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Prevalence of Glutathione S Transferase (GSTM1, GSTP1 and GSTT1) Genes Polymorphisms among Pediatric Sudanese Patients with Sickle Cell Anemia

Naif Taleb Ali[1*], Ozaz Yagoup Mohammed Ahmed[1], Fayad Osman Mohammed[2] and Huda Mohammed Haroun[1]

[1]Department of Hematology and Immunohematology, Faculty of Medical Laboratory Sciences, Gezira University, Sudan.
[2]Department of Molecular Biology, Institute of Endemic Diseases, University of Khartoum, Khartoum, 11111, Sudan.

Authors' contributions

All the authors have read and agreed to the final manuscript.

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ABSTRACT

Background: Sickle cell disease (SCD) is taken into account as one of the foremost types of anemia in Sudan, particulary in the western part of the country. The glutathione system plays a vital role in the removal of endogenous products of peroxidation of lipids, thus protecting cells and tissue against damage from oxidative stress. Impairment of the glutathione system as result of genetic polymorphisms of glutathione S-transferase (GST) genes is anticipated to increase the severity of SCD manifestations.
Aims/Objectives: This study was aimed to evaluate the rate of GSTM1, GSTT1 and GSTP1 gene polymorphisms among sickle cell anemia pediatric patients in Sudan.
Study Design: Case control study.
Place and Duration of Study: This study carried out in Khartoum town in Jafar Ibn Auf Pediatric Hospital / Khartoum during the period (June 2017 to June 2020).

**Corresponding author: E-mail: naiftalebali@gmail.com, naiftaleb@yahoo.com;*

Methodology: The total subjects of the confirmed diagnosis were 126 and 126 control. Among these cases of SCA 78 (61.9%) are males and 48 (38.1%) are female and for control 80 (63.5%) are male and 46 (36.5%) are female.
We measured the frequency distribution of the three GSTs gene polymorphisms, GSTM1 and GSTT1 genotypes were determined by polymerase chain reaction (PCR). GSTP1 genotyping was conducted with a PCR-restriction fragment length polymorphism assay, SPSS version 23 was used to analyze the data.
Results: The GSTM1 null genotype frequency was found to be slightly lower in the control group, (30.2% as opposed to 33.3% in SCA patients), but this difference was not considered to be statistically significant (OR = 1.16, 95% CI: 0.68-1.97; p- value = 0.5884), GSTT1 was found in 47.6% of SCA patients and 77.8% of the Control but the frequency of individuals carrying the GSTT1 null genotype was significantly higher among SCA patients, 52.4% compared to 22.2% of the Control; (OR = 3.85, 95% CI: 2.23- 6.65; p-value =0.0001). Individuals with a combined GSTM1 null/GSTT1 null genotype had an estimated 11.7-fold increased risk of SCA (OR=11.7; CI=2.67-51.2; p-value=0.0011).
The homozygous mutant type (Val/Val) of GSTP1 showed significant difference between patients and controls (OR= 6.53, 95% CI: 1.41-30.24; P-value = 0.0164).
Conclusion: The GSTT1 polymorphism and combined form of GSTM1 null/GSTT1 null genotype and the homozygous mutant type (Val/Val) of GSTP1 increase the risk of sickle cell anemia.

Keywords: Sickle cell disease; glutathione S transferase polymorphisms.

1. INTRODUCTION

Sickle cell disease (SCD) is an inherited blood disease with several complications in various populations worldwide. The disease is caused by a point mutation in the hemoglobin β (HBB) gene, which codes for β-globin, found on chromosome 11a. This mutation results in the substitution of the amino acid valine for glutamic acid in the globulin protein, and leads to the production of an abnormal form of the hemoglobin molecule, which is the hemoglobin S (HbS) [1].

The disease afflicts about 20 million people worldwide, with a high prevalence of about 12 million people living in Africa [2]. The mortality rate for children under 5 years with SCD is estimated at 75– 85% in Africa [3]. In Ghana, the prevalence of the disease is about 2% of births annually [4]. Vaso- occlusive crises (VOC) are a hallmark of SCD, which creates an economic burden and makes management of SCD patients difficult in developing countries [5]. The abnormal red blood cells, as a result of sickling, which is observed in SCD patients, may also lead to chronic intravascular hemolysis [6].

Glutathione S-transferases (GSTs) constitute a family of multifunctional enzymes that are coded for by at least eight distinct loci: alpha-(GSTA), mu (GSTM), theta-(GSTT), pi-(GSTP), sigma-(GSTS), kappa- (GSTK), omega-(GSTO), and zeta-(GSTZ), each one composed of one or more homodimeric or heterodimeric isoforms [7,8].

GSTs are involved in catalyzing reactions between glutathione (GSH) and a number of potentially toxic and carcinogenic electrophilic compounds [9].

GSTs also display peroxidase activity and can protect against oxidative damage through the combination with GSH, thereby maintaining cellular integrity [10,11].

Deficiency in the activity of this enzyme can be produced by inherited polymorphisms within specific GST genes, e.g., GSTT1 (22q11.23), GSTM1 (1q13.3), and GSTP1 (11q13) [12].

Polymorphisms of two isoforms of GST, GSTM1 and GSTT1, have been studied in many chronic diseases like cardiovascular and neurodegenerative diseases and these allelic variations have been linked to the inflammatory nature of these diseases [13].

In addition, genotypic and phenotypic variations in the activity of GSTs have been studied in relationship to the risk and prognosis of several cancers [14,15].

However, there are no data regarding the prevalence of GSTM1, GSTT1 and GSTP1 polymorphisms in pediatric with sickle cell anemia in Sudan. The results from our study will

facilitate future studies such as genetic susceptibility to disease.

2. PATIENTS AND METHODS

The study is a case control study, including 126 Sudanese SCD pediatric patients and 126 Sudanese controls of similar age that were healthy and without significant medical history. The study protocol was in accordance with the local hospital research guidelines and written informed consent was obtained from all patients.

This study was carried out in Khartoum town in Jafar Ibn Auf Pediatric Hospital / Khartoum. The complete blood count for the blood samples was carried out in the laboratory of Jafar Ibn Auf Pediatric Hospital by Sysmex KX21 Machine.

The DNA extraction and storage and molecular biology analysis were carried out performed in the department of Molecular biology Institute of Endemic Diseases (IEND) –University of Khartoum.

Clinical as well as laboratory data were obtained from medical records and interviews with the patients. Controls that were enrolled had normal complete blood counts, normal hemoglobin electrophoresis.

Diagnosis of SCD was based on hemoglobin electrophoresis, Sickling test and clinical examination.

2.1 Molecular Analysis

Blood samples and DNA extraction Blood samples of 5 mL were obtained from all participants, collected in sterile EDTA tubes, and then stored at −20°C until use.

DNA was extracted from EDTA blood samples by G-spin TM Total DNA extraction kit protocol intron biotechnology: the steps as the following, 200 µl of blood sample was placed in 1.5 ml micro centrifuge tube, 20 µl proteinase K and 5 µl RNase A solution were added. The solution was mixed gently by vortex, and then 200 µl of Buffer BL was added into sample and mixed thoroughly and placed at RT for 2 minutes. The lysate was incubated at 56C for10 mins and briefly centrifuged to remove drops from the inside of the lid. Then, 200 µlof absolute ethanol was added into the lysate and mixed gently by inverting 5-6 times or pipetting. The mixture was applied to the spin column (in a 2 ml collection tube) and centrifuged at 13,000 rpm for I min. The filtrate was discarded and the spin column was placed in a new 2 ml collection tube then 700 µl of buffer WA was added to the spin column, and centrifuged for 1 min at 13,000 rpm. The flow-through was discarded and 700 µl of buffer WB was added and centrifuged for 1 min at 13,000 rpm. The flow-through was discarded and the column was placed into a new 2ml collection tube, then again centrifuged for 1min to dry the membrane. Finally, the spin column was placed into a new 1.5 ml tube, and 40 µl of buffer CE was directly added onto the membrane, and incubated for 1 min at room temperature, DNA was then eluted by centrifugation for 1min at 13,000 rpm. DNA purity was quantified using a Nano Drop Spectro-photometer, and the DNA integrity was checked using agarose gelelectrophoresis.

Genotyping of GSTM1, GSTT1 and GSTP1 polymorphisms were determined by polymerase chain reaction (PCR) using a housekeeping B-globin gene as an internal control. The primer sequence and fragments produced are listed in Table 1.

The primers were synthesized by Sangon and PCR amplifications were carried out using the thermal cycler Applied QIAGEN (Rotor-Gene Q).

2.1.1 Genotyping of GSTM1 polymorphism

The following PCR protocol is designed for use with Optimase, the high-fidelity polymerase product from Transgenomic.

Table 1. Primers sequences used or GSTM1, GSTT1 and GSTP1 genotyping

Gene	Primers	Fragment size
GSTM1	F: GAACTCCCTGAAAAGCTAAAGC (Tm = 55.1ºC) R: GTTGGGCTCAAATATACGGTGG (Tm = 56.9ºC)	219 bp
GSTT1	F: TTCCTTACTGGTCCTCACATCTC (Tm = 57.2ºC) R: TCACCGGATCATGGCCAGCA (Tm = 58.3ºC)	480 bp
GSTP1	F: ACCCCAGGGCTCTATGGGAA (Tm = 58.3ºC) R: TGAGGGCACAAGAAGCCCCT (Tm = 58.3ºC)	176bp

Forward primer sequence: GAACTCCCTGAAAAGCTAAAGC (Tm = 55.1°C) Reverse primer sequence: GTTGGGCTCAAATATACGGTGG (Tm = 56.9°C) PCR product length: 219 bp

Protocol type: Simple 3-step PCR protocol Step 1: 95ºC, 2 min.

Step 2: 95°C, 30 sec.
Step3:59.0°C,30 sec.
Step4:72°C,30.0 sec.
Step 5: Repeat steps 2-4 35 more times
Step 6: 72°C, 5 min

PCR: Master mix (Maxime TM premix kit (i-Taq) lot number 302321651.

2.1.2 For genotyping of GSTT1 polymorphism

The following PCR protocol is designed for use with Optimase, the high-fidelity polymerase product from Transgenomic.

Forward primer sequence: TTCCTTACTGGTCCTCACATCTC Reverse primer sequence: TCACCGGATCATGGCCAGCA PCR product length: 480 bp

Protocol type: Simple 3-step PCR protocol Step 1: 95°C, 2 min.

Step 2: 95°C, 30 sec.
Step 3: 60.8°C, 30sec.
Step 4: 72°C, 50.0sec.
Step 5: Repeat steps 2-4 30 more times
Step 6: 72°C, 5 min

PCR: Master mix (Maxime TM premix kit (i-Taq) lot number 302321651.

The product obtained from each reaction was subjected to electrophoresis on a 2%agarose gel in an electric field of 10 V/cm, stained with 5 µg/mL ethidium bromide, and visualized and recorded with the aid of a video documentation system (Image Master VDS®, Amersham Pharmacia Biotech).

GSTM1 and GSTT1 genotypes were determined by the presence and absence (null) of bands of 157 and 480 bp, respectively, with an internal control of 268 bp (Figs. 1 and 2).

Table 2. PCR protocol for GSTM1

PCR reaction	Volume
Water for injection	16 µl
Primer F	1 µl
Primer R	1 µl
DNA	2 µl
Total	20 µl

2.1.3 For genotyping of GSTP1 polymorphism

GSTP1 (Ile105Val) polymorphism was determined with a polymerase chain reaction- restriction fragment length polymorphism assay [PCR-RFLP].

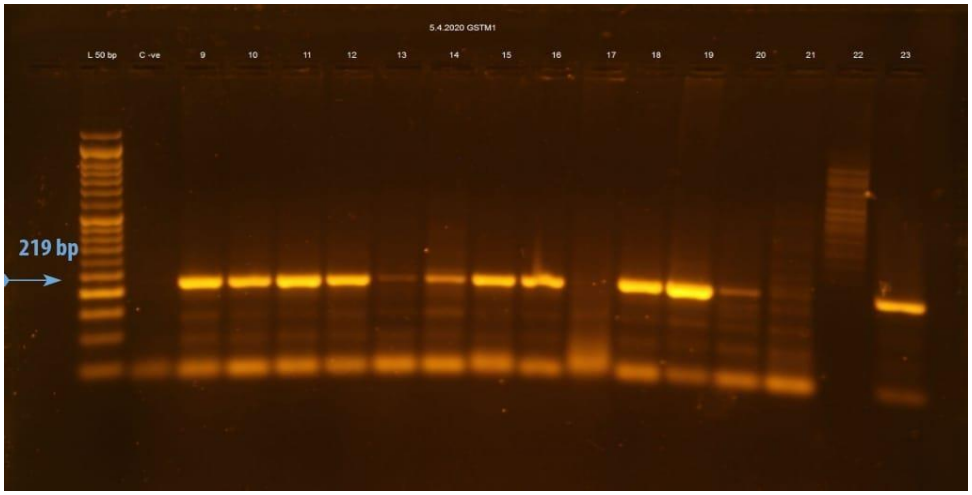

Fig. 1. Agarose gel electrophoresis for amplified PCR products of GSTM1(219bp) and B-globin (268 bpb) fragments

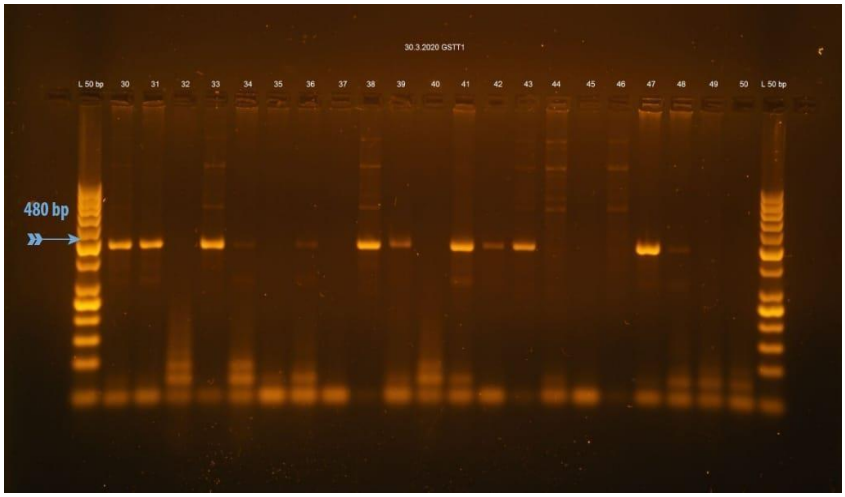

Fig. 2. Agarose gel electrophoresis for amplified PCR products of GSTT1 (480 bp) and B-globin (268 bpb) fragments

Table 3. PCR protocol for GSTT1

PCR reaction	Volume
Water for injection	16 µl
Primer F	1 µl
Primer R	1 µl
DNA	2 µl
Total	20 µl

The PCR primers were: 5'-ACC CCA GGG CTC TAT GGG AA-3' (F) and 5'-TGA
GGG CAC AAG AAG CCC CT-3' (R). PCR was carried out in a total volume of 20µl. It consists 2 µl genomic DNA, 1µl each primer, ready to load master mix (Maxime TM premix kit (i- Taq) and 16 µl distilled water. PCR condition includes initial denaturation at 95°C for 2 minutes, followed by 30 cycles at 95°C for 30 second, 61.3°C for 30 second, 72°C for 20 second and a last extension at 72°C for 5 minutes. PCR products were analyzed on a 2% Agarose gel stained with 0.3 µg/mL ethidium bromide, and visualized by gel documentation system (to check the presence of 176 pb ofGSTP1).

Then the PCR product was digested with the restriction endonuclease Alw261 (BsmAI) restriction enzyme {thermoscientific Alw261 (BsmA1) Lot Number 00743699} as follow: For each 7 µl of PCR product, 1 µl from 10X NEB

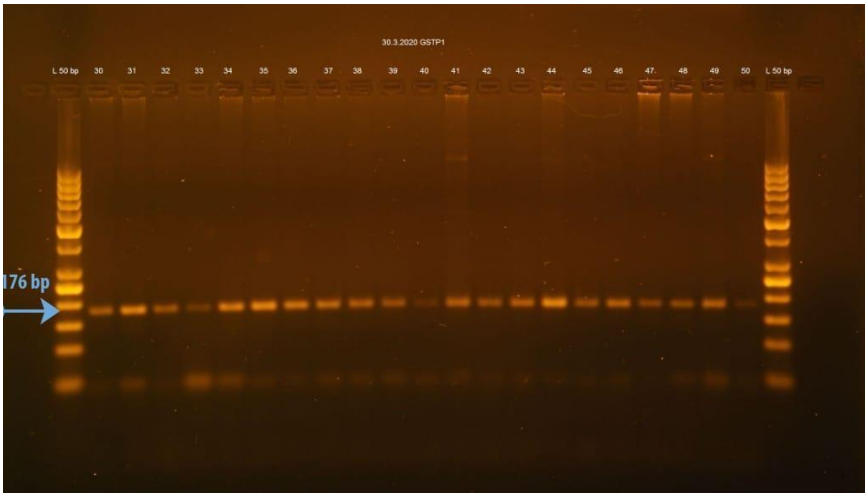

Fig. 3. Agarose gel electrophoresis for amplified PCR products of GSTP1 (176 bp) and B-globin (268 bpb) fragments

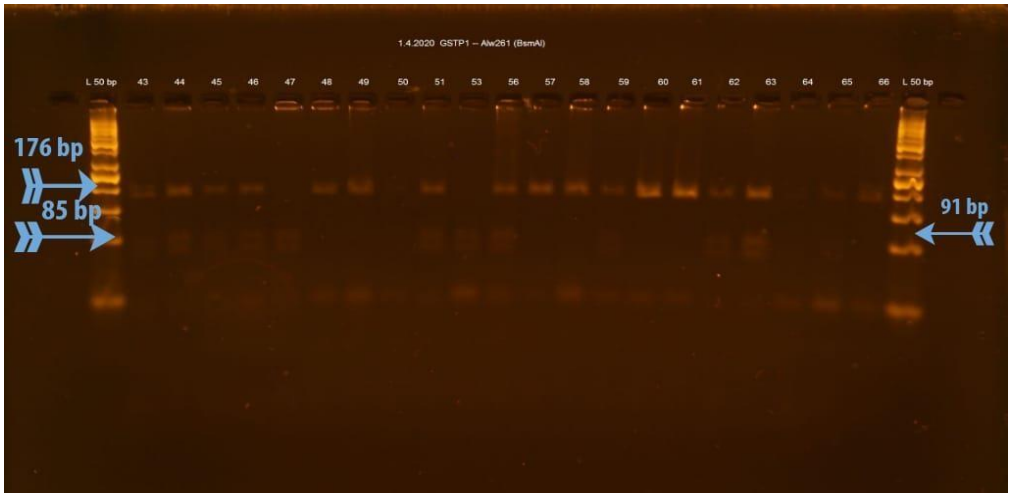

Fig. 4. DNA fragment digestion with Alw261 restriction enzyme

Lane DNA ladder: MW 100-1500 bp fragments, lane fragments at 176 bp and 85 bp indicates the presence of wild type (Ile/Ile), lane fragments at at176 bp, 91 bp and 85 bp indicates the presence of heterozygous mutant type (Ile/Val). Lane fragments at 91 bp and 85 bp indicates the presence of mutant type (Val/Val).

buffer and 0.5 µl from Alw261 restriction enzyme were added, then incubated at 37˚C for 20 hrs, followed by incubation at 65˚C for 20 minutes to inhibit the enzyme activity. The products are then resolved on 2% agarose gel electrophoresis containing ethidium bromide, then visualized using UV trans illuminator. The amplifiedfragment after digestion with Alw261 restriction enzyme, will give rise to: 2 fragments at 176 bp and 85 bp indicating the presence of wild type (Ile/Ile), appearance of 2 fragments at 91 bp and 85 bp indicates the presence of homozygous mutant type (Val/Val), while presence of 3 fragments at176 bp, 91 bp and 85 bp indicates the presence of heterozygous mutant type (Ile/Val). For quality control, genotyping of the samples was repeated blindly and were identical to the initial results (Fig. 4).

2.3 Statistical Analysis

Data were transferred to the Statistical Package for the Social Sciences (SPSS) Software program, version 23 to be statistically analyzed. Data were summarized using range, mean, and standard deviation and median for continuous variables and frequency and percentage for discrete ones. Comparison of continuous variables was performed using either independent sample t-tests or Mann–Whitney tests, while the comparison of discrete variables was conducted through chi square. P values less than

0.05 were considered statistically significant, and less than 0.01 were considered highly significant.

3. RESULTS

The obtained data are presented as frequencies, percentage, mean and standard deviation. A one-way ANOVA was performed to identify the significance between variables and the P- value of 0.05 was used.

The total subjects of the confirmed diagnosis were 126 and 126 control. All the recruited subjects to the hospital were diagnosed with homozygous sickle cell disease. The mean age of the study subjects was (8.0) years old; the minimum age was 10 months and the maximum one was 14.5 years. Among these cases of SCA 78 (61.9%) are males and 48 (38.1%) are female (Table 4) and for control 80 (63.5%) are male and 46 (36.5%) are female.

GSTM1, GSTT1 and GSTP1 genotypes in Sickle cell anemia patients and controls: The frequency of individuals carrying the GSTM1 in patients and controls were 44% and 21.7%respectively.

Table 4. Frequency of gender and age among (study population)

Gender	Mean	N	Std. deviation	Minimum	Maximum	percent
Male	7.5051	78	4.15573	.80	14.50	61.9
Female	8.8125	48	3.98201	.10	14.00	38.1
Total	8.0032	126	4.12392	.10	14.50	100

The GSTM1 null genotype frequency was found to be slightly lower in the control group, (30.2% as opposed to 33.3% in SCA patients), but this difference was not considered to be statistically significant (OR = 1.16, 95% CI: 0.68-1.97; p-value = 0.5884).

GSTT1 was found in 47.6% of SCA patients and 77.8% of the Control but the frequency of individuals carrying the GSTT1 null genotype was significantly higher among SCA patients, 52.4% compared to 22.2% of the Control; (OR = 3.85, 95% CI: 2.23- 6.65; p-value = 0.0001).

The distribution of the GSTP1, GSTM1 and GSTT1 genotypes in SCA patients and controls are shown in Table (5). The homozygous (Val/Val) of GSTP1, the heterozygous (Ile/Val) and the wild genotype of GSTP1 (Ile/Ile) forms were found in 9.7%, 35.5% and 54.8% of SCA cases, respectively.

In the Control, the homozygous (Val/Val) of GSTP1 Ile105Val, heterozygous (Ile/Val) and the wild genotype of GSTP1 (Ile/Ile) forms were 1.6%, 39.7% and 58.7%, respectively.

The homozygous mutant type (Val/Val) showed significant difference between patients and controls (OR= 6.53, 95% CI: 1.41-30.24; P-value = 0.0164).

The combined effects of GSTM1 and GSTT1 genotypes in SCA risk were also conducted. Individuals with a combined GSTM1 null/GSTT1 null genotype had an estimated 11.7-fold increased risk of SCA over individuals with a GSTM1 present /GSTT1 present genotype (OR=11.7; CI=2.67-51.2; p- value=0.0011).

The GSTM1 present /GSTT1 null genotype had an estimated 2.01-fold increased risk to over individuals with a GSTM1 present /GSTT1 present genotype (OR=2.01; CI=1.16-3.51; p-value=0.0135). in contrast, GSTM1 null/ GSTT1 present genotypes were not associated with a SCA risk (p-value= 0.071) Table (6).

Table 5. Distribution of GSTM1, GSTT1 and GSTP1 genotypes in SCA patients and control

Genotypes/ allele frequency		SCA N (%)	Control N (%)	OR	95%CI	P-value
GSTM1	Present	84 (66.7)	88(69.8)	Reference	0.68-1.97	0.5884
	Null	42 (33.3)	38(30.2)	1.16		
GSTT1	Present	60 (47.6)	98(77.8)	Reference	2.23-6.65	0.0001
	Null	66 (52.4)	28(22.2)	3.85		
GSTP1	AA	68 (54.8)	74 (58.7)	Reference		
	AG	44 (35.5)	50 (39.7)	0.96	0.57-1.61	0.971
	GG	12 (9.7)	2 (1.6)	6.53	1.41-30.24	0.0164

N, total number; OR, odd ratio; CI, confidence interval; Statistical significance (P- value) is 0.05

Table 6. Combination effect of GSTM1 and GSTT1 genotypes on SCA risk

Genotypes		SCA N (%)	Control N (%)	OR	95%CI	P-value
GSTM1	GSTT1					
Present	Present	38 (30.2)	62 (49.2)	Reference		
Present	Null	46 (36.5)	28 (22.2)	2.01	1.16-3.51	0.0135
Null	Present	22 (17.5)	34 (27)	0.572	0.31-1.05	0.071
Null	Null	20 (15.8)	2 (1.6)	11.7	2.67-51.2	0.0011

N, total number; OR, odd ratio; CI, confidence interval; Statistical significance (P- value) is 0.05

4. DISCUSSION

SCD is an inherited disorder that has as its cardinal features chronic hemolytic anemia and vaso-occlusion [16].

The clinical features of the disease ultimately result from mutated sickle cell hemoglobin (Hb S) within the red blood cells. Deoxygenation can induce a sickle-like deformation of the Hb S, in turn leading to microvascular occlusion [17].

Oxidative stress might play an important role in the pathophysiology of SCD, where impairment of reductive defense mechanisms could potentiate clinical manifestations of SCD [18].

In the reduced form, glutathione is the most important intracellular antioxidant scavenger, where it can act as a functional antagonist to the pro-inflammatory signaling evoked by hydrogen peroxide [19].

Previous studies have revealed a significant impairment of the glutathione system in SCA, as reflected by a reduction of total GSH, which is believed to underlie the effect of increased oxidative stress, resulting from HbS in stability, deoxygenation, denaturation, and precipitation. Furthermore, the higher free iron concentrations resulting from increased hemolysis might potentiate the clinical manifestations of SCD by magnifying the pro-oxidant burden [20].

The frequencies of GSTs polymorphic alleles especially GSTM1 and GSTT1 and GSTP1 have been reported in other anemia and there are several studies have been published on the relationship between GSTM1 polymorphism and various types of anemia. there are published reports about the association between GSTM1 and GSTT1null and GSTP1 polymorphism and Sickle cell anemia so this study will fill the gab regarding this polymorphism and its association With Sickle cell anemia in Sudanese patients.

In our study, we have observed the highest frequency distribution of GST null genotypes among SCD patients for GSTT1 (52.4%) followed by GSTM1 (33.3%) then combined GSTM1/T1 genotype (15.8%).

Wild-type GSTP1 (59%) was the commonest variant followed by the heterozygous (35%) and then homozygous (6%) polymorphism. There was statistically significant difference in the distribution of the three GSTs gene polymorphisms between SCD patients and controls.

Similar to our observation, a study by Aly RABAB and M. Hasaneen Bothina, (2013) in Egypt [21] in 37 Egyptian SCD patients concluded that there was no statistically significant difference in the prevalence of the combined GSTM1/T1 null genotype among Egyptian SCD patients compared to a control group.

like our results, the highest frequency of GST null genotypes was for GSTT1 (64.9%), which was statistically significantly higher when compared to the control group (42.5%), followed by the GSTM1 (37.8%) null genotype. In a Brazilian study involving 278 SCD patients by de Oliveira Filho et al., [22] GST null genotype distribution was not similar to our finding, with the highest frequency for the GSTM1 genotype (27.3%), followed by the GSTT1 null genotype (14.7%) and then the combined GSTM1/T1 null genotypes (11.1%).

However, unlike our finding, Anther study done in Egypt [23] by Hend N. Ellithy et.al (2015) in 100 Egyptian patients Observed the highest

frequency distribution of GST null genotypes among SCD patients for GSTM1 (42%) followed by GSTT1 (32%) then combined GSTM1/T1 genotype (16%).

Wild-type GSTP1 (59%) was the commonest variant followed by the heterozygous (35%) and then homozygous (6%) polymorphism. There was no statistically significant difference in the distribution of the three GSTs gene polymorphisms between SCD patients and controls.

Also like our finding, in another Brazilian compromising 76 Brazilian individuals, of whom [22]. had SCA, Silva et al. [24] found lower frequency of the GSTM1 null genotype in these patients, but the frequency of the GSTT1 null (50%) and both GSTM1 and GSTT1 null (17.9%) genotypes were higher.

Also like our finding in other study by Faisel Abu-Duhier and Rashid Mir, (2017) among the population of Tabuk, Saudi Arabia [25], there was a statistically significant difference between the frequency of GSTT1 null genotype among patients with SCD (P = 0.00001). It was observed that patients with SCD possess higher frequency of GSTT1 null genotype 26% than healthy controls (7.5%). However, only 10% of GSTM1 null genotype frequency was observed among patients compared to 16.5% of GSTM1 null genotype frequency among healthy controls (P = 0.129). While analyzing both the genotypes together, patients revealed significantly higher number of either GSTM1/GSTT1 null genotypes (34%) than healthy controls (24%).

Also like our finding in other study in India [26], by Pandey Sanjay et.al (2011), they showed the frequencies of GSTM1 (5%), GSTT1 (14%) and GSTT1/M1 (9%) were higher in patients than in controls where the GSTM1 frequency was 3%, GSTT1 was 5% and GSTT1/M1 frequency was 2%. The difference between groups for the GSTT1 null genotype was statistically significant (p-value = 0.05) while the differences between groups for the GSTM1 and GSTT1/M1 null genotypes were not (p-value > 0.05).

In contrast to all studies, Hala Fathy Shiba et al. [27] in Egypt, they found no statistically significant difference could be detected between patients and controls regarding prevalence of GSTM1, GSTT1, and GSTP1 genotypes (P 5 0.153, 0.469, and 0.110, respectively). The GSTM1 null genotype was the most prevalent genotype among controls as well as sickle cell patients (68% and 52%, respectively), followed by the mutant GSTP1 genotype (46% and 44%, respectively) and then the GSTT1 null genotype (18% and 26%, respectively).

Although most of studies like our finding, but the difference in the prevalence of GST polymorphisms within the same population may need more extensive studies which sample a larger number of SCD patients, as any reported differences between studies might be attributed to sampling error.

5. CONCLUSION

In this study, the GSTM1 null genotype frequency was found to be slightly higher in SCA patients. The GSTT1 null genotype and combined GSTM1 null/GSTT1 null genotype and GSTP1 homozygous mutant type (Val/Val) had increased risk of SCA.

Further studies are needed to confirm our findings.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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