

Breast Cancer Risk and the DNA Double-Strand Break End-Joining Capacity of Nonhomologous End-Joining Genes Are Affected by *BRCA1*

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ABSTRACT

A tumorigenic role of the nonhomologous end-joining (NHEJ) pathway for the repair of DNA double-strand breaks (DSBs) has been suggested by the finding of a significant association between increased breast cancer risk and a cooperative effect of single nucleotide polymorphisms (SNPs) in NHEJ genes. However, the lack of an association between hereditary breast cancer and defective NHEJ genes prevents conclusions from being drawn about a link between NHEJ and breast cancer development. Recently, *BRCA1*-deficient mouse embryonic fibroblasts were found to have significantly reduced NHEJ activity, suggesting an accessory role of *BRCA1* in NHEJ. The present study was performed to confirm this observation in human breast cancer cell lines and to examine whether the interaction between *BRCA1* and NHEJ was of tumorigenic significance. Support for this hypothesis came from the findings that (a) a case-control study (469 breast cancer patients and 740 healthy controls) showed that the breast cancer risk associated with high-risk genotypes of NHEJ genes was significantly modified by the *BRCA1* genotype. A significant increase in the cancer risk associated either with harboring one additional putative high-risk NHEJ genotype or with the joint effect of having reproductive risk factors (reflected by an interval of ≥ 12 years between menarche and first full-term pregnancy) and a higher number of high-risk genotypes of the NHEJ genes was only seen in women with at least one variant *BRCA1* allele (i.e., the *Glu/Gly* or *Gly/Gly* forms of *BRCA1 Glu¹⁰³⁸Gly*); and (b) a phenotype-based study measuring *in vitro* and *in vivo* NHEJ capacity showed that the precise end-joining capacity was different in breast cancer cell lines with different *BRCA1* statuses being higher in *BRCA1*-expressing MCF-7 cells than in HCC1937 cells (defective *BRCA1* expression). Furthermore, this end-joining capacity was decreased in MCF-7 cells in which *BRCA1* expression was blocked using small interfering RNA and increased in HCC1937 transfected with full-length *BRCA1*. Because *BRCA1* is a well-documented breast cancer susceptibility gene, this association between NHEJ and *BRCA1* not only suggests a role of *BRCA1* in NHEJ but also provides essential support for the tumorigenic contribution of NHEJ in breast cancer development.

INTRODUCTION

Human cancers are thought to result from inherent or somatically acquired genomic instability (1, 2). The capacity of cells to maintain their genomic stability by means of a variety of DNA repair mechanisms is therefore essential in preventing tumor initiation and progression (3). DNA double-strand break (DSB) is believed to be the most severe type of DNA damage (4, 5), and on the basis of our evidence that the frequency of DSB-related genomic deletion increases as tumors progress to poorer grades or later stages, we have proposed that breast cancer pathogenesis is driven by DSB-initiated chromosome instability and that the mechanisms involved in DNA

DSB repair are of particular etiological importance during breast tumorigenesis (6). In cells, DSBs are repaired by two major pathways, nonhomologous end-joining (NHEJ) and homologous recombination (HR), which differ in their requirement for a homologous DNA template during repair (4). The importance of HR during breast tumorigenesis has been unambiguously demonstrated by the identification of familial breast cancer susceptibility genes, including *BRCA1* and *BRCA2*, which are involved in HR repair of DNA DSBs (7, 8). It is therefore tempting to speculate that defects in the NHEJ pathway may be associated with breast tumor formation in humans. Given this, it is puzzling that no genetic evidence has been found to link defective NHEJ genes with the development of breast cancer. Of the NHEJ genes, only mutations in two have been found to predispose carriers to a higher rate of genetic diseases; these are *Ligase IV* and *Artemis*, which are associated, respectively, with Nijmegen breakage syndrome (NBS)-like syndrome and severe combined immunodeficiency, neither of which involves the development of cancer (9, 10). One possible explanation for this may be that because NHEJ is crucial for cells to maintain genetic stability, any severe defects (null mutants) in NHEJ-related genes such as those in NBS-like syndrome or severe combined immunodeficiency patients, would result in genomic instability and might be incompatible with life. Thus, for these high-penetrant NHEJ genes, only subtle defects arising from low-penetrance (risk) alleles (e.g., hypomorphic mutant or polymorphic variant) would escape cell-cycle checkpoint surveillance and allow the cell to survive and accumulate the unrepaired DNA damage required for tumor formation (11). Our finding of a significant association between increased breast cancer risk and a cooperative effect of single nucleotide polymorphisms (SNPs), the most subtle genetic variation in the genome (12), in NHEJ genes (11), is consistent with this hypothesis. However, the lack of an association between hereditary breast cancer and defective NHEJ genes still prevents conclusions to be drawn about a link between NHEJ and breast cancer. Recently, *BRCA1*-deficient mouse embryonic fibroblasts were found to have significantly reduced NHEJ activity (13, 14). Because *BRCA1* is a well-documented breast cancer susceptibility gene, if this association between NHEJ and *BRCA1* could be confirmed in humans and found to be important in determining breast cancer risk, this would provide strong support for the role of NHEJ in breast tumorigenesis. This study reports such an investigation, based (a) on a case-control study to estimate the breast cancer risk associated with harboring putative high-risk genotypes of NHEJ genes in women with different *BRCA1* genotypes and (b) on a phenotype-based study of the *in vitro* and *in vivo* NHEJ capacity of breast cancer cell lines with different *BRCA1* statuses.

MATERIALS AND METHODS

Genotype-Based Case-Control Study. The present study includes 469 breast cancer patients and 740 healthy controls recruited between September 2001 and June 2002. All subjects gave their informed consent. All breast cancer patients had pathologically confirmed primary infiltrating ductal carcinoma of the breast and 5% had a family history of breast cancer (mothers or sisters). These patients accounted for almost all (>90%)

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women with breast cancer attending our breast cancer clinics during the study period, the remaining patients being excluded because of a lack of suitable blood specimens. No significant differences in breast cancer risk factors were found between the included and excluded women. More importantly, because the clinics taking part in this study are three of the major breast cancer clinics in northern and central Taiwan, our patients accounted for a significant proportion (~20%) of all breast cancer cases diagnosed during the study period in these regions.

To avoid any differential recall bias of previous disease history, we deliberately randomly selected the controls from the health examination clinics of the same hospitals during the same study period. These controls accounted for ~80% of all women attending the clinics, and no significant differences were found in socioeconomic status between those included and those not included. The control subjects underwent a 1-day comprehensive health examination and showed no evidence of breast cancer, suspicious precancerous lesions of the breast, or other cancers.

Three experienced research nurses were assigned to administer a structured questionnaire to both cases and controls to collect all relevant information regarding risk factors of breast cancer. A 10-ml sample of peripheral blood, collected in acetate-citrate dextrose, was obtained from each breast cancer patient before operation and from each control subject. Buffy coat cells were immediately prepared and stored at -80°C until genomic DNA was prepared by conventional phenol/chloroform extraction, followed by ethanol precipitation, and stored at -20°C .

Three SNPs in the *BRCA1* gene, *Pro*⁸⁷¹*Leu*, *Glu*¹⁰³⁸*Gly*, and *Ser*¹⁶¹³*Gly*, were selected for study, as previous epidemiological studies had suggested a putative association between these specific SNPs and increased cancer risk (15). The SNPs in the five NHEJ genes (*Ku70*, *Ku80*, *DNA-PKcs*, *XRCC4*, and *Ligase IV*) used have been described previously (11). All SNPs were genotyped using a MassARRAY system (SEQUENOM, Inc., San Diego, CA) based on the primer extension protocol (16). The PCR primers and extension primers for these two SNPs were designed using Spectro-Designer software (SEQUENOM, Inc.).

The association between putative high-risk genotypes of these five NHEJ genes and breast cancer risk was evaluated with simultaneous consideration of established risk factors for breast cancer in a multivariate logistic regression model (17). Of particular interest was the possible modification by *BRCA1* of the risk associated with the number of high-risk NHEJ genotypes, which was examined using a stratified method (18, 19) and evaluated by calculating the risk (adjusted odds ratio and 95% confidence interval) of breast cancer in relation to harboring a higher number of high-risk NHEJ genotypes within women of different *BRCA1* genotypic status. We also used the same stratified method to estimate the joint effect of NHEJ genes and reproductive risk factors because reproductive risk factors reflect a longer period of or higher susceptibility to exposure to estrogen, which is known to initiate breast cancer development by causing DNA DSBs (20, 21). Thus, if *BRCA1* were involved in NHEJ, the joint effect would not be the same in women harboring different *BRCA1* genotypes.

In Vivo and in Vitro Functional Assays of End-Joining Capacity. To examine whether *BRCA1* was involved in NHEJ, we measured end-joining capacity *in vivo* and *in vitro* in human breast cancer cells with either normal *BRCA1* expression (MCF-7) or defective *BRCA1* expression (HCC1937). Furthermore, to determine whether the association between *BRCA1* and NHEJ was causal, *in vitro* and *in vivo* end-joining capacity was also measured in MCF-7 cells in which *BRCA1* expression was blocked using small interfering RNA (siRNA) and in HCC1937 cells transiently transfected with *BRCA1*.

Cell Culture and Reagents. The human breast cancer cell lines, MCF-7 and HCC1937, were purchased from the American Type Culture Collection (Manassas, VA) and grown in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 mg/ml streptomycin, and 100 units/ml penicillin. The cell lines were cultured at a constant temperature of 37°C in a 5% carbon dioxide-humidified atmosphere.

Transfection with siRNA. The siRNA duplexes consisted of 21 bp, including a two-deoxynucleotide overhang, the coding strand being GGAAC-CUGUCCACAAAGdTdT. Cells (MCF-7) were transfected with siRNA using the Oligofectamine protocol (Invitrogen-Life Technologies, Inc., La Jolla, CA). In brief, 24 h before transfection, the cells were seeded, without

antibiotics, at 1×10^6 cells/60-mm dish, corresponding to 40–50% confluency at the time of siRNA transfection. The cells were then incubated for 4 h at 37°C with various concentrations of siRNA plus Oligofectamine in Opti-MEM I or with Opti-MEM I alone, then fresh culture medium containing 30% fetal bovine serum was added in the ratio of 1 volume/3 volumes of the transfection medium. The cells were harvested 24 h after the beginning of the transfection period for *BRCA1* protein detection (by Western blotting) or *in vitro* NHEJ assay (13) or additionally transfected with pGL2 for the *in vivo* end-joining assay (Ref. 14; see below).

Full-Length *BRCA1* Plasmid Transfection. Parental HCC1937 cells were transfected with a pcDNA 3.1 plasmid containing the full-length *BRCA1* gene. For transient transfectants, cells were transfected with full-length *BRCA1* plasmid using the Lipofectamine 2000 protocol (Invitrogen). HCC1937 cells at 90–95% confluency were incubated for 4 h at 37°C with full-length *BRCA1* plasmid plus Lipofectamine in Opti-MEM I or with Opti-MEM I alone, then fresh culture medium containing 30% fetal bovine serum was added in the ratio of 1 volume/3 volumes of the transfection medium. The cells were then processed as in the siRNA experiment above.

Western Blot Analysis of *BRCA1* and Other DNA DSB Repair Proteins. Protein extracts were prepared, and equal amounts of protein (50 μg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL Nitrocellulose Membrane; Amersham Pharmacia Biotech, Piscataway, NJ). After addition of the blocking mixture, the membrane was incubated for 2 h at room temperature with primary antibody and washed three times with PBS, then bound antibody was detected using horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Antibodies against RAD51, Ku70, *BRCA1*, *XRCC4*, and DNA Ligase IV were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA), whereas the anti-NBS1 antibody was purchased from Oncogene (Oncogene Research Products, La Jolla, CA). The signal was detected using enhanced chemiluminescence (Santa Cruz Biotechnology) on Kodak BioMax films (Sigma Chemical Co., St. Louis, MO).

In Vitro NHEJ Assay. The cells were harvested and washed once in DMEM containing 10% FCS, three times in ice-cold PBS, and once in hypotonic lysis buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 5 mM DTT], then resuspended in 2 volumes of hypotonic buffer, incubated for 20 min at 0°C , and lysed by homogenization, then protease inhibitors (0.17 mg/ml phenylmethylsulfonyl fluoride, 0.01 trypsin inhibitor units/ml aprotinin, 1 $\mu\text{g}/\text{ml}$ pepstatin, 1 $\mu\text{g}/\text{ml}$ chymostatin, and 1 $\mu\text{g}/\text{ml}$ leupeptin) were added. After 20 min on ice, 0.5 volumes of high salt buffer [50 mM Tris-HCl (pH 7.5), 1 M KCl, 2 mM EDTA, and 2 mM DTT] were added and the extract centrifuged for 3 h. The supernatant was then dialyzed for 3 h against dialysis buffer [20 mM Tris-HCl (pH 8.0), 0.1 M KOAc, 20% (v/v) glycerol, 0.5 mM EDTA, and 1 mM DTT], fast-frozen, and stored at -70°C . The pBSK(+) duplex plasmid DNA (2.96 kb; Stratagene, La Jolla, CA) was linearized with *EcoRI*, dephosphorylated using calf intestinal phosphatase, and 5'-³²P end-labeled using polynucleotide kinase. Cell-free extracts (50–100 μg of protein) were incubated for 5 min at 37°C before being added to the reaction mix of 50 mM triethanolamine-HCl (pH 7.5), 0.5 mM Mg(OAc)₂, 60 mM KOAc, 2 mM ATP, 1 mM DTT, and 100 $\mu\text{g}/\text{ml}$ BSA containing 50 fmol of labeled linearized DNA. After incubation at 37°C for 1 h, the ³²P-labeled DNA products were deproteinized for 20 min at 37°C using 500 $\mu\text{g}/\text{ml}$ proteinase K and 1% SDS and analyzed by electrophoresis through 0.7% agarose gels.

The monomer and multimers bands (substrate and products) were detected on Kodak BioMax films (Sigma Chemical Co.). The data were quantitated by densitometry and, after normalization for loading and transfer, expressed as end-joining efficiency calculated as: intensity of end-joining products/total substrate \times 100%.

In Vivo End-Joining Assay. Plasmid pGL2 (Promega, Madison, WI) was completely linearized using either *HindIII* or *EcoRI*, as confirmed by agarose gel electrophoresis. The linearized DNA was subjected to phenol/chloroform extraction, ethanol-precipitated, dissolved in sterilized water, and used to transfect cells using Lipofectamine 2000 following the procedures described by the supplier (Invitrogen). The transfectants were harvested 48 h after transfection and assayed for luciferase activity as described previously (22).

Table 1 aOR of breast cancer development associated with having one additional high-risk genotype of the five NHEJ genes (Ku70, Ku80, DNA-PKcs, XRCC4, and Ligase IV), stratified by BRCA1 genotype^a

Genotypes of BRCA1 Glu ¹⁰³⁸ Gly	Cases (%)	Controls (%)	aOR (95% CI) ^b
Glu/Glu	179 (38.2)	265 (35.9)	1.04 (0.83–1.30)
Glu/Gly, Gly/Gly	290 (61.8)	473 (64.1)	1.28 (1.06–1.53)

^a aOR, adjusted odds ratio; NHEJ, nonhomologous end-joining; CI, confidence interval.

^b The aOR of breast cancer development associated with the number of high-risk genotypes of NHEJ genes was calculated in a multivariate logistic regression model containing the number of high-risk genotypes (5 versus 4, 4 versus 3, 3 versus 2, 2 versus 1, and 1 versus 0), age, a family history of breast cancer, reproductive risk factors, body mass index, and a history of hormone replacement therapy.

RESULTS

Women Harboring Different BRCA1 Genotypes Show Different Breast Cancer Risks Associated with Genotypic Polymorphisms of the NHEJ Genes

If the NHEJ genes were associated with breast cancer development via the hypothesized mechanism involving BRCA1, the relationship between cancer risk and the high-risk genotypes of the NHEJ genes would be expected to be different in different groups of women with different BRCA1 genotypes. The three SNP sites in BRCA1 were genotyped, but subsequent haplotype analysis provided little additional information because all three were found to be in strong linkage disequilibrium ($P < 0.01$, determined using the EH program available at the web site⁵ and the χ^2 test) in both cases and controls, and the frequency distribution based on haplotypes was similar to that based on individual SNPs. We therefore chose one SNP, Glu¹⁰³⁸Gly, which showed the most significant P , to represent the allelic status of BRCA1 and investigated the potential importance of BRCA1 in conjunction with the high-risk genotypes of the five NHEJ genes by the stratified method. The heterozygous and homozygous variant genotypes (*i.e.*, Glu/Gly, Gly/Gly) of BRCA1 were then grouped together and compared with the homozygous wild-type genotype (Glu/Glu); due to the small percentage of subjects with the homozygous variant genotype, this grouping provided increased statistical power. In agreement with our previous study using a smaller sample size (11), when the five NHEJ genes were considered as a whole, one additional putative high-risk NHEJ genotype was associated with a 1.17-fold (95% confidence interval, 1.01–1.34) increase in risk. However, interestingly, after stratification on the basis of BRCA1 genotypes, the risk (adjusted odds ratio) associated with harboring one additional high-risk NHEJ genotype was not the same in women with different BRCA1 genotypes, which was more significant in those with at least one variant allele of BRCA1 (Table 1). Furthermore, because estrogen is suggested to initiate breast tumorigenesis by causing DNA DSBs (20, 21), the significant modification by BRCA1 of breast cancer risk associated with NHEJ genotypic polymorphism prompted us to examine whether a higher risk of breast cancer due to a longer period of estrogen exposure [reflected by a longer interval of ≥ 12 years between the ages of menarche and first full-term pregnancy] and defective DSB repair (indicated by harboring a higher number of putative high-risk genotype of NHEJ genes) was affected by BRCA1. The results, shown in Table 2, demonstrated that a significant increase in cancer risk associated with the joint effect of having reproductive risk factors and a higher number of high-risk genotypes of the NHEJ genes was only seen in women with at least one variant BRCA1 allele.

Breast Cancer Cells of Different BRCA1 Genotype Statuses Show Different *In Vitro* and *In Vivo* End-Joining Capacities

Expression of BRCA1 and Other DNA DSB Repair Proteins in Breast Cancer Cell Lines with or without Active BRCA1. To determine whether BRCA1 was involved in NHEJ activity, we performed comparative *in vivo* and *in vitro* end-joining assays in BRCA1-expressing MCF-7 cells, MCF-7 cells transfected with BRCA1 siRNA (BRCA1 knockdown), BRCA1-deficient HCC1937 cells, and HCC1937 cells transiently transfected with full-length BRCA1 (BRCA1 restored). For quantitative comparison, whole cell extracts were normalized for total protein levels before immunoblot analyses. We confirmed the suppression of BRCA1 expression in the BRCA1-knockdown MCF-7 cells and the restoration of BRCA1 expression in the BRCA1-restored HCC1937 cells (Fig. 1). In addition, measurement of the expression of other DSB repair proteins, including NHEJ pathway proteins (Ku70, Ligase IV, and XRCC4), DSB trimming proteins (NBS1), and a HR pathway protein (RAD51), showed no significant differences, except that XRCC4 expression was slightly reduced in BRCA1-restored HCC1937 cells (Fig. 1). These results emphasize the fact that any changes in *in vivo* and *in vitro* end-joining activities observed in the subsequent analysis were more likely to be caused by BRCA1 than by the other DSB repair proteins, levels of which were not significantly affected by BRCA1 modification.

BRCA1 Affects *In Vitro* NHEJ Capacity. An *in vitro* NHEJ assay, in which the DNA end-joining capacity of the whole cell extract was measured by rejoining ³²P-labeled linear duplex DNA (13), was used to investigate the involvement of BRCA1 in NHEJ in breast cancer cell lines. After 1 h of incubation with the extract from control MCF-7 cells (BRCA1 positive), dose-dependent rejoining of DNA molecules was seen, rejoining of 25–35% of the input DNA molecules seen using 80 μ g of cell extract (Fig. 2A). To examine the role of BRCA1, we directly compared the NHEJ capacity of whole cell extracts of BRCA1-defective HCC1937 cells with that of MCF-7 and found that the HCC1937 cells showed a significant lower capacity, 80 μ g of HCC1937 extract having only one-third to one-fourth of the capacity of the MCF-7 extract (Fig. 2A). The validity of the assay was reconfirmed by the dose-response relationship between the amount of whole cell extract from HCC1937 and the *in vitro* end-joining capacity (Fig. 2A).

Having identified an association between the BRCA1 genotype of

Table 2 Risk of breast cancer (aOR) associated with the combination of the number of putative high-risk genotypes of NHEJ genes and a reproductive risk factor (years of estrogen exposure up to first full-term pregnancy), stratified by BRCA1 genotype^a

Reproductive risk factor/BRCA1 Glu ¹⁰³⁸ Gly	aOR (95% CI) ^b	
	No. of high-risk genotypes of NHEJ genes	
	0–2	3–5
Yrs of estrogen exposure up to first full-term pregnancy ^c		
BRCA1, Glu/Glu		
<12	1.00 (Ref.)	0.77 (0.45–1.32)
≥ 12	1.06 (0.60–1.87)	1.07 (0.55–2.09)
BRCA1, Glu/Gly, Gly/Gly		
<12	1.00 (Ref.)	1.30 (0.85–1.99)
≥ 12	1.43 (0.92–2.22)	2.55 (1.50–4.34)

^a aOR, adjusted odds ratio; NHEJ, nonhomologous end-joining; CI, confidence interval; Ref, reference group.

^b The aOR of breast cancer development associated with the number of putative high-risk genotypes and reproductive risk factor was estimated in a multivariate logistic regression model containing age, a family history of breast cancer, body mass index, and a group of dummy variables to represent the four different combinations of gene (number of putative high-risk genotypes) and reproductive risk factor status.

^c For postmenopausal nulliparous women, this index was age at menopause – age at menarche, whereas, for other women, it was age at first full-term pregnancy – age at menarche.

⁵ Internet address: <http://linkage.rockefeller.edu/soft>.

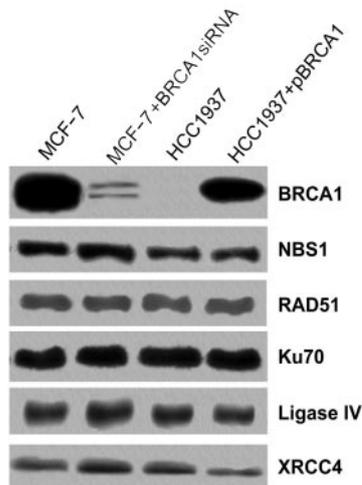


Fig. 1. Immunoblot analysis showing similar levels of a representative DNA double-strand breaks trimming/checkpoint protein (NBS1) and repair proteins (RAD51 in homologous recombination and Ku70, Ligase IV, and XRCC4 in nonhomologous end-joining) in cell lines assayed for *in vitro* and *in vivo* end-joining activity.

the cells and the *in vitro* end-joining capacity, we then used both loss-of-function and gain-of-function strategies to explore whether this association was causally linked. When *BRCA1* siRNA was transfected into MCF-7 cells to reduce *BRCA1* mRNA levels, marked *BRCA1* siRNA dose-dependent down-regulation of end-joining capacity was seen, 5 nM *BRCA1* siRNA eliminating 30–35% of the end-joining capacity and 20 nM *BRCA1* siRNA almost completely abolishing *in vitro* NHEJ capacity (Fig. 2B). In contrast, when the plasmid containing the full-length *BRCA1* gene was transfected into HCC1937, transfection with 8 μ g of plasmid caused an increase in end-joining capacity, whereas transfection with 16 μ g increased the activity in HCC1937 cells to ~70% of that in MCF-7 cells (Fig. 2B). The increasing and decreasing trends observed in these assays were all statistically significant ($P < 0.05$, examined by linear regression). Simultaneously performed immunoblots showed, as in Fig. 1, that the levels of *BRCA1* decreased or increased, respectively, in a dose-response manner using the loss-of-function or gain-of-function strategy (data not shown).

***BRCA1* Affects *in Vivo* NHEJ Capacity.** We then examined whether the role of *BRCA1* in affecting end-joining capacity was important *in vivo*. To detect end-joining capacity *in vivo*, a linearized pGL2 plasmid containing the endonuclease-digested *luciferase* reporter gene was transfected into breast cancer cell lines, the rationale being that the *luciferase* gene would only be expressed after the plasmid was re-end-joined as the circular form, and the relative end-joining efficiency could be calculated by comparing the luciferase activity in cells transfected with endonuclease-digested DNA with that in cells containing the uncut plasmid. The end-joining capacity detected using pGL2 digested with *HindIII* reflects overall end-joining (14) because this enzyme cleaves at the linker region between the promoter and the coding sequences, and any end-joining activity, even that resulting in small deletions or insertions, would not affect luciferase expression. However, because the *EcoRI* site is in the *luciferase* sequence and only precise end-joining would restore the original *luciferase*, the relative luciferase activity using this enzyme reflects precise end-joining capacity (14).

After the same strategy used to define the role of *BRCA1* in *in vitro* end-joining, we examined end-joining capacities in MCF-7 and HCC1937 breast cancer cell lines and found that, although their *in vivo* overall end-joining capacities were very similar, MCF-7 cells had significantly higher *in vivo* precise end-joining capacity than

HCC1937 cells (Fig. 3A). Consistent with the results for the *in vitro* assay, the *BRCA1*-deficient HCC1937 cells re-gained precise end-joining capacity on transfection with the full-length *BRCA1* plasmid, and the precise end-joining capacity of the *BRCA1*-expressing MCF-7 cells was eliminated by *BRCA1* siRNA inhibition, both in a dose-response manner (Fig. 3B). The increasing and decreasing trends observed in these assays were all statistically significant ($P < 0.05$, examined by linear regression). Neither manipulation had any effect on *in vivo* overall end-joining activity (data not shown).

The *in vivo* overall NHEJ is the sum of the precise and nonprecise end-joining activity, but the *in vitro* NHEJ mainly reflects precise end-joining activity (13, 14). This might explain why the HCC1937 cells showed a lower *in vitro* overall NHEJ capacity than the MCF-7 cells, whereas the *in vivo* overall end-joining activity was the same in both cells. More importantly, the difference between the *in vivo* and *in vitro* overall end-joining capacities suggests that *BRCA1* is only specifically involved in precise NHEJ. Finally, the use of the same restriction enzyme (*EcoRI*) for the preparation of the linear duplex DNA fragment (*in vitro* end-joining assay) and the linearized plasmid (*in vivo* precise end-joining assay) in the present study ensured that similar repair machinery was recruited and that the *in vivo* and *in vitro* results were comparable, and the fact that the two assays gave consistent results suggests that these findings are reliable.

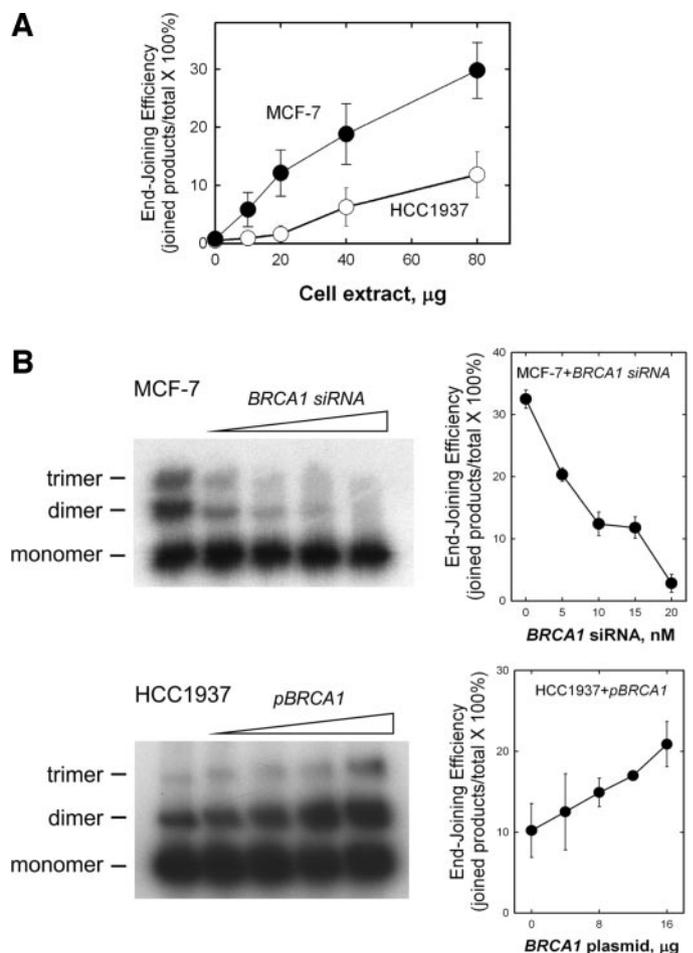
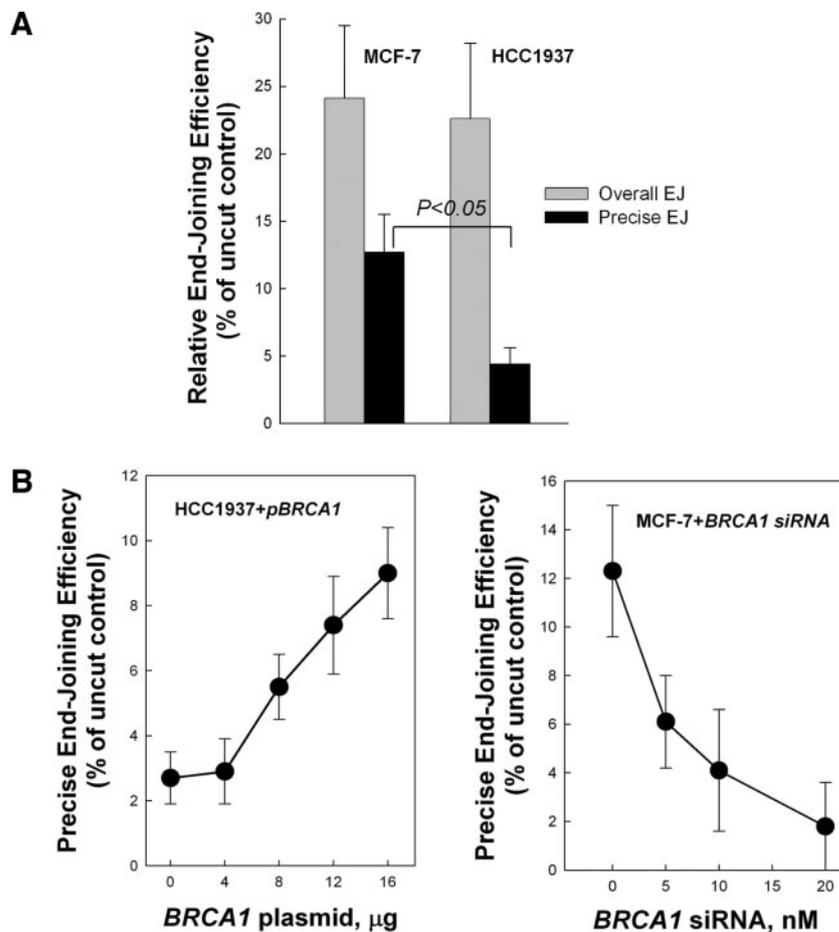


Fig. 2. *In vitro* end-joining activity in *BRCA1*-expressing (MCF-7) and *BRCA1*-defective (HCC1937) human breast cancer cell lines (A) or in MCF-7 cells in which *BRCA1* was knocked down using *BRCA1* small interfering RNA (siRNA) and in HCC1937 cells in which *BRCA1* was complemented by transient transfection of full-length *BRCA1* (B). End-joining activity was measured as [intensity of multimers/(multimers + monomer) \times 100%] by phosphoimage analysis of the gels. Each experimental point represented the average of at least three replicates \pm SE.

Fig. 3. *In vivo* overall and precise end-joining activities in *BRCA1*-expressing (MCF-7) and *BRCA1*-defective (HCC1937) human breast cancer cell lines (A) or *in vivo* precise end-joining activity in MCF-7, in which *BRCA1* was knocked down using *BRCA1* siRNA, and in HCC1937, in which *BRCA1* was complemented by transient transfection of full-length *BRCA1* (B). A plasmid containing the reporter gene (*luciferase* gene) and two unique restriction sites *Hind*III and *Eco*RI was transfected into different cell lines and used to measure overall end-joining activity and precise end-joining activity as described in "Materials and Methods." Each experimental point represented the average of at least three replicates \pm SE.



DISCUSSION

Through its involvement in DNA DSB repair, the NHEJ pathway plays an important role in maintaining genomic stability (4). While HR, the other DSB repair mechanism, uses the second, intact copy of DNA as the template for repairing sequences spanning the DSB, NHEJ involves the direct ligation of DNA ends by a ligation complex. Consequently, NHEJ often results in imprecise repair, yielding deletions or insertions (4, 23). Despite this, in contrast to the predominant role of HR in *Saccharomyces cerevisiae* and in the G_2 phase of the mammalian cell cycle, NHEJ plays the predominant role under most conditions in mammalian cells (24). This is mainly because in mammalian cells harboring a huge amount of a complex genome, searching for homologous sequences for precise DSB repair by HR does not seem to be efficient. Furthermore, because a significant portion ($\sim 70\%$) of the genome in mammalian cells consists of extragenic sequences that do not code for protein amino acids (25), the trade-off between the use of NHEJ to fix DSB, with the relatively minor cost of imprecise repair, and efficient DSB repair to avoid lethality caused by unrepaired damage becomes acceptable. However, it would be mechanistically reasonable if there were additional factors that could improve the fidelity of NHEJ, and interestingly, *BRCA1*, one of the key members of HR, has been suggested to possess this function in studies on mouse embryonic fibroblasts and in lymphoblastoid cell lines established from women carrying *BRCA1* mutation (13, 26). The present study is the first to confirm this accessory role of *BRCA1* in NHEJ in breast cancer cell lines, both *in vitro* and *in vivo*, and, given that *BRCA1* is a breast cancer susceptibility gene, the results are more biologically relevant than those in the previous studies on

embryonic fibroblasts and cell lines. More importantly, we have also provided evidence that the involvement of *BRCA1* in NHEJ is of tumorigenic significance and that the breast cancer risk associated with harboring a higher number of high-risk genotypes of NHEJ genes is significantly modified by *BRCA1* polymorphism.

Support for the role of *BRCA1* in DNA DSB repair is mainly based on the fact that *BRCA1* interacts directly or indirectly with the other proteins that play important roles in DSB repair (8, 27). Because it colocalizes with RAD51, an essential protein in regulating recombination and cofirms discrete nuclear foci with RAD51 in intranuclear structures after treatment with DSB-causing agents (28), *BRCA1* has been suggested to be involved in controlling HR in DSB repair (8, 23). In addition, when cells are subjected to ionizing radiation, wild-type *BRCA1* forms a complex with RAD50-MRE11-p95/NBS1 (MRN), both *in vitro* and *in vivo*, and the formation of the MRN complex is dramatically reduced in *BRCA1*-deficient breast cancer cells and restored by transfection with wild-type *BRCA1* (29). This suggests that *BRCA1* is important for cellular responses to DNA damage mediated by the MRN complex. However, whether and how MRN is involved in DSB repair by NHEJ is still not resolved (30), leading to speculation that *BRCA1* might not play a significant role in the NHEJ pathway. Different studies based on *in vivo* or *in vitro* assays of NHEJ capacity in cell lines of different tissue origins have yielded inconsistent results, which may be due to differences in the host cells used, the type of end-joining capacity detected, and the nature of the *Brcal* mutation in the mouse cells used (7, 23, 31). To resolve these inconsistencies, we propose the possibility that more than one NHEJ mechanism exists and that *BRCA1* is only required in some of these. As we demonstrated in the present study, the dependence of NHEJ on

BRCA1 is condition-specific because the *in vivo* studies showed that *BRCA1* was only required for precise end-joining.

The sample size of this case-control study is relatively large but, as many other association studies, might be still subject to possible underpower to detect the associations. However, because, in this study, the five NHEJ pathway genes were considered as a single unit (rather than as separate genes) and we did not separately examine which NHEJ gene was more important or which NHEJ gene interacted with *BRCA1*, we actually only examined two genetic risk factors, *i.e.*, one gene (*BRCA1*) and one pathway (NHEJ). Because of this, sample size and underpowering might not be a serious concern, if at all.

The genotypic polymorphisms at *BRCA1* and the NHEJ genes examined in the present study have been individually shown to be associated with breast cancer risk (11, 15). Although some polymorphisms are exonic and thus have the potential to alter the activity of enzymes, there is currently no evidence for genotype-phenotype associations, and thus, how the risk is affected by these SNPs remains unclear. It is possible that our epidemiological findings may be explained by alternative mechanisms such as (a) the polymorphisms may be in linkage disequilibrium with an exon change in the same gene that affects protein function, (b) intronic changes in gene sequences contain regulatory sequences such as enhancers, which affect the level of expression through transcriptional regulation, (c) the SNPs in these genes may be linked with alterations in other adjacent unidentified genes, increasing breast cancer risk. To exclude the third possibility, we attempted to use more than one SNP in these genes to assign the haplotypes and to examine haplotype effects on cancer risk, but the information generated by haplotype analysis was limited due to strong linkage disequilibrium between the SNPs in the same gene. Despite this, the strength of the present study is that it is based on the candidate gene approach, and, given the fact that all breast cancer susceptibility genes (including *ATM*, *p53*, *PTEN*, *BRCA1*, and *BRCA2*) are involved in DNA DSB checkpoint/repair pathway (6), the study of an association between breast cancer development and an interaction between DSB repair genes (such as *BRCA1* and NHEJ genes) is mechanistically and biologically reasonable. Although cell line studies and SNP-based epidemiological observations have provided evidence for this interaction between *BRCA1* and NHEJ, animal models using knockout mice and familial breast cancer syndromes have not reported any such relationship. We appreciate that this difference is important and consider that it may shed light on the nature of the critical mechanisms by which the interaction between *BRCA1* and NHEJ displays its tumorigenic contribution. We have proposed a hide-then-hit model (11), suggesting that, for these high-penetrant genes, only subtle defects arising from low-penetrance (risk) alleles (*e.g.*, polymorphic variant detected in the present study) would have the chance to escape cell-cycle surveillance without triggering checkpoint-induced lethality, thus leading to the accumulation of the essential genetic changes required for tumor formation. The epidemiological observations reported in this and our recent study (11) confirm such a hypothesis. It is also reasonable to speculate that mechanisms operating at the transcriptional level may be responsible for a similar low-penetrance effect in cancer development because they can switch genes on as well as off, thus modifying the level of expression of normal genes, instead of totally and permanently abolishing expression (32). Accordingly, as compared with the previous study using a *BRCA1*-knockout mouse cell line bearing a *p53* mutation to avoid cell death (13), the demonstration in the present study, using both gain-of-expression (using transfection to increase *BRCA1* expression) or loss-of-expression (using siRNA to knockdown *BRCA1* expression), that the interaction between *BRCA1* and NHEJ affects DSB repair capacity is more relevant to tumorigenesis. However,

because their effects are low penetrant, these alleles would predispose carriers to a higher risk of developing cancer but not necessarily cause cancer. The probability of manifesting the tumorigenic phenotype depends on the interaction between these alleles and the environment. Recently, the role of reproductive hormones in breast tumorigenesis has been greatly expanded to suggest that estrogen might be a complete carcinogen directly causing genetic alteration and tumor initiation, and breast cancer can be initiated by estrogen via exposure to estrogen metabolites, including catechol estrogen and catechols estrogen quinones (20, 21). These metabolites cause DNA DSBs formation, which may impose a selective microenvironment in the breast epitheliums, explaining why the increased cancer risk associated with the interaction between *BRCA1* and NHEJ varies in women with different profiles of estrogen-related risk factors.

The genetic evidence from familial cancer syndromes provides solid support for a causal relationship between mutated genes and the incidence of cancer, but not all cancer-associated genes can meet this requirement of linking germ-line defects to cancer development. However, as our understanding of tumorigenesis is extended from single-gene mechanisms to multigenic or to etiological pathway-wide networks, the consideration of whether there is a causal link between a putative cancer-associated gene and tumor development might be extended to whole tumorigenic networks. The most recent example is the discovery of the *BRCA2*-interacting protein, EMSY, which binds to *BRCA2* and turns off expression of *BRCA2*, and the demonstration that amplification of EMSY is frequently seen in sporadic breast cancer and is associated with worse survival (33). This finding has therefore been considered to provide the missing link between *BRCA2* and sporadic breast cancer development. Similarly, the finding that the interaction of NHEJ with *BRCA1* is associated with both increased risk of breast cancer and precise end-joining capacity not only suggests a role of *BRCA1* in NHEJ, but it also provides essential support for tumorigenic contribution of NHEJ during breast cancer formation.

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