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Manganese superoxide dismutase Ala-9Val polymorphism and risk of breast cancer in a population-based case–control study of African Americans and whites

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Abstract

Introduction: A polymorphism in the manganese superoxide dismutase (*MnSOD*) gene, Ala-9Val, has been examined in association with breast cancer risk in several epidemiologic studies. Results suggest that the Ala allele increases the risk of breast cancer and modifies the effects of environmental exposures that produce oxidative damage to DNA.

Methods: We examined the role of the *MnSOD* Ala-9Val polymorphism in a population-based case–control study of invasive and *in situ* breast cancer in North Carolina. Genotypes were evaluated for 2025 cases (760 African Americans and 1265 whites) and for 1812 controls (677 African Americans and 1135 whites).

Results: The odds ratio for *MnSOD* Ala/Ala versus any *MnSOD* Val genotypes was not elevated in African Americans (odds ratio = 0.9, 95% confidence interval = 0.7–1.2) or in whites (odds ratio = 1.0, 95% confidence interval = 0.8–1.2). Greater than additive joint effects were observed for the Ala/Ala genotype and smoking, radiation to the chest, and occupational exposure to ionizing radiation. Antagonism was observed between the Ala/Ala genotype and the use of nonsteroidal anti-inflammatory drugs.

Conclusions: The *MnSOD* genotype may contribute to an increased risk of breast cancer in the presence of specific environmental exposures. These results provide further evidence for the importance of reactive oxygen species and of oxidative DNA damage in the etiology of breast cancer.

Keywords: African Americans, breast cancer, manganese superoxide dismutase polymorphism

Introduction

A growing body of evidence suggests that reactive oxygen species (ROS) play a role in human cancer [1–3]. Free radical derivatives of molecular oxygen (superoxide, hydrogen peroxide, hydroxyl radical) are generated during normal metabolism, and are increased following ethanol consumption, cigarette smoking, and exposure to ionizing radiation. The consumption of antioxidants in foods, vitamin and mineral supplements, and the use of nonsteroidal anti-inflammatory drugs (NSAIDs) lower ROS levels [2]. Oxidative damage to DNA followed by mutation and alterations in gene expression are the principal mechanisms by which ROS contribute to carcinogenesis [1,4]. Several enzyme systems counteract oxidative damage, including catalase, glutathione peroxidases, and the superoxide dismutase family. Superoxide dismutases

AFFTP = age at first full-term pregnancy; CBCS = Carolina Breast Cancer Study; CI = confidence interval; ICR = interaction contrast ratio; MnSOD = manganese superoxide dismutase; NSAID = nonsteroidal anti-inflammatory drug; OR = odds ratio; PCR = polymerase chain reaction; ROS = reactive oxygen species.

convert superoxide (O_2^-) to peroxide (H_2O_2) and molecular oxygen (O_2). There are three superoxide dismutase enzymes: cytosolic superoxide dismutase, mitochondrial superoxide dismutase (manganese superoxide dismutase [MnSOD]), and extracellular superoxide dismutase [5].

The *MnSOD* gene on chromosome 6q25 encodes human MnSOD (also known as SOD2). MnSOD is synthesized in the cytoplasm as a precursor protein and is transported to the mitochondria, where it is processed and assembled into an active homotetramer. Studies of MnSOD protein expression showed higher levels in non-neoplastic breast epithelial cells compared with levels in invasive breast cancer [6]. MnSOD overexpression in breast cancer cell lines leads to upregulation of GAD153 (which is involved in the repair of double strand breaks in DNA) and to a variety of redox-sensitive transcription factors [7]. MnSOD also induces expression of the matrix metalloproteinase MMP-2, which helps to mediate cell migration and adhesion to the extracellular matrix [8]. Several investigators hypothesize that MnSOD may act as a tumor suppressor gene in breast epithelial cells [6,7,9].

A common polymorphism exists in the human *MnSOD* gene. This Ala-9Val polymorphism is a single nucleotide substitution of C → T at nucleotide 47, changing the encoded amino acid from Ala (GCT) to Val (GTT) [10,11]. The amino acid change occurs within the N-terminal mitochondrial targeting sequence, a 24-amino acid signal sequence that targets the MnSOD precursor protein for transport into the mitochondria. Mitochondrial localization of MnSOD is required to protect cells from ionizing radiation and other forms of oxidative damage [12]. The variant residue is nine amino acids upstream of the cleavage site, hence the polymorphism designation Ala-9Val. Computer models predicted that the Val allele would encode a beta-sheet conformation of the MnSOD precursor protein that exhibited impaired transport into the mitochondria, while the alpha-helical structure of the Ala-containing precursor would show correct transport [10]. Recent experiments by Sutton and colleagues [13] confirmed that, in rat liver, the human Val-containing MnSOD protein has difficulty crossing the mitochondrial inner membrane, leading to decreased formation of active MnSOD within the mitochondrial matrix. The MnSOD Ala-containing protein showed normal transport and generated 30–40% more active MnSOD protein than did the Val form of the enzyme.

Several epidemiologic studies have examined the association of the *MnSOD* Ala-9Val polymorphism and cancer. The Ala allele was associated with an increased risk of breast cancer in two populations [14,15] but not in another population [16]. The Ala allele was associated with an increased risk of prostate cancer [17] and of early-onset colorectal cancer [18], while the Val allele was associated with an increased risk of lung cancer [19] and of bladder cancer [20]. Studies of the role of MnSOD in noncancer outcomes also showed mixed results [21]. We conducted genotyping for *MnSOD* Ala-9Val genotypes in the Carolina Breast Cancer Study (CBCS), a population-based case–control study of breast cancer in African Americans and whites. We estimated odds ratios (ORs) for the *MnSOD* genotype and joint effects with several environmental exposures.

Materials and methods

Study design and participants.

The CBCS is a population-based case–control study of breast cancer conducted in North Carolina [22]. Participants provided informed consent using forms approved by the Institutional Review Board of the University of North Carolina School of Medicine, in compliance with the Helsinki Declaration. Breast cancer cases were identified in cooperation with the North Carolina Central Cancer Registry, and controls were identified using Division of Motor Vehicles lists (for women younger than age 65 years) and using Health Care Financing Administration lists (for women aged 65 years or older). Details of recruitment of participants and response rates have been published previously [23,24].

A total of 1803 cases of invasive breast cancer (787 African Americans and 1016 whites) and 1564 controls (718 African Americans and 846 whites) were enrolled between 1993 and 2001, and a total of 508 cases of *in situ* breast cancer (107 African Americans and 401 whites) and 458 controls (70 African Americans and 388 whites) were enrolled between 1996 and 2001. Controls were frequency matched to *in situ* cases based on age (± 5 years) and on race. In the invasive study, the response rates were 76.0% for cases and 55.0% for controls. In the *in situ* study, the response rates were 82.7% for cases and 65.2% for controls.

Home interviews were conducted to obtain blood samples and information on breast cancer risk factors. Response rates for blood draws that contained usable DNA combining all phases of the study were 89% for cases and 90% for controls. DNA samples were provided for a total of 2045 cases (768 African Americans and 1277 whites) and for 1818 controls (681 African Americans and 1137 whites).

Laboratory methods

DNA was extracted from whole blood using an automated ABI-DNA extractor (Applied Biosystems Nuclei Acid Purification System; Applied Biosystems, Foster City, CA, USA) in the University of North Carolina SPORE Tissue Procurement Facility. Genotyping for the *MnSOD* Ala-9Val

polymorphism (accession number S77127) was conducted using an ABI 7700 Sequence Detection System or the 'Taqman'™ assay (Applied Biosystems). PCR primers and probes were designed using Primer Express™ software (Applied Biosystems). The assay design and conditions were based on the allelic discrimination protocol from Applied Biosystems.

The Ala (C) allele-specific probe was labeled on the 5′ end with the VIC reporter dye and contained the nucleotide sequence 5′-CAAAGCCGGAGCC-3′, with 69.2% G–C content and a melting temperature of 65.1°C. The Val (T) allele-specific probe was labeled on the 5′ end with the FAM reporter dye and contained the nucleotide sequence 5′-CCAAAACCGGAGCC-3′, with 64.3% G–C content and a melting temperature of 65.5°C. Both probes contained the minor groove binding nonfluorescing quencher dye on the 3′ end. Forward and reverse primers were used to amplify the region surrounding the polymorphism. The nucleotide sequence for the forward primer was 5′-GGCTGTGCTTCTCGTCTTCA-3′, and the melting temperature was 59.2°C with 52.4% G–C content. The nucleotide sequence for the reverse primer was 5′-TTCTGCCTGGAGCCCAGAT-3′, and the melting temperature was 59.3°C with 57.9% G–C content.

PCR reactions were performed in a 15.0 µl reaction volume using the hot-start format. The reaction components were as follows: 1 × Taqman Universal PCR Master Mix, 900 nM each primer, 250 nM Ala (VIC), 150 nM Val (FAM) probe, and 15.0 ng genomic DNA. PCR reactions were run on a Perkin Elmer GenAmp® 9700 thermocycler (Perkin Elmer, Boston, MA, USA) using the 9600 mode under the following conditions: 50°C for 2 min (AmpErase® UNG activation), 95°C for 10 min (AmpliTaq® Gold activation), followed by 35 cycles of 95°C for 15 s (denature) and 57°C for 1 min (anneal/extend). Synthetic oligonucleotides of 73 base pairs corresponding to the *MnSOD-9Val* and *MnSOD-9Ala* alleles were included as positive controls in each plate. Samples that could not be scored were repeated.

MnSOD genotype data were obtained on a total of 3837 participants (2025 cases and 1812 controls). A total of 26 samples were not readable due to poor PCR amplification. Genotyping was repeated on a random 10% sample and results were identical to the original run.

Due to failure to observe Hardy–Weinberg equilibrium in a portion of the samples (see Results), the accuracy of the Taqman™ genotyping protocol was verified in three ways. First, genotyping was repeated on 20% of samples ($n = 772$) using the PCR-restriction fragment length polymorphism assay developed by Ambrosone and colleagues [14]. Five samples showed different results in the two assays (99.9% agreement). The five samples were repeated using the Taqman™ assay, and the results were identical to the original Taqman™ results. Only the Taqman™ results were therefore used in subsequent statistical analysis.

For the second verification method, 15 DNA samples from the Coriell Cell Repository (Camden, NJ, USA) were genotyped using the Taqman™ assay. The results corresponded to the direct DNA sequencing results provided by the NCI SNP500 project (http://SNP500cancer.nci.nih.gov).

Finally, we verified our Taqman genotyping procedure using direct DNA sequencing. Five DNA samples for each *MnSOD* genotype (Ala/Ala, Ala/Val and Val/Val) were amplified, were tagged with M13 forward and reverse universal primers, and were analyzed on the ABI 3730 DNA Analyzer using the ABI Prism™ BigDye™ Version 1.1 Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Applied Biosystems) (primer sequences and reaction conditions provided upon request). All direct DNA sequencing results corresponded to the previous genotyping results.

Statistical analysis

Differences in *MnSOD* allele frequencies between cases and controls were determined using a chi-square test. Departures from Hardy–Weinberg equilibrium were determined by comparing the observed genotype frequencies with expected genotype frequencies calculated using observed allele frequencies. Since deviations from Hardy–Weinberg equilibrium were observed (see Results), genotype frequencies in cases and controls were compared using the Cochrane–Armitage test for trend with a two-sided *P* value [25].

Age was based on age at diagnosis in cases or age at selection in controls. Race was classified according to self-report. Less than 2% of participants reported Native American or other race, and these were classified as whites. The stage at diagnosis in cases was classified according to the American Joint Committee on Cancer system based on review of medical records; data was missing for 107 cases. Additional covariates included family history (one or more first-degree relatives with breast cancer), menopausal status, duration of active cigarette smoking (years), any use of oral contraceptives, any use of hormone replacement therapy (postmenopausal women), a composite of age at first full-term pregnancy (AFFTP) and parity (nulliparous, parity = 1 and AFFTP <26 years, parity = 1 and AFFTP ≥26 years, parity ≥2 and AFFTP <26 years, and parity ≥2/AFFTP ≥26 years), age at menarche (continuous), lactation (ever, never), and alcohol use (ever, never). Body mass index (kg/m^2) and waist–hip ratio were calculated using measurements taken at the time of interview and were included as continuous variables.

High-dose radiation exposure to the chest was estimated by asking about medical procedures, including coronary catheterization, coronary angioplasty, and treatment of the upper body with radiation (excluding treatment or diagnosis for breast cancer). Occupational exposure was based upon a history of working for 6 months or longer at occupations with potential exposure to ionizing radiation (nurses, medical doctors, X-ray technicians and laboratory technicians) as described previously [26].

Participants were asked about any use of vitamin supplements for the previous 5 years, including multivitamins, vitamin A, vitamin C, vitamin E, and beta-carotene and calcium, as detailed previously [27].

The use of NSAIDs was determined by asking participants in phase 2 of the invasive study and the carcinoma *in situ* study about use of a variety of medications using color charts, as described previously [28]. Both prescription NSAIDs and nonprescription items, such as aspirin and acetaminophen, were included. Women who used NSAIDs for less than 3 months or who reported sporadic use (≤7 days per month) were classified as 'occasional users'. Women who used NSAIDs at least 8 days a month for 3 months or more were classified into two groups: those who used NSAIDs for less than 3 years' total duration were designated 'short-term users', while those who used NSAIDs for 3 years or longer were 'long-term users'.

Participants were asked about their usual servings per week of fruits and vegetables; a food frequency questionnaire or other comprehensive dietary history was not obtained. For this analysis, participants were classified according to the median consumption of fruits and vegetables in controls during summer months.

The ORs for breast cancer were calculated using unconditional logistic regression as implemented in the SAS software program (version 8.1; SAS Institute, Cary, NC, USA). Offset terms were incorporated into models using the SAS procedure, PROC GENMOD, to account for the sampling probabilities used to define eligible cases and controls [29]. Potential confounding was evaluated by selecting covariates for final models that resulted in a change of 10% or more for the beta coefficients for the *MnSOD* genotype or the relevant environmental exposure.

Interaction was evaluated on the multiplicative scale by comparing models with the main effects for the *MnSOD* genotype and environmental factors with models containing the main effects and interaction terms using a likelihood ratio test. $P < 0.10$ for likelihood ratio tests was considered statistically significant. Joint effects of the *MnSOD* genotype and environmental factors were estimated on an additive scale using a common referent group [30]. Interaction contrast ratios (ICRs) and 95% confidence intervals (CIs) were calculated in the SAS software program as described by Lundberg and colleagues [31]. The ICR is a measure of departure from additive joint effects: ICR > 0 implies greater than additive effects (positive interaction on an additive scale or synergy), ICR = 0 implies additive effects (no interaction), and ICR < 0 implies less than additive effects (antagonism) [30]. For the present study, we consider ICR ≥ 0.5 as evidence for a positive interaction on an additive scale. To estimate ORs for vitamin and mineral supplements, a common referent group of never users was employed as described previously [27].

Joint effects using an additive scale are presented for each exposure and *MnSOD* genotype. Stratified results using a multiplicative scale (two different referent groups) are also presented for NSAID use and the *MnSOD* genotype to assist in data interpretation.

Results

The characteristics of study participants and the response rates [24], as well as ORs for breast cancer and vitamin use, for smoking, for alcohol consumption, for exposure to ionizing radiation, for hormone use, and for NSAIDs have been previously published for the CBCS [26–28,32–35]. Briefly, modest inverse associations were observed for use of multivitamins, vitamin C, and vitamin E [27]. Weak positive associations were observed for smoking for longer than 20 years [32], for alcohol consumption [33], for high-dose radiation to the chest, and for occupational exposure to ionizing radiation [26]. No association was observed for hormone replacement therapy [34], and a weak positive association was found among younger women for the use of oral contraceptives [35]. An inverse association was observed for the use of NSAIDs [28]. No associations were found for summer or winter fruit and vegetable intake, and results have not been previously published for these variables.

Genotype and allele frequencies for *MnSOD* are presented in Table 1 for both African American and white study participants. The *MnSOD-9Val* allele was slightly more common among African American controls compared with white controls. The Val allele and the Val/Val genotype frequencies were higher among African American cases compared with controls, but the differences were not statistically significant. *MnSOD* allele and genotype frequencies were similar in cases compared with controls among whites. The *MnSOD* genotype frequencies did not differ according to stage at diagnosis of breast cancer in African American or white cases (data not shown).

The frequency of the Ala allele in white controls was within the previously reported range for Europeans and American

Table 1

Manganese superoxide dismutase (*MnSOD*) genotype and allele frequencies and odds ratios for breast cancer in African Americans and whites

MnSOD genotype and allele	Cases	Controls	Odds ratio (95% confidence interval)[a]
African Americans	$n = 760$	$n = 677$	
Genotype frequencies[b]			
Val/Val	259 (34%)	196 (29%)	Reference
Val/Ala	372 (49%)	357 (53%)	0.8 (0.6–1.0)
Ala/Ala	129 (17%)	124 (18%)	0.8 (0.6–1.1)
Cochran–Armitage trend test, $P = 0.08$			
Val/Val or Val/Ala	631 (83%)	553 (82%)	Reference
Ala/Ala	129 (17%)	124 (18%)	0.9 (0.7–1.2)
Allele frequencies[c]			
Val	0.59 (0.56–0.61)	0.55 (0.53–0.58)	
Ala	0.41 (0.39–0.44)	0.45 (0.42–0.47)	
Chi-square test, $P = 0.08$			
Whites	$n = 1265$	$n = 1135$	
Genotype frequencies[b]			
Val/Val	273 (21%)	266 (23%)	Reference
Val/Ala	681 (54%)	586 (52%)	1.2 (0.9–1.4)
Ala/Ala	311 (25%)	283 (25%)	1.1 (0.8–1.4)
Val/Val or Val/Ala	954 (75%)	852 (75%)	Reference
Ala/Ala	311 (25%)	283 (25%)	1.0 (0.8–1.2)
Cochran–Armitage trend test, $P = 0.59$			
Allele frequencies[c]			
Val	0.49 (0.47–0.51)	0.49 (0.47–0.51)	
Ala	0.51 (0.50–0.55)	0.51 (0.49–0.53)	
Chi-square test, $P = 0.60$			

[a] Adjusted for offsets and age. [b] Data presented as *n* (%). [c] Data presented as frequency (95% confidence interval).

whites (0.44–0.51) [14–18]. Tests for Hardy–Weinberg equilibrium showed a borderline, statistically significant departure from equilibrium distributions in African American controls ($P = 0.08$) and a significant departure in white cases ($P = 0.005$). In white cases, we observed an excess of Val/Ala heterozygotes (681 observed versus 631 expected). In African American controls, there was also an excess of heterozygotes (357 observed versus 335 expected). There was a nonsignificant excess of Val/Ala heterozygotes in African American cases ($P = 0.67$) and white controls ($P = 0.26$). In order to verify our Taqman™ genotyping results, we repeated *MnSOD* genotyping on 772 study participants using a PCR-restriction fragment length polymorphism assay (as described in Materials and methods); the results showed excellent agreement. In most previous studies, *MnSOD* genotypes appear to be in Hardy–Weinberg equilibrium, but these studies have not included populations with large numbers of African Americans.

The ORs for breast cancer and *MnSOD* genotypes in African Americans and whites are presented in Table 1. The ORs were close to the null value, with a slight inverse association in African Americans and a slight positive association in whites for Ala-containing genotypes compared with Val/Val. The OR for *MnSOD* Ala/Ala versus any *MnSOD* Val combining African American and white study participants was 1.0 (95% CI = 0.8–1.1), and the OR was similar for cases of carcinoma *in situ* versus controls (OR = 0.8, 95% CI = 0.6–1.1) and for invasive cancer cases versus controls (OR = 1.0, 95% CI = 0.9–1.2).

Table 2

Odds ratios for manganese superoxide dismutase (*MnSOD*) genotypes and breast cancer, according to menopausal status

MnSOD genotype	Cases	Controls	Odds ratio (95% confidence interval)[a]
Premenopausal	$n = 904$	$n = 783$	
Val/Val	224	187	Reference
Val/Ala	479	417	1.0 (0.8–1.2)
Ala/Ala	201	179	1.0 (0.7–1.3)
Val/Val or Val/Ala	703	604	Reference
Ala/Ala	201	179	1.0 (0.8–1.2)
Postmenopausal	$n = 1121$	$n = 1029$	
Val/Val	308	275	Reference
Val/Ala	574	526	1.0 (0.8–1.2)
Ala/Ala	239	228	0.9 (0.7–1.2)
Val/Val or Val/Ala	882	801	Reference
Ala/Ala	239	228	0.9 (0.8–1.2)

[a] Adjusted for offsets, age and race.

The ORs for *MnSOD* stratified on menopausal status are presented in Table 2. Values close to the null were observed in each group. The ORs did not differ among *in situ* or invasive breast cancer cases versus controls, and they did not change after adjustment for known breast cancer risk factors or any of the covariates presented in Materials and methods (data not shown).

The ORs estimating the joint effects of the *MnSOD* genotype and several environmental exposures are presented in Table 3. The ORs for combinations of *MnSOD* genotype and vitamin use, for alcohol use, and for use of oral contraceptives were close to the null value, and the ICRs were close to zero. The ORs were elevated for combinations of *MnSOD* Ala/Ala genotype and for smoking duration longer than 20 years, for high-dose radiation to the chest, and for occupational exposure to ionizing radiation. The ICRs suggested positive interaction on an additive scale for each of these exposures and the *MnSOD* Ala/Ala genotype.

A slight inverse association was observed for the combination of the *MnSOD* Ala/Ala genotype and hormone replacement therapy, but the ICR was close to zero. *P* values for likelihood ratio tests were less than 0.10 only for the interaction of the *MnSOD* genotype and high-dose radiation to the chest ($P = 0.009$). Results were similar in African Americans and whites, and in premenopausal women and postmenopausal women (data not shown). ICRs were close to zero for the joint effects of summer vegetable consumption and the *MnSOD* genotype (data not shown).

The ORs for the joint effects of the *MnSOD* genotype and NSAID use are presented in Table 4 for participants in phase 2 of the invasive study and in the carcinoma *in situ* study. ORs for combinations of the *MnSOD* genotype and NSAID use are presented on a multiplicative scale and on an additive scale. On a multiplicative scale, a strong inverse association of NSAID use and breast cancer was observed among participants with any *MnSOD* Val genotype, but not the *MnSOD* Ala/Ala genotype. The *P* value for the likelihood ratio test was 0.09.

Joint effects were calculated on an additive scale using a common referent group of any *MnSOD* Val genotype and never using NSAIDs. ICRs >0 were observed for each category of NSAID use and the *MnSOD* Ala/Ala genotype. The ICR for ever using NSAIDs (occasional + short term + long term) and the *MnSOD* Ala/Ala genotype was 0.5 (95% CI = 0.1–0.9). Since the ORs for the *MnSOD* Ala/Ala genotype and NSAID use alone are both less than 1.0, ICR >0 for the combination of both exposures is consistent with antagonism between NSAID use and the *MnSOD* Ala/Ala genotype [30]. The antagonistic effects of NSAID use and *MnSOD* genotype can be seen more clearly on a multiplicative scale (Table 4), where the protective effect of NSAID use was abolished among participants with the *MnSOD* Ala/Ala genotype.

To compare our results with the two previous studies of *MnSOD* genotypes and breast cancer, we calculated the ORs for the *MnSOD* genotype and environmental factors using the methods of Ambrosone and colleagues [14] and Mitrunen and colleagues [15].

Table 3

Joint effects of the manganese superoxide dismutase (*MnSOD*) genotype and environmental risk factors on breast cancer

	Val/Val or Val/Ala *MnSOD* genotype		Ala/Ala *MnSOD* genotype		
	Cases/controls	Odds ratio (95% CI)	Cases/controls	Odds ratio (95% CI)	ICR (95% CI)
Vitamin use[a]					
No vitamin use	626/543	Referent	148/143	0.9 (0.7–1.2)	
Any vitamin use	959/862	0.9 (0.8–1.1)	292/264	0.9 (0.7–1.1)	0.1 (–0.2, 0.4)
Multivitamin use	791/695	1.0 (0.8–1.1)	235/200	0.9 (0.7–1.2)	0.1 (–0.2, 0.4)
Vitamin A	58/48	1.1 (0.7–1.7)	17/16	0.9 (0.4–1.9)	–0.1 (–1.0, 0.7)
Vitamin C	405/363	1.0 (0.8–1.2)	129/103	1.1 (0.8–1.5)	0.3 (–0.1, 0.7)
Vitamin E	401/325	1.1 (0.9–1.3)	123/101	1.1 (0.8–1.5)	0.1 (–0.3, 0.6)
Beta-carotene	59/58	1.0 (0.7–1.6)	17/15	1.1 (0.5–2.4)	0.2 (–0.8, 1.1)
Calcium supplements	413/412	0.8 (0.7–1.0)	137/123	0.9 (0.7–1.3)	0.2 (–0.2, 0.6)
Smoking[b]					
No active or passive	311/268	Referent	87/87	0.8 (0.6–1.2)	
Passive only	540/478	1.0 (0.8–1.3)	143/144	1.0 (0.7–1.3)	
Duration of active smoking					
≤ 10 years	186/180	0.9 (0.7–1.3)	59/56	0.9 (0.6–1.3)	
> 10 years to 20 years	175/166	1.0 (0.8–1.4)	52/49	0.8 (0.6–1.4)	
> 20 years	366/309	1.2 (0.9–1.5)	96/71	1.5 (1.0–2.2)	0.5 (–0.1, 1.0)
Alcohol consumption[c]					
No	507/454	Referent	122/139	0.7 (0.6–1.0)	
Yes	1076/949	1.0 (0.8–1.2)	318/268	1.0 (0.8–1.3)	0.3 (0.0, 0.6)
High-dose radiation to chest[a]					
No	1468/1299	Referent	395/387	0.9 (0.8–1.1)	
Yes	116/106	1.1 (0.8–1.4)	45/20	2.3 (1.3–4.1)	1.3 (0.0, 2.7)
Occupational exposure to ionizing radiation[a]					
No	1497/1334	Referent	413/391	0.9 (0.8–1.1)	
Yes	88/71	1.1 (0.8–1.6)	27/16	1.6 (0.8–3.1)	0.6 (–0.5, 1.7)
Oral contraceptives (regular use)[a]					
No	561/511	Referent	136/146	0.9 (0.6–1.1)	
Yes	1016/885	1.1 (0.9–1.4)	304/258	1.1 (0.9–1.4)	0.1 (–0.2, 0.5)
Hormone replacement therapy (postmenopausal women, regular use)[a]					
No	436/375	Referent	117/106	1.0 (0.7–1.3)	
Yes	446/426	0.8 (0.7–1.0)	122/122	0.7 (0.5–1.0)	–0.1 (–0.5, 0.3)

CI, confidence interval; ICR, interaction contrast ratio. [a] Adjusted for offsets, age, race, family history, age at menarche, age at first full-term pregnancy (AFFTP)/parity composite, smoking duration, and alcohol use. [b] Adjusted for offsets, age, race, family history, age at menarche, AFFTP/parity composite, and alcohol use. [c] Adjusted for offsets, age, race, family history, age at menarche, AFFTP/parity composite, and smoking duration.

Ambrosone and colleagues [14] calculated ORs for the *MnSOD* genotype after stratifying on environmental exposures, using a separate referent group of the *MnSOD* Val/Val genotype within each stratum. ORs were calculated for the *MnSOD* genotype across strata of fruit and vegetable intake and antioxidant use in premenopausal women and postmenopausal women. In the present study, ORs for the *MnSOD* genotype varied only slightly

Table 4

Odds ratios for non-steroidal anti-inflammatory drug (NSAID) use stratified on the manganese superoxide dismutase (*MnSOD*) genotype (multiplicative scale) and joint effects (additive scale) (phase 2 invasive and CIS studies only)

	Val/Val or Val/Ala *MnSOD* genotype		Ala/Ala *MnSOD* genotype		
Use of NSAIDs	Cases/controls	Odds ratio (95% CI) [a]	Cases/controls	Odds ratio (95% CI) [a]	ICR (95% CI)
Multiplicative scale					
Never	105/48	Referent	19/16	Referent	
Occasional	511/420	0.6 (0.4–0.8)	142/127	0.8 (0.4–1.9)	
Short term	148/141	0.4 (0.3–0.7)	43/36	0.9 (0.4–2.4)	
Long term	218/200	0.4 (0.3–0.7)	58/58	0.9 (0.4–2.1)	
Additive scale					
Never	105/48	Referent	19/16	0.5 (0.2–1.3)	
Occasional	511/420	0.5 (0.4–0.8)	142/127	0.5 (0.3–0.7)	0.4 (–0.1, 0.9)
Short term	148/141	0.4 (0.3–0.7)	43/36	0.5 (0.3–1.0)	0.5 (–0.1, 1.1)
Long term	218/200	0.4 (0.3–0.7)	58/58	0.4 (0.2–0.7)	0.4 (–0.1, 0.9)

CI, confidence interval; ICR, interaction contrast ratio. [a] Adjusted for offsets, age, race, family history, age at menarche, age at first full-term pregnancy/parity composite, smoking duration, alcohol use, lactation, menopausal status, oral contraceptive use, education, body mass index, and waist–hip ratio.

according to fruit and vegetable intake, and vitamin use, and did not differ in premenopausal versus postmenopausal women. The greatest difference noted was an adjusted OR of 1.1 (95% CI = 0.8–1.4) for the *MnSOD* Ala/Ala genotype versus any *MnSOD* Val genotype among participants with low summer vegetable intake, compared with an OR of 0.9 (95% CI = 0.7–1.1) for participants with high vegetable intake.

Mitrunen and colleagues [15] calculated the OR for *MnSOD* genotypes (Ala/Ala versus Val/Val) after stratifying on alcohol use, on smoking (ever/never) and use of antioxidants, on oral contraceptives, and on hormone replacement therapy. In the present study, adjusted ORs for the *MnSOD* Ala/Ala genotype versus the *MnSOD* Val/Val genotype were 1.1 (95% CI = 0.8–1.4) among ever smokers and 0.8 (95% CI = 0.6–1.1) among never smokers, and were 1.0 (95% CI = 0.8–1.3) among ever drinkers and 0.8 (95% CI = 0.6–1.1) among never drinkers. ORs for the *MnSOD* genotype were close to the null value across the strata of the remaining covariates.

Discussion

Using data from a population-based case–control study of invasive and *in situ* breast cancer in North Carolina, we did not observe an association between the *MnSOD* genotype and breast cancer (ignoring environmental factors) in African American or white women, nor among premenopausal or postmenopausal women. Ambrosone and colleagues [14] reported a positive association for the *MnSOD* Ala/Ala genotype and breast cancer in New York state that was stronger in premenopausal women than in postmenopausal women. Mitrunen and colleagues [15] reported a weak positive association in women from Finland that was similar in premenopausal and postmenopausal women, while Egan and colleagues [16] observed no association in premenopausal or postmenopausal women from Massachusetts and New Hampshire.

The number of participants in the CBCS was larger than in previous studies. The ORs for genotypes tend to converge towards the null as the sample size increases in association studies [36–38], and the phenomenon has been seen previously for several loci in breast cancer [39].

We did not observe strong joint effects for the *MnSOD* genotype and vitamin use or summer vegetable and fruit consumption. Ambrosone and colleagues [14] reported elevated ORs for the *MnSOD* Ala/Ala genotype in women with low consumption of fruits and vegetables and dietary sources of carotenoids, ascorbic acid, and alpha-tocopherol. Egan and colleagues [16] did not observe strong differences in the ORs for *MnSOD* stratified according to fruit and vegetable intake. Mitrunen and colleagues [15] reported that the OR for the *MnSOD* genotype was stronger in persons who took vitamin A, vitamin C, and vitamin E supplements. Fruit and vegetable intake and dietary intake of vitamin A, vitamin C, and vitamin E in the studies of Ambrosone and colleagues [14] and of Egan and colleagues [16] were estimated using detailed food frequency questionnaires. Mitrunen and colleagues [15] did not collect information on dietary intake of fruits and vegetables, and in the present study only a few basic questions were asked about fruit and

vegetable intake. The latter two studies thus had insufficient power to fully investigate the joint effects of the *MnSOD* genotype and intake of dietary antioxidants.

Modest joint effects for the *MnSOD* genotype and smoking were observed in our study. Mitrunen and colleagues [15] reported an elevated OR for the *MnSOD* genotype among smokers, while Egan and colleagues [16] reported a stronger association for the *MnSOD* genotype in never smokers than in ever smokers. Neither study addressed the duration of smoking.

We did not observe evidence for joint effects of the *MnSOD* Ala/Ala genotype and use of alcohol, of oral contraceptives, or of hormone replacement therapy. Mitrunen and colleagues [15] reported elevated ORs for the *MnSOD* genotype in ever drinkers, in users of oral contraceptives, and for postmenopausal estrogen use. Egan and colleagues [16] reported a slightly elevated OR for the *MnSOD* genotype in women who used oral contraceptives, but only a weak association among users of hormone replacement therapy. We did not observe strong joint effects for the *MnSOD* genotype and exogenous hormone use in the present study. One explanation may be that we did not observe strong main effects (ignoring *MnSOD* genotypes) for oral contraceptives [35] or for hormone replacement therapy [34] in the CBCS. We also did not observe greater than additive joint effects for the *MnSOD* genotype and indices of increased endogenous hormone exposure (early age at menarche, nulliparity, late age at first pregnancy, late age at menopause; data not shown). Relatively few persons reported high levels of alcohol consumption in our study population [33], decreasing the power to observe joint effects with the *MnSOD* genotype.

We observed moderately elevated joint effects of exposure to ionizing radiation and the *MnSOD* Ala/Ala genotype. Previous studies of the *MnSOD* genotype and breast cancer did not investigate interactions with radiation exposure. Our results are consistent with laboratory evidence that MnSOD expression plays a role in adaptive responses to ionizing radiation [40]. Green and colleagues [41] recently showed, however, that the Ala-9Val polymorphism in *MnSOD* was not correlated with sensitivity to radiotherapy in breast cancer patients. The present results suggest that the *MnSOD* genotype could influence the risk of breast cancer associated with low-dose occupational and medical sources of ionizing radiation, but additional epidemiologic studies are needed.

We observed evidence suggesting that the *MnSOD* Ala/Ala genotype antagonizes a protective effect of NSAID use and breast cancer. Several previous epidemiologic studies of breast cancer, as well as the CBCS, demonstrated protective effects of NSAID use (for a review, see [28]). In the CBCS, an inverse association was observed for occasional and regular users, and for users of prescription NSAIDs as well as nonprescription NSAIDs. The effects were strongest among women with the longest NSAID use [28]. Proposed mechanisms for the protective effect of NSAID use include decreased production of inflammatory cytokines, decreased cell proliferation, and reduced production of ROS. Interactions between NSAIDs and the *MnSOD* genotype suggest that both factors operate on a common biochemical pathway or series of pathways. Reduced ROS and decreased oxidative damage may thus underlie the protective effect of NSAID use and breast cancer. The gene–environment interaction also provides evidence that the protective effect of NSAIDs and breast cancer observed in the present study and in previous epidemiologic studies may be causal, and may not be due to confounding by lifestyle or other factors.

Epidemiologic studies of the *MnSOD* Ala-9Val polymorphism are difficult to interpret in light of recent data suggesting that the Val allele leads to impaired protein transport and to reduced MnSOD activity, while the Ala allele has normal activity [13]. Indeed, while studies of breast cancer have consistently implicated the Ala allele in increasing risk, studies of other health outcomes show differing associations [13,17,18,21]. The transport studies of Sutton and colleagues [13] were conducted in rat liver, which may not be generalizable to human breast tissue or other tissues. Since MnSOD removes the superoxide anion, a potential source of DNA damage, one would predict that that the *MnSOD* Val allele would lead to an increased risk of cancer. On the other hand, MnSOD also generates hydrogen peroxide that can be toxic if not removed [13].

As suggested by Wang and colleagues [19], the effects of the *MnSOD* polymorphism may depend upon the tissue and tumor site, and may perhaps even depend on the host species. The effects of the *MnSOD* genotype also appear to depend upon environmental exposures that increase or decrease the levels of ROS, and such exposures may differ across populations. Finally, there may be unidentified variants in linkage disequilibrium with the *MnSOD-9Val* allele that contribute to transport and/or enzymatic activity. The complete story of the *MnSOD* genotype is probably quite complex, a situation that has proven true for many or most single-nucleotide polymorphisms [42,43].

There are several weaknesses to our study. *MnSOD* genotypes were not in Hardy–Weinberg equilibrium. One possible explanation for this is laboratory error [44]. We compared our genotyping results with a 'gold standard' of direct DNA sequencing, and the results were identical. Genotyping error can thus be practically excluded.

A second weakness of our study is that we had limited power to examine joint effects of the *MnSOD* genotype and dietary intake of antioxidants (due to lack of data) and alcohol use (due to low levels of alcohol consumption in our study population). Estimates of the joint effects of the *MnSOD* genotype and exposure to ionizing radiation were imprecise due to the small number of exposed participants.

Finally, the *MnSOD* Ala-9Val polymorphism may interact with polymorphisms in other genes involved in modulating levels of oxidative damage that were not measured in our study. We did not observe evidence for joint effects of the *MnSOD* genotype and polymorphisms in the glutathione S-transferases *GSTM1*, *GSTT1*, or *GSTP1* (data not shown). Genotyping for additional loci that could interact with *MnSOD* was not conducted for study participants.

Conclusions

In a population-based case–control study of invasive and *in situ* breast cancer in African American women and in white women, we observed weak joint effects for the *MnSOD* Ala/Ala genotype and several environmental exposures. Our results are in agreement with previous epidemiologic studies of breast cancer that showed no main effects for the *MnSOD* genotype [16] and interactions between the *MnSOD* Ala/Ala genotype and environmental exposures that alter endogenous levels of ROS species [14–16]. The present study adds to previous knowledge by including exposure to ionizing radiation as a potential environmental exposure that may be mediated in part by *MnSOD* genotype. We also identified a possible antagonistic interaction between NSAID use and the *MnSOD* genotype.

Taken together, these four studies help to implicate ROS as important biologic intermediates in the etiology of breast cancer. However, the increased risk associated with the *MnSOD-9Ala* allele is difficult to interpret given that the Val allele (but not the Ala allele) is associated with reduced MnSOD enzymatic activity in rat liver model systems [13].

As pointed out by St Clair and Kasarskis [21], more functional studies of the *MnSOD-9* polymorphism are needed. Epidemiologic studies of *MnSOD* will shed additional light on the role of ROS in the etiology of breast cancer and other diseases, and could help to understand the mechanisms of NSAIDs and other medications that reduce the risk of breast cancer.

Authors' contributions

RM, PM, VV, and TK participated in the interpretation of results and writing of the manuscript.

C-KJT conducted the statistical analyses.

JP, ARdC and GP conducted the laboratory analyses.

Competing interests

None declared.

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