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MnSOD Genotype and Prostate Cancer Risk as a Function of *NAT* Genotype and Smoking Status

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Abstract. *Background: Cigarette smoke contains carcinogenic aromatic and heterocyclic amines that are metabolized by N-acetyltransferase (NAT). These carcinogens also produce reactive oxygen species that are metabolized by manganese superoxide dismutase (MnSOD). The association between prostate cancer (PCA) and the polymorphism of MnSOD and NAT, and cigarette smoking was investigated. Patients and Methods: DNA samples from 187 PCA patients and 175 age-matched controls were genotyped for MnSOD, NAT1 and NAT2 by PCR restriction fragment length polymorphism analysis and DNA sequencing. Results: MnSOD AA genotype, as compared to MnSOD VV and VA, was associated with PCA (odds ratio, 1.65; 95% confidence interval, 1.03-2.66. There was no association of PCA with NAT or smoking. Results of exploratory analyses of the data suggest that the association of PCA and MnSOD exists only in the subpopulation of rapid NAT1 genotypes and smokers. Conclusion: The present study demonstrates the association of PCA and MnSOD. Oxidative stress and cigarette smoking may play an important role in the carcinogenesis of the prostate in those who have MnSOD AA and rapid NAT1 genotypes.*

Prostate cancer (PCA) is the most prevalent non-skin malignancy in the US male and it is estimated that 1 in 6 males probably will develop invasive PCA in his lifetime (1). The etiology of PCA is multifactorial and it is suspected that environmental factors contribute to the majority of the cancers, including familial PCA (2). Chronic inflammation has been associated with the development of malignancy in several organs such as the esophagus, stomach, colon, liver and urinary bladder (3). Epidemiological data have correlated PCA with prostatitis and sexually transmitted diseases (4). Inflammation invariably produces reactive oxygen species (ROS), which can damage both mitochondrial and nuclear DNA (5, 6). Oxidative damage to nuclear DNA may result in the arrest or induction of transcription, induction of signal transduction pathways, replication errors or genomic instability, all of which may result in carcinogenesis (7, 8).

Approximately 2% of oxygen in the body is converted to superoxide radicals and its reactive metabolites in the mitochondria (9). Manganese superoxide dismutase (MnSOD) is present in the mitochondria and plays an important role in protection from ROS-mediated DNA damage. MnSOD converts superoxide radical to oxygen and hydrogen peroxide (10). The human *MnSOD* gene has a polymorphism at codon 16, which encodes for either alanine (A) or valine (V) (11). This polymorphism has been reported to be a risk factor for several malignancies. The A allele has been reported to be a risk factor for breast cancer (12, 13). It was further observed that breast cancer risk was elevated in premenopausal women who had the AA genotype with low consumption of dietary antioxidants (13). The risk was also increased in women with the AA genotype who smoked (14). A cohort study of Finnish male smokers suggested that men with the AA genotype had a 1.70-fold greater risk for PCA as compared to those with other *MnSOD* genotypes (15). However, this finding was not confirmed (16). Furthermore, it was reported that the AA genotype was a risk factor only for individuals with aggressive PCA who had low serum antioxidant levels, but not for the entire PCA cohort (17). Therefore, further investigation is needed to elucidate this association.

Cigarette smoking is a well-known risk factor for a variety of cancers, but there is no convincing evidence that it is associated with PCA (18). *N*-Acetyltransferases (NAT) are

Abbreviations: MnSOD, manganese superoxide dismutase; NAT, N-acetyltransferase; PCA, prostate cancer; PSA, prostate-specific antigen; ROS, reactive oxygen species.

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involved in the metabolic activation of carcinogenic aromatic and heterocyclic amines present in cigarette smoke (19). *NAT1* and *NAT2* genotypes are polymorphic in humans and they are generally described as rapid or slow acetylators (20, 21). Polymorphism of *NAT* as a risk factor for PCA has been under investigation (22). Cigarette smoke also produces ROS (23). In the present study, we investigated the association of the *MnSOD* genotype and prostate cancer risk as a function of *NAT* genotype and smoking status.

Patients and Methods

Study participants. The study was approved by the Institutional Review Board, SUNY Upstate Medical University. Patients were recruited between 1998 and 2001 from the Urology Clinic of University Hospital of SUNY Upstate Medical University, Syracuse, NY. The clinic serves the population of the rural central New York and the Syracuse area. Patients were either recently diagnosed with PCA or had PCA and were under follow-up or treatment. Age-matched controls were recruited from healthy males seeking annual urological examinations and from prostate cancer-screening programs. Individuals with normal digital rectal examination and prostate-specific antigen (PSA) <4 ng/ml were considered for inclusion as controls. Participants were questioned as to how many cigarettes and for how long they had smoked. Those who had smoked more than 100 cigarettes in their lifetime were categorized as smokers (24).

Genotyping. DNA was extracted from buccal swabs using QIAamp DNA Blood Mini Kits (Qiagen, Valencia, CA, USA) according to the supplier's instructions. *MnSOD* was PCR amplified with a primer pair (5'-GTGCTTTCTCGTCTTCAGCA-3' and 5-AACGCCTCCTGGTACTTCTCC-3') for 30 thermocycles: 94˚C for 30 s, 55˚C for 30 s, 72˚C for 30 s. PCR products were digested with BsaWI (60˚C, 1 h; New England BioLabs, Beverly, MA, USA). Digested products were visualized on a 2.5% agarose gel stained with ethidium bromide. Fragment patterns specific for three *MnSOD* genotypes were VV (GTT; 176 bp, 37 bp), VA (GTT/GCT; 213 bp, 176 bp, 37 bp) and AA (GCT; 213 bp).

NAT1 was PCR amplified from codon 124 through the polyadenylation region using a primer pair (5'-TGGGTTTGG ACGCTCATA-3' and 5'-TCACCAATTTCCAAGATAAC-3') with the following thermal program: 94˚C for 1 min, 60˚C for 2 min, 72˚C for 1 min for 40 cycles. The amplicon was sequenced using primers (5'-GTGGCAGCCTCTGGAGT-3' and 5'-GACTCT GAGTGAGGAAGAAATA-3') with a Perkin Elmer ABI 310 automatic DNA sequencer. Genotypes were assigned according to the DNA sequences for the known *NAT1* alleles reported by Deitz *et al*. (25). Eight *NAT1* acetylator alleles (NAT1*3, NAT1*4, NAT1*10, NAT1*11, NAT1*14, NAT1*15, NAT1*16, and NAT1*18) and 14 genotypes were identified. Genes which contained at least one *10 allele were categorized as rapid *NAT1* (*NAT1R*) genotypes and others were categorized as slow *NAT1* (*NAT1S*) genotypes.

The entire 873 base-coding region of *NAT2* was PCR amplified using a primer pair (5'-GATCATGGACATTGAAGCATATT-3' and 5'-ATACATACACAAGGGTTTATTTTGT-3') with the same thermal program. *NAT2* was sequenced using nested primers (N109F and N440F; 5'-TTTGAACCTTAACATGCAT-3' and 5'-CTTGCATTTTCTGCTTGACA-3'). Nested primer N109F gave the *NAT2* sequence starting from base +109 and extending well past base +440. Likewise, nested primer N440F gave the sequence from the base +440 of *NAT2* and continued into the polyadenylation region. There were 12 discernable individual *NAT2* alleles (NAT2*4, NAT2*4A, NAT2*5A, NAT2*5B, NAT2*5C, NAT2*6A, NAT2*6B, NAT2*7B, NAT2*12A, NAT2*12B, NAT2*13 and NAT2*14B) found in this study. Analysis of the two sequenced portions of *NAT2* genomic DNA showed it was not possible to differentiate between allele combinations *4/*5B and *5A/*12A, *4/*6A and *6B/*13, *4/*7B and *7A/*13, *4/*12B and *12A/*13, *5A/*12B and *5B/*13, and *6A/*12A and *6B/*12B. However they all are rapid genotypes as alleles *4, *12A, *12B and *13 give rapid *NAT2* (*NAT2R*) phenotypes.

The laboratory personnel were unaware of the case-control status. To assess genotyping reproducibility, 25% of the samples were randomly selected for repeated determination and all genotypes were as initially determined.

Statistical analysis. Unconditional logistic regression models were used for statistical analyses using the software package JMP version 3.2.1, SAS Institute, Inc. (Cary, NC, USA). The 95% confidence interval (95% CI) was determined at the level of α=0.05 for type I error. The Pearson's chi square test or Fisher exact test was applied when the number in each sub-population was too small for logistic regression analysis after the population was stratified according to *NAT* genotype and smoking status. Likelihood ratio statistics were used when applicable. The analysis was adjusted for age, race and smoking status if possible; race and smoking status were treated as category variables and age as a numerical variable. Pearson's chi square test was applied to the Hardy-Weinberg equilibrium.

Results

This study consisted of 175 controls and 187 PCA patients whose genomic DNA was successfully genotyped for *MnSOD*. There were 145 Caucasians, 27 African-Americans and 3 other races in the control group and 162, 22 and 3, respectively, in the cancer group. The age was 62.3±9.9 and 63.3±8.0 years (mean±S.D.) for the control and cancer groups, respectively (p=0.842). There were 103 (61%) and 97 (52%) smokers in the control and cancer groups, respectively (p=0.182).

The genotype frequencies of *MnSOD* for VV, VA and AA were 0.229, 0.549 and 0.223 in the control group, and 0.219, 0.460 and 0.321 in the PCA group, respectively (Table I). The distribution of the genotypes in both the control group (p=0.438) or the cancer group (p=0.627) were consistent with the Hardy-Weinberg equilibrium. The observed numbers of these genotypes in the cancer group and the expected numbers of these genotypes derived from the observed frequencies in the control group were significantly different (p<0.01). As shown in Table I, the PCA group had a greater frequency of *MnSOD* AA genotype than did the control group.

Of the samples that were successfully genotyped for *MnSOD*, 170 control samples were successfully genotyped for both *NAT1* and *NAT2*, and 179 and 180 PCA samples were successfully genotyped for *NAT1* and *NAT2*, respectively. The results of logistic regression analysis

Table I. *Association between prostate cancer, MnSOD and NAT genotypes and smoking.*

	No. of individuals		Crude		Adjusted	
	Controls	Patients	OR	95% CI	OR	95% CI
MnSOD						
VV	40	41	1 (Ref.)			
VA	96	86	0.93	0.72-1.21	0.82*	0.48-1.41
AA	39	60	1.50	0.83-2.72	1.43*	0.79-2.62
VA+AA	135	146	1.06	0.64-1.73	1.01*	0.61-1.67
VA+VV	136	127	1 (Ref.)			
AA	39	60	1.65	1.03-2.64	1.65*	1.03-2.66
NAT1						
S	106	122	1 (Ref.)			
R	64	57	0.77	0.50-1.20	0.84*	0.54-1.33
NAT2						
S	92	111	1 (Ref.)			
R	78	69	0.73	0.48-1.12	0.68*	0.44-1.05
Smoking status						
No	72	90	1 (Ref.)			
Yes	103	97	0.75	0.49-1.14	0.75†	0.49-1.15

Data were analyzed with logistic regression adjusted for: *age, race and smoking; †age and race. MnSOD, manganese superoxide dismutase; NAT, *N*-acetyltransferase; OR, odds ratio; CI, confidence interval; S, slow NAT; R, rapid NAT.

Table II. *Association between prostate cancer and MnSOD AA according to NAT1 and NAT2 genotypes.*

Genotype		No. of individuals		Crude		
NAT1	*MnSOD*	Controls	Patients	OR	95% CI	*p*-Value*
NAT1R	VA+VV	53	37	1 (Ref.)		
	AA	11	20	2.60	1.12-6.0	0.025
NAT1S	VA+VV	79	87	1 (Ref.)		
	AA	27	35	1.18	0.65-2.11	0.586
	MnSOD-NAT1 interaction, p=0.131					
NAT2R	VA+VV	63	52	1 (Ref.)		
	AA	15	17	1.37	0.63-2.99	0.428
NAT2S	VA+VV	70	73	1 (Ref.)		
	AA	22	38	1.66	0.89-3.07	0.109
	MnSOD-NAT2 interaction, p=0.713					

*Analyzed with Pearson's chi square test. Similar *p*-values were obtained with likelihood ratio test. Abbreviations as Table I.

suggested that neither *NAT1* nor *NAT2* was associated with PCA (Table I). Additionally, smoking did not appear to be a risk factor for PCA (Table I).

Table III. *Association between prostate cancer and MnSOD AA according to smoking status.*

Smoking status		No. of individuals		Crude		Adjusted*	
	MnSOD	Controls	Patients	OR	95% CI	OR	95% CI
Yes	VA+VV	81	63	1 (Ref.)		1 (Ref.)	
	AA	22	34	1.98	1.06-3.71	1.89	1.00-3.57
No.	VA+VV	55	64	1 (Ref.)		1 (Ref.)	
	AA	17	26	1.31	0.65-2.66	1.45	0.69-3.03
	MnSOD-smoking interaction, p=0.393						

*Analysis with logistic regression adjusted for age and race. Abbreviations as Table I.

Table IV. *Association between prostate cancer and MnSOD AA according to smoking status in the NAT1R group.*

Smoking status		No. of individuals		Crude		
	MnSOD	Controls	Patients	OR	95% CI	*p*-Value*
Yes	VA+VV	36	16	1 (Ref.)		
	AA	5	11	4.26	1.03-13.9	0.015
No	VA+VV	22	21	1 (Ref.)		
	AA	6	9	1.57	0.49-5.02	0.456
	MnSOD-Smoking interaction, p=0.251					

*Analyzed with Pearson's chi square test. Likelihood ratio tests yielded similar *p*-values. Abbreviation as Table I.

The distribution of *MnSOD* genotypes was stratified according to *NAT1* genotype. There was an association between *MnSOD* AA and PCA in the rapid *NAT1* group (odds ratio, OR=2.6; Table II). In contrast to the rapid NAT1, *MnSOD* AA was not associated with PCA in the slow *NAT1* group (Table II). When stratified according to *NAT2* genotype, the association between *MnSOD* and PCA was not significant (Table II).

The distribution of *MnSOD* was stratified according to smoking status and it appeared that the *MnSOD* AA genotype was a risk factor for PCA in smokers but not in nonsmokers (Table III). The OR was greatly increased in smokers who also had a rapid *NAT1* genotype (Table IV). It appeared that the association between *MnSOD* AA and PCA was present in the subpopulation with *NAT1R* genotype who were also smokers. However, there was no interaction between *MnSOD*, *NAT1* and smoking.

Discussion

The association between *MnSOD* and PCA has been investigated. Woodson *et al*. (15) suggested the *MnSOD* AA genotype was associated with PCA in smokers, especially in patients having a high-grade tumor. Li *et al*.

(17) suggested that the *MnSOD* AA genotype was not associated with all PCA, but was associated with PCA in those patients who had low serum levels of antioxidants, such as selenium, lycopene and α-tocopherol. Their results suggest that those who with the *MnSOD* AA genotype are at risk for PCA under a condition of high ROS or low blood level of antioxidants. However, Choi *et al*. (16) suggested that there was no association between the *MnSOD* AA genotype and PCA, and smoking status did not modify associations. In the present study, we found an association between PCA and the *MnSOD* AA genotype, particularly in men who were smokers and/or had rapid *NAT1* genotypes.

Although the function of the *MnSOD* polymorphism is not well understood, Sutton *et al*. (26) showed that the A-containing MnSOD is transported more efficiently through the mitochondrial membrane. Therefore, individuals with the AA genotype may have higher MnSOD activity as compared to those with other genotypes. In mitochondria, superoxide radical is converted by MnSOD into oxygen and hydrogen peroxide, which is further detoxified into water by glutathione peroxidase. The rate of hydrogen peroxide decomposition is proportional to both the glutathione level and the activity of glutathione peroxidase. Under physiological conditions, the ability to decompose hydrogen peroxide is thousands of times greater than is the generation of superoxide radical (27). However, at a high concentration of peroxide, the step of NADP reduction becomes rate-limiting, and the overall reaction rate of the detoxification of peroxide decreases by 100 times (27). Thus, high activity of MnSOD may lead to metabolic imbalance and induce toxicity if the rate of hydrogen peroxide decomposition decreases. Furthermore, cigarette smoke can produce superoxide radical (23), which is involved in the redox-cycling of Fenton reagents such as ferric/ferrous ions (28, 29). The regeneration of oxidized Fenton reagents provides continuous conversion of hydrogen peroxide to hydroxyl radical that can modify DNA.

It has been suspected that heterocyclic amines that are present in cooked meat and metabolized by NAT may contribute to the carcinogenesis of prostate (19, 30, 31). Therefore, several attempts have been made to correlate NAT with PCA. It has been reported that *NAT1R* is a risk factor for PCA (22, 30), but the results from another study do not support this conclusion (32). The *NAT2S* genotype has also been reported to be a risk factor, particularly for high grade PCA (33), but other studies have not shown the *NAT2* genotype to be a risk factor for PCA (32, 34). We found that those individuals with *NAT1S* and *NAT2S* had a slightly increased risk for PCA but this was not statistically significant.

Despite numerous studies, cigarette smoking has not been conclusively shown to be a risk factor for PCA (18). We did not find an association between smoking and PCA either. However, we did find an association between *MnSOD* and PCA in smokers but not in nonsmokers. Furthermore, in the NAT1R/smoker group, those who with the *MnSOD* AA genotype had a 4-fold increased risk of PCA than those with other *MnSOD* genotypes (Table IV). That smoking increased the risk for PCA in individuals with *NAT1R* genotypes is consistent with the potential for acetyltransferase-mediated transformations of tobacco-derived carcinogenic aromatic and heterocyclic amines to metabolites capable of modifying DNA in the prostate (19). This is novel and important information, which may explain how cigarette smoking-derived carcinogens in urine may act directly by causing genetic damage and indirectly by causing inflammation that results in oxidative stress. Therefore, individuals with genetic variations which result in less efficient detoxification of carcinogens and handling of superoxide radicals would be at the greatest risk for developing PCA.

Our study has several limitations. Controls did not undergo biopsies to exclude PCA. This is an important aspect of verification bias that is inherent in most case-control studies, because even a negative prostate biopsy cannot definitively rule out occult PCA. When autopsied prostates were evaluated for cancer based on PSA <4 ng/ml, negative biopsy or both criteria, the occult cancer rates were 22% , 15% and 12% , respectively (35). Moreover, there was an association between PCA and the polymorphism of *MnSOD* in the autopsy cases where the control group was verified to be free of occult PCA (35). These data suggest that the contamination of occult cancer in the control group can result in false-negative results (36). The level of statistical significance of the present study may be diminished by the inclusion of as many as 22% occult cancer cases in the control group.

Our subpopulation of men who were smokers with *NAT1R* and *MnSOD* AA genotypes was a stratification of our larger study population, which was the basis of our original sample size calculation. Nevertheless, these observations provide important insight into the possible mechanism of PCA development which may be tested in larger cohorts.

In conclusion, our results suggest that the *MnSOD* AA genotype is a risk factor for PCA, particularly in smokers and in those who have rapid *NAT1* genotypes. The risk is greatly increased in those who have both factors. MnSOD is involved in the metabolism of ROS, therefore, conditions that elicit inflammation and ROS may contribute to the carcinogenic process. Our data may help elucidate why studies focusing on the individual role of smoking, NAT and MnSOD may be inconclusive in their association with PCA, while our study investigating a combination of these risk factors was able to identify a subpopulation of individuals at an increased risk.

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