50-bp Ins/Del Polymorphism of SOD1 is Associated with Increased Risk of Cardiovascular Disease

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Abstract- Compelling evidence suggests that the oxidative stress plays a key role in the pathophysiology of cardiovascular disease (CVD). Superoxide dismutase (SOD) enzymes play a major role in detoxification of reactive oxygen species and protection against oxidative stress. We examined the possible association between a 50-bp insertion/deletion in the SOD1 promoter 1684-bp upstream of the SOD1 ATG with CVD in an Iranian population. A total of 400 individuals including 200 CVD patients and 200 healthy subjects from the same ethnic background as the control group were participated in this study. Genomic DNA from all subjects was screened for the 50-bp SOD1 promoter deletion using a polymerase chain reaction (PCR) assay. Our finding showed an association between SOD1 DEL/DEL (9% vs. 2.5%) and INS/DEL genotypes and risk of CVD and these genotypes increased the susceptibility to CVD (OR=2.096, 95% CI: 1.336-3.286, P= 0.001 for the INS/DEL genotype; OR=4.811, 95% CI: 1.734-13.346, P= 0.003 for the DEL/DEL genotype). Additionally, the DEL allele of the SOD1 variation was found to be more prevalent in the CVD patients with the frequency of 26.3% and 13.5% in cases and controls, respectively, and this difference reached statistical significance (OR=2.281, 95% CI: 1.586-3.279, P= 0.001). The analysis of SOD1 genotypes according to patients' characteristics revealed that the SOD1 Ins/del and Del/Del genotypes were more prevalent in CVD patients with a history of CVD or hypertension or DM ($P<0.05$), whereas the majority of Ins/Ins genotype carriers had no history of these diseases. Overall, our results demonstrated that SOD1 50-bp Del/Del and Ins/Del genotypes, as well as Del, allele, were associated with an increased risk of CVD.
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Introduction

Cardiovascular disease (CVD) is the principal cause of mortality worldwide, with latest statistics proposing that it is responsible for 17.5 million deaths annually with several fold higher numbers thought to suffer from CVD related morbidity (1). A wealth of evidence suggests that the oxidative stress plays a key role in the pathophysiology of several cardiovascular diseases including hypertension, myocardial infarction, stroke, atherosclerosis, and ischaemia–reperfusion injury and heart failure (2,3). In endothelial and smooth cells of the vascular wall and in myocardial cells, the oxidative stress revealed to induce cell proliferation, hypertrophy,

apoptosis and inflammation leading to pathophysiological processes and damage to cellular components (4). Oxidative stress is defined as an imbalanced redox state where pro-oxidants overwhelm antioxidant capacity, resulting in increased availability of ROS (reactive oxygen species) (5).

ROS including free radicals such as superoxide anion ($O2-$) and nonradical species such as hydrogen peroxide, nitric oxide (NO), are produced unceasingly in all cells as part of the normal cellular metabolism (4). Oxidative stress occurs once production of ROS surpasses local antioxidant capacity. In this situation, ROS can stimulate oxidation of LDL, forming ox-LDL, which is not recognized by the LDL receptor leading to

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foam cell formation (6). Additionally, ROS can activate formation of advanced glycation end products (AGEs) (7), polyol pathway, hexosamine pathway and PKC, involved in the pathogenesis of vascular complications (7). Supraphysiological levels of ROS severely damage DNA, lipids, proteins, and disrupt cardiovascular reactivity (8,9). Superoxide dismutase (SOD), glutathione peroxidase (GPX1) and catalase (CAT) compose the primary defense system which removes extra ROS and maintains balance between oxidative and antioxidative activity under normal physiological conditions (10).

SODs are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide (11). They play an important role in antioxidant mechanism in nearly all cells exposed to oxygen. Three isoforms of SOD are expressed in humans, encoded by different genes. Cu-Zn SOD1 is present in the cytosol, nucleus, and the intermembrane space of mitochondria, and is dependent on copper (Cu) and Zinc (Zn) for its activity; SOD2 (MnSOD), a manganese containing enzyme, is present in the mitochondrial matrix, and SOD3 is the extracellular member of the SOD family (EC-SOD), and like SOD1, its metal cofactors are Cu and Zn (12). In blood vessels, SOD1 accounts for ~85% of the total cellular SOD activity of most mammalian cells where it preserves NO release from the endothelium (4,13). Mice deficient in SOD1 show increased superoxide and vascular dysfunction, whereas SOD1 overexpression decreases oxidative stress, attenuates induction and activation of matrix metalloproteinase-9 (MMP-9), and protects against vascular dysfunction (14,15).

The human SOD1 gene (Entrez Gene ID 6647) is located on chromosome 21q22.11, and it codes for the monomeric SOD1 polypeptide. The coding region consists of five exons interrupted by four introns. Several polymorphisms have been identified in SOD1 gene, mainly distributed in the regulatory regions, including promoter, UTRs, and introns (16). A 50 bp deletion polymorphism in SOD1 promoter (1684 bp upstream of the ATG start codon) has been recognized. In-vitro analyses have demonstrated that the SOD1 50 bp deletion is associated with decreased promoter activity and low-mRNA levels in cells, which is caused by the loss of two Sp1 binding sites (17). Associations of SOD1 gene variants with type 2 diabetes (18) (4), and cardiovascular-related risk factors (19) have been observed, but no data regarding the association of SOD1 50-bp deletion variant with CVD is available. Therefore, the present study aimed to evaluate the association of SOD1 50-bp deletion

polymorphism in CVD patients and compare that with healthy individuals in a south-east Iranian population.

Materials and Methods

Patients and clinical data collection

A total of 400 subjects including 200 CVD patients and 200 healthy individuals were enrolled for the genotyping of SOD1 50bp deletion polymorphism. Blood samples were collected after a 12-hour overnight fast before cardiovascular procedures. The CVD patients were described as the occurrence of recognized myocardial infarction, coronary insufficiency (unstable angina with demonstrated ischemic electrocardiographic changes), death due to coronary heart disease, or atherothrombotic stroke. At the time of enrollment, participants completed questionnaires on race/ethnic status, demographics, history of cigarette smoking, medical history and medications. In addition, use of lipid-lowering drugs (LLD), family history of hypertension, diabetes mellitus (DM), serum levels of total plasma cholesterol (TC), total triglycerides (TG), LDL-C and HDL-C was recorded (Table 1).

Hypertension was determined as systolic blood pressure >140 mm Hg, diastolic blood pressure >90 mm Hg or therapy for hypertension. DM was diagnosed as a fasting blood glucose level above 7 mmol/L or current use of hypoglycemic agents (19). TC, TG, LDL-C and HDL-C levels were measured as mg/dl using a standard enzymatic kit (Pars Azmoon Co, Iran). The study was approved by the institutional review board of the cardiovascular institute, and the participating hospitals. All subjects provided their written informed consent and were self-reported as the Zahedan University of Medical Sciences.

DNA preparation, PCR

Blood samples were collected in EDTA-containing tubes, and genomic DNA was isolated from peripheral blood leukocytes using salting-out method as described previously (20). Genotyping was performed by polymerase chain reaction (PCR) using one forward primer: AATTCCTTACCCCTGTTCTA and one-reverse primer: GGCAGATTTCAGTTCATTGT. The reaction mixture was subjected to denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 30 secs, 62°C for 30 secs, 72°C for 25 secs, then by a final extension at 72°C for 10 min. The resultant polymerase chain reaction (PCR) products yielded 2 DNA fragments of 297 and 247 bp for the Ins and Del alleles, respectively, on 2% agarose gel as presented in figure 1.
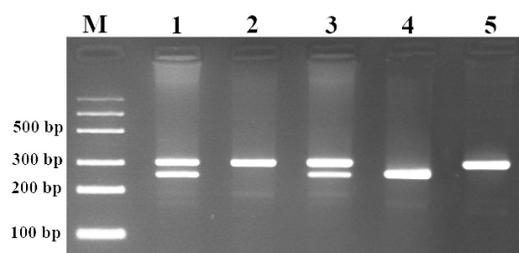
Figure 1. Photograph of the PCR products of the 50 bp ins/del of SOD1 gene on 2% agarose gel. M: DNA marker; Lane 1, 3: Ins/del; Lanes 2, 5: Ins/Ins; Lane 4: del/del

Statistical analysis

All statistical analyses were performed using the SPSS software for Windows, version 15.0 (SPSS Inc, Chicago IL, USA). Comparisons between groups were analyzed by Student t, and ANOVA tests with 95% confidence interval when appropriate for quantitative variables. For genetic comparisons, Hardy–Weinberg equilibrium and differences in allele and genotype frequencies were evaluated using the χ2 test. Logistic regression was used for computation of ORs and 95% CIs. A two-tailed P-value less than 0.05 was considered to be statistically significant.

Results

In the present study, we found that several clinical characteristics of participants were statistically different between CVD patients and healthy controls such as sex, LLD usage, ethnicity, smoking status, history of CVD, hypertension, DM, serum levels of TG, TC, LDL-C and HDL-C (table1). Since in this study only a fraction of CVD patients took LLD, therefore compared with control, their serum TG, TC and LDL-C levels were significantly higher, and HDL-C level was significantly lower in CVD patients (*P*<0.01).

The allele and genotype frequencies of SOD1 gene 50bp variant were compared between 200 CVD cases and 200 controls (table 2). A significant difference was found between two groups regarding allelic and genotyping distribution of the SOD1 50bp DEL variant. In patients the difference reflected a significant increase in the DEL/DEL (9% vs. 2.5%) and INS/DEL (34.5% vs. 22.0%) genotypes and a significant decrease in INS/INS genotype (56.5% vs. 75.5%) (OR=2.096, 95% CI: 1.336-3.286, *P*=0.001 for the INS/DEL genotype; OR=4.811, 95% CI: 1.734-13.346, *P*=0.003 for the DEL/DEL genotype). In addition, the DEL allele of this variation was found to be more prevalent in the CVD patients with the frequency of 26.3% and 13.5% in cases and controls, respectively and this difference reached statistical significance (OR=2.281, 95% CI: 1.586-3.279, *P*= 0.001). Adjustments according to the age, sex was done in genotype analysis, but the associations between Del/Del and Ins/Del genotypes and the CVD risk remained significant. The observed genotype frequency of SOD1 del variant was statistically consistent with the expected distributions according to Hardy–Weinberg equilibrium in all the studied groups (*P*=0.123 and 0.412 in patients and controls, respectively).

Table 1. Clinical and biochemical data in CVD cases and controls

	CVD case subjects	Control subjects	*P*
n	200	200	--
Age (years)	60.84±10.85	61.32±8.98	0.641
Men n (%)	129 (66.2)	83 (41.7)	<0.001
Ethnicity	--	--	<0.001
Fars	112(58.0)	131 (65.5)	--
Balouch	79 (40.9)	21(10.5)	--
Afqan	2 (1.0)	48(24.0)	--
LLD Users n (%)	74 (37.0)	6 (3.2)	<0.001
Current smoking n (%)	34 (19.2)	16 (8.1)	0.006
History of CVD n (%)	66 (34.7)	7(3.5)	<0.001
History of hypertension n (%)	78 (51.3)	7 (3.6)	<0.001
History of Diabetes n (%)	77 (44.0)	3(1.5)	<0.001
TG level (mg/dl)	214.02±40.56	119.29±41.41	<0.001
Cholesterol level (mg/dl)	209.99±39.27	174.71.±28.42	<0.001
Cholesterol LDL (mg/dl)	130.39±29.99	112.36±33.53	<0.001
Cholesterol HDL (mg/dl)	37.57±5.85	49.61±8.14	<0.001

Clinical characteristics of age, LDL-C, HDL-C, TC and TG values are given as mean±SD; and other values as number and percent of individuals

Table 3 represents demographic characteristics of subjects according to SOD1 genotypes. From the table, it can be inferred that the sex, history of CVD, DM and hypertension were different among three SOD1 genotypes. The frequencies of Ins/del and Del/Del genotypes among CVD patients with a history of CVD or hypertension or DM were much higher than that in patients negative for these risk factors. Although, the Del/Del carriers (137.4±27.9) held elevated levels of LDL-C compared to individuals with Ins/Ins and Ins/Del genotypes (127.4±27.9 and 133.3±33.6, respectively), but the difference was not statistically significant (*P*=0.067).

Table 2. Distribution of SOD1 genotypes between CVD patients and healthy individuals

Genotypes	CVD n (%)	Control n (%)	OR (95% CI)	P-value	*Adjusted OR (95% CI)	Adjusted *P*-value
INS/INS	113 (56.5)	151 (75.5)	-	Ref.	-	Ref.
INS/DEL	69 (34.5)	44 (22.0)	2.096 (1.336-3.286)	0.001	2.288 (1.414-3.702)	0.001
DEL/DEL	18 (9.0)	5 (2.5)	4.811 (1.734-13.346)	0.003	5.898 (2.061-16.880)	0.001
Allele						
INS	295 (73.7)	346 (86.5)	-	Ref.	-	Ref.
DEL	105 (26.3)	54 (13.5)	2.281 (1.586-3.279)	0.001		

ORs and 95% CIs were computed with the use of binary logistic regression analyses. * Adjusted ORs were stratified by age, sex

Table 3. Demographic characteristics of subjects according to SOD1 genotypes

	SOD1 50-bp del variants			
	Ins/Ins	**Ins/Del**	**Del/Del**	***P***
Age (years)	61.13	60.37	60.75	0.909
Sex				
Men n (%)	83 (64.3)	34 (26.4)	12 (9.3)	
Female n (%)	27 (40.9)	33 (50.0)	6 (9.1)	0.003
Ethnicity				
Fars	65 (58.0)	39 (34.8)	8 (7.1)	
Balouch	46 (58.2)	25 (31.6)	8 (10.1)	0.213
Afghan	0 (0.0)	1 (50.0)	1 (50.0)	
LLD Drugs Usage				
Positive n (%)	34 (45.9)	28 (37.8)	12 (16.2)	0.101
Negative n (%)	20 (69.0)	7 (24.1)	2 (6.9)	
Current smoking				
Positive n (%)	18 (52.9)	11 (32.4)	5 (14.7)	0.439
Negative n (%)	81 (56.6)	51 (35.7)	11 (7.7)	
History of CVD				
Positive n (%)	23 (20.7)	35 (53.0)	8 (12.1)	0.0001
Negative n (%)	83 (71.6)	25 (21.6)	8 (6.9)	
History of hypertension				
Positive n (%)	28 (35.9)	34 (43.6)	16 (20.5)	0.0001
Negative n (%)	51 (68.9)	22 (29.7)	1 (1.4)	
History of Diabetes				
Positive n (%)	35 (45.5)	34 (44.2)	8 (10.4)	0.023
Negative n (%)	64 (65.3)	25 (25.5)	9 (9.2)	
TG level (mg/dl)	214.4±38.35	214.2±44.7	207.9±37.8	0.754
Cholesterol level (mg/dl)	210.5±40.0	212.0±41.8	199.1±21.4	0.137
Cholesterol LDL (mg/dl)	127.4±27.9	133.3±33.6	137.4±27.9	0.067
Cholesterol HDL (mg/dl)	37.6±5.90	37.4±5.98	37.6±5.23	0.821

Clinical characteristics of age, LDL-C, HDL-C, TC and TG values are given as mean±SD; and other values as number and percent of individuals

Discussion

Oxidative stress plays a significant role in the pathogenesis of cardiovascular disease and coronary artery disease (CAD) by altering vasomotor tone (21), enhancing atherosclerosis (22) and contributing to hypertension (23, 24). In vascular cells, excess generation of ROS including hydrogen peroxide, nitric oxide (NO) and O2-, catalysed by enzymes such as reduced nicotinamide adenine (phosphate) (NAD(P)H)

oxidase, can induce vascular and myocardial structural damage and result in a hypertrophic phenotype (25).

In the current study, it was revealed that SOD1 50 bp Ins/Del and Del/Del genotypes, as well as the Del allele, were associated with an increased risk of CVD. The carriers of the Del/Del genotype had a 5.9-fold higher risk of CVD than those carrying the Ins/Ins genotype. Additionally, the Del allele was more frequent in CVD patients than in controls, with the frequency of 26.3% and 13.5% in patients and control, respectively.

SOD1 accounts for ~85% of the total cellular SOD activity of most mammalian cells and is highly active in the human kidney and in the vascular wall (4). It has been shown that SOD1 is an important mediator of post-ischaemic injury in the heart (26) and that increasing intracellular SOD1 protects the heart from this injury (14).

The SOD1 promoter region is of great importance for the fine-tuned modulation of SOD1 mRNA levels as it harbors the binding sites for several transcription factors [8] and sequence differences in these cis-acting responsive elements may be causative for varied mRNA expression. In vitro analysis demonstrated that the 50 bp deletion polymorphism in SOD1 promoter (1684 bp upstream of the ATG start codon) is associated with reduced promoter activity and deficient SOD1 expression in cells, because of the loss of two Sp1 binding sites. (4). SOD1 deficiency is believed to result in increased levels of vascular superoxide (27), hypertrophy of arteries and peroxynitrite and impaired endothelium-dependent relaxation in both large arteries and microvessels (18) (28).

SOD has been proposed to be involved in atherogenesis through suppression of oxidative alterations caused by O2-, prevention of O2- -mediated removal of NO, thereby facilitating endothelium-dependent vasorelaxation inhibition of leukocyte adhesion to the vascular endothelium, and altered vascular cellular responses (11). Impaired expression of SOD1 is associated with acute or chronic oxidative injury, including atherosclerosis (29). Growing evidence has suggested that atherosclerosis could be thought of as a chronic inflammatory disease with the basic abnormality lying in the redox-state of the vascular wall cells (30). (14) (11). The earliest events in the pathogenesis of atherosclerosis are thought to be changes in endothelial functions, in turn triggered by ROS-induced oxidative modification of low-density lipoproteins (LDL), leading to the formation of oxidized LDL (oxLDL), a molecule that is not recognized by the LDL receptor and contributes to foam cell formation

(18). Besides, oxLDL would act as a pro-atherogenic stimulus by activating NADPH oxidase, and thus promoting the production of ROS, which in turn has a positive feedback effect on further oxLDL production. ROS can promote monocyte recruitment and infiltration into the intima, as well as foam cell formation, vascular smooth-muscle cell proliferation, leucocyte and smooth-muscle cell chemotaxis. The accumulation of atherosclerotic plaque in the lining of the arterial wall produces narrowing of the vessel, which contributes to the development of cardiovascular diseases (22,31).

Oxidative stress has also been implicated in the pathogenesis of hypertension (32). Hypertension is associated with increased superoxide anion, the major vascular ROS, which inactivates NO and result in a reduction in NO bioavailability thus impairing relaxation (2, 33). The reaction product between $O2 −$ and NO, peroxynitrite ($OONO−$), constitutes a strong oxidant molecule, which is able to oxidize proteins, lipids and nucleic acids, causing cell damage in multiple organs including the brain, the vasculature and the kidney (2,5). SOD is very important in NO bioavailability, and NO-induced vascular relaxation is associated with regulation of blood pressure (11,13). Mechanisms by which SOD improves hypertension include modulation of vasodilation (34), vasoconstriction (35), vascular remodeling (36), cardiac hypertrophy (37) and neuronal control of sympathetic activity (38). In accord with these facts, our results certify the protective role of SOD1 against hypertension, since the SOD1 Ins/Del and Del/Del genotypes, which were proposed to be associated with low levels of SOD1, were more prevalent in patients with a history of hypertension, whereas the majority of SOD1 Ins/Ins carriers had no hypertension history.

It is well known that sympathetic activity is enhanced in patients with essential hypertension or secondary hypertension in chronic kidney disease (39), diabetes (40), or obesity (41), and a variety of experimental hypertensive models, such as spontaneously hypertensive rats (SHR) (27). Superoxide anions in the paraventricular nucleus (PVN) play an important role in excessive sympathetic activation (27,42). Central SOD1plays an important role in the modulation of sympathetic nerve activity. In fact, SOD1 overexpression in the PVN reduces arterial blood pressure, attenuates sympathetic activity, and improves myocardial and vascular remodeling in SHR (38).

The analysis of SOD1 genotypes according to patient's characteristics also revealed that the SOD1 Ins/Del genotype was more common in CVD patients

with a history of DM, whereas the majority of Ins/Ins genotype carriers had no history of DM. Our findings in SOD1 gene supports the known fact that serum SOD activity is significantly reduced in patients with DM (43). SOD1 deficiency in DM is thought to be caused by either genetic alterations such as 50 bp deletions or by non-enzymatic glycation, known as the Maillard reaction (44,45). There is strong evidence that hyperglycaemia causes oxidative stress, which can influence multiple systems linked to diabetic complications (45,46). Increased production of ROS in hyperglycemic conditions has been reported to decrease NO bioavailability (9). NO produced by vascular endothelium regulates vasodilation, anti-coagulation, leukocyte adhesion and smooth-muscle proliferation in the vasculature (4).

In conclusion, in the present study it was demonstrated that SOD1 50bp Del/Del and Ins/Del genotypes, as well as Del, allele were associated with an increased risk of CVD, and could possibly contribute to susceptibility to CVD. To the best of our knowledge, this is the first report regarding association of SOD1 50 bp deletion variant and susceptibility to CVD. Our results need to be replicated in other nationalities and confirmed with larger samples size.

Acknowledgement

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