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Is the Val16Ala Manganese Superoxide Dismutase Polymorphism Associated With the Aging Process?

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Oxidative stress has been related to aging. Recent evidences suggest that a genetic dimorphism that encodes for either alanine or valine in superoxide dismutase (SOD2) is involved with oxidative stress. However, the current literature is still controversial, and the potential role of the Ala16Val polymorphism in human aging needs to be established. Here we investigated the role of the SOD2 polymorphism in: a) age-related mortality, b) morbidity (breast and prostate cancer), c) immunological markers, and d) DNA damage in peripheral blood cells. We did not find an association between SOD2 polymorphisms and mortality. However, the AA genotype was associated with increased risk for prostate and breast cancer, immunosenescence profile, as well as DNA damage. These data suggest that SOD2 presents characteristics that support the free radical theory of aging.

AGING is a complex multifactor biological process associated with progressive degeneration of biological functions, increased susceptibility to diseases, and higher probability of death within a given period of time (1). However, the causative factors associated with this process are still obscured. Several theories have been postulated to explain this process, including the free radical theory of aging that states that the age-related degenerative process is, to a large extent, the consequence of radical oxidative species (ROS) damage (2). Age-related ROS damage is supported by several lines of evidence that show association among diseases with oxidative stress (such as neoplasias and atherosclerosis) and neurodegenerative, infectious, ophthalmologic, and metabolic diseases (3).

ROS and reactive nonradical species are derived from radicals that are generated from cells and tissues of aerobic organisms in response to both external and internal stimuli. The major source of ROS generation is the mitochondria respiratory chain. However, several lines of evidence suggest that low levels of ROS are pivotal in many biological processes. For this reason, there are antioxidant molecules (endogenous and exogenous) that regulate the tissue-specific ROS concentration (1,2). We know that a delicate balance between the advantageous and detrimental effects of ROS is important to life. Endogenous defenses against ROS include glutathione peroxidase, catalase, and superoxide dismutase enzymes (4). The regulation between ROS production and clearance is known as oxidant–antioxidant balance.

The superoxide anion is the first ROS produced from the mitochondria respiratory chain. The SODs convert superoxide enzymically into hydrogen peroxide. The hydrogen peroxide is then converted to water by catalase and glutathione peroxidase action.

Studies performed in experimental models showed that SOD levels are related to life span. *Drosophila* fruit fly studies (5) indicated that flies selected for increased longevity have elevated levels of SOD and increased resistance to oxidative stress. Long-lived mutant worms are also resistant to oxidative stress, and show an age-dependent increase in SOD and catalase activity. For instance, the *daf-2* mutation in *Caenorhabditis elegans* is associated with increased manganese SOD (MnSOD) expression (6,7). In contrast, the increase of SOD levels could lead to oxidative imbalance. Patients with Down Syndrome show increased DNA damage induced by hydrogen peroxide (H_2O_2) and Cu(II); this increase could be related to increased risk to cancer (8).

There are three forms of SOD: cytosolic and extracellular Cu-Zn SOD (SOD1 and SOD3) and mitochondrial MnSOD (named also as SOD2). Several lines of evidence suggest that SOD2 could be biologically important for life, as one study reported increased neonatal lethality in SOD2 knock-out mice (9). This would explain why only a very limited number of mutations have been described in SOD2. One interesting example is the human polymorphism (Ala-9Val) in the mitochondrial targeting sequence (MTS) of this radical-scavenging enzyme. A genetic dimorphism encodes for either alanine (Ala) or valine (Val) in the MTS of human manganese superoxide dismutase (MTS-SOD2), and has been reported to modulate the risk for disease (10,11). Surprisingly, the association between MTS-SOD2 polymorphism and disease is not related to only one allele. There are studies suggesting an association between the Ala variant

and breast (12), prostate (13), and colon (14) neoplasias. There are also some studies associating the Val variant and nonfamilial idiopathic dilated cardiomyopathy (15) and degree of carotid atherosclerosis (16). This apparent paradox could be explained by oxidative imbalance related to homozygous expression. There is evidence supporting this hypothesis. Sutton and colleagues (17) have shown that Ala-MnSOD is more active than the Val-SOD enzyme. This study was performed in vitro with chimeric proteins composed of either one of the MTS-SOD2 fused to the mouse dehydrofolate reductase protein, and analyzes of the import of the two human SOD2 precursor variants into rat liver mitochondria. When compared with Ala proteins, the Val-MnSPD/MTS-dehydrofolate reductase precursor and Val-SOD2 precursor were both partly arrested within the inner mitochondrial membrane. The results obtained from Sutton and colleagues (17) showed that the Ala-SOD2 precursor generated 30%–40% more of the active, original SOD2 enzyme than did Val-SOD2; however, their results should be carefully interpreted because they were based purely on a semiquantitative gel assay and have not been confirmed.

We sought to investigate in this study whether the higher SOD2 production in AA genotypes and the lesser SOD2 production in VV genotypes could cause an oxidative imbalance and thus explain the association of these genotypes to age-related diseases. This was achieved by four integrated approaches: the possible association between MTS-MnSOD polymorphism with mortality, morbidity (breast and prostate cancer), the modulation of immunological markers, and the DNA damage in healthy elderly carriers of different MTS-SOD2 genotypes.

Methods

Four general approaches were conducted here to test the hypothesis that MTS-MnSOD polymorphism could be associated to human aging: a) one study aimed at verifying whether the MTS-MnSOD polymorphism frequencies change during aging (association with mortality); b) an analysis designed to investigate whether the MTS-MnSOD polymorphism is associated with healthy conditions, comparing healthy groups (young adults and healthy elderly persons) to elderly persons with degenerative diseases (prostate and breast cancer); c) an analysis to verify whether different MTS-MnSOD alleles could be related to immunological markers known to be important in the age-related degenerative process; and d) the analysis of DNA damage, because it is influenced by oxidative imbalance and age-related diseases such as cancer.

Populations

The study protocol was approved by both scientific and ethics committees (Pontifical Catholic University of Rio Grande do Sul, PUCRS, Porto Alegre, Brazil) and written informed consent was obtained from all participants. The study was structured considering the checklist for reporting and appraising of gene–disease associations proposed by Little and colleagues (18). In the first analysis, the study design was similar to that described in Da Cruz and colleagues (19), and included 489 volunteers divided into three groups: newborns, individuals between 21 and 79 years old, and individuals between 80 and 105 years old (oldest-old). The population examined was composed of individuals mixed from different ethnic groups (Native South American and European Caucasian) originating a new genetic pool, termed here as Gaucha population. The newborn group was selected from pregnant women that underwent prenatal supervision at Hospital Femina, Porto Alegre, Brazil. The newborn's blood sample was obtained from the umbilical cord. The participants in the two other age groups were recruited by random selection from the Health and Social Assistance Program of Gravataí, Brazil. Details about ethnicity of the sample could be based on studies by Alves-Silva and colleagues (20) and Parra and colleagues (21) about Brazilian ancestrally that included the population of Southern Brazil. The authors emphasize that this population is descended from massive interethnic crosses, occurring during 500 years of Brazilian history, between people from three continents (European colonizers, autochthonous Amerindians, and African slaves), and underline that this population does not represent isolated ethical groups. Parra and colleagues (21) estimated that the mtDNA lineages ancestry found a quite homogeneous miscegenated population (admixture estimated in 0.13 ± 0.08 using Estimate $\pm$ *SE* method and 0.17 using Collins–Schramm method) formed by a 66% European, 22% Amerindian, and 12% African mtDNA ancestrally. For this reason, we consider the sample source as a unique population (Gaucha population), and population stratification is not shown here. Blood samples were collected to isolate DNA. First- or second-degree relatives of participants previously included in research protocols were excluded to avoid genetic frequency bias.

To perform the second analysis, elderly participants (>60 years) were recruited from local community centers and registered at the Office for Social Care in Gravataí (Rio Grande do Sul). This elderly population corresponded both ethnically and socioeconomically to the general population of our State (Rio Grande do Sul). All participants took part in the GENESIS Program for the study on the genetic–environmental interactions on human aging. Participants were divided into two groups: 1) controls, comprised of healthy elderly persons (HE group); and healthy adults (HA group), appraised by the SENIEUR protocol (22) that defines rigorous criteria for selecting healthy individuals in immunogerontological studies. The health conditions were checked accordingly to accurate clinical investigations and to hematological and biochemical parameters. Details regarding the selection of these healthy groups are described in a study by Luz and colleagues (23). The second group (EC group) comprised elderly persons (>60 years) with previous documented history of later breast or prostate cancer, without other morbidities that could interfere in the analyses. For instance, participants were excluded for cardiovascular or metabolic diseases including moderate hypertension (>160/90 mmHg), diabetes mellitus type II, clinical depression, and autoimmune diseases. The EC group was recruited in the Clinical Services Department of the Hospital São Lucas of PUCRS, Brazil.

The third analysis was performed in the HE group only. None of the donors was on a drug regimen known to affect the immune system or had a prior history of neoplasias or

infectious or autoimmune disease. Peripheral blood was collected by venipuncture in the morning (between 9 AM and 10 AM), and samples were stored in lithium–heparin tubes prior to analyses. The immunological markers were analyzed by two-color flow cytometry (fluorescence-activated cell sorting; FACS) as previously described (24). Immunophenotyping was carried out using a direct immunofluorescence technique with the following monoclonal antibodies (BD Pharmingen, San Jose, CA): CD25, CD69, CD95, CD45RA, CD45RO, and CD28, and the appropriate isotype controls. Antibodies for cell-adhesion molecules (CD11c, CD31, CD49d, and CD62L) were also used. The samples were promptly analyzed using a FACSCalibur (BD Pharmingen). A minimum of 10,000 lymphocytes gated by size (FSC) and granularity (SSC) were analyzed using CellQuest software (BD Pharmingen).

The fourth analysis included an investigation of DNA damage in peripheral blood cells of the HE group. For such analysis, the alkaline comet assay was performed as described by Singh and colleagues (25). Briefly, peripheral blood cells (5 μl) were embedded in 95 μl of 0.75% low-melting-point agarose. After solidification, the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris, pH 10.0–10.5, with freshly added 1% Triton X-100 and 10% dimethyl sulfoxide) for a minimum of 1 hour and a maximum of 2 weeks. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 20 minutes. The DNA was subjected to electrophoresis for 15 minutes at 25 V (0.90 V/cm) and 300 mA, and the buffer was then neutralized with 0.4 M Tris (pH 7.5). Finally, the DNA was stained with ethidium bromide (2 μg/ml). The slides were coded for blind analysis.

To demonstrate the electrophoresis conditions and efficiency of this methodology, negative and positive controls of human blood cells were used for each electrophoresis treatment. For a positive control, 50 μl of whole blood was mixed with 13 μl of methyl methanesulfonate (MMS-M4016; Sigma, St. Louis, MO) at 8×10^{-5} M. This mixture was then incubated for 2 hours at 37°C. The result of each electrophoresis was considered only if the negative and positive controls yielded negative and positive results, respectively.

Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed from each person, by using a fluorescence microscope equipped with an excitation filter of BP546/12 nm and a barrier filter of 590 nm. Cells were also scored visually according to tail size, into five classes, from no tails (0) to maximally long tails (4); this visual scoring resulted in a single DNA damage score for each study group. Therefore, the group damage index was calculated and ranged from 0 (all cells with no tails, 100 cells $\times$ 0) to 400 (all cells with maximally long tails, 100 cells $\times$ 4) (26,27). Damage frequency (%) was calculated by dividing the number of cells with tails by the number of cells without tails. Blood samples were incubated for 1 and 24 hours at 37°C.

MTS-SOD2 Genotyping

Genomic DNA was isolated from peripheral blood leukocytes by using a GFX Genomic Blood DNA Purification Kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K.). Polymerase chain reaction amplifications were done at a total volume of 50 μl containing 5.0 μl of 10× buffer, 1.0 μl of 25 mM $MgCl_2$, 1.25 μl of 10 mM dNTP, 0.5 μl of Taq Polymerase (Gibco), 1.0 μl of each primer (40 pmol), 3.0 μl (0.25 μg) of genomic DNA, and 34.5 μl of ddH_2O. The amplification primers (Gibco) for a 110-bp fragment of the human SODs gene were 5′-ACCAGCAGGCAGCT-GGCGCCGG-3′, (sense-strand) and 5′-GCGTTGATGT-GAGGTTCCAG-3′ (antisense-strand) with thermocycler parameters comprised of an initial cycle of 95°C for 5 minutes, 35 cycles at 95°C for 1 minute, and one cycle at 61°C for 1 minute. The final cycle was followed by an extension period of 2 minutes at 72°C. The polymerase chain reaction product (10 μl) was digested with Hhae III (15 U; 37°C, 6 hours, Gibco). Digested products (23 and 85 bp) were visualized on a 6% agarose gel (Amersham Biosciences) stained with ethidium bromide. A mutation was introduced by a primer mismatch to create a restriction cut site for Hhae III in the –9 codon, though the following genotypes were observed: –9Ala/Ala (23 and 85 bp); –9Ala/Val (23, 85, and 110 bp); and –9Val/Val (110 bp).

Statistical Analysis

Allele frequencies were estimated by the gene-counting method. Chi-square analysis was used to estimate the Hardy–Weinberg equilibrium and to compare genotypic and allelic frequencies among the ethnic groups. The age distribution of the samples was analyzed by the Kolmogorov–Smirnov test, and showed normal distribution. For this reason, parametric tests were used to verify the association between MTS-SOD2 polymorphism and age. Chi-square analysis was used to compare genotype frequencies among the various study groups, considering sex as possible intervenient variable. Chi-square analyses were also used to determine the differences in distribution of the MTS-SOD2 genotypes and alleles between groups. Odds ratios and 95% confidence intervals were calculated using unconditional logistic regression to evaluate associations between MTS-SOD2 genotypes and/or alleles separately for prostate and breast cancer. Subgroups were compared by one-way analysis of variance. Multiple comparisons among group mean differences were checked with Bonferroni's post hoc test. Statistical differences for the comet assay were determined by the nonparametric Kruskal–Wallis test followed by a post hoc Mann–Whitney U test based on the score of each participant (27). All significant levels were two-tailed. The alpha value considered was $p = .05$. A computer statistics package (SPSS 11.0; SPSS, Chicago, IL) was used for the statistical analyses in this study.

Results

MTS-MnSOD Polymorphism as Function by Age

The mean age of all participants in the first study was 62.17 ± 10.56 years. The sex distribution included 40.5% males ($n = 198$) and 59.5% females ($n = 291$). Table 1 shows the MTS-SOD2 genotypes and allele frequencies for the entire sample and among the three age groups. The genetic frequencies were in Hardy–Weinberg equilibrium.

Table 1. Ala16Val MTS-MnSOD Polymorphism Frequencies in Gaucha Population

		Frequencies			
Genotype	*N*	Total	Newborns (65)	Adults (296)	Oldest Old (75)
AA	71	14.5	18.5 (12)	13.5 (47)	16.0 (12)
VV	122	24.9	20.0 (13)	24.9 (87)	29.3 (22)
AV	296	60.6	61.5 (40)	61.6 (215)	54.7 (41)

Note: MTS-MsSOD = mitochondrial targeting sequence-manganese superoxide dismutase; A = alanine allele; V = valine allele.

The A and V allele frequencies were 0.4478 and 0.552, respectively. The three age groups presented similar genotype frequencies ($\chi^2 = 2.795$, $p = .593$).

As shown in Table 2, participants of various SOD2 genotypes did not differ in age ($F = .15$, $p = 8.66$) considering sex as intervenient variable ($F = 2.86$, $p = 3.78$).

The age distribution by percentiles was also calculated; we did not find any statistical differences when elderly participants (>60, ≥70, and ≥80 years old) were compared with young participants (≤60, ≤50, and <40 years old) (data not shown). These data suggest that MTS-MnSOD polymorphism frequencies do not change as function by age.

MTS-SOD2 Polymorphism and Morbidity

The second analysis included healthy young adults (HA; 37.28 ± 6.98 years, $n = 115$), healthy elderly persons (HE; 67.10 ± 6.45 years, $n = 51$), and elderly persons with breast or prostate cancer ($n = 120$; 66.40 ± 7.39 years).

The MTS-SOD2 genetic comparison is described in Table 3. The MTS-SOD2 frequencies were similar between prostate and breast cancer (data not shown). For this reason we combined the prostate and breast cancer in only one case group. We found that MTS-SO2 frequencies differed significantly between the healthy and case groups ($\chi^2 = 11.996$, $df = 6$, $p < .03$).

We then investigated the dose effect of the alleles among the various groups to confirm the association among the polymorphism and the morbidities studied. The results showed that the odds ratio for the AA genotype was 2.50-fold higher (95% confidence interval = 1.24–5.05) than that in the case group (patients with breast and prostate cancer).

Table 2. Age Comparison Between Sex and Ala16Val MTS-MnSOD Polymorphism in Gaucha Population

Genotype	Sex	Mean	*SD*
VV	Male	58.7	9.3
	Female	65.3	9.3
	Total	62.9	9.8
AV	Male	58.5	11.6
	Female	63.8	9.7
	Total	61.5	10.9
AA	Male	61.6	11.5
	Female	65.8	7.1
	Total	64.4	8.9
Total	Male	58.9	11.1
	Female	64.5	9.2

Note: MTS-MnSOD = mitochondrial targeting sequence-manganese superoxide dismutase.

Table 3. Comparison of the Allelic and Genotypic Frequencies of the Superoxide Dismutase 2 Ala16Val Polymorphism Among the Investigated Groups

	HA	HE	EC
Genotypes			
AA	8 (7.0)	5 (9.8)	18 (15.0)
VV	29 (25.2)	11 (21.6)	24 (20.0)
AV	78 (67.8)	35 (68.6)	78 (65.0)
Alleles			
A	0.409	0.441	0.475
V	0.591	0.559	0.525

Note: HA = Healthy adults; HE = healthy elderly persons; EC = elderly persons with breast or prostate cancer.

These data indicate a positive association between the AA genotype and prevalence of malignancy.

MTS-SOD2 Polymorphism and Immunological Markers

A large panel of cell surface markers was investigated in peripheral blood cells to search for any association with MTS-SOD2 polymorphisms. When each genotype was considered separately, the only association found was for CD45RO expression ($F = 6.231$, $p = .007$). In this case, the AA genotype of HE group presented lower expression of CD45RO (87.72 ± 14.78) than VV (197.97 ± 20.59). The AV genotype presented intermediary values (130.77 ± 11.07).

A second analysis was then performed to test the possible allelic dose effect. To investigate for AA dose, AV and VV participants were grouped; to test for VV dose, AV and AA participants were grouped (Table 4).

The AA group presented significantly lower frequencies of CD95 (APO1/Fas) and CD11c than did the VV+AV group, whereas the VV group presented significantly higher frequencies of CD4 than did the AA+VV group. In addition, the AA genotype group presented lower densities (expression) of CD49 than did the VV+AV genotype group. The VV genotype group presented higher densities of CD45RO and lower densities of CD31 than did the AA+AV group. These data suggest that MTS-SOD2 polymorphism has a genetic dominant effect on these immunological markers.

MTS-SOD2 Polymorphism and DNA Damage

Figure 1 shows the smear of peripheral whole blood cells analyzed by comet assay. Our results showed that basal DNA damage was related to SOD2 genotype in 1-hour culture. In particular, the basal damage index was significantly higher in AA participants (17.00 ± 8.00) compared to that in VV (7.00 ± 6.00) and AV (5.83 ± 1.09) participants ($p = .034$). After 24 hours, the DNA damage index indicated a tendency for higher levels in VV participants (35.01 ± 12.0) than AA (17.50 ± 9.50) and AV (9.37 ± 2.03) participants, but this only approached statistical significance ($p = .09$).

DISCUSSION

The results described here showed a positive association between human MTS-SOD2 polymorphism and elderly morbidity, immunological markers, and basal DNA damage but not with mortality. The AA genotype presented

Table 4. Frequency (% ± *SEM*) of Lymphocyte Subsets Expressing Cell Surface Markers Considering MTS-MnSOD Allelic Dose Effect in Healthy Elderly Sample

Markers	Genotypes	Mean	*SE*	Genotypes	Mean	*SE*
CD4	AA	32.97	5.34[a]	AA	822.11	84.47
	VV	39.84	2.83[b]	VV	833.59	75.00
	AV	28.91	2.04[a]	AV	759.15	42.33
	Total	31.67	1.84	Total	785.68	33.77
CD8	AA	14.46	2.63[a]	AA	803.47	144.23
	VV	16.39	2.34[a]	VV	850.15	126.59
	AV	16.18	2.05[a]	AV	709.92	46.97
	Total	15.85	1.42	Total	753.33	46.61
CD25	AA	0.54	0.27[a]	AA	245.07	169.01
	VV	1.41	1.04[a]	VV	100.77	33.91
	AV	0.89	0.36[a]	AV	178.75	31.96
	Total	0.90	0.28	Total	179.51	37.40
CD95	AA	1.08	0.34[a]	AA	58.77	19.56
	VV	12.56	7.22[c]	VV	76.79	20.60
	AV	4.55	3.53[b]	AV	114.97	15.97
	Total	5.11	2.58	Total	97.62	12.12
CD45RA	AA	6.77	3.14[a]	AA	153.58	18.11
	VV	15.61	9.76[b]	VV	444.07	282.76
	AV	10.13	3.10[b]	AV	183.86	32.48
	Total	10.33	2.52[a]	Total	218.07	48.01
CD69	AA	9.12	4.77[a]	AA	458.03	65.32
	VV	5.08	4.28[a]	VV	311.95	109.56
	AV	3.84	1.94[a]	AV	349.41	77.15
	Total	5.05	1.67	Total	372.22	48.68
CD45RO	AA	22.64	7.28[a]	AA	52.37	12.35
	VV	15.53	8.39[a]	VV	107.92	52.72
	AV	22.30	4.03[a]	AV	112.28	29.46
	Total	21.32	3.16	Total	100.09	21.00
CD28	AA	38.40	8.24[a]	AA	87.73	14.80
	VV	30.34	9.87[a]	VV	197.97	20.59
	AV	28.95	4.79[a]	AV	130.77	11.07
	Total	30.98	3.75	Total	130.22	10.25
CD11	AA	0.88	0.30[a]	AA	162.12	14.35
	VV	1.69	1.04[b]	VV	193.93	42.34
	AV	4.95	1.67[b]	AV	150.36	7.44
	Total	3.39	1.02	Total	159.33	8.49
CD31	AA	38.19	8.48[a]	AA	164.73	48.21
	VV	27.75	4.97[a]	VV	215.07	115.46
	AV	35.41	4.54[a]	AV	143.26	18.64
	Total	34.73	3.41	Total	160.13	23.76
CD49	AA	37.16	12.15[a]	AA	187.53	58.96
	VV	35.63	10.95[a]	VV	87.38	19.47
	AV	23.98	5.07[a]	AV	157.50	33.02
	Total	28.20	4.32	Total	152.25	24.29
CD62	AA	59.79	3.08[a]	AA	57.96	10.21
	VV	58.21	7.87[a]	VV	81.01	12.51
	AV	43.86	4.93[b]	AV	173.32	55.39
	Total	49.67	3.52	Total	132.24	34.62

Notes: Different letters indicate significant differences ($p < .05$) from one-way analysis of variance followed by a post hoc Bonferroni's test.

SE = standard error; MTS-MsSOD = mitochondrial targeting sequence-manganese superoxide dismutase.

increased risk to develop breast or prostate cancer and to present immunosenescence markers and higher DNA damage. It is remarkable that AA genotype frequency does not decrease during aging.

At first glance, these results may represent a paradox. However, our data agree with previous results described by

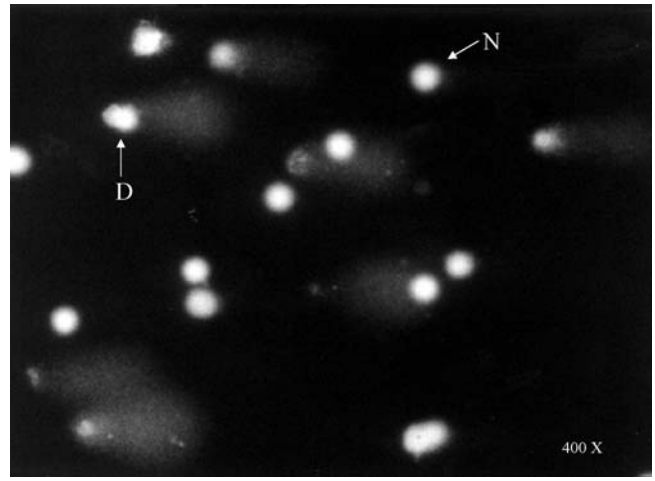

Figure 1. Smear of whole blood cells analyzed by comet assay (original magnification, ×400). N = normal undamaged cells; D = damaged cells.

Van Remmen and colleagues (28) in mice heterozygous for the *sod2* gene (*sod2* +/– mice) studied for the phenotype of lifelong reduced SOD2 activity. These authors described that the *sod2* +/– mice have reduced MnSOD activity (50%) in all tissues throughout life, and have increased oxidative damage in all tissues and in mitochondrial DNA. The increased oxidative damage to DNA in the *sod2* +/– mice is associated with a 100% increase in tumor incidence (the number of mice with tumors) in old *sod2* +/– mice compared with old wild type mice. In this case, the life spans (mean and maximum survival) of the *sod2* +/– and wild type mice were identical. Van Remmen and colleagues (28) showed that lifelong reduction of MnSOD activity leads to increased levels of oxidative damage to DNA and to increased cancer incidence, but it does not appear to influence aging. Considering the studies in *sod2* +/– mice and in humans, our data corroborate with a previous investigation made in Northern and Southern Italian centenarians that did not find association between MTS-SOD2 polymorphism with extreme longevity (29). However, what hypothesis could explain this apparent paradox? Several integrated questions need to be discussed to explain these results.

The lack of association between MTS-SOD2 polymorphism and human longevity described here is difficult to reconcile with the data available now (29). However, we can speculate that this could be due to the progress of human civilization on biological response to environmental stress factors (30).Variation in the intensity of stress can be expressed on a fitness–stress continuum, where fitness is inversely related to stress level, and stress resistance is an adaptive process resisting mortality (31–33).

During ancient times, the biological protection for some types of stressors such as infections was the main condition for survival. In contrast, now human aging is determined by the struggle to deal with increasing rates of chronic degenerative diseases. In this way, how could a change in biological response related to oxidative stress (e.g., MTS-SOD2) be associated with highly prevalent morbidities in elderly persons and not be related to longevity?

To answer this question, we need to consider the relationship between oxidative balance and immune system as well as the physiological role of ROS modulation during organism development: the REDOX regulation is involved with several physiological functions including the immune system. Activated macrophages and neutrophils can produce large amounts of superoxide and its derivatives via the phagocytic isoform of NADPH oxidase. During inflammation, hydrogen peroxide is produced by activated macrophages. The massive production of antimicrobial ROS in an inflammatory environment is called the ''oxidative burst,'' and plays an important role as a first line of defense against environmental pathogens (1,34). For this reason, individuals who produce more effective SOD2 (Ala carriers) could potentially generate higher levels of hydrogen peroxide and thus present a stronger innate immunity. However, the development of civilization is associated with a better control of infections via use of vaccines and drug treatments. Unfortunately, we cannot test this hypothesis at the present moment. In contrast, the control of infections contributed to increased human survival rates at older ages. As the VV genotype does not produce a defective enzyme but just produces less active SOD2, the hypothesis postulated here could be acceptable. Although it seems difficult to provide epidemiological evidence for this hypothesis, the stress theory of aging considers that the control of aging trajectories includes measures of longevity in contrast to assessment of survival rate, frequently used as a monitor of longevity in natural populations. This theory considers that the primary or operative factor is oxidative stress; therefore, individuals with the potential for a long life should carry genes for oxidative stress resistance that correlate with resistance to a wide array of stress (33). Taking into account this theory, we can not discard MTS-SOD2 as a candidate gene for aging and its association with diseases that are highly prevalent in elderly populations. The positive association between AA genotype and breast and prostate cancer described here support this hypothesis, as well as did other studies that found a similar association between AA genotype and neoplasias (10,12–14). However, other studies did not find this association (35–37).

As the immune system is pivotal to disease control, the possible role of oxidative imbalance caused by MTS polymorphism could help us to understand recently obtained evidence. For this reason we performed an additional study analyzing a large number of immune cell surface markers in healthy elderly groups. Only strictly healthy (SENIEUR) elderly participants took part in this analysis, because elderly patients present immune modifications associated with cancer treatment and the young adults group potentially included individuals who will develop cancer in early or at later ages. We found differences related to antitumoral activities in cell surface markers in healthy elderly persons with different MTS-SOD2 genotypes.

The data produced here showed that VV individuals had fewer senescent immune surface markers (e.g., CD95 apoptosis receptor) than did AA individuals. Apoptosis and successful aging has been studied extensively during the last five years. For instance, Kolbus and colleagues (38) studied mouse fibroblasts derived from fetuses lacking c-Jun, and found that the resistance to apoptosis was associated with impaired expression of CD95 ligand (CD95-L), a well-known apoptosis inducer. When we consider the results described by Kolbus and colleagues (38), the lower CD95 expression in the AA genotype could be associated with higher cancer risk in these individuals, as described in the literature and in our results. Because we found an association between the AA genotype and breast and prostate cancer, we can not discard the possible differential immune modulation related to MTS-MnSOD polymorphism.

We also demonstrated that AA individuals presented a higher DNA damage index in peripheral blood cells. However, when the DNA damage index was calculated in cells cultured for 24 hours, we found that VV presented more DNA damage after 24 hours, whereas AA maintained the same DNA damage level. A previous study (39) has shown that DNA damage increased after a 24-hour culture. Probably for this reason we found high levels of DNA damage in VV and maintenance of higher DNA damage in AA individuals (39). Further studies should be performed to test whether these data are related to cancer risk. Taking into account the results described here, we could propose that MTS-SOD2 is a candidate gene for human aging, and presents characteristics that support the free radicals theory of aging.

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