GSTT1 (rs4025935) null genotype is associated with increased risk of sickle cell disease in the populations of Tabuk—Northwestern region of Saudi Arabia

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ABSTRACT

Background: Glutathione system plays an important role in the protection of cells and tissue against damage from oxidative stress. Impairment of the glutathione system due to genetic polymorphism of GST genes may increase the risk and severity of sickle cell disease (SCD). Present study was, therefore, undertaken to examine the relative impact of the genetic polymorphism of GSTT1 and GSTM1 (rs4025935 and rs71748309) on susceptibility and hematological aspects of the patients with SCD.

Methods: Present study included 100 patients with SCD and 200 healthy controls from northwestern region of Saudi Arabia. GSTM1 and GSTT1 (rs4025935 and rs71748309) genotypes were investigated by using single-tube multiplex PCR technique.

Results: It was observed that patients with SCD possessed significantly higher frequency of GSTT1 null genotype (26%) than healthy controls (15%), (P = 0.00001). Compared to the presence of GSTT1 genotype, the OR for the GSTT1 null genotype were estimated to be 4.3 (2.17–8.64, P = 0.00001). However, such association was not observed with respect to the presence of GSTM1 null genotype. In addition, it was observed that SCD in patients with GSTT1 genotype, the mean percentage levels for HbF and HbS were 0.48 and 35.4%, respectively; however, among SCD patients with GSTT1 null genotype, the mean percentage levels were significantly higher 1.62% (P = 0.004) and 39.38% (P = 0.02), respectively.

Conclusion: GSTT1 null genotype is significantly associated with increased risk of SCD among the population of northwestern region of Saudi Arabia. In addition, it may be one of the important factors responsible for hematological manifestations of SCD.

KEYWORDS

Sickle cell disease; GSTT1 null and GSTM1 null genotypes; Null genotype; ROS

Introduction

Sickle cell disease (SCD) is a global public health disorder that affects millions of people across the globe. It is a monogenic disorder caused by an A-to-T point mutation in the hemoglobin β-globin gene that produces abnormal hemoglobin S (HbS), which polymerizes in the deoxygenated state, resulting in physical deformation or sickling of erythrocytes.1 This mutation causes hemoglobin to polymerize when deoxygenated, resulting in ‘sickle’ shaped erythrocytes that adhere to the blood vessel walls. Individuals homozygous for the mutation (HbSS) have SCD and may experience chronic anemia, aplastic crisis, severe pain, cerebrovascular accidents, and splenic and renal dysfunction: carrying only a single copy of the HBB rs334 polymorphism (HbAS) confers sickle cell trait, which is usually asymptomatic.2–5

The prevalence of SCD in Saudi Arabia is patchy and probably underestimated, but studies have reported that SCD is a relatively common genetic disorder in this part of the world. The prevalence of SCD in Saudi Arabia varies significantly in different parts of the Kingdom, with the highest prevalence is in the Eastern province, followed by the southwestern provinces.6,7 The reported prevalence for sickle cell trait ranges from 2 to 27%, and up to 2.6% will have SCD in some areas. Clinical and hematological variability exists in SCD in Saudi Arabia with two major phenotypes: a mild phenotype and a severe phenotype.8,9 The information on impact of SCD mortality in Saudi Arabia is absent and studies on mortality patterns are limited. However, hospital-based studies from the Eastern province show that 73% of deaths occur under the age of 30 years, with acute chest syndrome followed by infection as the major cause of deaths.10 Rapid advances made in understanding the molecular genetics of SCD in the early part of the twentieth century have not been matched by comparable progress toward understanding its clinical complications, and developing effective therapies.

Antioxidant enzymes have an important mechanism by which cells limit the damage caused by ROS. Glutathione S-transferases (GSTs) is a family of genes with a critical function in the protection against electrophiles and the products of oxidative stress.11 GST enzymes are responsible for the detoxification of chemicals.
found in the environment and naturally synthesized metabolites, and they play an important role in protecting tissue from oxidative damage.12,13 The relation between GST gene polymorphism and SCD has been investigated in various studies, which demonstrated that the risk of SCD increases in populations with GST gene polymorphism.14–17 The present study was, therefore, undertaken to examine the relative impact of the genetic polymorphism at the gene loci glutathione S-transferase theta 1 (GSTT1) and glutathione S-transferase mu 1 (GSTM1) on susceptibility and hematological aspects of the patients with SCD from northwestern part of Saudi Arabia.

Methods

Study population

Selection criteria of patients

- The subjects of consecutive patients with clinically confirmed SCD from Tabuk region of Saudi Arabia. All patient specimens were timed around routine blood drawn that was the part of routine workout, and hence will not require additional phlebotomy. The specimens obtained were newly diagnosed, pathologically and HPLC confirmed cases of SCD patients.
- The study included of 200 healthy controls ranging from 20 to 50 years of age, visiting King Khaled Hospital, Tabuk, Saudi Arabia for routine checkup. The controls were enrolled from the general population of the same geographical region. Routine medical check-up was conducted (CBC, KFT, LFT, etc.) and history of illness was recorded by a health practitioner. Those who appeared apparently healthy without any history of any significant disease or other chronic diseases were considered as normal. The study was carried out with the approval of The Research Ethics Committee, the University of Tabuk. A standard questionnaire was used to document the socio-demographical characteristics such as age, sex, and lifestyle.

Genotype analysis

- Peripheral blood samples were collected in EDTA vials from the patients with SCD as well as from healthy controls. Genomic DNA from blood was isolated using DNA extraction kit (QiAmp DNA Blood Mini Kit 51106) and stored at −20°C until PCR. Quality and integrity of DNA was checked by NanoDrop™ (Thermo Scientific, Wilmington, DE, USA).
- GSTM1 and GSTT1 genotypes were analyzed using single-tube multiplex PCR assay which was performed in a reaction volume of 25 µl containing template DNA (50 ng), 0.25 µl of 25 pmol of each primers (listed in Table 1) and 12.5 µl from GoTaq® Green Master Mix (Promega, Madison, WI, USA) which is composed of GoTaq® DNA Polymerase, 2X Green GoTaq® Reaction Buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, and 3 mM MgCl₂. Final volume of 25 µl was adjusted by adding nuclease free ddH₂O.
- The reaction mixture was subjected to initial denaturation at 94°C for 10 minutes, followed by 35 cycles of 94°C for 45 seconds, 62°C for 45 seconds, and 72°C for 45 seconds with final extension of 72°C for 5 minutes.
- The amplification products were separated by electrophoresis through 2% agarose gel stained with ethidium bromide. GSTM1 and GSTT1 genotypes yielded 215 and 480 bp bands sizes, respectively, while the internal positive control (CYP1A1) PCR product corresponded to 312 bp size as depicted in Fig. 1.
- Presence of all the three band sizes (215, 480, and 312 bp) indicates both GSTM1 and GSTT1 positive genotypes; a single band size of 312 bp indicates both GSTM1 and GSTT1 null genotypes; band sizes of 215 and 312 bp indicates GSTM1 positive/GSTT1 null genotypes; and band sizes of 480 and 312 bp indicates GSTT1 positive/GSTM1 null genotypes.

Statistical analysis

- The distribution of the GSTM 1 and GSTT1 genotypes among the study groups were evaluated using Chi-square or Fisher exact probability tests. Odds ratio and confidence interval were used to estimate risk of the SCD among the population. Student t test was applied to compare the genotypes with the various laboratory characteristics of the SCD patients. Epi info 6 and SPSS version 16 statistical software’s were used to analyze the data. A P value <0.05 was considered significant.

Table 1 Primers used for GSTM1 and GSTT1 genotype analysis

| Gene   | Primer sequence | Product size |
|--------|-----------------|--------------|
| GSTM1  | F-5'-GAACCTCCTGAAAAGCTAAAGC-3' | 215 bp       |
|        | R-5'-GGGATCCCATATGATAGCTTG-3' |              |
| GSTT1  | F-5'-TTCCCTAGGGTCTCCTCCTC-3' | 480 bp       |
|        | R-5'-CATGCGATATGAGCAGCGAC-3' |              |
| CYP1A1 | F-5'-GAATCTCGCCACCTCAGGCGT-3' | 312 bp       |
|        | R-5'-CAGCTGATTTGGAAGGTGC-3'  |              |
Results

Laboratory variables

Various laboratory variables were obtained from medical records of patients (n = 100) with SCD and are as described in Table 2. Among the SCD patients, the mean percentage for HbA1, HbA2, and HbF were 63.4, 4.06, and 0.77%, respectively. SCD patients had the HbS level in the range from 25 to 82.4% with overall mean value of 36.5%.

GSTM1/T1 genotype distribution

Compared to healthy controls 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%). In addition, it was observed that among the 100 cases only one patient showed both GSTM1 and GSTT1 null genotypes, whereas such genotype was not observed in healthy controls (Tables 3–5).

GSTM1/T1 genotypes and risk of SCD

In order to estimate the association between the GSTM1/T1 genotypes and risk of SCD among Saudi population, a multivariate analysis based on logistic regression like odds ratio, risk ratio, and risk difference with 95% confidence intervals was calculated for each group. With respect to GSTT1 genotype, it was observed that compared to the presence of GSTT1 genotype, the OR, RR, and RD for the GSTT1 null genotype were estimated to be 4.3 (2.17–8.64), 2.0 (1.30–2.94), and 34.8 (19.1–50.58) with statistically significant difference (P = 0.00001), respectively (Table 3). However, with respect to the presence of GSTM1 genotype, the OR, RR, and RD for the GSTM1 null genotype were estimated to be 0.56 (0.26–1.19), 0.85 (0.70–1.02), and −11.76 (−25.67–2.15) with statistically non-significant difference (P = 0.129), respectively (Table 4). While taking both the GSTM1/T1 genotypes together in consideration, it was observed that compared to the presence of both the GSTM1 and GSTT1 genotype, the OR, RR, and RD for genotypes either null for GSTM1 or GSTT1 were estimated to be 1.7 (0.98–2.80), 1.2 (0.98–1.46), and 11.5 (−0.77–23.79) with P = 0.05, respectively (Table 5). However, such association was not calculated for the genotypes both null for GSTM1 and GSTT1, because only a single SCD patient showed such genotype.

GSTM1/T1 genotypes and laboratory characteristics

Various important laboratory characteristics of patient’s with SCD were compared with the GSTM1/T1 genotypes. It was observed that SCD patients with GSTM1 genotype, the mean percentage levels for HbA1, HbA2, HbF, and HbS were 64.53, 4.13, 0.73, and 35.82%, respectively; however, among SCD patients with GSTM1 null genotype, the mean percentage levels were 53.18, 3.38, 1.18, and 42.31%, respectively. The difference was statistically significant with respect to HbA1 and HbS levels (P = 0.011 and 0.008, respectively). Similarly, it was observed that SCD patients with GSTT1 genotype, the mean percentage levels for HbA1, HbA2, HbF, and HbS were 64.03, 4.24,
0.48, and 35.4%, respectively; however, among SCD patients with GSTT1 null genotype, the mean percentage levels were 61.57, 3.54, 1.62, and 39.38%, respectively. The difference was statistically significant with respect to HbF and HbS levels (P = 0.004 and 0.02, respectively).

While considering both the GSTM1/T1 genotypes together, it was observed that SCD patients with both GSTM1 and GSTT1 genotypes, the mean percentage levels for HbA1, HbA2, HbF, and HbS were 64.68, 4.36, 0.48, and 35.16%, respectively; however, among SCD patients with either GSTM1 null or GSTT1 null genotypes, the mean percentage levels were 60.89, 3.47, 1.34, and 38.99%. The difference was statistically significant with respect to HbF and HbS levels (P = 0.02 and 0.014, respectively). Interestingly, HbS level was observed to be 84.3% in the patient which possessed both GSTM1 and GSTT1 null genotypes (not mentioned in the Table 6).

Several studies have reported that the homozygous deletions of GSTM1 and GSTT1 (rs4025935 and rs71748309) result in a complete loss of enzyme activity and leads to toxicity and consequently can change an individual’s susceptibility to carcinogens and toxins as well as affect the toxicity and efficacy of certain drugs and is also linked with an increase in a number of diseases likely due to an increased susceptibility to environmental toxins and carcinogens.

**Discussion**

SCD is a global public health disorder that affects millions of people across the globe. Clinical manifestations of SCD result from sickling of HbS due to oxidation, which is augmented by accumulation of oxygen-free radicals. Deficiencies in normal antioxidant protective mechanism might lead to clinical manifestations of SCD like vasoocclusive crisis and acute chest syndrome. 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 SCD; polymorphisms of these genes may be related to the different manifestations between individuals. GSTs have been expected to increase the severity of SCD manifestations. GSTS have been reported to constitute the major defensive antioxidant system against oxidative stress by reducing ROS, one of the major factors leading to a risk status, to less reactive metabolites. The glutathione system plays an important role in the removal of endogenous products of peroxidation of lipids, thus protecting cells and tissue against damage from oxidative stress.

**Table 3** Distribution of GSTT1 genotypes in SCD patients and healthy controls

| Variables | Controls (n = 200) | Cases (n = 100) | OR (95% CI) | RR (95% CI) | RD (95% CI) | P value |
|-----------|-------------------|----------------|-------------|-------------|-------------|---------|
| GSTT1 present | 185 (92.5%) | 74 (74%) | 1.00 | 1.00 | | |
| GSTT1 null | 15 (7.5%) | 26 (26%) | 4.3 (2.17–8.64) | 2.0 (1.30–2.94) | 34.8 (19.1–50.58) | 0.00001 |

**Table 4** Distribution of GSTM1 genotypes in SCD patients and healthy controls

| Variables | Controls (n = 200) | Cases (n = 100) | OR (95% CI) | RR (95% CI) | RD (95% CI) | P value |
|-----------|-------------------|----------------|-------------|-------------|-------------|---------|
| GSTM1 present | 167 (83.5%) | 90 (90%) | 1.00 | 1.00 | | |
| GSTM1 null | 33 (16.5%) | 10 (10%) | 0.56 (0.26–1.19) | 0.85 (0.70–1.02) | −11.76 (−25.67–2.15) | 0.129 |

**Table 5** Distribution of double combinations of GST genotypes in SCD patients and healthy controls

| GSTM1 and GSTT1 | Controls (n = 200) | Cases (n = 100) | OR (95% CI) | RR (95% CI) | RD (95% CI) | P value |
|-----------------|-------------------|----------------|-------------|-------------|-------------|---------|
| Both present | 152 (76%) | 65 (65%) | 1.00 | 1.00 | | |
| Either null | 48 (24%) | 34 (34%) | 1.7 (0.98–2.80) | 1.2 (0.98–1.46) | 11.5 (−0.77–23.79) | 0.05 |
| Both null | 0 (0.0%) | 1 (1%) | – | – | – | |

**Table 6** Distribution of single and double combinations of GST genotypes in SCD patients with respect to laboratory parameters

| Variables | HbA1 (mean ± SD) | P value | HbA2 (mean ± SD) | P value | HbF (mean ± SD) | P value | HbS (mean ± SD) | P value |
|-----------|------------------|---------|------------------|---------|----------------|---------|----------------|---------|
| GSTM1 present | 64.53 ± 12.59 | 0.011 | 4.13 ± 4.06 | 0.562 | 0.7300 ± 1.67 | 0.444 | 35.82 ± 6.07 | 0.008 |
| GSTM1 null | 53.18 ± 17.134 | | 3.38 ± 0.47 | | 1.18 ± 2.44 | | 42.31 ± 14.24 | |
| GSTT1 present | 64.03 ± 9.68 | 0.426 | 4.24 ± 4.45 | 0.430 | 0.48 ± 0.37 | 0.004 | 35.44 ± 3.84 | 0.020 |
| GSTT1 null | 61.57 ± 20.95 | 3.54 ± 0.86 | 1.62 ± 3.29 | | | | 39.38 ± 12.82 | |
| Both present | 64.68 ± 10.03 | 0.183 | 4.36 ± 4.69 | 0.48 ± 0.38 | 0.020 | | | 35.16 ± 3.90 | 0.014 |
| Either null | 60.89 ± 18.31 | 3.47 ± 0.76 | 1.34 ± 2.91 | | | | 38.99 ± 11.23 | |
The risk of developing SCD was observed to be more than four times in association with the GSTT1 null genotype compared to the presence of GSTT1 genotype. However, such association was not observed with respect to the presence of GSTM1 null genotype. Several epidemiological studies have reported that the GSTT1 null genotypes results in a lack of functional protein which may cause increased vulnerability to oxidative DNA damage and excessive ROS generation and may ultimately result in increased susceptibility to various diseases associated with oxidative stress including SCD.22

The distribution of GSTM1 and GSTT1 null genotypes varies among different ethnic groups. Several population-based studies have reported a prevalence ranging from 47 to 58% for the GSTM1 null genotype and from 13 to 25% for the GSTT1 null genotype among white Europeans23 and 27.6% among the US white population.24

Limited data exist about status of GSTM1 and GSTT1 polymorphism among patients with SCD especially among the population of northwestern region of Saudi Arabia. Present study observed that patients with SCD possess 26% and 10% frequency of GSTT1 null GSTM1 null genotypes, respectively.

Among the various important laboratory characteristics of patient’s with SCD, it was observed that SCD patients with GSTT1 null or GSTM1 null genotype possessed significantly higher percentages of HbS levels as compared to the patients with the normal respective genotypes. Higher level of HbS was observed in the patient which possessed both GSTM1 and GSTT1 null genotypes. The individuals with the GSTM1 and GSTT1 null genotypes showed a higher chance of developing acute chest syndrome, malleolar ulcer, and aseptic necrosis of the femoral head. The absence of GSTT1 and/or GSTM1 was an important risk factor for increasing the morbidity of SCD, especially in regard to acute chest syndrome.25,26

Clinical implications
The absence of common functional GST enzymes yields a slower clearance of ROS, which may increase the risk for end organ damage. The lack of a survival benefit or detriment by GST genotype implies that administering an intervention to reduce toxicity based on GST genotype should not adversely affect survival.27,28

Additional studies to confirm the association of GSTM1 null genotype and toxicity and to further study the mechanisms on the role of GST enzymes in endothelial damage after medication, chemotherapy and/or radiation treatment are warranted. GSTT1 is involved in activation and detoxification reactions and catalyzes the conjugation of industrial chemicals, e.g. ethylene oxides, with glutathione.29 Homozygous deletions of the GSTM1 and GSTT1 genes are common and result in a complete loss of enzyme activity. These genetic variations can change an individual’s susceptibility to carcinogens and toxins as well as affect the toxicity and efficacy of certain drugs. Null mutations of this class mu gene have been linked with an increase in a number of diseases likely due to an increased susceptibility to environmental toxins and carcinogens.30,31 The risk of individuals with the GSTT1 null genotype developing acute chest syndrome and aseptic necrosis of the femoral head were, respectively, 10 and 6.3 times higher compared with the individuals with normal GSTT1 genotype.16

Conclusion
In conclusion, the GSTT1 null genotype is significantly associated with increased risk of SCD. In addition, GSTT1 null genotype may be one of the important factors responsible for hematological manifestations of SCD. This is the first study which revealed the status of null genotypes among the GST genes among the patients with SCD from the northwestern region of Kingdom of Saudi Arabia. However, further studies with larger sample sizes are needed in order to validate our findings.

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