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FAST TRACK

Breast Cancer Risk Associated With Genotype Polymorphism of the Catechol Estrogen-Metabolizing Genes: A Multigenic Study on Cancer Susceptibility

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Estrogen has been suggested to trigger breast cancer development via an initiating mechanism involving its metabolite, catechol estrogen (CE). To examine this hypothesis, we carried out a multigenic case-control study of 469 incident breast cancer patients and 740 healthy controls to define the role of important genes involved in the different metabolic steps that protect against the potentially harmful effects of CE metabolism. We studied the 3 genes involved in CE detoxification by conjugation reactions involving methylation (catechol-*O*-methyltransferase, *COMT*), sulfation (sulfotransferase 1A1, *SULT1A1*), or glucuronidation (UDP-glucuronosyltransferase 1A1, *UGT1A1*), one (manganese superoxide dismutase, *MnSOD*) involved in protection against reactive oxidative species-mediated oxidation during the conversion of CE-semiquinone (CE-SQ) to CE-quinone (CE-Q), and 2 of the glutathione *S*-transferase superfamily, *GSTM1* and *GSTT1*, involved in CE-Q metabolism. Support for this hypothesis came from the observations that (*i*) there was a trend toward an increased risk of breast cancer in women harboring a greater number of putative high-risk genotypes of these genes ($p < 0.05$); (*ii*) this association was stronger and more significant in those women who were more susceptible to estrogen [no history of pregnancy or older (≥26 years) at first full-term pregnancy (FFTP)]; and (*iii*) the risks associated with having one or more high-risk genotypes were not the same in women having experienced different menarche-to-FFTP intervals, being more significant in women having been exposed to estrogen for a longer period (≥12 years) before FFTP. Furthermore, because CE-Q can attack DNA, leading to the formation of double-strand breaks (DSB), we examined whether the relationship between cancer risk and the genotypic polymorphism of CE-metabolizing genes was modified by the genotypes of DSB repair genes, and found that a joint effect of CE-metabolizing genes and one of the two DSB repair pathways, the homologous recombination pathway, was significantly associated with breast cancer development. Based on comprehensive CE metabolizing gene profiles, our study provides support to the hypotheses that breast cancer can be initiated by estrogen exposure and that increased estrogen exposure confers a higher risk of breast cancer by causing DSB to DNA.

Key words: breast cancer; estrogen; catechol estrogen; polymorphism; molecular epidemiology

An increased risk of breast cancer due to prolonged exposure to estrogen has been well documented by epidemiological observations showing that estrogen-related risk factors, including age at menarche, age at menopause, parity and age at first full-term pregnancy (FFTP), are significantly associated with breast cancer risk.[1,2] Why estrogen exposure should increase breast cancer risk is an intriguing question that has not been conclusively answered. One simple explanation is that the proliferative effect of estrogen on breast epithelium promotes the growth of tumor cells, leading to progression of breast cancer.[3–5] For a tumor to form, however, the essential step of tumor initiation, during which the genes regulating cell growth, differentiation and death are damaged, must occur before tumor cell outgrowth. No single carcinogen has been identified conclusively as causing the DNA damage responsible for breast cancer initiation.[6] Estrogen has attracted considerable attention recently because that there is evidence that it can cause DNA damage and mutation.[6–8] In this initiating mechanism, a critical role is played by the oxidative metabolism of estrogen, which occurs in 2 stages, first to the 4-hydroxyestradiol form of catechol estrogen (CE), then, *via* the E2-3,4-semi-quinones (CE-SQ), to further oxidized metabolites, the E2-3,4-quinones (CE-Qs), because *in vitro* experiments have shown that CE-Qs can bind to DNA to form adducts, leading to DNA depurination, the major type of genetic damage implicated in mutation and strand break of DNA during tumorigenesis.[9,10] In addition, the generation of reactive oxidative species (ROS) during the conversion of CE-SQ to CE-Qs can also lead to oxidative DNA damage.[6,10] To examine whether these various steps in estrogen metabolism were of significance in breast tumorigenesis and to gain an insight into the initiating role of estrogen during breast tumorigenesis, we carried out a multigenic case-control study to define the role of important genes involved in the different metabolic steps that protect against the potentially harmful effects of CE metabolism, namely those that directly detoxify CE, thus avoiding the subsequent stages of CE metabolism, and those that protect against the effects of ROS generated during the conversion of CE-SQ to CE-Qs or detoxify CE-Qs. The genes examined were catechol-*O*-methyltransferase (*COMT*), sulfotransferase 1A1 (*SULT1A1*), and UDP-glucuronosyltransferase 1A1 (*UGT1A1*), involved in CE detoxification by, respectively, methylation, sulfation, or glucuronidation, manganese superoxide dismutase (*MnSOD*), involved in protection against ROS-mediated oxidation, and *GSTM1* and *GSTT1*, 2 of the glutathione *S*-transferase (GST) superfamily, involved in CE-Q inactivation.[11] Our aims were to determine whether genotypic polymorphism in these genes was associated with an increased risk of breast cancer, and whether the association between genotypes and risk was modified by estrogen exposure. Finally, because the genotypic polymorphism of the genes involved in DNA double-

Abbreviations: aOR, adjusted odds ratio; BMI, body mass index; CE, catechol estrogen; CE-Q, CE-quinone; CE-SQ, CE-semiquinone; CI, confidence interval; COMT, catechol-*O*-methyltransferase; DSB, double strand break; DSBR, double strand break repair; FFTP, first full-term pregnancy; GST, glutathione *S*-transferase; HR, homologous recombination; MnSOD, manganese superoxide dismutase; NHEJ, non-homologous end joining; ROS, reactive oxidative species; SNP, single nucleotide polymorphism; SULT1A1, sulfotransferase 1A1; UGT1A1, UDP-glucuronosyltransferase 1A1.

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strand break repair (DSBR) has already been defined in the cases and controls used in our present study,[12,13] we had the unique opportunity of using this information to explore a possible interaction between CE metabolism and DSBR. If estrogen were associated with breast cancer development via the hypothesized mechanism involving the formation of a CE-Q depurination adduct leading to DNA double-strand breaks (DSB), the relationship between cancer risk and CE metabolism would be expected to be modified by the genotypes of the DSBR genes.

Material 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,[12–19] that is characterized by low incidence,[14] early tumor onset,[15] hormone dependency[16] and novel genomic alterations.[17,18] This and all our previous studies had been approved by the ethics committee of the Institute of Biomedical Sciences, Academia Sinica. Because of the low incidence of breast cancer, which suggests an overall lower effect of common risk factors, and its homogenous genetic background, the Taiwanese population has certain advantages for studying the effects of subtle genetic variations,[20] such as genotypic polymorphisms. Using data from 254 female incident breast cancer patients and 379 healthy female controls recruited between September 2001 and March 2002, we reported recently an association between breast cancer risk and genotypic polymorphisms of the DSBR genes.[12] Since then, we have continued to enroll study subjects and the present study therefore includes 469 incident breast cancer patients and 740 healthy controls recruited up to October 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). Their ages ranged from 35–80 years. These patients accounted for almost all (>90%) 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 our study are 3 of the major breast cancer clinics in northern and central Taiwan, our patients accounted for a significant proportion of all breast cancer cases diagnosed during the study period in these regions, and no significance difference of demographic features was found between the cases included in our study and the remaining cases in the study region.

To avoid any differential recall bias of previous disease history, we purposely randomly selected the controls from the health examination clinics of the same hospitals during the same study period. These controls accounted for about 20% of all women attending the clinics, and no significant differences were found in socioeconomic status between those included and those not included. Their ages ranged from 33–83 years, being very similar to that of cases. The control subjects underwent a 1-day comprehensive health examination and the women showing any evidence of breast cancer, suspicious precancerous lesions of the breast, or other cancers were excluded.

Questionnaire

Three experienced research nurses were assigned to administer a structured questionnaire to both case and control subjects. The information collected included demographic characteristics (ethnic background, residence area, family income, and educational level), reproductive risk factors [age at menarche or menopause, age at first full-term pregnancy (FFTP), number of pregnancies, parity, history of breast feeding, and menopausal status], medical history [age at diagnosis of breast cancer, family history of breast cancer (first-degree relatives), history of breast biopsy, and history of breast screening], and exogenous hormone exposure [use of oral contraceptives and hormone replacement therapy (HRT)]. Body mass index (BMI) and a history of alcohol consumption or cigarette smoking 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.

Specimen and DNA preparation

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.

Genotypic polymorphism and genotyping

The polymorphisms of individual CE metabolizing genes selected for genotyping were chosen because previous genotype-phenotype association studies had documented a defective or suboptimal function related to a specific allele. These were: (*i*) *Val108Met* in *COMT* (resulting from a G-to-A transition); the *Met* allele encodes a heat-sensitive form of COMT and is defined as the slow activity allele[21]; (*ii*) *Arg213His* in *SULT1A1* (resulting from a G-to-A transition); the *His* allele was found to be associated with low thermal stability and low SULT activity in an *in vitro* study;[22] and (*iii*) the repeat number polymorphism in the A(TA)nTAA motif of the TATA box in the promoter region of *UGT1A1*; functional analyses of the transcriptional activity have shown that the transcription activation of this gene is correlated inversely with repeat number, and alleles with 7 or 8 repeats show low expression[23]; (*iv*) the T-to-C transition leading to replacement of Val with Ala in the mitochondrial targeting sequence of *MnSOD*; the resulting amino acid change is predicted to alter the secondary structure of the protein and may affect the cellular allocation and mitochondrial transport of this anti-oxidative enzyme[24]; (*v*) genetic deletion polymorphisms of *GSTM1* and *GSTT1*; the deleted alleles result in no enzyme activity.[25]

The single nucleotide polymorphisms (SNP) of *COMT* and *MnSOD* were genotyped by a MassARRAY system (SEQUENOM, Inc., San Diego, CA), based on the primer extension protocol.[26] The PCR primers and extension primers for these 2 SNP were designed using Spectro-Designer software (SEQUENOM, Inc.). A PCR-based RFLP assay[27] was used to determine the SNP of *SULT1A1*. To genotype deletion polymorphisms in *GSTM1* and *GSTT1*, a multiplex-PCR procedure was used, the primer sets being based on those described previously by Arand *et al.*[28] Our genotyping protocol for the promoter region containing the TA repeats polymorphism of *UGT1A1* was similar to that described previously,[23] using an ABI Prism 3100 DNA sequencer and GENESCAN3.1 and GENOTYPER 3.7 software. To ensure that the observed polymorphisms were specific and not the results of experimental variation, the results were confirmed by repeating 15% of the assays and by sequencing 10% of the specimens.

To examine possible interaction between CE-metabolizing genes and DSBR genes on breast cancer risk, we incorporated the SNP results we genotyped previously[12,13] into our present study. These include the SNP of individual genes involved in 2 DSBR pathways, *i.e.*, the non-homologous end-joining (NHEJ) pathway [*Ku70*(*G46922A*), *Ku80*(*G69506A*), *DNA-PKcs*(*C55966T*), *XRCC4*(*T1394G*) and *Ligase IV*(*C4044T*)] and the homologous recombination (HR) pathway [*ATM*(*C98125G*), *p53*(*Arg72Pro*), *RAD51*(*G135C*), *BRCA1*(*A3232G*), and *BRCA2*(*A1342C*)], which were all genotyped by the MassARRAY system.

Statistical analysis

The following statistical analyses were sequentially carried out. (*i*) 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 served as important indices to estimate the estrogen exposure level or susceptibility to estrogen exposure in the later anal-

ysis; (*ii*) the genotypic frequency of each polymorphism of the individual CE-metabolizing genes was compared between cases and controls. Differences in frequency and the association between susceptibility genotypes and breast cancer risk were tested using multivariate logistic regression models[29] with simultaneous consideration of known risk factors of breast cancer, and the adjusted odds ratio (aOR) and the corresponding 95% confidence interval (95% CI) for the association were estimated. In our present study, increased exposure to CE-Qs was hypothesized to contribute to an increased risk of breast cancer, and women harboring putative high-risk alleles [the *COMT Met* (slow activity) allele, the *SULT1A1 His* (low activity) allele, the low expression allele of *UGT1A1* (*i.e.*, the allele containing 7 or 8 TA repeats), the *MnSOD Ala* allele, and the null (no activity) alleles of *GSTM1* and *GSTT1*] were considered to be at higher risk of cancer. A backward elimination procedure[29] was used to select the optimal model. (*iii*) A joint contribution of individual CE-metabolizing genes to increased breast cancer risk was explored in several ways, based on known CE metabolic mechanisms.[11] Using a dummy variable coding scheme,[30] we estimated breast cancer risk (aOR) in women harboring different numbers of putative high-risk genotypes. The strength of this approach is that it is more mechanistically reasonable at the cellular level, because it is not dependent on the assumption that the difference in risk between women with the same difference in the number of high-risk genotypes will be the same, irrespective of the actual number of high-risk genotypes. Furthermore, we carried out a conventional logistic regression, a test evaluating whether a trend to an increase in the number of putative high-risk genotypes in all CE-metabolizing genes with increasing breast cancer risk (measured by the β estimates from this regression model) was statistically significant. We also investigated separately the joint effect (reflected by the number of putative high-risk genotypes) of genes involved in CE detoxification and the joint effect of genes involved in protection against ROS damage during the conversion of CE-SQ to CE-Qs and in CE-Qs/SQ inactivation. (*iv*) Of particular interest was the relationship between CE-metabolizing genes and breast cancer risk in women with different levels of estrogen exposure or with different degrees of estrogen susceptibility, which was examined using joint and stratified methods. In the joint method,[31,32] we calculated the risk of breast cancer associated with the combination of the number of putative high-risk genotypes of CE-metabolizing genes and a reproductive risk factor. Using β estimates from the logistic regression model, in which we used a set of dummy variables, representing different combinations of genes (*i.e.*, the number of putative high-risk genotypes) and risk factors, we assessed the risk associated with harboring different numbers of putative high-risk genotype within risk factor strata. In the stratified method, possible modification of risk associated with CE-metabolizing genes by estrogen exposure was evaluated by calculating the aOR of breast cancer in relation to the number of high-risk genotypes within different levels (categories) of estrogen-related risk factors. (*v*) If estrogen initiates breast tumorigenesis by causing DNA DSB, the relationship between breast cancer risk and CE-metabolizing genes would not be the same in women harboring different DSBR genotypes; this was evaluated by calculating the risk (aOR) of breast cancer associated with genotypic polymorphisms of CE-metabolizing genes in women with a higher or a lower number of DSBR susceptibility (putative high-risk) genotypes.

Results

The risk profile of this series of study subjects was similar to that reported in our previous studies.[12,13,16] Of the various reproductive risk factors, pregnancy-related risk factors were consistently found to be highly associated with an increased risk. Compared to controls, cases had a lower frequency of a history of full-term pregnancy (no history *vs.* having at least one full-term pregnancy, aOR = 1.54; 95%CI = 1.00–2.38) and were older at FFTP (>25 years *vs.* ≤25 years, aOR = 1.70; 95%CI = 1.28–2.26). The significant protection conferred by pregnancy against the development of breast cancer has been suggested to be due to its causing permanent differentiation of the vulnerable breast stem cells, thus reducing susceptibility to estrogen exposure.[33] We then estimated the effect of estrogen exposure during the critical period between menarche and FFTP and found that the risk was increased in those women who had been exposed to estrogen for a longer period before FFTP (≥12 years *vs.* <12 years, aOR = 1.41, 95% CI = 1.07–1.86). 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 (BMI ≥24 kg/m^2) showed a significantly higher risk (aOR = 1.57; 95% CI = 1.24–1.99).

To explore a possible association between breast cancer and individual polymorphisms in *COMT*, , *SULT1A1*, and *MnSOD*, the heterozygous and homozygous variant genotypes of these genes were grouped together and compared to the homozygous wild-type genotype. Due to the small percentage of subjects with the homozygous variant genotype (<10%) for some genes, this grouping provided increased statistical power for detecting the main effect (an association between genes and breast cancer risk). For *GSTM1* and *GSTT1*, the PCR assay used could only detect null (*i.e.*, the homozygous deletion variant) and non-null genotypes (homozygous wild-type and heterozygous wild-type), and, as in other studies,[34,35] we made no attempt to differentiate between the 2 genotypes in the non-null groups. When the genotypic distribution of polymorphisms of individual CE-metabolizing genes was compared between cases and controls and the effects of breast cancer risk factors simultaneously adjusted in the multivariate logistic regression model, with the exception of *SULT1A1*, all the high-risk genotypes showed an increase in risk (Table I), but only *COMT* polymorphism shows a significant association with breast cancer development. To comprehensively assess the relative contribution of these CE-metabolizing genes in the association with breast cancer development, we carried out logistic regression analysis considering the combined effects of individual genes, and, consistent with the findings in Table I, the high-risk *COMT* genotypes were found to be significantly associated with increased breast cancer risk (aOR = 1.30; 95%CI = 1.02–1.66). The putative high-risk *SULT1A1* genotypes were still negatively not correlated significantly with breast cancer (aOR = 0.92; 95% CI = 0.55–1.52), and this gene was therefore not included in the subsequent analysis.

Given that individual CE-metabolizing genes participate cooperatively in CE metabolism[11] and that an increased risk of cancer due to a combined effect of genes belonging to a common antitumor pathway has been demonstrated in a mouse model,[36] we examined whether a joint effect of these CE-metabolizing genes was associated with breast cancer development by determining the breast cancer risk associated with harboring different numbers of putative high-risk genotypes (Fig. 1). The grouping schemes used in this figure were based on basic statistical consideration, *i.e.*, the sample sizes of individual subgroups should be comparable. To exclude a false combination effect due to an unequal contribution of individual genes, using a dummy variable coding scheme and women with all putative low-risk genotypes as the reference group, we separately estimated the risk associated with harboring different numbers of putative high-risk genotypes. The results were consistent with the presence of a joint effect, a higher risk being observed in women harboring a higher number of high-risk genotypes (Fig. 1*A*). We then divided the CE-metabolizing pathway into the 2 pathways of (*i*) CE detoxification and (*ii*) anti-oxidation and CE-Q inactivation, and looked for a possible joint effect among genes within each pathway. The results showed a significant increase in cancer risk with a higher number of putative high-risk genotypes and were consistent with a joint effect of genes involved in either pathway (Fig. 1*b*,*c*). To address the possibility that the sample size was too small to assess the multiple gene-gene interactions, we used a more conservative definition of the joint effect, only considering the contribution of genotypic

TABLE I – DISTRIBUTION OF GENOTYPE POLYMORPHISMS OF THE CATECHOL ESTROGEN (CE)-METABOLIZING GENES AND THE ESTIMATED ODDS RATIO (OR) AND ADJUSTED ODDS RATIO (aOR) FOR BREAST CANCER

Genotype of CE-metabolizing gene	Cases (%)[1]	Controls (%)[1]	OR (95%CI)	aOR (95%CI)[2]
COMT				
Val/Val	237 (50.5)	420 (56.8)	1.00 (Ref.)[3]	1.00 (Ref.)
Val/Met	197 (42.0)	262 (35.4)	1.32 (1.03–1.67)	1.32 (1.04–1.68)
Met/Met	35 (7.5)	58 (7.8)		
SULT1A1				
Arg/Arg	439 (93.8)	693 (93.6)	1.00 (Ref.)	1.00 (Ref.)
Arg/His	27 (5.8)	47 (6.4)	0.81 (0.48–1.40)	0.83 (0.49–1.42)
His/His	2 (0.4)	0 (0.0)		
UGT1A1				
6/6	369 (78.8)	584 (78.9)	1.00 (Ref.)	1.00 (Ref.)
6/7	93 (19.9)	151 (20.4)	1.07 (0.80–1.44)	1.05 (0.78–1.41)
7/7	6 (1.3)	5 (0.7)		
MnSOD				
Val/Val	343 (73.1)	545 (73.7)	1.00 (Ref.)	1.00 (Ref.)
Val/Ala	115 (24.5)	183 (24.8)	1.04 (0.79–1.36)	1.06 (0.80–1.38)
Ala/Ala	11 (2.4)	11 (1.5)		
GSTT1				
Non-null	238 (51.6)	400 (54.3)	1.00 (Ref.)	1.00 (Ref.)
Null	223 (48.4)	336 (45.7)	1.13 (0.89–1.44)	1.13 (0.89–1.44)
GSTM1				
Non-null	231 (49.7)	371 (50.6)	1.00 (Ref.)	1.00 (Ref.)
Null	234 (50.3)	362 (49.4)	1.06 (0.82–1.37)	1.00 (0.78–1.26)

[1]Minor difference of sample size in individual comparisons was due to a lack of DNA specimens in study subjects.–[2]The adjusted odds ratios and 95% confidence intervals were calculated by logistic regression models containing age, a family history of breast cancer, pregnancy-related risk factor, and body mass index.–[3]Ref, reference group.

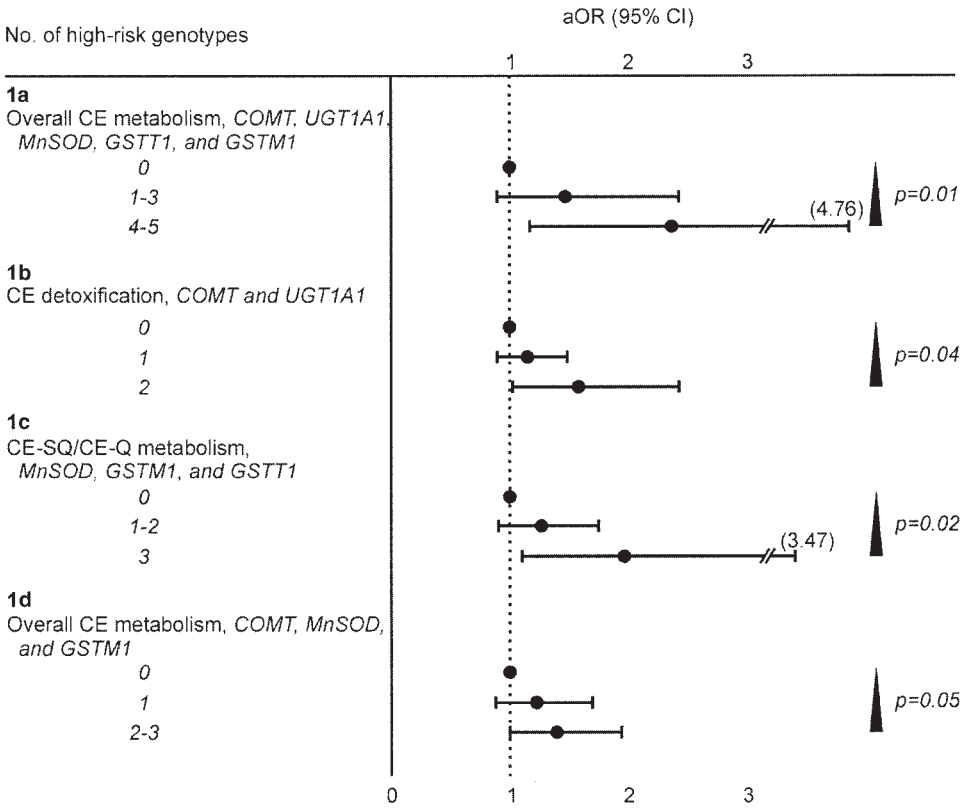

FIGURE 1 – Breast cancer risk (aOR and 95% CI) associated with the number of putative high-risk genotypes of catechol estrogen-metabolizing genes. The women harboring the same numbers of high-risk genotypes of individual genes in CE-metabolism, CE detoxification and CE-SQ/CE-Q metabolism were grouped. The risks were estimated using the women harboring no high-risk genotypes as the reference.

polymorphisms of three genes, *COMT*, *MnSOD*, and *GSTM1*, which individually represent protective effects due to CE detoxification, ROS scavenging, and CE-Qs inactivation, and found that the results using this conservative definition (Fig. 1*d*) were consistent with those based on all 5 genes.

If these susceptibility genes were associated with breast cancer development via the hypothesized mechanism involving estrogen metabolism, the relationship between cancer risk and susceptibility genotypes would be expected to be more significant in that subset of women who had been exposed for a longer time to estrogen (longer menarche to FFTP interval) or were expected to be more susceptible to estrogen (no history of pregnancy or older at FFTP). We, therefore, investigated the potential importance of estrogen exposure in conjunction with these susceptibility genotypes by

both the joint method (Table II) and the stratified method (Table III). For the analysis using the joint method, we first classified our women into 2 groups, those with no putative high-risk genotypes of the CE-metabolizing genes and those with at least one putative high-risk genotype in all CE-metabolizing genes or in those genes involved in either of the 2 pathways described above, because such a definition would give sufficient statistical power to address the question. The reference group consisted of women with no putative high-risk genotype and having been less susceptible to estrogen exposure (having a history of pregnancy or younger at FFTP). Our hypothesis was supported by the finding that, in the absence of the estrogen-related risk factors, the harboring of at least one putative high-risk genotype was associated with a small and not significant increase in risk, whereas, in the presence of these risk factors, the harboring of at least one putative high-risk genotype was associated with a much greater and more significant combined risk of breast cancer (Table II). In the stratified method, on the other hand, we examined whether breast cancer risk associated with at least one putative high-risk genotype was modified by estrogen-related risk factors (Table III). A consistent significant association between an increased cancer risk and harboring at least one putative high-risk genotype was only seen in those women who had been exposed to estrogen for ≥12 years before FFTP (Table III). In contrast, among women with a short menarche to FFTP interval (<12 years), there was no significant association (all $p > 0.05$).

The significant interaction between CE-metabolizing genes and estrogen exposure in contributing to breast cancer risk prompted us to explore a possible joint effect of these genes and those involved in DSBR, because the protective effects of the CE-metabolizing genes should prevent estrogen metabolites (*i.e.*, CE-Qs) from forming depurinating adducts, leading to DNA DSB.[10–12] Previously, we had simultaneously genotyped

TABLE II – RISK (ADJUSTED ODDS RATIO, aOR[1]) OF BREAST CANCER ASSOCIATED WITH THE COMBINATION OF PUTATIVE HIGH-RISK GENOTYPES OF CATECHOL ESTROGEN (CE)-METABOLIZING GENES AND THE REPRODUCTIVE RISK FACTORS OF A HISTORY OF PREGNANCY, OR AGE AT FIRST FULL-TERM PREGNANCY (FFTP)

No. of high-risk genotypes	Pregnancy history	CE detoxification (*COMT, UGT1A1*)	CE-SQ/CE-Q metabolism[2] (*MnSOD, GSTM1, GSTT1*)	Overall CE metabolism (all 5 genes)	Overall CE metabolism (*COMT, MnSOD, GSTM1*)
0	Yes	1.00 (Ref.)[3]	1.00 (Ref.)	1.00 (Ref.)	1.00 (Ref.)
>0	Yes	1.17 (0.90–1.50)	1.27 (0.92–1.76)	1.50 (0.92–2.46)	1.26 (0.92–1.73)
0	No	1.21 (0.58–2.52)	1.69 (0.63–4.54)	0.75 (0.15–3.79)	1.13 (0.41–3.09)
>0	No	1.97 (1.12–3.48)	1.78 (1.01–3.11)	2.38 (1.23–4.60)	1.97 (1.12–3.45)
No. of high-risk genotypes	**Age at FFTP, year**				
0	<26	1.00 (Ref.)	1.00 (Ref.)	1.00 (Ref.)	1.00 (Ref.)
>0	<26	0.95 (0.67–1.35)	1.26 (0.77–2.05)	1.42 (0.71–2.82)	1.39 (0.88–2.18)
0	≥26	1.34 (0.89–2.02)	1.58 (0.86–2.91)	1.46 (0.56–3.83)	1.85 (1.03–3.34)
>0	≥26	1.91 (1.31–2.79)	2.14 (1.28–3.56)	2.38 (1.18–4.81)	2.28 (1.41–3.67)

[1]The adjusted odds ratio (aOR) for breast cancer development associated with putative high-risk genotypes and reproductive risk factors 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 genotype) and reproductive risk factor status.–[2]CE-SQ, CE-semiquinone; CE-Q, CE-quinone.–[3]Ref, reference group.

TABLE III – INCREASED RISK (aOR) OF BREAST CANCER ASSOCIATED WITH THE NUMBER OF PUTATIVE HIGH-RISK GENOTYPES OF CATECHOL ESTROGEN (CE)-METABOLIZING GENES STRATIFIED BY THE ESTROGEN-RELATED RISK FACTORS [YEARS OF THE MENARCHE TO FIRST FULL-TERM PREGNANCY (FFTP) INTERVAL]

Estrogen exposure before FFTP[4]	No. of high-risk genotypes	Cases (%)[1]	Controls (%)[1]	aOR (95%CI)[2]
CE detoxification (*COMT, UGT1A1*)				
≥12 yrs	0	78 (35.9)	163 (43.7)	1.00 (Ref.)[3]
	>0	139 (64.1)	210 (56.3)	1.46 (1.02–2.08)
<12 yrs	0	102 (43.4)	159 (43.9)	1.00 (Ref.)
	>0	133 (56.6)	203 (56.1)	1.01 (0.72–1.41)
CE-SQ/CE-Q metabolism[5] (*MnSOD, GSTM1, GSTT1*)				
≥12 yrs	0	41 (15.7)	97 (26.2)	1.00 (Ref.)
	>0	171 (84.3)	273 (73.8)	1.52 (1.00–2.33)
<12 yrs	0	37 (18.0)	59 (17.2)	1.00 (Ref.)
	>0	195 (82.0)	296 (82.8)	0.99 (0.62–1.57)
Overall CE metabolism (*COMT, UGTIA1, MnSOD, GSTM1, GSTT1*)				
≥12 yrs	0	12 (5.7)	40 (10.8)	1.00 (Ref.)
	>0	200 (94.3)	330 (89.2)	2.10 (1.06–4.16)
<12 yrs	0	15 (6.5)	29 (8.2)	1.00 (Ref.)
	>0	217 (93.5)	326 (91.8)	1.35 (0.69–2.64)
Overall CE metabolism (*COMT, MnSOD, GSTM1*)				
≥12 yrs	0	34 (15.8)	85 (22.9)	1.00 (Ref.)
	>0	181 (84.2)	286 (77.1)	1.71 (1.08–2.71)
<12 yrs	0	48 (20.5)	75 (21.0)	1.00 (Ref.)
	>0	186 (79.5)	282 (79.0)	1.02 (0.67–1.55)

[1]Difference of sample size in individual comparisons was due to (1) exclusion of study subjects without the history of pregnancy and (2) a lack of DNA specimens in study subjects.–[2]The adjusted odds ratio (aOR) for breast cancer associated with the number of putative high-risk genotypes was estimated in a multivariate logistic regression model containing age, a family history of breast cancer and body mass index.–[3]Ref, reference group.–[4]For postmenopausal nulliparous women, this index = age at menopause − age at menarche; for other women, this index = age at first full-term pregnancy − age at menarche.–[5]CE-SQ, CE-semiquinone; CE-Q, CE-quinone.

multiple SNP of the genes involved in 2 DSBR pathways, *i.e.*, the non-homologous end-joining (NHEJ) pathway (*Ku70*, *Ku80*, *DNA-PKcs*, XRCC4, and Ligase IV) and the homologous recombination (HR) pathway (*ATM*, *p53*, *RAD51*, *BRCA1* and *BRCA2*).[12,13] We have found that significant joint effects leading to an increased risk of breast cancer were seen in women harboring a higher number of high-risk genotypes of the NHEJ or HR genes, with aOR of 1.17 [95% CI = 1.01–1.34] and 1.16 (95%CI = 1.00–1.35) for having one additional high-risk genotype of NHEJ or HR genes, respectively. Genotypic polymorphisms of the CE-metabolizing genes and HR genes had a joint effect in increasing breast cancer risk, because a significant increase in aOR was seen in women harboring a higher number of putative high-risk genotypes of both the CE-metabolizing genes and HR genes (Fig. 2), further confirming the hypothesis that breast cancer is initiated by exposure to estrogen metabolites that cause DSB. Unexpectedly, the NHEJ genes and CE-metabolizing genes did not have a joint effect on breast cancer development, because the aOR for a higher number of putative high-risk genotypes of both the NHEJ genes and CE-metabolizing genes was almost the same as that for a higher number of either alone (Fig. 2). To further verify this finding, we used the stratified method to examine whether the breast cancer risk contributed by the genes from the individual DSBR pathways would be affected differently by polymorphism of the CE-metabolizing genes and found that this was the case. The results (Fig. 3) demonstrated that a significant increase in cancer risk associated with one or more high-risk genotypes of the HR genes was only seen in women with a higher number of high-risk genotypes of the CE-metabolizing genes, whereas a similar increase in risk associated with a higher number of high-risk genotypes of the NHEJ genes was only seen in women with a lower number of putative high-risk genotypes of the CE-metabolizing genes.

Discussion

Our study addresses the issue of catechol estrogen metabolism in relation to breast cancer risk in a multigenic model. This approach should allow a more precise evaluation of the risks associated with individual susceptibility genes and a more comprehensive insight into tumorigenesis initiated by estrogen exposure. In addition to the well-established mechanism by which estrogen triggers tumor promotion by binding to the estrogen receptor, activating the signaling pathway leading to proliferation,[3,4] the mechanism by which estrogen causes DNA damage, thus initiating breast cancer development, is becoming clearer. The ability of estrogen metabolites (*i.e.*, CE-Qs) to bind to DNA, leading to the formation of depurinating adducts and DNA damage, has been demonstrated.[6,7,8] The fact that the same transformation phenotypes and similar genomic alteration profiles are seen in human breast epithelial cells treated with either estrogen metabolites or well-defined chemical carcinogens is also evidence for the mutagenic effect of estrogen.[37] Furthermore, breast cancer progression was found to be associated with an increasing frequency of DNA DSB suggested to result from the carcinogenic effect of estrogen-induced ROS.[18] As a result, a possible protective effect of the frequent intake of antioxidants (*e.g.*, vitamin C) has been suggested.[38] In line with these findings, we have demonstrated recently that the association between breast cancer risk and risk factors reflecting greater susceptibility to estrogen is more significant in women harboring a higher number of putative high-risk genotypes of DSBR genes.[12,13] In our present study, we used a different approach to examine the initiating role of estrogen by exploring whether breast tumorigenesis was linked to inherited variants in genes involved in the metabolism of mutagenic estrogen metabolites. Such a design, based on the genetic background, ensures a more valid inference to be made about the temporal sequence[16] between an impaired ability to detoxify mutagenic

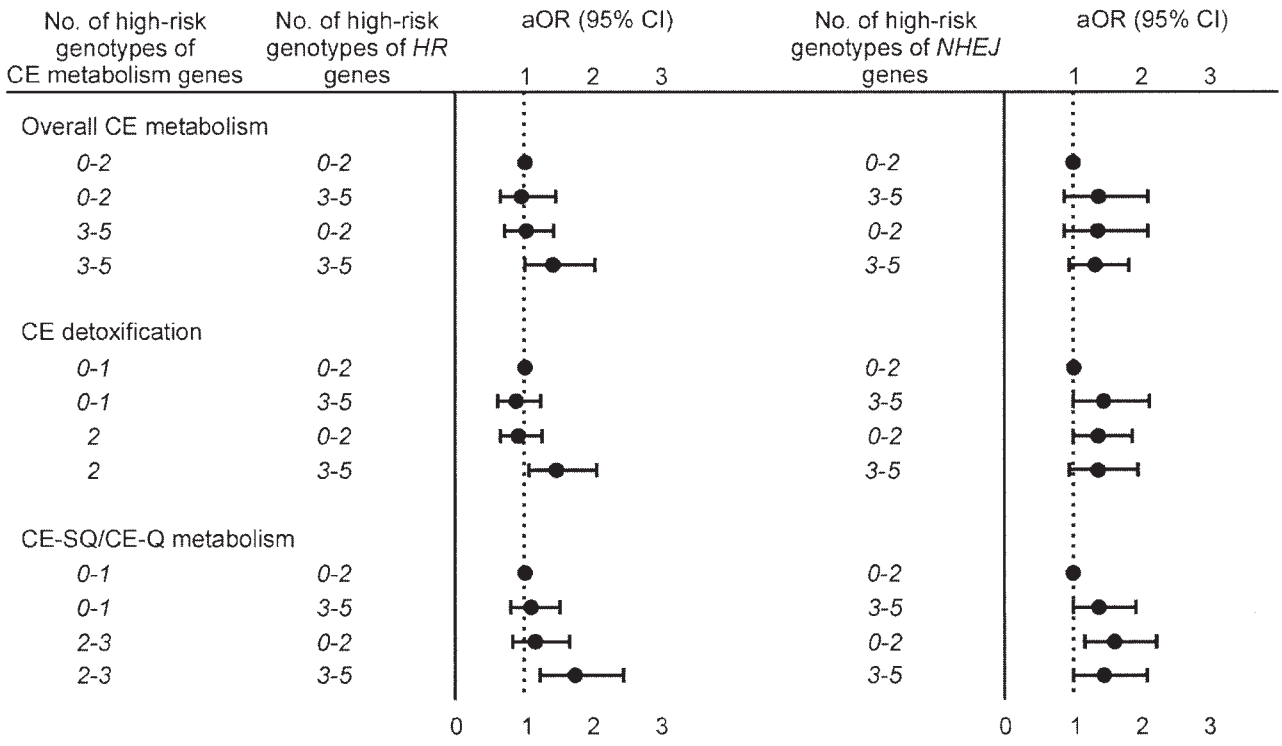

FIGURE 2 – Breast cancer risk (aOR and 95% CI) associated with the combination of putative high-risk genotypes of catechol estrogen (CE)-metabolizing genes and high-risk genotypes of homologous recombination (HR) genes or nonhomologous end-joining (NHEJ) genes. The women harboring the same numbers of high-risk genotypes of individual genes in both CE-metabolism and DNA double-strand-break repair (*i.e.*, HR or NHEJ) were grouped; the risks were estimated using the women harboring low number of high-risk genotypes as the reference.

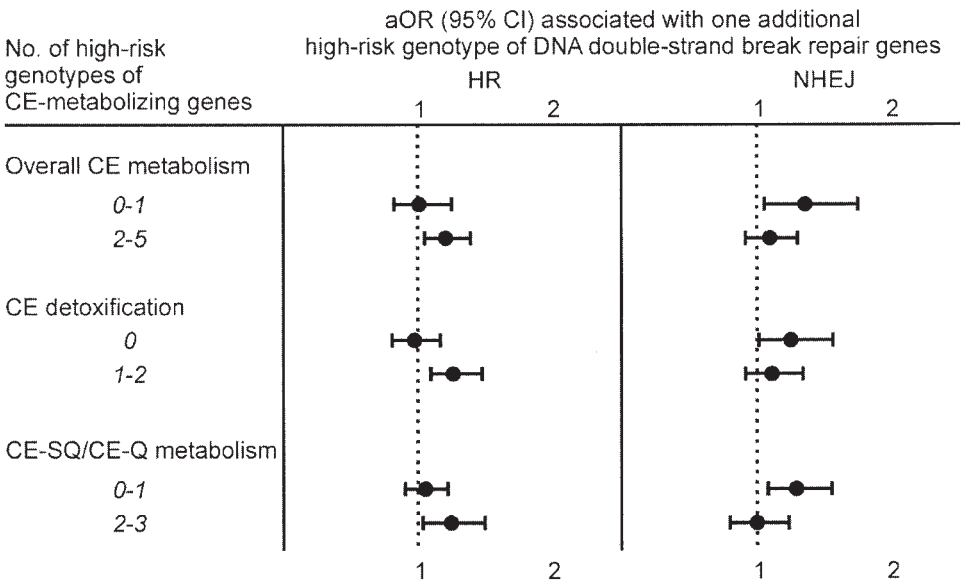

FIGURE 3 – Breast cancer risk (aOR and 95% CI) associated with one additional high-risk genotype of the genes involved in either homologous recombination (HR) or non-homologous end-joining (NHEJ) stratified by the number of high-risk genotypes of the catechol estrogen (CE)-metabolizing genes.

CE-Qs and increased breast cancer risk. Our main findings include: (*i*) there was a trend toward an increased risk of breast cancer in women harboring a greater number of high-risk genotypes of CE-metabolizing genes (Fig. 1); (*ii*) this association between risk and the number of putative high-risk genotypes was stronger and more significant in women more susceptible to estrogen (no history of pregnancy or older at FFTP) (Table II); and (*c*) the risks associated with harboring a higher number of high-risk genotypes were not the same in women having different risk factors reflecting estrogen exposure (Table III). These results provide support for the idea that increased estrogen exposure confers a higher risk of breast cancer by causing DNA damage.

The genetic evidence from familial cancer syndromes provides support for a causal relationship between mutated genes and the incidence of cancer.[39] Given this, it is puzzling that no genetic evidence has been found to link defective CE-metabolizing genes and breast cancer development. Of the CE-metabolizing genes, only mutations in 2 have been found to predispose carriers to a higher rate of genetic diseases; these are *COMT* and *UGT1A1*, which are associated, respectively, with schizophrenia or Gilbert's syndrome (manifested as neonatal hyperbilirubinemia),[40,41] neither of which have an estrogen-associated etiology. It is now recognized, however, that apparently disparate disease spectra can be caused by mutated (high-penetrance) and hypomorphic/polymorphic (low-penetrance) variants of the same gene.[42] More importantly, how, and in which organs, low-penetrance alleles display specific pathological phenotypes would depend on the cellular microenvironment generated by exposure to exogenous and endogenous hormones, as well as on genetic interactions with other functionally-related genes.[12] This provides clues to why carriers of mutated CE-metabolizing genes do not develop breast cancer, and why breast cancer risk is particularly associated with specific combinations of polymorphic alleles of these genes in women exposed to estrogen for a longer period or more susceptible to estrogen because of having no history of pregnancy. Because these CE-metabolizing genes participate in multiple physiologic functions in cells in different tissues, any severe mutation affecting them would result in severe disease in the tissues in which the genes play an active role. Thus, in tumor formation, which is distinct from other genetic diseases in that it requires an extended period of time to accumulate the genetic changes needed for its development, only cells harboring subtle defects arising from low-penetrance variants would have the chance to grow without triggering serious pathological outcomes. Because of their suboptimal capacity to detoxify CE, however, these cells would be subject to a higher rate of DNA damage caused by CE metabolites. This suggestion is consistent with our recently proposed "hide-then-hit model"[12] to explain the tumorigenic contribution associated with SNP of the high-penetrant NHEJ genes. The findings of the present study yield important insights into the relationship between genetic susceptibility due to multiple low-penetrance alleles of functionally-related genes and the development of cancers.

The multigenic approach used in our study provided a unique opportunity to evaluate the relative importance of individual CE-metabolizing genes in breast cancer development and led to *COMT* being identified as the most significant gene. Interestingly, in our previous study exploring the tumorigenic contribution of the estrogen metabolism pathway by simultaneous consideration of genes involved in estrogen biosynthesis (*CYP17*), hydroxylation (*CYP1A1*), and inactivation of reactive metabolites (*COMT*), *COMT* was also found to play the most significant role.[16] The reasons why *COMT* is apparently the most important in the estrogen-metabolizing genes are therefore of interest. Carcinogen dosage has been suggested to affect the association of cancers with polymorphisms of susceptibility genes.[43] Given that an association between increased breast cancer risk and *COMT* has been consistently found in many epidemiological studies based on different ethnic groups, variations in serum estrogen levels among populations[44] cannot explain the unique role of *COMT*. It is therefore more likely that its importance is due to *O*-methylation being a major contributor to CE detoxification *in vivo*. In line with this expectation, it has been shown that *O*-methylation results in a faster clearance rate than sulfation or glucuronidation and that 2-methoxyestrone (produced by COMT-mediated *O*-methylation of estrogen) is one of the most abundant estrogen metabolites in plasma and urine.[45] It should be noted that 2-methoxyestrone is a very potent inhibitor of tumor cell proliferation,[46] indicating the dual anti-cancer function of *COMT* in actively suppressing tumor growth and in passively preventing mutation. Although the genes involved in the CE-metabolizing pathway as a whole contributed significantly to preventing breast cancer formation, our findings also suggest that the anti-oxidation effect of *MnSOD* and the CE-Q-inactivating conjugation reactions (mediated by *GST* genes) are not as important as the detoxification reaction mediated by *COMT*. We propose the following two possibilities to explain this observation. First, because there are other mechanisms for the scavenging of ROS or preventing their mutagenic effect (*e.g.*, the genes of base excision repair, which repair ROS-modified DNA damage), any anti-tumor effect associated with *MnSOD* may be attenuated significantly. Secondly, the *GST* genes are involved in

the metabolism not only of CE, but also of a wide range of possible carcinogens, resulting in activation, as well as detoxification, and therefore in principle, *GST* gene deletion could be associated with either a decreased or increased risk of cancer.[47]

The finding that genotypic polymorphisms of the CE-metabolizing genes interact differently with the genes of the two DSBR pathways in relation to breast cancer risk is of particular significance. This might, at least in part, reflect which DSBR pathway the breast epithelium uses to repair the DNA damage caused by CE. The HR and NHEJ pathways differ in their requirement for a homologous DNA template,[48] HR using an intact sister duplex as the template, whereas NHEJ does not require a template. Thus, the relative contribution of each pathway depends on whether the cell is actively replicating, HR making a greater contribution in replicating cells, because the sister chromatid is readily available.[48] Women more exposed to estrogen have a greater chance of their breast cells undergoing cell division, and, at the same time, accumulating more DNA damage, and thus the interaction between HR genes and CE-metabolizing genes would be critical in determining breast cancer risk. Our findings certainly do not exclude a tumorigenic contribution of a sub-optimal NHEJ pathway, but the association between CE metabolism and HR in breast cancer formation provides additional support for the possibility that breast cancer is initiated by estrogen exposure, which causes DNA DSB.

The hormonal risk factors associated with breast cancer are well established. As a result, many models have been developed on the basis of the estrogen-related risk factor profile and are currently used to predict the risk of developing breast cancer, but few can account for the large variation in risk found in the general population. This is mainly because the majority of women fall within a narrow range of variability in age at menarche, total number of full-term pregnancies, and age at FFTP. It would therefore be very helpful to be able to identify additional parameters, and our study suggests that genotype polymorphisms of the CE-metabolizing genes may help in risk prediction. The simultaneous genotyping of all the genes involved in breast tumorigenic pathways would certainly be advantageous, but is not practical; however, it has been demonstrated that, even if the contribution of only a few genes could be identified, a significant benefit in defining risk might be gained.[49] Because the most plausible etiological mechanisms in breast tumorigenesis are taken into account, the findings of the present study and our recent identification of breast cancer risk associated with DSBR genes could potentially have important impact on the prediction of breast cancer risk. These findings may result in significant improvements in the efficacy of population-based programs for the prevention of, and intervention for, breast cancer.

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