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Regular paper

The relation between glutathione S-Transferase M1 null-genotype and cardiac problems in beta-thalassemia

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This work was carried out to investigate the role of Glutathione S-Transferase M1 (GSTM1) null genotype frequency in prognosis of β-thalassemia, and to detect the correlation between GSTM1 null genotype and appearance of cardiac complications in β-thalassemia. Materials and Methods. The studied groups in the present work were divided to three groups (group I: 20 healthy subjects, group II: 56 β-thalassemic patients and group III: 16 β-thalassemic patients with cardiac complications were taken from group II). The measurement of human high sensitive C-reactive protein (hs-CRP) was performed using nephelometry. GSTM1 genotype was detected by Polymerase Chain Reaction (PCR) and cardiac complications were determined by using Echocardiography. Results. A statistically significant increase in hs-CRP and interleukin-6 (IL-6) levels was found in β-thalassemic patients with cardiac complications compared to normal subjects. Results showed no relation between GSTM1 null genotype frequency neither with β-thalassemia nor with cardiac complications appearance, where the interaction between GSTM1 null genotype in β-thalassemic patients with cardiac complications and healthy subjects were insignificant compared to subjects with GSTM1 non-null genotype. Conclusions. GSTM1 null genotype frequency has no role in β-thalassemia or cardiac complications appearance.

Key words: GSTM1, β-thalathemia, Cardiac complications, hs-CRP, IL-6

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INTRODUCTION

β-thalassemias are a group of hereditary blood disorders characterized by abnormal synthesis of the beta chains of hemoglobin resulting in variable phenotypes ranging from severe anemia to clinically asymptomatic individuals (Matin *et al.,* 2015). The reduced amount (beta+) or absence (beta0) of beta globin chains result in a relative excess of unbound alpha globin chains that precipitate in erythroid precursors in the bone marrow, leading to their ineffective erythropoiesis and hence to premature death (Galanello & Origa, 2009). The degree of globin chain reduction is determined by the nature of the mutation at the beta globin gene located on chromosome 11 (Jha R & Jha S, 2014).

An important issue that has evolved during past decades is the age of onset of heart failure. In the 1960s, before the initiation of regular blood transfusions and iron chelation, heart failure occurred as early as during the second decade of life, with an average age of onset of 16 years (Kremastinos *et al.,* 2010).

Glutathione S-transferase (GST) enzymes belong to a super family of multifactorial isoenzymes that in addition of being well known detoxification agents are also involved in excretion processes of toxic molecules as well (Sclafani *et al.,* 2013). Evidence suggests that the GST expression level is a crucial factor in determining cell sensitivity to a broad spectrum of toxic chemicals, as GST genes are up regulated in response to oxidative stress (Hayes & Pulford, 1995). Genetically determined variations cause changes in activity level and/or expression of some GST and may cause decreased defense capacity against oxidative stress (Sclafani *et al.,* 2013).

Inflammatory biomarkers, including CRP and IL-6 are increased in various inflammatory conditions and are useful in studying thalassemia and other disease states, including heart disease (Cantisani *et al.,* 2012).

Our objective from this work to investigate the role of Glutathione S-Transferase M1 (GSTM1) null genotype frequency in prognosis of β-thalassemia and to detect the correlation between GSTM1 null genotype and appearance of cardiac complications in β-thalassemia.

MATERIALS AND METHODS

Subjects. This study was conducted at the National Research Centre (NRC) in Cairo, Egypt. Written consent or their guardian's approval was obtained according to rules of the medical research ethics committee at NRC (number: 10–172). This study included 76 subjects (20 healthy subjects and 56 patients), their ages ranged from 3 to 25 years, groups were divided into: Group I: included 20 healthy subjects matched in age and gender. Group II: included 56 β-thalassemic patients under treatment. Group III: included 16 β-thalassemic patients with cardiac complications were taken from group II (subgroup from group II).

Blood samples were collected by a well trained nurse from each thalassemic patient just before a scheduled transfusion of packed red blood cells. Three ml venous blood samples were obtained from each subject and di-

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Abbreviations: CVD, cardiovascular disease; CBC, complete blood count; GST, Glutathione S-transferase; GSTM1, Glutathione S-Transferase M1; IL-6, interleukin-6; hs-CRP, high sensitive C-reactive protein; MCV, mean corpuscular volume; PCR, Polymerase Chain Reaction; RBC, red blood cell; SPSS, Statistical Package for Social Science

Table 1. Hematological profile between healthy subjects (group I) and β-thalassemic patients (group II & III).

Parameters	Healthy subjects (n=20)		β-thalassemic Patients (n=56)		*P*-value
	Median	Range	Median	Range	
Age (years)	13.52	5–25	11.73	3–25	0.11
Hb (g/dL)	13.67	11.2–15.6	7.62	5.3–9.2	0.000**
RBCs (x10^{12}/L)	4.91	3 .77–5.41	3.37	2.5–4	0.011*
MCV (fl)	88.14	76.8–95.3	71.42	52–83	0.001*
Retics (%)	1.23	0.8–1.9	4.85	2.4–11.7	0.000**
Gender (Male)	7	35%	25	44.5%	0.315
(Female)	13	65%	31	55.5%	

*Statistically significant (P < 0.05). **High Statistical significance (P < 0.01)

vided into EDTA tube (2.0 ml) with smooth shaking and vaccutainer plain tube (1.0 ml). Vaccutainer plain tubes were left for short time to allow blood to clot, and then clear serum samples were obtained by centrifugation (B. Bran-Sigma 2K15, USA) at 4000 rpm for 10 minutes. The separated serum was sealed and stored at –20°C until the time of performing the analysis. The frozen serum samples were thawed at 4–8°C then mixed by gentle shaking at room temperature prior to be used to determine serum levels of CRP and IL-6. EDTA tube (whole blood samples), was used fresh for DNA extraction by salting out method (Adinarayana & Kakara, 2013). DNA was frozen at –20°C for PCR.

Diagnosis of beta-thalassemia. Complete blood count (CBC) is critical for the diagnosis of thalassemias. The key components of the CBC include: Hb, red blood cell (RBC) number, mean corpuscular volume (MCV).

Cardiac evaluation was performed by clinical assessment, in addition to the results of hs-CRP and IL-6.

Method. Measurement of human hs-CRP in serum was performed using the nephelometric technique (Minineph TM, the Binding Site Ltd, PO Box 11712, Birmingham, B14 4ZB, U.K.) (Erlandsen & Randers, 2000). Human IL-6 was measured by AviBion Human ELISA kits (Orgenium Laboratories Business Unit, Vantaa, Finland) according to the manufacturer's recommendations (Cantisani *et al.,* 2012).

Genomic DNA was extracted from EDTA whole blood samples by using the salting out method. Detection of glutathione S-transferase M1 gene polymorphism was done by Polymerase Chain Reaction (PCR) technique using the following sequence specific primers: Forward: 5'-GAA CTC CCT GAA AAG CTA AAG C-3' and Reverse: 5'-GTT GGG CTC AAA TAT ACG GTG G-3'. The housekeeping gene Cd 57/58 was simultaneously detected using sequence specific primers as follows; Forward: 5'-ATG TGG AGA CAG AGA AGA CTC TTG GGT T-3' and Reverse: 5'-TCA TTC GTC TGT TTC CCA TTC TAA AC-3'. In total volume 25 µl including (taq, dntps, primer, Housekeeping gene, buffer, Q-solution and DNA sample), the reaction was incubated at 95°C for 5 min and subjected to 35 cycles of 95°C for 60 s, 60°C for 60 s and 72°C for 60 s, then a final 72°C-extension for 5 min. Next, PCR aliquots were electrophoresed on 2% agarose gels stained with ethidium bromide. The internal standard fragment of housekeeping gene was 430 bp in length, whereas the amplified gene products of GSTM1 were 215 bp (Silverman *et al.,* 2009).

Statistical methods. Data were expressed as median with range (min–max). Statistical significance of the difference was analyzed using Statistical Package for Social Science (SPSS) Version 15.0, SPSS Inc. (Chicago, IL, USA). The nonparametric Mann-Whitney test was used for comparison of medians. P values of < 0.05 were considered statistically significant. The correlation coefficient (r) which is a measure of the degree of closeness of the linear relationship between two variables (X and Y) was determined; r always lies between –0.01 and +0.01. Description of qualitative variables was in the form of numbers (no.) and percentage (%). Comparisons between quantitative variables were carried out after data were explored for normality using Kolmogorov-Smirnov test of normality. The results of the test indicated that the data were normally distributed; the student's t-test was then used for the comparisons of means between groups. Chi-Square test ($\chi 2$) was used to detect correlations between qualitative variables, *p*-value < 0.05 was considered to be statistically significant.

RESULTS

Table 1 showed statistically significant decrease in Hb, MCV and RBCs in β-thalassemic patients (median was 7.62 g/dL, 71.42 fl and 3.37×10^{12}/L) respectively

Table 2. hs-CRP and IL-6 compared in healthy subjects (group I) and β-thalassemic patients with cardiac complications (group III).

Parameters	Group I (n=20)		Group III (n=16)		*P*-value
	Median	Range	Median	Range	
hs-CRP (mg/L)	3.6	3.6-4.4	4.4	3.6-14.0	0.000**
IL-6 (pg/ml)	43.80	20-68	57.77	28-92	0.004**

*Statistically significant (*P* < 0.05). **High Statistical significance (*P* < 0.01)

Table 3. Comparison between GSTM1 null genotype (– ve gene) and GSTM1 non-null genotype (+ ve gene) in beta-thalassemic patients group II and healthy subjects group I.

PCR		Group II	Group I	Chi-Square	*P* value	Odds Ratio (95% Confidence Interval)
GSTM1 + ve gene	No	28	11	0.144	0.704	0.815 (0.283–2.347)
	%	52.8	57.9			
GSTM1 – ve gene	No	25	8			
	%	47.2	42.1			

Table 4. Comparison between GSTM1 null genotype (– ve gene) and GSTM1 non-null genotype (+ ve gene) in β-thalassemic patients with cardiac complications (group III) and healthy subjects (group I).

PCR		Group III	Group I	Chi-Square	*P* value	Odds Ratio (95% Confidence Interval)
GSTM1 + ve gene	No	7	11	0.322	0.497	0.566 (0.120–3.222)
	%	44	57.8			
GSTM1 – ve gene	No	9	8			
	%	56	42.2			

as compared to healthy subjects (median was 13.67 g/dL, 88.14 fl and 4.91×10^{12}/L). Moreover, reticulocytes showed statistically significant increase in β-thalassemic patients (median was 4.85%) respectively as compared to healthy subjects (median was 1.23%), whereas no significant difference in age and gender were recorded.

Table 2 showed that hs-CRP and IL-6 showed a significant increase in β-thalassemic patients with cardiac complications (group III) compared to healthy Subjects (group I) ($P > 0.05$).

The GSTM1 null polymorphisms were analyzed in parallel PCR reactions, using CD57/58 as a reference gene in each of the reactions. In the electrophoresis gel of the PCR amplification products; individuals homozygous for GSTM1 null polymorphism (GSTM1 null genotype), showed only one band visible at 430 bp corresponding to the PCR product of the reference gene, whereas the electrophoresis of the PCR products from patients with functional wild-type alleles of GSTM1 (GSTM1 non-null genotypes) presented two bands: 215 bp band of GSTM1 PCR product and 430 bp band of the reference gene (Fig. 1). From 76 subjects enrolled in the current study, only 72 samples were eligible for DNA extraction (whole blood samples were available), 53 β-thalassemic patients and 19 healthy subjects (control). There were twenty five β-thalassemic patients (47.2%) homozygous for the GSTM1 null allele (GSTM1 null genotype), whereas twenty eight β-thalassemic patients (52.8%) had the functional wild-type allele (GSTM1 non-null genotypes) in β-thalassemic patients. Nevertheless, there were eight healthy subjects (42.1%) homozygous for the GSTM1 null allele (GSTM1 null genotype), whereas eleven healthy subjects (57.9%) had functional wide-type allele (GSTM1 non-null genotypes).

Table 3 showed no association between null genotype of GSTM1 and β-thalassemia. The interaction between GSTM1 null in β-thalassemic patients and healthy subjects was statistically insignificant (*P*-value = 0.704 and OR = 0.815) (appeared in compared with subjects with the GSTM1 non-null genotype).

Table 4 showed no association between GSTM1 genotype and β-thalassemic patients with cardiac complications, where the interaction between GSTM1 null genotype (– ve gene) in β-thalassemic patients with cardiac complications (group III) and healthy subjects (group I) was insignificant (*P* value = 0.497 and OR = 0.566) compared to subjects with GSTM1 non-null genotype (+ ve gene).

DISCUSSION

Our results showed statistically a significant decrease in Hb, MCV and RBCs in β-thalassemic patients as compared to healthy subjects. Moreover, a statistically significant increase was found in reticulocytes between the two groups, whereas no significant difference was observed in age and gender.

All β-thalassemic patients' Hb concentrations were significantly decreased compared to healthy controls ($p < 0.05$). This is expected in thalassemic patients, where normal Hb synthesis is impaired (Abdalla *et al.,*

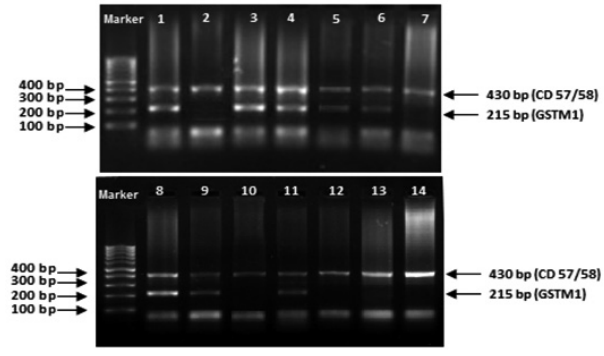

Figure 1. Electrophoretic separation of PCR product for GSTM1 exhibiting a 215bp fragment. Marker is 100 bp DNA ladder (Jena Bioscience Gmbh, Germany).
Lane 1: Positive control. Lane 2: Negative control. Lane 3, 4, 5, 6, 8, 9 & 11: Positive bands for GSTM1 (215bp), housekeeping gene (430 bp). Lane 7, 10, 12, 13 & 14: Negative bands for GSTM1 (215bp). (GSTM1 null genotype), positive for housekeeping gene (430 bp)

2011). Clarke and Higgins (2000) showed that primary blood indices including red blood cell count and hemoglobin were significantly decreased in thalassemia cases compared to the controls. It is accepted that the number of red blood cells is proportional to the degree of decrease in hemoglobin concentration (Clarke & Higgins, 2000). MCV and MCH values are invariably low in β-thalassemia when counted with well calibrated hematology analyzer (Yousafzai *et al.,* 2010). The percentage reticulocytes count of thalassemic patients was significantly higher than those of normal subjects (Lamchiagdhase *et al.,* 2000).

Our results showed that, hs-CRP and IL-6 were elevated in beta-thalassemic patients with cardiac complications compared to healthy subjects ($P > 0.05$).

Rajaram *et al.* (2011) found that, the most important finding of the study is the strong association between diastolic dysfunction (DD) and elevated hs-CRP levels, and LV hypertrophy. Moreover, Shi *et al.* (2010) suggested that assessment of hs-CRP level, may help to refine cardiovascular disease (CVD) risk.

Aggeli *et al.* (2005) indicated that, an increase in the circulating level of IL-6 among thalassemic patients as important component of the pro-inflammatory response. Nevertheless; Vopato *et al.* (2001) noted that systemic inflammation, as measured by IL-6, may be related to the clinical evolution of patients with CVD.

Regarding GSTM1, our study found twenty five β-thalassemic patients (47.2%) homozygous for the GSTM1 null allele (GSTM1 null genotype), whereas twenty eight β-thalassemic patients (52.8%) had functional wild-type allele (GSTM1 non-null genotypes) in β-thalassemic patients, out of 53 beta-thalassemic patients and 19 healthy subjects (controls). Nevertheless, there were eight healthy subjects (42.1%) homozygous for the GSTM1 null allele (GSTM1 null genotype), and eleven healthy subjects (57.9%) had functional wild-type allele (GSTM1 non-null genotypes) among 19 healthy subjects.

Our results showed no association established between GSTM1 null genotype frequency and β-thalassemia, where the interaction between GSTM1 null genotype in β-thalassemic patients and healthy subjects was statistically insignificant (P-value = 0.704 and OR = 0.815 with 95% confidence Interval: 0.283–2.347) compared to subjects with the GSTM1 non-null genotypes.

Our results agree with Sclafani *et al.* (2013) who found that, the frequency of the GSTM1-null genotype range from 23 to 62% in different population around the world (Sclafani *et al.,* 2013). However, Rabab *et al.* (2013) reported that GSTM1 null genotype was 44% in Egyptian population; on the other hand, GSTM1 deletion polymorphism for African-Americans was found to be 23–35% and for Chileans was 21% (Rabab *et al.,* 2013).

In our study, no association was established between GSTM1 null genotype frequency and β-thalassemic patients with cardiac complications, where the interaction between GSTM1 null genotype in β-thalassemic patients with cardiac complications and healthy subjects was (P-value = 0.497 and OR = 0.566 with 95% confidence Interval: 0.120–3.222) compared to subjects with the GSTM1 non-null genotypes.

The study results agree with Origa *et al.* (2008) who observed that, Sardinian healthy controls and thalassemia patients with expected cardiac iron overload did not show a statistically different GSTM1 null frequency (Origa *et al.,* 2008). Therefore, GST gene polymorphisms may not play an important role in the development of endocrine, liver, and cardiac dysfunction in patients with β-thalassemia major (Wu *et al.,* 2006). GSTM1 null genotype was less frequent in the acute myocardial infarction group than in controls (Wilson *et al.,* 2000). In contrast, Chakarov *et al.* (2014) have shown that, the frequency of GSTT1 null genotypes was significantly higher in β-thalassemic patients with myocardial siderosis than in controls (Chakarov *et al.,* 2014).

CONCLUSIONS

GSTM1 null genotype frequency has no role in β-thalassemia or cardiac complications appearance.

REFERENCE

Abdalla MY, Fawzi M, Al-Maloul SR, El-Banna N, Tayyem RF, Ahmad IM (2011) Increased oxidative stress and iron overload in Jordanian β-thalassemic children. *Hemoglobin* **35**: 67–79. doi: 10.3109/03630269.2010.544624.

Adinarayana KPS and Kakara RR (2013) Estimation of homozygote recessive and heterozygous CDK3 distribution in randomly selected cancer subjects. *J Evol Med Dental Sci* **45**: 8818–8822.

Aggeli C, Antoniades C, Cosma C, Chrysohoou C, Tousoulis D, Ladis V (2005) Endothelial dysfunction and inflammatory process in transfusiondependent patients with beta-thalassemia major. *Int J Cardiol* **105**: 80–84.

Cantisani M, Vitiello M, Falanga A, Finamore E, Galdiero M, Galdiero S (2012) Peptides complementary to the active loop of porin P2 from Haemophilus influenzae modulate its activity. *Int J Nanomedicine* **7**: 2361–2371. doi: 10.2147/IJN.S30467.

Chakarov I, Vlaykova T, Slavov E, Marinov R, Chakarova P (2014) Role of serum pro-hepcidin and GSTM1 and GSTT1 null polymorphisms for estimation of the risk of myocardial siderosis in children and "young adults" with β-thalassemia major. *Compe Clin Pathol* **23**: 725–733. doi: 10.1007/s00580-013-1677-9

Clarke G, Higgins T (2000) Laboratory Investigation of Hemoglobinopathies and Thalassemias: review and update. *Beckman Conference Clin Chem* **46**: 1284–1290.

Erlandsen EJ, Randers E (2000) Reference interval for serum C-reactive protein in healthy blood donors using the Dade Behringm N Latex CRP mono assay. *Scand J Clin Lab Invest* **60**: 37–43. doi: 10.1080/00365510050185029.

Galanello R, Origa R (2009) Management of thalassaemia. *Iron* **11**: 264–285. doi: 10.1186/1750-1172-5-11.

Hayes JD, Pulford DJ (1995) The glutathione S-Transferase supergene family: regulation of GST and the contribution of the lsoenzymes to cancer chemoprotection and drug resistance part II. *Crit Rev Biochem Mol* **30**: 521–600.

Jha R, Jha S (2014) Beta thalassemia-a review. *J Pathol Nepal* (JPN) **4**: 663–671. doi: http://dx.doi.org/10.3126/jpn.v4i8.11609.

Kremastinos DT, Farmakis D, Aessopos A, Hahalis G, Hamodraka E, Tsiapras D, Keren A (2010) β-Thalassemia cardiomyopathy history, present considerations, and future perspectives. *Circulation: Heart Failure* **3**: 451–458. doi: 10.1161/CIRCHEARTFAILURE.109.913863.

Lamchiagdhase P, Pattanapanyasat K, Muangsup W (2000) Reticulocyte counting in thalassemia using different automated technologies. *Laboratory Hematology* **6**: 73–78.

Matin S, Jahromi MG, Karemizadeh Z, Haghpanah S, De Sanctis V, Soliman A, Dehbozorgian J, Majd Z, Rezaei N, Karimi M (2015) The frequency of adrenal insufficiency in adolescents and young adults with thalassemia major *versus* thalassemia intermedia in Iran. *Mediterr J Hematol Infect Dis* **7**: e2015005. doi: 10.4084/MJHID.2015.005.

Origa R, Satta S, Matta G, Galanello R (2008) Glutathione S-transferase gene polymorphism and cardiac iron overload in thalassaemia major. *Brit J Haematol* **142**: 143–145. doi: 10.1111/j.1365-2141.2008.07175.x.

Rabab MA, Bothina MH (2013) GSTM1 and GSTT1 polymorphism in Egyptian sickle cell anemia patients. *Int J Hematol Oncol/UHOD: Uluslararasi Hematoloji Onkoloji Dergisi* **23**: 269–275.

Rajaram V, Evans AT, Caldito GC, Kelly RF, Fogelfeld L, Black HR, Doukky R (2011) High sensitivity C-reactive protein is associated with diastolic dysfunction in young African Americans without clinically evident cardiac disease. *The Open Cardiovasc Med J* **5:** 188–195. doi: 10.2174/1874192401105010188.

Sclafani S, Calvaruso G, Agrigento V, Maggio A, Nigro VL, D'Alcamo E (2013) Glutathione S transferase polymorphisms influence on iron overload in β-thalassemia patients. *Thalassemia Reports* **3**, e6: 20–22. doi: 10.1007/s10528-015-9687-8.

Shi B, Ni Z, Cai H, Zhang M, Mou S, Wang Q, Qian J (2010) High-sensitivity C-reactive protein: an independent risk factor for left ventricular hypertrophy in patients with lupus nephritis. *J Biomed Biotechnol* 1–5. doi: 10.1155/2010/373426.

Silverman RH, Klein EA, Weight CJ, Nguyen CT, Gupta JD (2009) Method for detection of xmrv. *U.S. Patent Application* **12/645**, 181: 1–15.

Vopato S, Guralnik JM, Ferruci L (2001) Cardiovascular disease, interleukin-6, and risk of mortality in older women. The Women's Health and Aging Study. *ACC Curr J Rev* **10**: 26–27. doi: 10.1161/01.CIR.103.7.947.

Wilson MH, Grant PJ, HARDIE LJ, Wild CP (2000) Glutathione S transferase M1 null genotype is associated with a decreased risk of myocardialinfarction. *The FASEB J* **14**: 791–796. PMID: 10744635.

Wu KH, Chang JG, Ho YJ, Wu SF, Peng CT (2006) Glutathione Stransferase M1 gene polymorphisms are associated with cardiac iron deposition in patients with beta thalassemia major. *Hemoglobin* **30**: 251–256. PMID: 16798650.

Yousafzai YM, Khan S, Raziq F (2010) Beta-thalassaemia trait: Haematological parameters. *J Ayub Med Coll Abbottabad* **22**: 84–86. PMID: 22455269.