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Original article

Association of the manganese superoxide dismutase polymorphism with vasospastic angina pectoris

Hajime Fujimoto (MD, PhD)[a,*], Hisae Kobayashi[b], Ken Ogasawara (MD)[c], Minoru Yamakado (MD, PhD)[d], Minoru Ohno (MD, PhD, FJCC)[a]

[a] *Department of Cardiovascular Medicine, Toranomon Hospital, 2-2-2, Toranomon, Minato-ku, Tokyo, Japan*
[b] *Okinaka Memorial Institute for Medical Research, Toranomon Hospital, Tokyo, Japan*
[c] *The Cardiovascular Institute, Tokyo, Japan*
[d] *Mitsui Memorial Hospital, Tokyo, Japan*

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Summary
Background: Vasospastic angina (VSA) is closely related to endothelial dysfunction caused by oxidative damage. Manganese superoxide dismutase (MnSOD) is an antioxidant enzyme that functions in mitochondria. There are two genetic variants of MnSOD arising from a substitution of an alanine for a valine in the signal peptide. We previously reported that the valine allele of MnSOD decreases the mitochondrial MnSOD (mtMnSOD) activity. Here, we investigated the association of the MnSOD polymorphism (Ala16Val) with VSA.
Methods and results: Blood samples were collected from 618 healthy subjects who did not have any symptoms or other evidence suggesting angina pectoris, and 228 patients who underwent coronary angiography on suspicion of angina, and were diagnosed to have VSA by acetylcholine test. MnSOD genotype of each subject was determined by real-time polymerase chain reaction. The valine allele frequency was higher in the VSA patients (0.890) than in the healthy subjects (0.839) [odds ratio (OR) = 1.55, $p = 0.0085$]. In healthy subjects the MnSOD genotype distribution was as follows: alanine/alanine 1.9%, alanine/valine 28.3%, and valine/valine 69.8%, and in VSA patients the prevalence was: alanine/alanine 1.3%, alanine/valine 19.3%, and valine/valine 79.4%. Thus, the valine allele was closely associated with VSA ($p = 0.019$). Multivariate logistic regression analysis showed valine/valine homozygosity to be an independent risk factor for VSA (OR = 2.02, 95% CI 1.43, 2.85; $p = 0.0012$).
Conclusion: The valine variant of MnSOD signal peptide increases the risk of VSA.

Introduction

Vasospastic angina (VSA) plays an important role in the pathogenesis of ischemic heart disease, and sometimes leads to dire consequences such as acute coronary syndrome

* Corresponding author. Tel.: +81 3 3588 1111; fax: +81 3 3582 7068.
E-mail address: hafujimoto-circ@umin.ac.jp (H. Fujimoto).

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or arrhythmic cardiac arrest [1—4]. VSA is a common disease all over the world, but it is more prevalent in the Japanese and Korean populations than in Caucasians [5,6].

VSA is closely related to the endothelial dysfunction caused by oxidative damage [7—9]. Manganese superoxide dismutase (MnSOD) is an antioxidant enzyme that functions in mitochondria [10,11]. A number of reports have suggested that MnSOD protects endothelial function [12,13], and reduces the damage inflicted on vascular wall cells by oxidized low-density lipoprotein (oxLDL) [14,15]. There are two genetic variants of MnSOD arising from a substitution of an alanine for a valine in the signal peptide. We previously reported that the valine allele of MnSOD decreases the mitochondrial MnSOD (mtMnSOD) activity, and the tolerance of the cells against oxLDL, thereby increasing the risk of coronary artery disease [16]. It is known that the distribution of MnSOD genotype is associated with ethnicity, and the prevalence of the valine/valine genotype of MnSOD is higher in the Japanese population than in Westerners [11,17,18]. Based on these findings, we hypothesized that the MnSOD genetic polymorphism may be related to the prevalence of VSA.

In this study, we investigated the association of MnSOD gene polymorphism with VSA.

Materials and methods

Subjects

This study complied with the principles of the Declaration of Helsinki concerning the participation of human subjects in clinical studies. The study protocol was approved by the Ethics Committee of each hospital participating in the study. The study was explained to every subject and written informed consent was obtained.

To investigate the association between the MnSOD polymorphism and VSA, a total of 846 Japanese subjects were enrolled consisting of 618 healthy subjects, 38—76 years old, and 228 VSA patients, 31—83 years old. The 618 healthy subjects were recruited at their annual health examination at Mitsui Memorial Hospital, Tokyo, Japan, between May and November 2000, and did not have any chest symptoms or electrocardiogram (ECG) abnormalities suggesting CAD, or a medical history of CAD. The 228 VSA patients were recruited at the Cardiovascular Institute Hospital, Tokyo, Japan, and were enrolled by the examination of 828 subjects who underwent coronary angiography (CAG) between November 1999 and July 2000 for the first time in their lives on suspicion of coronary artery disease because of chest symptoms or ECG abnormalities. Acetylcholine test was performed for the 353 patients who had no lesions with a percent diametric stenosis of 50% or more in their coronary arteries. Acetylcholine chloride dissolved in 5 ml of 0.9% saline was injected in incremental doses of 25, 50, and 100 μg into the left coronary artery and then 25 and 50 μg into the right coronary artery. VSA was diagnosed when total or sub-total occlusion of coronary artery accompanying the symptoms of myocardial ischemia was induced by intra-coronary injection of the drug. Acetylcholine test was positive in 228 of the 353 patients. Therefore, we enrolled the 228 patients in the study.

MnSOD genotyping

MnSOD genotypes were analyzed as previously described [16]. In brief, a 5 ml blood specimen was collected into a tube containing ethylenediaminetetraacetic acid (EDTA; final concentration 5 mM), from every subject. The genomic DNA was extracted from leukocytes using QIAamp blood kit (Qiagen, Tokyo, Japan). The genotype was determined by a fluorescence-based allele-specific polymerase chain reaction and melting curve analysis using a Light Cycler™ System (Roche Diagnostics, Mannheim, Germany). The polymerase chain reaction was performed using two amplification primers (forward primer: 5′-AGCCCAGCCGTGCGTAGA-3′, and reverse primer: 5′-GCGTGGTGCTTGCTGTGG-3′). An initial denaturation of DNA was accomplished at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 63 °C for 10 s, and elongation at 72 °C for 8 s. Melting curve analysis was performed using an upstream fluorescent probe (5′-GCAGGTCGGGGAGGCTGTGCTTCTGCCTGGAGCCCA-3′-Fluorescein) and a downstream probe (LightCyclerRed640-5′-ATACCCCAAAACCGGAG-3′-phosphate). The DNA samples were selected randomly and assessed in the same batch by a laboratory technician blinded to the patient origin of the samples.

Statistical analysis

Quantitative data are presented as mean ± standard deviation, and the categorical data as frequencies (percentage). Continuous variables were compared using the unpaired *t*-test. Binary variables were compared by means of the Fisher exact test, and the variables comprising more than two categorical factors were compared by means of the chi-square test. To identify the risk factors of VSA, univariate logistic regression analysis was performed using the valine/valine genotype and conventional coronary risk factors of VSA such as gender, age, hypertension, hyperlipidemia, diabetes, and smoking, as independent variables. Then, a multivariate logistic regression model was used to test the significance of the genotype after controlling for the other variables listed above. Odds ratios (ORs) were calculated as an estimate of relative risk of VSA associated with the valine/valine genotype. A *p*-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using JMP 5 software (SAS Institute, Cary, NC, USA).

Results

Assay of genetic polymorphisms

The d*F*/d*T* (*F*: fluorescence signal, *T*: temperature, d*F*/d*T*: the rate of change of the fluorescent signal with temperature) versus temperature curve of DNA corresponding to each MnSOD genotype is shown in Fig. 1. The alanine/alanine genotype had one peak centered at about 49.5 °C; the valine/valine genotype had one peak centered at about 59.5 °C, and the alanine/valine genotype had two peaks centered at about 49.5 °C and 59.5 °C. Among all subjects, we could not identify the genotypes of two samples from the

Table 1 Characteristics of the subjects enrolled to investigate the association between manganese superoxide dismutase polymorphism and VSA.

	Healthy subjects				VSA patients			
	Total 100.0 (616)	Ala/Ala 1.9 (12)	Ala/Val 28.3 (174)	Val/Val 69.8 (430)	Total 100.0 (228)	Ala/Ala 1.3 (3)	Ala/Val 19.3 (44)	Val/Val 79.4 (181)
Male (%) (*n*)	70.6 (435)	75.0 (9)	70.1 (122)	70.7 (304)	75.4 (172)	100.0 (3)	72.7 (32)	75.7 (137)
Age (year)	57.8 ± 9.7	61.0 ± 9.2	58.3 ± 10.1	57.5 ± 9.6	61.0 ± 57.8[†]	64.7 ± 6.0	60.6 ± 10.6	61.0 ± 10.1
Hypertension (%) (*n*)	21.1 (129)	25.0 (3)	19.5 (34)	21.4 (92)	28.1 (64)[*]	33.3 (1)	27.3 (12)	28.2 (51)
Hyperlipidemia (%) (*n*)	25.6 (158)	25.0 (3)	24.1 (42)	26.3 (113)	47.8 (109)[†]	33.3 (1)	47.7 (21)	48.1 (87)
Diabetes mellitus (%) (*n*)	5.0 (31)	8.3 (1)	3.4 (6)	5.6 (24)	11.4 (26)[*]	0.0 (0)	13.6 (6)	11.0 (20)
Hyperuricemia (%) (*n*)	8.8 (54)	8.3 (1)	9.2 (16)	7.9 (34)	9.2 (21)	0.0 (0)	11.4 (5)	8.8 (16)
Body mass index (kg/m^2)	22.9 ± 2.6	23.0 ± 3.0	22.9 ± 2.6	22.8 ± 2.7	23.1 ± 3.2	26.0 ± 1.7	23.9 ± 5.3	22.8 ± 2.4
Smoking (%) (*n*)	21.8 (134)	16.7 (2)	21.3 (37)	22.1 (95)	40.8 (93)[†]	33.3 (1)	40.9 (18)	40.9 (74)

VSA, vasospastic angina; Ala, alanine; Val, valine.
[*] $P < 0.05$ compared with healthy subjects.
[†] $P < 0.001$ compared with healthy subjects.

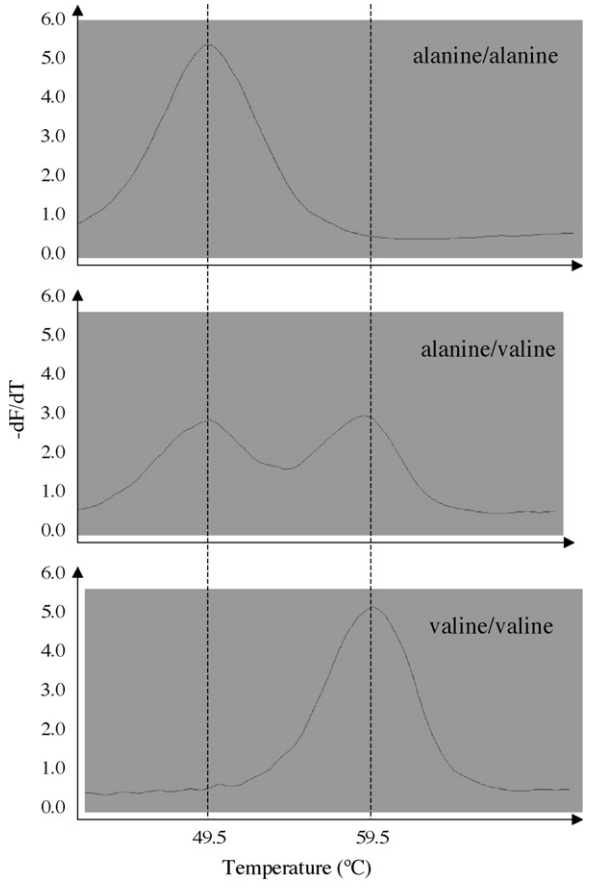


Figure 1 A plot of temperature versus −d*F*/d*T* for the detection of manganese superoxide dismutase genotype by Light Cycler analysis. *F*, intensity of fluorescent signal from LCRed; *T*, temperature; d*F*/d*T*, the rate of change of the fluorescent signal with temperature.

healthy subjects because we could not find a peak at either 49.5°C or 59.5°C. The genotyping error rate was 0.24%. In total, 616 healthy subjects and 228 VSA patients were included in the analysis.

Characteristics of the enrolled subjects

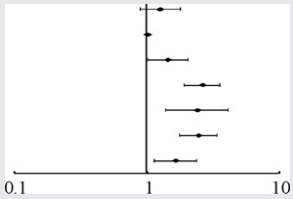
The characteristics of the subjects enrolled in the study are shown in Table 1. The prevalence of hypertension, diabetes mellitus, hypercholesterolemia, and smoking was higher in the VSA patients than in the healthy subjects. No significant difference could be found in the distribution of risk factors between the three genotypes both in healthy subjects and VSA patients.

Distribution of MnSOD genotypes in healthy subjects and in VSA patients

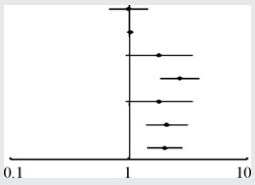
The MnSOD genotype distribution among the 616 healthy subjects was as follows: alanine/alanine 12 subjects (1.9%), alanine/valine 174 subjects (28.3%), and valine/valine 430 subjects (69.8%), and was compatible with Hardy—Weinberg equilibrium. This distribution was similar to that previously reported [11,18].

Table 2 Logistic regression analysis for risk factors and for the valine/valine genotype in relation to VSA.

	OR (95% CI)	*P*
A. Univariate logistic regression analysis		
Male	1.28 (0.90, 1.81)	0.167
Age (per year)	1.03 (1.02, 1.05)	<0.0001
Hypertension	1.46 (1.03, 2.07)	0.033
Hyperlipidemia	2.66 (1.94, 3.64)	<0.0001
Diabetes mellitus	2.43 (1.41, 4.19)	0.0014
Smoking	2.48 (1.79, 3.43)	<0.0001
Valine/valine	1.67 (1.16, 2.40)	0.0060



	OR (95% CI)	*P*
B. Multivariate logistic regression analysis		
Male	1.00 (0.68, 1.46)	0.99
Age (per year)	1.03 (1.01, 1.05)	0.0007
Hypertension	1.49 (0.94, 2.38)	0.092
Hyperlipidemia	2.71 (1.85, 3.95)	<0.0001
Diabetes mellitus	1.80 (0.94, 3.48)	0.079
Smoking	2.10 (1.39, 3.17)	0.0005
Valine/valine	2.02 (1.43, 2.85)	0.0012



By contrast, the MnSOD genotype distribution in the 228 VSA patients was: alanine/alanine 3 patients (1.3%), alanine/valine 44 patients (19.3%), and valine/valine 181 patients (79.4%). The distribution of MnSOD genotype was closely associated with VSA ($p = 0.019$ by chi-square analysis), that is, the valine allele was related to the presence of VSA.

The valine allele frequency was higher in the VSA patients (0.890) than in the healthy subjects (0.839) (OR = 1.55, $p = 0.0085$ by chi-square analysis).

Logistic regression analysis

Next, we performed logistic regression analysis to investigate the role of the valine/valine genotype as a risk factor of VSA. According to the univariate logistic regression analysis, the valine/valine genotype was associated with VSA with an OR of 1.67 (95% CI 1.16, 2.40; $p = 0.0060$, Table 2A). Furthermore, a multivariate logistic regression analysis revealed that the valine/valine genotype as well as age, smoking, and hypercholesterolemia, were independent risk factors for VSA (OR = 2.02, 95% CI 1.43, 2.85; $p < 0.0012$, Table 2B).

Distribution of MnSOD genotypes in the patients who underwent CAG and were diagnosed not to have VSA

To exclude the selection bias, we analyzed the distribution of the MnSOD genotypes in the 125 patients who underwent CAG, and were diagnosed to have neither atherosclerotic coronary artery disease nor VSA by CAG. These 125 patients had no stenotic lesions with percent diametric stenosis of 50% or more. All of them underwent acetylcholine test or ergonovine test, and the results were negative. The MnSOD genotype distribution in these 125 patients was: alanine/alanine 3 patients (2.4%), alanine/valine 38 patients (30.4%), and valine/valine 84 patients (67.2%). The distribution of MnSOD genotype was closely associated with VSA ($p = 0.040$ by chi-square analysis), that is, the valine allele was related to the presence of VSA (Table 3). The valine allele frequency in the 125 non-VSA patients was 0.824, and significantly lower than that in the 228 VSA patients (OR 1.73, $p = 0.013$, by chi-square analysis; Table 3).

Discussion

The major finding of our study is the significant association of the MnSOD polymorphism with VSA. The valine allele was closely related to VSA, and the valine/valine genotype was found to be an independent genetic risk factor of VSA. Although the prevalence of VSA in the world has not been precisely investigated, it is well known that the distribution of VSA has a significant association with ethnicity. That is, VSA is more common in the Japanese and Korean populations than in Caucasians [5,6]. Thus far, several genetic

Table 3 Distribution of manganese superoxide dismutase genotypes in the patients diagnosed not to have vasospastic angina (VSA) by coronary angiography (CAG).

Genotype	Non-VSA patients[a] ($n = 125$)	VSA patient ($n = 228$)
Alanine/alanine (%) (*n*)	2.4 (3)	1.3 (3)[†]
Alanine/valine (%) (*n*)	30.2 (38)	19.3 (44)[†]
Valine/valine (%) (*n*)	67.2 (84)	79.4 (181)[†]
Allele frequency (alanine/valine)	0.176/0.824	0.110/0.890[‡]

[a] Those who underwent CAG, but were diagnosed to have neither severe stenotic lesions nor vasospasm.
[†] $P = 0.040$ by chi-square analysis.
[‡] Odds ratio 1.73, $P = 0.013$ by chi-square analysis.

polymorphisms have been reported to have an association with VSA [19—23]. But many of these polymorphisms could not explain the distribution of VSA with respect to ethnic origin. MnSOD genotype distribution is also associated with ethnicity, and the valine allele frequency is higher in the Japanese than in Caucasians [11,17,18]. Thus, our data will provide an important clue to clarify the ethnicity association of VSA.

In this study, univariate and multivariable logistic regression analysis showed that hyperlipidemia, smoking, and aging to be the most meaningful risk factor for VSA as previously reported [24,25]. Our data revealed that the valine/valine genotype of MnSOD as well as hyperlipidemia, smoking, and aging are independent risk factors of VSA. Despite the finding that the odds ratio of the valine/valine genotype for VSA is lower than the other conventional risk factors, our data provide evidence that oxidative damage plays an important role in the pathogenesis of VSA.

There are many basic and clinical data that suggest that endothelial dysfunction plays a key role in the pathogenesis of vasospasm [7—9,26,27]. There are also several reports that superoxide radicals damage endothelial function, and superoxide dismutase acts protectively for the endothelial cells [28—30]. OxLDL enhances coronary vasoconstriction by increasing the activity of specific protein kinase C isoforms in coronary smooth muscle [31]. From these data, it may be suggested that oxidative damage in endothelial cells and smooth muscle cells is closely associated with vasoconstriction. Hypercontraction of smooth muscle cells also plays an important role in vasospasm [32]. It is reported that smooth muscle cell hypercontraction is mediated by oxidative stress-related effectors such as Rho-kinase [33]. Therefore, there is a possibility that the reduction in the tolerance against oxidative stress by the valine allele of MnSOD may be related to the hypercontraction of smooth muscle cells, thereby increasing the risk of VSA. We previously reported that the valine allele of MnSOD is associated with reduction of the mitochondrial MnSOD activity and the tolerance of macrophages against apoptosis induced by oxLDL [16]. We did not investigate the differences in the function of endothelial cells and smooth muscle cells for each genotype group because of the difficulty in sampling these cells. But since the intracellular signaling pathway appears to be similar for various types of vascular wall cells, the MnSOD polymorphism may also be associated with the tolerance of both endothelial cells and smooth muscle cells against oxidative damage, thereby modifying the risk of atherosclerosis and vasospasm.

Study limitation

We enrolled 618 healthy subjects with no signs of angina pectoris. But we did not demonstrate that they did not have atherosclerotic coronary artery disease or VSA by examinations such as exercise test or CAG. Thus, there is a possibility that unsymptomatic VSA patients are included in the 618 healthy subjects. Despite this, we confirmed the association of MnSOD polymorphism with VSA by another analysis enrolling 228 VSA patients and 125 subjects demonstrated not to have VSA by acetylcholine test. A study with a larger population comparing the VSA patients and control subjects rigorously diagnosed not to have VSA is necessary to obtain more convincing evidence.

Conclusions

Our data indicate that the valine variant of MnSOD signal peptide increases the risk of VSA, and the valine/valine genotype is an independent genetic risk factor of VSA. Our results provide an important clue to clarify the role of oxidative stress in VSA and the distribution of VSA with respect to ethnic origin.

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