Published in final edited form as:

Breast Cancer Res Treat. 2010 November ; 124(2): 433–439. doi:10.1007/s10549-010-0840-0.

Manganese superoxide dismutase polymorphism, treatment-related toxicity and disease-free survival in SWOG 8897 clinical trial for breast cancer

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Abstract

To date, the few studies of associations between a functional polymorphism in the oxidative stress-related gene *SOD2* and breast cancer survival have been inconsistent. In a homogeneous patient population from a large cooperative group trial (SWOG 8897), we evaluated this polymorphism in relation to both treatment-related toxicity and disease-free survival (DFS). Among 458 women who received cyclophosphamide-containing adjuvant chemotherapy, those with variant C alleles, related to higher antioxidant activity, experienced less grade 3–4 neutropenia (OR=0.52, 95% CI=0.29–0.92) but had worse DFS (HR=1.59, 95% CI=0.99–2.55) than women with TT genotypes. No associations were observed among 874 women who were followed without adjuvant therapy. Our results are consistent with the hypothesis that women with higher *SOD2* antioxidant activity may experience less treatment-related toxicity but shorter time to disease recurrence or death after breast cancer

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Conflict of interest/disclosure

The author(s) indicated no potential conflicts of interest.

adjuvant chemotherapy, supporting the modifying effects of oxidative stress-related enzymes on cancer treatment toxicity and efficacy.

Keywords

SOD2; polymorphism; SNP; toxicity; disease-free survival

Introduction

The *SOD2* gene encodes for manganese superoxide dismutase (MnSOD), a key enzyme in the oxidative stress metabolism pathway. Recently, Glynn et al found significant relationships between a well characterized polymorphism (rs4880 or Val16Ala) in *SOD2* and breast cancer survival after cyclophosphamide-containing chemotherapy (1). The direction of the observed association was consistent with the proposed functional effects of the polymorphism, indicating that higher antioxidant activity may lead to poorer survival after chemotherapy. However, the patient population in that study was pooled from the United States and Norway, and included patients with varied tumor characteristics and who received differing treatment regimens. The heterogeneity of the population could obscure associations for specific treatment regimens, and may limit generalizability. In addition, potential associations between the *SOD2* polymorphism and treatment-related toxicities have not been examined.

There is strong rationale to examine associations between polymorphisms in *SOD2* and treatment-related outcomes. Oxidative stress mediated by excessive reactive oxygen species (ROS) causes massive cellular damage such as mitochondrial permeabilization, leading to cell apoptosis (2). It represents a common cytotoxic mechanism for a number of anti-cancer drugs such as cyclophosphamide and anthracyclines, causing cell death in both normal and malignant tissues (3). MnSOD is a key mitochondrial antioxidant enzyme in defense against ROS. It catalyzes the dismutation of two superoxide radicals ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and oxygen (O_2). H_2O_2 can either be neutralized to H_2O and O_2 by catalase (CAT) and glutathione peroxidase (GPX), or it can be further converted by myeloperoxidase (MPO) to hypochlorous acid (HOCl), a potent oxidizing radical causing secondary oxidative damage. Therefore, *SOD2* represents a divergent point in the oxidative stress pathway, and the ultimate endogenous ROS levels may depend, in part, upon a delicate balance between enzymatic activities of *SOD2*, MPO, CAT and GPX (Figure 1).

Single nucleotide polymorphism (SNP) rs4880 in codon 16 of *SOD2* introduces a valine to alanine amino acid change in the mitochondrial targeting sequence, and therefore a conformational change from β -sheet to α -helix in the protein secondary structure, which may increase the mitochondrial import of the C allele (4). In two *in vitro* studies, a delayed mitochondrial import of the T allele caused degradation of the precursor protein and mRNA and therefore 30–40% lower enzyme activity (5,6). However, there is also evidence that does not support these proposed functions of the polymorphism. Among 231 healthy college volunteers, those carrying the T allele had 33% higher enzyme activity than those with C alleles, as measured in mitochondria isolated from red blood cells (7). We previously examined this SNP in relation to breast cancer outcomes among a small, heterogeneous population (8), as well as with risk of breast cancer (9). In those two studies, carriers of C alleles had better overall survival than women with TT genotypes, and CC genotypes were also associated with increased risk of premenopausal breast cancer, particularly among those with lower consumption of fruits and vegetables.

Because of the heterogeneity of patient populations as well as treatment received in previous studies, we examined potential relationships between *SOD2* genotypes and acute

hematological toxicity as well as DFS in the context of a completed Southwest Oncology Group (SWOG) clinical trial, S8897. Women with available DNA extracted from normal lymph nodes were eligible for this ancillary study, including a treated group of 458 women who were at higher risk of recurrence and randomized to cyclophosphamide, doxorubicin, and 5-fluorouracil (CAF) or cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) adjuvant chemotherapy with or without tamoxifen treatment, and an untreated group of 874 women who were at lower risk of recurrence and did not receive adjuvant therapy. We hypothesized that, in the treated group, women carrying *SOD2* rs4880 C alleles, with better localization to the mitochondrion and thus higher antioxidant activity, would have less treatment-induced toxicity but poorer DFS; we anticipated that there would be no effects of this SNP in the untreated group.

Results

Characteristics of patients in the treated and untreated groups from whom adequate DNA samples were available are summarized in Table 1. As expected, women in the treated group (higher risk of recurrence) were younger and more likely to be premenopausal, and had larger tumors than women in the untreated group, who were at lower risk of recurrence. The distribution of *SOD2* genotypes was similar between the treated and untreated groups. Because S8897 was conducted prior to the development of colony stimulating factor (CSF) for support during chemotherapy, more than sixty percent of the patients developed acute grade 3 and 4 neutropenia and leucopenia. A total of 129 and 257 disease events (recurrence or death) occurred in the treated and untreated groups, respectively.

Among women receiving adjuvant chemotherapy, those who were homozygous for C alleles had half the risk of grade 3 and 4 neutropenia than women with TT genotypes (adjusted OR=0.52, 95% CI=0.29–0.92, p-value=0.03) (Table 2). There was a similar reduced risk of grade 3 and 4 leucopenia among women with CC genotypes (adjusted OR=0.63, 95% CI=0.36–1.10, p-value=0.11), although not statistically significant. The associations did not differ between chemotherapy arms or between women with or without tamoxifen treatment.

SOD2 genotypes also appeared to affect DFS among women in the treated group. Kaplan-Meier survival curves revealed a modestly worse DFS among women with TC genotypes than those with TT genotypes, although the log-rank test was not statistically significant (Figure 3). In multivariate models, women with TC genotypes had a borderline significantly increased risk of disease recurrence or death (adjusted HR=1.59, 95% CI=0.99–2.55, p-value=0.057) compared to women with TT genotypes. The association was not statistically significant, however, for women who were homozygous for C alleles (adjusted HR=1.40, 95% CI=0.81–2.44, p-value=0.23), and genetic effects did not appear to be dose-dependent. Tests for interaction revealed no impact of chemotherapy regimen (CAF vs CMF) or tamoxifen treatment (yes vs no) on the relationship between *SOD2* genotypes and DFS (interaction of genotypes with CAF/CMF p=0.83; interaction of genotypes with tamoxifen p=0.95; data not shown). No associations between *SOD2* genotypes and DFS were observed among women in the untreated group.

Discussion

In this ancillary study to a completed clinical trial with 1,332 breast cancer patients receiving standardized treatment and follow-up, we found the C variant of *SOD2*, which results in better mitochondrial import, was associated with lower risk of acute hematological toxicity but with higher risk of disease recurrence or death. The effects were specific to adjuvant therapy since no significant associations were found in the observational cohort of women who did not receive adjuvant therapy. The findings are consistent with our hypothesis that high antioxidant

activity protects cells from oxidative damage caused by cancer therapy, reducing drug toxicity to normal tissues but compromising treatment efficacy to cancer cells.

Among 279 women who received varied adjuvant chemotherapy, we previously reported better survival, although not statistically significant, among women with CC genotypes, compared to those with CT and TT genotypes (HR=0.70, 95% CI=0.40–1.24) (8). A similar trend was also reported in a small study with 95 patients treated for metastatic breast cancer, where the TT genotype was associated with worse overall (HR=2.52, 95% CI=1.31–4.85) and breast cancer-specific survival (HR=1.92, 95% CI=1.03–3.57) compared to those with CC genotypes (10). However, due to small sample size and mixed therapy modalities, neither study was able to investigate drug-specific effects. Using data from a larger number of patients receiving standardized cyclophosphamide-containing treatment, our results conflict with those previous studies: women with TC or CC genotypes had worse DFS than those with TT genotypes. Our results, nevertheless, are consistent with the recent findings from Glynn et al among breast cancer patients pooled from the United States and Norway, wherein they observed that women with CC genotypes had poorer breast cancer-specific survival than those with TT genotypes (HR=2.47, 95% CI=1.46–4.19) (1). When analyses were restricted to a small subset of patients (n=142) who received cyclophosphamide-containing chemotherapy regimens, results were strengthened (HR=22.0, 95% CI=5.22–92.9), further refining the comparability of their results to ours, in which all patients received cyclophosphamide treatment. The small number of patients in their study may explain the higher HR and wider confidence interval than the results from our study. Moreover, both studies observed relationships between the *SOD2* polymorphism and survival only among women who received adjuvant chemotherapy and not among those who did not receive treatment, supporting the hypothesis that *SOD2* plays an important role in regulating chemotherapy-induced oxidative stress.

We also noted that women with CC genotypes had almost half the likelihood of developing grade 3 and 4 neutropenia, and were also less likely to develop high grade leucopenia than those with TT genotypes. These results are consistent with our previous findings among 446 women who received radiation therapy following lumpectomy for breast cancer (11,12). In that study, CC genotypes were associated with reduced risk of both acute skin radiotoxicity (OR=0.61, 95% CI=0.32–1.20) and chronic skin radiotoxicity (OR=0.79, 95% CI=0.42–1.48), although the results were not statistically significant. The fact that the C allele was associated with both less toxicity and poorer survival, which would support the hypothesis that lower oxidative stress reduces cell damage in both normal and malignant tissues, strengthens our confidence in the findings.

We previously examined germline polymorphisms in other oxidative stress-related genes in relation to breast cancer treatment outcomes. Among 279 women receiving varied breast cancer therapy, those with the *MPO* GG genotypes had almost a two-fold reduction in hazard of death than those with AA genotypes (HR=0.60, 95% CI=0.38–0.95) (8). Those findings were recently validated among 453 women in this cohort (HR=0.41, 95% CI=0.21–0.77) (13). In the same study, we also reported modifying effects of nitric oxide synthase variants on DFS after breast cancer chemotherapy (14). Together, the growing body of evidence underscores the importance of inter-patient variations in oxidative stress metabolic capacity in determining response to breast cancer chemotherapy.

Given its position in the ROS metabolic pathway, the ultimate role of *SOD2* in ROS metabolism may be complicated by other enzymes in the same pathway. Because hydrogen peroxide, the ROS intermediate produced by *SOD2*, can either be neutralized to non-toxic H₂O and oxygen by CAT and GPX, or be subsequently converted to potent toxic radical hypochlorous acid by MPO, increased endogenous *SOD2* activity may protect against oxidative damage if activity of CAT and GPX dominates, or may lead to oxidative damage if activity of MPO dominates

(Figure 1). It was shown in one study that overexpression of SOD2 in breast cancer cell lines induced resistance to Adriamycin treatment (15); while in another study high expression of SOD2 was linked to better survival among esophageal and gastric cancer patients (16). The apparently contradictory results may reflect a shifting balance between non-toxic neutralization and oxidative radical conversion, and may provide an alternative explanation for the inconsistent findings on the *SOD2* polymorphism and breast cancer survival as noted above.

Intake of exogenous antioxidants may also influence the balance between endogenous antioxidant enzymes and treatment outcomes. We previously reported associations between increased risk of premenopausal breast cancer risk and CC genotypes only among women below the median for consumption of fruits and vegetables and dietary ascorbic acid and α -tocopherol, indicating a shift towards generating potent oxidative radicals when exogenous antioxidant intake is inadequate (9). Similarly, among men from the Physician's Health Study who carried the more active CC genotype, high levels of antioxidants including selenium, lycopene, and α -tocopherol were associated with significantly lower risk of prostate cancer risk, but the associations were not significant among those carrying CT or TT genotypes (17). The interactions between exogenous antioxidants and endogenous antioxidant enzyme activity may also influence cancer treatment outcomes and are currently under investigation in another study.

In summary, in an ancillary study to SWOG 8897 clinical trial, we found that the *SOD2* C alleles with better mitochondrial import were associated with less acute hematological toxicity but worse disease-free survival compared to T alleles. Our findings support a beneficial role of SOD2 in reducing oxidative damage caused by cancer therapeutic drugs to normal tissues, but also provide a caveat that high endogenous antioxidant activity may compromise treatment efficacy to cancer cells. The accumulating data showing that gene variants associated with higher oxidative stress (*MPO*, *NOS* and *SOD2*) provide clues to identifying predictive genetic markers for commonly used breast cancer chemotherapeutic drugs by focusing on the oxidative stress pathway.

Methods

Patient population

This ancillary study was conducted within a completed clinical trial led by the Southwest Oncology Group (SWOG) for The Breast Cancer Intergroup (TBCI) of North America (S8897, INT0102). Details regarding the clinical trial design, treatment regimens, patient characteristics and follow-up were previously reported (13,14,18). In brief, 3,965 eligible women with early stage (T1 to T3a) node-negative breast cancer were enrolled from 1989 to 1993. As shown in Figure 2, they were assigned initially to one of three groups (high risk, low risk, and intermediate risk) with different recurrence risk based on tumor size and hormone receptor (ER and PR) status. Women in the intermediate risk group were subsequently re-assigned to either the high risk or low risk group based on S phase fraction of their primary cancers as determined by flow cytometry. Those in the high risk group (n=2,691) were randomized to 6 cycles of oral cyclophosphamide, intravenous doxorubicin and 5-fluorouracil (CAF) (19) or 6 cycles of oral cyclophosphamide, intravenous methotrexate and 5-fluorouracil (CMF) (20). Upon completion of adjuvant chemotherapy, patients were subsequently randomized to tamoxifen for 5 years or not. During the study period, prophylactic growth factors were not administered. Women in the low risk group (n=1,208) did not receive adjuvant chemotherapy or hormone therapy, but were followed as an observational cohort. After a median 10.8 years of follow-up, the study concluded with no significant difference in disease-free survival (DFS) between women receiving CAF and CMF regimens (18).

For this ancillary study, uninvolved lymph node tissues were available from women who were initially in the low risk and intermediate risk groups, since protocol-mandated tissue procurement was restricted to these patient groups. Since all women with hormone-receptor negative breast cancer were classified as high risk initially, nodal tissue was not collected. Therefore, this analysis is restricted to women who were either ER-positive and/or PR-positive. The study also predates routine testing of HER2 status so HER2 status is also unknown. Written consent was obtained from patients for use of their tissues for ancillary research, and this study was approved by the Institutional Review Board at Roswell Park Cancer Institute. According to REMARK criteria (21), a diagram illustrating the number of specimens collected and number of patients with available DNA is included (Figure 1). Results of the relationships between genetic variations in oxidative stress pathways and treatment outcomes have been previously reported based on the same study population (13,14).

Biospecimens, DNA, and genotyping

Genomic DNA was extracted from two 5 mm paraffin-embedded formalin-fixed slides from archived normal lymph nodes following a modified protocol as previously described in detail (22). Sufficient DNA specimens for genotyping were obtained from 458 patients in the treated group and 874 patients in the untreated group. Genotyping of rs4880 was performed by Sequenom matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Qualified genotyping call rate was 94.3% for the treated group and 95.2% for the untreated group, with failures due to DNA inadequacy. The concordance was 100% among 5% duplicate pairs included in the assays.

Statistical analysis

An analytic plan for testing associations between *SOD2* variants and clinical outcomes was prospectively planned in a formal, written protocol. Two primary endpoints were assessed in the analyses: toxicity and DFS. For high grade acute hematological toxicity, we considered grade 3 and 4 neutropenia and leucopenia. S8897 was conducted prior to development of the NCI Common Toxicity scales, and therefore standard SWOG toxicity criteria were applied (23). In brief, grade 2–4 neutropenia was defined by count of neutrophils between 1,000–1,500/mm³, 500–1,000/mm³, and <500/mm³, respectively; and grade 2–4 leucopenia was defined by count of leukocytes between 2,000–3,000/mm³, 1,000–2,000/mm³, and <1,000/mm³, respectively. We only included hematological toxicity because the other toxicities were too rare to be evaluated. DFS was defined per the protocol as the time from the date of randomization to the date of death due to any cause or the first local, regional, or distant recurrence or a new breast primary, but not a new non-breast primary cancer.

For toxicity analysis in the treated group, unconditional logistic regression models were used to estimate odds ratios (OR) and 95% confident intervals (CI) for grade 3 and 4 toxicity under an assumption of co-dominant genetic effects, with adjustment for age, menopausal status, treatment arm (CMF vs CAF, tamoxifen vs not), and time from initial surgery to randomization. Survival analysis was performed for the treated and untreated groups separately. Kaplan-Meier curves for DFS were generated and the difference by genotypes was contrasted by log-rank test. Cox proportional hazards models were used to estimate adjusted hazard ratios (HR) and 95% CIs after controlling for age, menopausal status, treatment arm (CMF vs CAF, tamoxifen versus not) for the treated group, and time from initial surgery to randomization for the untreated group. To examine if associations varied by chemotherapy regimen or tamoxifen assignment in the treated group, interactions between genotypes and chemotherapy arm and between genotypes and tamoxifen treatment were tested.

Acknowledgments

This investigation was supported in part by the following R01 and PHS Cooperative Agreement grant numbers awarded by the National Cancer Institute, DHHS: CA095222, CA32102, CA38926, CA02599, CA13612, CA22433, CA27057, CA37981, CA46282, CA20319, CA35431, CA76447, CA45560, CA12644, CA14028, CA58416, CA04919, CA35090, CA35176, CA58686, CA58861, CA46113, CA58882, CA35128, CA74647, CA46136, CA45450, CA35261, CA35192, CA12213, CA16385, CA58658, CA46441, CA58723, CA45377, CA35119, CA42777, CA73590, CA114558-02, CA35178 and CA35262. Drs. Ambrosone, Hayes, Hortobagyi and Rae are recipients of funding from the Breast Cancer Research Foundation. Dr. Yao was partially supported by a Department of Defense award DAMD W81XWH-08-1-0223.

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Figure 1. Reactive oxidative species (ROS) metabolism pathway

Variant: non-synonymous coding SNP rs4880 of *SOD2* gene, T (Valine) ➡ C (Alanine)

Function: C variant associated with better mitochondrial targeting

Hypothesis: C variant associated with less toxicity but poorer survival after treatment

Figure 2. Schema of S8897 trial design and tissues available for genotyping

Abbreviations: CMF, cyclophosphamide, methotrexate, and 5-fluorouracil; CAF, cyclophosphamide, Adriamycin, and 5-fluorouracil; TAM, tamoxifen.

Figure 3. Kaplan-Meier disease-free survival curves by rs4880 genotypes in treated group in SWOG 8897.

Table 1

Characteristics of patients with adequate DNA samples in treated and untreated groups in SWOG 8897

Characteristics	% treated group (n=458)	% untreated group (n=874)
Age		
<40	15	8
40–49	37	31
50–59	23	25
60–69	19	24
≥70	6	12
Median (range), years	49 (27–85)	54 (24–89)
Race		
Non-Hispanic White	88	92
Other	12	8
Menopausal status		
Premenopausal	51	38
Postmenopausal	49	62
Postmenopausal estrogen		
Yes	14	18
No	86	82
Primary treatment		
BCS, delayed RT	18	23
BCS, RT prior to registration	12	12
Mastectomy	70	64
Tumor size, cm		
≤1	0	50
1.1–1.9	100	50
Neutropenia		
≤2	33	-
3	22	-
4	45	-
Leucopenia		
≤2	38	-
3	48	-
4	14	-
SOD2 rs4880 (Val16Ala)		
TT (Val/Val)	25	24
TC (Val/Ala)	50	50
CC (Ala/Ala)	25	26
# Events (recurrence or death)	129	257

Abbreviations: BCS, breast conserving surgery; RT, radiation therapy.

Table 2

Estimated odds ratios and 95% confidence intervals of acute hematological toxicity associated with *SOD2* genotypes in treated group in SWOG 8897

Genotype	# of grade 3 and 4 vs grade 2	Adjusted OR (95% CI)	P-value
Neutropenia			
TT	79/30	1.00	
TC	149/68	0.81 (0.48–1.36)	0.42
CC	61/45	0.52 (0.29–0.92)	0.03
Leucopenia			
TT	68/41	1.00	
TC	141/76	1.07 (0.66–1.74)	0.79
CC	56/50	0.63 (0.36–1.10)	0.11

Factors adjusted in the multivariable models include age, menopausal status, treatment arm (CMF vs CAF, tamoxifen vs not), and time from initial surgery to randomization.

8897 Estimated hazard ratios and 95% confidence intervals of disease-free survival associated with *SOD2* genotypes in treated and untreated groups in SWOG

Table 3

Genotype	CAF or CMF ± TAM			No adjuvant chemotherapy		
	# event/total	Adjusted HR (95% CI)	P-value	# event/total	Adjusted HR (95% CI)	P-value
TT	23/109	1.00		55/203	1.00	
TC	67/217	1.59 (0.99–2.55)	0.06	122/414	1.10 (0.80–1.52)	0.55
CC	29/106	1.40 (0.81–2.44)	0.23	68/215	1.20 (0.84–1.71)	0.32

Factors adjusted in the multivariable models include age, menopausal status, and treatment arm (CMF vs CAF; tamoxifen versus not) and time from initial surgery to randomization for treated group. Abbreviations: CAF, cyclophosphamide, Adriamycin, and 5-fluorouracil; CMF, cyclophosphamide, methotrexate, and 5-fluorouracil; TAM, tamoxifen.