Pathol. Oncol. Res. (2009) 15:19–24
DOI 10.1007/s12253-008-9064-6

ORIGINAL PAPER

MnSOD Gene Polymorphism Association with Steroid-Dependent Cancer

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Received: 14 April 2008 / Accepted: 6 May 2008 / Published online: 14 June 2008

Abstract Oxidative stress enhances carcinogenesis due to DNA damage. Manganese superoxide dismutase (MnSOD) Val16Ala polymorphism has been recently associated with breast and prostate cancer. The role of oxidative stress in male breast cancer is poorly investigated due to the low prevalence of this neoplasia. We studied the relationship between prostate cancer (PC), male (MBC) and female breast cancer (FBC) and this polymorphism in a case–control study. Human genetic polymorphism Val16Ala of MnSOD was obtained from blood and paraffin-embedded tumor samples. The polymorphism was determined in 11 cases of MBC, 51 cases of PC, 89 cases of FBC and 372 age-adjusted healthy controls by polymerase chain reaction–restriction fragment length polymorphism techniques using restriction enzyme *Hae III*. Chi-square or Fisher test were used to compare the MnSOD frequency distribution. The observed genotypic frequencies of all samples were AA=9.6% (n=50), VV=25.4% (n=133) and AV=64% (n=340), all at Hardy–Weinberg equilibrium. Breast and prostate cancer risk was elevated in male and female patients with the Ala/Ala genotype compared to controls (p=0.006, odds ratio=2.5, 95% confidence interval 1.393–4.541). Even though the frequency of the Ala allele was low (9.6%) in the studied population, these data support the hypothesis that MnSOD and oxidative stress play a significant role in breast cancer risk both in males and females and also brings new information on the role of this polymorphism in prostate cancer. This is the first study which provides some evidence that genetic polymorphism in the MnSOD gene may be associated with an increased risk of male breast cancer. Studies with a larger sample size are needed to confirm the findings.

Keywords MnSOD polymorphism · Male breast cancer · Female breast cancer · Prostate cancer · Steroid metabolism · Oxidative stress

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Introduction

Induction of high levels of reactive oxygen species (ROS) produce a state of oxidative stress (OS) in cells, which may damage cellular DNA, proteins, and lipids resulting in cell-cycle arrest, cellular senescence, and cell death [1]. Chronic OS has been implicated in neoplastic transformation [2] and promotion of tumorigenesis [3], including steroid influenced tumors such as prostate and breast cancer [4, 5].

Within mitochondria, manganese superoxide dismutase (*MnSOD*) provides a major defense against oxidative damage by reactive oxygen species. A diallelic polymorphism (Ala-9Val) in the mitochondrial targeting sequence (MTS) of human *MnSOD* has been previously reported. Calculation of a helix forming potential predicted the typical amphiphilic helical structure in -9Ala allele and its disruption in -9Val allele. This mutation may reflect

functional polymorphism of mitochondrial transport of human *MnSOD*. Ala16Val is implicated in decreased efficiency of *MnSOD* transport into target mitochondria in V allele carriers [6]. A study performed by Sutton et al. [6] suggested that the Ala-*MnSOD* precursor generated 30–40% more of the active, matricial, processed *MnSOD* homotetramer than the Val-*MnSOD* precursor. These results showed that the Ala-*MnSOD*/MTS allows efficient *MnSOD* import into the mitochondrial matrix, while the Val-variant cause partial arrest of the precursor within the inner membrane and decreased formation of the active *MnSOD* tetramer in the mitochondrial matrix. Although the AA genotype presents highest *MnSOD* activity, several studies have described association between this genotype and cancer including breast [7–9], prostate [9, 10], lung [11] and colorectal carcinoma [12]. However, others studies did not find the same results [13, 14], probably because of gene–gene, gene–environmental and physiological interactions associated with *MnSOD* polymorphism and cancer.

It is not yet known whether physiological interactions such as steroids metabolism could influence the interaction between *MnSOD* polymorphism and breast or prostate cancer risk. Since the major risk factors for female breast cancer are associated with prolonged exposure to increased levels of estrogen, enhanced cell proliferation induced either by endogenous or exogenous estrogens, would increase the number of cell divisions and thereby the possibility for mutation. Recently, Mitrunen and Hirvonen [15] studied the potential role of polymorphic genes encoding for enzymes involved in estrogen biosynthesis and conversion of the estrogen metabolites and their by-products as *MnSOD* in modulating individual susceptibility to breast cancer. These authors suggested that although some of these genes showed a low-penetrance they appeared as good risk factors candidates for sporadic breast cancer.

Although there are several studies demonstrating the association between prostate or female breast cancer and *MnSOD* polymorphism, so far no such report has been published about male breast cancer (MBC).

Male breast cancer is an uncommon disease, accounting for less than 1% of all breast tumors and less than 0.17% of all malignancies in men [16]. Due to its rarity, there are few studies regarding the biological mechanisms implicated in this disease. Most of our knowledge on this tumor has been extrapolated from female breast cancer, since these both diseases are hormone-dependent. All indications suggest that MBC shares a common etiology and similar natural history to its female counterpart. Risk factors, such as hyperoestrogenism, oxidative stress, obesity, alcohol and radiation exposure are similar in both genders [16, 17].

If the interaction between *MnSOD* and cancer is indeed influenced by estrogen/androgen metabolism studies performed in male and female breast cancer and prostate could help to elicit this question. On the basis of that, we performed a case–control study comparing the *MnSOD* polymorphism in males and females affected by breast and prostate carcinomas.

Materials and Methods

Study Participants

A case–control study was performed comparing prostate cancer (PC), male breast cancer, female breast cancer (FBC) and healthy control groups (male and females). The sample included 523 subjects distributed in five groups: male breast cancer (MBC=11), male prostate cancer (PC=51), female breast cancer (FBC=89), female controls (FC=217) and male controls (MC=155).

Control individuals were recruited from an existing database of 746 socially active healthy subjects who took part in a community-based research named GENESIS Program that investigates genetic–environmental interactions on human ageing [18, 19]. This investigation was structured considering the checklist for reporting and appraising of gene-diseases associations proposed by Little et al. [20].

Healthy controls were selected according to clinical general health status and were frequency matched for age to the expected age distribution of the cases. The control exclusion criteria included: infections, acute or chronic inflammation, autoimmune diseases, heart disease, under-nourishment, anemia, leucopenia, clinical depression, neurodegenerative disease, and other previous neoplasias. The exclusion of subjects affected by these diseases was based on the reported association between V allele and coronary disease [21].

The case individuals were pooled from Breast Cancer Centers and Pathology Laboratories from three hospitals localized in Porto Alegre, Brazil (Hospital Santa Rita, Hospital Conceição and Hospital São Lucas). The inclusion criteria included patients with pathological diagnosis of ductal or lobular breast carcinoma or prostatic adenocarcinoma. Other types of neoplasia were excluded.

As there were few cases in the male breast cancer group (n=11) due to its low prevalence, we applied a rigorous age-stratification (≥50 years) selection in the male healthy control group.

The study protocol was approved by the Institutional Ethics Committees of all relevant institutions and informed consent was obtained from all individuals whose information was collected prospectively.

MnSOD Genotyping

Genomic DNA was isolated from peripheral blood leukocytes (controls and cases) and from tissue samples (cases)

using a DNA Mini Kit Purification (Mo Bio) (BMC). Polymerase chain reaction (PCR) amplifications were done at a total volume of 50 μl containing 10×buffer 5.0 μl, 25 mM $MgCl_2$ 1.0 μl, 10 mM dNTP 1.25 μl, Taq Polymerase 0.5 μl (Gibco Inc, Co.), 40 pmol each primer 1.0 μl, Genomic DNA 3.0 μl (0.25 μg), ddH2O 34.5 μl. The amplification primers (Gibco Inc, Co.) for a 110 bp fragment of the human *MnSOD* gene were 5′-ACCAGC AGGCAGCTGGCGCCGG-3′, (sense-strand) and 5′-GCGTTG ATGTGAGGTTCCAG-3′ (antisense-strand) with thermocycler parameters comprised of an initial cycle of 95° for 5 min followed by 35 cycles at 95° for 1 min, 61° for 1 min. The final cycle was followed by an extension period of 2 min at 72°. PCR product (10 μl) was digested with *Hae III* (15U; 37°, 6 h, Gibco. Inc, Co.). Digested products (23 and 85 pairs of base) were visualized on a 4% agarose gel (Amersham Biosciences Inc, Co.) stained with ethidium bromide. A mutation was introduced by a primer mismatch to create a restriction cut site for *Hae III* in the -9 codon, though the following genotypes were observed: -9Ala/Ala (23 and 85bp); -9Ala/Val (23, 85 and 110 bp); and -9Val/Val (110 bp) (Fig. 1).

Statistical Analysis

Allele frequencies were estimated by the gene-counting method. Chi-square (χ^2) analysis was used to estimate the Hardy–Weinberg equilibrium. The allelic and genotype frequencies were compared among groups using chi-square statistical test or Exact Fisher test. All significant levels were two-tailed. The alpha value considered was $p=0.05$. A computer statistics package (SPSS 11.0, Chicago, USA) was used for statistical analysis in this study.

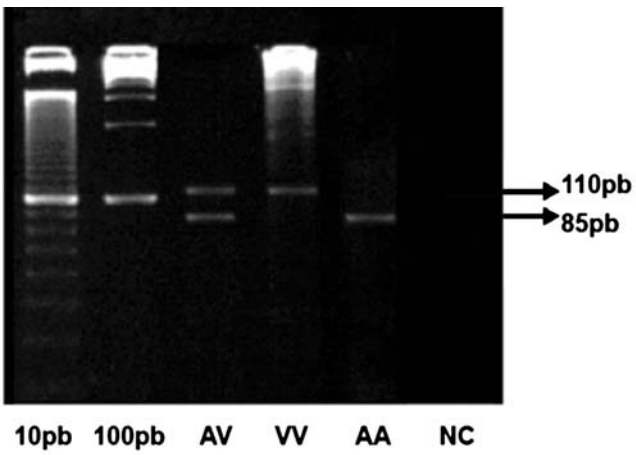

Fig. 1 *MnSOD* polymorphism by PCR–restriction fragment length polymorphism techniques. *pb* Pairs of base, *NC* negative control

Table 1 Mean age comparison among breast, prostate and control groups

Groups	Number	Mean age±SD
MBC	11	64.25±11.63
PC	51	66.35±7.98
FBC	89	63.74±8.52
FC	217	64.24±7.60
MC	155	63.16±7.05

MBC Male breast cancer, *PC* prostate cancer, *FBC* female breast cancer, *FC* female control, *MC* male control

Results

The study included 151 subjects aged between 52.62 and 75.88 years, who were diagnosed with breast or prostate cancer and 372 control individuals aged between 56.64 and 71.87 years-old.

The mean age was statistically similar ($p=0.160$) in all samples as shown in Table 1.

MnSOD genotypes and allele frequencies considering all samples (cases and controls) together were AA=9.6% ($n=50$), VV=25.4% ($n=133$) and AV=64.0% ($n=340$) and A=0.410 and V=0.590 respectively. The genetic frequencies were in Hardy–Weinberg equilibrium.

MnSOD polymorphisms frequencies comparing among breast, prostate and control groups are shown in Table 2. The polymorphisms frequencies are statistically different among groups by chi-square analysis ($p=0.05$).

Therefore, we grouped neoplasias cases and compared with control groups. The statistical difference were maintained using chi-square analysis ($p=0.006$).

The frequencies of the Ala allele were 15.9% and 7.0% in cases and control individuals, respectively. Genotypic frequencies were 25.8% (Val/Val), 58.3% (Ala/Val), and 15.9% (Ala/Ala) for cases, and the respective frequencies were 25.3%, 67.7%, and 7.0% for control individuals (Table 3).

Table 2 *MnSOD* polymorphism frequencies comparison among breast, prostate and control groups

Groups	*MnSOD*			Total
	AA	VV	AV	
MBC	1 (9.0%)	5 (45.5%)	5 (45.5%)	11 (100%)
PC	9 (17.6%)	10 (19.6%)	32 (62.8%)	51 (100%)
FBC	14 (15.7%)	24 (27.0%)	51 (57.3%)	89 (100%)
FC	18 (8.3%)	52 (24.0%)	147 (67.7%)	217 (100%)
MC	8 (5.2%)	42 (27.1%)	105 (67.7%)	155 (100%)
Total	50 (9.6%)	133 (25.4%)	340 (65.0%)	523 (100%)

MBC Male breast cancer, *PC* prostate cancer, *FBC* female breast cancer, *FC* female control, *MC* male control

Table 3 *MnSOD* polymorphism frequencies comparison among cancer and control groups

Genotypes	Groups		
	Cases	Controls	OR (CI 95%)
	n (%)	*n* (%)	
AA	24 (15.9)	26 (7.0)	
VV	39 (25.8)	94 (25.3)	
AV	88 (58.3)	252 (67.7)	
AA	24 (15.9)	26 (7.0)	2.515 (1.393–4.541)
VV+AV	127 (84.1)	346 (93.0)	
VV	39 (25.8)	94 (25.3)	1.030 (0.668–1.588)
AA+AV	112 (74.2)	278 (74.7)	

AA×(AV+VV), Pearson chi-square=9.850, p=0.002); VV×(AV+AA), Pearson chi-square=0.018, p=0.894)
AA -9Ala/Ala, *VV* -9Val/Val, *AV* -9Ala/Val, *OR* odds ratio, *95% CI* confidence interval

Additionally we investigated it there was dominance between *MnSOD* alleles. We observed that the cancer is related with AA genotype (Table 3, Fig. 2). Male breast cancer presented AA frequency intermediary between control and prostate cancer groups. However, the male breast cancer frequency was similar to female control group.

Discussion

In this population-based case–control study we found that the *MnSOD* Ala/Ala genotype was associated with a significantly elevated risk of male and female breast and prostate cancer compared to controls. To our knowledge, this is the first report in the medical literature to suggest an association of male breast cancer and *MnSOD* polymorphism.

The positive association between male breast cancer and prostate cancer with the A allele could be explained by some biochemical evidences reported in the literature [22]. The degree of ROS in a cell is dependent on the balance between ROS anabolism and catabolism. There are a number of reports, which establish a link between estrogen-induced breast cancer and oxidative stress [23–25]. In fact, a variety of estrogens are capable of acting as complete carcinogens through a mechanism that involves oxidative stress in the kidney, liver and breast tissues of various rodent models [24, 26]. Furthermore, oxidative DNA damage is reportedly increased in breast cancer tissues relative to normal breast, with a strong correlation to estrogen receptor (ER) status [27]. The enzyme 8-oxo-20-deoxyguanosine (8-oxo-dG) triphosphatase has also been found to be induced in the tumor tissues of patients with breast cancer, and the base excision repair products of 8-oxo-dG have been reported to increase in the urine of cancer patients [10, 12].

Some ROS may be generated from estrogen metabolism through catechol redox cycling. Mitrunen and coworkers conducted a case–control study among 483 cases and 482 controls in a Finnish Caucasian population, and reported that the Ala allele was associated with breast cancer risk, with an odds ratio (OR) of 1.5 in the Ala/Ala or Val/Ala groups compared with the Val/Val group [7]. Postmenopausal women who had used estrogen replacement therapy and carried either the Ala/Ala or Val/Ala genotype had a 2.5-fold higher risk for breast cancer. Women who had used oral contraceptives and carried the Ala/Ala or Val/Ala genotype had a 3.0-fold higher risk for breast cancer. More recently, Egan and coworkers [28] conducted a population-based case–control study among 476 cases and 502 controls in an American population. Overall, relative risks were not significantly elevated in women with one (OR 1.27, 95% confidence interval (CI) 0.91–1.77) or two (OR 1.18, 95% CI 0.81–1.73) Ala alleles, as compared with the Val/Val genotype. Risk, however, was increased among premenopausal women carrying the Val/Ala genotype (OR 1.88), but not among women carrying the Ala/Ala genotype (OR 0.94). Women carrying the Ala/Ala or Val/Ala genotype who had used oral contraceptives or had higher body mass index also showed an increased risk for breast cancer.

It has been suggested that oxidative stress resulting from metabolic activation of carcinogenic estrogens plays a critical role in estrogen-induced carcinogenesis, however the involved mechanisms are not fully understood. An animal study [29] using an estrogen-induced hamster renal tumor model, a well established animal model of hormonal carcinogenesis, demonstrated that tumors can be induced by subchronic treatment with a combination of a noncarcinogenic estrogen and a chemical known to produce oxidative stress. In vitro study using the ER-alpha-positive hamster kidney tumor (H301) and the human breast cancer (MCF-7) cell lines treated with estrogens with differing carcinogenic potentials suggested that metabolic activation and subsequent generation of oxidative stress may play critical roles in estrogen-induced carcinogenesis [30].

Similar mechanisms were reported in human studies. Hong et al. studied the possible association between breast tissue density, malondialdehyde and cytochrome P_{450} 1A2

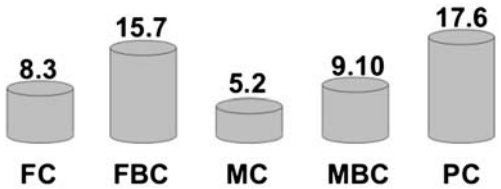

Fig. 2 AA genotype prevalence in control, breast and prostate cancer groups (%). *FC* Female control, *FBC* female breast cancer, *MC* male control, *MBC* male breast cancer, *PC* prostate cancer

(CYP1A2) levels. Mammographically dense breast tissue is a strong predictor of breast cancer risk, and is influenced by both mitogens and mutagens. One enzyme that is able to affect both the mitogenic and mutagenic characteristics of estrogens is CYP1A2 which is principally responsible for the metabolism of 17β-estradiol. The authors studied 146 premenopausal and 149 postmenopausal women and the results provided evidence that variation in the activity level of enzymes involved in estrogen metabolism is related to levels of mammographic density and potentially to breast cancer risk [31].

Several studies have evaluated the association of the *MnSOD* Val-9Ala polymorphism with other cancers, although the results were inconsistent among cancer sites. Recently, Woodson and coworkers reported that the Ala/Ala genotype was associated with a 1.7-fold (95% CI 0.96–3.08) increased risk for prostate cancer as compared with the Val/Val genotype [14].

This study suggests a possible association between the *MnSOD* Ala/Ala genotype and a significantly elevated risk of male and female breast and prostate cancer. However, the study is limited by the small numbers of individuals with male breast cancer, a rare disease, which may result in unstable OR estimates and limited statistical power for stratified analyses. Studies with a larger sample size are needed to confirm the findings.

Acknowledgments To GENESIS Gravatai Project team to help us in data collection and in the molecular biology analysis, and to CNPq and CAPES for grants and fellowships.

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