

Polymorphisms in Genes Related to Oxidative Stress (*CAT*, *MnSOD*, *MPO*, and *eNOS*) and Acute Toxicities from Radiation Therapy following Lumpectomy for Breast Cancer

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Abstract Purpose: Because radiotherapy exerts cytotoxic effects via generation of massive oxidative stress, we hypothesized that *catalase*, *manganese superoxide dismutase*, *myeloperoxidase* (*MPO*), and *endothelial nitric oxide synthase* (*eNOS*) genotypes might result in greater risk of radiotoxicity.

Experimental Design: Cases ($n = 446$) were Caucasian women with breast cancer who received radiotherapy following lumpectomy. Genotypes were determined by matrix-assisted laser desorption/ionization time-of-flight. The development of acute reactions (moist desquamation) associated with genotypes was modeled using the Cox proportional hazards model, accounting for cumulative biologically effective radiation dose.

Results: Genotypes associated with higher levels of reactive oxygen species (ROS) were not associated with risk of radiotoxicity. However, relationships between overweight/obesity [body mass index (BMI), >25] and radiotoxicity risk seemed to be modified by *eNOS* and *MPO* genotypes associated with higher generation of nitric oxide and ROS, respectively. Women with high BMI (>25) and *eNOS* GG genotypes were at more than a 6-fold increase in risk (hazard ratio, 6.39; 95% confidence interval, 2.53-16.15) compared with those with BMI <25 , and for *MPO*, those with high BMI (>25) and GG genotypes also had greater risk of radiotoxicity (hazard ratio, 3.61; 95% confidence interval, 1.78-7.35) compared with those with BMI <25 . Overweight/obesity was not a strong risk factor among women with other *eNOS* and *MPO* genotypes. Exploratory analysis using classification and regression trees indicated that total number of risk alleles contributed, in part, to acute toxicity outcomes among a subgroup of women.

Conclusions: Associations between BMI and radiotoxicity risk may be most apparent among women with genotypes related to higher levels of oxidative stress. Regression trees may be useful in future studies to examine the contributions of multiple factors to individual susceptibility to adverse effects of cancer treatment.

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Radiation therapy exerts antitumor effects through increased formation of reactive oxygen species (ROS; ref. 1). Oxidative stress, resulting from an excess of ROS and/or a decrease in antioxidant levels, provokes cell death through massive cellular damage to macromolecules, such as lipids, proteins, and nucleic acids, or indirectly through triggering abnormal signaling and cell cycle regulation (2). Radiation therapy can also cause mitochondrial permeabilization due to the enhanced generation of ROS, triggering apoptosis (3). Although radiotherapy precisely targets the tumor cells with high energy beams of radiation to minimize radiation exposure to normal tissue, normal tissues that rapidly proliferate, such as skin, gastrointestinal mucosa, and hematopoietic cells, are also relatively radiosensitive (4). Thus, it is likely for patients to develop acute toxicities from radiotherapy in these tissues. Radiation may differentially affect normal and tumor tissue because there is considerable heterogeneity with respect to different growth states and cellular composition between tumor and normal tissue (5). Although radiotoxicity may not be directly related to patients' survival, it merits close attention. Minimizing acute

toxicity will significantly improve quality of life and may be related to better treatment compliance. Thus, it is of paramount importance to have a better understanding of the factors related to radiation-induced side effects.

As reviewed by Harper et al. (4), both treatment- and patient-related factors influence acute toxicities during and following breast cancer irradiation. Treatment-related factors include fraction size (the dose delivered with each treatment), the total dose delivered, the volume of tissue treated, the type of radiation, and the use of chemotherapy. Patient-related factors include breast size, smoking habits, axillary lymph drainage before treatment, and interindividual genetic susceptibility. Twardella et al. (6) reported previously that, in this study population, overweight/obesity [body mass index (BMI), >25] and alcohol consumption were associated with an increased risk of acute radiotoxicity [hazard ratio (HR), 2.86; 95% confidence interval (95% CI), 1.74-4.73 for BMI >25 versus BMI ≤25; HR, 1.88; 95% CI, 1.00-3.53 for ever alcohol use versus never use]. However, current smoking status (HR, 0.86; 95% CI, 0.44-1.70 for smoker versus nonsmoker), age (HR, 0.98; 95% CI, 0.96-1.01 as a continuous value), lymph node involvement (HR, 1.27; 95% CI, 0.66-2.47), and hormonal therapies given (HR, 1.54; 95% CI, 0.77-3.09) were not associated with risk of radiotoxicity.

Interindividual genetic susceptibility due to variability in enzymes related to oxidative stress may be particularly relevant to radiotoxicity because the cytotoxic effects of radiotherapy are through an oxidative stress mechanism. Catalase (CAT) and manganese superoxide dismutase (MnSOD) neutralize ROS, whereas myeloperoxidase (MPO) and endothelial nitric oxide (NO) synthase (eNOS) generate ROS. MnSOD catalyzes the dismutation of two superoxide radicals, producing H₂O₂ and oxygen in the mitochondrion. A T>C polymorphism (rs#1799725, Ex2+24T>C) of MnSOD in the mitochondrial targeting sequence (7) affects the mitochondrial transport of MnSOD into the mitochondrion, where it is biologically available (8). CAT is an endogenous antioxidant enzyme that neutralizes ROS by converting H₂O₂ into H₂O and O₂. There is a common -329T>C (also known as -262) polymorphism (rs#1001179) in the promoter region of the human CAT gene, and we showed previously that individuals with the common CC genotype have significantly higher CAT activity compared with those with the variant TC or TT genotypes (9). On the other hand, MPO generates ROS endogenously by catalyzing a reaction between H₂O₂ and chloride to generate hypochlorous acid, a potent oxidizing agent (10). A functional MPO -642G>A (also known as -463) polymorphism (rs#2333227) in the 5' upstream region exists, and the MPO variant A allele confers a lower transcriptional activation than the -642 common G allele *in vitro* due to the disruption of the SP1 binding site (11). eNOS catalyzes the production of the free radical, NO (12). A G>T polymorphism (rs#1799983, Ex8-63G>T) results in a Glu²⁹⁸Asp substitution that leads to reduced NO levels (13). We reported previously that some of these genetic polymorphisms related to higher levels of oxidative stress were associated with better breast cancer survival (14). Because enzyme variability resulting from polymorphisms may determine the ultimate levels of ROS in the breast, a comprehensive analysis of these genotypes may provide a better estimate of oxidative stress resulting from radiotherapy.

Classification and regression tree (CART) analysis is a tree-building technique, based on the recursive partitioning method (15, 16). All genetic and nongenetic predictor variables are screened simultaneously for the one that best splits the data into two more homogeneous nodes based on acute radiotoxicity status. Subsequent partitioning proceeds in each daughter node guided by specific splitting rules. Unlike other types of modeling, CART does not require assumptions about underlying distributions or values of the predictor variables. As such, CART is a powerful tool allowing the exploration of many predictor variables. Importantly, it has the potential to uncover complex interactions that are difficult or impossible to model using traditional multivariate technique.

We examined potential relationships between polymorphisms in oxidative-stress related genes and the risk of radiotoxicity, as well as potential interactions between genotypes and lifestyle factors in a prospective cohort of breast cancer patients receiving radiotherapy following lumpectomy, using traditional multivariate models. In an exploratory analysis, we also evaluated potential higher-order gene-gene or gene-lifestyle factor interactions using CART analysis.

Materials and Methods

Study population

We recruited Caucasian female breast cancer patients receiving primary radiotherapy of the breast after breast conserving surgery at radiotherapy units of the Women's Clinic in Heidelberg, the St. Vincentius Clinic, the City Hospital of Karlsruhe, and the University Hospital of Mannheim from June 1998 to March 2001. Details of the choice of patients and data collection have been reported previously (6). Women were excluded if they were currently or ever treated with chemotherapy to avoid potential confounding. There were no age limitations for participation. The study was approved by the ethical committee of the University of Heidelberg, the Institutional Review Board at Roswell Park Cancer Institute, and the U.S. Army Medical Research and Materiel Command Human Subjects Research Review Board. Informed consent was obtained from all patients enrolled.

Measurements

Biologically effective radiotherapy dose. All patients were administered a common breast radiation treatment, including computed tomography-based planning, simulation, verification, and quality assurance, and received conformal tangential irradiation with lateral and medial wedge fields. As described previously (6), all regimens included irradiation of the entire breast. At three units, women received either 50 Gy given in 5 × 2.0 Gy fractions weekly or 50.4 Gy in 5 × 1.8 Gy fractions weekly followed by a boost radiation from 6 to 25 Gy. At the fourth radiation department, 56 Gy of whole breast irradiation were applied in 5 × 2.0 Gy fractions weekly without a boost. The biologically effective radiotherapy dose (BED) was calculated to account for differences in fractionation and overall treatment time, using the formula

$$BED = nd \left(1 + \frac{d}{\alpha/\beta} \right) - \frac{\gamma}{\alpha} (T - T_0),$$

given the number of fractions n , the fraction size of d , an α/β ratio of 10 Gy for acute skin reactions (17), a time factor γ/α of 0.7 Gy daily, the overall treatment time of T , and a starting time for compensatory proliferation T_0 of 21 days (18).

Acute toxicities. Clinical radiation reaction developing in the skin within the radiation field of the breast was documented four times during the study: (a) before the beginning of radiotherapy and at a cumulative dose of (b) 36 to 42 Gy, (c) 44 to 50 Gy, and (d) about

60 to 66 Gy (end of radiotherapy). The severity of acute side effects was assessed using a modified classification system based on the common toxicity criteria of the NIH (19). For more detailed categorization of skin reaction, grade 2a was defined as tender/bright erythema or moderate edema, grade 2b as severe erythema, and grade 2c as at least one area of moist desquamation or interruption of radiotherapy due to toxicity. Development of acute side effects of grade 2c and above was considered as acute radiotoxicity in our study (20).

A total of 478 patients participated with complete clinical and epidemiologic information, of whom 84 developed acute clinically relevant toxicity by the end of radiotherapy. Blood samples collected before starting radiotherapy were available for 446 patients (average age, 60.3 ± 9.0 years) who were included in this analysis. Of these patients, 77 presented with acute toxicity grade 2c or above. The average biologically effective radiotherapy dose by censoring was 54.0 ± 4.8 Gy with a range of 35.5 to 64.5 Gy.

Genotyping. Genomic DNA from pretreatment blood samples was extracted from lymphocytes using the QIAamp DNA Blood Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All DNA preparations were stored at 4°C until use. Polymorphisms for *MnSOD*, *CAT*, *MPO*, and *eNOS* were assessed using Sequenom's (San Diego, CA) high-throughput matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (21). Controls for each genotype were included on each plate, as well as two nontemplate controls per plate, and laboratory staff were blinded to case/control status.

Other exposure assessment. Clinical data on tumor characteristics and therapy regime were abstracted from patient records. Participants completed a self-administered questionnaire at the first visit before beginning of radiotherapy, which elicited information on demographic factors, medical history, family history of cancer, and lifestyle factors.

Data analysis

The effects of the genetic variants on the risk of developing clinical radiotoxicity were evaluated using Cox proportional hazards models (version 8.2 of the SAS PHREG procedure, SAS Institute, Inc., Cary, North Carolina). The event of interest was the occurrence of acute radiotoxicity, defined as skin reactions scoring grade 2c or above. Because the risk of skin toxicity increases with BED, we modeled its occurrence in relation to BED received, instead of time in days of radiotherapy. In this way, we adjusted for differences in radiation dose when a toxicity of grade 2c or more was recorded and for the total dose received. Heterozygotes and variant homozygotes were assessed both separately and jointly in relation to the common genotype as a reference category. The differences in treatment by various hospitals were adjusted by including hospital (four clinics) and photon beam energy for whole breast (two categories) and for boost irradiation (no boost and four categories) in the minimally adjusted model. To consider the potential confounding factors, fully adjusted models were further adjusted for BMI, smoking status, alcohol consumption, and hormonal therapies received. Comorbidities, such as diabetes, were not found previously to be a risk determinant of acute toxicity (6) and were not considered in this analysis.

To explore whether genotypes modified the associations between lifestyle factors and the risk of radiotoxicity, data were stratified by *CAT*, *MnSOD*, *MPO*, and *eNOS* genotypes, and effects of those factors on risk were evaluated within each stratum. Genotypes were collapsed into two categories: homozygous (common genotypes) versus heterozygous + variant homozygous. Lifestyle factors that we evaluated were BMI, alcohol consumption (yes versus no), smoking status (never versus current and past), and hormonal therapy (yes versus no). BMI [weight (kg)/height (m)²] was calculated, and we set the cutoff index for overweight as the index recommended by the WHO: BMI: ~25, 25-30, and 30+ (22). To test statistical interactions on a multiplicative scale, a cross-product term of the ordinal score for each genotype and the risk factor variables (i.e., genotype \times lifestyle factor) was included in the multivariate models. The log-likelihood statistic for models with a multiplicative interaction term was compared with those without. Tests

for trends were conducted using the ordinal values for those lifestyle factors.

To evaluate potential higher-order gene-gene or gene-environment interactions, classification trees as implemented in CART version 5.0 were grown to complete exploratory nonparametric analysis of the data (15, 16). Models were similar to those used for Cox proportional hazards modeling and included the outcome variable of acute skin toxicity and genotypic explanatory variables for *MnSOD*, *MPO*, *CAT*, and *eNOS* coded as a continuous aggregate score (0-8), indicating an individual's total carriage number of risk alleles (*CAT* T, *MnSOD* C, *MPO* G, and *eNOS* G alleles). Other explanatory variables in the models were alcohol consumption (categorical variable), BMI (coded either as a three-level categorical or a continuous variable), hospital (categorical variable), photon beam energy and boost irradiation (categorical variables), smoking status (categorical variable), and hormone therapy use (categorical variable). We used three different prior class probabilities: matched to the observed class frequencies in the data (prior probabilities of 17% for an acute toxic event and 83% for no event), equal to each other (50% prior probability for both an acute toxic event and no event), and the average of the two previous schemes (prior probabilities of 33.5% for an acute toxic event and 66.5% for no event).

Results

Population characteristics. The characteristics of the study population have been published previously (6). All women were Caucasians, and the majority of women had early-stage (67% stage I; 23% stage II) and node-negative (77%) breast cancer. Mean and SD of age were 60 and 9 years, respectively (range, 26-87 years). The characteristics of the study population were similar when the analyses were restricted to respondents who donated blood or for those without DNA available for these analyses (data not shown). The observed allele frequencies for all genetic loci were similar to those reported previously in Caucasian subjects. Seventy-seven (17%) of the 446 participants developed acute radiotoxicity by the end of treatment.

Association between *CAT*, *MnSOD*, *MPO*, and *eNOS* genotypes and acute toxicity. Relationships between *CAT*, *MnSOD*, *MPO*, and *eNOS* genotypes and risk of acute radiotoxicity are shown in Table 1. There were no significant associations between risk of acute radiotoxicity and any of the genotypes, when hospital and photon beam quality for whole breast and boost irradiation were included in the models. These estimates remained essentially unchanged when we further adjusted for other potential confounding factors (i.e., smoking status, alcohol consumption, BMI, and hormonal therapy).

Associations between lifestyle factors and the risk of radiotoxicity by genotypes. When we evaluated whether associations between lifestyle factors (i.e., BMI, alcohol consumption, smoking status, and hormone therapy use) and the risk of radiotoxicity were modified by genotypes (Table 2), we observed that the associations between BMI and the acute radiotoxicity were more pronounced among women with *eNOS* GG or *MPO* GG genotypes, related to higher generation of NO or ROS. Compared with women with low BMI, those who were overweight/obese (BMI, >25) and had *eNOS* GG genotypes had more than a 6-fold increase in risk (HR, 6.39; 95% CI, 2.53-16.15) of radiotoxicity. Among women with *eNOS* GT and TT genotypes, overweight/obesity was associated with a lower, nonsignificant increase in risk (HR, 1.72; 95% CI, 0.88-3.38).

Table 1. Associations between *CAT*, *MnSOD*, *MPO*, and *eNOS* genotypes and the risk of radiotoxicity ($n = 447$)

Genotypes	No.*	Toxicities	Minimally adjusted HR [†] (95% CI)	Fully adjusted R [‡] (95% CI)
CAT				
CC	233	43	1	1
CT	162	31	0.92 (0.56-1.51)	0.95 (0.59-1.54)
TT	22	1	0.33 (0.05-2.47)	0.35 (0.05-2.59)
CC	233	43	1	1
CT+TT	184	32	0.86 (0.53-1.40)	0.90 (0.56-1.45)
(Lower removal of ROS or NO)				
MnSOD				
TT	113	24	1	1
TC	204	34	0.75 (0.43-1.31)	0.71 (0.41-1.20)
CC	111	17	0.71 (0.36-1.38)	0.61 (0.32-1.20)
TT	113	24	1	1
TC+CC	315	51	0.74 (0.44-1.24)	0.68 (0.41-1.12)
(Lower removal of ROS or NO)				
MPO				
GG	251	44	1	1
AG	133	22	0.89 (0.52-1.51)	0.90 (0.54-1.53)
AA	13	3	1.26 (0.38-4.19)	1.45 (0.43-4.86)
GG	251	44	1	1
AG+AA	146	25	0.92 (0.55-1.53)	0.95 (0.57-1.56)
(Higher generation of ROS)				
eNOS				
GG	187	33	1 (1)	1
GT	174	32	0.95 (0.57-1.59)	0.82 (0.49-1.37)
TT	65	9	0.72 (0.34-1.53)	0.64 (0.30-1.35)
GG	187	33	1	1
GT+TT	239	41	0.89 (0.55-1.44)	0.77 (0.48-1.23)
(Higher generation of NO)				

*Genotype data were missing 30, 19, 50, and 21 for *CAT*, *MnSOD*, *MPO*, and *eNOS*, respectively.

[†]Minimally adjusted model: adjusted for hospital (four clinics) and photon beam quality for whole breast (two categories) and boost irradiation (no boost and four categories).

[‡]Fully adjusted model: adjusted for hospital (four clinics), photon beam quality for whole breast (two categories) and boost irradiation (no boost and four categories), BMI, current smoking status, alcohol consumption, hormone therapy use.

Similar relationships were noted for *MPO*. Overweight/obesity (BMI, >25) was associated with more than a 3-fold increase in risk of radiotoxicity (HR, 3.61; 95% CI, 1.78-7.35) among women with *MPO* GG genotypes, related to higher generations of ROS; overweight/obesity was associated with a lower nonsignificant increase in risk (HR, 2.21; 95% CI, 0.91-5.39) among women with *MPO* GA and AA genotypes. Multiplicative interactions between genotypes and BMI were not statistically significant (*eNOS* genotype; $P = 0.25$ and *MPO* genotype; $P = 0.27$). *CAT* and *MnSOD* genotypes did not modify associations between BMI and the risk of acute toxicity (Table 2). Finally, when we combined *MPO* and *NOS* genotypes, the effect of high BMI (BMI, ≥ 25) was greatest among women with both *MPO* GG and *NOS* GG genotypes. Among those women, overweight/obese women had the greatest increased risk (adjusted HR, 18.84; 95% CI, 2.50-142.00), although cell size is small and the 95% CI is wide. There was no modification of associations between risk of acute toxicity and alcohol consumption, smoking status, or hormonal therapy by any of the genotypes (data not shown).

Exploratory CART analysis. We evaluated potential higher-order gene-gene and gene-environment interactions using CART as an exploratory analysis. No trees were grown when the prior class probabilities matched the observed class frequencies in the data (i.e., 17% prior probability of an acute

toxic event). When the prior class probabilities were set to equal each other (i.e., 50% prior probability of an acute toxic event), the majority of trees grown were two-node trees that split on the variable of BMI (≤ 25 versus 26 or more when categorical and ≤ 25.18 versus 25.19 or more when continuous). The terminal node that included women with high BMI was consistently classified as the event node, thus supporting the findings of Twardella et al. (6) as expected.

When prior class probabilities were averaged across the two previously mentioned schemes (i.e., 33.5% prior probability of an acute toxic event) and models were limited to the 356 women for whom we had complete genotyping data at all four loci, a 21-node tree was grown. Total carriage number of risk alleles persisted as a splitting variable through four prunings of the tree that resulted in a 13-node tree (Fig. 1). This decision tree indicated that total carriage number of risk alleles in *CAT*, *MnSOD*, *MPO*, and *eNOS* contributed, in part, to acute toxicity outcomes among some of the 35 women who had BMI >25, received boost irradiation of 12, 15, or 18 mV electrons, attended one of the four enrollment clinics, drank alcohol, never smoked, and had hormone therapy. Although exploratory and based on small numbers, these results suggest a complex, context-dependent interaction of ROS genotypes. Subsequent pruning produced an eight-node tree that did not contain the genotype variable as a splitter.

Discussion

We evaluated associations between genetic polymorphisms related to oxidative stress (i.e., CAT, MnSOD, MPO, and eNOS) and the risk of acute radiotoxicity, defined as moist desquamation of the skin, among breast cancer patients who received radiotherapy. None of these genotypes was associated with the risk of radiotoxicity. However, among women with eNOS GG and MPO GG genotypes, the associations between BMI and the risk of radiotoxicity were most pronounced. To our knowledge, this is the first study to evaluate comprehensively the associations between oxidative stress-related genetic polymorphisms and the risk of radiotoxicity in a population with relatively homogeneous treatment.

In this appraisal of the effects of polymorphisms involved in an oxidative stress mechanism on acute toxicity from radio-

therapy, findings did not support our *a priori* hypothesis that genotypes related to higher oxidative stress would be associated with an increase in risk of acute radiotoxicity. There are several possible explanations for the null associations in this analysis. One possible explanation is that slight effects of variability in oxidative stress-related genotypes are overwhelmed by the massive generation of ROS caused by radiation therapy. It is unlikely that null findings are due to a lack of relevance of the polymorphisms evaluated. All genetic polymorphisms are functional, and we previously reported differential associations by three of these genotypes with the risk of breast cancer (23–25). We found previously that MnSOD and MPO genotypes related to higher oxidative stress were associated with a better breast cancer survival among women who were treated for cancer, possibly related to better radiotherapy and/or chemotherapy efficacy (14). It is also possible that null

Table 2. Associations of BMI and the risk of radiotoxicity by CAT, MnSOD, MPO, and eNOS genotypes (n = 447)

BMI	No.	Toxicities		HR* (95% CI)	No.	Toxicities		HR* (95% CI)	
		CAT CC (better removal of ROS or NO), n = 233				CAT CT+TT (lower removal of ROS or NO), n = 184			
<25	111	10		1	99	10		1	
25-30	83	21		2.98 (1.35-6.57)	57	15		2.87 (1.24-6.63)	
30+	39	12		4.43 (1.77-11.09)	28	7		2.46 (0.86-7.08)	
<25	111	10		1	99	10		1	
≥25	122	33		3.33 (1.57-7.06)	85	22		2.73 (1.25-5.96)	
<i>P</i> _{trend}				0.0005				0.06	
<i>P</i> _{multiplicative interaction} [†]								0.56	
				MnSOD TT (better removal of ROS or NO), n = 113		MnSOD TC+CC (lower removal of ROS or NO), n = 315			
<25	57	8		1	166	14		1	
25-30	38	10		2.40 (0.90-6.37)	101	24		2.65 (1.32-5.31)	
30+	18	6		3.74 (1.20-11.72)	48	13		3.49 (1.58-7.69)	
<25	57	8		1	166	14		1	
≥25	56	16		2.76 (1.13-6.76)	149	37		2.90 (1.52-5.55)	
<i>P</i> _{trend}				0.0015				0.01	
<i>P</i> _{multiplicative interaction} [†]								0.57	
				MPO AG+AA (lower generation of ROS), n = 146		MPO GG (higher generation of ROS), n = 251			
<25	74	8		1	127	11		1	
25-30	46	11		2.37 (0.92-6.13)	92	24		3.38 (1.60-7.14)	
30+	26	6		1.90 (0.59-6.07)	32	9		4.30 (1.75-10.59)	
<25	74	8		1	127	11		1	
≥25	72	17		2.21 (0.91-5.39)	124	33		3.61 (1.78-7.35)	
<i>P</i> _{trend}				0.18				0.0002	
<i>P</i> _{multiplicative interaction} [†]								0.27	
				eNOS GT+TT (lower generation of NO), n = 239		eNOS GG (higher generation of NO), n = 187			
<25	125	15		1	96	6		1	
25-30	73	13		1.23 (0.56-2.70)	69	22		6.13 (2.38-15.75)	
30+	41	13		2.76 (1.27-6.01)	22	5		8.17 (2.29-29.18)	
<25	125	15		1	96	6		1	
≥25	114	26		1.72 (0.88-3.38)	91	27		6.39 (2.53-16.15)	
<i>P</i> _{trend}				0.006				0.0001	
<i>P</i> _{multiplicative interaction} [†]								0.25	

*Adjusted for hospital (four clinics) and photon beam quality for whole breast (two categories) and boost irradiation (no boost and four categories).

[†]To test statistical interactions on a multiplicative scale, a cross-product term of the ordinal score for each genotype and BMI was included. The log-likelihood statistic for models that included a multiplicative interaction term was compared to those that did not.

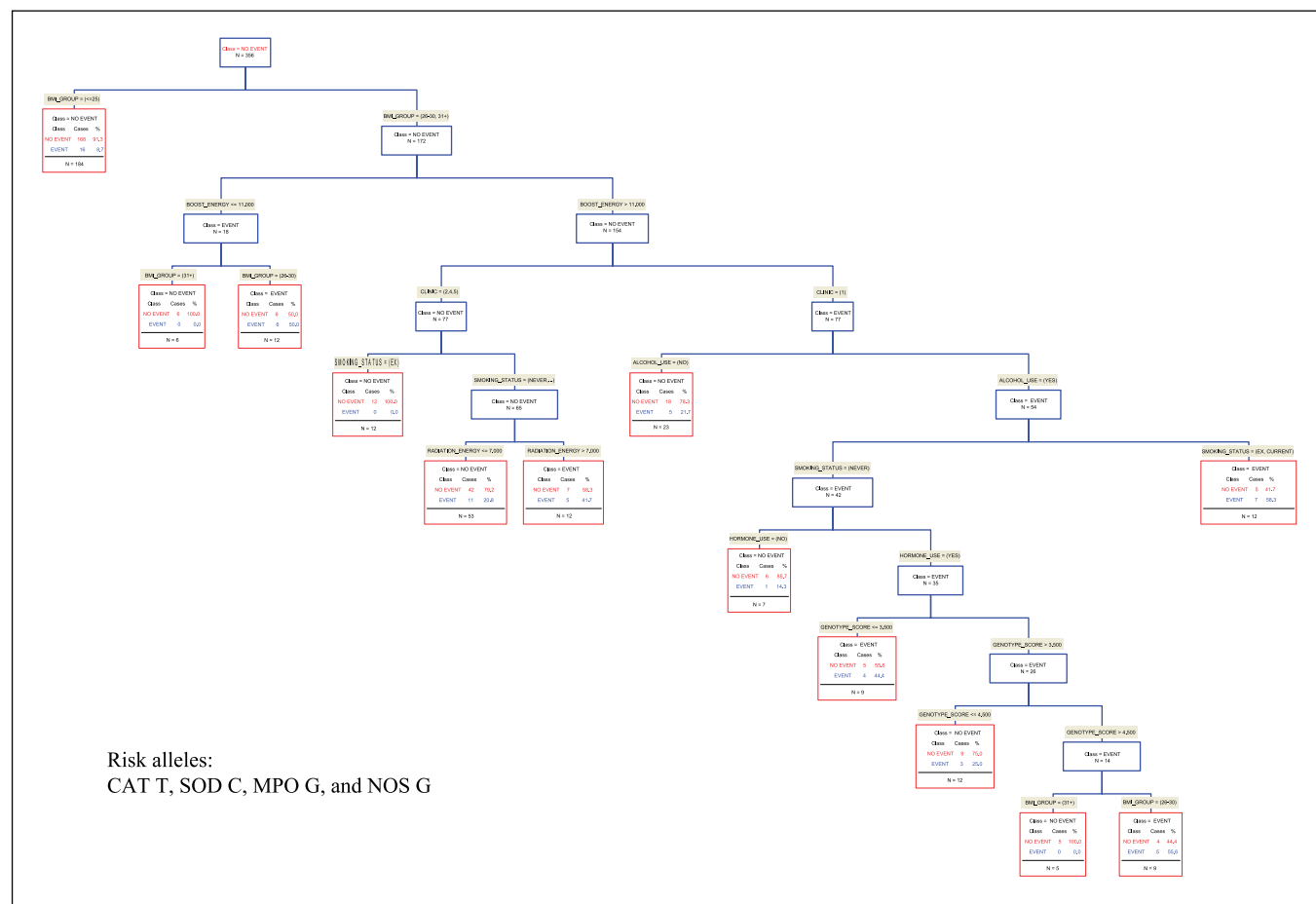


Fig. 1. CART analysis among 356 women with complete genotyping information for *MnSOD*, *CAT*, *MPO*, and *eNOS*. Events indicate the outcome of acute radiotoxicity. Splitting variables are indicated in grayed boxes along with the splitting value. Terminal nodes contain the number of observations within each class assignment (event/no event) followed by an underline and the total number of observations in the node. Splitting variables and values predicting acute radiotoxicity include the following: BMI (BMI_GROUP: ≤25 versus 26-30 versus 31+), radiation boost energy (BOOST_ENERGY: ≤11 versus >11), clinic [CLINIC: (clinic) 1 versus (clinics) 2, 3, and 4], smoking status (SMOKING_STATUS: ex-smoker versus current or never smokers), alcohol (ALCOHOL_USE: yes versus no), photon beam energy (RADIATION_EHEREARE_ENERGY: ≤7 versus >7), hormone therapy use (HORMONE_USE: yes versus no), and total carriage number of risk alleles for *MnSOD*, *CAT*, *MPO*, and *eNOS* (GENOTYPE_SCORE: 0-3 versus 4 versus 5-8).

findings for toxicity, in contrast to positive findings for survival in our previous study, could be due to differential effects of ROS on normal versus tumor tissue (5). However, further studies are needed to clarify exact cellular mechanisms.

In our analysis, development of acute side effects of grade 2c and above was considered an indication of increased sensitivity to radiotoxicity. By choosing grade 2c (moist desquamation of the skin or interruption of radiotherapy because of side effects) as an indicator of increased acute toxicity, we selected an indicator that is less prone to variability in classification. When we used different cutoff points [i.e., 2b or higher (35% of study population) and 2a or higher (70% of study population)], the estimates remained unchanged (data not shown). Thus, our findings are robust.

We found that associations between the risk of radiotoxicity and BMI were more notable among women with *eNOS* GG or *MPO* GG genotypes, related to higher generation of NO and ROS, than among those with at least one *eNOS* T allele or at least one *MPO* A allele. The higher risk of acute toxicity among overweight/obese women was somewhat expected because overweight women tend to have a large breast size, and radiotherapy of large breasts requires a special radiation

protocol with tangential radiation fields, which often results in an increased maximum radiation dose at the surface. Among obese women, this may result in difficulty in delivering a homogeneous dose. To determine if the effects of genotypes among overweight women were influenced by heterogeneous dosing, we reanalyzed the data with maximum dose of BED and found that estimates were unchanged from those used in the primary analyses. Thus, it is unlikely that the observed interaction was due to nonhomogeneous dose distribution.

Overweight/obesity is associated with higher systemic body oxidative stress, and several studies showed that levels of oxidative stress or inflammation biomarkers were higher in overweight women (26, 27). Adipocytes produce cytokines (i.e., interleukin and tumor necrosis factor) in response to lipopolysaccharides, catecholamines, or intracellular triglycerides, and these cytokines stimulate neutrophils and eosinophils to produce ROS. *MPO* and *NOS* are enzymes that generate ROS in response to these cytokines (28). Thus, it is plausible that higher BMI, which is related to higher oxidative stress and inflammation, may be associated with an increased risk of radiotoxicity particularly among women with endogenously higher generation of ROS by having *NOS* GG and *MPO* GG

genotypes. In this study population, the inverse associations between DNA repair XRCC Gln (Ex10-4A>G, rs#25487) or APE1 Glu (Ex5+5T>G, rs#3136820) alleles and risk of radiotoxicity were more pronounced among normal weight women, compared with overweight (29), consistent with our findings.

There is support in the literature for differential findings for BMI and acute radiotoxicity by eNOS genotypes. Both mRNA and protein levels of eNOS were significantly higher in s.c. adipose tissues of obese humans than nonobese humans (30). NO has been shown to modulate glucose transport and lipoprotein hydrolysis and thus may be positively associated with BMI (31). This is consistent with our findings that high BMI was associated with increased radiotoxicity risk, particularly among women with high activity eNOS genotype. However, cell sizes were small and risk estimates were somewhat unstable; thus, the results may also be attributable to chance.

Alternatively, these findings could indicate that the effects of ROS are greater in a higher hormonal milieu (i.e., higher BMI) and that eNOS expression may play a role in the complex relationships between oxidative stress, hormonal factors, and the risk of radiotoxicity. Estradiol stimulates eNOS activity through an Akt/protein kinase B–dependent pathway (32); in postmenopausal women, reduced endometrial expression of eNOS has been reported, which is reversible by hormone replacement therapy (33). Although evidence is limited, there are indications that steroid hormones regulate MPO expression. For example, circulating variations in MPO are positively associated with estrogen levels during the menstrual cycle (34), and hormone replacement therapy increases MPO release from neutrophils in postmenopausal women (35). Thus, a hormonal mechanism could explain relationships between high activity of eNOS or MPO, high BMI, and the risk of acute toxicity, although this is speculative. However, hormonal therapy did not modify associations between genotypes and the risk of acute toxicity in our population, and the associations among eNOS or MPO, BMI, and risk of radiotoxicity did not differ by menopausal status (data not shown).

CART provided an exploration of many predictor variables that could be related to risk of radiotoxicity and, as an exploratory analysis, uncovered complex high-order interactions that are difficult or impossible to model using traditional multivariate techniques. CART results confirmed previous findings that BMI is an important risk factor for radiotoxicity. Although CAT, MnSOD, MPO, and eNOS genotypes were not associated with the risk of toxicity in the overall population, total carriage number of those risk alleles contributed, in part, to acute toxicity outcomes among a subgroup of women who had BMI >25, received boost irradiation of 12, 15, or 18 electrons, attended one of the four enrollment clinics, drank alcohol, never smoked, and took hormone therapy. This is one advantage of CART analysis because it can find the subgroup that is otherwise hidden by using traditional modeling

methods. However, interpretation of CART results should be cautious because the tree structure was somewhat complicated, and only small numbers of women were in the subgroup. Nonetheless, these analyses provide a model for future evaluations of multiple factors that may predict prognosis among patients receiving therapy for cancer.

Although results could be affected by confounding, this is unlikely because we tested possible confounding factors (i.e., tumor stage, node status, metastatic status, hormone therapy use, age, smoking, BMI, smoking status, and alcohol use), finding that none of them changed the estimated effect by 10% or more when those variables were included in the model. Thus, estimates are less likely to be confounded by those variables. It is also possible that nondifferential misclassification bias due to genotyping error could deflate the estimates toward the null (36, 37). However, the likelihood of genotyping error is low; genotyping was done with matrix-assisted laser desorption/ionization time-of-flight technology, which is a highly accurate genotyping method, and there was a concordance rate of 100% in the 8% of randomly selected duplicates that were included for quality control purposes. Thus, it is unlikely that null associations were due to nondifferential information bias or negative confounding. Because cell sizes were small and risk estimates were somewhat unstable in the stratified analysis, the results may also be attributable to chance. Finally, assessment of late reactions for determination of clinical radiotoxicity needs to be further investigated.

We present one of the first reports to evaluate the complex role of genetic and nongenetic factors in relation to the risk of acute radiotoxicity. Our data provide the first report that overweight women with high-activity eNOS or MPO genotypes have a higher risk of acute toxicity from radiotherapy. By using CART analysis, we explored variables related to oxidative stress comprehensively and uncovered high-order interactions among those predictor factors, although interpretation of results should be cautious. This preliminary report needs to be replicated in a larger study. Although few studies have been conducted in this area, a better understanding of the factors related to radiotoxicity could have an important effect on improving quality of life among breast cancer patients undergoing therapy.

These data provide further support for a link between genes, BMI, and toxicity from radiation therapy and contribute to a better understanding of the role of BMI and eNOS and MPO genotypes in predicting treatment outcomes. It is encouraging to note that maintaining normal weight can help to minimize radiotoxicity, particularly for women with higher endogenous generation of NO or ROS. These findings underscore public health recommendations for maintaining healthy weight as an approach not only to reduce risk of postmenopausal breast cancer (38) but also to reduce toxicity associated with radiation therapy for breast cancer.

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