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Breast Cancer Risk Associated with Genotypic Polymorphism of the Nonhomologous End-Joining Genes: A Multigenic Study on Cancer Susceptibility[1]

Yi-Ping Fu, Jyh-Cherng Yu, Ting-Chih Cheng, M. Ann Lou, Giu-Cheng Hsu, Chia-Yun Wu, Sou-Tong Chen, Hurng-Sheng Wu, Pei-Ei Wu, and Chen-Yang Shen[2]

Institute of Biomedical Sciences [Y-P. F., T-C. C., C-Y. W., P-E. W., C-Y. S.] and Life Science Library [C-Y. S.], Academia Sinica, Taipei, 11529, Taiwan, Graduate Institute of Epidemiology, National Taiwan University, Taipei, Taiwan [Y-P. F., T-C. C., C-Y. W.]; Departments of Surgery [J-C. Y.] and Radiology [G-C. H.], Tri-Service General Hospital, Taipei, Taiwan, Department of Surgery, Cardinal Tien Hospital and Fu-Jen Catholic University, Taipei, Taiwan [M. A. L.]; Department of Surgery, Changhua Christian Hospital, Changhua, Taiwan [S-T. C.]; and Department of Surgery, Show Chwan Memorial Hospital, Changhua, Taiwan [H-S. W.]

ABSTRACT

The role of the familial breast cancer susceptibility genes, *BRCA1* and *BRCA2*, in the homologous recombination pathway for DNA double-strand break (DSB) repair suggests that the mechanisms involved in DNA DSB repair are of particular etiological importance during breast tumorigenesis. However, there is currently no evidence for an association between breast cancer and the other DSB repair pathway, the nonhomologous end-joining (NHEJ) pathway. It is possible that, because this DNA repair pathway is so crucial for mammalian cells to maintain genomic stability, any severe defects in it would result in serious outcomes, such as genomic instability and cell death, and block subsequent cell outgrowth and tumor formation. Thus, only subtle defects arising from low-penetrance alleles would escape lethality accumulating essential genetic changes and be associated with cancer formation, and the tumorigenic contribution of these alleles would become more obvious if individual putative high-risk genotypes of each NHEJ gene act jointly. Furthermore, this joint effect might be modified by specific environmental factors, and we hypothesized that estrogen exposure might be one such factor because estrogen is suggested to cause DNA DSBs, triggering breast tumorigenesis. Because single nucleotide polymorphisms (SNPs) are the most subtle genetic variation in the genome, to examine these hypotheses, we have genotyped 30 SNPs in all five NHEJ genes (*Ku70, Ku80, DNA-PKcs, Ligase IV,* and *XRCC4*) in 254 primary breast cancer patients and 379 healthy controls. Support for these hypotheses came from the observations that (*a*) two SNPs in *Ku70* and *XRCC4* were associated with breast cancer risk ($P < 0.05$); (*b*) a trend toward increased risk of developing breast cancer was found in women harboring a greater number of putative high-risk genotypes of NHEJ genes (an adjusted odds ratio of 1.46 for having one additional putative high-risk genotype; 95% confidence interval, 1.19–1.80); (*c*) this association between risk and the number of putative high-risk genotypes was stronger and more significant in women thought to be more susceptible to estrogen, *i.e.*, those with no history of full-term pregnancy; and (*d*) the protective effect conferred by a history of full-term pregnancy was only significant in women with a lower number of putative high-risk genotypes of NHEJ genes. Based on comprehensive NHEJ gene profiles, this study provides new insights to suggest the role of the NHEJ pathway in breast cancer development and supports the possibility that breast cancer is initiated by estrogen exposure, which causes DNA DSBs.

INTRODUCTION

Cancer is believed to result from a series of genetic alterations leading to progressive disorder of the normal mechanisms controlling growth, differentiation, cell death, or genomic instability. The response of the cell to genetic injury and its ability to maintain genomic stability by means of a variety of DNA repair mechanisms are therefore essential in preventing tumor initiation and progression. Familial cancer syndromes, including xeroderma pigmentosum and hereditary nonpolyposis colorectal cancer, which are, respectively, causally linked to defective nucleotide excision repair and mismatch repair (1), emphasize the importance of DNA repair mechanisms during tumorigenesis. The fact that the family breast cancer susceptibility genes, *BRCA1* and *BRCA2*, are involved in the homologous recombination pathway for DNA DSB[3] repair (2) supports the idea that breast cancer pathogenesis is driven by DSB-initiated chromosome instability (3), and the mechanisms involved in DNA DSB repair are of particular etiological importance during breast tumorigenesis. However, it is intriguing that there is currently no evidence for the involvement in breast cancer of the other DSB repair pathway in mammalian cells, the NHEJ pathway (4–6). Recently, germ-line mutations in *Ligase IV*, one of the genes involved in NHEJ, have been identified in patients presenting with a novel syndrome, NBS-like syndrome, which resembles A-T and NBS and is characterized by developmental delay and immunodeficiency (7). An important characteristic of both A-T and NBS is the elevated incidence of cancer (1), and *ATM* (the gene responsible for A-T) and *NBS1* (the gene responsible for NBS) play critical roles in maintaining a normal checkpoint response to DSB (4, 5, 8). However, *Ligase IV*-mutated patients do not develop cancer (7). 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 patients, would result in genomic instability and trigger cell death by cell cycle checkpoint surveillance. 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 checkpoint surveillance and accumulate the unrepaired DNA damage required for tumor formation. The first aim of this study was therefore to investigate this possibility. Because SNP is the most frequent and most subtle genetic variation in the human genome and has great potential for application to association studies of complex diseases (9), we used SNPs in the NHEJ genes *Ku70*, *Ku80*, *DNA-PKcs*, *XRCC4*, and *Ligase IV* to define their tumorigenic contribution to breast cancer development.

Phenotypic variation is often seen in many apparently simple single-gene disorders, and some of these differences occur in subjects with the same mutation, indicating the presence of modifying factors. Such modification is expected to be relatively stronger in the case of an effect contributed by low-penetrance genes (alleles), and the modifying factors are probably both genetic and environmental. For example, the greatest incremental lung cancer risk (7-fold) for the

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[2] To whom requests for reprints should be addressed, at Institute of Biomedical Sciences, Academia Sinica, Taipei, 11529, Taiwan. Fax: 886-2-2782-3047; E-mail: bmcys@ccvax.sinica.edu.tw.

[3] The abbreviations used are: DSB, double-strand break; aOR, adjusted odds ratio; BMI, body mass index; CI, confidence interval; LD, linkage disequilibrium; NHEJ, nonhomologous end-joining; SNP, single nucleotide polymorphism; NBS, Nijmegen breakage syndrome; A-T, ataxia telangiectasia.

high-risk genotype of *CYP1A1* (the phase I gene involved in the carcinogen detoxification pathway) is seen in light smokers because heavy smokers with this genotype have less than twice the risk of heavy smokers without this genotype (10). On the other hand, the greater cancer risk associated with the high-risk *CYP1A1* genotype is particularly significant in the subgroup of subjects harboring the low-activity (high-risk) genotype of *GSTM1* (the phase II gene involved in the carcinogen detoxification pathway; Ref. 11), suggesting that the risk might also be modified by the status of other functionally linked genes. More importantly, as our understanding of cancer development is extended from single-gene disorders to multigenic disorders and etiological pathway-wide abnormalities, genes involved in the same mechanistic pathway are expected to modify the tumorigenic effects contributed by the other partners, and thus it becomes important to determine whether the joint effects of several defective genes in a common antitumorigenic pathway are associated with an increased cancer risk (second study aim). We were especially interested in knowing whether the association between the joint effect of NHEJ genes and breast cancer was modified by reproductive risk factors reflecting susceptibility to estrogen exposure (third study aim). The rationale underlying this hypothesis is that estrogen metabolites may initiate breast tumorigenesis by causing various forms of oxidative DNA damage, including DSB formation (12, 13). Because NHEJ is one of the major mechanisms involved in DSB repair, elucidating an interaction between NHEJ genotypes and reproductive risk factor on cancer risk might yield valuable clues on the association of breast tumorigenesis with estrogen.

MATERIALS AND METHODS

Study Population. This case-control study is part of an ongoing cooperative study aimed at understanding the causes of breast cancer in Taiwan (3, 14–18), which is characterized by low incidence (14), early tumor onset (15), hormone dependency (16), and novel genomic alterations (17, 18). Two hundred and fifty-four female breast cancer patients and 379 healthy female controls, all of whom gave their informed consent, were enrolled. All breast cancer patients had pathologically confirmed primary infiltrating ductal carcinoma of the breast, and all were diagnosed and treated at the Tri-Service General Hospital or the Cardinal Tien Hospital between September 2001 and March 2002; these patients accounted for almost all (>90%) women with breast cancer attending our breast cancer clinics during the study period; the remaining patients were excluded because of a lack of adequate blood specimens. No significant differences in breast cancer risk factors were found between the included and excluded women. More importantly, because the breast cancer clinics taking part in this study are two of the major breast cancer clinics in northern Taiwan, our patients accounted for a significant proportion (about 40%) of all breast cancer cases diagnosed during the study period in northern Taiwan.

To avoid any differential recall bias of previous disease history, we purposely randomly selected the controls from the health examination clinic of the same hospital during the same study period. These controls accounted for about 75% of all women attending the clinic, and no significant differences were found in terms of 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, any suspicious precancerous lesions of the breast, or other cancers.

Questionnaire. Two experienced research nurses were assigned to administer a structured questionnaire to both case and control subjects. The information collected included age at diagnosis, family history of breast cancer (first-degree relatives), history of breast biopsy, history of breast screening, age at menarche and/or menopause, parity, age at first full-term pregnancy, number of pregnancies, history of breast feeding, use of oral contraceptives, hormone replacement therapy, history of drinking alcohol and smoking cigarettes, ethnic background, residence area, family income, and education level. The BMI and menopausal status were also recorded. Women younger than 55 years who had undergone hysterectomy but not bilateral oophorectomy were classified as unknown in terms of menopausal status.

SNP Selection and SNP Genotyping. SNP information was obtained from the following five databases: (*a*) the SNP Consortium;[4] (*b*) National Center for Biotechnology Information;[5] (*c*) GeneSNPs Public Internet Resource;[6] (*d*) GeneCards;[7] and (*e*) Japan SNP Database.[8] We selected six SNPs for each NHEJ gene, and a total of 30 SNPs were genotyped. Because there have not been any reports of an association between genotypic and phenotypic changes in the SNPs of the NHEJ genes, these selected SNPs were used as markers to reflect possible LD between themselves and different alleles of the gene of undefined phenotypic variation. We used more than one SNP per gene to have an unbiased definition of the allelic and haplotypic statuses of each gene.

All SNPs were genotyped using a MassARRAY (SEQUENOM, Inc., San Diego, CA). The PCR primers and extension primers for all SNPs were designed using Spectro-Designer software (SEQUENOM, Inc.). To ensure that the observed polymorphisms were specific and not the results of experimental variation, the results were confirmed by repeating 25% of the assays and by directly sequencing 10% of the specimens.

Statistical Analysis. The following sequential statistical analyses were used. (*a*) Univariate and multivariate analyses were used to determine risk factors and to establish background risk profiles for breast cancer in this series of study subjects. Significant reproductive risk factors would serve as important indices to estimate the estrogen exposure level or susceptibility to estrogen exposure in the later analysis. (*b*) Genotypic frequencies of each SNP of the individual NHEJ genes were compared between cases and controls. The hypothesis of differences in frequencies of low-penetrance alleles between cases and controls (first study aim) was first tested using multivariate logistic regression models with simultaneous consideration of known risk factors of breast cancer, and adjusted *P*s for the association were estimated. (*c*) A joint contribution of individual NHEJ genes to increased breast cancer risk (second study aim) was explored in several ways. We performed a conventional logistic regression (19), a test evaluating whether a trend toward an increase in the number of putative high-risk genotypes in all NHEJ genes with increasing breast cancer risk (measured by the β estimates from this regression model) was statistically significant. Furthermore, because, in NHEJ, a DSB is first recognized by the end-binding DNA-PK complex (consisting of Ku70, Ku80, and DNA-PKcs), which subsequently recruits the ligase IV/XRCC4 complex, thereby enhancing its ligation activity in DSB rejoining (20, 21), we investigated separately the joint effect (reflected by the number of putative high-risk genotypes) of the genes participating in these two different steps of NHEJ, *i.e.*, we separately looked for joint effect of *Ku70*, *Ku80*, and *DNA-PKcs* and for the joint effect of *Ligase IV* and *XRCC4*. (*d*) Because we were especially interested in the relationship between the joint effect of NHEJ genes and breast cancer risk within categories of risk factors representing different levels of estrogen exposure or different degrees of susceptibility to estrogen exposure (third study aim), we calculated the risk of breast cancer associated with the combination of the number of putative high-risk genotypes of NHEJ genes and a reproductive risk factor. Using β estimates from the logistic regression model, in which we used a set of dummy variables (22, 23) representing different combinations of genes (*i.e.*, the number of putative high-risk genotypes) and risk factors, we assessed the relative excess risk from harboring different numbers of putative high-risk genotype within risk factor strata (22, 24). (*e*) If estrogen initiates breast tumorigenesis by causing DSB formation, then the relationship between breast cancer risk and reproductive risk factors would not be the same in women harboring different NEHJ genotypes; this was evaluated by calculating the risk (aOR) of breast cancer associated with reproductive risk factors in women with a higher or a lower number of NHEJ susceptibility (putative high-risk) genotypes.

RESULTS

The risk profile of this series of study subjects was similar to that reported in our previous studies (14, 16), and, using multivariate

[4] http://snp.cshl.org/.
[5] http://www.ncbi.nlm.nih.gov/SNP/index.html.
[6] http://www.genome.utah.edu/genesnps/.
[7] http://bioinformatics.weizmann.ac.il/cards/.
[8] http://snp.ims.u-tokyo.ac.jp/.

logistic regression analysis, an increased but not significant risk (multivariate aOR, 1.3; 95% CI, 0.8–2.1) was found to be conferred by a family history of breast cancer in female first-degree relatives. In terms of reproductive risk factors, compared with controls, cases were younger at menarche (≦13 years *versus* >13 years, aOR, 1.1; 95% CI, 0.8–1.5) and had a lower frequency of a history of full-term pregnancy (no history *versus* having at least one full-term pregnancy, aOR, 2.5; 95% CI, 1.5–4.2). Significant protection was conferred by a greater number of full-term pregnancies (history of ≥3 full-term pregnancies *versus* no history of full-term pregnancy, aOR, 0.5; 95% CI, 0.3–0.8). No association was found between cancer risk and smoking status, radiation exposure, hormone replacement therapy, or dietary intake of specific kinds of foods or vegetables, but obese women (women with a BMI > 24 kg/m^2) showed a significantly higher risk (aOR, 2.3; 95% CI, 1.3–4.1).

Thirty SNPs of five NHEJ genes described in SNP databases were genotyped for initial screening in 192 cases and 192 controls. Of these, 16 were not observed, and 2 were infrequent (frequency of the less frequent allele < 0.01), so these 18 SNPs were not genotyped in the rest of the samples. The remaining 12 SNPs (3 for *Ku70*, 2 for *Ku80*, 1 for *DNA-PKcs*, 3 for *Ligase IV*, and 3 for *XRCC4*) were genotyped in all cases and controls. All 12 SNPs were in Hardy-Weinberg equilibrium in the controls. To explore a possible association between breast cancer and individual SNPs of the NHEJ genes, the heterozygous and homozygous variant genotypes were grouped together and compared with the homozygous wild-type genotype. Because of the small percentage of individuals among our subjects with the homozygous variant genotype (<5%) for some genes (*e.g., Ku80*), this grouping resulted in increased statistical power in detecting the main effect (an association between NHEJ genes and breast cancer risk). When the genotype distributions of the 12 SNPs were compared between cases and controls and the effects of breast cancer risk factors were simultaneously adjusted in the multivariate logistic regression models, two SNPs, one in *Ku70* and the other in *XRCC4*, were found to show statistically significant differences (Table 1). Because all SNPs in the same gene were in strong LD ($P < 0.01$, examined by the EH program available online[9]) in both cases and controls and because the frequency distribution based on haplotypes was similar to that based on individual SNPs, we chose one SNP, which showed the most significant *P*s in the multivariate logistic regression analyses, to represent the allelic status for individual NHEJ genes. Furthermore, in the subsequent analyses, to look for an association between breast cancer risk and genotypic polymorphism of NHEJ genes, we defined the susceptibility (high-risk) genotypes on the basis of the findings of the present study. This definition is genetically reasonable because possible LD between these SNPs and functionally significant alleles is expected to differ in populations. Thus, the heterozygous and homozygous variants of *Ku80(G69506A)* or *DNA-PKcs(C55966T)* and the homozygous wild-type of *Ku70(C-61G)*, *Ligase IV(C4062T)*, or *XRCC4(T1394G)* were considered as putative high-risk (susceptibility) genotypes.

Because Ku70, Ku80, and DNA-PKcs are known to form a trimeric complex (DNA-PK) with a DSB end-binding function to prevent degradation of DSB, thus facilitating subsequent DSB ligation during NHEJ (20, 25), the prediction was that any defect due to missense variants in individual genes of this complex would act by dominantly interfering with the function of the normal allele of the other partner. Thus, to determine whether a joint effect of these DSB end-binding genes may be associated with breast cancer development, we examined the breast cancer risk associated with the number of these putative high-risk genotypes, using women with all three putative low-risk genotypes as the reference group. As shown in Table 2, the risk of breast cancer increased significantly with the number of putative high-risk genotypes ($P = 0.01$). Furthermore, on the basis that the heterodimer of Ku70 and Ku80 first binds to the ends of a DSB and then recruits DNA-PKcs, activating its kinase activity to phosphorylate target proteins that then trim the DSB ends, making them ligatable (20, 25), we then dissected the pathway into its two components and looked for a possible interaction between Ku (composed of Ku70 and Ku80) and the catalytic subunit (DNA-PKcs). We generated a two (disease status, *i.e.*, case and control)-by-four (genotype status of both *DNA-PKcs* and *Ku*, reflected by *Ku70* and *Ku80*) table (24), in which women with putative low-risk genotypes of both *DNA-PKcs* and *Ku* (both *Ku70* and *Ku80* being putative low-risk

Table 1 *Genotype frequencies of sequence variants of the NHEJ genes, Ku70, Ku80, DNA-PKcs, Ligase IV, and XRCC4, in breast cancer cases and controls and the adjusted P value in relation to breast cancer risk*

SNP and genotype[a]	No. of cases (%)	No. of controls (%)	P^b
Ku70, C-61G (rs2267437)			
CC	192 (75.6)	261 (68.9)	0.03[c]
CG	55 (21.7)	106 (27.9)	
GG	7 (2.7)	12 (3.2)	
Ku70, G43009T, (Gly593Gly) (rs132788)			
GG	148 (58.3)	191 (50.4)	N.S.
GT	85 (33.4)	161 (42.5)	
TT	21 (8.3)	27 (7.1)	
Ku70, A46922G (rs132793)			
GG	229 (90.2)	329 (87.0)	N.S.
GA	23 (9.0)	47 (12.5)	
AA	2 (0.8)	2 (0.5)	
Ku80, G69506A (rs3835)			
GG	210 (82.7)	333 (87.9)	N.S.
GA	42 (16.5)	45 (11.9)	
AA	2 (0.8)	1 (0.2)	
Ku80, G69632A (rs3834)			
GG	213 (83.9)	330 (88.0)	N.S.
GA	39 (15.3)	44 (11.7)	
AA	2 (0.8)	1 (0.3)	
DNA-PKcs, C55966T (rs2231178)			
CC	200 (78.7)	300 (39.6)	N.S.
CT	54 (21.3)	73 (19.4)	
TT	0 (0.0)	4 (1.0)	
Ligase IV, A6008G (Ile591Val) (rs2232641)			
AA	246 (96.8)	365 (97.6)	N.S.
AG	8 (3.2)	8 (2.1)	
GG	0 (0.0)	1 (0.3)	
Ligase IV, C4062T (rs1805388)			
CC	137 (54.2)	198 (52.7)	N.S.
CT	100 (39.5)	150 (39.9)	
TT	16 (6.3)	28 (7.4)	
Ligase IV, C4044T (rs1805389)			
CC	192 (75.6)	284 (74.9)	N.S.
CT	56 (22.0)	87 (23.0)	
TT	6 (2.4)	8 (2.1)	
XRCC4, A245G (Gln82Gln) (rs1805377)			
GG	135 (53.8)	196 (51.7)	N.S.
GA	102 (40.6)	159 (42.0)	
AA	14 (5.6)	24 (6.3)	
XRCC4, T1394G (rs2075685)			
TT	207 (81.5)	272 (72.0)	0.02[d]
GT	44 (17.3)	97 (25.6)	
GG	3 (1.2)	9 (2.4)	
XRCC4, C1475T (rs2075686)			
CC	103 (40.6)	168 (44.3)	N.S.
CT	110 (43.3)	172 (45.4)	
TT	41 (16.1)	39 (10.3)	

[a] The numbers represent the position (measured in bp) from the transcription site, and the letters represent nucleotide changes. If the SNPs are in exons, the position of the amino acid is also given. The number shown in parentheses is the NCBI SNP cluster ID of each SNP.

[b] Multivariate *P* were estimated in logistic regression models containing breast cancer risk factors, including age, family history of breast cancer, history of full-term pregnancy, and BMI. In these regression models, heterozygous and homozygous variants were grouped together and compared to the homozygous wild-type. N.S., not significant.

[c] Unadjusted $P = 0.06$ estimated by χ^2 test.

[d] Unadjusted $P = 0.01$ estimated by χ^2 test.

[9] http://linkage.rockefeller/edu/soft.

Table 2 *aOR of breast cancer development associated with the number of putative high-risk genotypes of the genes involved in DNA DSB NHEJ pathway*

No. of high-risk genotypes	No. of cases (%)	No. of controls (%)	aOR (95% CI)[a]
DSB end-binding complex (*Ku70*, *Ku80*, and *DNA-PKcs*)			
None	39 (15.4)	89 (23.6)	1.00 (Ref.)[b]
One	145 (57.0)	202 (53.6)	1.76 (1.10–2.82)
Two	65 (25.6)	78 (20.7)	2.14 (1.24–3.67)
Three	5 (2.0)	8 (2.1)	2.17 (0.69–7.86)
With one additional putative high-risk genotype			1.35 (1.07–1.71) *P* for trend = 0.01
DSB end-ligation complex (*Ligase IV* and *XRCC4*)			
None	46 (18.1)	104 (27.8)	1.00 (Ref.)[b]
One	201 (79.1)	263 (70.3)	1.64 (1.07–2.49)
Two	7 (2.8)	7 (1.9)	2.42 (0.78–7.52)
With one additional putative high-risk genotype			1.61 (1.10–2.36) *P* for trend = 0.01
NHEJ (*Ku70*, *Ku80*, *DNA-PKcs*, *Ligase IV*, and *XRCC4*)			
With one additional putative high-risk genotype			1.46 (1.19–1.80) *P* for trend = 0.0003

[a] The aOR of breast cancer development associated with the number of putative high-risk genotypes was estimated in a multivariate logistic regression model containing age, family history of breast cancer, history of full-term pregnancy, BMI, and either (*a*) a group of dummy variables to represent women harboring different numbers of putative high-risk genotypes (to specifically calculate the aOR of individual groups) or (*b*) the number of putative high-risk genotypes (to calculate the risk associated with having one additional putative genotype and the *P* for the trend).

[b] Ref., reference group.

genotypes) served as the reference group. When arranged in this way, the data showed the aOR for the putative high-risk genotype of *Ku* or putative high-risk genotype of *DNA-PKcs* alone to be 1.82 and 1.41, respectively, whereas the aOR for both together was 2.11 (95% CI, 1.18–3.78), a result consistent with a joint effect of two DNA-PK subunits on breast cancer risk. It should be noted that although *DNA-PKcs* genotypic polymorphism was not significantly associated with breast cancer in the single gene analysis, the joint effect analysis suggests that it acts in association with *Ku* to increase the risk of breast cancer. In the downstream subpathway of NHEJ, XRCC4 and Ligase IV are known to form a complex and are functionally linked with each other, with Ligase IV being responsible for DSB end rejoining and XRCC4 enhancing this activity (21). Our epidemiological observation that the presence of a greater number of putative high-risk genotypes of these two genes was significantly associated with an increased cancer risk (Table 2) is in line with the possibility that a joint effect of individual genes in this subpathway is related to breast cancer development. Finally, when the NHEJ was considered as a whole, the observed association was, as expected, consistent with those demonstrated in the two upstream/downstream subpathways, with an additional putative high-risk NHEJ genotype associated with a 1.46-fold increase in risk (95% CI, 1.19–1.80; Table 2).

This significant association between a joint effect of individual NHEJ genes and breast cancer prompted us to explore interactions between NHEJ genotypes and the established significant risk factor for breast cancer, namely, no history of full-term pregnancy, and to examine an initiating role of estrogen exposure, via DSB formation, because this risk factor is known to be an indicator of increased susceptibility to estrogen exposure. Full-term pregnancy is protective against breast cancer development, possibly because it results in permanent differentiation of the vulnerable breast stem cells, altering subsequent susceptibility to estrogen (26, 27). To carry out this analysis, we first classified our women into two groups: those with >1 and those with ≤ 1 putative high-risk genotype in the five NHEJ genes, because such a definition would give sufficient statistical power to address relevant questions. The reference group consisted of women harboring ≤ 1 putative high-risk genotype and having a history of full-term pregnancy. In the absence of the reproductive risk factor, the harboring of a higher number (>1) of putative high-risk genotypes of the NHEJ genes was associated with a significant but modest increase in risk. However, in the presence of the reproductive risk factor, the harboring of a >1 putative high-risk genotype of NHEJ genes was associated with a much greater combined risk of breast cancer (Table 3). These results indicate the presence of an interaction between the NHEJ pathway and women's susceptibility to estrogen exposure during breast tumorigenesis.

In the above analyses, we summed the number of putative high-risk genotypes for all five NHEJ genes, and a greater combined effect was seen with a higher number of putative high-risk genotypes. We next used a more conservative definition of the joint effect, only considering the contribution of genotypic polymorphisms of the two genes, *Ku70* and *XRCC4*, that showed a significant association with breast cancer risk in the single gene analysis, and we found that the results using this conservative definition were totally consistent with those based on the sum for all five genes. Harboring an additional putative high-risk genotype of *Ku70* or *XRCC4* was significantly associated with a 1.5-fold increase in risk (95% CI, 1.07–2.24). Using women with no putative high-risk genotypes of *Ku70* and *XRCC4* and with a history of full-term pregnancy as the reference group, women with both risk factors (*i.e.*, ≥ 1 putative high-risk genotype and no history of full-term pregnancy) demonstrated a higher risk (aOR, 3.75; 95% CI, 1.85–7.63) than women with either only a higher number of high-risk genotypes (aOR, 1.54; 95% CI, 1.02–2.35) or only no history of full-term pregnancy (aOR, 2.14; 95% CI, 0.74–6.14). The hypothesis that a joint effect of two NHEJ genes and risk factors reflecting increased susceptibility to estrogen exposure was linked to breast cancer development was therefore confirmed by these findings based on this conservative definition.

Finally, we investigated the potential importance of a protective

Table 3 *Risk of breast cancer associated with the combination of the number of putative high-risk genotypes of NHEJ genes and a reproductive risk factor (history of full-term pregnancy)*

	aOR (95% CI)[a]	
	No. of high-risk genotypes of NHEJ genes	
Reproductive risk factor	$\leqq 1$	>1
Having a history of full-term pregnancy		
Yes	1.00 (Ref.)[b]	2.42 (1.61–3.65)
No	2.75 (0.92–8.26)	5.29 (2.67–10.49)

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

[b] Ref., reference group.

Table 4 *Decreased risk (aOR) of breast cancer development associated with a history of full-term pregnancy, stratified by the number of putative high-risk genotypes of NHEJ genes*

Pregnancy history	aOR (95% CI)[a]	
	≦1 high-risk genotype	>1 high-risk genotype
Having one additional full-term pregnancy	0.83 (0.71–0.98)	0.92 (0.83–1.03)
No. of full-term pregnancies		
0	1.00 (Ref.)[b]	1.00 (Ref.)[b]
1–2	0.45 (0.16–1.23)	0.65 (0.35–1.23)
≧3	0.27 (0.09–0.83)	0.53 (0.26–1.06)

[a] The aOR of breast cancer development associated with having one additional full-term pregnancy or the number of full-term pregnancies was calculated in a multivariate logistic regression model containing age, family history of breast cancer, BMI, and the number of full-term pregnancies or a set of dummy variables to represent groups with different numbers of full-term pregnancies.

[b] Ref., reference group.

effect of the number of full-term pregnancies in conjunction with putative low-risk genotypes of the five NHEJ genes. The aORs associated with an additional full-term pregnancy within strata of the number of putative high-risk NHEJ genotypes were estimated. Modification of the protective effect was supported by our findings, shown in Table 4, which demonstrate that a significant decrease in cancer risk associated with the number of full-term pregnancies was seen only in women with a lower number of putative high-risk genotypes. Furthermore, to confirm this result, we specifically estimated the risk associated with different numbers of full-term pregnancies in women with different high-risk NHEJ genotypes. Within the >1 putative high-risk genotype stratum, there was only a modest, nonsignificant decrease in risk of developing breast cancer with three or more full-term pregnancies. However, in women with a lower number (≦1) of putative high-risk genotypes of NHEJ genes, a significant decrease in breast cancer risk (about 4-fold; aOR, 0.27; 95% CI, 0.09−0.83) was seen with a history of three or more full-term pregnancies. The possibility of a difference in statistical power in the detection of cancer risk due to different sample sizes in the subsets of women can be excluded because there were actually fewer study subjects with ≦1 putative high-risk genotype of individual genes than with >1 putative high-risk genotype.

DISCUSSION

On the basis of a multigenic model, the present study has comprehensively examined the tumorigenic contribution to breast cancer development of critical genes participating in NHEJ, one of the two DSB repair pathways. Furthermore, to the best of our knowledge, this is the first study to address the issue of an interaction between DSB repair and estrogen-related risk factor in relation to breast cancer risk. Our study should permit a more precise evaluation of the risks associated with individual susceptibility genes and a better insight into breast tumorigenesis initiated by estrogen exposure and how this is modified by DNA repair capacity. The results, that the SNPs of two NHEJ genes, *Ku70* and *XRCC4*, are found to be associated with increased breast cancer risk, support our hypothesis that certain high-penetrance genes, originally thought to confer an extremely high risk for hereditary diseases, could play an etiological role via the effect of low-penetrance alleles. Distinct from high-penetrance alleles, these polymorphic alleles of NHEJ genes would *predispose* carriers to a higher risk of developing cancer but would not necessarily *cause* cancer. More importantly, our results suggest that the possibility of manifesting the tumorigenic phenotype depends not only on the joint effect of individual NHEJ genes but also on the interaction between genotypic polymorphisms of NHEJ genes and a reproductive risk factor, possibility reflecting susceptibility to estrogen exposure. These results are consistent with the finding that established risk factors for breast cancer, such as reproductive history, might influence cancer risk in women with *BRCA1* or *BRCA2* mutation (28). They explain why the increased cancer risk associated with variant alleles varies in different populations. For example, there are discrepancies between our results and those of a recent case-control study (29), based on a large Caucasian group, which examined the contribution of 13 DSB repair genes predisposing to breast cancer but only found evidence for a significant relationship between cancer risk and a SNP in *Ligase IV*. Because Asian women have, on average, 20% lower serum estradiol levels than Western women (30), the demonstration of ethnicity-specific effects of genetic polymorphisms of DSB repair (NHEJ) genes on breast cancer risk in Taiwanese women is not surprising and might yield unique and valuable clues about the association of breast tumorigenesis with DNA repair and estrogen.

NHEJ mutant mice exhibit a relatively long latency (*Ku70*, *Ku80*, and *DNA-PKcs* knockout mice) or even absence (*Ligase IV* and *XRCC4* knockout mice) of tumorigenesis, which can probably be explained by highly efficient apoptosis because inhibition of apoptosis by a *p53* mutation, in addition to the NHEJ gene mutation, results in rapid tumor development (4, 6). The importance of these findings is that escaping checkpoint surveillance is a critical element in the pathogenesis of cancer resulting from defective DNA repair mechanisms (Fig. 1*A*), and it is probable that only mild phenotypic defects, such as slightly increased genomic instability resulting from suboptimal repair capacity associated with SNPs of repair genes, could meet this "hide-then-hit" requirement (Fig. 1*B*). Our results showing an association between breast cancer risk and SNPs of NHEJ genes (including the genes *Ligase IV* and *XRCC4*, which do not show malignant phenotypes in knockout mice) lend support to this possibility. On the other hand, our demonstration of breast tumorigenic

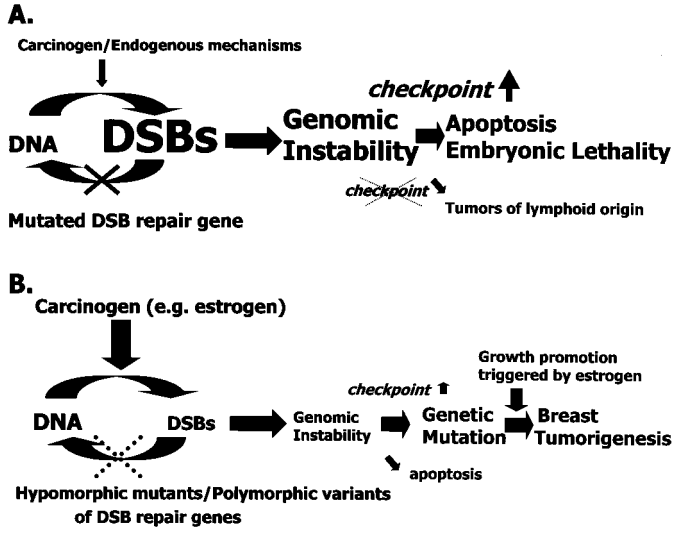

Fig. 1. Hypothesized model of tumorigenic effect contributed by NHEJ genes. *A*, because DNA DSB repair pathways, including NHEJ, are crucial for cells to maintain genetic stability, any severe defects (null mutants) in these genes would result in high levels of genomic instability and subsequently lead to cell death triggered by cell cycle checkpoint (*e.g., p53*) surveillance. Only in the case of checkpoint inactivation do tumors of lymphoid origin have the chance to develop because these cells depend on proper DSB repair to differentiate and thus are particularly susceptible to impaired DSB repair. *B*, for these high-penetrant DSB repair genes, only mild defects (low levels of DSBs) resulting from suboptimal repair capacity associated with low-penetrance (risk) alleles (*e.g.*, hypomorphic mutant or polymorphic variant) would escape checkpoint surveillance and accumulate the unrepaired DNA damage (genetic mutation) required for tumor formation. The tumorigenic effect contributed by low-penetrance genes (alleles) would become stronger in the case of a modification, and the modifying factors are probably both genetic (*e.g.*, multiple polymorphic variants of genes of the same repair pathway) and environmental (*e.g.*, exposure to DSB-causing agents, such as estrogen, or cell outgrowth in breast triggered by estrogen).

contribution of low-penetrance alleles of NHEJ genes is also consistent with the suggestion that apparently disparate spectrum of malignancies can be differently caused by the mutated form or by hypomorphic/polymorphic variants of the same genes (31). During B- and T-cell differentiation, the genes that encode immunoglobulins and T-cell receptors have to be assembled into active genes by V(D)J recombination, which proceeds through a DSB intermediate and requires NHEJ proteins for completion (6). Accordingly, it appears mechanistically reasonable that B-cell or T-cell tumors are the dominant malignant phenotypes observed in NHEJ gene knockout mice bearing a *p53* mutation (4, 6). In contrast, possible genomic defects resulting from low-penetrance NHEJ variants are expected to be minor and may not be great enough to initiate tumors at primary sites [*i.e.*, tumors of lymphoid origin (a hypothesized model is shown in Fig. 1)]. Consequently, cancers would develop in other tissues but would require a long period of time to accumulate essential genetic defects, and tumorigenesis would be prompted by selective exogenous or endogenous environmental factors (32). The results of the present study lead us to suspect that increased exposure of breast epithelium to estrogen may be one such factor, allowing breast cells with a suboptimal NHEJ capacity to accumulate sufficient DSBs in cancer-causing genes and consequently to display a growth advantage, leading to tumors.

This study, which shows that the joint effect of individual SNPs of NHEJ genes, measured by the number of putative high-risk genotypes, is significantly associated with breast cancer risk, suggests that NHEJ genes may *act together*, leading to breast cancer development. Recently, evidence has emerged for the cooperative involvement of different genes in disease etiology and cancer development. The combination of heterozygous abnormalities in different but functionally related genes is known to play a causal role in the pathogenesis of certain genetic syndromes (33). Evidence for an increased risk of cancer due to a combined effect of genes belonging to a common antitumor pathway has been provided in a mouse model (34). At the cellular level, the amount of DNA damage present in lymphoblastoid cell lines of healthy people not exposed to a carcinogen is directly related to the number of variant alleles of the genes involved in the base excision repair pathway (35). Furthermore, support for a joint carcinogenic effect comes from observational studies. For instance, there is a trend toward increased risk of developing breast cancer in women harboring a greater number of putative high-risk genotypes of major estrogen-metabolizing genes (16). Interestingly, Pharoah *et al.* (36) recently developed a model indicating that familial breast cancers that cannot been explained by *BRCA1* or *BRCA2* are caused by the combined effect of a large number of codominant alleles, each of which is associated with a small increase in risk.

In addition to *in vitro* experiments (12, 13), many studies on breast cancer patients that have focused mainly on different steps of estrogen metabolism have demonstrated the potential of estrogen to cause DNA damage. Inherited variants in genes involved in the detoxification of mutagenic estrogen metabolites, including those coding for catechol-*O*-methyltransferase (16, 37), glutathione *S*-transferase (reviewed in Ref. 38), and uridine diphospho-glucuronosyltranserase (39), have been suggested to be associated with an increased risk of breast cancer. In addition, given that oxidation mediated by reactive oxygen species is thought to be the mechanism involved in the carcinogenic effect of estrogen, possible protective effects of the frequent intake of antioxidants (*e.g.*, vitamin C; Ref. 40) or a predisposition due to harboring high-activity antioxidant enzymes (*e.g.*, Manganese superoxide dismutase; Ref. 41) have been suggested. The present study tested this hypothesis using a different approach in which we explored whether breast tumorigenesis was linked to DNA DSB repair. The design, based on genotypic polymorphism, provides a reliable estimate of the lifetime influence of impaired DNA repair on the risk of developing breast cancer. In addition, such a design, based on the genetic background, is not subject to bias due to incorrect interpretation of the temporal sequence between defective repair and tumor initiation. Our findings that (*a*) the increased cancer risk associated with no history of pregnancy, supposedly resulting in increased susceptibility to estrogen exposure, was even more pronounced in women harboring a higher number of putative high-risk genotypes of NHEJ genes (Table 3) and (*b*) the protective effect conferred by full-term pregnancy varied according to the genotype status of the NHEJ genes (Table 4) support the notion that increased estrogen exposure confers a higher risk of breast cancer by generating DSB damage in DNA. These results shed light on our understanding of breast tumorigenesis because, although a link between common carcinogens, including cigarette smoke, and breast cancer has been suggested, nothing definite is known about the DNA-damaging agents causing breast cancer. Our findings certainly do not exclude the well-established mechanisms by which estrogen triggers cell proliferation and tumor promotion. Rather, the dual role of estrogen as both an initiator, causing DNA DSB damage, and a promoter, leading to cell proliferation, provides a more direct explanation for breast cancer development.

One of the most important issues in using SNPs in an association study is the interpretation of the association identified between SNPs and a given trait. The present study used a candidate gene approach, based on SNPs located in the genes of the NHEJ pathway. Because most of the SNPs analyzed in our multivariate models are in introns or do not affect amino acid coding and therefore probably do not affect protein function, the observed associations between breast cancer risk and SNPs should be interpreted as the presence of LD between these SNPs and other SNPs in exons, resulting in functional polymorphism. We have 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 is limited, due to strong LD between SNPs in the same gene. However, these SNPs, which probably have no functional effect, may be of particular methodological importance in addressing the tumorigenic contribution of NHEJ genes to breast cancer risk. Although NHEJ is essential for mammalian cells to repair DSB, it is intrinsically an error-prone mechanism (4, 5), which suggests that the balance and interaction between the activities of the two DSB repair pathways, NHEJ and homologous recombination (an error-free mechanism), may be as important as the individual pathways (42). Thus, the use of SNPs with no functional effects enabled us to examine putative associations without resorting to an *a priori* hypothesis proposing that a decreased NHEJ capacity is related to an increased risk of cancer.

To search for mutators responsible for the genomic instability leading to breast tumorigenesis, in our recent studies (3, 17, 18), we have attempted to identify specific molecular mechanisms whose functional aberrations are related to advanced pathological/clinical manifestations. This approach is supported by the fact that, instead of being a single-gene disease, cancer arises from aberrations in a complex, interconnecting network of multiple regulatory genes involved in normal growth control processes and the maintenance of genomic stability. Given the high frequency of chromosomal abnormalities and mutations found in human cancers, the hypothesis that cancer is caused by a mutator phenotype was proposed (43) and has been confirmed by germ-line mutation of defective DNA repair genes causally leading to the initiation of hereditary cancer syndromes (1). The present study followed the same hypothesis and demonstrated that the combination of genotypic polymorphisms of NHEJ genes and a reproductive risk factor is associated with breast cancer risk. This result not only supports the hypothesis of a carcinogenic role of

estrogen in causing DNA DSBs in breast cancer development but also demonstrates the possible tumorigenic contribution of low-penetrance alleles of genes participating in the DSB repair pathway. However, given the number of comparisons and the sample size of the present study, the conclusions should be interpreted with caution and confirmed by other studies. On the basis of a larger sample size, we are genotyping more SNPs for individual NHEJ genes, by which haplotype analysis would become possible. Furthermore, other important genes/pathways known to be involved in DSB repair, including the genes of DSB sensing (*ATM*, *RAD50*, *MRE11*, and *NBS1*) and homologous recombination [*RAD51*, *RAD52*, *hCHK2*, *BRCA1*, and *BRCA2* (4–6)], may also play a role and are currently under investigation.

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