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RESEARCH COMMUNICATION

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50 bp deletion in promoter superoxide dismutase 1 gene and increasing risk of cardiovascular disease in Mashhad stroke and heart atherosclerotic disorder cohort study

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Abstract

Cardiovascular disease (CVD), one of the main mortality causes worldwide is considered to be affected by general oxidative stress and inadequacy antioxidant system. Superoxide dismutase 1 (SOD1), a cytosolic antioxidant enzyme has a key role in neutralizing the excessive prooxidant by scavenging the super oxide anions. SOD1 polymorphic variants exhibit the altered activity properties. In the current study, we are aimed to investigate the association between the SOD1 polymorphism and CVD prevalence. A 6-years case control follow up study was designed to genotype the 526 participants (311 controls and 215 cases) for studying the 50 bp INS/DEL polymorphism at SOD1 promoter gene and analyze their blood lipid profile and anthropometric characteristics. Among the two possible alleles of

Abbreviations: CVD, cardiovascular disease; SOD1, superoxide dismutase1; MASHAD study, Mashhad stroke and heart atherosclerotic disorder; W, wild type; M, mutant; INS/DEL, insertion/deletion; Bp, base pair; ROS, reactive oxygen species.

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the SOD1 gene (Wild [W] and Mutant [M]) the meaningful association was detected between the Mutants' frequency and the prevalence of CVD patients (p-value <.001). The W and M allele refer to inserted and deleted 50 bp in the polymorphic site of the SOD1 promoter, respectively. The WM and MM genotypes' frequency which indicate the wild heterozygotes and Mutant homozygotes, respectively, were significantly correlated with the prevalence of cardiovascular disease (p-value <.001). The present study has the potential to introduce the 50 bp INS/DEL polymorphism of SOD1 genotyping as a novel unique diagnostic approach for screening the high risk CVD.

KEYWORDS

cardiovascular disease, deletion, oxidative stress, superoxide dismutase 1 variant

1 | INTRODUCTION

Oxidative stress is due to an imbalance between the production and breakdown of reactive oxygen species (ROS) or reactive nitrogen species.[1] Antioxidant systems may be related to the development of oxidative alterations in arterial cells[2] and lead to the pathogenesis of cardiovascular disease (CVD).[3] Superoxide dismutase (SOD) is an antioxidant enzyme, involved in the catabolism of the superoxide anion.[4–6]

A particularly important pathophysiological event related to ROS is oxidation of lipids, in particular of low-density lipoprotein (LDL),[7] a process that is central to atherosclerotic lesion formation.

Coronary artery disease (CAD) is the main cause of mortality in the world.[8] Despite recognition of traditional risk factors for CAD including smoking, hypertension, diabetes mellitus, obesity, metabolic syndrome, physical inactivity, and dyslipidemia,[9,10] the occurrence of CAD is growing. Atherosclerosis is now recognized as a chronic inflammatory disease that can lead to CVD. Atherosclerotic plaque formation and vascular endothelial cell injury results from high levels of ROS.

SOD is an enzyme that catalyzes the dismutation of the superoxide ($O2^-$) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2),[11] and plays a pivotal role in balancing the concentration of ROS. Three isoforms of SOD are reported in humans, that encoded by different genes. SOD1 isoform in blood circulation explaining 85% of the SOD activity related to SOD polypeptide.[12,13] The gene encoding SOD1 polypeptide is located on human chromosome 21q22.11 and there are five exons and four introns in this area, and the sod1 promoter has a high GC-rich region, as well as TATA box and CCAAT box (Figure 1). Regulation of the SOD genes may play a role in modulating the concentrations of ROS. The compartmentalization and control of SODs at both expression and activity levels contribute to the level of SOD activity and consequently local ROS concentrations.[14]

Several studies have identified several polymorphisms in SOD1 gene, that are mainly located in the regulatory regions, including the promoter region, UTRs, and introns.[15] A 50 bp deletion variation in SOD1 promoter (1,684 bp upstream of the ATG start codon) has been identified and is associated with reduced mRNA expression.[16] Some investigators have shown that the association between the SOD1 gene variants and type 2 diabetes[12,17] and cardiovascular risk factors,[18] but few studies have been done on SOD1 50-bp deletion variant with respect to CVD risk. Accordingly, in this study, we investigated the serum SOD1 activity and test the hypothesis that the demonstrated association between hetero and homozygosity for 50 bp deletion in the SOD1 promoter 1,684 bp upstream of the SOD1 ATG and risk of CVD in a large well-defined Mashhad stroke and heart atherosclerotic disorder (MASHAD) cohort study patients.

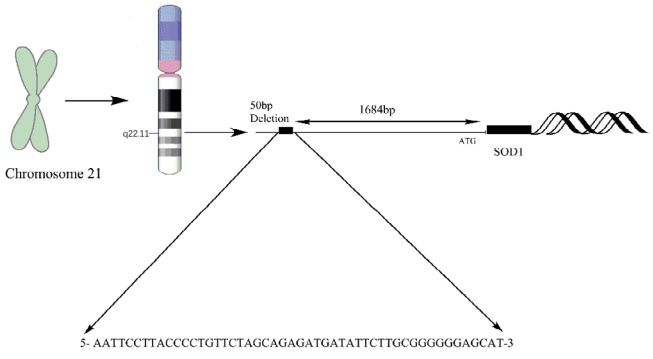

FIGURE 1 The location of the 50 bp-deletion relative to the SOD1 gene. SOD1, superoxide dismutase1

2 | METHODS

2.1 | Study population

The MASHAD cohort study is a cohort study of cardiovascular risk factors; it started in 2010 and will continue until 2020. The total population that was recruited was 9,761 using a stratified cluster random sampling technique.[19] The exclusion criteria for recruitment at baseline were CVD risk factors such as diabetes, hyperlipidemia, and hypertension and CVD history.

2.2 | Demographic, anthropometric, and metabolic data

Body weight and height of participants were measured using with calibrated digital balance in kilogram scale (SECA 813, Hamburg, Germany) to the nearest 0.1 kg and centimeters and the nearest 0.1 cm with a stadiometer (SECA 217, Hamburg, Germany); also Waist and hip circumference were measured using centimeters.

Systolic and diastolic blood pressure were measured using a sphygmomanometer twice in exactly the same manner that it explained previously.[19]

2.3 | Follow-up

Participants were followed up by the phone twice in 2011 and 2014 and 646 subjects self-reported a possible CVD event; they were invited for verification of their status in 2015–2016. According to this follow up 235 CVD events were confirmed that included 208 who survived the event and 27 who died. They were registered according to the International Classification of Diseases, according to the ICD-10. Four hundred and eleven subjects did not have a confirmed CVD event. DNA for subjects with confirmed event (stable and unstable angina, MI and CVD sudden death) and no event were extracted and the 50 bp deletion in promoter SOD1 gene was determined. Finally, 200 subjects with a confirmed event and 271 without an event were compared and were well matched for the prevalence of diabetes mellitus, dyslipidemia and hypertension, and the relative risk for CVD associated with the 50 bp deletion in these subjects was evaluated (Figure 2).

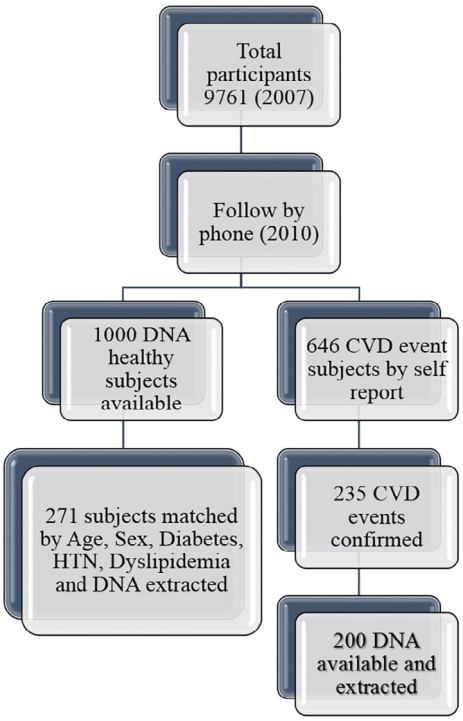

FIGURE 2 Determination of analytic sample size, The MASHAD Study cohort. MASHAD study, Mashhad stroke and heart atherosclerotic disorder

2.4 | Blood sample

2.4.1 | Routine biochemical analysis

After a 14 hr overnight fasting, blood sample was collected between 8 and 10 a.m. by venipuncture of an antecubital vein from subjects in a sitting position.

Fasting blood glucose, serum total cholesterol, LDL and high-density lipoprotein (HDL) cholesterol, triglycerides, and serum SOD1 activity were measured.

2.4.2 | DNA preparation, PCR

Blood samples were collected into EDTA-containing tubes, and genomic DNA was derived from peripheral blood leukocytes using Fermentas DNA extraction kit. The DNA for 471 subjects including 200 subjects with event and 271 healthy subjects were available. Genotyping was carried out 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 7 min, followed by 35 cycles at 95°C for 30 s, 62°C for 30 s, 72°C for 25 s, then by a final extension at 72°C for 10 min. The resultant PCR products for the Del alleles were separated using a 2.5% agarose gel (Figure 3).

2.4.3 | SOD1 activity assay

The assay buffer contained Tris–cacodylic acid buffer and a pyrogallol solution. Tris–cacodylic acid buffer (0.05 M, pH 8.2) including 0.001 M diethylenediamine Penta acetic acid (DTPA) was prepared by adding Tris (0.05 M, containing 0.001 M DTPA) to cacodylic acid (0.05 M,

containing 0.001 M DTPA) until pH 8.2 was got. Before use, the buffer was balanced in air for 1 hr; for preparing the pyrogallol solution (0.2 mM) a stock 0.02 M (100) pyrogallol solution was made in water, flushed with nitrogen for 1 hr to remove soluble oxygen, aliquoted (100 µl per aliquot), and frozen until use.

Each serum event and healthy samples (20 µl/well) was added in duplicate wells. Immediately before measurement, pyrogallol stock solution (0.02 M) was diluted 1:100 with equilibrated assay buffer and added to the wells (180 µl/well) using a multichannel pipettor. The reactions were read in a plate reader at 405 nm in 5-min intervals during 1 hr. The blocking of pyrogallol oxidation was determined for every dilution of SOD. A level of SOD that prevented the oxidation of pyrogallol by 50% (relative to the control) was defined as a unit of SOD activity under the situations explained.[20]

SPSS version 18 (SPSS Inc. Chicago, IL) was used for all statistical analyses. The normally distribution of the data was checked using the Kolmogorov–Smirnov test. Data were presented as Mean ± *SD* or interquartile range. T-student test or Mann–Whitney U and analysis of variance (ANOVA), or Kruskal–Wallis have been done.

Kaplan–Meier survival curves were applied to explain the outcomes in the follow-up period according to genotypes and allele. Relative risks (RRs) and 95% confidence intervals (CIs) of event from all cases for genotypes and allele were examined by using extended Cox's proportional hazard model after adjusted by age. GraphPad Prism 6 and Adobe Illustrator CC |Graphic Design Software for figures were used. Hardy Weinberg disequilibrium was check.

3 | RESULT

3.1 | Demographic and anthropometric characteristics

During the 6 years follow up, 235 CVD events (2.7%) were confirmed among the MASHAD cohort. Baseline Clinical and biochemical data in subjects with an event and the no event group after follow up are shown in Table 1.

The mean of age of the healthy subjects was 50.39 ± 7.82 years and in group with an event was 50.8 ± 6.68 years ($p > .05$). 44.5% of the subjects with an event were male and 55.5% of subjects were female. In addition, 59.5, 17.2, and 23.3% subjects with an event were non-smokers, ex-smokers, and current smoker, respectively (Table 1). About 35.1% of healthy subjects and 39.4% of subjects with an event had a family history of CVD. The mean systolic and diastolic blood pressures were 120.68 ± 18.09 and 78.57 ± 11.01 mmHg in healthy subjects, and 123.4 ± 19.69 and 80.17 ± 10.4 mmHg in subjects with an event, respectively (Table 1); these were not significantly different between the two groups ($p > .05$).

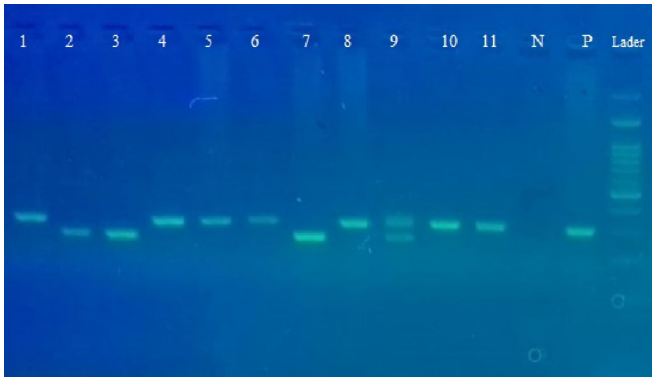

FIGURE 3 Photograph of the PCR products of the 50 bp of SOD1 del on 2.5% agarose gel; P, positive control; N, negative control; 1, 4, 5, 6, 8, 10, 11 lane WW; 9 lane WM, and 2,3,7 MM

TABLE 1 Clinical and biochemical data in CVD cases and controls

	No event *n* (%)	Event *n* (%)	*p*-value
n	271	200	526
Age, year	50.39 ± 7.82	50.8 ± 6.68	.58
Sex			
Male	133 (41.5%)	89 (44.5%)	.17
Female	138 (58.5%)	111 (55.5%)	
Smoking			
Non-smoker	213 (68.8%)	127 (59.5%)	.06
Ex-smoker	34 (11.3%)	36 (17.2%)	
Current smoker	62 (19.9%)	49 (23.3%)	
CVD family history *n* (%)	79 (35.1%)	43 (39.4%)	.25
Systolic blood pressure (mmHg)	120.68 ± 18.09	123.4 ± 19.69	.12
Diastolic blood pressure (mmHg)	78.57 ± 11.01	80.17 ± 10.4	.24
Serum triglyceride (mg/dl)	118 (93–161)	124 (95–217)	.15
Serum total cholesterol (mg/dl)	189.8 ± 39.54	190.62 ± 42.44	.83
LDL cholesterol (mg/dl)	80.33 ± 36.08	82.88 ± 36.08	.56
HDL cholesterol (mg/dl)	55.12 ± 9.84	54.06 ± 9.08	.54
Serum glucose (mg/dl)	96.32 ± 38.34	99.6 ± 62.98	.52
Serum SOD activity (U/ml)	2.45 ± 1.7	2.07 ± 1.27	.11

Note: Data presented as Mean ± *SD* or inter quartile range. T-test or Mann–Whitney U has done for comparing two groups.
Abbreviations: CVD, cardiovascular disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SOD, superoxide dismutase.

TABLE 2 Clinical and biochemical data according to SOD1 genotypes

	WW	WM	MM	*p*-value
n	289	159	23	
CVD family history n (%)	100 (82.0%)	18 (14.8%)	4 (3.2%)	.57
Systolic blood pressure (mmHg)	122.74 ± 18.96	122.72 ± 20.06	125.91 ± 19.22	.822
Diastolic blood pressure (mmHg)	79.33 ± 11.58	78.95 ± 10.02	83.8 ± 8.53	.27
Serum triglyceride (mg/dl)	120 (91.5–163.5)	116 (88.25–167.5)	116 (86–168.25)	.69
Serum Total cholesterol (mg/dl)	189.23 ± 39.34	184.34 ± 45.44	211.88 ± 42.4	.056
LDL cholesterol (mg/dl)	82.84 ± 36.32	73.51 ± 37.72	91.13 ± 38.89	.071
HDL cholesterol (mg/dl)	54.38 ± 9.72	57.11 ± 9.08	55.52 ± 9.36	.167
Serum glucose (mg/dl)	98.79 ± 45.32	91.17 ± 62.33	98.79 ± 47.28	.42
Serum SOD activity (U/ml)	2.66 ± 1.42	2.34 ± 1.19	1.65 ± 1.07[a]	.003

Note: Data presented as Mean ± *SD*, inter quartile range; or percentage. Chi-square test or ANOVA or Kruskal–Wallis has been done.
Abbreviations: CVD, cardiovascular disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SOD, superoxide dismutase.

3.2 | Biochemical characteristics

The mean of serum total cholesterol, LDL and HDL in healthy subjects were 189.8 ± 39.54, 80.33 ± 36.08 and 55.12 ± 9.84 mg/dl, respectively and in subjects with an event were 190.62 ± 42.44, 82.88 ± 36.08 and 54.06 ± 9.08 mg/dl, respectively ($p > .05$) (Table 1). Also the median of triglyceride in healthy subjects was 118 (93–161) mg/dl and in subjects with event was 124 (95–217) mg/dl (Table 1). The mean of cholesterol, LDL, and the median of triglyceride in subjects with event were higher but not significant than healthy subjects ($p > .05$). The mean serum glucose in subjects with an event, was 99.6 ± 62.98 mg/dl, higher than healthy subjects, 96.32 ± 38.34 mg/dl, ($p > .05$) but it is not significant. In addition, serum SOD1 activity in healthy subjects was 2.45 ± 1.7 (U/ml) and in subjects with event was 2.07 ± 1.27 (U/ml) and it was not significant ($p > .05$).

3.3 | Clinical and biochemical data according to SOD1 genotypes

According to SOD1 genotypes 121, 76, and 7 of 204 male had WW, WM, and MM genotypes, respectively; 168, 83, and 16 of 267 female had WW, WM, and MM genotypes, respectively (Table 2).

The 82.0% of subjects with CVD family history had WW genotype, 14.8% had WM genotype and 3.2% had MM genotype

According to WW, WM, and MM genotypes, mean of systolic blood pressure were 122.74 ± 18.96, 122.72 ± 20.06, and 125.91 ± 19.22, respectively; and the mean of diastolic blood pressure was 79.33 ± 11.58, 78.95 ± 10.02, and 83.8 ± 8.53 in WW, WM, and MM genotypes, respectively.

The mean of cholesterol, LDL, and HDL according to WW genotypes were 189.23 ± 39.34, 82.84 ± 36.32, and 54.38 ± 9.72, respectively. These were 184.34 ± 45.44, 73.51 ± 37.72, and 57.11 ± 9.08 according to WM genotype, respectively; and 211.88 ± 42.4, 91.13 ± 38.89, and 55.52 ± 9.36 according to MM genotype, respectively. The mean of cholesterol in MM genotype was higher than WW and WM genotype but not significant ($p > .05$). In addition, the mean of glucose according to WW, WM, and MM genotypes was 98.79 ± 45.32, 91.17 ± 62.33 and 98.79 ± 47.28, respectively; the mean of glucose was not significant in these groups ($p > .05$).

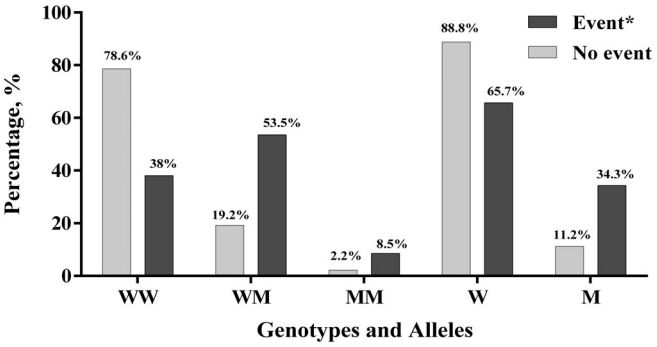

FIGURE 4 Prevalence and frequency of genotypes and alleles according to subjects with event and healthy participants. SOD1, superoxide dismutase1

Mean of serum SOD1 Activity was 2.66 ± 1.42, 2.34 ± 1.19, and 1.65 ± 1.07 (U/ml) according to WW, WM, and MM genotypes, respectively; our finding showed that the serum SOD1 activity in WM and MM groups were significantly lower than the WW genotype ($p < .001$).

3.4 | Risk of CVD associated with SOD1 genotypes

The genotype distributions were in Hardy–Weinberg equilibrium in all sample sets examined. According to our

TABLE 3 Estimation of RR for allele and genotypes effect during the time

	RR (95% CI)				
	1 month	**36 month**	**60 month**	**84 month**	**120 month**
Genotypes					
WW	Reference				
WM	0.561 (0.207–1.525)	0.885 (0.663–1.182)	1.209 (0.269–5.419)	1.652 (0.296–9.207)	2.636 (0.339–20.49)
MM	1.19 (0.581–2.438)	1.876 (0.739–4.85)	2.562 (0.815–8.061)	3.501 (0.905–13.54)	5.59 (1.041–29.994)*
Allele					
W	Reference				
M	1.494 (0.981–2.365)	2.401 (1.84–3.123)*	3.281 (2.529–4.254)*	6.659 (3.05–6.586)*	7.156 (3.811–13.43)*

Note: Extended Cox regression has been done; *$p < .05$.

findings, the prevalence of WW in healthy subjects was 78.6% and in subjects with events was 38%; the prevalence of the WM genotype in healthy subjects was 19.2% and in subjects with events was 53.5%; and prevalence of MM in healthy subjects was 2.2% and in subjects with events was 8.5% (Figure 4). The prevalence of WM and MM genotypes in subjects with an event was significantly higher than healthy subjects ($p < .001$). The frequency of the W allele in healthy subjects was 88.8% and in subjects with event was 65.7% (Figure 4); and the frequency of M allele in healthy subjects was 11.2% and in subjects with event was 34.3% (Figure 4). The frequency of M allele in subjects with event significantly was higher than healthy subjects ($p < .001$).

Our results demonstrated that with increasing the follow up time, relative risk in subjects with WM and MM genotypes had increased, but it just was significant after 120 months in MM genotype (5.59 [1.041–29.994]) ($p < .05$) (Table 3, Figure 5). Also in subjects with M allele were showed that with increasing the time relative risk was significantly elevated and it was 7.156 (3.811–13.43) after 120 months (Table 3, Figure 5).

4 | DISCUSSION

To the best of our knowledge, this is the first cohort study investigating the association between 50 bp deletion variant of SOD1 gene promoter and the risk of cardiovascular disease incidence in a representative Iranian population. Our results demonstrate that subjects carrying the WM or MM genotypes of the 50 bp deletion variant as well as the M allele had an increased relative risk for events ($p < .001$). Moreover, we observed that subjects carrying the Mutant genotype had a decreased activity of SOD1 gene.

There is a growing body of data showing an association between oxidative stress and the development of CVD

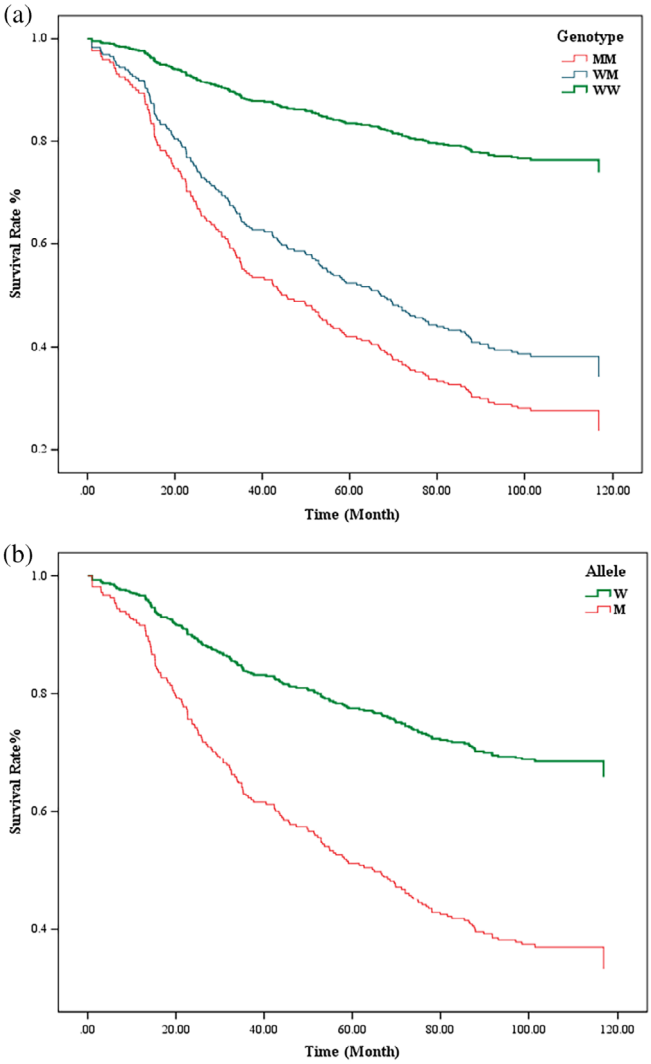

FIGURE 5 Kaplan–Meier survival curves from (a) genotypes and (b) allele SOD1 gene. SOD1, superoxide dismutase1

disease.[21] In our previous study we also found a relationship between risk factors of CVD including MetS and inflammatory markers with prooxidant–antioxidant balance.[1]

Moreover several studies have shown that antioxidant factors including the SOD1 enhanced levels play an important role in protecting the postischemic heart disease.[22,23]

The SOD1 gene has several polymorphic regions in its regulatory regions such as the promoter, introns, and UTRs.[15] The association of some SOD1 single nucleotide polymorphisms (SNPs) with the progression of cardiovascular disease has been shown previously.[24,25] In this regard, Yoichiro Otaki and et al., showed that SOD1 gene polymorphisms (rs1041740 and rs17880487)are associated with cardiovascular death.[26] Moreover, in other studies the two SOD1 35 A/C and SOD2 A16V (C/T) gene polymorphisms were found to be associated with the presence of type 2 diabetes and risk factors of cardiovascular disease.[17,18,27]

Our recent analysis showed that the 50 bp deletion variant of SOD1 gene promoter was associated with an increased risk of cardiovascular disease incidence, and subjects carrying the WM (RR = 3.51, CI: [2.5–4.93]) and MM (RR = 4.765, CI: [2.692–8.426]) genotypes had an increased relative risk for events (p-value <.001). Ebrahim Eskandari-Nasab and et al. have also demonstrated a significant association between the 50 bp INS/DEL polymorphism of SOD1 gene and an increased risk of CVD in a case–control study.[28] These results showed subjects carrying the MM (OR = 2.096, 95% CI: 1.336–3.286, $p = .001$) and WM and (OR = 4.811, 95% CI: 1.734–13.346, $p = .003$) genotypes had a higher risk of CVD, respectively. They found that the frequency of MM, MW, and WW genotypes were 95, 34.5, and 56.5% in the CVD group, while these frequencies in control group were 2.5, 22, and 75.5%, respectively. Several studies were also found similar results in the sporadic amyotrophic lateral sclerosis (SALS) case and control groups.[29,30] Of note, the frequencies of the 50 bp deletion variant in our population were more or less similar with this population.

Additionally, we demonstrated that the serum SOD1 activity in healthy subjects (2.45 ± 1.7 [U/ml]) was higher than subjects with event (2.07 ± 1.27 [U/ml]) but it was not statistically significant ($p > .05$). Weinbrenner and coworkers showed that SOD activity and plasma oxLDL levels were lower in healthy subjects than in CHD patients.[31] Moreover high level of serum SOD activity may be involved in the protection mechanism against CVD.[32] Several studies showed that prooxidant–antioxidant imbalance have a main role in atherosclerotic plaque formation and other cardiovascular diseases.[32]

We also found that serum SOD1 activity in WM (2.34 ± 1.19 [U/ml]) and MM (1.65 ± 1.07 [U/ml]) groups were significantly lower than WW (2.66 ± 1.42 [U/ml]) genotype ($p < .001$). In the other words, it has been shown that the mutant genotypes of 50 bp deletion genetic variant due to the loss of two transcription factor binding sites (SP1) at their SOD1 promoter region display the reduction in both promoter activity and SOD1 expression.[12] It is expected that the deficiency of SOD1 gene can lead to the enhancement of vascular superoxide levels[33] and thus their deformity and dysfunction.[34] According to literature review there is no study about association between 50 bp INS/DEL polymorphism and CVD event except for Eskandari et al. in which did not mention the racial difference. Then based on Iranian heterogeneous population, it is suggested in future study this polymorphism assess in different ethnicity.[28]

5 | CONCLUSION

Previous studies have reported an association between the 50 bp INS/DEL polymorphism and CVD in the cross-sectional studies. In the current study, we have focused on the analysis of the functionality of SOD1 gene and investigate its association with the risk of CVD incidence in a MASHAD cohort study patients. The results suggest that the 50 bp deletion variant has an important role in the regulation of serum SOD1 activity. In addition, our results showed that serum SOD1 activity is decreased in subjects with MM genotype of the 50 bp deletion variant and also subjects carrying WM genotype had a high risk of CVD incidence in a large cohort study.

In particular, we showed higher relative risk in subject carrying W/M–M/M genotypes and M allele while the follow up time is increasing. Regarding to our finding, investigation of 50 bp deletion variant in promoter SOD1 gene can be as a predictive factor for susceptibility of CVD.

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CONFLICT OF INTEREST

The authors have no conflict of interest to disclose

AUTHOR CONTRIBUTIONS

M.G.-M., A.H. M., G.A.F., and H.E.: study design. A.A., A.T., P.Z., and H.G.: design primer, done PCR. and S.D.: SOD assay. F.S., Z.A., N.F., and B.T.: wrote paper. M.T,

N.A.S., and S.D.: data analysis. M.M., F.S., Z.A., and S.D.: follow up the subjects.

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