**Yeast strain**

The diploid strain RS112 of the yeast S. cerevisiae (MATa/MATa ura3-52/ ura3-52 leu2-3,112/leu2-A98 trp5-27/trp5 ade2-40/ade2-101 ilv1-92/ ilv1-92 arg4-3/ARG44 his3A50/hs3A30/his3-A200 lys2/lys2-801) obtained from Robert Schiestl [University of California, Los Angeles (UCLA), Los Angeles, CA, USA] was used to assess the effect of BRCA2 expression on homologous recombination. The RS112 strain was transformed with the plasmids carrying the BRCA2 wild type or the selected variants under the control of the galactose-inducible yeast promoter Gal1p.
The haploid yeast strain W8702 (MATa, YFP-RAD51 trp1, leu2, ura5 from Rodney Rothstein) was used to determine the effect of BRCA2 expression on DNA damage-induced RAD51 nuclear foci. The haploid yeast strain RSY12 (MATa leu2-3,112 his11-15 URA3::HIS3) was transformed with pWJ1344 and therefore used to determine the effect of BRCA2 expression on DNA damage-induced RAD52 nuclear foci. Media preparation and yeast culturing were carried out according to standard techniques. Yeast was transformed with plasmid DNA using the lithium acetate method with single-stranded DNA as carrier, following the procedure described in (27). Transformants were selected in solid medium lacking uracil (SC-URA). Colonies were grown for 4 days at 30°C and analysed further.

**Recombination assay**

The RS112 strain was constructed from the haploid RSY6 and consequently carries the same intra-chromosomal recombination substrate as RSY6 (29). 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, that share 400 bp of homology. These two alleles are separated by the ade2-40 and ade2-101, located in two homologous chromosomes that allow an ade2-40 and ade2-101, located in two homologous chromosomes that allow

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**Table I. Description of the BRCA2 variants analysed**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Nucleotide</th>
<th>Codon</th>
<th>Base change</th>
<th>AA change</th>
<th>Designation</th>
<th>BIC</th>
<th>Score</th>
<th>PolyPhen</th>
<th>Align GV/GD</th>
<th>IARC</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1084</td>
<td>286</td>
<td>T&gt;C</td>
<td>Ser&gt;Pro</td>
<td>S286P</td>
<td>5</td>
<td>0.31</td>
<td>0.102</td>
<td>Class 0</td>
<td>NC</td>
</tr>
<tr>
<td>11</td>
<td>3007</td>
<td>972</td>
<td>A&gt;G</td>
<td>Met&gt;Val</td>
<td>M972V</td>
<td>–</td>
<td>0.28</td>
<td>2.055</td>
<td>Class 0</td>
<td>NC</td>
</tr>
<tr>
<td>11.4</td>
<td>3260</td>
<td>1011</td>
<td>C&gt;G</td>
<td>Thr&gt;Arg</td>
<td>T1011R</td>
<td>4</td>
<td>0.00</td>
<td>1.804</td>
<td>Class 0</td>
<td>NC</td>
</tr>
<tr>
<td>11.4</td>
<td>3263</td>
<td>1019</td>
<td>C&gt;G</td>
<td>Leu&gt;Val</td>
<td>L1019V</td>
<td>22</td>
<td>0.17</td>
<td>0.157</td>
<td>Class 0</td>
<td>NC</td>
</tr>
<tr>
<td>11</td>
<td>5866</td>
<td>1878</td>
<td>C&gt;G</td>
<td>Asn&gt;Lys</td>
<td>N1878K</td>
<td>7</td>
<td>1</td>
<td>1.165</td>
<td>Class 0</td>
<td>NC</td>
</tr>
<tr>
<td>11</td>
<td>5973</td>
<td>1915</td>
<td>C&gt;T</td>
<td>Thr&gt;Met</td>
<td>M1915T</td>
<td>9</td>
<td>0.13</td>
<td>1.616</td>
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<td>NC</td>
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<tr>
<td>11</td>
<td>6249</td>
<td>2006</td>
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<td>Ser&gt;Arg</td>
<td>S2006R</td>
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<td>0.41</td>
<td>1.467</td>
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<td>NC</td>
</tr>
<tr>
<td>11</td>
<td>6550</td>
<td>2108</td>
<td>C&gt;T</td>
<td>Arg&gt;Cys</td>
<td>R2108C</td>
<td>125</td>
<td>0.36</td>
<td>2.188</td>
<td>Class 15</td>
<td>NC</td>
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<tr>
<td>18</td>
<td>9477</td>
<td>2748</td>
<td>G&gt;A</td>
<td>Gly&gt;Asp</td>
<td>G2748D</td>
<td>97</td>
<td>0.08</td>
<td>1.947</td>
<td>Class 65</td>
<td>5</td>
</tr>
<tr>
<td>22</td>
<td>9079</td>
<td>2951</td>
<td>G&gt;A</td>
<td>Ala&gt;Thr</td>
<td>A2951T</td>
<td>40</td>
<td>0.05</td>
<td>1.413</td>
<td>Class 0</td>
<td>NC</td>
</tr>
<tr>
<td>25</td>
<td>9499</td>
<td>3091</td>
<td>G&gt;A</td>
<td>Val&gt;Ile</td>
<td>V3091I</td>
<td>1</td>
<td>0.34</td>
<td>0.088</td>
<td>Class 0</td>
<td>NC</td>
</tr>
</tbody>
</table>

NC, variants not present in LOVD IARC database.

* Number of times the variant has been described in Breast Cancer Information Core.

* Score < 0.05, Deleterious Substitution.

* Score 1.5–2, Probably Damaging Substitution; score > 2, Damaging Substitution.

* Class 0, less likely to interfere with function; class 65, most likely to interfere with function. Multiple alignment with mammalian BRCA2. The variants in bold are the controls chosen *neutral, **deleterious.

* From class 1 neutral to class 5 pathogenic following recommendations reported in Human Mutation 29(11), 1282 (2008) and registered in LOVD-IARC database (http://brca.iarc.fr/LOVD/).
vital cells and onto solid medium lacking histidine or adenine to determine the frequency of intra-chromosomal and inter-chromosomal recombinations, respectively. Results are reported as mean of at least four independent experiments ± standard deviation. Data are statistically analysed by the Student t-test comparing the recombination frequency to the negative control (pYES2, empty vector).

As previously reported, a significant increase in recombination higher than 2-fold as compared with the negative control has to be considered biologically relevant (12, 31).

Results

In this study, we aimed to understand if the yeast S. cerevisiae is a good model to study the role of BRCA2 in DNA recombination and repair and to characterise the unclassified BRCA2 missense variants. Therefore, we have expressed the wild-type BRCA2 in yeast and determined the effect of BRCA2 on yeast homologous recombination, MMS-induced Rad51 and Rad52 foci and MMS sensitivity. Finally, we have also expressed several missense BRCA2 variants and determined the effect on yeast homologous recombination.

BRCA2 is expressed in yeast

Yeast cells carrying the pYES-BRCA2 plasmid were grown at 30°C under constant shaking for 17–24 h and then shifted in 5% galactose medium for 4–6 h. As the BRCA2 protein is very large, we checked first if the BRCA2 cDNA was efficiently transcribed by RT–PCR. We extracted the total RNA from the clones transformed with the plasmid carrying the BRCA2 alleles and performed RT–PCR as reported in the Materials and methods section. As shown in Figure 3a and 3b, BRCA2 wild type and the missense variants are transcribed. Although the pYES2 vector is widely used to produce high levels of exogenous proteins in yeast (http://tools.invitrogen.com/content/sfs/manuals/pyes2man.pdf), we had very hard time to visualise the protein by western blot. Therefore, we extracted the total proteins from the yeast strains expressing BRCA2 wild type, the S286P and the G2748D variants and performed immunoprecipitation using the anti-flag antibody. Although the protein seems to degrade very easily, the results shown in Figure 3c indicated that yeast is able to express BRCA2. Moreover, we carried out immunofluorescence in order to visualise the BRCA2 protein inside the yeast cells. Results reported in the Figure 4a and 4b clearly confirm that yeast is able to express BRCA2. Interestingly, BRCA2 localises both in the nucleus and in the cytoplasm.

Expression of wild-type BRCA2 increases homologous recombination and resistance to MMS in yeast and has no effect on number of nuclear foci

As BRCA2 is mainly involved in homologous recombination, we determined whether the expression of BRCA2 wild type may affect the frequency of intra- and inter-chromosomal recombinations in yeast after 4-h induction in 5% galactose. The expression of BRCA2 wild type induced a highly significant (P < 0.001) increase in both intra- and inter-chromosomal recombinations; the increase was as much as 5.2-fold and 9.5-fold over the negative control (strain carrying the empty pYES2 grown in galactose) for intra- and inter-chromosomal recombinations, respectively (Table II). BRCA2 has been reported to interact with RAD51 and participate in homologous recombination DNA repair (32). In addition, Rad52 interacts with Rad51 and promotes both strand annealing and exchange (33). We, therefore, checked whether BRCA2 expression may affect the number of MMS-induced Rad51 and/or Rad52 nuclear foci. The strains W8702 carrying the pYES-BRCA2 and the RSY12 carrying both pWJ1344 carrying the RAD52-YFP and the pYES-BRCA2 were grown in galactose for 4 h and then treated with MMS for 2 h to induce nuclear foci. Then, spheroplasts were fixed and the proteins were visualised as described in the Materials and methods section. MMS increased the number of cells carrying Rad51 and Rad52 foci although BRCA2 had no effect on the total number of rad51 and rad52 nuclear foci as compared with the control (Figure 4a and 4b). BRCA2 is localised in the nucleus. Moreover, the expression of BRCA2 induced a statistically significant increase in the survival to MMS in both the W8702 and the RSY12 strains, as reported in the Table III.
To investigate whether yeast can be exploited to develop a functional assay to characterise the BRCA2 missense variants not yet classified, we expressed two neutral BRCA2 variants and the deleterious variant G2748D and determined the effect on both intra- and inter-chromosomal yeast recombinations. The expression of the neutral variants A2951T and M1975T induced a significant increase ($P < 0.05$) in intra-chromosomal recombination (Figure 5a). Specifically, the M1975T increased recombination by 1.9-fold as compared with the control; this value is statistically significant and very close to the biological relevancy (31). Moreover, the other neutral variant increased recombination by 2.5-fold as compared with the negative control. The expression of deleterious missense variant G2748D did not increase both events in yeast (Figure 5a and 5b). The expression of the M927V, N1878K and R2108C also increased both homologous recombination events, although less significantly than the wild type (Figure 5a and 5b; Table II). The variants A2951T and V3091I significantly increased intra-chromosomal recombination (Figure 5a), but not inter-chromosomal recombination (Figure 5b). On the other hand, the expression of the variants S286P, L1019V and N1878K significantly increased inter-chromosomal recombination (Figure 5b). Moreover, expression of the variants T1011R and S2006R did not induce any recombination event in yeast (Figure 5a and 5b). To confirm whether the increase in recombination was indeed due to the expression of BRCA2 variants, we performed experiments to determine the frequency of homologous recombination after growing the strain carrying the vector with the BRCA2 missense variants in glucose, where the protein expression is not taking place because the GAL1 promoter is repressed (15). The frequency of both recombination events was not significantly different from that one determined in the strain carrying the empty pYES2 (Table II); intra-chromosomal recombination was ranging from 1.27 to 1.66 $\times 10^4$ vital cells, inter-chromosomal recombination was ranging from 0.43 to 0.91 $\times 10^{-5}$ vital cells. In conclusion, the neutral variants increased recombination although at lesser extent than the wild type, whereas the deleterious variant did not affect recombination, differing from the result obtained when the BRCA2 wild type was expressed. Importantly, six out of eight selected variants that are classified as neutral (Table I) increased at least one recombination, event in yeast suggesting that yeast can be useful to develop a functional assay helping to classify BRCA2 missense variants.

Expression of the BRCA2 missense variants differentially affects yeast recombination

To investigate whether yeast can be exploited to develop a functional assay to characterise the BRCA2 missense variants not yet classified, we expressed two neutral BRCA2 variants and the deleterious variant G2748D and determined the effect on both intra- and inter-chromosomal yeast recombinations. The expression of the neutral variants A2951T and M1975T induced a significant increase ($P < 0.05$) in intra-chromosomal recombination (Figure 5a). Specifically, the M1975T increased recombination by 1.9-fold as compared with the control; this value is statistically significant and very close to the biological relevancy (31). Moreover, the other neutral variant increased recombination by 2.5-fold as compared with the negative control. The expression of deleterious missense variant G2748D did not increase both events in yeast (Figure 5a and 5b). The expression of the M927V, N1878K and R2108C also increased both homologous recombination events, although less significantly than the wild type (Figure 5a and 5b; Table II). The variants A2951T and V3091I significantly increased intra-chromosomal recombination (Figure 5a), but not inter-chromosomal recombination (Figure 5b). On the other hand, the expression of the variants S286P, L1019V and N1878K significantly increased inter-chromosomal recombination (Figure 5b). Moreover, expression of the variants T1011R and S2006R did not induce any recombination event in yeast (Figure 5a and 5b). To confirm whether the increase in recombination was indeed due to the expression of BRCA2 variants, we performed experiments to determine the frequency of homologous recombination after growing the strain carrying the vector with the BRCA2 missense variants in glucose, where the protein expression is not taking place because the GAL1 promoter is repressed (15). The frequency of both recombination events was not significantly different from that one determined in the strain carrying the empty pYES2 (Table II); intra-chromosomal recombination was ranging from 1.27 to 1.66 $\times 10^4$ vital cells, inter-chromosomal recombination was ranging from 0.43 to 0.91 $\times 10^{-5}$ vital cells. In conclusion, the neutral variants increased recombination although at lesser extent than the wild type, whereas the deleterious variant did not affect recombination, differing from the result obtained when the BRCA2 wild type was expressed. Importantly, six out of eight selected variants that are classified as neutral (Table I) increased at least one recombination, event in yeast suggesting that yeast can be useful to develop a functional assay helping to classify BRCA2 missense variants.
Although hundreds of BRCA2 truncating mutations have been associated with increased risk of cancer, the contribution of other BRCA2 variants remains largely undefined and is an important obstacle to identify individuals at risk of cancer and to provide appropriate health care options and counseling. Classification of BRCA2 variants as cancer predisposing or neutral has proven problematic because it is not known whether these subtle changes alter the function of the proteins sufficiently to predispose to cancer and there is insufficient information from family studies of these rare variants to allow classification using the likelihood model. Because of this problem, functional assays that assess the impact of amino acid changes on BRCA2 protein function have been proposed as an alternative approach to the classification of the cancer (11).

BRCA2 deficiency is associated with a defect in homologous recombination and a high degree of chromosome instability, including chromosome breaks and radial chromosomes (4, 34, 35). The direct interaction between BRCA2 and RAD51 and their co-localisation in nuclear foci after DNA damage was the first evidence for a role for BRCA2 within DNA double-strand break repair by homologous recombination (36). In this study, we demonstrated that expression of the BRCA2 wild type confers a hyper-recombination phenotype, which could be exploited to develop a new functional assay to characterise UCVs. Moreover, we showed that BRCA2 may get involved in the DNA repair pathways because we demonstrated that the BRCA2 expressing yeast cells were more resistant to MMS than control. Recently, full-length BRCA2 was purified from yeast and mammalian cells (37–39). Moreover, purified BRCA2 has been shown to bind yeast Rad51 in vitro (39). This result was somehow expected, given the high homology between yeast and human RAD51 (39). Therefore, yRAD51 and BRCA2 may also interact in the cells leading to a complex that could be less active in repairing endogenous DNA breaks. This may result in the accumulation of recombination intermediates that may explain the increase in recombination. The yeast strain used here contains two different recombination substrates that allow to measure the intra-chromosomal deletion events between DNA repeats and inter-chromosomal recombination events between two homologous chromosomes (31). These two events have been proposed to occur by different mechanisms.

<table>
<thead>
<tr>
<th>Number of Rad51-YFP foci</th>
<th>control</th>
<th>MMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYES-BRCA2</td>
<td>1.7 ± 0.3</td>
<td>50.0 ± 0.2</td>
</tr>
<tr>
<td>empty vector</td>
<td>1.1 ± 0.1</td>
<td>52.5 ± 5.0</td>
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<table>
<thead>
<tr>
<th>Number of Rad52-YFP foci</th>
<th>control</th>
<th>MMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYES-BRCA2</td>
<td>3.6 ± 0.1</td>
<td>15.8 ± 2.0</td>
</tr>
<tr>
<td>empty vector</td>
<td>4.7 ± 1.1</td>
<td>15.6 ± 3.1</td>
</tr>
</tbody>
</table>

Table II. Effect of BRCA2 protein expression on intra- and inter-chromosomal recombination in the RS112 strain of S. cerevisiae

<table>
<thead>
<tr>
<th></th>
<th>HIS3 colonies/10^4 cells</th>
<th>ADE2 colonies/10^5 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYES2, glucose</td>
<td>2.49 ± 0.81</td>
<td>0.61 ± 0.20</td>
</tr>
<tr>
<td>pYES2, galactose</td>
<td>1.59 ± 0.25</td>
<td>0.63 ± 0.25</td>
</tr>
<tr>
<td>pYES-BRCA2, glucose</td>
<td>2.68 ± 0.49</td>
<td>1.38 ± 0.56</td>
</tr>
<tr>
<td>pYES-BRCA2, galactose</td>
<td>8.23 ± 1.94*</td>
<td>6.02 ± 0.25*</td>
</tr>
</tbody>
</table>

Single colonies were inoculated in 5 ml SC-URA 2% glucose and incubated at 30°C for 24 h while shaking. Thereafter, cultures were induced in rich medium containing 5% galactose for 4 h at 30°C under constant shaking. Then, cells were washed and plated as described in the Materials and methods section. Data are reported as the mean of four independent experiments ± the standard deviation. Results were statistically analysed by comparing the data obtained in galactose using Student’s t-test. *P ≤ 0.001.
mechanisms and may require different genetic factors (40). Previously, we have reported that the expression of human p53 affects homologous recombination in the same yeast strain confirming again that yeast provides an excellent genetic system to uncover the molecular mechanisms involved in cancer (41). The effect of over-expression of BRCA2 in spontaneous and DNA double-strand break repair was determined in human cells and gave quite contrasting results (10,12,20). We have recently shown that the over-expression of full-length BRCA2 weakly affects homologous recombination in HeLa cells; on the other hand, BRCA2 pathogenic variants increased recombination in the same assay indicating that the HeLa recombination assay may help to classify missense variants (12). However, BRCA2 over-expression stimulates DNA double-strand break repair by homologous recombination in human cells, whereas the pathogenic variants did not (10,20). Maybe, spontaneous and induced homologous recombination occurs by different mechanism in human cells; DNA double-strand breaks could not be required for spontaneous recombination in human cells, but it has been shown to initiate spontaneous recombination in yeast (42). This could be the reason why BRCA2 increases spontaneous recombination in yeast and double-strand break repair in human cells.

We previously showed that the expression of deleterious or potentially tumorigenic BRCA1 variants increases homologous recombination in the RS112 yeast suggesting that yeast genetics could be helpful in characterising potentially deleterious BRCA1 variants (15).

To address whether yeast could also potentially be useful to distinguish a BRCA2 mutated protein from a neutral polymorphism, we have expressed two neutral (M1915T and A2951T) and one pathogenic variant (V3091I) in yeast. We found that the expression of BRCA2 increases spontaneous recombination in yeast, whereas BRCA2 pathogenic variants did not (10,20). This could be the reason why BRCA2 increases spontaneous recombination in yeast and double-strand break repair in human cells.

### Table III. Effect of BRCA2 protein expression on survival to MMS in the strain W8702 and RSY12 of *S. cerevisiae*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control</th>
<th>MMS</th>
<th>Control</th>
<th>MMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYES2, galactose</td>
<td>100.0</td>
<td>45.2±2.0</td>
<td>100.0</td>
<td>66.0±2.7</td>
</tr>
<tr>
<td>pYES-BRCA2, galactose</td>
<td>100.0</td>
<td>63.5±8.8*</td>
<td>100.0</td>
<td>87.8±7.0*</td>
</tr>
</tbody>
</table>

Cells were grown for 24 h at 30°C in selective medium URA-glucose and then 107 cells were induced in SC-URA containing 4% galactose for 24 h. Then they were treated with 200 µg/ml MMS in 5ml of galactose medium for 4 h at 30°C under shaking. Cells were washed in water, counted and plated in complete medium. Data are reported as mean of four independent experiments±standard deviation. Results were statistically analysed by comparing the MMS data using the Student’s *t*-test. *P ≤ 0.005.

![Fig. 5](image_url)  
**Fig. 5.** Effect of BRCA1 missense variant expression on yeast homologous recombination. Cells of the RS112 strain containing the plasmid expressing BRCA2 missense variants were first pre-grown in glucose. Then, cells were inoculated galactose medium for 24 h at 30°C. As described in the Materials and methods section, cells were counted and plated to score for cell-surviving fraction, the frequency of HIS3 intra-chromosomal recombination and ADE2 inter-chromosomal recombination. (A) Effect of BRCA2 protein expression on intra-chromosomal recombination. (B) Effect of BRCA2 protein expression on inter-chromosomal recombination. Data are reported as mean of 4–6 independent experiments ± standard deviation. Results were statistically analysed using the Student’s *t*-test. *P < 0.05, **P < 0.005.
BRC2 pathogenic variant G2748D. The neutral M1915T and A2951T variants indeed significantly increased one homologous recombination event in yeast although at lower level than the wild type. On the other end, the pathogenic variant G2748D did not increase HR in yeast and, consequently, confers a phenotype very different from the wild-type BRC2. We selected eight variants that are classified as not deleterious (class 0) by in silico methods. We determined the effect of the expression of these variants on yeast recombination and we showed that six out of eight BRC2 variants are able to induce at least one recombination event in our assay. These results may suggest that the variants that increase HR in our system are presumably not deleterious because they confer the same phenotype as the BRC2 wild type. This may indicate that yeast could be a very promising genetic system to characterise BRC2 missense variants and to understand the genetic basis of BRC2-driven cancer.

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Conflict of interest statement: The authors declare that they have no conflict of interest.

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