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13. ABSTRACT (Maximum 200 Words)

Recent evidence suggests that the breast and ovarian cancer susceptibility gene product BRCA1 is involved in the DNA damage response. However, the precise biochemical function of BRCA1 has remained unknown. Initially, we identified a naturally-occurring allele of BRCA1 which codes for a single amino acid substitution from arginine to tryptophan at residue 1699 (R1699W) and displays temperature-sensitive activity in transcription. The transcription activity of the mutant protein appears to undergo complex regulation, as temperature-sensitive (TS) transcription activation was found to be cell type specific and not dependent on the tissue of origin. We have characterized the behavior of this mutant and isolated several stable cell lines. In addition, through a screen in yeast we identified 11 more unique TS mutants and determined a preferential location for these mutations in the first BRCT domain of BRCA1. We have also generated a series of mutations at residue 1699 and characterized them for transcription activity in different temperatures. Our results provide a framework to engineer conditional mutants of BRCA1 that will aid in the dissection of its in vivo function.

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INTRODUCTION

Linkage analysis revealed that mutations in the *BRCA1* locus account for the majority of families with multiple members affected with breast and ovarian cancer [1]. The cloning and sequencing of *BRCA1* did not immediately yield clues to its cellular function(s). The gene codes for a product with little resemblance to other proteins of known function [2]. The observation that BRCA1 colocalized to Rad51 foci during S phase and that these foci underwent a dynamic reorganization following DNA damaging insults provided the first link to the DNA damage response pathway [3,4]. It soon became evident that BRCA1 played an important role in the cellular response to DNA damage because BRCA1 deficiency compromised homologous recombination, transcription-coupled repair, microhomology end-joining, and non-homologous end-joining [5-10]. Taken together, these data firmly placed BRCA1 in the DNA damage response pathway[11] *but its precise biochemical role has continued to elude researchers.*

In the 10 years since it was cloned, BRCA1 has been proposed to play a role in ubiquitination [12,13], chromatin remodeling [14-16], and several aspects of transcriptional regulation such as repression [17], activation [18,19], polyadenylation [20] and elongation [21]. There is a large body of evidence implicating BRCA1 directly or indirectly in many aspects of transcription and although it is unlikely that it acts as a classical transcription activator, the evidence is consistent with a co-activator function [22,23]. As described in our original proposal, we identified a cancer-predisposing temperature-sensitive mutation in BRCA1. This missense mutation, R1699W, a single amino acid substitution from arginine to tryptophan represents a naturally-occurring allele which was found to segregate with disease [24]. We observed that this mutation was able to activate transcription at the permissive temperature of 30°C but not at 37°C. Based on these observations, we proposed that the activity of this mutant could be modulated as a function of temperature to more clearly define the function(s) of BRCA1.

BODY

During the past three years we achieved the goals described in the three specific aims of our proposal. The aims in this proposal were performed by two fellows: Blase Billack (2002 and 2003) who left for a faculty position in breast cancer research and Vesna Dapic (2004). During our characterization of the R1699W mutant and of the other temperature-sensitive mutants isolated in yeast [25,26] we found that these mutants were not yet widely applicable as tools. Some of the problems included a relative high activity (30-50% of wild type) at the restrictive temperature and a permissive temperature that was too low to allow robust growth of cells. Therefore, we decided to explore other alternatives to better engineer our TS-mutant by characterizing additional mutations at residue R1699. Therefore we revised item (b) of Task 3 (see revised work statement sent on July 2003):

Task 1. Confirm that the full-length cDNA encoding the R1699W mutation is temperature-sensitive for physiologically relevant transcription activity in HCC1937 cells (months 0-2).

a. Develop mammalian expression constructs containing tagged, full-length wild-type and mutant BRCA1 cDNA. The selected mutants are R1699W, R1699Q, M1775R and Y1853X.
b. Transiently transfect the expression constructs and measure transcription activity at permissive and non-permissive temperatures from the mdm2promoter-luciferase reporter construct.

Task 2. Establish and characterize stable cell lines (in HCC1937 cells) expressing the BRCA1 constructs created in Task 1 (months 2-26).

a. Count the number of cells in growing cultures for each cell line at permissive and non-permissive temperatures to obtain growth curves.
b. Perform western blots of lysates harvested above to assess protein expression levels.
c. Perform immunohistochemistry on cells to determine the intracellular localization of BRCA1 proteins.

Task 3. Use stable cell lines cultured at appropriate temperatures to assess BRCA1 function in DNA repair and protein-protein interactions (months 26-36).

a. Perform colony-forming assays on cultures treated with specific doses of ionizing radiation to assess double strand DNA repair.

b. *Transiently transfect the mdm2 promoter reporter construct that has been damaged by UV irradiation for specific periods of time into cells and assay transcription activity to assess nick DNA repair.* ***This item was revised as*** *(according to revised statement of work sent on July 2003):*

b. Introduce the following amino acid changes at residue R1699: R1699E, R1699K, R1699F, R1699G, R1699L, R1699I, R1699H, and fully characterize them for temperature-sensitive activity in a transcriptional assay. The mutants that show TS-activity will be cloned into a full-length context. In addition, we will select a subclone of HCC1937 cell line that can withstand higher temperatures.

KEY RESEARCH ACCOMPLISHMENTS *(summary bulleted list can be found at the end of this section).*

Task 1a and b.

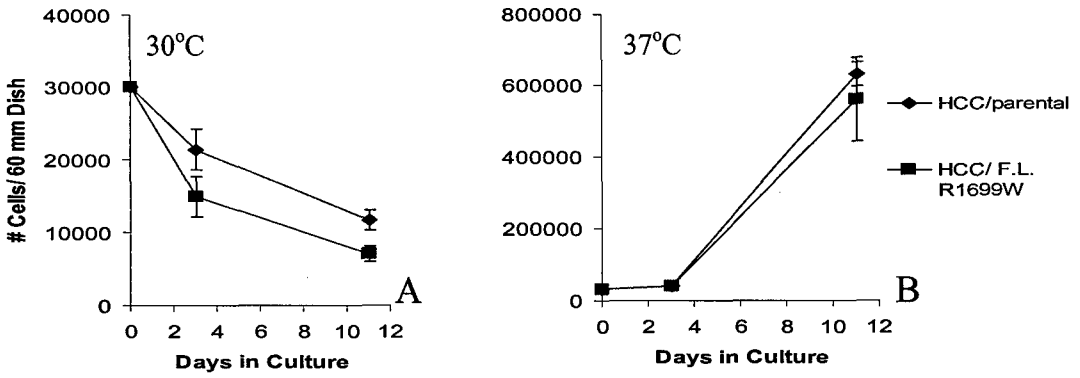
- Full-length constructs (aa. 1-1863) of mutant BRCA1 R1699W, R1699Q, M1775R or Y1853X were obtained. These constructs were then used to measure transcription activation at permissive (30°C) and non-permissive temperatures (37°C) from the *mdm2* promoter-luciferase reporter construct. Initial studies using transient transfection assays examined transcriptional activation in the full-length context using human 293T (BRCA1-proficient) or HCC1937 (BRCA1-deficient) cells co-transfected with wild-type or mutant full-length BRCA1 and the mdm2-luciferase reporter. No significant difference were observed among the different constructs in transcriptional activation of the reporter at all times tested after transfection. It has been well-documented that wild-type BRCA1 can potentiate the activation of p53-dependent and interferon γ-dependent gene transcription [27-29]. The lack of differences in transcriptional activity among the wild-type and mutant constructs of BRCA1 is probably due to levels of expression too low to observe effects on transcriptional activity. Studies in our laboratory have demonstrated that expression of a full-length fusion construct of wild-type BRCA1:GAL4-DBD is significantly lower than expression of the wild-type C-terminus:GAL4-DBD [30].

Task 2a, b and c.

- Several stable cells lines of HCC1937 expressing full length constructs of R1699W were established, as determined by western blotting. They stably express the full-length R1699W mutation and grow at similar rates as the parental cells at 37°C. At 30°C, both parental and stably-expressing cells grow significantly more slowly than at 37°C and are dramatically affected by extended growth at 30°C. L56BR [31] cells which stably express the R1699W mutation were also established. Western blot analysis of full length M1775R in stably transfected HCC1937 cells revealed similar expression levels as cells stably transfected with wild-type (data not shown). Stable cell lines of HCC1937 expressing Y1853X were not isolated despite several attempts. We next assessed the ability of the stably transfected HCC1937 and L56BR cells to grow at permissive and nonpermissive temperatures. After 11 days in culture at the permissive temperature of 30°C, both parental and the stably-transfected cells were reduced to approximately one third of the original number plated (Fig. 1). Cells expressing the R1699W mutant were significantly more sensitive to the detrimental effects of growth at 30°C than were the untransfected parental cells (Fig. 1A). At 37°C, growth of both parental cells and cells expressing the R1699W full length mutant construct was unaffected (Fig. 1B). Our observations indicate that the increased transcription activity in the HCC/RW10.8B cells at the permissive temperature correlates with diminished survival of cells. This may be explained in part by the observation that ectopic overexpression of BRCA1 can trigger apoptosis [32]. Similar results at 30°C were obtained using L56BR cells that stably express the full length R1699W mutant construct (data not shown).

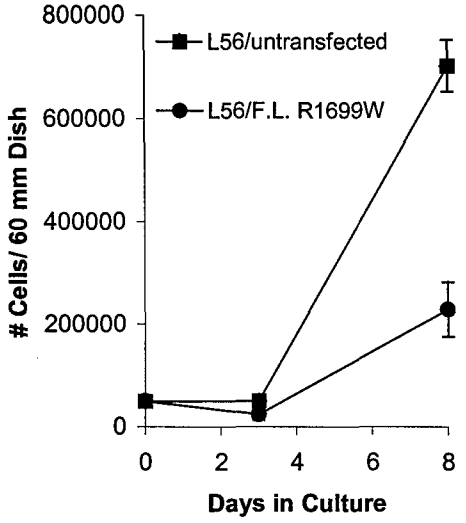
L56BR cells expressing the R1699W mutant grow more slowly than the untransfected parental cells at 37°C (Fig. 2). This is of particular interest because this is the nonpermissive temperature observed for transcription activity in 293T and HCC1937 cells. The reduction in growth rate in R1699W-expressing L56BR cells, when compared to the untransfected parental cells, suggested that the R1699W mutant has residual activity at this temperature. We were unable to measure reporter gene expression in L56BR cells transfected with or without the R1699W fusion construct, due to the low transfection efficiency of these cells. To explore the possibility that the R1699W mutation could have activity at 37°C, we analyzed the transcription activity as a function of temperature in several human-derived cancer cell lines and found that the R1699W mutant is regulated in a complex manner and is cell-type dependent and not directly related to the tissue of origin[26].

Figure 1: Effect of R1699W mutation on growth of HCC1937 cells.



HCC1937 parental and HCC/RW10.8B cells stably expressing the full length R1699W mutant construct of BRCA1 were plated onto 60 mm culture dishes and grown at 30°C (Panel A) or 37°C (Panel B). Data points represent the mean ± the standard deviation of triplicate samples.

Figure 2:Effect of R1699W mutation on growth of L56BR cells



L56BR untransfected parental cells and L56BR/RW cells stably expressing the full length R1699W mutant construct of BRCA1 were plated onto 60 mm culture dishes and grown at 37°C. Data points represent the mean ± the standard deviation of triplicate samples.

Figure 3: Cellular localization of R1699W.

HCC1937 cells expressing the R1699W allele were cultured at 37°C, fixed and probed with BRCA1-specific antibody. The figure demonstrates that the R1699W protein is primarily nuclear.

Immunohistochemical analysis of BRCA1 expression using an antibody that recognizes a common epitope in the wild type and R1699W protein revealed that at both permissive and restrictive temperatures the respective proteins are predominately localized to the nucleus of HCC1937 cells (Fig. 3 and not shown). These data indicate that the cellular distribution of the full length mutant protein is normal.

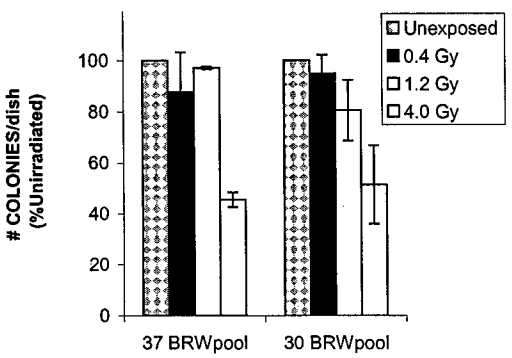
Task 3a and revised b.

We also developed a mouse fibroblast cell line which was BRCA1 deficient (gift from Thomas Ludwig, Columbia University) and engineered to stably express the human R1699W allele. Using these cells, we examined the ability of the R1699W protein to mediate double strand break repair following DNA damage by radiation. Figure 4 depicts the ability of cells treated with or without increasing amounts of γ irradiation to recover and grow as colonies on cell culture dishes. 293T cells with wild-type BRCA1 levels served as the positive control. When compared to the BRCA1-proficient 293T cells, the mouse cells expressing the mutant protein (BRW) were significantly more resistant to doses of 1.2 and 4.0 Gy. Mouse cells showed no differences in DNA repair activity at either 30°C or 37°C. These data suggest that either the activity of the R1699W mutant protein at both temperatures is enough to mediate DNA repair or that the regulation of this protein is different in mouse cells than in 293T cells.

The experiments described in this report led us to the conclusion that the mutants and cell lines (HCC1937 and L56Br) used in the experiments were inadequate to serve as a adequate tool. We therefore

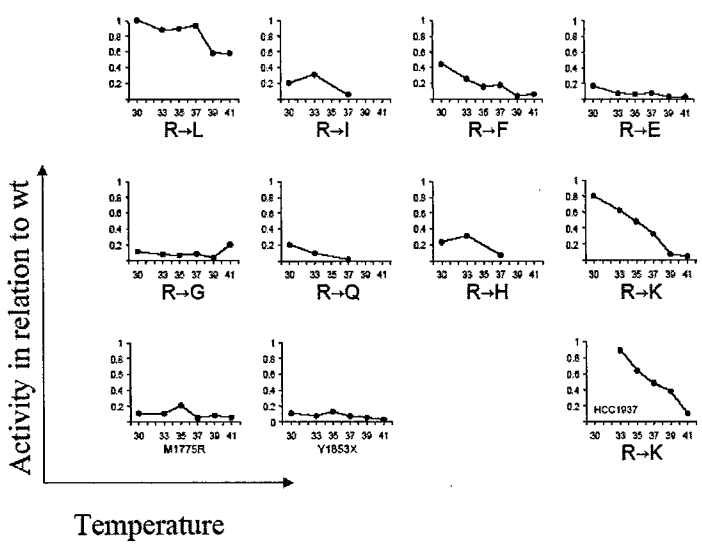
modified our approach as follows. First, we generated a series of new mutants of residue 1699 of BRCA1 (From Arg to: Leu, Ile, Phe, Glu, Gly, His and Lys). We have tested them in a series of temperatures in at least two cell lines and found that only mutant Arg1699Lys is adequate showing ~95% of wild-type activity at 33°C and <10% activity at 41°C in HCC1937 cells (Figure 5). To cover all the bases we have also generated (but not tested yet) mutants in residue 1836 (From Glu to: Asp, Gly and Lys) that is involved in the predicted salt bridge with residue R1699. We are also in the process of generating double mutants combining the mutants in residue 1699 with those in residue 1836. Second, to resolve the problem of HCC1937 slow growth in high temperatures we attempted (and are repeating) to isolate clones that are resistant to higher temperatures but they have been unsuccessful. We have then obtained two novel BRCA1-null cell lines isolated in Steven Ethier's Lab: SUM-149 and SUM-1315. These cells display high transfection efficiency and we are now planning to perform experimenst with these cells. As a complementary approach to using the temperature-sensitive mutants to modulate BRCA1 function, we have developed an expression plasmid coding for a short hairpin RNA that results in the breakdown of BRCA1 through RNA interference.

Figure 4: Effect of R1699W on colony forming ability following irradiation.



Cells were plated on 60 mm culture dishes at low density and on the following day exposed to increasing amounts of γ irradiation. Cells were then grown at 30°C or 37°C for 10 days. Bars represent the mean ± the standard deviation of triplicate samples.

Figure 5. Temperature sensitive activity of BRCA1 Mutants



Cells (293T or HCC1937) were co-transfected with a GAL4 fusion of BRCA1 c-terminal (aa 1560-1863) with the corresponding mutants (depicted in one-letter amino acid code) and a GAL4 responsive luciferase reporter. An constitutive reporter was also transfected to serve as an internal control. Cells were transfected for 2 h and then transfered to different temperatures for 24-48 h when cells were harvested and luciferase activity measured. Activity is plotted in relation to activity of a parallel control of wt BRCA1.

SUMMARY OF KEY RESEARCH ACCOMPLISHMENTS

- *Developed and tested constructs containing mutants of BRCA1 that confer temperature-sensitivity.*
- *Developed stable cell lines and tested their radiation sensitivity at different temperatures.*
- *Determined that BRCA1 containing temperature-sensitive mutations is correctly localized to the nucleus.*
- *Isolated through a yeast-based screen 11 novel temperature-sensitive mutants.*
- *Mapped the location in the BRCT domains of BRCA1 in which temperature-sensitive mutations are preferentially located (N-terminal BRCT).*
- *Determined that mutations in the residues that participate in the salt bridge that stabilizes the BRCT domains (R1699 and E1843) are an adequate target to engineer new temperature-sensitive reagents.*

REPORTABLE OUTCOMES

- Constructs of GAL4 fused to C-terminus of wild-type BRCA1 and mutant constructs of R1699 to Trp, Leu, Ile, Phe, Glu, Gly, His and Lys; M1775R and Y1853X have been developed.
- Constructs of full length wild-type BRCA1 and mutant constructs of R1699W; M1775R and Y1853X have been developed. Constructs are HA-tagged and contained within the pCDNA3 mammalian expression vector.
- We established several cell lines of HCC1937 and L56Br expressing mutant full-length BRCA1 R1699W.
- Isolation of 11 unique BRCA1 temperature-sensitive mutants froma screen in yeast (see manuscript below).
- Abstract and poster presentation entitled, "A Conditional Allele of BRCA1" at the DoD Era of Hope Meeting in Orlando, FL. September 2002 (P22-4).
- Abstract and poster presentation entitled, "Mutations in the hydrophobic core of the BRCT domain confer temperature sensitivity to BRCA1 in transcription activation" at the DoD Era of Hope Meeting in Orlando, FL. September 2002 (P20-12).
- Manuscript entitled, "A Naturally-Occurring Allele of *BRCA1* Coding for a Temperature-Sensitive Mutant Protein" by Worley et al. *Cancer Biology and Therapy*, **1:** 497-501 (*Appendix*).
- Manuscript entitled, "Mutations in the BRCT domain confer temperature sensitivity to BRCA1 in transcription activation" by Carvalho, Billack et al. *Cancer Biology and Therapy*, **1:** 502-508 (*Appendix*). Both of these articles were featured in the cover and in a Commentary article by Jeff Parvin.
- Development of RNA intereference Expression Plasmid for BRCA1.
- Review entitled "Linking breast cancer susceptibility and the DNA damage response" by Dapic et al. *Cancer Control Journal,* In press (*Appendix*).
- Faculty Position for the postdoctoral fellow (Blase Billack) obtained in the Department of Pharmaceutical Sciences at St. John's University, Queens, NY. Blase, the first fellow in this fellowship has started his laboratory focused exclusively on Breast Cancer Research.

CONCLUSIONS

In the past three years we have characterized temperature-sensitive mutations in BRCA1 and started to understand the structural determinants of the BRCT domain. This has been a technically challenging project and we are still in the process of identifying an ideal temperature-sensitive mutant to be used in experiments to dissect the function of BRCA1. Nevertheless, we still believe that this project is innovative and will bear fruits by allowing researchers to probe the function of BRCA1 in real time. This view was also expressed in an editorial commentary on our papers [33]. Another caveat in our strategy was the possibility that isolating mutants by testing its transcription activation activity might not be generalized to other potential functions of BRCA1. In fact, several independent studies have shown that the BRCT domains of BRCA1 can specifically recognize phosphoserine-containing peptides, suggesting a mechanism by which these domains recruit phosphorylated signaling factors or complexes [34-36]. Importantly, the residues involved in phosphopeptide binding are strikingly similar to the ones defined to be part of a functional site, and identified in our laboratory,[37] implicated in transcription regulation. These findings provide a structural basis for our working model that transcription assays are a bona fide monitor for the structural integrity of the BRCT domains and conclusions drawn from them are likely to be generalizable.

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Ref Type: Conference Proceeding

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Appendixes:

1) "A Naturally-Occurring Allele of *BRCA1* Coding for a Temperature-Sensitive Mutant Protein" by Worley et al. *Cancer Biology and Therapy*, **1:** 497-501.

2) "Mutations in the BRCT domain confer temperature sensitivity to BRCA1 in transcription activation" by Carvalho, Billack et al. *Cancer Biology and Therapy*, **1:** 502-508.

3) "Linking breast cancer susceptibility and the DNA damage response" by Dapic et al. *Cancer Control Journal, In press.*

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Research Paper

A Naturally Occurring Allele of BRCA1 Coding for a Temperature-Sensitive Mutant Protein

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KEY WORDS

BRCA1, Yeast, Transcription, Temperature-sensitive mutants, BRCT domain, Ovarian cancer

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ABSTRACT

Recent evidence suggests that the breast and ovarian cancer susceptibility gene product BRCA1 is involved in at least two fundamental cellular processes: transcriptional regulation and DNA repair. However, the mechanism of action of BRCA1 in either of these processes is still unknown. Here, we report the characterization of a disease-predisposing allele of BRCA1, identified in a family with several cases of ovarian cancer, coding for a protein that displays temperature-sensitive activity in transcriptional activation. The mutant protein differs from the wild type protein at a single amino acid, R1699W that occurs in a region at the N-terminal BRCT domain that is highly conserved among BRCA1 homologs. When the C-terminus of the mutant protein (aa 1560–1863) was fused to a heterologous GAL4 DNA-binding domain and expressed in yeast or mammalian cells, it was able to activate transcription of a reporter gene to levels observed for wild type BRCA1 at the permissive temperature (30°C) but exhibited significantly less transcription activity at the restrictive temperature (37°C or 39°C). Our results indicate that the transcriptional activity of the R1699W mutant can be modulated as a function of temperature and provide a novel experimental approach which can be utilized to dissect the molecular mechanism(s) of BRCA1 in processes related to transcription.

INTRODUCTION

Individuals carrying inactivating germline mutations in BRCA1 (OMIM 113705) have an increased predisposition to breast and ovarian cancer.[1,2] A growing body of evidence indicates that the breast and ovarian cancer susceptibility gene product BRCA1 is involved in at least two fundamental cellular processes: transcriptional regulation and DNA repair.[3-5] However, the mechanism of action of BRCA1 in either of these processes is still unknown.

During our analysis of BRCA1 germline missense mutations we came across a naturally occurring BRCA1 allele, identified in a family with several cases of ovarian cancer in which the mutation segregates with disease.[6] This allele carries a single point mutation (nucleotide C5214T), leading to a change from an arginine to a tryptophan residue at position 1699, located at the C-terminal region of BRCA1. The BRCA1 C-terminus (aa 1560–1863) has the ability to activate transcription when fused to a heterologous DNA binding domain (DBD) and introduction of disease-associated germ-line mutations impair transcription activation, while benign polymorphisms do not.[6-10] Missense mutations in BRCA1 that abolish transcription activation or disrupt the N-terminal RING finger structure also affect the ability of BRCA1 to interact with the RNA polymerase II holoenzyme in vitro and in vivo.[11,12] The C-terminal region encompasses two BRCT domains in tandem (BRCT-N [aa 1649–1736]; BRCT-C [aa 1756–1855])[13-15] and disruption of these domains is linked to cancer susceptibility. Interestingly, the ability to activate transcription does not seem to be a general characteristic of BRCT domains since BRCT domains isolated from other proteins, with the exception of RAP1, do not possess such activity.[16]

We previously observed that the R1699W mutant retains wild-type transcriptional activity in yeast but displays a loss-of-function phenotype when transcription activity is assessed in human cells.[6] Considering the conservation of basal transcription machinery in yeast and human cells,[17] we hypothesized that the discrepancy in transcription activity was due to differences at the temperature in which the cells were being cultured; 30°C for yeast cells and 37°C for human cells. In the present study we demonstrate that the cancer-predisposing R1699W variant of BRCA1 acts as a temperature-sensitive mutant in both yeast and human cells in transcription activation assays.

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MATERIALS AND METHODS

Yeast. *Saccharomyces cerevisiae* strain EGY48 [*MATα, ura3, trp1, his3*, 6 lexA operator-*LEU2*] contains a LexA-responsive *LEU2* gene, which when activated permits growth in the absence of leucine.[18] Transformations were performed using the yeast transformation system based on lithium acetate (Clontech) according to the manufacturer's instructions.

Yeast Expression Constructs. Constructs containing the fusion GAL4 DBD:BRCA1 wild type (amino acids 1560–1863) or mutants R1699W, M1775R and Y1853X were previously described.[6] BRCA1 inserts (wild type and mutants) were subcloned into pLex9 in-frame with the DBD of LexA. A *TRP1* selectable marker is present in pLex9, allowing growth in medium lacking tryptophan.

Yeast Growth Assay. Cells were transformed with the LexA DBD fusion constructs and plated in solid medium lacking tryptophan. At least three independent colonies for each construct were inoculated into liquid medium lacking tryptophan and grown to saturation (OD_{600} ~1.5) at 30°C. Saturated cultures were used to inoculate fresh medium lacking tryptophan or medium lacking tryptophan and leucine to an initial OD_{600} of 0.0002. Parallel cultures were then incubated at 30°C or 37°C and growth was assessed by measurement at OD_{600} after 38 hr.

Transcription Assay in Mammalian Cells. The region comprising the GAL4 DBD fused to BRCA1 C-terminus containing the R1699W mutation was excised from pGBT9 backbone[6] with *Hind*III and *Bam*H1 and ligated into pCDNA3. Constructs in pCDNA3 containing fusion of GAL4 DBD and wild type BRCA1, M1775R or Y1853X variants were previously described.[6] We used the reporter pG5E1bLuc, which contains a firefly luciferase gene under the control of five GAL4 binding sites[19] and transfections were normalized with an internal control, pRL-TK, which contains a *Renilla* luciferase gene under a constitutive TK basal promoter using a dual luciferase system (Promega). Human 293T cells were cultured in DMEM supplemented with 5% calf serum and plated in 24-well plates at ~60% confluence the day before transfection. Transfections were carried out in triplicates using Fugene 6 (Roche, Indianapolis, IN) at 37°C for 12 hr. Cells were then incubated at 30°C, 37°C or 39°C and harvested 16 hr later. Human cell lines NIH-OVCAR-3 and SKOV-3, both derived from ovarian adenocarcinoma, and CAOV-2, derived from the malignant ascites of a patient with progressive adenocarcinoma of the ovary, were kindly provided by Jeff Boyd (Memorial Sloan Kettering).

Western Blotting. Yeast cells were grown in selective media to saturation and OD_{600} was measured. Cells were harvested and lysed in cracking buffer (8M Urea; 5% SDS; 40 mM Tris-HCL [pH6.8]; 0.1 mM EDTA; 0.4 mg/ml bromophenol blue; use 100 ml per 7.5 total OD_{600}) containing protease inhibitors. The samples were boiled and separated on a 10% SDS-PAGE. The gel was electroblotted on a wet apparatus to a PVDF membrane. The blots were blocked overnight with 5% skim milk using TBS-tween, and incubated with the a-pLexA (for LexA constructs) monoclonal antibody (Clontech) using 0.5% BSA in TBS-tween. After four washes, the blot was incubated with the a-mouse IgG horseradish peroxidase conjugate in 1% skim milk in TBS-tween. The blots were developed using an enhanced chemiluminescent reagent (NEN, Boston, MA).

RESULTS

A Temperature-Sensitive Phenotype in Yeast. We previously observed that the R1699W mutant (Fig. 1) retains wild-type transcriptional activity in yeast but displays a loss-of-function phenotype when transcription activity is assessed in human cells.[6] This discrepancy was not due to differential protein stability, vector background or promoter stringency in the reporter.[6] To further investigate this phenomenon, we generated yeast cell lines with an inducible GAL4 DBD: R1699W BRCA1 fusion integrated in the yeast genome as a single copy. In this context, the R1699W variant also displays activity comparable to wild type BRCA1, ruling out the possibility that the results were due to abnormally high levels of the protein expressed by an episomal plasmid (results not shown).

We then reasoned that differences in transcription activation could be due to differences in the temperature at which the cells were being cultured; 30°C for yeast cells and 37°C for human cells. To test this idea directly, we transformed *Saccharomyces cerevisiae* EGY48 with cDNAs coding for fusions of LexA (DBD) and the wild type C-terminal region of BRCA1 (aa 1560–1863) or constructs carrying either the R1699W mutant or one of two other germline disease-associated mutations, M1775R and Y1863X, as negative controls. Transcription activity was quantified at 30°C and 37°C using an integrated reporter gene (6 *lexA* binding sites; *LEU2*) that, when activated, allows growth in the absence of leucine. Cells carrying the wild-type BRCA1 construct were able to grow in selective medium lacking leucine at both temperatures (Fig. 2A). Conversely, cells carrying the two disease associated mutants did not show any detectable growth at either temperature (Fig. 2A). Interestingly, cells carrying the R1699W mutant were able to grow at levels comparable to the wild type at 30°C but growth was dramatically impaired at 37°C, indicating a marked reduction in transcriptional activity (Fig. 2A). Expression was comparable for the R1699W and the wild type protein at both temperatures (Fig. 2B).

The R1699W Variant Displays Temperature-Dependent Activity in Human Cells. To confirm the temperature-dependent activity of the R1699W variant, we cotransfected human kidney 293T cells with a luciferase reporter gene driven by GAL4-responsive promoter and cDNAs coding for fusions of GAL4 DBD with the wild type C-terminal region of BRCA1 (aa 1560-1863), the R1699W mutant or either of the two disease-associated mutations, M1775R and Y1863X, as negative controls. We then incubated cells in parallel at two temperatures: 30°C and 37°C. The disease-associated mutants M1775R and Y1853X display a small increase in relative activity at 30°C but their activity is significantly lower than the wild type BRCA1 (Fig. 3A, left panel). Interestingly, the transcriptional activity of the R1699W mutant was restored to wild type levels when cells were cultured at 30°C, indicating that this mutant acted as a temperature-sensitive allele of BRCA1 in transcription activation (Fig. 3A, left panel). Although at 37°C the R1699W mutant displays residual activity, experiments conducted at 39°C indicated a further reduction in activity (Fig. 3A, right panel). Expression was comparable for R1699W, M1775R and the wild type protein at both temperatures (not shown).

A Complex Regulation in Breast and Ovarian Cancer Cells. Considering the occurrence of multiple ovarian cancers (but not breast cancer) in the family in which the R1699W was identified, Lund 279, we next asked whether ovarian cancer cell lines were different from breast cancer cell lines with respect to the temperature-sensitive phenotype. We tested transcriptional activation in two

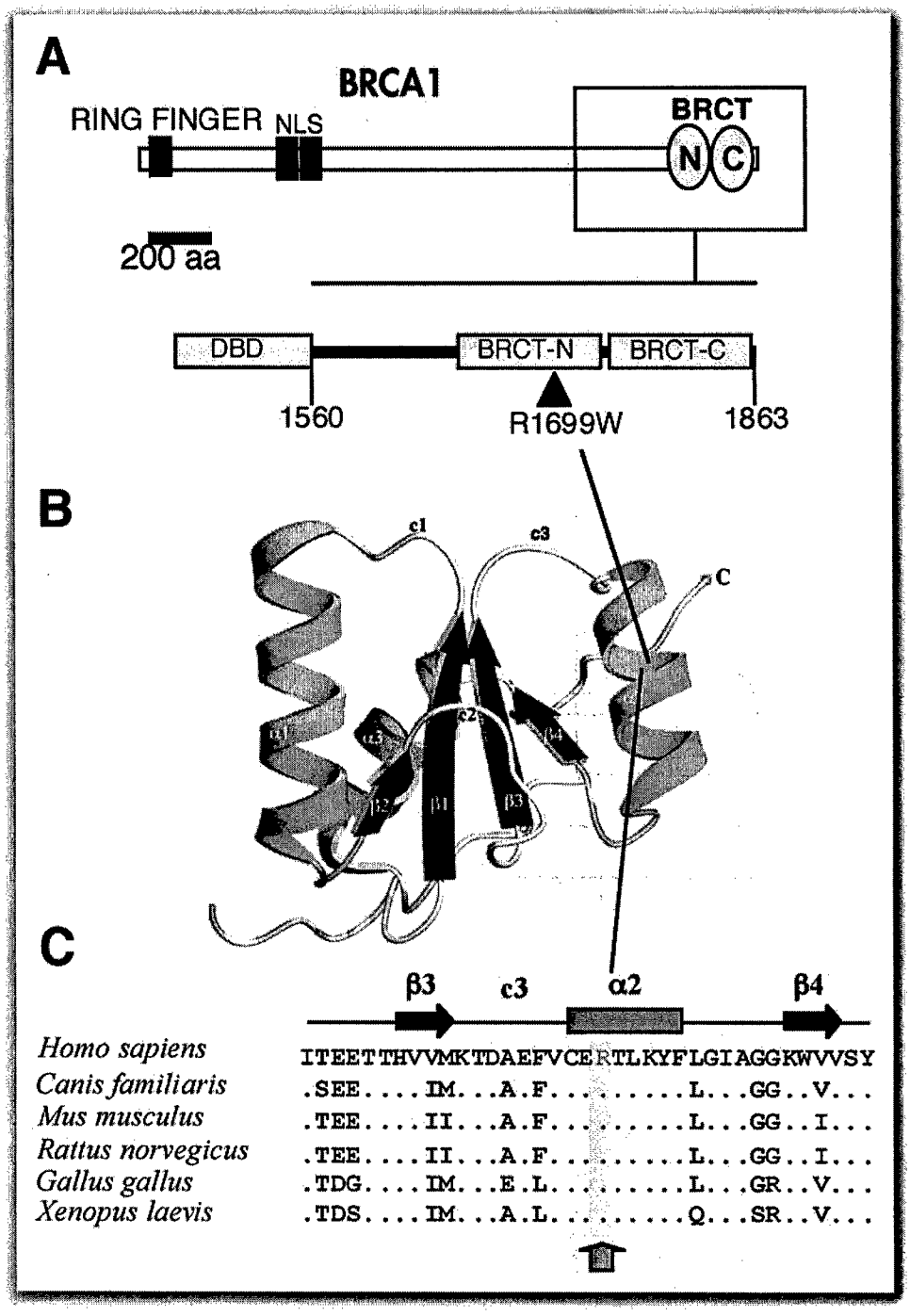

Figure 1. A temperature-sensitive BRCA1 mutant in transcription activation. (A) Top panel. Schematic representation of full length BRCA1 protein featuring: the RING domain in the N-terminus; the BRCT domains in the C-terminus (gray circles) and the nuclear localization signals (NLS). The region analyzed in this study is contained in the box, which is enlarged and represented in the bottom panel. Bottom panel. GAL4- and LexA-DNA binding domain (DBD) fusions to BRCA1 C-terminus (aa 1560–1863). The location of the R1699W mutation is indicated by a filled triangle. (B) Model of three-dimensional structure of BRCA1 BRCT (from ref. 30 by permission from Oxford University Press) indicating the location of the R1699 residue. (C) Alignment of BRCA1 homologs with secondary structures indicated on top. Dots represent identical amino acids. Location of R1699 residue is indicated with a red arrow.

breast cancer cell lines (MCF-7 and HCC1937) and three ovarian cancer cell lines (OVCAR-3, CaOV-2 and SKOV-3)(Fig. 3B). Surprisingly, the experiments revealed a complex regulation of this mutant in different human cancer cell lines. In MCF-7 and Caov-2, the R1699W allele was able to activate transcription to levels comparable to wild type BRCA1 at permissive and restrictive temperatures (Fig. 3B). In HCC1937 and NIH-OVCAR-3, the R1699W displayed temperature-dependent activity with normal activity at 30°C and loss of function at 37°C, consistent with our previous observation in 293T cells (Fig. 3A, B). Interestingly, in SKOV-3 cells the R1699W displayed a loss-of-function phenotype at both temperatures (Fig. 3B). In conclusion, our results did not reveal any correlation of the temperature-sensitive phenotype and tissue of origin and suggest that the R1699W variant may have a cell-type specific temperature-sensitive phenotype.

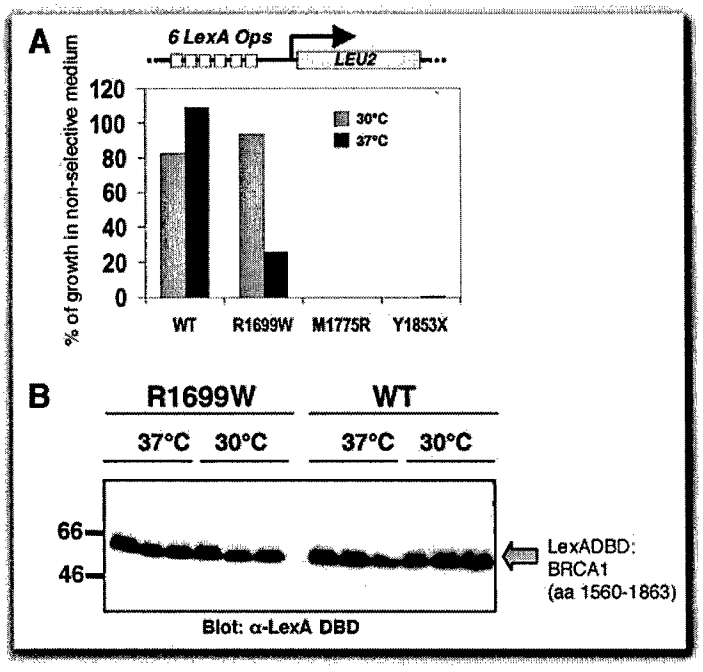

Figure 2. Transcriptional activity of BRCA1 R1699W at different temperatures in yeast. (A) Activity in yeast cells as measured by activation of an integrated LEU2 gene. Cells were cultured in non-selective and selective medium (lacking leucine) at 30°C and 37°C for 38 hr. Growth was measured by OD_{600}. Growth in non-selective medium was considered 100%. M1775R and Y1853X mutants were used as negative controls. (B) Mutant R1699W is expressed in yeast at the same level as wild type (gray arrow) at both temperatures. Three independent clones are shown for each condition. Blot was probed with α-LexA DBD monoclonal antibody.

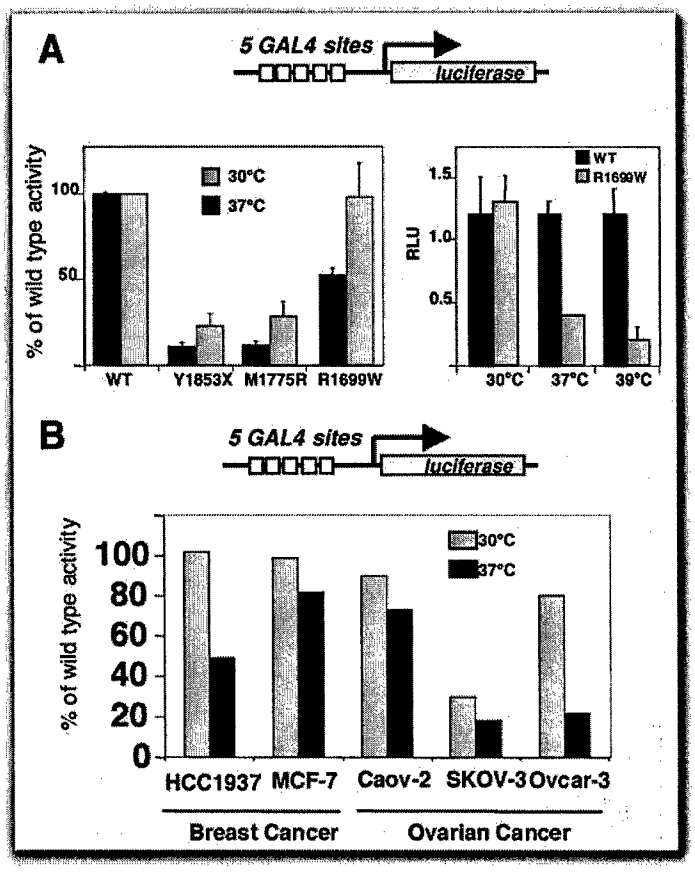

Figure 3. Transcriptional activity of BRCA1 R1699W at different temperatures in human cells. (A) Left panel. Transcriptional activity of the wild type BRCA1, R1699W, Y1853X and M1775R mutants at 30°C (gray bars) and 37°C (black bars). Activity of the R1699W variant in 293T cells is comparable to wild type at 30°C but markedly reduced at 37°C. Right panel. Transcriptional activity of the wild type BRCA1 and the R1699W mutant at different temperatures. RLU, relative luciferase units. Represents the ratio between Firefly luciferase and Renilla luciferase (internal control). (B) Temperature-sensitive activity of R1699W is cell type-specific. Average of three independent experiments. HCC1937, MCF-7 are breast cancer cell lines; CaOV-2, SKOV-3, NIH-OVCAR-3 are ovarian cancer cell lines. Structures of the reporters are depicted on top of the graphs. Transfections are normalized with a constitutive Renilla luciferase reporter.

DISCUSSION

The biochemical function of BRCA1 has remained elusive and the current evidence suggests that BRCA1 may have a pleiotropic function in the DNA damage response pathway and may be able to influence several activities that revolve around DNA damage resolution.[3,5,20-23] Alternatively, BRCA1 being a large multifunctional protein may have a wide range of unrelated biochemical activities in the cell. One approach to understand the function of a protein with tumor suppressor action is to analyze naturally occurring mutations that cause cancer predisposition.

In our study of missense mutations found in individuals with high risk for breast and ovarian cancer we came across a naturally occurring BRCA1 allele identified in a family from Lund that displayed unusual behavior in the transcription activation assay.[6] The clinical data suggests that the R1699W mutation (Arg to Trp substitution at codon 1699; see also Breast Cancer Information Core Database at http://research.nhgri.nih.gov/bic/) is likely to have a deleterious effect in vivo and predispose carriers to cancer. Disease association is further emphasized by the presence of the R1699W mutation in a large pedigree with several women diagnosed with ovarian cancer (Tom Frank, personal communication). Nonetheless, our initial transcription-based tests of this variant showed that it retained wild type activity in yeast but not in mammalian cells.[6] Previous studies had shown a complete concordance between results in yeast and mammalian cells.[7,8,24] This apparent divergence could not be explained by vector background, promoter stringency or abnormally high levels of the protein expressed by an episomal plasmid but rather was due to temperature differences at which the assays were carried out. Our results demonstrate that the R1699W variant display a temperature-sensitive phenotype in transcription activation and therefore may represent the first conditional mutant of BRCA1 to be described (Fig. 2).

The R1699W mutation is located in the BRCT domain, a region that is involved in binding to many different proteins that associate with BRCA1[3] and crucial for transcriptional activity.[10] The mutation, R1699W, replaces an arginine residue involved in a salt bridge that is thought to stabilize the packing of the two BRCT domains.[25] It occurs in a region at the N-terminal BRCT domain that is highly conserved among BRCA1 homologues (Fig. 1C). Interestingly, this region is not found in other BRCT motifs and seems to be unique to BRCA1 BRCT.[14,15] In fact, the predicted α-helix 2, in which the mutation resides, is conserved in all known BRCA1 homologs (Fig. 1C) and has been proposed to be responsible for determining functional specificity of the BRCT domains.[26]

Interestingly, Lund 279 presents almost exclusively ovarian cancer cases and no breast cancer cases raising the possibility of

differential effects of this mutant in breast versus ovary epithelia. That observation led us to test the temperature-sensitiveness in breast cancer and ovarian cancer cell lines. Intriguingly, our results indicate that the temperature-sensitive phenotype of the R1699W mutant is cell type specific but no tissue correlation was apparent with the cell lines tested (Fig. 3). At this point we can only speculate on the nature of such behavior and propose the following possible scenarios:

1. Residue 1699 is involved in binding to a factor required for transcription activation by BRCA1. The mutation would cause a marked decrease in binding affinity that is less severe at lower temperatures, therefore making it susceptible to variations in the concentration of this factor. For example, in cells where the factor is abundant, the R1699W variant would still be able to bind enough of the factor to promote transcription at both temperatures. Conversely, in cells in which the factor was at very low concentrations the R1699W variant would not be able to recruit the factor at either temperature. At intermediate concentrations of the factor, the R1699W variant would be able to bind it at 30°C but its ability to recruit the factor would be extremely reduced at 37°C.
2. Alternatively, it is also possible that instead of required for transcription, the function of this factor is to confer stability to the mutated protein (e.g., chaperone). In this case, the scenario of varying concentrations described above, or differential expression would also be applicable. This scenario is consistent with the observed increase in activity of the other mutants at 30°C (Fig. 2C).

Recently, mutants of the Xeroderma pigmentosum group D (XPD) helicase subunit of TFIIH displaying a temperature-sensitive phenotype in transcription and DNA repair have been isolated from patients with trichothiodystrophy (TTD). Patients carrying the mutant allele have a fever-dependent reversible deterioration of TTD features.[27,28] In this case, the phenotype manifests predominantly in the skin, hair and nails for reasons that are not well understood.[27] This finding raises important questions about the implications of the R1699W variant to the clinical phenotype. It is possible that small differences in temperature between breast and ovary may be responsible for an increased incidence of ovarian cancer. It remains to be seen if other families carrying this allele also display a preferential occurrence of ovarian cancer. These data also suggest that conditional mutants of BRCA1 in transcription may also have a conditional phenotype in the DNA damage response. We are currently exploring these possibilities.

In parallel with the present study we performed a random mutagenesis screen in yeast and identified 11 additional temperature-sensitive mutants of BRCA1.[29] Different from the variant described in this study, R1699W, which is an exposed surface residue, these additional TS mutants localized primarily to the hydrophobic core of the BRCT-N domain of BRCA1.[29] Further characterization is needed to assess whether these conditional mutations of BRCA1 may serve as experimental tools to dissect the precise molecular role of BRCA1 in processes related to transcriptional regulation.

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Research Article

Mutations in the BRCT Domain Confer Temperature Sensitivity to BRCA1 in Transcription Activation

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ABSTRACT

BRCA1 is a tumor suppressor gene and germ line mutations account for the majority of familial cases of breast and ovarian cancer. There is mounting evidence that BRCA1 functions in DNA repair and transcriptional regulation. A major hurdle to dissect the role of BRCA1 is the lack of molecular reagents to carry out biochemical and genetic experiments. Therefore, we used random mutagenesis of the C-terminus of BRCA1 (aa 1560–1863) to generate temperature-sensitive (TS) mutants in transcription activation. We obtained 11 TS mutants in transcription that localized primarily to the hydrophobic core of the BRCT-N domain of BRCA1. One of the mutants, H1686Q, also displayed temperature-dependent transcription activation in human cells. These conditional mutants represent valuable tools to assess the role of BRCA1 in transcription activation.

INTRODUCTION

Germ-line mutations in BRCA1 confer high risk for breast and ovarian cancer.[1,2] The molecular function of BRCA1 is not yet known but there is increasing evidence that it is involved in DNA damage repair and gene transcription.[3,4] Several lines of evidence support a direct role for BRCA1 in transcription. When fused to a heterologous DNA binding domain (DBD) the C-terminus of BRCA1 activates transcription from a reporter gene and the introduction of cancer-associated mutations, but not benign polymorphisms, abolish[25] activation.[5-7] In addition, BRCA1 interacts with the RNA polymerase II and with several complexes involved in chromatin remodeling.[8-11] Ectopic expression of BRCA1 results in the transcription of genes involved in cell cycle control and DNA damage repair.[12-17] Interestingly, BRCA1 also interacts with CsTF50 in a complex that regulates mRNA processing pointing to a pleiotropic role in transcription.[18]

Despite the absence of BRCA1 homologs in its genome, yeast has been an important model system to study BRCA1 as well as the function of several mammalian transcription factors.[19] Yeast has been utilized to perform structure-function analysis of BRCA1 in transcription as well as to probe its mechanisms of activation based on the correlation with the clinical data.[5,7,20-22] In addition, overexpression of human BRCA1 in yeast generates a small colony phenotype that has been proposed as a method to classify uncharacterized mutations in BRCA1.[23,24] Thus, despite its limitations, yeast is a defined system to analyze BRCA1 function and is adequate for the rapid screening of large mutant libraries.

A major hurdle to define the function(s) of BRCA1 is the lack of molecular tools. Temperature-sensitive (TS) mutants would be particularly useful for this analysis. Recently, we have identified a BRCA1 allele in a family with familial ovarian cancer that displays a temperature-sensitive phenotype in mammalian cells (refs. 21, 41). Therefore, we hypothesized that a differential screen in yeast based on random mutagenesis would allow us to isolate additional TS mutants. We followed the same procedure we had previously used to generate loss-of-function mutants in transcription activation and performed parallel screens at 30°C and 37°C.[20] We utilized this yeast-based system to identify and characterize 11 TS mutations and 15 loss-of-function (LF) mutants of BRCA1. One of the TS mutations identified in the yeast screen was found to exhibit a similar phenotype in human cells. These mutants will allow the study of BRCA1 function in yeast and provide a basis for the development of novel conditional mutants for mammalian cells.

METHODS

Yeast. *Saccharomyces cerevisiae* strain EGY48 [*MATα, ura3, trp1, his3, 6 lexA* operator-*LEU2*] was co-transformed with the LexA fusion vectors and reporter plasmid pSH18–34, which has lacZ under the control of 8 LexA operators.[25,26] The LexA DBD fusion of wild type human BRCA1 C-terminus

(aa 1560–1863) and two germ-line mutants of BRCA1, Y1853X and M1775R were used as controls. Competent yeast cells were obtained using the yeast transformation system (Clontech).

Error-Prone PCR Mutagenesis and Screening. A 30-cycle PCR reaction (94°C denaturation; 55°C annealing; 72°C extension) was performed using *Taq* polymerase, p385-BRCA1 plasmid as a template and oligonucleotide primers (S9503101, 5′-CGGAATTCGAGGGAACCCCTTACCTG-3′; S9503098, 5′-GCGGATCCGTAGTGGCTGTGGGGGAT-3′). PCR products were gel purified and co-transformed in an equimolar ratio with an *NcoI*-linearized wild-type pLex9 BRCA1 (aa 1560–1863) plasmid and pSH18-34. Transformants carrying the mutagenized cDNAs were plated at 37°C or 30°C on plates lacking tryptophan and uracil and containing 80 mg/L X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside), 2% Galactose, 1% Raffinose, 1X BU salts (1L of 10X BU salts: 70g $Na_2HPO_4.7H_2O$, 30g NaH_2PO_4). The X-gal plates allowed direct visualization and were scored after 6 days. Clones were recovered from yeast and sequenced.

Mammalian Cell Reagents. A region comprising the BRCA1 coding region containing the TS mutation in pLex9 vector was excised with *EcoR*1 and *Bam*H1 and subcloned in pGBT9 in frame with GAL4 DBD. The fusion GAL4 DBD: BRCA1 was then cut with *Hind*III and *Bam*H1 and ligated into pCDNA3. We used the reporter pG5E1bLuc, which contains a firefly luciferase gene under the control of five GAL4 binding sites and transfections were normalized using a dual luciferase system (Promega). For the mammalian two-hybrid system the pCDNA3 GAL4 DBD: BRCA1 (aa 1560–1863) and the constructs carrying different TS mutations were used as bait to test interaction against CtIP. The construct containing CtIP (aa 45–897) fused to the herpesvirus VP16 transactivation domain (aa 411–456) was used as target and the VP16 vector was used as negative control (gift from Richard Baer, Columbia University). Human 293T cells were cultured in DMEM supplemented with 5% calf serum and plated in 24-well plates at ~60% confluence the day before transfection. Transfections were carried out in quadruplicates using Fugene 6 (Roche, Indianapolis, IN) at 37°C for 12 hr. Cells were then incubated at 30°C or 37°C and harvested 16 hr later.

RESULTS

Screen for TS Mutants of BRCA1 in Transcription Activation. We screened ~ 3 x 10^6 independent clones and recovered 1,302 putative LF mutants at 37°C (Fig. 1A). These colonies were then plated on fresh plates and incubated at 37°C and 30°C for confirmation (Fig. 1B). All plates contained yeast expressing wild-type cDNA to control for the different activity of β-galactosidase at both temperatures. Several clones turned out to display either a loss-of-function (white clones) or wild-type (blue clones) phenotype at both temperatures. Plasmids were recovered, retransformed into yeast and their activity confirmed. Clones that failed to display a reproducible activity were discarded. Plasmids representing 38 clones (3 were not recovered) were analyzed by restriction digest and although no clone had detectable deletions/insertions by gel analysis, sequencing revealed that 12 had nucleotide deletions or nonsense mutations and were not analyzed further. The remaining clones were processed for sequencing and the mutation identified. Eleven clones displayed markedly reduced activity at 37°C and wild-type activity at 30°C (TS clones; Table 1) and 15 had reduced activity at both temperatures (LF clones, Table 2).

TS Mutants in Yeast. Our screen resulted in the isolation of 11 TS mutants (8 unique) in transcription activation in yeast (Table 1). Seven clones displayed only one missense mutation and four clones displayed two missense mutations (Table 1). It is unclear whether the two mutations are required for the TS phenotype or not. At least in one case, TS32 (S1722F/K1667E), we know this is not the case because a similar mutation was found independently in another clone, TS25. Mutations causing TS activity were found in exons 16–20 and 24. Interestingly, conserved hydrophobic residues were found to be a major target of mutations followed by mutations in serine residues (Table 1 and Fig. 2). With three exceptions, S1631N, L1639S and E1836G, all mutations occurred either in the N-terminal BRCT region or in the interval between the N- and C-terminal BRCTs (Table 1 and Fig. 2).

Loss-of-Function (LF) Mutants. Due to the experimental design, several clones proved not to be TS mutants but instead LF mutants at both temperatures tested (Table 2). These mutations also targeted hydrophobic

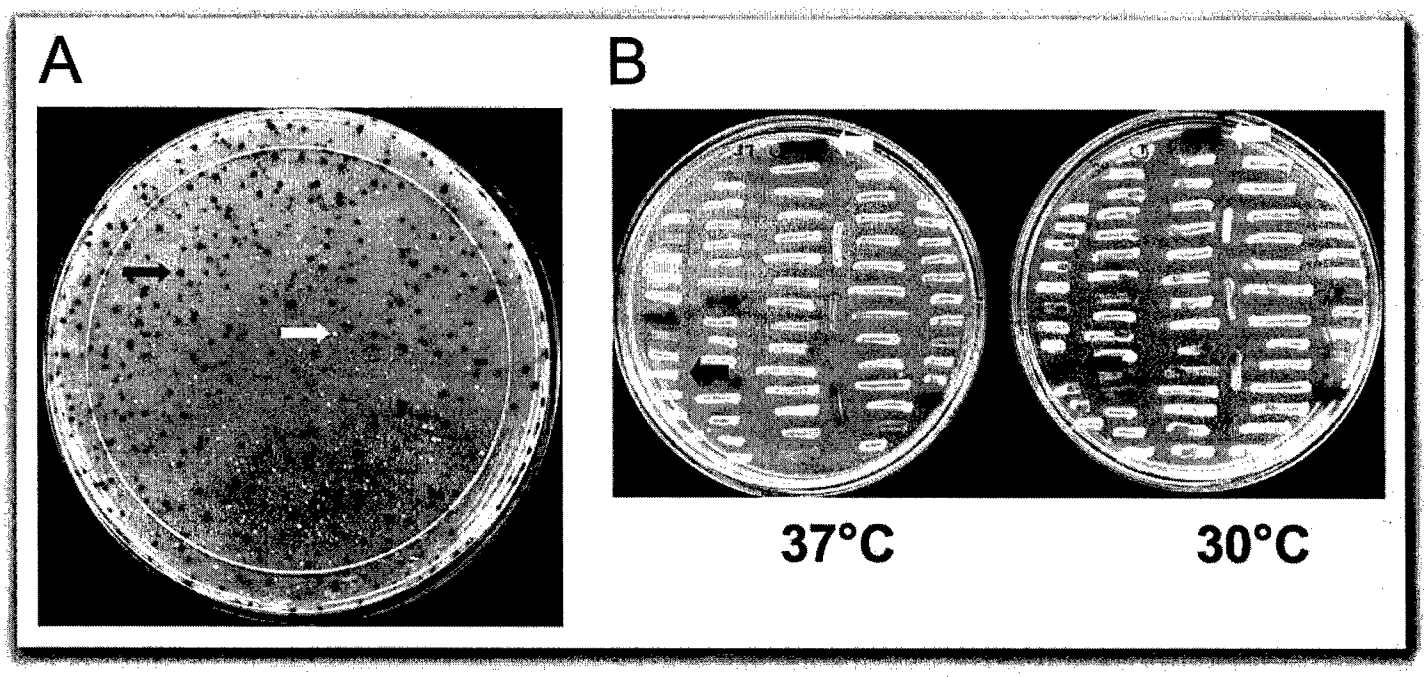

Figure 1. Screening for temperature-sensitive mutants of BRCA1 in transcription. (A) Primary screening at 37°C. Transformants carrying BRCA1 with wild-type activity appear as blue colonies (blue arrow) and transformants carrying loss-of-function mutants at 37°C appear as white colonies (white arrow). White colonies were replated in parallel and incubated at 30°C and 37°C. (B) Plates containing replicas of each white clone isolated from primary plates. A transformant carrying a wild-type BRCA1 is included at the top of each plate (white arrow). Clones that were consistently white at 37°C and blue at 30°C were isolated as temperature-sensitive mutants (blue arrow). Clones that were white at both temperatures were isolated as loss-of-function mutants.

Table 1 **TEMPERATURE-SENSITIVE MUTANTS OF BRCA1 (AA 1560–1863) IN TRANSCRIPTION**

Clone	Exon	Mutation	Nucleotide Change[a]	Allowed Residues[b]	Secondary Structure[c] and Comments	Activity[d] 30°C	37°C
TS1	17	F1668S	T5122C	F	BRCT-N α-helix 1	+++	-
	24	E1836G	A5626G	DE	BRCT-C α-helix 3		
TS4	16	L1605L	T4932C	silent	unknown	+++	-
	17	V1687A	T5179C	V	BRCT-N β-sheet 3		
	19	K1727E	A5298G	KRQ	BRCT-N/BRCT-C interval		
TS6	16	L1639S	T5053C	LV	unknown	+++	-
TS19	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
	20	E1735E	A5324G	silent	BRCT-N/BRCT-C interval		
TS25	16	S1610S	T4949C	silent	unknown	+++	-
		S1722F	C5284T	S	BRCT-N α-helix 3		
TS26	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS30	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS32	17	K1667E	A5118G	KR	BRCT-N α-helix 1	+++	-
		S1722F	C5284T	S	BRCT-N α-helix 3		
TS33	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS36	16	S1631N	G5011A	SI	unknown	+++	-
	18	V1713A	T5257C	VI	BRCT-N β-sheet 4; uncharacterized variant found as a germline mutation[e]		
TS50	17	H1686Q	T5177A	H	BRCT-N β-sheet 3	+	-

[a]Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession #U14680; [b]Residues that are found in the same position in an alignment of human (U14680), chimpanzee (AF207822), dog (U50709), rat (AF036760), mouse (U68174), chicken (AF355273) and frog (AF416868) homologs. [c]According to the BRCA1 BRCT crystal structure;[37] [d]Activity was scored in plates after 6 days. (+++) activity comparable to wild-type BRCA1; [e]As described in the Breast Cancer Information Core (BIC) database.

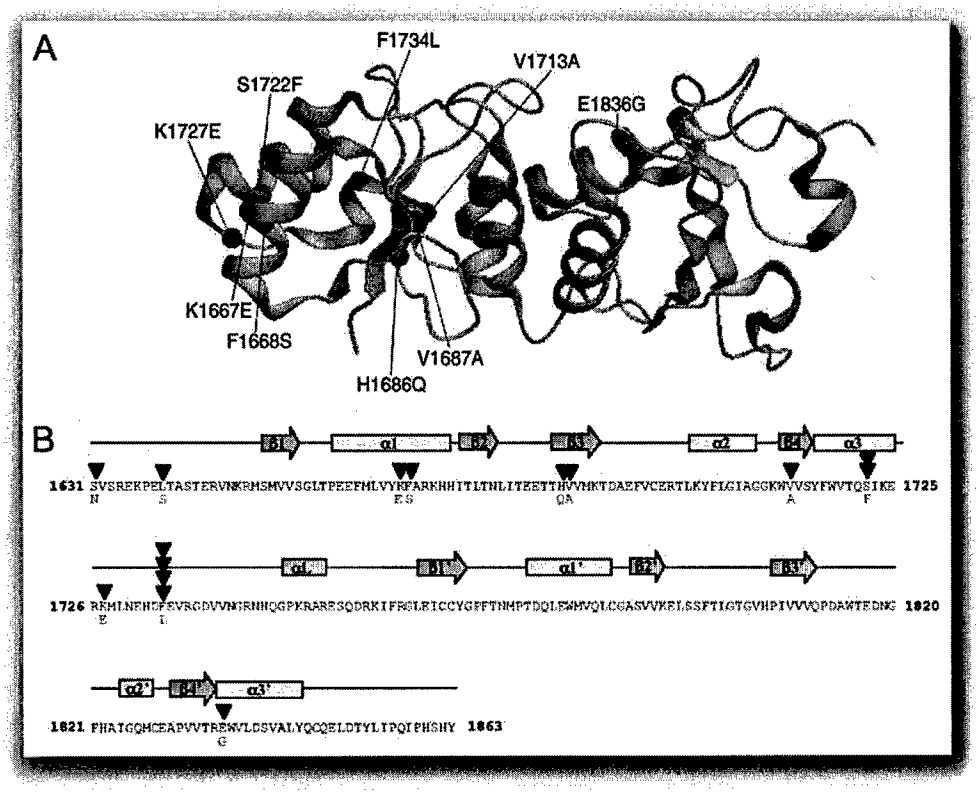

Figure 2. TS mutations localize primarily to the BRCT-N domain. (A) The location of the eight unique TS mutations is shown in the BRCT-N and BRCT-C domains of human BRCA1 according to the crystal structure of human BRCA1 BRCT region.[37] Red spheres represent the only coding change in a single clone and blue spheres represent changes that are in clones with multiple mutations. Note that with exception of S1631N, L1639S (which precede the BRCT domains and are not shown) and E1836G (TS1), all other TS mutations map to the BRCT-N domain. (B) Secondary structure elements according to crystal structure of BRCA1 BRCT region[37] are depicted above the sequence. Interval region, separating BRCT-N and BRCT-C is represented by a dotted line with a α-helix (αL; purple). Residue positions mutated in TS clones are shown for clones containing one (red triangle) or two changes (blue triangle). Changes are indicated below the sequence.

Table 2 **LOSS-OF-FUNCTION MUTANTS OF BRCA1 (AA 1560-1863) IN TRANSCRIPTION**

Clone	Exon	Mutation	Nucleotide Change[a]	Allowed Residues[b]	Secondary Structure[c] and Comments	Activity[d] 30°C	Activity[d] 37°C
LF2	23	Q1811R	A5551G	Q	BRCT-C β3-α2 loop; uncharacterized variant found as a germline mutation[e]	-	-
	23	P1812S	C5553T	P	BRCT-C β3-α2 loop		
	24	A1843P	G5646C	AS	BRCT-C α-helix 3; uncharacterized variant found as a germline mutation[e]		
LF3	17	L1671L	A5132G	silent		-	-
	18	E1694G	A5201G	E	BRCT-N β3-α2 loop		
	24	V1842A	T5644C	VIL	BRCT-C α-helix 3		
LF5	16	L1657P	T5089C	L	BRCT-N β1-α1 loop	-	-
LF8	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF15	24	A1843T	G5646A	AS	BRCT-C α-helix 3	-	-
LF20	17	T1691T	A5192G	silent	unknown	-	-
	17	F1668S	T5122C	F	BRCT-N α-helix 3		
	24	R1835R	A5624G	silent			
	24	P1856T	C5685A	PQS	unknown		
LF22	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF23	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF24	20	G1743R	G5346A	G	BRCT-N/BRCT-C interval	-	-
LF27	16	L1636L	T5034C	silent		-	-
	16	L1657P	T5089C	L	BRCT-N β1-α1 loop		
	17	L1664L	C5110T	silent			
LF28	16	S1577P	T4848C	S	unknown	-	-
	16	S1655P	T5082C	S	BRCT-N β1-α1 loop; residue mutated in the germline (S1655F)[e]		
LF35	21	M1775R	T5443A	M	BRCT-C β1-α1 loop; cancer-associated mutation found in the germline[e]	-	-
	22	Q1779Q	A5456G	silent			
LF34	23	I1807S	T5539G	IVL	BRCT-C β-sheet 3	-	-
	23	H1822H	T5585C	silent			
LF38	16	E1660G	G5098A	EKSC	BRCT-N α-helix 1	-	-
LF47	16	R1649R	A5066G	silent		-	-
	18	F1704S	T5230C	F	BRCT-N α-helix 2		

[a]Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession #U14680; [b]Residues that are found in the same position in an alignment of human (U14680), chimpanzee (AF207822), dog (U50709), rat (AF036760), mouse (U68174), chicken (AF355273) and frog (AF416868) homologs. [c]According to the BRCA1 BRCT crystal structure;[36] [d]Activity was scored in plates after 6 days. (+++) activity comparable to wild-type BRCA1; [e]As described in the Breast Cancer Information Core (BIC) database.

residues in the BRCT domains. Interestingly, we recovered a recurring cancer-associated mutation of BRCA1, M1775R (LF35; Table 2).[29] Also, Q1811R and A1843P, found together in LF2, are unclassified variants listed in the Breast Cancer Information Core database (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/). Two mutations, F1704S and L1657P, were found in 4 and 2 independent clones, respectively. Mutations causing LF phenotype were found in all exons examined with the exception of exon 19.

TS Mutants in Human Cells. All unique TS clones had their activity measured in human cells using a fusion to GAL4 DBD and a luciferase reporter driven by a GAL4-responsive promoter. Negative controls used were two cancer-associated mutants, M1775R and Y1853X.[29,30] In four independent experiments, one of the mutants (TS50) reproducibly displayed significant activity at the permissive temperature. Whereas at 30°C it exhibited approximately 30% of wild-type activity, at 37°C it did not activate transcription of the reporter (Fig. 3). Western blot analysis

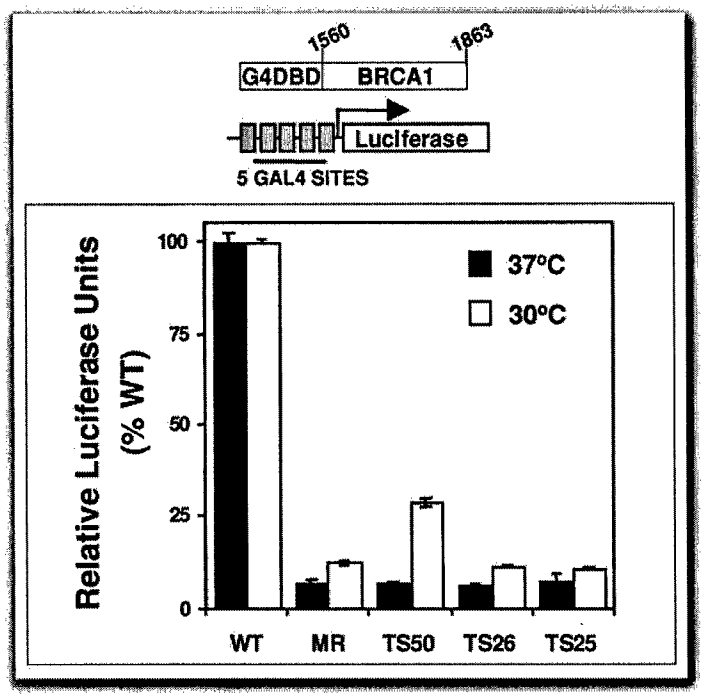

Figure 3. Transcriptional activity of TS mutants in mammalian cells. Upper panel shows a schematic representation of the GAL4-DBD:BRCA1 (aa 1560–1863) fusion protein and of the luciferase reporter gene driven by five GAL4 binding sites. Lower panel depicts the activation of luciferase expression by wild type and mutant BRCA1 constructs in 293T cells at 37°C (solid bars) or 30°C (open bars). Data were normalized to the percentage of wild-type activity at each temperature. MR, BRCA1 (aa 1560–1863) carrying the cancer-associated M1775R mutation used as negative control.

revealed that all mutant constructs were being expressed, albeit at lower levels than the WT protein (not shown).

Mammalian Two-Hybrid System. Recent reports have demonstrated that CtIP, a protein involved in transcriptional repression and a substrate of ATM, interacts with the BRCT domains of BRCA1.[28,31-34] We next examined whether the TS mutants could interact with CtIP in a temperature-dependent manner in a mammalian two-hybrid assay. We reasoned that this assay would provide a complementary approach to assess the temperature-sensitive phenotype of the mutants. Our results confirm previous reports that CtIP interacts with the carboxy-terminal region of BRCA1 and show that this interaction also occurs at 30°C (Fig. 4).[28,31] Interestingly, TS26 and TS50 were found to interact with CtIP only at 30°C. The fold induction relative to the activity of the TS mutants transfected with the VP16 transactivation domain alone (7-fold and 10-fold, respectively) was less than fold induction obtained with the WT and CtIP:VP16, suggesting that the interaction at 30°C is only partially restored. Although we observed that TS26 interacts with CtIP, it failed to activate transcription at either temperature (Fig. 3).

DISCUSSION

The function of BRCA1 has remained elusive despite extensive effort to characterize its biochemical activities. It has been implicated in DNA repair, transcription activation and repression, transcription-coupled repair, mRNA processing, cell cycle checkpoint regulation and ubiquitination.[3,4,18,35,36] We reasoned that the isolation of conditional mutants would be an important addition in the experimental armamentarium to study BRCA1. Here we developed a screening strategy to isolate mutants of the BRCA1 C-terminus that display a TS phenotype.

Our screen isolated 11 unique loss-of-function (LF) mutants (Table 2), extending our analysis of mutants that affect transcription

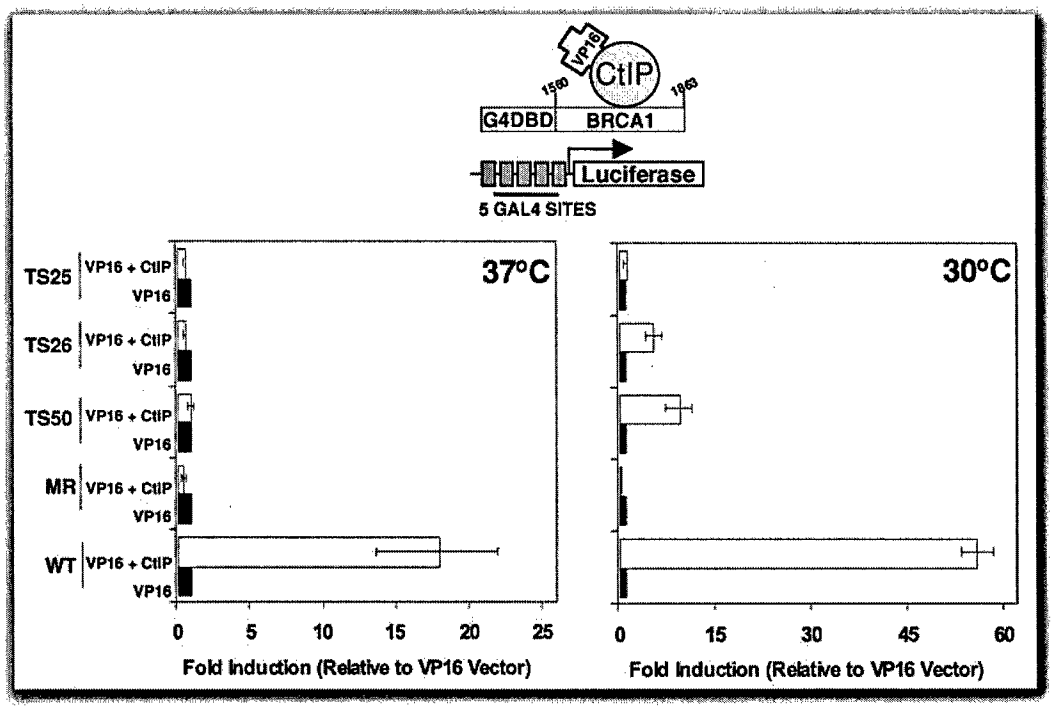

Figure 4. Mammalian two-hybrid system reveals a temperature-dependent interaction between BRCA1 TS mutants and CtIP. The upper panel shows a schematic representation of the GAL4-DBD:BRCA1 (aa 1560–1863) fusion protein used as bait, the CtIP:VP16 fusion protein used as target and the luciferase reporter gene driven by five GAL4 binding sites. The lower panel depicts the activation of the reporter gene at 37° C or 30° C by wild type and mutant BRCA1 constructs in 293T cells cotransfected with empty VP16 vector (that codes for the VP16 transactivation domain alone) or vector containing CtIP (that codes for the CtIP:VP16 fusion protein). The data were normalized to show the fold induction of transcriptional activity for each TS mutant relative to its activity when transfected with VP16 vector alone.

activation by BRCA1 and allowing us to have a more detailed picture of the structure-function features of the C-terminal region of BRCA1.[20,21] The LF mutants recovered were localized primarily in conserved hydrophobic residues at the BRCT-N and the BRCT-C domains.

We have also isolated 8 unique TS mutants using the yeast screening (Table 1, Fig. 2). One mutation, F1734L, was found in 4 independent clones in our TS set and two mutations, F1704S and L1657P, were found in 4 and 2 independent clones, respectively, in the loss-of-function set. These findings suggest that the screen might have reached saturation and therefore the mutants recovered identify important regions for the regulation of BRCA1. To understand the functional consequences of these mutations we mapped the mutations onto the crystal structure of the BRCT domain region of BRCA1 (Fig. 2A).[27] Two mutations (S1631N and L1639S) mapped to regions outside the BRCT domain and were excluded from our analysis. Significantly, all other mutations leading to temperature sensitivity, with one exception (E1836G) mapped to secondary structures in the BRCT-N and to the interval region (Fig. 2B) and cluster preferentially at the hydrophobic core of the domain (Fig. 2A). The reason for this clustering is not known but it is possible that mutations the BRCT-C have more dramatic consequences for the general folding and therefore are not stable even at lower temperatures. Alternatively, the BRCT-N may provide an important binding site to the RNA polymerase II holoenzyme, an idea that is corroborated by in vitro binding studies of BRCA1 and RNA helicase A.[9] Therefore, for mutations in the BRCT-C to affect transcription their effect has to be more dramatic allowing us to isolate only loss of function mutations.

Interestingly, mutations in residues located at hydrophobic cores in the catalytic domain of tyrosine kinases as well as in SH3 domains have been demonstrated to confer temperature-sensitivity.[38] In three clones (TS1, TS4 and TS36) two mutations were found and only one of them may be important for temperature sensitivity. Alternatively, as found in TS mutants of v-Src, two mutations may be required.[39,40]

One of the TS mutants isolated in yeast, H1686Q, displayed a temperature-dependent activation of transcription when tested in human cells (Fig. 3). This observation indicates that residue H1686 is located at a critical position for the stability of the BRCT domains (Fig. 2). In addition to the ability of TS50 to activate transcription only at the permissive temperature in mammalian cells, we found that its interaction with CtIP also occurred in a temperature-dependent manner (Fig. 4). Intriguingly, mutant TS26 interacts with CtIP at the permissive temperature but is unable to activate transcription at either 30°C or 37°C. Based on these observations we propose that TS50 can be used to clarify the physiological relevance of the BRCA1/CtIP interaction.

The inability of most of these clones to behave as TS mutants in mammalian cells may be due to inherent differences in the range of temperatures and metabolism of yeast versus the mammalian system. Alternatively, this may reflect the fact that the reporter used in the screen is not stringent. We tend to favor the latter explanation because there are documented examples of TS mutants isolated in yeast screens at 25°C and 33°C, permissive and restrictive temperature respectively, that turned out to display TS activity in mammalian cells at 34°C and 40.5°C.[38] This is a striking example in which the permissive temperature in mammalian cells was even higher than the restrictive temperature in yeast suggesting that the mutants adapt to the range of temperatures used in a particular host. The use of a low-stringency reporter is important at the restrictive temperature to guarantee the selection of mutants with the lowest possible activity. However, when screened at the permissive temperatures it will allow the selection of clones that may have low activity. We are currently exploring these different possibilities.

Although only one of the mutant clones displayed a mammalian TS phenotype in transcription, the other clones isolated here are candidates to become molecular biological tools in yeast to dissect the function of BRCA1 in transcription and to guide further efforts to isolate more relevant TS mutants in mammalian cells. If we apply a conservative interpretation of the transcriptional assay, i.e., that it is a measure of the integrity of the BRCT domain, then it is possible that the data collected here may serve as a basis to rationally design conditional mutants to other proteins that present BRCT domains in their structure. It is important to stress that the TS mutants recovered are inactive at 37°C and are likely to represent cancer-associated variants if found as germ-line mutations.

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Linking Breast Cancer Susceptibility and the DNA Damage Response

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Abbreviations: AT, ataxia telangiectasia; BC, breast cancer; B/OC, breast and ovarian cancer; DSB, double strand breaks; HR, homologous recombination; LFS, Li-Fraumeni syndrome; LOH, loss of heterozygosity; NHEJ, non-homologous end joining; OMIM, Online Mendelian Inheritance of Man; SNP, single nucleotide polymorphism; ssDNA, single strand DNA.

Abstract:

Background: In the past ten years a number of genes involved in breast cancer susceptibility were identified and characterized. These genes include *BRCA1, BRCA2, ATM, TP53, CHEK2* and *PTEN*. Despite the recent advances in identifying predisposition genes and in understanding their molecular function a large percentage of families with high risk for breast cancer still cannot be accounted for mutations in these genes, indicating the existence of additional loci in the genome that play a role in conferring susceptibility. Until these loci are identified attempts to develop a comprehensive strategy of early detection for breast cancer susceptibility will not be successful.

Methods: In order to search for clues to novel predisposition genes we review the current literature on: a) the molecular function of the known breast cancer susceptibility genes and; b) mutation screening and association (case-control) studies of candidate breast cancer predisposition genes.

Results: The present picture suggests that any particular locus, in isolation, is not going to make a contribution to breast cancer susceptibility substantial enough to be detected by classic linkage methods. Interestingly, mutations in DNA damage response genes other than *BRCA1* and *BRCA2* confer an increased risk for breast cancer and account for inherited susceptibility to a certain degree. However, candidate gene studies focusing on other DNA damage responsive genes have largely been disappointing.

Conclusions: Most of the breast cancer predisposition genes identified so far seem to play key roles in the signal transduction pathways that make up the cellular response to DNA double strand breaks (DSB). Thus, we propose that a comprehensive look at gene products involved in the DSB arm of the DNA damage response pathway might be important to determine additional predisposition genes. Importantly, the relative failure of recent candidate gene studies to identify any major predisposition gene may reflect the fact that they have been largely targeted to genes whose products are involved in the process of DNA repair whereas the known predisposition genes seem to play roles in sensing and signaling damage.

Introduction

Breast cancer is a disease caused by a complex combination of genetic and environmental factors and is one of the most common types of cancer affecting women in the Western world. In 2004, it is estimated that there will be over 200,000 new cases diagnosed and over 40,000 deaths from breast cancer in the United States alone. Linkage analysis of families with a high risk of breast cancer has identified two major susceptibility genes: *BRCA1* and *BRCA2* [1,2]. In the context of large multiple case families, the *BRCA1* and *BRCA2* genes are numerically the most important, accounting for more than 80% of families with six or more cases of both early-onset breast cancer and ovarian cancer [3]. However, the probability of harboring a mutation is much lower in families with fewer cases of the disease, and population studies have demonstrated that these genes only account for a minority of the overall familial risk of breast cancer. In fact, as many as 60% of families with site-specific female breast cancer cannot be explained by mutations in *BRCA1* and *BRCA2* [4,5]. In addition, mutations in these genes are relatively rare in the general population and together they account for less than 10% of all breast cancer cases [4,6] (Fig. 1). Therefore the challenge we face today is how to identify individuals at risk for the remaining cases. Conceivably, if we are able to identify the major genetic factors that contribute to breast cancer risk we would be able not only to provide comprehensive early identification of individuals at risk but also to tailor prevention and treatment regimens to adequately address specific molecular changes in these cancers.

BRCA1 and *BRCA2* were identified and isolated by linkage analysis and positional cloning [1,2], a strategy that works well for highly penetrant genes. To date, few additional candidate breast cancer susceptibility loci have been identified in families not attributable to any of the known genes. Recently, Kainu et al.[7] reported evidence for a novel breast cancer susceptibility locus on chromosome 13q21. However, posterior studies concluded that if a susceptibility gene does exist at this locus, it would only account for a small proportion of non-*BRCA1/2* families with multiple cases of early-onset breast cancer [8]. These findings illustrate the difficulties inherent in efforts to identify additional susceptibility genes for a highly prevalent disease and suggest that the traditional linkage approach may have reached its limit. Indeed, if current models are correct, the remaining predisposition genes are likely to

have lower penetrance or be part of a polygenic effect and therefore difficult to isolate by linkage [9]. Some of the candidate low penetrance genes have been proposed to be proto-oncogenes, genes involved in metabolic, estrogen and immunomodulatory pathways [10,11]. In particular, genes in hormonal metabolism pathways have received increased attention but research on the impact of these genes on breast cancer risk is still at an early stage.

In the ten years since the cloning of the first breast cancer susceptibility gene, *BRCA1*, we have made significant progress in our knowledge of breast cancer [1,12]. It is possible that understanding the function of the genes identified so far may allow us to make better informed guesses of candidate genes to be studied. Besides *BRCA1* and *BRCA2,* several other genes whose inactivation predisposes to breast cancer have been identified such as *ATM, TP53, CHEK2* and *PTEN.* Although many of these genes are associated with rare hereditary diseases such as Li-Fraumeni syndrome (*TP53* and *CHEK2)*, Cowden disease (PTEN) and Ataxia Telangiectasia (ATM) and therefore unlikely to be major contributors to risk in the general population they highlight a common characteristic: several of them play a role in the cellular response to DNA damage (Fig. 2). In a simplistic view one could look at the DNA damage response as composed of processes sensing and signaling the presence of damage and processes involved in the actual repair of the DNA strands. In this view, the known breast cancer predisposition genes seem to be involved in sensing and signaling damage rather than being directly involved in DNA repair.

Damage of genomic DNA occurs spontaneously and constantly throughout the life of an organism and can be further enhanced by exogenous DNA damaging factors. Therefore an efficient response to DNA damage is essential for cellular life. Spontaneous DNA damage results from errors in fundamental cellular processes, such as DNA replication. Exogenous DNA damage factors include environmental pollution, ionizing radiation (IR), ultraviolet rays (UV), and chemotherapeutic drugs. The most detrimental form of DNA damage is chromosomal double-strand break (DSB), which is lethal to the cell if not repaired. DNA DSB can be induced by ionizing radiation, DNA replication errors, or cell oxidative metabolism. Two major pathways for the repair of DSBs in mammalian cells include homologous recombination repair (HRR), which essentially provides an error-free repair by using a

homologous template (the homologous chromosome or the sister chromatid) and the more error-prone non-homologous end joining (NHEJ)[13,14]. Independent of which mechanism is used, mistakes may introduce mutations that in some cases will promote tumorigenesis. Both pathways consist of a complex network of events that trigger cell cycle checkpoints to prevent cells from progressing through the cycle with damaged DNA and activate a specific DNA repair mechanism (Fig. 2). A number of genes involved in the DNA DSB repair pathway have been implicated as breast cancer susceptibility genes. Below we review what is known about the function of these genes in an attempt to understand how it impinges on breast cancer risk and to propose other genes that may be involved in predisposition.

BRCA1 [OMIM 113705] *and BRCA2* [OMIM 600185]

One defective copy of *BRCA1* or *BRCA2* in the germ-line is sufficient for breast cancer predisposition, but the loss of the second allele is required for cancer development, but very little is known about the mechanisms by which the wild type allele is lost. Surprisingly, despite the association with inherited predisposition, somatic mutations in *BRCA1* and *BRCA2* are extremely rare in sporadic breast cancer [15,16]. *BRCA1* and *BRCA2* encode very large nuclear proteins, widely expressed in different tissues, markedly during S and G2 phases. They bear little resemblance to one another or to other proteins of known function [17]. Orthologs are not found in the yeast or fly, but a *BRCA1* ortholog in the worm *Caenorhabditis elegans* has been recently reported, suggesting a very peculiar track in evolution [18].

Both BRCA1 and BRCA2 have been consistently linked to various processes involved in the DNA damage response. These include the repair of double-strand breaks by homologous recombination (HR), the repair of oxidative damage by transcription-coupled repair and a possible role in non-homologous end joining (NHEJ) [19-21].

BRCA1 and BRCA2 are also implicated in the maintenance of chromosome stability, possibly through their function in recombination [reviewed in [12,17,22]]. Mouse and human cells null for *BRCA1* and *BRCA2* suffer from chromosome instability and have a heightened sensitivity to DNA lesions that

are normally repaired by HR [23,24]. Models have also been proposed to explain the roles of BRCA1 and BRCA2 in maintenance of chromosome instability through functions in DNA replication [25]. Stalled replication forks caused by a variety of mechanisms such as base lesions, DNA breaks or strand gaps, are thought to require HR to restart replication. If HR is dysfunctional then stalled replications forks may lead to persistent DNA breaks and ultimately to gross chromosomal rearrangements including translocations. These are indeed frequently seen in cells lacking *BRCA1* and *BRCA2* [24]. Such rearrangements may well provide the raw material for the further genetic changes required for tumor progression [26]. Alternatively, chromosomal instability initiated by *BRCA* deficiency may be the result of incorrect routing of double strand breaks (DSB) processing down an inappropriate pathway, rather than the failure of repair *per se* [17]. In this model, DSB in *BRCA*-deficient cells are rerouted for repair by mechanisms that are potentially error-prone (NHEJ or single strand annealing) because the preferred mode of (error-free) processing by HR is unavailable.

The exact molecular function(s) of BRCA1 in the DNA damage response has remained elusive. Although the classification of the proteins involved in the DNA damage response as sensors, transducers and effectors is arbitrary and complicated by overlapping roles it is helpful for a systematic analysis [27]. In this view, BRCA1 is likely to participate as a sensor or transducer rather than directly as a repair factor (effector) [27]. Some hints can be gleaned from the protein-protein interaction partners of BRCA1. BRCA1 interacts with Rad51 and the MR11/RAD50/Nbs1 protein complex which participates in DSB repair [28-30]. BRCA1 may also have local activities at DSB sites through its interaction with enzymes that alter chromatin and DNA structure. BRCA1 interacts with SWI/SNF and other proteins that remodel chromatin, such as regulators of acetylation/deacetylation and with DNA helicases, including the RecQ homolog encoded by the Bloom´s syndrome gene, *BLM*, and the novel helicase BACH1 [31-35]. These data suggest a role for BRCA1 as a scaffold or platform to coordinate different activities needed for repair. The molecular role of BRCA2 is somewhat better understood. BRCA2 interacts with and regulates the function of RAD51, the mammalian homolog of *E. coli* RecA that has a catalytic activity central to HR [36]. RAD51 coats single-strand DNA (ssDNA) to form a nucleoprotein filament that invades and pairs with a homologous DNA duplex, initiating strand

exchange between the paired DNA molecules. The interaction involves a substantial proportion of total cellular pool of each protein, suggesting that BRCA2 works directly to regulate the availability and activity of RAD51 in this key reaction [37]. Taken together these observations place BRCA1 and BRCA2 firmly in DNA damage response pathway and suggest a pleiotropic role in this pathway.

***ATM* [OMIM 607585]**

The ATM (Ataxia-Telangiectasia Mutated) protein was identified as the product of the gene mutated in the rare human autosomal recessive disorder ataxia-telangiectasia (AT) [38]. ATM is a serine/threonine protein kinase that belongs to the phosphatidyl inositol-3-kinase super family. The ATM kinase plays a central role in response to DSB and loss of ATM abolishes the checkpoints at the G1-S transition, in S phase and at the G2-M boundary [27]. AT is characterized by neurodegeneration, immunodeficiency, genomic instability, hypersensitivity to ionizing radiation, and increased cancer predisposition [39]. It is estimated that about 1-2% of the general population may be heterozygote carriers of the ATM gene, but do not show any of the major disease symptoms. However, certain types of ATM mutations in heterozygous carriers seem to increase cancer predisposition, particularly breast cancer [40-42].

The majority of mutations identified in the gene are truncating mutations resulting in unstable, truncated protein products, leaving heterozygous carriers of such mutations with a reduced level of functional ATM protein produced by a wild type allele [43]. However, *ATM* truncations do not contribute to early onset breast cancer [44]. The early studies of the relationship between *ATM* heterozygosity and breast cancer risk were inconclusive, and neither linkage analyses nor mutation studies provided supporting evidence for a role of *ATM* in breast cancer predisposition. An explanation that clarified these initial findings came from a missense mutation model [45]. The model defines two groups of *ATM* heterozygous mutations in the general population that cause different degrees of cancer predisposition. One group has a truncated allele and a second group has a missense mutation. These missense mutations allow production of full size, stable, but functionally inactive ATM protein, and act as dominant negative mutations interfering with the function of the normal allele. Carriers of these

mutations have a high predisposition for breast cancer. This explanation is further supported by linkage and penetrance analysis of *ATM* mutations among breast cancer cases [46]. Additional support for this model came from a study of *ATM* "knock-in" heterozygous mice harboring an in-frame deletion corresponding to the human 7636del9 mutation [47]. The *ATM* "knock-in" showed an increased susceptibility to developing tumors. In contrast, no tumors were observed in the *ATM* heterozygous ($ATM^{+/-}$) mice.

A mechanistic understanding of the different roles for truncating and missense mutations in breast cancer predisposition came from the elegant work of Bakkenist et al. [48]. They found that ATM molecules exist as dimers or higher-order multimers in undamaged cells where the kinase domain of each monomer is bound to an internal domain of another neighboring ATM molecule containing the catalytic site. While in this state, ATM is inactive and unable to phosphorylate its substrates. After DNA damage, the kinase domain of one ATM molecule phosphorylates another ATM molecule in the dimer complex, and the phosphorylated ATM dissociates from the complex to phosphorylate other substrates. Thus, kinase inactive and non-phosphorylatable missense mutants of ATM are locked in the inactive complex. This mechanism of activation provides an explanation for the dominant-negative effect of ATM heterozygous missense mutations. However despite of the AT mutations prevalence in the population the risk conferred by AT heterozygosity is still too low to account for a large proportion of familial breast cancers. The degree to which the *ATM* gene contributes to sporadic breast cancer will require further studies and mutation screening. Nevertheless, its central role in the DNA damage response reinforces the notion that this pathway may be intrinsically linked to breast cancer predisposition.

***TP53* [OMIM 191170]**

Breast cancer is a major component of the rare Li-Fraumeni syndrome (LFS), in which germline mutations of the *TP53* gene have been documented [49,50]. LFS is an autosomal-dominant disease characterized by early occurrence of multiple cancers, such as sarcomas, breast cancer, brain tumors, leukemia, and adrenal cortical tumors [50]. It is estimated that 50% of women who survive

childhood cancers will develop breast cancer by the age of 50, and lifetime penetrance approaches 100% [51]. Although highly penetrant, the Li-Fraumeni genes account for less then 1% of breast cancer cases [51].

TP53 is a tumor suppressor gene encoding a nuclear phosphoprotein that acts as a transcription factor involved in the control of cell cycle progression, repair of DNA damage, genomic stability and apoptosis [52]. In response to DNA damage, the p53 protein arrests cells in the G1 phase of the cell cycle, allowing the DNA repair mechanism to proceed prior to DNA synthesis. Loss of p53 function abolishes this growth arrest response to DNA damage. Interestingly, p53 mutations are frequently found in *BRCA1*-linked tumors and several studies have suggested that the status of *BRCA1/BRCA2* influences the type and distribution of *TP53* mutations in breast cancer [53-55].

In conclusion, mutations in p53 are a rare cause of breast cancer except for those associated with Li-Fraumeni syndrome. While *TP53* is one of the most commonly mutated genes in human tumors, among sporadic breast tumors only a small fraction carries a *TP53* mutation [56]. Importantly, p53 is a key regulator of the response to DNA damage and, similar to BRCA1, a substrate for damage-induced ATM phosphorylation.

CHEK2 [OMIM 604373]

Germline mutations in *CHEK2* (*CHK2*), gene have also been implicated in the etiology of Li-Fraumeni syndrome [57]. This gene encodes the human ortholog of yeast checkpoint kinases Cds1 and Rad53 in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, respectively [58]. In mammalian cells, CHEK2 is phosphorylated by ATM in response to DSB [59]. Activated CHEK2 phosphorylates a number of target proteins that in turn prevent cellular entry into mitosis and activate DNA repair pathways. In addition, CHEK2 also acts in the G1/S checkpoint by phosphorylating p53 and mediating activation and stabilization of p53 by ATM [60,61]. In another important connection, CHEK2 phosphorylates Cdc25C and BRCA1 [58,62]. Mutation screening of the *CHEK2* gene among LFS cases revealed a deletion mutation *CHEK2 1100delC*, that inactivates the kinase activity of the protein [57]. This allele has also been proposed to be a low-penetrance breast cancer susceptibility

allele [63,64]. Additional screening of *CHEK2* variants did not identify any other variant that occurs at significantly elevated frequency, indicating that *1100delC* may be the only *CHEK2* allele with a significant contribution to breast cancer susceptibility [65]. Interestingly, *CHEK2 1100delC* is associated with breast cancer only in non-carriers of *BRCA1* and *BRCA2* [63]. A recent search for new breast cancer susceptibility genes among families with no *BRCA1* and *BRCA2* mutation, suggested a model in which *CHEK2 1100delC* interacts with an as yet unknown gene to increase breast cancer risk [66].

Although the *CHEK2 1100delC* allele confers moderate risk, its prevalence suggest that it may be a more significant player in breast cancer incidence than genes associated with breast cancer only in the context of rare hereditary syndromes. Again, the cross talk between CHEK2 and the other breast cancer predisposition gene products in the DNA damage pathway is evident.

***PTEN/MMAC1* [OMIM 601728]**

PTEN (also known as *MMAC1*) was originally identified as a tumor suppressor gene defective in a variety of human cancers [67,68]. Germ-line mutations in *PTEN* are associated with Cowden disease, a rare autosomal dominant inherited cancer syndrome characterized by a high risk of breast, thyroid, and endometrial carcinomas [69-71]. Most cancer-associated *PTEN* mutations are truncations, which cause a 25-50% lifetime breast cancer risk among women affected with Cowden disease [72,73]. *PTEN* mutations are rare in sporadic breast cancer and have been found only in 5% of the sporadic cases [74,75]. However, 29-48% of sporadic breast cancer cases show loss of heterozygosity (LOH) at the *PTEN* locus, while no alterations have been found in the remaining allele [76]. In addition, about 40% of breast cancers show a decrease or absence of PTEN protein levels.

PTEN is a phosphatase with dual specificity for proteins and major cellular lipids. Its tumor suppressor function has been linked to its lipid phosphatase activity, which is specific for the position 3 of major cellular lipids phosphatidylinositol 3,4,5-trisphosphate (PIP3), and phosphatidylinositol 3,4,-bisphosphate, both byproducts of the lipid kinase activity of the phosphoinositide 3-kinase (PI3K) [77]. The PI3K pathway regulates cell growth and survival through signaling to its downstream effectors, the protein kinases AKT and PDK1. Among numerous AKT kinase substrates are members of the

FOXO forkhead transcription factors subfamily [78]. Activated AKT kinase promotes phosphorylation and subsequent inactivation of the FOXO family members. Interestingly, in PTEN-deficient cells the FOXO transcription factors are aberrantly localized to the cytoplasm and cannot activate transcription [79]. In addition, PTEN-mediated down regulation of AKT stimulates transcription of the cyclin-dependent kinase inhibitors $p27^{Kip1}$, $p21^{Waf1/Cip1}$, and $p57^{Kip2}$ [79]. Importantly, the FOXO transcription factors modulate expression of several genes that regulate cellular response to DNA damage linking PTEN/PI3K/AKT pathway to DNA damage repair pathway [80].

Searching for additional genes

It is clear that mutations in *BRCA1* and *BRCA2* genes cause defects in DNA repair after DSB and predispose carriers to breast cancer. In addition, other known breast cancer susceptibility genes such as *ATM*, *CHEK2* and *TP53* also function in the DNA damage response pathway. Inactivation of *PTEN* although less clear than the above mentioned genes also seem to impinge on the ability of cells to respond to damage. While it is possible that the apparent clustering of predisposition genes in this pathway may be restricted to the rare hereditary syndromes described above, it is plausible to think that they reveal important common characteristics in the biology of breast cancer susceptibility. Why would this ubiquitous pathway be specifically tied to breast cancer predisposition is a lingering question. Although there is no clear explanation for that, recently several hypotheses, at least in the context of *BRCA1* inactivation, have been formalized and can now be experimentally tested[12,25,81,82].

If the DNA damage response pathway were a major target of inactivation in breast cancer we would predict that other known genes would be targets of germline or somatic mutations. In fact, screens to identify mutations in DNA damage response genes as well as association studies using candidate polymorphisms have been undertaken (Table 1). While no clear major target emerged from these studies, they provide just enough evidence to keep the issue alive. The reasons for the inability to identify any major additional gene are unknown. Interestingly, although we still have an incomplete understanding of the biochemistry involved in DNA damage response and repair it could be argued that many of the studies (Table 1) have focused on proteins involved in the DNA repair process while

the known predisposition genes seem to be involved in sensing, signaling and amplifying the damage signal [27]. Therefore, a candidate gene approach focusing on genes whose products are involved in signaling DNA damage such as *CHK1*, *Claspin*, *53BP1* and *ATRIP* may prove more fruitful when combined with a better understanding of the biochemistry of the DNA damage response. Another possible reason for the relative failure of the candidate gene approach may be due to the fact that inactivating mutations in these genes have in isolation, only small effects on risk. The implication is that significant increases in risk are only going to be apparent when combined with mutations in additional genes. This scenario would be analogous to synthetic lethality in yeast where two mutations in separate genes are viable as single mutations but lethal when combined. Interestingly, several of the association studies mentioned here (Table 1) suggest that this is the case. For example, while mutations in certain genes had marginal or no association with risk when studied in isolation, they showed significant association when combined with variant alleles in other genes [83,84]. Importantly, novel methods to identify synthetic gene interactions in multicellular organisms have only now become possible by exploiting RNA interference [85]. These approaches will certainly bring exciting results in the near future.

It is also extremely important not to be limited to the usual suspects. Genome-wide association studies, using single nucleotide polymorphisms (SNPs) are unbiased in that there is no preconceived idea about which genes are likely to be involved in the disease process and are going to be instrumental in identifying other candidate pathways [86,87]. However, large data sets and appropriate SNP genome coverage are needed, putting this approach beyond the reach of smaller laboratories. It is expected that as technology improves and costs decrease this approach will have widespread use.

In the near future, we can look forward to the identification of novel breast cancer predisposing genes due to rapid advancement of gene discovery technologies. The identification and functional characterization of such genes will have a significant impact on breast cancer research and early detection. A major challenge that will keep researchers busy for years to come will be to understand the complicated mechanisms and changes that lead to the development and progression of breast cancer and to apply this knowledge to breast cancer detection, prevention and treatment.

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Figure 1. Breast cancer susceptibility genes. Hereditary breast cancer (right) constitutes only about 5-10% of all breast cancer cases (left). Germ line mutations in the two major susceptibility genes *BRCA1* and *BRCA2* account for less than 5% of all breast cancer cases, while mutations in genes such as *ATM*, *CHEK2*, *PTEN* and *TP53* account only for about 1% of all breast cancer cases. The genetic factors underlying sporadic breast cancer cases are largely unknown.

Figure 2: Breast cancer susceptibility gene products and the DNA damage response pathway. In this simplified view, ATM is activated by the presence of DNA double strand breaks (DSB) and phosphorylates CHEK2, BRCA1 and p53. Activated CHEK2 also phosphorylates TP53 and BRCA1. Phosphorylation of these proteins seems to be required for the efficient activation of various cell cycle checkpoints. BRCA2 regulates the function of the RAD51 protein, which bridges the interaction between BRCA1 and BRCA2. Another protein implicated in breast cancer predisposition, PTEN, mediates down regulation of AKT. Pointed arrowheads indicate activation, and flat arrowheads indicate inhibition.

Table 1. Mutations in DNA damage repair genes and breast cancer risk.

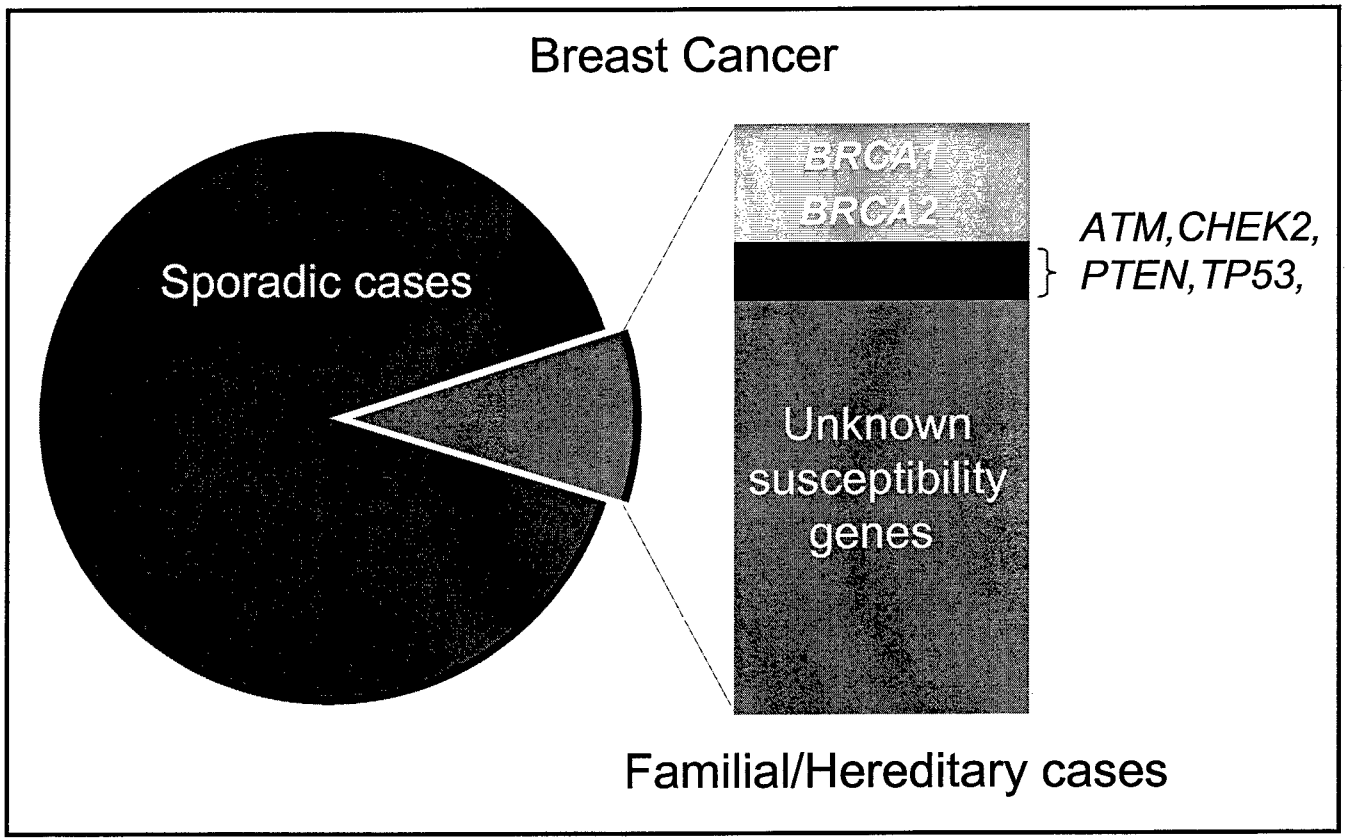
Gene	Polymorphism[a]	Type of study/ Population[b]	Results[a]	Reference
BACH1	Pro919Ser; G64A	Kin-cohort study of 2430 relatives (190 with BC and 2240 without).	Only Pro919Pro was associated with increased risk.	Sigurdson et al.[88]
	Pro919Ser; Val193Ile; Arg173Cys; Glu879Glu and 21 other variants.	MS. 21 families with inherited B/OC not associated with *BRCA1/2*, 58 early-onset BC patients and 30 controls.	No variant could be clearly related to BC risk.	Rutter et al.[89]
	Pro919Ser; Pro1034Leu G2637A; C3411T	MS. 214 B/OC patients from 151 families with hereditary B/OC.	No variant could be clearly related to BC risk.	Karpinnen et al.[90]
	Pro47Ala; Met299Ile; Val193Ile; Pro919Ser; G2637A; C3411T	MS. 65 early-onset BC patients not associated with *BRCA1/2* and 200 controls.	Pro47Ala and Met299Ile found only in cases. Functional evidence suggests that it may be pathogenic.	Cantor et al.[35]
	Arg173Cys; Glu879Glu; Pro919Ser; Tyr1137Tyr	MS. 25 BC and B/OC families and 95 familial BC cases not associated with *BRCA1/2.*	No variant could be clearly related to BC risk.	Luo et al.[91]
BARD1	Ser378Arg; His506His; Val507Met; Cys557Ser and three other variants	MS. 126 hereditary BC and B/OC families.	Only the Cys557Ser was seen at elevated frequency in cases as compared to controls.	Karppinen et al.[92]
	None detected in BC	MS. 50 breast tumors.	Somatic mutation Val695Leu was found but could determine disease-association.	Thai et al.[93]
	Asn295Ser; Lys312Asn; Cys557Ser; 1144del21bp	MS. 40 hereditary BC and B/OC families that were BRCA1/2 non-carriers and 20 early-onset sporadic BC cases.	Segregation analysis of a family with Cys557Ser had near-borderline significance in linkage to disease.	Ghimenti et al.[94]
	Ser241Cys; Arg378Ser; Asn470Ser; His506His; Val507Met; 1139del21bp	MS. 60 familial BC patients not associated with *BRCA1/2.* Followed by a case-control study with 143 BC cases and 155 controls.	Asn470Ser was the only one was not observed in controls. Case-control study showed its association with increased BC risk in postmenopausal women.	Ishitobi et al.[95]
DNA-PKc	C55966T	AS. 192 BC cases and 192 controls.	No significant association.	Fu et al.[84]
GADD45	None detected	MS. 59 familial BC.	None detected.	Sensi et al.[96]
H2AX	None detected	Mutation screening. 101 hereditary BC not associated with BRCA1/2.	None detected.	Monteiro et al.[97]
Ku70	Gly593Gly	AS. 2205 BC and 1826 controls.	No association was found.	Kuschel et al.[98]
	Gly593Gly; A46922G; C61G	AS. *See Fu et al. above.*	Only C61G had a statistically significant difference between cases and controls suggesting that it is associated with BC.	Fu et al.[84]
Ku80	G69506A; G69632A	AS. *See Fu et al. above.*	No significant association.	Fu et al.[84]
Ligase IV	C299T (5'UTR); Asp501Asp	AS. 1004 BC cases and 1385 controls.	No overall association with BC risk.	Han et al.[99]

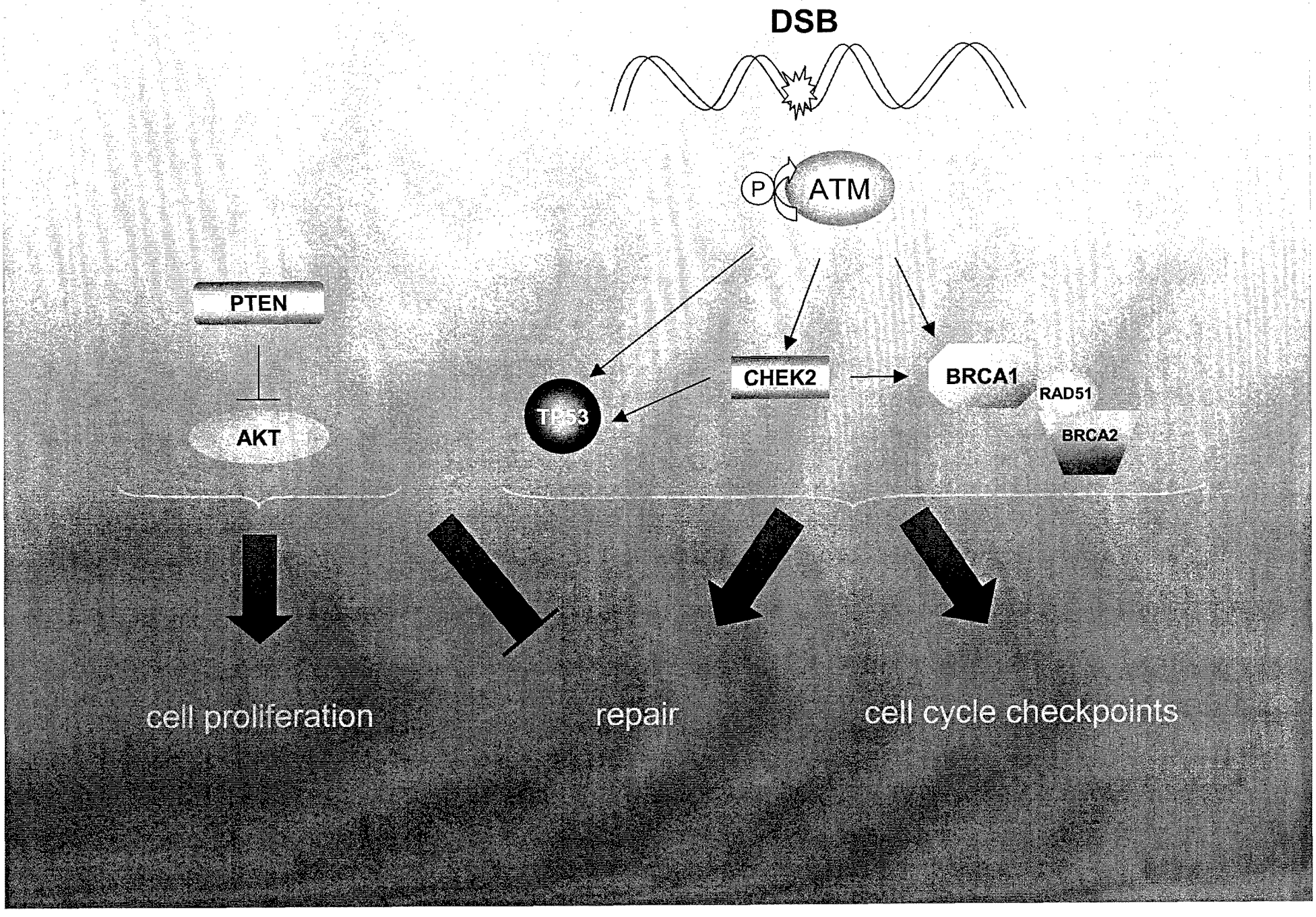
Gene	Polymorphism[a]	Type of study/ Population[b]	Results[a]	Reference
	Asp501Asp	AS. *See Kuschel et al. above.*	Associated with a decrease in BC risk.	Kuschel et al.[98]
	Ile591Val; C4062T; C4044T	AS. *See Fu et al. above.*	No significant association.	Fu et al.[84]
Mre11	Arg305Trp	MS.151 families with hereditary breast/ovarian cancer.	Found in 1/151 patients but not in controls.	Heikkinen et al.[100]
NBS1	Leu34Leu; Glu185Gln; Asp399Asp; Pro672Pro	AS. *See Kuschel et al. above.*	No association was found.	Kuschel et al.[98]
	Glu185Gln	AS. 223 Finnish BC patients and 172 Polish familial BC cases.	Frequency distribution was similar in cases and controls.	Forsti et al.[101]
	R215W; 657del5	AS. 224 BC patients and 1620 controls.	657del5 found 3 times more frequently in cases but could not prove the significance of increased BC risk.	Steffen et al.[102]
	657del5	AS. 150 early onset BC patients, 80 familial BC and 530 controls.	Frequency distribution and LOH analysis suggests that it is associated with BC.	Gorski et al.[103]
	Leu34Leu; Leu150Phe; Glu185Gln; Asp399Asp; Leu574Ile; Pro672Pro	MS. *See Heikkinen et al. above.*	Only Leu150Phe was considered potentially pathogenic.	Heikkinen et al.[100]
RAD50	His68His; 687delT; Ile94Leu; Arg224His	MS. *See Heikkinen et al. above.*	Only 687delT is likely to be disease-associated.	Heikkinen et al.[100]
RAD51	Gln150Arg	MS. 20 hereditary and 25 sporadic BC patients.	Present in 2/45 patients with hereditary BC but not in 200 with sporadic BC.	Kato et al.[104]
	None detected	MS. 120 patients with early onset BC.	No sequence variation detected.	Bell et al.[105]
	G135C (5'UTR)	AS. Ashkenazi Jewish BRCA1/2 carriers; 164 with BC and 93 without.	Elevated risk for BRCA2 but not for BRCA1 carriers.	Levy-Lahad et al.[106]
		AS. 309 BRCA1/2 carriers; 166 non-carriers BC cases; 155 controls.	Elevated risk for BRCA2 but not for BRCA1 carriers or non-carriers.	Kadouri et al.[107]
		AS. *See Kuschel et al. above.*	No increased risk.	Kuschel et al.[98]
		AS. 83 pairs of female carriers of *BRCA1* 5382insC mutation.	Reduced risk for BRCA1 5382insC mutation carriers.	Jakubowska et al.[108]
		MS and AS. BRCA1/2 carriers with and without BC.	Elevated risk for BRCA2 but not for BRCA1 carriers.	Wang et al.[109]
RAD52	Ser346ter; Tyr415ter	AS. 160 members of B/OC families and 128 healthy controls.	No increased risk for BC.	Tong et al.[110]
	Ser346ter; Tyr415ter	MS. *See Bell et al. above.*	No increased risk for BC.	Bell et al.[105]
	Ser346ter	AS. 727 BC cases and 969 controls.	No increased risk for BC.	Han et al.[111]
	C2259T (3'UTR)	AS. *See Kuschel et al. above.*	No increased risk.	Kuschel et al.[20]

Gene	Polymorphism[a]	Type of study/ Population[b]	Results[a]	Reference
RAD54	Gly325Arg	MS. 93 BC patients and 100 controls.	Not possible to determine pathogenicity.	Matsuda et al.[112]
	Cys657Ser	MS. *See Bell et al. above.*	Not possible to determine pathogenicity.	Bell et al.[105]
XPF	Arg415Gln	AS. 253 BC cases and 268 controls.	Found at elevated frequency in cases as compared to controls.	Smith et al.[83]
XPG	Asp1104His	AS. 220 BC cases and 308 controls	Marginally significant increased frequency in cancer cases.	Kumar et al.[113]
XRCC1	Arg194Trp; Arg280His; Arg399Gln	AS. 254 BC cases and 312 controls.	Only Arg280His was associated with increased risk.	Moullan et al.[114]
	Arg194Trp; Arg399Gln	AS. 412 BC cases and 400 controls (Arg194Trp); 639 BC cases and 647 controls (Arg399Gln).	Arg399Gln was associated with risk only among African Americans but not among whites.	Duell et al.[115]
	Arg194Trp; Arg399Gln	AS. 253 BC cases and 268 controls.	Only Arg194Trp was associated with increased risk.	Smith et al.[83]
	Arg399Gln	AS. 1088 BC cases and 1182 controls (Shanghai).	No overall association with BC risk.	Shu et al.[116]
	Arg194Trp; Arg399Gln	AS. 162 BC cases and 302 controls.	Weak association of Arg194Trp with risk.	Smith et al.[117]
	Arg399Gln	AS. 402 cases and 402 controls (Ontario).	No overall association with BC risk.	Figueiredo et al.[118]
	Arg194Trp; Arg280His; Arg399Gln	Kin-cohort study. *See Sigurdson et al. above.*	Increased risk was noted for homozygous carriers of Arg194Trp and Arg399Gln.	Sigurdson et al.[88]
	Arg194Trp; Arg399Gln	AS. *See Forsti et al. above.*	No overall association with BC risk.	Forsti et al.[101]
XRCC2	Arg188His	AS. *See Han et al. above.*	No overall association with BC risk.	Han et al.[99]
	IVS-16bp; Leu31Val	MS. 105 B/OC families not associated with BRCA1/2 and 200 controls.	Leu31Val was detected only once in cases but LOH and segregation analysis indicates this variant is not pathogenic.	Rodriguez-Lopez et al.[119]
	Arg188His	AS. *See Kuschel et al. above.*	The association was marginally significant.	Kuschel et al.[98]
	Arg188His	AS. 521 BC patients and 895 controls	The association was of borderline statistical significance.	Rafii et al.[120]
XRCC3	Thr241Met	AS. *See Smith et al. above.*	Thr241Met homozygotes may have increased BC risk.	Smith et al.[117]
	Thr241Met and two other variants.	AS. *See Han et al. above.*	No overall association with BC risk.	Han et al.[99]
	Thr241Met	AS. *See Figueiredo et al. above.*	Thr241Met homozygotes were marginally associated with risk.	Figueiredo et al.[118]
	Thr241Met	AS. *See Forsti et al. above.*	Borderline significance in Finnish cohort and not significant in Polish cohort.	Forsti et al.[101]
	Thr241Met	AS. (Danish prospective cohort). 426 cases and 424 controls	No overall association with BC risk.	Jacobsen et al.[121]
	Thr241Met	AS. *See Kuschel et al. above.*	No overall association with BC risk.	Kuschel et al.[98]

Gene	Polymorphism[a]	Type of study/ Population[b]	Results[a]	Reference
XRCC4	Gln82Gln; T1394G; C1475T	AS. *See Fu et al. above.*	Only C61G had a statistically significant difference between cases and controls suggesting that it is associated with BC.	Fu et al.[84]

[a] Missense changes are shown in three-letter code for amino acids. Noncoding changes are indicated by the nucleotide change. [b] Type of study: MS, mutation screening; AS, association study. [c] The results displayed here are a summary of the overall results and are only confined to the findings as pertaining to breast cancer. Results differ for the association of a certain SNP with other cancers, or for combinations with other genetic and environmental factors but that is not listed here. Readers are encouraged to consult the original papers for a full analysis and discussion as well as a review by Goode et al. discussing polymorphisms in DNA repair genes and cancer in general. BC, breast cancer; B/OC, breast/ovarian cancer.


Breast Cancer
Sporadic cases
BRCA1
BRCA2
ATM, CHEK2,
PTEN, TP53,
Unknown
susceptibility
genes
Familial/Hereditary cases


DSB
P
ATM
PTEN
AKT
TP53
CHEK2
BRCA1
RAD51
BRCA2
cell proliferation
repair
cell cycle checkpoints