

Manganese superoxide dismutase (MnSOD) polymorphism, α -tocopherol supplementation and prostate cancer risk in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (Finland)

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Abstract

Objective: Manganese superoxide dismutase (MnSOD) is a mitochondrial enzyme that plays a key role in protecting the cell from oxidative damage. A polymorphism in the mitochondrial targeting sequence (a valine to alanine substitution), thought to alter transport of the enzyme into mitochondria, has been associated with increased risk for breast cancer with a more pronounced association among women with low intake of dietary antioxidants. We examined the role of MnSOD in the development of prostate cancer in a large, randomized cancer prevention trial of male smokers, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. We hypothesized that MnSOD may be associated with prostate cancer and that long-term antioxidant supplementation (α -tocopherol 50 mg/day for five to eight years) could modify the effect on risk.

Methods: Logistic regression was used to estimate these associations among 197 cases and 190 controls genotyped and matched for age, intervention group, and clinic.

Results: Men homozygous for the MnSOD ala allele had a 70% increase in risk over men homozygous for the val allele (odds ratio, OR = 1.72, 95% confidence interval, CI = 0.96–3.08, $p = 0.07$). Supplementation with α -tocopherol had no impact on the MnSOD–prostate cancer association. Although there was no difference in the association with disease stage, men homozygous for MnSOD ala (compared to MnSOD val/val or val/ala) showed a three-fold risk increase for high-grade tumors (OR = 2.72, 95% CI: 1.15–6.40, $p = 0.02$).

Conclusion: These data suggest an effect of the MnSOD ala/ala genotype on the development of prostate cancer. Our observation of a stronger association with high-grade tumors may have prognostic implications that should also be pursued.

Introduction

Oxidative damage induced by the generation of reactive oxygen species (ROS) by exogenous and endogenous

exposures is thought to be involved in prostate carcinogenesis [1, 2]. Oxidative stress can result in DNA damage including breakage, as well as lipid peroxidation, protein modification, membrane disruption, and mitochondrial damage [3, 4]. Manganese superoxide dismutase (MnSOD, also referred to as SOD2) is the primary antioxidant enzyme in the mitochondria that plays a key role in the detoxification of superoxide free radicals and protects cells from oxidative stress [5]. MnSOD is a nuclear-encoded protein that is transported

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into the mitochondria *via* an amino-terminal targeting sequence that is cleaved upon entry into the mitochondria [6]. As mitochondria are considered the principal source of ROS generation, with mitochondria consuming >95% of the oxygen in most cells [7], MnSOD likely plays a pivotal role in protecting cells from ROS-induced oxidative damage.

A polymorphism in the MnSOD gene in the mitochondrial target sequence that changes the amino acid codon at -9 from valine (GTT) to alanine (GCT) was recently described [8]. This change was predicted to alter the secondary structure of the protein and to affect the transport of the enzyme into mitochondria, although this has not been demonstrated experimentally. The polymorphism was found to be prevalent in the population (the frequency of the alanine allele was ~50% among Caucasian women) and the homozygous alanine genotype was associated with a four fold increased risk for breast cancer among premenopausal women [9]. The effect of the polymorphism was more pronounced among women who reported low dietary intake of antioxidants, suggesting that dietary antioxidants may compensate for lowered MnSOD activity. An association between MnSOD and breast cancer was confirmed in a subsequent study of Finnish women, although only a 50% risk increase was observed. The authors found that supplementation with antioxidants had no effect on the MnSOD-breast cancer association, however [10]. Thus far, the association of MnSOD with prostate cancer has not been reported.

We evaluated the association between the MnSOD polymorphism and prostate cancer risk in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study. Because the trial results showed a 32% reduction in the incidence of prostate cancer and a 41% reduction in mortality in response to daily supplementation with α -tocopherol (vitamin E) [11], we were also interested in whether long-term α -tocopherol supplementation (or baseline dietary and serum antioxidant status) modified the effect of MnSOD on prostate cancer risk.

Subjects and methods

Study population

We conducted a nested case-control study within the ATBC Study conducted in Finland. This was a randomized, placebo-controlled prevention trial that tested the efficacy of five to eight years of supplementation with α -tocopherol (50 mg/day), β -carotene (20 mg/day), or both in reducing the incidence of lung, prostate and other cancers. The ATBC Study cohort consisted of

29,133 white male smokers of at least five cigarettes daily. Participants were recruited between 1985 and 1988 and followed during the active trial period until death or April 30, 1993. Men were also followed post-intervention. The overall design, rationale, and objectives of this study have been published as have the main trial findings [11, 12]. The trial showed a 16% increase in lung cancer incidence among subjects in the β -carotene supplemented group and a 32% reduction in prostate cancer in the α -tocopherol group. General medical history, diet, smoking, and other background data along with a fasting blood sample was collected from all subjects at baseline. The dietary information was gathered using a validated, self-administered food-use questionnaire given to all participants prior to randomization. The questionnaire was linked to the food composition database of the National Public Health Institute of Finland. The ATBC Study was approved by the institutional review boards of the National Cancer Institute (US) and the National Public Health Institute of Finland.

Selection of cases and controls

Incident cases of prostate cancer cases (ICD9-185) diagnosed between 1983 and December 31, 1994 ($n = 208$) with a whole blood sample available (collection of bloods for genotyping took place between 1992 and 1993, $n = 20,305$ men) were identified through the Finnish Cancer Registry and the Register of Causes of Death. The medical records of the cases were centrally reviewed independently by two study oncologists and the histopathologic specimens by one or two pathologists. Information on disease stage and grade were available for 99 and 85% of the subjects, respectively. Sixty-four percent of the cases were diagnosed with localized disease (stage 0-II), 11% with regional (stage III), and 25% with remote disease (stage IV). About 17% of the cases had tumors considered to be poorly differentiated (grade 3, roughly equivalent to Gleason grade 8-10). Controls were selected from the same cohort of men with blood available for genotyping and were matched 1:1 to cases by age (± 5 years), intervention group, and study clinic.

DNA isolation and MnSOD genotyping analysis

DNA was isolated from whole blood samples as previously described [13]. MnSOD genotyping was conducted using matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Sequenom, San Diego, CA). This method distinguishes different alleles by generating allele-specific

products with mass differences that can be determined using mass spectrometry. The method includes an initial step of PCR amplification of MnSOD gene region containing the polymorphism followed by allele-specific primer extension using extension primers that create two products differing by one to three nucleotides 3' to the single nucleotide polymorphism (SNP). The allele-specific extension products are then distinguished by mass spectrometry. PCR of the MnSOD gene containing the polymorphic regions was conducted using 5'-ACGTTGGATCTGTGCTTTCTCGTCTTCAG (sense primer) and 3'-ACGTTGGATTTCTGCCTGGAGCC-CAGATAC (antisense primer). All PCR reactions were performed in 5 μ l volume using 2.5 ng of genomic DNA, 1 \times PCR buffer (Qiagen), 2.5 mM MgCl₂, 200 μ M of each dNTP, 0.1 unit of Taq Polymerase (Qiagen) and 200 nM each primer. Thermal conditions were 95 °C for 15 min, 45 cycles of 95 °C for 20 s, 56 °C for 30 s and 72 °C for 2 min with a final extension of 72 °C for 3 min. Purified PCR products were used for the primer extension reaction after addition of extension mix (9 μ l), containing 600 nM extension primer (5'-GGAGCCAGATACCCCAA), 50 μ M each of ddATP, ddCTP, ddTTP, and dGTP, 1 \times buffer, and 0.064 unit of ThermoSequenase (Sequenom, San Diego, CA). Primer extension reactions were performed at 94 °C for 2 min followed by 40 cycles of 94 °C for 5 s, 52 °C for 5 s and 72 °C for 5 s. Primer extension products (alanine allele: 5'-GGAGCC-CAGATACCCCAA; and valine allele: 5'-GGAGCC-CAGATACCCCAAAGC) were purified using SpectroCLEAN resin (Sequenom). Approximately 14 μ l of each extension product was transferred onto a silicon microchip forming a crystalline matrix. The matrix was pulsed with ultraviolet light causing ionization of the analyte molecules. The raw spectral data was transferred from the SpectroREADER Biflex detector (Sequenom) into a pre-programmed template using SpectroTYPER software (Sequenom). The software assigned genotype probabilities using the programmed assay definitions and calculated mass differences. Genotyping was performed in batches containing equal number of cases and controls, and negative controls (PCR reagents without DNA) were included with each batch. A random sample of 10% of the study samples were repeated for quality control and showed 98% concordance. Genotyping was successful for 197 cases and 190 controls, the final samples used for this analysis.

Statistical analyses

All statistical analysis were performed using statistical analysis systems (SAS) software package (SAS Corp,

Cary, NC). The associations between the various risk factors and the development of prostate cancer were evaluated using logistic regression. Differences between cases and controls for selected baseline characteristics and MnSOD genotype status were examined using the Wilcoxon rank sum test for continuous variables and the χ^2 test for categorical variables. Multivariate models were developed including age as a continuous term and intervention group as an indicator variable for supplementation with α -tocopherol or no α -tocopherol (reference group). Intervention assignment was assessed as a dichotomous variable comprised of α -tocopherol supplemented (includes α -tocopherol only and α -tocopherol plus β -carotene) versus non-supplemented groups (includes placebo and β -carotene only) to increase power in accordance with the trial's factorial design [12]. Other study factors were assessed as confounders by evaluating whether their inclusion into the multivariate model changed the odds ratios by more than 10% or led to a significant change in the likelihood ratios ($p < 0.05$). Effect modification of the MnSOD-prostate cancer association by α -tocopherol intervention group and other selected covariates was tested by including the cross-product interaction term of the MnSOD genotype and each covariate in the multivariate regression models and by stratified analyses.

Results

The characteristics of prostate cancer cases and controls are shown in Table 1. There were essentially no differences in demographic, dietary, or serum antioxidants between cases and controls. The distribution of the MnSOD genotype among the controls was 0.26 for val/val, 0.53 for the val/ala, and 0.21 for ala/ala (Table 2). The genotype distribution of the controls are in Hardy-Weinberg equilibrium ($p = 0.82$) and the distribution is similar to that observed in a population of US Caucasian women [9] and in Finnish women [10]. Due to the potential effects of MnSOD on many chronic diseases or conditions (e.g., neurological, cardiac, and inflammation [14]) and since our population was composed of a clinical trial population of older males who were long-term heavy smokers, we were concerned that the MnSOD polymorphism may influence overall survival. That is, men with the MnSOD ala/ala polymorphism might have developed a smoking-related disease earlier in life and were not available to participate in the trial or ineligible due to smoking-related illness. We therefore evaluated MnSOD genotype in another Finnish population consisting of younger, healthy volunteers recruited from a blood bank. The distribution of the genotype

Table 1. Baseline characteristics according to prostate case-control status, ATBC Study, Finnish men

	Mean and standard deviation		p-Value ^a
	Cases (n = 197)	Controls (n = 190)	
Age at baseline, year	60.8 (5.2)	60.6 (5.0)	0.80
Age at diagnosis, year	66.2 (5.2)	–	–
Body mass index ^b	26.4 (3.4)	25.8 (3.4)	0.10
Energy intake ^c	2711 (521)	2819 (820)	0.20
Dietary antioxidants ^c			
α -Tocopherol (mg/day)	10.4 (4.9)	10.8 (5.3)	0.45
β -Carotene (μ g/day)	2127 (1371)	2096 (1329)	0.83
Vitamin C (mg/day)	95.0 (39.7)	95.9 (42.4)	0.84
Serum antioxidants			
α -Tocopherol (mg/l)	11.9 (2.4)	11.9 (2.6)	0.88
β -Carotene (μ g/l)	234 (184)	209 (141)	0.13

Table 2. Association between MnSOD polymorphism and prostate cancer risk, ATBC Study, Finnish men

	Cases # (%)	Controls # (%)	OR (95% CI) ^a	p-Value
All cases				
MnSOD val/val	43 (21.6)	49 (25.7)	1.00 (ref.)	–
MnSOD val/ala	98 (49.3)	102 (53.4)	1.12 (0.68–1.85)	0.65
MnSOD ala/ala	58 (29.2)	40 (20.9)	1.72 (0.96–3.08)	0.07
α -Tocopherol group ^b				
MnSOD val/val	19 (23.2)	21 (26.2)	1.00 (ref.)	–
MnSOD val/ala	32 (39.0)	39 (48.8)	0.91 (0.42–1.98)	0.81
MnSOD ala/ala	31 (37.8)	20 (25.0)	1.72 (0.74–3.97)	0.21
No α -tocopherol group				
MnSOD val/val	23 (22.6)	28 (25.4)	1.00 (ref.)	–
MnSOD val/ala	66 (57.4)	63 (57.3)	1.29 (0.67–2.50)	0.44
MnSOD ala/ala	26 (22.6)	19 (17.3)	1.68 (0.75–3.78)	0.21

^a Odds ratio and 95% confidence interval adjusted for baseline age and α -tocopherol intervention group.

^b p-Interaction based on interaction term of MnSOD ala/ala versus MnSOD val/val or val/ala and α -tocopherol intervention group ($p = 0.55$).

between the two populations was similar, suggesting that MnSOD did not influence overall survival among our trial population (data not shown).

The risk of prostate cancer associated with the MnSOD ala/val heterozygous genotype and the MnSOD ala/ala genotype are presented in Table 2. There was no risk increase for the heterozygous genotype, whereas the homozygous variant was associated with a 70% risk increase ($p = 0.07$). We assessed whether supplementation with α -tocopherol (vitamin E) (study subjects were supplemented between five and eight years in a randomized trial) modified the effect of the MnSOD genotype on prostate cancer and observed no differences in risk between the α -tocopherol supplemented versus no supplement groups (Table 2). Further, we did not observe modification of the MnSOD–prostate cancer

association by β -carotene intervention, baseline dietary antioxidant status (α -tocopherol, β -carotene, and vitamin C) or serum antioxidant status (α -tocopherol and β -carotene) (data not shown).

Results of the subset analyses based on tumor grade/stage of disease are shown in Table 3. In order to improve precision for the subset analyses, we included the heterozygous individuals in the reference category (since our data presented in Table 2 demonstrate no association with the heterozygous genotype). There was a 2.7-fold increased risk for a high-grade tumor (poorly differentiated) among men homozygous for the ala allele compared to men heterozygous or homozygous for val allele. However, there were no differences in the association between MnSOD genotype and prostate cancer risk according to stage of disease.

Table 3. Association of MnSOD ala/ala polymorphism and prostate cancer risk overall and according to stage and grade of disease, ATBC Study, Finnish men

	OR (95% CI) ^a	<i>p</i> -Value
Total cases (n = 197)		
MnSOD val/val or ala/val ^b	(ref.)	
MnSOD ala/ala	1.57 (0.98–2.50)	0.06
Grade, degree of differentiation ^c		
Well (n = 69)	1.49 (0.78–2.83)	0.23
Moderate (n = 70)	1.33 (0.69–2.55)	0.38
Poor (n = 28)	2.72 (1.15–6.40)	0.02
Stage ^c		
Localized (0–II) (n = 125)	1.58 (0.93–2.69)	0.08
Regional (III) (n = 22)	2.00 (0.77–5.20)	0.15
Remote (IV) (n = 49)	1.42 (0.68–2.98)	0.35

^a Odds ratio and 95% confidence interval for MnSOD ala/ala compared to MnSOD val/val and val/ala (reference group).

^b Genotypes adjusted for baseline age and α -tocopherol intervention.

^c Information on stage of disease and tumor grade only available for 167 and 196 cases, respectively.

We also assessed effect modification by other study factors including age, age at cancer diagnosis, body mass index, total caloric and fat intake, alcohol consumption and smoking and observed no significant interactions.

Discussion

We evaluated the association between a polymorphism in the MnSOD gene and prostate cancer risk in a subset of participants of the ATBC Cancer Prevention Study, with a particular interest in whether the association was modified by α -tocopherol supplementation. We observed a 70% increase in overall prostate cancer risk associated with the MnSOD ala/ala genotype. Long-term α -tocopherol supplementation did not, however, appear to modify the effect of MnSOD on prostate cancer risk. Interestingly, individuals with the MnSOD ala/ala genotype had a three-fold increased risk for a high-grade tumors, a histopathologic indicator of more aggressive disease.

The MnSOD ala/ala genotype has previously been associated with a four-fold increase in risk for breast cancer among pre-menopausal women only, particularly among women who consumed lesser amounts of dietary antioxidants and in whom a six-fold risk increase was shown for the MnSOD ala/ala genotype [9]. A second study found a significant but less substantial increase (1.5-fold increase) for breast cancer among Finnish

women, with no real difference between pre- and post-menopausal women [10]. This study also showed no modification of the MnSOD-breast cancer association by antioxidant supplements.

Our interest in MnSOD and prostate cancer risk in the ATBC Study cohort was in part motivated by our observation of a significant 32% reduction in prostate cancer incidence among the trial participants assigned to the α -tocopherol group. Further, MnSOD may be particularly important in the prostate since the gland is thought to be rich in mitochondria (mitochondria synthesize citrate, a major constituent of seminal fluid) [15]. In this setting, α -tocopherol supplementation might compensate for lower MnSOD activity, particularly among men possessing the homozygous variant genotype, by reducing free radicals produced in the mitochondria. We observed no such modification of the MnSOD–prostate cancer association by α -tocopherol supplementation in the present study, however.

Although the functional significance of the amino acid substitution from a valine to alanine in the mitochondria targeting sequence has not been demonstrated experimentally, it was predicted (by computer simulation) to alter transport of the enzyme into the mitochondrion [8]. This might result in the reduced capacity to scavenge free radicals in mitochondria which would have the potential to disrupt critical cellular processes and increase the risk for neoplastic transformation. It is thought that ROS at low concentrations can activate signal transduction pathways and alter the expression of growth- and differentiation-related genes whereas ROS at high concentrations are detrimental to the cell [16]. Antioxidant enzymes such as MnSOD serve as ROS scavengers and have been shown to prevent malignant transformation *in vitro* and *in vivo*. These experiments demonstrated that increasing MnSOD levels can suppress the malignant phenotype as evidenced by slower cell growth rate, lower colony formation, and less tumor formation in nude mice [17–20].

The stronger association of MnSOD with high-grade tumors suggest that MnSOD activity may have prognostic implications, given that poorly differentiated tumors are associated with recurrence, metastatic disease and poor survival [21, 22]. Studies evaluating MnSOD protein expression in tumors support an association between MnSOD activity and cell differentiation, with several cancer types showing reduced MnSOD levels [23–25] and lower levels correlating with de-differentiation [17]. It would be tempting to speculate that the MnSOD polymorphism may have potential prognostic importance, especially if these findings are borne out in other larger studies in diverse populations.

In conclusion, our data support a role for the MnSOD ala/ala genotype in the development of prostate cancer. Neither high intake of dietary antioxidants nor long-term supplementation with α -tocopherol appeared to modify the effect of MnSOD on prostate cancer risk.

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