Mediterranean Journal of Hematology and Infectious Diseases

Original Article

Impact of Superoxide Dismutase Genetic Polymorphism (SOD2 Val16Ala) and Superoxide Dismutase Level on Disease Severity in a Cohort of Egyptian Sickle Cell Disease Patients

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Competing interests: The authors declare no conflict of interest.

Abstract. ***Background:*** **Oxidative stress plays a pivotal role in the pathophysiology of sickle cell disease (SCD) and its associated disease complications. Superoxide Dismutases (SODs) are protective enzymes against oxidative stress. SOD2 deficiency results in the accumulation of oxidized red cell proteins, increased rate of hemoglobin oxidation, decreased red cell membrane deformability, and subsequently decreased red cells survival.**
Objective: **The current study was designed to determine the effect of SOD2 Val16Ala gene polymorphism (rs4880) on SOD2 level and their possible impact on SCD disease severity in a cohort of Egyptian SCD patients.**
Methods: **Genotyping SOD2 Val16Ala polymorphism by TaqMan allelic discrimination assay for hundred SCD patients and a hundred age-sex matched healthy controls revealed the genotypic and allelic frequencies of the studied polymorphism in the SCD patients were close to that of the controls.**
Results: **Serum SOD2 level was significantly lower in those having the polymorphic genotypes (p=0.005). SOD2 level inversely correlates with the annual rate of hospitalization (r=-0.023, p= 0.038).**
Conclusion: **SOD2 Val16Ala polymorphism was associated with low serum SOD2 level that may predict disease severity.**

Keywords: SOD2, rs4880, SCD, Egypt.

Citation: Khorshied M.M., Shaheen I.A., Selim Y.M.M., Elshahawy A.O., Youssry I. Impact of Superoxide Dismutase genetic polymorphism (SOD2 Val16Ala) and Superoxide Dismutase level on disease severity in a cohort of Egyptian sickle cell disease patients. Mediterr J Hematol Infect Dis 2022, 14(1): e2022037, DOI: http://dx.doi.org/10.4084/MJHID.2022.037

Published: May 1, 2022 **Received: January 29, 2022** **Accepted: April 13, 2022**

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Introduction. Sickle cell disease (SCD) is coupled with a state of chronic inflammation, ischemia-reperfusion injury and increased production of Reactive Oxygen species (ROS) such as superoxide and hydrogen peroxide up to twice the normal.[1] This state is often aggravated when patients are exposed to pathologic conditions like acidosis, infections and dehydration.[2,3] Oxidative stress plays a pivotal role in the

pathophysiology of SCD and its associated complications.[4,5] This high oxidative stress and an unbalanced oxidant/antioxidant status could contribute to the pathogenesis of SCD related complications.[5,6]

Superoxide Dismutase (SOD) is a key enzyme in the dismutation of superoxide radicals which result from cellular oxidative metabolism into hydrogen peroxide. It is an indispensable antioxidant defense system, especially in the cells involved in aerobic cellular metabolism.[7] There are three distinct types of superoxide dismutase (SOD) in human cells: a homodimeric cytosolic Cu/Zn-SOD, an extracellular homotetrameric glycosylated SOD, and a mitochondrial matrix homotetrameric Mn-SOD.[8] Mitochondrial manganese superoxide dismutase (Mn-SOD), encoded by the SOD2 gene, represents a major cellular antioxidant. Its deficiency results in the accumulation of oxidized red cell proteins, increased rate of hemoglobin oxidation, decreased red cell membrane deformability and subsequently decreased red cells survival.[9,10]

Previous reports showed that sickled red blood cells produce twice as much superoxide, hydrogen peroxide, and hydroxyl radicals as normal healthy controls.[11] The antioxidant defense systems in sickle patients are suboptimal, thus ineffectively neutralizing the excess pro-oxidant produced.[12,13] Furthermore, individuals presenting a genetic predisposition to have lower SOD levels would have difficulties compensating for the ROS triggered by sickling cells.[14] The current study was designed to determine the effect of SOD2 Val16Ala gene polymorphism (rs4880) on SOD2 level and their possible impact on SCD disease severity in a cohort of Egyptian SCD patients.

Materials and Methods.

Study population. The current cross-sectional study included 100 Egyptian SCD patients in a steady-state, which was defined as the period free of crisis extending from at least 3 weeks since the last clinical event and 3 months or more since the last blood transfusion to at least one week before the start of a new clinical event. Patients were selected from the Hematology Outpatient Clinic of the New Cairo University Children's Hospital. Consanguinity was found in 49% of the studied patients, and their ages ranged between 1 and 37 years, with a mean age of 17.56± 6.77 years, 58 were males, and 42 were females, with a male to female ratio of 1.3. In addition, demographic, clinical and laboratory data were retrieved from patients' files. Diagnosis and phenotyping of SCD patients was based on Alkaline Hemoglobin Electrophoresis and High-Performance Liquid Chromatography (HPLC). Fifty-five (55%) patients had sickle cell anemia (HbSS), forty-three (43%) were double heterozygous for sickle-beta-thalassemia (HbSβ), one (1%) patient was double heterozygous for hemoglobin C & S (HbSC) and one (1%) patient was double heterozygous for hereditary persistence of fetal hemoglobin & Hemoglobin S (HbS/HPFH).

Twenty-eight (28%) patients were transfusion-dependent, and 14% were splenectomized. More than 90% of our patients were on hydroxyurea therapy, and 43% received iron chelation therapy in the form of deferoxamine (7/43), deferiprone (35/43) and deferasirox (6/43); some were on combined therapy with a median serum ferritin level of 540 ng/mL (IQR 171-992 ng/mL). Disease severity score was calculated, and SCD patients were stratified into three groups; mild (<3), moderate (>3 ≤ 5) and severe cases (>5) (**Table 1**).[15]

Hundred healthy age and sex-matched children with normal hematological parameters were included in the study as a control group. The study was approved by the Research Ethics Committees of the Clinical Pathology Department and Department of Paediatrics, Institutional Review Board (IRB) - Faculty of Medicine, Cairo University. Informed consent was obtained from the patients or their guardians before enrolment in the study. All procedures performed were in accordance with the recommendation of the Declaration of Helsinki, 1964 and its later amendments or comparable ethical standards.

Table 1. SCD severity score.

Item	Determinant	Score
Anaemia score	Hb ≥ 10 g/dl	0
	Hb ≥ 8 < 10 g/dl	1
	Hb ≥ 6 < 8 g/dl	2
	Hb ≥ 4 < 6 g/dl	3
	Hb < 4 g/dl	4
White cell count score	Count < 9×10^9 /L	0
	Count ≥ 9 < 11×10^9 /L	1
	Count ≥ 11 < 15×10^9 /L	2
	Count ≥ 15×10^9 /L	3
Complications' score	Nephropathy	2
	Stroke	2
	Other complications	1
Transfusion score	Calculated from the equation; Total number of units of blood/ Age	

Hb: Hemoglobin.

Genotyping of SOD2 Val16Ala polymorphism. ACCORDING TO THE MANUFACTURER'S INSTRUCTIONS, genomic DNA was extracted from whole blood samples using GeneJET Whole Blood Genomic DNA Purification Mini Kit (cat no #K0781, Thermo Scientific, USA). The concentration and purity of the DNA were assessed by spectrophotometer, and samples were stored in the elution buffer at -20°C until being used. Genotypic analysis was done by Applied Biosystem step one Real-Time PCR System allelic discrimination assay. As previously described, the assay was designed using Taq-Man SNP Genotyping Assays

(Assay ID: C_8709053_10, Applied Biosystems, Thermofisher, Foster City, CA, USA).[14] 20% of the samples were randomly chosen concerning case/control status and reanalyzed to validate our results. The results were interpreted by different observers and were found to be 100% concordant.

Measurement of SOD2 level in SCD. Determination of serum SOD2 level in SCD patients was performed by Enzyme-Linked Immunosorbent Assay (ELISA) (Catalog No: SG-10188, SinoGeneclon, YuHang, China) according to manufacturers' instructions.

Statistical methods. Data were statistically described in terms of mean ± Standard Deviation (± SD), median and range (or IQR), or frequencies (number of cases) and percentages when appropriate. Comparing numerical variables between the study groups was done using the Mann Whitney U test for independent samples for comparing two groups and the Kruskal Wallis test for comparing more than two groups. For comparing categorical data, Chi-square ($\chi 2$) test was performed. The exact test was used instead when the expected frequency was less than 5. Correlation between various variables was done using Pearson moment correlation equation for linear relation of normally distributed variables and Spearman rank correlation equation for non-normal variables/non-linear monotonic relation. Two-sided p values less than 0.05 were considered statistically significant. All statistical calculations were done using the computer program IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA), release 22 for Microsoft Windows.

Results. Genotypic analysis showed no statistically significant difference in the genotypic and allelic distribution of SOD2 Val16Ala polymorphism between SCD patients and controls **(Table 2).**

Table 2. Genotypic and allelic distribution of SOD2 Val16Ala SNP in SCD patients and controls.

SOD2 Val16ALA polymorphism	SCD Patients No. (%)	Control group No. (%)	P value
TT	32 (32%)	34 (34%)	1 (Reference)
TC	45 (45%)	44 (44%)	0.89
CC	23 (23%)	22 (22%)	0.87
TT vs TC + CC	68 (68%)	66 (66%)	0.76
T allele	0.55	0.56	0.76
C allele	0.45	0.44	

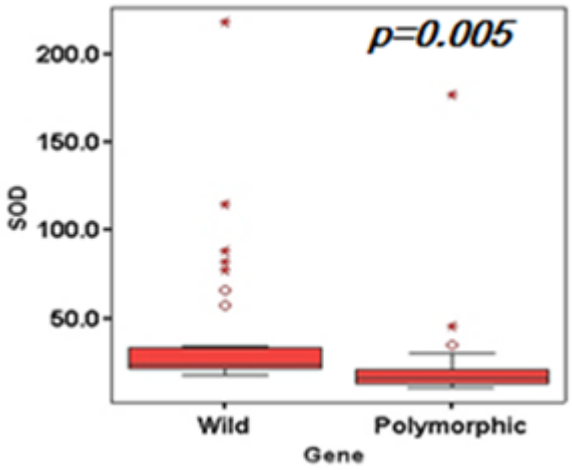

Figure 1. Boxplot of median serum SOD level in SCD patients having the wild and the polymorphic genotypes of SOD2 Val16Ala SNP.

A comparison between SCD patients having wild and the polymorphic genotypes of SOD2 Val16Ala SNP revealed that hepatobiliary complications (hepatomegaly and gall stones) were significantly lower in polymorphic patients' genotypes (p=0.03). Neurological complications (strokes and transient ischemic attacks) were reported in six patients, and all of them had the homozygous polymorphic genotype (CC). SOD level was significantly lower in patients having the polymorphic (heteromutant and homomutant) genotypes (p=0.005) **(Figure 1).** Otherwise, there was no statistically significant difference between the two patients' groups regarding their demographic, clinical and laboratory data **(Table 3**). Based on the disease severity score, 27 patients (27%) were classified as mild, 39 patients (39%) were classified as moderate, and 34 patients (34%) were classified as severe. Comparing SOD2 levels between the SCD patients stratified according to this score was statistically insignificant (p=0.075) **(Table 4).** No significant correlation was found between SOD2 level and any of the different hemolytic indicators and disease-associated complications apart from an inverse correlation between SOD level and the annual rate of hospitalization (r=-0.023, p= 0.038) (**Figure 2**).

Discussion. There are contradictory reports on the level of various antioxidants in patients with SCD, which warrant further investigation for confirmation.[17] The antioxidant defense system is significantly diminished through decreased expression and activity levels of antioxidant enzymes, including superoxide dismutases (SOD), catalases, and glutathione peroxidases.[1,12] Oppositely, Das and Nair[17] stated that SOD levels were

increased in sickle cell anemia patients and postulated that this increase might be an adaptive defense mechanism to counteract the increased oxidative stress. This imbalance between oxidative stress and antioxidant

Table 3. Comparison between SCD patients having the wild and the polymorphic genotypes of SOD2 Val16Ala polymorphism as regards their demographic, clinical and laboratory characteristics.

Item	Wild genotype (TT) (n=32) No. (%)	Polymorphic genotypes (TC & CC) (n=68) No. (%)	P- value
Gender Male/Female	18/14 (56% / 44%)	39/29 (57% / 43%)	0.92
Splenic sequestration	4 (12.5%)	4 (5.9%)	0.26
Hepatobiliary complications	15 (46.9%)	17 (25%)	**0.03***
Bone / joints affection	17 (53.1%)	37 (54.4%)	0.9
Pulmonary complications	12 (37.5%)	18 (26.5%)	0.28
Infections	7 (21.9%)	20 (29.4%)	0.4
Neurological complications	0 (0%)	6 (8.8%)	0.092
Leg ulcers	1 (3.1%)	3 (4.4%)	0.76
Priapism	1 (3.1%)	0 (0%)	0.296
Renal affection	0 (0%)	1 (1.5%)	0.68
Vasoocclusive crises (VOC):			
Severe VOC	16 (50%)	29 (42.6%)	0.6
Mild/moderate VOC	14 (43.8%)	32 (47%)	
Transcranial Doppler (TCD):			
Normal	29 (90.6%)	57 (83.8%)	
Conditional	2 (6.2%)	5 (7.4%)	0.56
Abnormal	1 (3.1%)	6 (8.8%)	
SCD phenotypes:			
SS	15 (46.9%)	40 (58.8%)	
Sb thalassemia	16 (50%)	27 (39.7%)	0.296
SC	1 (3.1%)	0 (0%)	
S/HPFH	0 (0%)	1 (1.5%)	
Disease severity grade:			
Mild	11 (34.4%)	16 (23.5%)	
Moderate	11 (34.4%)	28 (41.2%)	0.52
Severe	10 (31.2%)	24 (35.3%)	
Severity score Median (IQR)	5 (2-7)	5 (3-8)	0.39
Blood transfusion (age of start/years) Median (IQR)	3 (2-4)	3 (2-4)	0.23
Blood transfusion (Units/life time) Median (IQR)	3.5 (2-9)	4 (3-18)	0.25
Hospitalization Median (IQR)	2 (1-5)	3 (2-6)	0.69
Laboratory data Median (IQR)			
Serum SOD2 level (U/mL)	26.1 (21.2-57.5)	16.7 (13 – 21.3)	**0.005***
Haemoglobin level (g/dl)	8 (7.8-9)	8.3 (7.4-10)	0.73
Hematocrite %	27 (23-28)	25.9 (23-28)	0.46
WBCs ($x10^3/cm^3$)	7.55 (6.3-12.6)	7.7 (5.8-12.9)	0.26
Plts ($x10^3/cm^3$)	403.5 (261-560)	274 (202-422)	0.62
Reticulocyte count (%)	3 (2-3)	4 (2-6)	0.38
Total bilirubin (mg/dl)	1.55 (1.1-1.9)	1.5 (0.9-2.2)	0.62
Direct bilirubin(mg/dl)	0.3 (0.2-0.4)	0.3 (0.2-0.43)	0.57
LDH (U/L)	334 (253-586)	470 (310-734)	0.25
Serum Ferritin (ng/ml)	228.5 (126-1242)	562 (274-980)	0.33

Hb: hemoglobin, RBCs: red blood cells, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, TLC: total leucocytic count, Plt: platelet count, T.bil: total bilirubin, D.bil: direct bilirubin, LDH: lactate dehydrogenase, S.ferritin: serum ferritin, SOD: superoxide dismutase, SS: homozygous sickle, Sß: sickle beta thalassemia, IQR: Inter quartile range.

Table 4. Comparison of serum SOD level between the different disease severity grades.

Disease severity grade	Number	Range	Median	P value
Mild	27 %	11.1 – 176.5	23.65	0.075
Moderate	39%	11.1 - 77.5	17.3	
Severe	34%	10.8 - 217.7	20	

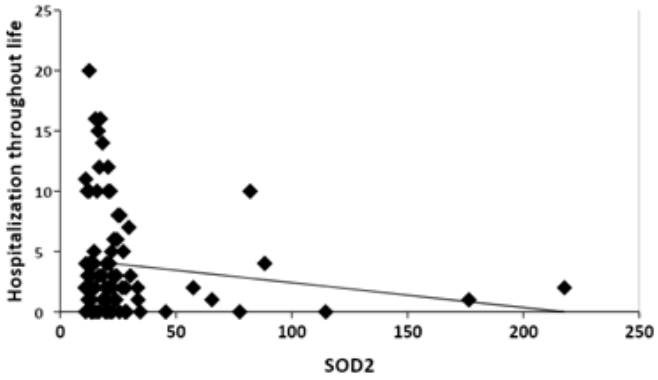

Figure 2. Correlation between serum SOD level and the frequency of hospitalizations throughout life.

defenses in SCD warranted further investigations.

In our study, SOD2 Val16Ala polymorphic (C) allele was 0.44 in healthy Egyptian controls. Similarly, it was present in 45% of people with African ancestry[18,19] and close to that of Caucasians, being 0.5.[20] SOD2 polymorphism has a wide range of allelic frequencies depending on ethnicity being 0.65 in Latinos, 0.54 in South Asians, 0.52 in Europeans (non-Finnish) and 0.16 in East Asians.[1] In SCD patients, the heteromutant (TC) and homomutant (CC) genotypes were detected in 45% and 23% of patients, respectively. The genotypic and allelic frequencies of SOD2 Val16Ala polymorphism in our cohort of Egyptian SCD patients were close to that reported in Brazilian patients.[14] The polymorphic (C) allele frequency in our SCD patients was 0.44, while it was 0.35 in Turkish patients[20] and 0.52 in Brazilian patients.[14] The Turkish and Brazilian studies and ours showed no statistical difference in SOD2 allelic and genotypic frequencies between SCD patients and healthy controls.

The high frequency of the SOD2 Val16Ala polymorphism and reported associations with sickle complications emphasize the fundamental role that this polymorphism could play in contributing to the phenotypic spectrum of the disease.[1] In the current study, all the patients with neurological complications had the homozygous polymorphic genotype (CC); nevertheless, there was no statistically significant difference in the frequency of the neurological complications between patients having the wild and those having the polymorphic genotypes. However, Domingos et al.[22] found that SOD2 Val16Ala polymorphism was independently associated with risk of stroke (Odds ratio: 1.98; 95% confidence interval [CI]: 1.18-3.32; P = 0.009) and with the long term cumulative incidence of stroke (hazard ratio: 2.24, 95% CI: 1.3-3.9; P = .004) in adults with SCD.

Hepatobiliary complications (hepatomegaly and gall stones) were significantly lower in patients with polymorphic genotypes (TC and CC). It has been reported that human peripheral blood mononuclear cells from the carriers of TT genotype of SOD2 produced more proinflammatory cytokines such as IL-1, IL-6, TNF-α and IFN-γ than those having the CC genotypes, which could affect the hepatocyte's function.[23] The pathology of chronic hemolytic status among SCD patients is a major contributing factor to gall stone formation.

The frequency of patients with moderate and severe disease severity scores was higher in patients with polymorphic genotypes than those with wild ones, yet the difference did not reach a statistically significant level. However, a recent study in Brazil on a pediatric SCD cohort revealed that SOD2 polymorphism favors sickle vasoocclusive crises and acute splenic sequestration.[13] The median serum SOD2 level in our patients was 20.2 U/mL, almost two-fold that reported in Nigerian sickle patients being 9.45±3.39 U/ml.[14] In our study, the SOD2 level in patients with the polymorphic genotypes of the studied SNP was higher than in the study of Farias et al..[14] This difference could explain the association between decreased serum SOD2 levels and both vasoocclusive crises (VOC) and acute splenic sequestration crises found in their study and not in ours. The level of enzymatic antioxidants such as SODs, catalases and glutathione peroxidases were reduced in SCD.[24-26] In the present study, serum SOD2 level was significantly lower in patients with polymorphic genotypes (TC & CC). This datum is in line with the study of Farias and coworkers.[14] A decreased SOD2 enzymatic level has also been observed in cryopreserved human hepatocytes[27] and isolated human erythrocytes with the Val16Ala polymorphism.[28] Studies about the effects of SOD2 as a marker of oxidative stress suggest a protective role of the T allele.[29,30] Individuals carrying MnSOD TC/CC genotypes have increased DNA damage induced by ROS compared with the TT genotype. Thus, although these studies did not measure the functional enzyme activity, they support the idea that the T allele could be a marker for ROS protection. Our correlation studies revealed a significant inverse relationship between SOD level and the annual rate of hospitalization, which may reflect the protective role of SOD in SCD patients. A low SOD level could suggest antioxidant therapies[31]. However, although VOC is an important index of disease severity in sickle patients,[32] there was

no statistical correlation between SOD2 level and VOC or other disease-associated complications in our study. Moreover, it did not correlate with disease severity grades.

On the contrary, Okocha et al.[15] found an inverse correlation between SOD2 level and disease severity score in Nigerian patients. The hemolytic indicators and serum ferritin did not correlate with SOD2 level or SOD2 Val16Ala polymorphism in this study. Armenis and Al[33] reported that too SOD2 mRNA levels were significantly lower in SCD patients than controls, which, differently from our results, correlated with red blood cell count, reticulocyte count, platelet count, and C-reactive protein, ferritin, and brain natriuretic peptide values**.**

In conclusion, our study revealed that SOD2 Val16Ala polymorphism was associated with low serum SOD2 levels. The combined analysis of SOD2 Val16Ala polymorphism and serum SOD2 level strongly suggests that the studied SNP could significantly impact the serum levels and subsequently influence the annual rate of hospitalization, which could be considered a predictor for disease complications. For a greater understanding of the pathogenesis of SCD, larger studies are needed to evaluate whether genetic variations of the Mn-SOD gene contribute to the disease-associated complications. In addition, further research is warranted in larger sample sizes with other antioxidant enzymes to clarify their impact on SCD associated morbidities.

Limitation of the study. It will be desirable to replicate this study with more SCD patients in both steady-state and crises.

Funding information. The authors did not receive support from any organization for the submitted work.

Conflict of interest. The authors declare no conflict of interest.

Ethical approval. The study was approved by the Research Ethics Committees of the Clinical Pathology Department and Department of Paediatrics, Institutional Review Board (IRB)- Faculty of Medicine, Cairo University.

Consent to participate. Informed consent was obtained from the patients or their guardians before enrolment in the study. All procedures performed were in accordance with the recommendation of the Declaration of Helsinki the 1964 and its later amendments or comparable ethical standards.

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