BRCA1 induces DNA damage recognition factors and enhances nucleotide excision repair

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Published online: 26 August 2002, doi:10.1038/ng953

Inheritance of a mutation in the gene BRCA1 confers on women a 50–85% lifetime risk of developing breast cancer1,2. Mutations in the TP53 tumor-suppressor gene are found in 70–80% of BRCA1-mutated breast cancer but only 30% of those with wildtype BRCA1 (ref. 3). The p53 protein regulates nucleotide excision repair (NER) through transcriptional regulation of genes involved in the recognition of adducts in genomic DNA. Loss of p53 function results in deficient global genomic repair (GGR), a subset of NER that targets and removes lesions from the whole genome4–6. Here we show that BRCA1 specifically enhances the GGR pathway, independent of p53, and can induce p53-independent expression of the NER genes XPC, DDB2, and GADD45. Defects in the NER pathway in BRCA1-associated breast cancers may be causal in tumor development, suggesting a multistep model of carcinogenesis.

Studies of the effect of BRCA1 overexpression on NER were performed in human U2OS osteosarcoma cell lines, allowing for tetracycline-regulated expression of BRCA1 (ref. 7). Cell line UBR60 carries wildtype p53. Cell line E621, derived from UBR60, stably expresses a transfected human papillomavirus cDNA, the product of which targets p53 for degradation6, effectively making the E621 cells deficient for p53. Analysis of inducible BRCA1 protein expression demonstrated tight regulation, with a fourfold protein induction in both cell lines 24 hours after removal of tetracycline (Fig. 1). Induction of p53 was detected in the p53-wildtype UBR60 cells after UV irradiation, but not in the p53-deficient E621 cells (Fig. 1). These cell lines served as models to probe the independent effects of BRCA1 and p53 on NER.

NER is subdivided into two pathways: GGR and transcription-coupled repair (TCR), which preferentially removes lesions from the transcribed strand of expressed genes8,9. Loss of p53 function results in deficient GGR, but does not affect TCR following UV irradiation4–6. GGR and non-transcribed strand repair of cyclobutane pyrimidine dimers (CPDs) is reduced by more than half in human cells dysfunctional in p53 (refs 4–6,10). Thus, we anticipated that in the presence of tetracycline (no BRCA1 induction), repair of CPDs in the p53-deficient E621 cell line would be lower than in the p53-wildtype UBR60 cell line, and this is, in fact, what we observed (28% versus 3% repair at 24 hours; Fig. 2). Overexpression of BRCA1 led to greater GGR of CPDs (42% versus 28%) in p53-wildtype UBR60 cells by 24 hours; this difference, however, was not statistically significant (P = 0.75; Fig. 2a). The effect of BRCA1 was more dramatic in p53-deficient E621 cells: 34% of CPDs were repaired at 24 hours when BRCA1 was overexpressed versus 3% without BRCA1 induction (P = 0.008; Fig. 2b). Both p53-deficient E621 and p53-wildtype UBR60 cells repaired nearly 100% of UV-induced 6–4 photoproducts, and this was not affected by overexpression of BRCA1 (data not shown).

Analysis of NER within either strand of the expressed gene DHFR confirmed our results in regard to GGR by demonstrating that BRCA1 selectively affects repair of the non-transcribed strand independent of p53. Both cell lines preferentially repaired the transcribed strand over the non-transcribed strand of DHFR, and exhibited similar efficiency (Fig. 3b,c). Predictably, the p53-wildtype UBR60 cells exhibited a higher level of repair of the non-transcribed strand of DHFR than did the p53-deficient E621 cell line in the absence of BRCA1 induction (29% versus 2% of CPDs repaired at 24 hours; Fig. 3a,c). Consistent with the GGR results, overexpression of BRCA1 in p53-wildtype UBR60 cells resulted in a further increase in CPD repair in the non-transcribed strand to 36%, but this was not statistically significant (P = 0.21; Fig. 3a,c). Also consistent with the GGR results (Fig. 2b), p53-deficient E621 cells repaired only 2% of CPDs in the non-transcribed strand, but overexpression of BRCA1 increased repair to 30% at 24 hours (P < 0.0009; Fig. 3c). There was no effect of BRCA1 overexpression on TCR in the p53-wildtype UBR60 cells (Fig. 3b). Repair of CPDs in the transcribed strand in p53-deficient E621 cells with normal BRCA1 expression was 34% at 24 hours and 53% with overexpression of BRCA1, but this difference was not statistically significant (P = 0.056; Fig. 3d).

We have previously shown that GADD45 (a gene involved in growth arrest and DNA damage), DDB2 (a gene defective in xeroderma pigmentosum group E cells and encoding the p48
protein) and XPC (the gene defective in xeroderma pigmentosum group C) are regulated by p53 and required for efficient GGR. BRCA1 regulates transcription of GADD45 (refs 7,14,15), providing a potential mechanism for the effect of BRCA1 on NER. Therefore, we evaluated the effect of BRCA1 on expression of additional NER genes.

Overexpression of BRCA1 protein enhanced mRNA expression of both DDB2 and GADD45 in p53-deficient E621 cells (Fig. 4). Consistent with previous results in other cell lines11, p53-wildtype UBR60 cells showed a threefold higher basal level of DDB2 mRNA compared to p53-deficient E621 cells. Overexpression of BRCA1 did not further enhance DDB2 expression in p53-wildtype UBR60 cells in the presence or absence of UV irradiation. A 1.9-fold induction of DDB2 was observed with overexpression of BRCA1 in p53-deficient E621 cells in the absence of irradiation. In the absence of BRCA1 overexpression, UV irradiation had no effect on DDB2 expression, regardless of p53 status. The p53-wildtype UBR60 cells exhibited twofold higher basal levels of GADD45 than did p53-deficient E621 cells. We observed a 1.8-fold increase and a 2-fold increase of GADD45 expression with overexpression of BRCA1 in unirradiated and UV-irradiated p53-deficient E621 cells, respectively. Therefore, overexpression of BRCA1 restored expression of DDB2 and GADD45 in p53-deficient cells to levels seen in p53-wildtype cells, supporting our finding of a strong effect of BRCA1 on GGR in p53-deficient E621 cells.

Overexpression of BRCA1 also induced the expression of XPC (xeroderma pigmentosum group C complementing protein) in p53-wildtype UBR60 cells, particularly at 48 hours after BRCA1 induction, but this effect was not observed after UV irradiation (Fig. 5a). Overexpression of BRCA1 without UV irradiation resulted in increased expression of XPC in p53-deficient E621 cells (Fig. 5b). With UV irradiation, XPC induction also occurred. This effect was not dependent on expression of BRCA1, but was enhanced by it at 48 hours (Fig. 5b). Levels of xeroderma pigmentosum group A complementing protein (XPA), which functions downstream of p48 and XPC in excision of damaged DNA, did not change with overexpression of BRCA1 in cells either wildtype or deficient for p53 (Fig. 5a,b).

We have previously shown that p53 is necessary for efficient mammalian GGR through p53-dependent transcriptional regulation of genes involved in NER4–6,11–13. We now extend this hypothesis to include BRCA1 as a transcriptional regulator of NER genes. Further support for our finding comes from several in vivo observations in breast cancers. Substrates for the NER pathway include UV photoproducts as well as carcinogens such as polyaromatic hydrocarbons and DNA adducts formed from cisplatin chemotherapy16,17. BRCA1-deficient cells are hypersensitive to cisplatin, suggesting a defect in NER of cisplatin adducts18. The NER substrate benzo[a]pyrene-7,8-diol-9,10-epoxide is more...
prevalent in breast cancer than in benign tissue, and carcinogens other than polyaromatic hydrocarbons have been found to react with DNA in breast epithelial cells\textsuperscript{29,30}. Clinical support of a role for BRCA1 in GGR comes from an analysis of the distribution of TP53 mutations in BRCA1-mutated breast cancers\textsuperscript{31}. This study found a strong bias for mutations arising at thymines in the non-transcribed strand of TP53 in BRCA1-mutated tumors compared to sporadic breast cancers, consistent with our finding that BRCA1 expression induces repair of the non-transcribed strand. Furthermore, 2\% of the BRCA1-mutated breast cancers exhibited CC\textrightarrow TT tandem transition mutations in TP53 compared to only 0.1\% of sporadic breast cancer cases. This mutation is thought to be a signature of defective NER\textsuperscript{22}. Although seen mainly in skin cancers and thought to be secondary to UV exposure, TP53 tandem CC\textrightarrow TT mutations have also been identified in breast and ovarian cancer cases of unknown BRCA status and thus may be involved in breast carcinogenesis\textsuperscript{21}. Taken together, these data strongly suggest a defect in GGR in BRCA1-mutated tumors underlying the mutational spectrum observed for TP53.

The BRCA1 protein may be involved in several DNA repair pathways, including TCR of oxidative DNA damage. Mouse Brca1\textsuperscript{−/−} embryonic stem cells and mouse embryonic fibroblasts null for both Brca1 and Trp53 are deficient in TCR of oxidative DNA damage but not UV-induced damage compared to parental cells\textsuperscript{3,24}. Repair of the non-transcribed strand of Dhhp is not affected by Brca1 after either UV or oxidative DNA damage in these studies\textsuperscript{25}. The effect of Brca1 on GGR may, however, be more difficult to detect in mouse cells than human cells because the rate and overall level of GGR of CPDs is much lower in rodents than in humans\textsuperscript{25}.

Our work suggests a mechanism for the effect of BRCA1 on GGR involving transcriptional regulation of NER genes. BRCA1 can activate the GADD45 promoter independent of p53, supporting the notion that BRCA1 activates GADD45 and maintains GGR in p53-deficient cells\textsuperscript{15}. We and others have demonstrated that alterations in the expression of GADD45, p48, and XPC can lead to biological changes in NER\textsuperscript{26–28}. We observed a significant effect of BRCA1 on GGR in p53-deficient cells and on the induction of NER genes in contrast to p53-wildtype cells, which maintain higher basal levels of p48, XPC, and GADD45.

Our findings point to an important role for BRCA1 in DNA repair and the maintenance of genomic stability, and suggest a specific mode of action for BRCA1 in DNA damage response pathways. We suggest a model for the effect of BRCA1 and p53 on NER in BRCA1-associated breast cancers (Fig. 6). Loss of heterozygosity (LOH) of BRCA1 leads to an initial decline in NER activity, resulting in an accumulation of additional mutations, particularly in the non-transcribed strand of TP53. Although sporadic breast cancers rarely contain mutations in BRCA1, promoter hypermethylation causing silencing of BRCA1 occurs in 20\% of such cancers and may initiate a similar set of downstream genetic events\textsuperscript{29}. Loss of p53 function leads to even more genomic instability, resulting in additional genetic alterations leading to an invasive cancer phenotype.

**Methods**

**Cell lines.** The p53-wildtype U2OS osteosarcoma founder cell line UATS, which stably expresses a tetracycline-responsive transactivator, was used to generate subclones\textsuperscript{2}. These cell lines were generously provided by D. Haber (Massachusetts General Hospital, Boston). The clone named

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**Fig. 4** Overexpression of BRCA1 induces expression of DDB2 and GADD45 mRNA in p53-wildtype and p53-deficient cells. E621 (lanes a–e) and UBR60 (lanes f–j) cells were either induced 24 h before irradiation, by removing tetracycline (tet) and washing three times with PBS (lanes b, c, e, g, h, j) or maintained in tetracycline to suppress BRCA1 expression (lanes a, d, f, i). BRCA1-induced (lanes e and j) and BRCA1-uninduced (lanes d and i) cells were exposed to 10 J m\textsuperscript{2} UV, incubated for 24 h and harvested. Relative mRNA expression was adjusted for loading based upon GAPDH levels, and fold induction for DDB2 and GADD45 is expressed relative to the unirradiated, uninduced band for E621 (lane a) and UBR60 (lane f) cells. UBR60 cells exhibited threefold and twofold higher basal uninduced levels of DDB2 and GADD45 expression, respectively, than did E621 cells (lanes f versus a).

**Fig. 5** Overexpression of BRCA1 induces expression of XPC but not XPA in p53-wildtype and p53-deficient cells. a, p53-wildtype UBR60 cells were induced 24 h before irradiation by removing tetracycline (tet) and washing three times with PBS (lanes d–g) or maintained in tetracycline to suppress BRCA1 expression (lanes a–c). Both BRCA1 induced (lanes f and g) and uninduced (lanes b and c) cells were exposed to 10 J m\textsuperscript{2} of UV and either harvested immediately for a 0-h time point (lanes b and f) or incubated in media and harvested 24 h later (lanes c and g). b, p53-deficient E621 cells were induced 24 h before irradiation by removing tetracycline and washing three times with PBS (lanes c–f) or maintained in tetracycline to suppress BRCA1 expression (lanes a and b). p53-deficient E621 cells, both induced (lanes e and f) and uninduced (lane b), were exposed to 10 J m\textsuperscript{2} of UV and either harvested immediately for a 0-h time point (lane e) or incubated for 24 and then harvested (lanes b and f).
UBR60 exhibits tightly controlled inducible expression of wildtype BRCA1. This cell clone was subsequently transfected with the human papillomavirus gene E6 that targets p53 for ubiquitin-mediated degradation; this p53-deficient cell line is called E621. Cells were treated with 1 µg ml−1 tetracycline daily to suppress BRCA1 induction. BRCA1-inducible cell lines UBR60 and E621 lost inducible BRCA1 expression over time due to promoter hypermethylation, and expression was restored by treating with 2 µg/ml 5-azacytidine (Sigma) for 24 h on days 1 and 4, with tetracycline removed on day 6. BRCA1 induction was seen on day 7. In all experiments BRCA1 expression was induced 24 h before UV irradiation by removing tetracycline from the medium and washing with phosphate-buffered saline (PBS) three times.

Immunoprecipitations and western blotting. Protein expression in the UBR60 and E621 cell lines after UV irradiation, BRCA1 induction or both was assayed by immunoblot analysis. Total cellular protein was obtained by lysing cells in RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride) supplemented with Complete mini protease inhibitors (Roche). The cell lysate was centrifuged at 15,000 g for 15 min and the supernatant collected. Equal amounts of supernatant from immunoprecipitations were incubated with C-20 rabbit polyclonal BRCA1 antibody overnight along with protein A/G–Sepharose beads (Santa Cruz Biotechnology). Recovered proteins were separated on an 8% polyacrylamide gel and the gel was probed with an antibody against BRCA1 (MS110, Santa Cruz Biotechnology; 1:1,000 in TBS-Tween (TBS-T)-2% Blotto) and proteins detected using mouse monoclonal antibodies against tubulin simultaneously. The membranes were blocked in TBS-T with 5% skim milk and proteins detected by chemiluminescent substrate (Pierce) and autoradiography or CDC chemiluminescent digestion. Recovered proteins were separated on an 4–20% gradient polyacrylamide gel (GENEMate, ICS Bioexpress), transferred to nitrocellulose membranes and probed for XPC, XPA and tubulin simultaneously. The membranes were blocked in TBS-T with 5% Blotto and proteins detected using mouse monoclonal antibodies against XPC (1:5 in TBS-T; from E. Lee, University of Texas, San Antonio), XPA (1:500 in TBS-T-2% Blotto; 12F-5, Neomarkers), BRCA1 (1:1,000 in TBS-T-2% Blotto; MS110, Santa Cruz Biotechnology), tubulin (1:200,000 in TBS-T-2% Blotto; Clone B-5-1-2, Sigma) and p53 (1:1,000; DO1, Santa Cruz Biotechnology). Protein bands were detected using the Supersignal chemiluminescent substrate (Pierce) and autoradiography or CDC chemiluminescence imaging (Eastman Kodak).

Northern blotting. BRCA1 protein expression was induced in cells 24 h before UV irradiation by removing tetracycline from the medium. Both induced and uninduced cells were exposed to 10 µg/ml 5-azacytidine (Sigma). For northern hybridizations, 5 µg of total RNA was separated on a 1% glyoxal gel (Ambion). A linear amplification technique (Strip-Ez PCR, Ambion) was used to make a DNA probe from a vector containing a full-length cDNA of DD2 that we previously cloned from W138 normal human fibroblast cells. Primer sequences are available upon request. GADD45 and GAPDH mRNA probes were generated using a plasmid containing a T7 promoter (GeneStorm and Ambion, respectively).

Radioactivity was detected with a phosphorimager (Bio-Rad). Band density was calculated using Quantity One Software (Bio-Rad). Blots were stripped after exposure using Strip-EZ (Ambion) and rehybridized to a different probe.

Global genomic NER immunoblot assay. Repair of CPDs and 6–4 photoproduc-tants in the various cell lines at different times after UV irradiation was measured using an immunoblot assay, as previously described4–6. Monoclonal antibodies specific for either CPDs (1:1,000) or 6–4 photoproduc-tants (1:500) were kindly supplied by T. Mori (Nara Medical University, Japan30). Data from triplicate DNA samples from these three different biological experiments were averaged. Statistical analysis of differences in DNA repair curves due to expression of BRCA1 were performed using the unpaired t-test.

Analysis of strand-specific DNA repair. To determine the rate of removal of CPDs from the transcribed or non-transcribed strand of a specific gene fragment, strand-specific RNA probes were used to evaluate the frequency of CPDs in an HpaI restriction fragment of 17 kb or a PstI restriction fragment of 7 kb spanning the central region of the DHFR gene. Purified DNA was digested with HpaI or PstI and treated or mock-treated with bacterio- phage T4 endonuclease V (generously supplied by R.S. Lloyd, University of Texas–Galveston). Southern blotting and analysis were carried out as previously described4–6. Data from triplicate DNA samples from three different biological experiments were averaged. Statistical analysis of differences in DNA repair curves due to expression of BRCA1 were performed using the unpaired t-test.

Acknowledgments
We thank P. Hanawalt, A. Ganesan, F. Stockdale, C. Lopez and members of the Ford Laboratory for helpful discussions and critical reading of the manuscript, and I. Cross for expert technical assistance. This work was supported in part by a National Institutes of Health RO1 Award, a Sidney Kimmel Foundation for Cancer Research Scholar Award and a Burroughs Wellcome Fund New Investigator Award in Toxicological Sciences (to J.M.F.). A.R.H. is an ASCO Clinical Scholar and is supported by the California Breast Cancer Research Program.

Competing interests statement
The authors declare that they have no competing financial interests.

Received 25 April; accepted 11 July 2002.


Fig. 6 A model for the involvement of BRCA1 and TP53 in NER in BRCA1-deficient breast cancers. LOH for BRCA1 leads to an initial decline in NER activity, resulting in an accumulation of additional mutations, particularly in the non-transcribed strand of TP53. Loss of TP53 function results in defective cell-cycle checkpoints, apoptosis and DNA repair, and leads to even more pronounced genomic instability, resulting in additional genetic alterations leading to an invasive cancer phenotype. Breast tissue from non-BRCA1 carriers may have promoter hypermethylation of BRCA1, resulting in gene silencing and a similar downstream chain of events leading to invasive breast cancer.


