Original Research Article

The relation between Glutathione S Transferase (GSTM1, GSTP1 and GSTT1) polymorphisms and clinical diversity of Sickle cell disease among pediatric Sudanese patients

Abstract

Background: Sickle cell disease (SCD) is a highly variable condition, with some patients being asymptomatic and others frequently admitted to hospital. Impairment of the glutathione system due to genetic polymorphisms of glutathione S-transferase (GST) genes is expected to influence on the severity of SCD manifestations.

Objectives: This study aimed to investigate the possible association between the presence of GSTM1, GSTT1 and GSTP1 gene polymorphisms and SCD severity, diversity and complications.

Study design: prospective cross-section hospital based 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).

Methodology: The total subjects of the confirmed diagnosis were 126 patients, 78 (61.9%) are males and 48 (38.1%) are female.

GSTM1 and GSTT1 genotypes were determined by polymerase chain reaction (PCR), GSTP1 genotyping was conducted with a PCR-RFLP, and the data analyzed by SPSS version 23.

Results: The GSTM1null genotype was found to be present in male more than female (OR =2.6 and p = 0.002) and trend to be protective from development of Dactylitis (OR =0.313 and p = 0.006) and reduce risk to develop ACS (OR =0.23 and p = 0.002) while this polymorphism increase requirement for blood exchange (OR =1.1 and p = 0.044), the GSTT1null genotype found to be present in female more than male (OR =2.6 and p = 0.012) and this polymorphism reduce requirements for blood transfusion (OR =0. 137 and p < 0.001) and annual hospitalization (OR =0.436 and p = 0.029), and reduce risk to development of stroke (OR =0.125 and p = 0.008), polymorphism of both GSTM1 and GSTT1 found to be associated with appearance of disease before one year of age (OR =1.43 and p = 0.004) and trend to be protective from development of Dactylitis (OR =0.124 and p = 0.002),and there are no statistically significance association between GSTP1 gene polymorphism and gender variability and clinical manifestations of SCD.

Conclusion: Some GST genes polymorphisms were significantly associated with increased risk and some trend to have protective effect on clinical manifestations of SCD.

Key words: SCD, GST, GSTM1, GSTT1, GSTP1, polymorphisms, ACS, Sudan

Introduction

Sickle cell diseases (SCD) is a disorder caused by a mutation that results in a single substitution of amino acid valine for glutamic acid in the sixth position on the beta subunit of hemoglobin resulting in abnormal hemoglobin, hemoglobin (Hb) S [1]. In its deoxygenated state, the HbS molecules become polymerized and deform the red blood cells, causing

oxidative damage, cellular dehydration, abnormal phospholipid asymmetry, and increased adhesion to vascular endothelium [2].The multifactorial nature of the SCD involves several changes in erythrocyte sickling, vaso-occlusive episodes, hemolysis, activation of inflammatory mediators, oxidative stress and endothelial dysfunction, which apparently result from HbS instability, generating oxygen radicals [3]. Among the major complications of SCD, stroke, acute chest syndrome, infections, osteoarticular lesions, lower limbs ulcers and priapism are among the most common [4,5]. Patients with SCA are subject to increased oxidative stress, especially during vaso-occlusive crises and acute chest pain. Oxidative stress in SCA can cause profound damage to erythrocytes, reducing their life-span and can be attributed mainly to the instability of HbS [6]. The autooxidation of Hb is related to the ability of an electron bound to heme iron to become mismatched. The Hb molecule, particularly the nonpolar region, requires iron in the ferrous form (Fe^{2+}), which supports the reversible transport of oxygen. Any change in the chemical complex that protects the heme group may enable access by small ions or water molecules with displacement of electrons from heme and, thus, formation of superoxide radicals, triggering the oxidation of hemoglobin [7, 8].The formation of this new Hb type (HbS) in SCD may alter the delicate balance of free-radical generation and anti-oxidant defense systems in red blood cells, which are a significant source of free radicals in biological systems [9]. In the deoxygenated state, the HbS tends to aggregate into rod like polymers, resulting in the deformed sickle shape and rigidity of red blood cells (RBCs) characteristic of this condition [10]. Normal RBCs are usually subjected to oxidative stress as a result of continuous reactive oxygen species (ROS) production that accompanies Hb autoxidation. Autoxidation leads to SCD-derived oxidative stress consequences that include an increase in membrane lipid peroxidation levels and alterations in anti-oxidant defense system [11, 12]. An altered glutathione (GSH) metabolism in association with increased oxidative stress has been implicated in the pathogenesis of many diseases [13]. Alterations in GSH concentration have been demonstrated in many pathological conditions including SCD [14]. Glutathione S-transferases (GST) are a family of enzymes involved in phase-II detoxification of endogenous and xenobiotic compounds. Polymorphisms in GST genes have been associated with susceptibility to different diseases [15]. The clinical severity and hematological manifestations of sickle cell anemia are varied and are influenced by the participation of several genes in modulating the phenotype of sickle cell disease; polymorphisms of these genes may be related to the different manifestations between individuals [16].Many genetic factors such as α-thalassemia, fetal hemoglobin synthesis, and ß-globin haplotype have been identified, but none of these can fully explain the differences in clinical expression observed in SCD patients. Probably, there are other elements that contribute to the many phenotypes of the disease [17, 18]. In SCD, previous reviews have reported that several of these polymorphisms are associated not only with the degree of anemia, but also with pain rate, prevalence of stroke, leg ulcers, pulmonary hypertension, hepato-biliary complications and priapism, among other several clinical aspects [19].

The study of GSTM1, GSTT1 and GSTP1 gene polymorphisms in Sickle cell disease patients are the first step toward helping the understanding of the pathophysiology of disease, enabling predictive medicines and providing clinically useful pharmacogenomics, so this study was aimed to investigate the possible association between the presence of GSTM1, GSTT1 and GSTP1 genes polymorphism and SCD severity, diversity and complications.

Materials and methods:

This study is prospective cross-section hospital based study; the current study included 126 pediatric patients with sickle cell disease. Patients were invited to participate in the study during their regular follow up visits to Jafar Ibn Auf Pediatric Hospital / Khartoum. The

study protocol was in accordance with the local hospital research guidelines and informed consent was obtained from all patients. The data and blood samples collection were carried out in Khartoum town in Jafar Ibn Auf Pediatric Hospital / Khartoum.
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 data were obtained from medical records and interviews with the patients. Diagnosis of SCD was based on hemoglobin electrophoresis, Sickling test and clinical examination.

Molecular analysis:
5 ml of blood 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: briefly, a total of 200 µl of blood sample was placed in1.5ml micro centrifuge tube, 20 µl proteinase K and 5µl of RNase 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 Room temperature for 2 minutes. The lysate was incubated at 56 C for 10 min and briefly centrifuged to remove drops from the inside of the lid. Thereafter, 200 µl of 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 forI min. The filtrate was discarded and the spin column was placed in a new 2ml 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 2 ml 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 (Thermo Scientific2000) and the DNA integrity was checked using agarose gel electrophoresis.
Genotyping of GSTM1and GSTT1 polymorphisms:
The primers were synthesized by Sangon and PCR amplifications were carried out using the thermal cycler Applied QIAGEN (Rotor-Gene Q).
For GSTM1 genotype, the following pair of primers was used in the genotyping analysis: Forward primer: GAACTCCCTGAAAAGCTAAAGC-3, Reverse primer: 5-GTTGGGCTCAAATATACGGTGG -3. PCR was carried out in a total volume of 20 µl. It consists of 2 µl of genomic DNA, 1 µl from each primer, Master mix (Maxime TM premix kit (i-Taq) and 16 µl distilled water. PCR was initiated by denaturation step at 95°C for 2 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing temperatures ranged between 59 °C for 30 second and 72 °C for 30 second, and final extension at 72°C for 5 minutes.
For GSTT1 genotype, the following pair of primers was used in the genotyping analysis: Forward primer: TTCCTTACTGGTCCTCACATCTC -3, Reverse primer: 5-TCACCGGATCATGGCCAGCA -3. PCR was carried out in a total volume of 20 µl. It consists of 2 µl of genomic DNA, 1 µl from each primer, Master mix (Maxime TM premix kit (i-Taq) and 16 µl distilled water. PCR was initiated by denaturation step at 95°C for 2 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing temperatures ranged between 60 °C for 30 second and 72 °C for 50 second, and final extension at 72°C for 5 minutes. 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 219 and 480 bp respectively (Figs. 1 and 2).

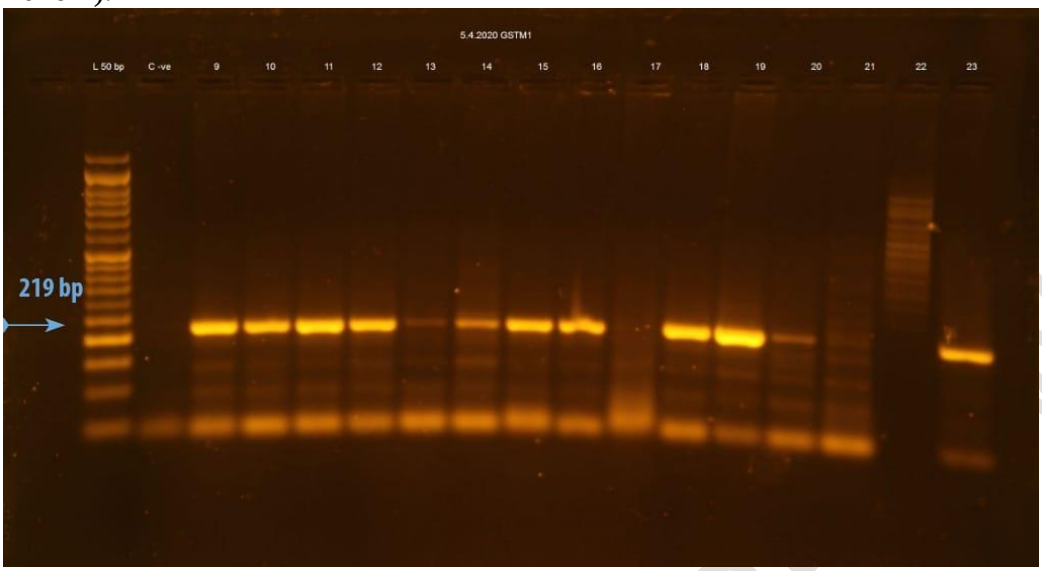

Figure 1: Agarose gel electrophoresis for amplified PCR products of GSTM1 (219bp) fragments

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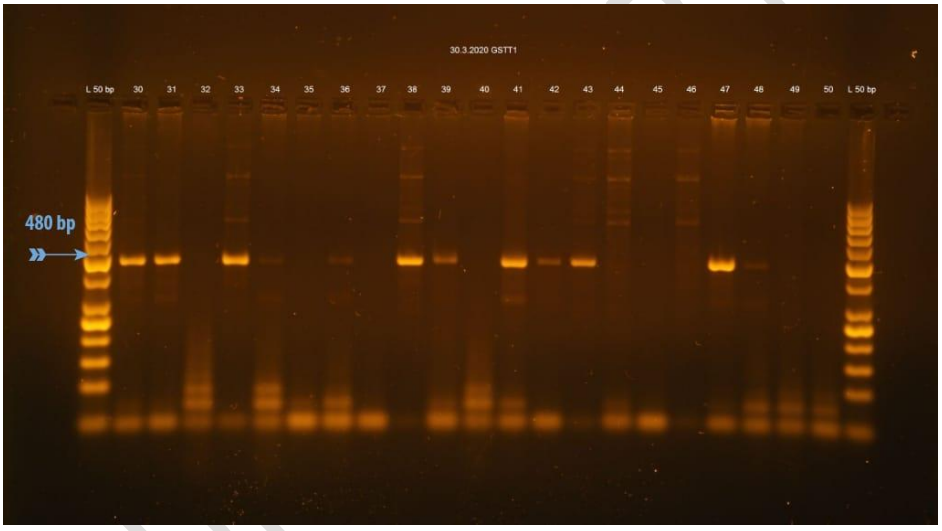

Figure 2: Agarose gel electrophoresis for amplified PCR products of GSTT1 (480 bp) fragments.

For Genotyping of GSTP1 polymorphism

GSTP1 (Ile105Val) polymorphism was determined with a polymerase chain reaction- restriction fragment length polymorphism assay [PCR-RFLP]. The PCR primers were: 5′-ACCCCAGGGCTCTATGGGAA-3′ (F) and 5′- TGAGGGCACAAGAAGCCCCT -3′ (R). PCR was carried out in a total volume of 20µl. It consists 2µl genomic DNA, 1µl each primer, ready to load master mix (Maxime TM premix kit (i-Taq) and 16µl 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 of GSTP1) (Fig. 3).

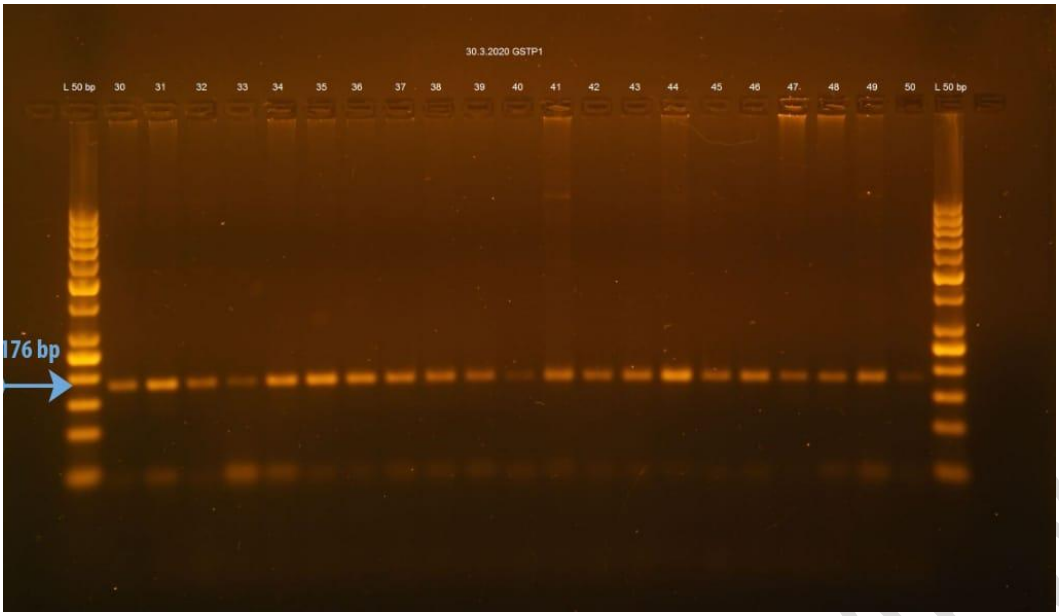

Figure 3: Agarose gel electrophoresis for amplified PCR products of GSTP1 (176 bp) fragments.

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 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 amplified fragment after digestion with Alw261 restriction enzyme, will give rise to: 2 fragments at 176 bp and 85 bp indicating the presence of wild type (IIe/IIe), appearance of 2 fragments at 91 bp and 85bp 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 were repeated blindly and were identical to the initial results. (Fig. 4)

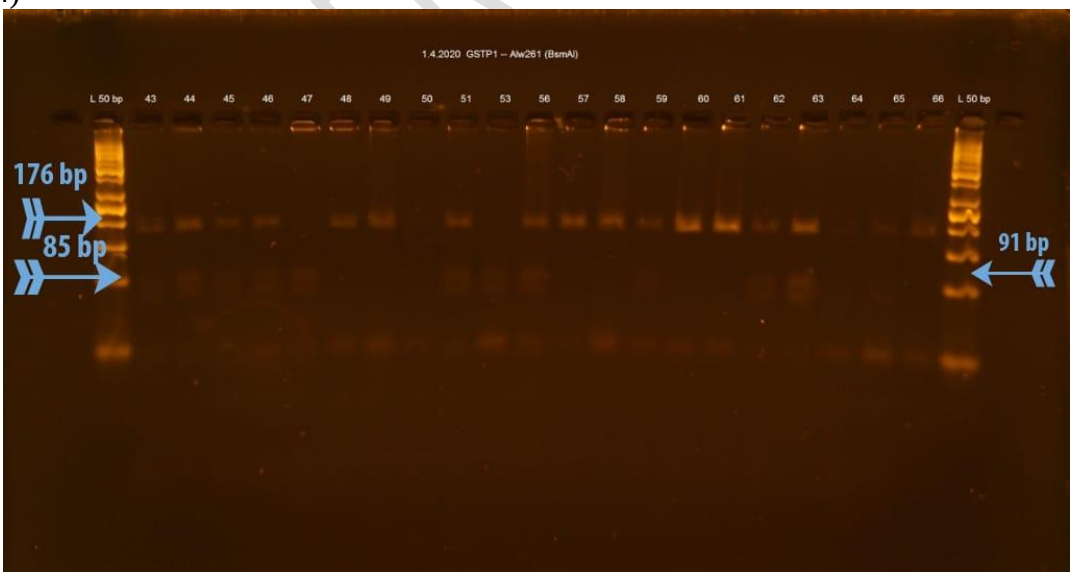

Figure 4: DNA fragment digestion with Alw261 restriction enzyme
Lane DNA ladder: MW 100-1500 bp fragments, lane fragments at 176 bp and 85 bp indicates the presence of wild type (IIe/IIe), lane fragments at at176 bp, 91 bp and 85 bp indicates the presence of heterozygous mutant type (Ile/Val). Lane fragments at 91 bp and 85 bp indicates the presence of mutant type (Val/Val).

Statistical analysis:
Data were transferred to the Statistical Package for the Social Sciences (SPSS) Software program, version 23 to be statistically analyzed. The obtained data are presented as frequencies, percentage, mean and standard deviation, Descriptive and analytic statistics and crosstabulation were performed, Data were summarized using Chi-square or Fisher exact probability tests. Associations between the GSTM1, GSTT1 and GSTP1 polymorphisms and clinical manifestations of SCD patients were estimated using odd ratio (OR) and 95% confidence intervals (95% CIs). Odd ratio and confidence interval were used to estimate risk of the SCD among the population; the lowest accepted level of significance was 0.05 or less.

Ethical consideration:
Approval was received from ministry of health in Khartoum/Sudan and Jafar Ibn Auf Pediatric Hospital.

Results:
The total subjects of the confirmed diagnosis were 126 patients, 78 (61.9%) are males and 48 (38.1%) are female, 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.

Frequency of clinical manifestations among SCD patients:
In the study subjects, 94 (74.6%) were diagnosed with SCD when they were less than one year and 32 (25.4%) were diagnosed when their age one year or more, the frequency of blood transfusion for these patients 82 (65.1%) were diagnosed with SCD had blood transfusion less than two times per year and 44 (34.9%) had blood transfusion more than two times per year, for the frequency of VOC 50 (39.7 %) were diagnosed with SCD had crises less than two times per year and 76 (60.3%) had VOC more than two times per year. Regarding the frequency of annul hospitalization 46 (36.5%) were diagnosed with SCD had less than two times per year and 80 (63.5%) had more than two times per year.
And out of whole subjects 14 (11.1%) were diagnosed with SCD had stroke and 52 (41.3%) had Dactylities, the frequency of ACS 78 (61.9%) were diagnosed with SCD had less than two times per year and 48 (38.1%) had more than two times per year.
And out of whole subjects 8 (6.3%) were diagnosed with SCD had bones problems and 4 (3.2%) had splenomegaly and 2 (1.6%) had blood exchange.

(GSTM1, GSTT1 and GSTP1) with clinical manifestations:
The GSTM1null genotype was found to be present in male more than female (Odd ratio =2.6, confidence interval 95% = 1.324 – 5.168 and p. value 0.002) and trend to be protective from development of Dactylitis (Odd ratio =0.313, confidence interval 95% = 0.136 – 0.716 and p. value 0.006) and reduce risk to develop ACS (Odd ratio =0.259, confidence interval 95% = 0.107 – 0.625 and p. value 0.002) while this polymorphism increased requirements to blood exchange (Odd ratio =1.050, confidence interval 95% = 0.981 – 1.123 and p. value 0.044).
Also we found there are association between GSTM1null genotype increase risk to stroke but this association not statistically significant (Odd ratio =3.33, confidence interval 95% = 0.710 – 15.643 and p. value 0.109).
This study found no significant association between GSTM1 genotype and time of anemia appearance, frequency of blood transfusion, annual hospitalization, VOC, bone problems and splenomegaly (Table 1).

Table 1: Association between GSTM1 null genotype and clinical manifestations	
Clinical manifestations	GSTM1

	Present	Null	Chi-square	Odd ratio	Confidence interval 95%	p. value
Gender						
Male	44	34	9.692	2.6	(1.324 – 5.168)	0.002
Female	40	8				
Anemia appearance						
< 1 year	62	32	0.084	0.881	0.372 – 2.083	0.772
≥ 1 year	22	10				
Blood transfusion						
< 2 per year	54	28	0.070	0.900	0.412 – 1.966	0.792
≥ 2 per year	30	14				
VOC						
< 2 per year	32	18	0.265	0.821	0.386 – 1.743	0.607
≥ 2 per year	52	24				
Annual hospitalization						
< 2 per year	30	16	0.068	0.903	0.420 – 1.942	0.794
≥ 2 per year	54	26				
Stroke						
Yes	12	2	2.571	3.33	0.710 – 15.643	0.109
No	72	40				
Dactylities						
Yes	42	10	7.924	0.313	0.136 – 0.716	0.006
No	42	32				
ACS						
< 2 per year	44	34	9.692	0.259	0.107 – 0.625	0.002
≥ 2 per year	40	8				
Bone problems						
Yes	6	2	0.267	1.54	0.297 – 7.971	0.605
No	78	40				
Splenomegaly						
Yes	2	2	0.516	0.488	0.066 – 3.590	0.472
No	82	40				
Blood exchange						
Yes	0	2	4.065	1.050	0.981 – 1.123	0.044
No	84	40				

The GSTT1null genotype found to be present in female more than male (Odd ratio =2.6, confidence interval 95% = 1.224 – 5.472 and p. value 0.012) and this polymorphism may reduce requirements to blood transfusion (Odd ratio =0.137, confidence interval 95% = 0.059 – 0.318 and p. value < 0.001) and annual hospitalization (Odd ratio =0.436, confidence interval 95% = 0.206 – 0.924 and p. value 0.029) and reduce risk to development of stroke (Odd ratio =0.125, confidence interval 95% = 0.0267 – 0.585 and p. value 0.008).Also this study found there are association between GSTT1null genotype and increase frequency of VOC, ACS, bone problems and splenomegaly but this association not statistically significant (Odd ratio =1.34, confidence interval 95% = 0.654 – 2.738 and p. value 0.424) , (Odd ratio =1.47, confidence interval 95% = 0.713 – 3.044 and p. value 0.294) , (Odd ratio =2.9, confidence interval 95% = 0.562 – 14.96 and p. value 0.203) and (Odd ratio =1.1, confidence interval 95% = 0.151 – 8.088 and p. value 0.923) respectively. And there are no significant association between GSTT1 null genotype and time of anemia appearance and Dactylities (Table 2).

Table 2: Association between GSTT1 null genotype and clinical manifestations

Clinical manifestations	GSTT1					
	Present	Null	Chi-square	Odd ratio	Confidence interval 95%	p. value
Gender						
Male	**44**	**34**	**6.344**	**2.6**	**1.224 – 5.472**	**0.012**
Female	**16**	**32**				
Anemia appearance						
< 1 year	**42**	**52**	**1.281**	**0.628**	**0.280 – 1.410**	**0.258**

≥ 1 year	**18**	**14**				
Blood transfusion						
< 2 per year	**26**	**56**	**23.84**	**0.137**	**0.059 – 0.318**	**0.000**
≥ 2 per year	**34**	**10**				
VOC						
< 2 per year	**26**	**24**	**0.638**	**1.34**	**0.654 – 2.738**	**0.424**
≥ 2 per year	**34**	**42**				
Annual hospitalization						
< 2 per year	**16**	**30**	**4.786**	**0.436**	**0.206 – 0.924**	**0.029**
≥ 2 per year	**44**	**36**				
Stroke						
Yes	**12**	**2**	**9.164**	**0.125**	**0.0267 – 0.585**	**0.008**
No	**48**	**64**				
Dactylities						
Yes	**30**	**22**	**3.602**	**0.500**	**0.243 – 1.027**	**0.059**
No	**30**	**44**				
ACS						
< 2 per year	**40**	**38**	**1.101**	**1.47**	**0.713 – 3.044**	**0.294**
≥ 2 per year	**20**	**28**				
Bone problems						
Yes	**2**	**6**	**1.752**	**2.9**	**0.562 – 14.96**	**0.203**
No	**58**	**60**				
Splenomegaly						
Yes	**2**	**2**	**0.009**	**1.103**	**0.151 – 8.088**	**0.923**
No	**58**	**64**				
Blood exchange						
Yes	**2**	**0**	**2.235**	**0.967**	**0.922 – 1.013**	**0.135**
No	**58**	**66**				

Polymorphism of both GSTM1 and GSTT1 found to be associated with appearance of disease before one year of age (Odd ratio =1.43, confidence interval 95% = 1.264 – 1.623 and p. value 0.004) and trend to be protective from development of Dactylitis (Odd ratio =0.124, confidence interval 95% = 0.027 – 0.563 and p. value 0.002).
Also, there are no significance association between GSTM1/ GSTT1null genotype and gender, frequency of blood transfusion, hospitalization, VOC, stroke, ACS, bone problems, splenomegaly and blood exchange (Table 3).

Table 3: Association between GSTM1/GSTT1 null genotype and some clinical manifestations

	GSTM1/GSTT1					
	Present	**Null**	**Chi-square**	**Odd ratio**	**Confidence interval 95%**	**p. value**
Anemia appearance						
< 1 year	74	20	8.093	1.432	1.264 – 1.623	0.004
≥ 1 year	32	0				
Dactylities						
Yes	50	2	9.591	0.124	0.027 – 0.563	0.002
No	56	18				
ACS						
< 2 per year	62	16	3.301	0.352	0.110 – 1.123	0.078
≥ 2 per year	44	4				

The present study found no statistically significance between GSTP1 gene polymorphism and gender variability and clinical manifestations of SCD.

Discussion:

Sickle cell anemia (SCA) is a chronic and progressively debilitating medical condition featuring ongoing hemolytic anemia and recurrent acute vaso-occlusive events [20]. It is characterized by a clinical course highly variable, ranging from death in early childhood [21] to a normal life span with few complications [22].

The complex pathophysiology of SCA which can be affected by a number of modifying factors including haplotype of β-globin gene cluster [23], coinheritance of polymorphisms associated with clinical aspects [24,25] and treatment response [16], Hemoglobin fetal (Hb F) levels [26], chronic inflammation and oxidative states [27,28] as well as gender [23].

Human GSTs have been well characterized as ethnic-dependent polymorphism frequencies and largely divergent among populations around the world [29, 30].

There are published reports about the association between GSTM1 and GSTT1 and GSTP1 polymorphisms and Sickle cell diseases but to date no study published in Africa except in Egypt, so this study aimed to fill the gap by investigating the possible association between the presence of GSTM1, GSTT1 and GSTP1 genes polymorphisms and SCD severity, diversity and complications in pediatric Sudanese patients.

In present study, GSTM1null genotype was found to be present in male more than female (OR =2.6 and p = 0.002) and this agreed with another study done in Sudan [31], they observed male had GSTM1 null genotype more than female (58.8% and 41.7%) respectively.

Also in this study, the GSTT1null genotype found to be present in female more than male (OR =2.6, p = 0.012), and this agreed with meta-analysis study [32] did report a significantly higher frequency of GSTT1 deletion among healthy Caucasian females, yet was not able to explain it on biological grounds, since GSTT1 gene is not located on the sex chromosome and [33] observed the female (68.6%) to male ratio (31.4%) was high which might explain the higher frequency of GSTT1 deletion among female controls. Difference between males and females may also be related to gender-associated expression of the GST family enzymes [34, 35], or the influence of sex hormones, importance of which GST regulation is well established in rodent models [36, 37].

Also, the GSTM1null genotype trend to be protective from development of Dactylitis (OR = 0.313 and p = 0.006) and associated with increase requirement to blood exchange (OR =1.050 and p = 0.044), to date no any published reports agreed or contrast this finding.

Also, GSTM null genotype reduce risk of ACS (OR = 0.259 and p = 0.002), in contrast to our finding, in Egypt [38], and in Brazil [39], they observed the GSTM1 null genotype was significantly associated with ACS.

We also observed the GSTM1null genotype associated with increased risk to stroke (OR =3.33, and p = 0.109), and this association not statistically significant, this agreed with study done in Brazil [39], they observed the patients with GSTM1 null showed a risk 3.9 times higher to develop stroke, Vasculopathy has been implicated in the development of pulmonary hypertension, stroke, leg ulcers and priapism, particularly associated with hemolytic severity.

In this study, there are no significant association between GSTM1 null genotype and the time of disease appearance, annual hospitalization and splenomegaly, to date, no study involving the polymorphism of GSTM1 gene and these clinical manifestations of SCD has been published, and no significant association between GSTM1 null genotype and the frequency of blood transfusion, and this agreed with another studies in Egypt [38, 40] and on significant association with VOC, bone problems.

The GSTM1 gene contains four alleles and most widely studied, GSTM1 polymorphism M1*A 0.2 is associated with decreased risk of bladder and breast cancer in Caucasians, M1*B 0.2 with decreased risk of pituitary adenomas; M1*0 0.59 has been shown to increase the risk of lung, colon, bladder, and post-menopausal breast cancer. GSTM1*A has been associated with a decreased risk of bladder cancer and has an allele frequency of 20% [41].

Evolution from the basic identification of polymorphic sites has provided the tools to discover the genetic complexity that affects genotype–phenotype correlation [16].
For GSTT1 null genotype, this study found no significant association with the time of anemia appearance, Dactylities and splenomegaly.
In this study, the GSTT1null genotype associated with decrease requirements to blood transfusion (OR =0.137 and $p < 0.001$), in contrast to our finding, in Egypt [38], found the GSTT1 null genotype was associated with significantly increased requirement of blood transfusion, and in the contrast for both, another studies in India [42], observed the requirement of blood transfusion is not dependent on GST deletions, and in Egypt [40] found no significant association between GST genotypes and transfusion frequency.
Also GSTT1 null genotype associated with reduce frequency of annual hospitalization (OR=0.436 and $p = 0.029$), but we don't find any published reports studied this association to confirm or contrast our finding.
And GSTT1 null genotype associated with reduce risk to development of stroke (OR =0.125 and $p = 0.008$) and this agreed with [39] found (OR =0.55 and $p = 0.45$) but no associated significance.
Also, in present study, GSTT1 null genotype associated with increased risk of ACS and bone problems and VOC (OR=1.47 and $p = 0.294$), (OR =1.47 and $p = 0.294$) and (OR =1.34 and $p = 0.424$) respectively, and this association not statistically significant and this agreed with another studies [38, 39, 6].
The previous reports demonstrated that patients with SCD are subject to increased oxidative stress mainly in ACS, Acute chest syndrome in SCA is defined as a new infiltrate on chest radiograph associated with one or more symptoms, such as fever, cough, hypoxia, tachypnea, dyspnea, the pathophysiology of this injury is complex, Microbial infection, vaso-occlusion, ischemia, necrosis of marrow or thromboembolism can initiate the process [43].
Polymorphism of both GSTM1 and GSTT1 genes found to be associated with appearance of disease before one year of age (OR =1.43 and $p = 0.004$) and trend to be protective from development of Dactylitis (OR =0.124 and $p = 0.002$) but we don't find published reports confirm or contrast this finding.
Also, this study found no statistically significance between GSTM1/ GSTT1null genotype and gender variability, annual hospitalization, splenomegaly and blood exchange, no previous reports confirm or contrast this finding.
Also, no statistically significance with VOC, frequency of blood transfusion, ACS, stroke and bone problems, and this agreed with previous studies [38, 39, 40, 44], except [39], contrast our finding only in ACS.
In this study, there are no statistically significance between GSTP1 gene polymorphism and gender variability and clinical manifestations of SCD, and this agreed with another studies in Egypt [38, 40], they found the non-wild-type GSTP1 polymorphism was not associated with clinical manifestations of SCD.
The difference between the clinical manifestations of this study and previous studies make complexity in detecting the association between these polymorphisms and SCD severity and diversity, this study were the first in the studying of some clinical manifestations to date no study published before such as, the time of disease appearance, frequency of annual hospitalization, Dactylities, splenomegaly and blood exchange in SCD patients.
The unexpected clinical diversity in a monogenic disease such as SCD has led to countless genetic studies and current knowledge has evolved, together with technological development in molecular biology [44].
Moreover, since GST is a multigene family, one or two single polymorphisms and null genotype expression may not be sufficient to alter the overall enzymatic and antioxidant capacity [45].

Previous studies have shown that there is marked geographical and ethnic variation in the distribution of genes for polymorphic GSTs [46], and this study was the first in Africa except Egypt.

The sickle mutation is found in different distinct haplotypes. Four in Africa designated Senegal, Benin, Central African Republic and Cameroon. The Senegal type is the most benign one [47, 48]. Bantu haplotype has been associated with more severe disease outcome and a high organ damage incidence, Benin haplotype has been associated with intermediate disease severity. On the other hand, Senegal and Indian-Arab haplotypes have been associated with milder disease severity [49, 50] due to their higher Hb F levels related to the C→T mutation at position -158 XmnI in the Gγ-globin gene promoter region [51].

In Sudan, the most frequent haplotypes were the Cameroon followed by the Benin [51].

The difference between studies variables, geographical and ethnic variation and between studies make the interpretations more complex, Some studies reported no association detected or associated with clinical manifestations not included in this study, for example, in Egypt [40, 44] they didn't find any significant association between both GSTT1 and GSTMT1 null genotypes and clinical severity of the disease in SCD patients, also in Egypt [39] found GSTM1 null associated with pulmonary hypertension and VOC and the absence of GSTT1 was associated with increased risk of pulmonary hypertension and ACS but was not statistically significant and VOC and both GSTM1 and GSTT1 null genotypes were significantly predisposed to pulmonary hypertension, and In a Brazilian cohort [52] they found no association between GSTM1 and GSTT1 gene polymorphism and oxidative stress parameters in SCD patients, also [53] Pooled analysis of GSTT1 and GSTP1 polymorphisms revealed significantly increased risk of complications in SCD, while GSTM1 null genotypes did not show association with SCD complications. Significant between study heterogeneity (I2 > 50%) was observed for in all three polymorphisms (GSTM1 = 68.7%), (GSTT1 = 71.6%), (GSTP1 = 83%).These contradictory results may be explained by the fact that Silva and colleagues studied the relation between GST gene polymorphisms and biochemical markers of oxidative stress, not the clinical manifestations of SCD. Thus, biochemical markers of oxidative stress may not be an accurate marker for measuring SCD severity. In addition, different ethnicity could explain this contradiction [39].

The protective effect found in this study in some clinical manifestations and differences in the associations or lack of them in the previous studies may be explained by many factors, there are evidence on gene–gene interactive effects on GSTs and genotype–phenotype correlation, previous studies postulated, there are another mechanism for defense against oxidative stress, erythrocyte shave a self- sustaining activity of antioxidant defense enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), in addition to low-molecular-weight antioxidants, such as glutathione(GSH) and vitamins E and C [54], RBC superoxide dismutase (SOD) activity has been shown to increase in some SCD studies [55–56], Nur et al. [9] Demonstrated an increased GSSG efflux in sickle erythrocytes that can be a protective action, because GSSG is an oxidant itself and its enhanced excretion under oxidative conditions prevents the potentially toxic effects of its intracellular accumulation [57]. But increased GSSG efflux could play an important role in GSH depletion in these cells. Erythrocytes are not only a main ROS source in SCD but also endure the brunt of the intracellular oxidative stress [11, 58].The primary antioxidant enzymes against superoxide radicals include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [59].

TBARS is one of the existing biomarkers, and this evaluation is an indirect quantification of lipid peroxidation processes, which makes it a good indicator of pro-oxidant stimuli [60]. In accordance with results reported previously [61, 52, 62], and [63], observed significantly higher levels of TBARS in patients with sickle cell disease than in the controls [60].Also, G6PD is an important enzyme related to the antioxidant defense in erythrocytes [52], Higher activity of this enzyme in patients with sickle cell disease was found than in the control group, previously reported that

erythrocytes from patients with sickle cell disease have an increased percentage of reticulocytes, while the activity of G6PD in reticulocytes is normal, but declines exponentially as the red cells age [63].Also [64] observed higher CAT activity in the plasma of SS children compared to AA controls, GPX activity would be more representative of the current severity of the disease since children with no hospitalized VOC had lower GPX activity than children with a positive history of VOC over the last 2 years. Faes et al. [65] found higher GPX activity in young adults with SCA compared to healthy individuals.

GST polymorphisms not only increase risk to disease, but they also appear to influence protective effect and good prognosis in some diseases, in hematological diseases, some reports found that, GSTT1 and GSTM1 polymorphisms showed a trend towards protection from having ALL [66], Krajinovic et al [67] reported an increase risk of ALL with GSTM1 null genotype but not with the GSTT1 null genotype, In a report by Davies et al, [68] however, no association was noted for both null genotypes. The differences in the associations or lack of them in the previous studies may be explained by other factors like the heterogeneity in environmental toxin exposure and the effect of other detoxifying genes [66].

Dufour et al (2005) data indicate that genetic polymorphisms of the detoxifying enzymes GSTT1, GSTM1, GSTP1, CYP3A4 and NQO1 are not associated with the risk of developing idiopathic aplastic anemia, suggesting that these polymorphisms do not play a major role in the pathogenesis of idiopathic aplastic anemia in Caucasian patients, It is worth noting that in the cases of GSTM1 and GSTT1, the null polymorphism is a gene deletion that causes total absence of the enzyme, thus the lack of correlation of the above polymorphisms with aplastic anemia also indicates that the absence of these detoxifying systems does not affect the risk of developing this disease in Caucasian subjects [69], Homozygous deletion of GSTM1 may interfere with iron chelation therapy and lead to slow unloading of liver iron [70].

Also these polymorphisms had protective effect in other diseases; some reports found that, the decreased GST activity served to protect the host erythrocytes against the invading malarial parasite by up-regulating oxidative defense mechanisms [71].GST polymorphisms not only influence susceptibility to disease, but they also appear to to influence responsiveness to cancer chemotherapeutic agents [72].

In some studies GSTM1-null genotype play a protective role for cancer [73], Diabetic retinopathy in type 1 diabetes [74], Diabetic retinopathy in people with type 2 diabetes [75, 76], Cilensˇek I et al. (2012) proposed a protective effect for GSTM1 null genotype against retinopathy explained by an up-regulation of other antioxidant enzymes such as manganese superoxide dismutase which become more effective in detoxification of atherogenic compounds [75], and the high activity of cytochrome P450 system in such patients might offer another explanation [74].

Also another proposed that mutant detoxification enzymes such as GSTs may result in the induction of other GST transferases and antioxidant proteins, which represent compensation to increase in the intracellular levels of antioxidative products [77].Protected against colon cancer, due to the potentially slower excretion of isothiocyanates [78], protection against hearing impairment in testicular cancer patients [79], that the GSTT1 null genotype had a protective effect on the development of schizophrenia and the combination of null genotypes of the GSTT1 and GSTM1 genes was made at a lower risk of schizophrenia [80, 81], protective role of the GSTT1-null genotype against coronary artery disease [82], also null genotypes of GSTM1 and GSTT1 confers protective effect regarding male infertility[83,84].Only one collective of authors has reported that null genotype is associated with disease protection [85], it may be explained by several speculative possibilities. First, the absence of GST may upregulate other antioxidant genes like superoxide dismutase [86]. Second, GST enzymes are normally involved in the synthesis of inflammatory mediators, leukotrienes and prostaglandins [87], so lack of GST activity may lead to decrease in the inflammatory response and to protection against T1D. Third, an unknown compound may be metabolized by GST into a toxic form, so null genotype would be protective,

such as dihaloalkanes are bioactivated by increased activity of GSTT1 into more genotoxic metabolites [88] or GST pi knockout mice are protected against acetaminophen toxicity as acetaminophen is not activated into its toxic metabolite [89].
Due to little papers like this study are published and there are difference in clinical manifestations and population, limited information about this subject has been published, the absence of data obtained on phenotypic effects of GST family genes, Future large studies evaluating GST genes in addition to other antioxidant genes are needed to provide evidence on gene–gene interactive effects on SCD, which makes further functional studies a necessity to determine the exact genotype-phenotype correlation.
Most of studies done before agreed with our study in most finding in the same clinical manifestations which done by this study and previous studies, and for some clinical manifestations, this study was the first, as time of disease appearance, frequency of annual hospitalization, dactylities, splenomegaly and blood exchange, the diversity of the clinical manifestations and severity among patients with similar GST polymorphisms may be due to the presence of other modifying genes effects on these genes, this may need more extensive studies which sample a larger number of SCD patients, also any reported differences between studies might be attributed to sampling error.

Conclusion:

In conclusion, this study found, The GSTM1null genotype was found to be present in male more than female and trend to be protective from development of Dactylitis and may reduce risk to develop ACS, while this polymorphism may increase requirements to blood exchange.
The GSTT1null genotype found to be present in female more than male and this polymorphism may reduce requirements to blood transfusion and annual hospitalization and may reduce risk to development of stroke.
Polymorphism of both GSTM1 and GSTT1 found to be associated with appearance of disease before one year of age and trend to be protective from development of Dactylitis.
Also, there are no statistically significance between GSTP1 gene polymorphism and gender variability and clinical manifestations of SCD.
This study were the first in the studying of some clinical manifestations to date no study published before, as time of disease appearance, frequency of annual hospitalization, Dactylities, splenomegaly and blood exchange in SCD patients and was the first done in Africa except Egypt.
The polymorphism of GSTM1, GSTT1 and GSTP1 genes were identified as important risk factors for developing severe hematological and clinical manifestations of SCD. Future large studies evaluating GST genes in addition to other antioxidant genes are needed to provide evidence on gene–gene interactive effects on SCD, which makes further functional studies a necessity to determine the exact genotype-phenotype correlation.
Identification of GSTM1, GSTT1 and GSTP1 null genotype as risk factors for developing severe manifestations of SCD may help to categorize SCD patients whom are genetically at risk of developing severe manifestations due to defective anti-oxidative defense mechanism, in order to minimize severity of their symptoms by using prophylactic antioxidants and other measures that improve their reductive defense mechanisms.

ESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also,

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66**.** Genetic Polymorphisms of Glutathione-S-Transferase P1,T1 and M1 in Pediatric Patients with Acute Lymphocytic Leukemia in a Philippine Tertiary Hospital Maria Melanie Liberty B. Alcausin1, Pamela D. Fajardo1,Catherine Lynn T. Silao1,2, Amy Goleta-Dy1,Eufrosina A. Melendres1, Eva Maria C. Cutiongco-dela Paz1,2, Carmencita David-Padilla1,2

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