Glutathione S transferase polymorphisms influence on iron overload in β-thalassemia patients

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Abstract

In patients with β-thalassemia iron overload that leads to damage to vital organs is observed. Glutathione S transferase (GST) enzymes have an antioxidant role in detoxification processes of toxic substances. This role is determined genetically. In this study, we correlated GSTT1 and GSTM1 genotypes with iron overload measured with direct and indirect non-invasive methods; in particular, we used serum ferritin and signal intensity of the magnetic resonance image (MRI) in 42 patients with β-thalassemia, which were regularly subjected to chelation and transfusion therapy. Multiplex polymerase chain reaction was used to determine the genotype. The loss of both alleles leads to a decreased value of liver and heart MRI-signal intensity with a consequent iron accumulation in these organs; the loss of only one allele doesn't lead to relevant overload. Serum ferritin doesn't appear to be correlated to iron overload instead.

Introduction

Regular blood transfusions and iron chelation therapy are standard treatments for thalassemia major and for some cases of thalassemia intermedia.

Patients with β-thalassemia major usually suffer from iron overload as a consequence of recurrent transfusions and ineffective erythropoiesis. Iron has a catalytic role to produce powerful reactive oxidant species (ROS) and free radicals, which lead to oxidative damage. Antioxidants play an essential role in protection of the cells from oxidative damage.[1]

They include several agents such as enzymes (glutathione peroxidase, superoxide dismutase, catalase), large molecules (ferritin, albumin), and small molecules (uric acid, glutathione, bilirubin, ascorbic acid, tocopherol, and vitamin E). Their defense mechanism in biological system involves chain breaking and preventive mechanisms.[2,3]

Same studies had shown that the erythrocytes of thalassemia patients are exposed to higher oxidative stress and a possible consequential accelerated apoptosis because of the high concentration of ROS.[4] ROS elimination involves glutathione transferase own, so these enzymes play an important physiological role.

Glutathione S transferase (GST) enzymes belong to a superfamily of multifactorial isoenzymes that in addiction of being well known detoxification agents are involved in excretion processes of toxic molecules as well.

Evidence suggests that GST expression level is a crucial factor in determining cells sensitivity to a broad spectrum of toxic chemicals.[5]

This antioxidant role comes from the fact that these isozymes catalyze conjugation reaction of glutathione with electrophiles exogenous (carcinogens, drugs) and endogenous substrates (various catabolism products) contributing to disposal of many xenobiotics.[6]

Genetically determined variations cause changes in activity level and/or expression of some GST and may cause decreased defense capacity against oxidative stress.

GST genes in fact are up regulated in response to oxidative stress.[7]

These enzymes are encoded by 16 polymorphic genes divided into 5 classes: α (*GSTA*), π (*GSTP*), μ (*GSTM*), θ (*GSTT*), ζ (*GSTZ*).[8] In particular, the genetic human loci GSTM1 and GSTT1 are highly polymorphic and the deletion of both (genotype null) abolishes enzyme activity by increasing susceptibility to oxidative stress. It has been observed that a member of this family (GSTM1) plays an important role in detoxification processes in cancer.

Homogenous deletion of GSTM1 results in the lack of GSTM1 enzyme activity and it is associated with tumors of the lung, bladder, prostate and other districts.[9] In this study we have correlated the GSTT1 and GSTM1 genotypes with iron overload measured as serum ferritin and as signal intensity of the magnetic resonance imaging (MRI) in patients with beta thalassemia regularly subjected to chelation therapy. All patients were chelated from the beginning of transfusion therapy, so we supposed that differences in response to oral chelation may be caused by the presence of these genetic polymorphisms.

Materials and Methods

Patients

We analyzed *GSTM1* and *GSTT1* genes polymorphisms in 42 randomly selected patients referred to our center, 35 with thalassemia major, 7 thalassemia intermedia. We also analyzed 62 healthy subjects (64% female, mean age 38) randomly selected from the general population as control. Clinical, laboratory and instrumental data are shown in Table 1. All patients were regularly transfused and underwent to chelation therapy.

GSTT1 GSTM1 genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells with phenol-chloroform method. Genotyping GSTM1 and GSTT1 were performed with a multiplex polymerase chain reaction (PCR). Primers used are the following: GSTM1 forward primer: 5' AGG AAC TCC CTG AAA AGC TA AA; GSTM1 reverse primer: 5' TGG GCT CAA ATA TAC GGT GGA G; GSTT1 forward primer: 5' CTT ACT GGT CCT-CAC ATC TCC TT; GSTT1 reverse primer: 5' AGC TCA CCG GAT CAT GGC CA. In multiplex PCR was added to an internal control of amplification represented by CYP 1A using following primers: CYP 1A 1.1: 5 'GAA CTG CCA CTT CAG CTG TCT; CYP 1A 1.2: 5 'CAG CTG CAT TTG GAA GTG CTC. PCR mix (50 μL) contained 200 ng of genomic DNA, 2.5 μL of 10 pmol primers, 5 μL buffer 10x, 1.5 μL of $MgCl_2$ 50 mmol, 2 μL

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Key words: glutathione S transferases, β-thalassemia, iron overload.

Funding: this research was supported by Sicilian Health Regional Project T18/6.

Acknowledgements: we thank *Piera Cutino* Association and Foundation Research for its continuous support.

Contributions: ED'A, study planning and supervision, manuscript revising; SS, laboratory work, manuscript writing; VA, laboratory work; GC, patients recruitment, clinical data collection, manuscript writing; VLN, statistical analysis; AM, scientific supervision, research implementation.

Conflict of interests: the authors declare no conflict interests.

Received for publication: 29 April 2013.
Revision received: Not requested.
Accepted for publication: 2 September 2013.

Thalassemia Reports 2013; 3:e6
doi:10.4081/thal.2013.e6

of 4 mmol dNTP and 2.5 U of enzyme Taq Polimaresi (Invitrogen Corp., Carlsbad, CA, USA). Thermal amplification protocol included: 94° for 10' (1 cycle); 94° for 1', 58° for 1', 72° for 5' (35 cycles), and a final cycle at 72° for 10'. PCR-products of co-amplification of GSTM1 (215 bp), GSTT1 (480 bp) and amplification control CYP A1 (312 bp) were analyzed on an ethidium bromide-stained 3% agarose gel and displayed to the ultraviolet lamp (Figure 1).

Iron overload evaluation: methods

A retrospective analysis based on history, laboratory data and imaging studies was carried out to assess the extent of iron overload. In particular we collected mean serum ferritin and signal intensity of the cardiac and hepatic MRI. MRI T2* is a parameter simple, fast and robust to measure iron accumulation; it is the time needed for the organ to lose approximately two-thirds of its signal and is measured in milliseconds (ms). T2* is shortened with increasing iron concentration.[10] A shortening of myocardial T2* to <20 ms (implying increased myocardial iron above normal) is associated with an increased likelihood of decreased left ventricular ejection fraction (LVEF), whereas patients with T2* values >20 ms have a very low likelihood of decreased LVEF.[11,12] Hepatic MRI T2* provides a semi-quantitative method for non-invasive estimation of parenchymal iron levels, depending on the intensity of the T2* signal is classified as mild, moderate and severe. Serum ferritin measurement may be the only available method of assessing iron burden in developing countries. It is useful for close and frequent patient monitoring to indicate changes in iron burden. More accurate measurements of iron stores are performed at less frequent intervals. Although serum ferritin has been used to establish when to start chelation therapy, it is now known to be an inaccurate indicator of cardiac iron or of total body iron burden. Serum ferritin also fluctuates in response to inflammation, abnormal liver function, and ascorbate deficiency.[13]

Statistical analysis

It was calculated genotypes frequency. It was considered serum ferritin value and its frequency distribution considering three categories (<500, between 500 and 1000, and <1000). Data about ferritin, heart and liver MRI are reported as mean±standard deviation. It was observed bi-variate distribution of variables heart MRI-genotype, liver MRI-genotype and genotype-ferritin value was calculated correlation index.

Results

Garte *et al.*[14] made a study on Caucasian population and found that GSTM1 null frequency is 53%. This frequency is similar to that found in our healthy controls (54%) but in patients group we found a prevalence of 43%. Prevalence of genotype GSTM1+/GSTT1+ is 35% and 33.3% in patients and controls respectively, prevalence of genotype GSTM1+/GSTT1- is 16.2% and 3.3% in patients and controls respectively, finally prevalence of genotype GSTM-/GSTT- (double null) genotype is 5.2% in patients and 10% in controls. Table 2 summarizes data concerning patients' number with various genotypes and it highlights the average ferritin values and magnetic resonance.

Table 1. Clinical, laboratory and instrumental data of the patients studied.

Parameter	Value
No patients	42
Females (%)	69
Mean age (years)	32
Mean ferritin (ng/mL)	1549
Blood transfused annually (mL)	172.43
Treatment	33.3% DFP; 45.2% DFX; 14.2% DFP+DFO; 7% DFP-DFX
MRI T2* heart (mean)	33.27 ms
MRI T2* hepatic (mean)	4.2 ms

DPF, dereriprone; DFX, deferoxamine; DFO, deferasirox; MRI, magnetic resonance imaging.

Table 2. Comparison of magnetic resonance data collection and ferritin values with different genotypes.

	GSTM1 Null	GSTM1+/GSTT1+	GSTT1 Null	GSTM1-/GSTT1-
Patients	16	13	6	2
Serum ferritin (ng/mL)	1770.4±1237.74	1826.5±916.66	970.6±665.9	1590.5±936.9
SIR of cardiac MRI (ms)	36.9±9.8	35.07±9.05	30±12.96	24.5±9.19
SIR of hepatic MRI (ms)	4.2±3.03	5.33±5	4.33±2.4	2.25±0.07

GST, glutathione S trasferase; SI, signal intensity; MRI, magnetic resonance imaging.

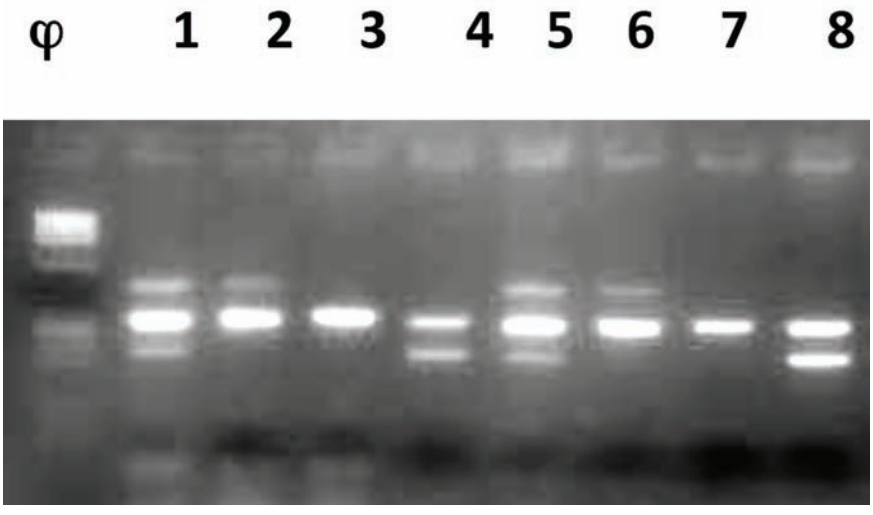

Figure 1. Polymerase chain reaction products of co-amplyfication of glutathione S transferase (GST)M1 (215 bp), GSTT1 (480 bp) and amplification control CYP A1 (312 bp) tested on 3% agarose gel. Lane 1 and 5 genotype GSTM1+/GSTT1+. Lane 2 and 6 GSTM1-/GSTT1+ (GSTM1 Null). Lane 3 and 7 GSTM1-/GSTT1-. Lane 4 and 8 genotype GSTM1+/GSTT1- (GSTT1 Null).

Conclusions

Glutathione-S-transferase enzymes work as antioxidants and their activity is determined genetically. The frequency of the GSTM1-null genotype range from 23 to 62% in different population around the world. The GSTT1-null genotype was found in 15-30% Caucasian and more than 50% of Chinese people.[10] Iron overload due to frequent blood transfusion in patients with beta-thalassemia is the major causes of long term complications, including cardiac dysfunction, liver impairment and endocrine dysfunction. Iron overload generates oxygen-free radicals and peroxidative tissue injury. Therefore, levels of antioxidant can be used to determine the susceptibility of individual patients to such complications. Wu *et al.* (2006)[15] analyzed the polymorphisms of two endogenous antioxidant enzymes, glutathione S-transferase M1 (GSTM1) and glutathione S-transferase T1 (GSTT1). They found that the GSTM1 null (delected) genotype was associated with a decreased signal intensity ratio on MRI, an index of increased myocardial iron, suggesting that genetic variations of GSTM1 enzyme are associated with cardiac iron deposition. The aim of Wu's study was to evaluate whether the GSTM1 null genotype is a predisposing factor for myocardial and iron overload in beta thalassemia patients with low body iron, as assessed by lifelong serum ferritin levels. We correlated GSTT1 and GSTM1 genotype with iron overload measured as serum ferritin and as signal intensity of the MRI in patients with β thalassemia regularly subjected to transfusion and chelation therapy. We analyzed genotype for GSTM1 and GSTT1 in 42 randomly selected patients referred to our center, 35 had thalassemia major, 7 thalassemia intermedia. We also analyzed 62 normal subjects (62% female, 41 mean age years) randomly selected from the general population as control. The fact that even in adequately chelated patients from the beginning of transfusion therapy is found an accumulation of hepatic iron, suggests that genotype null o loss of one allele is a predisposing accumulation factor. Genotypes GSTM+/GSTT+ rarely show accumulation. In 92% of case, patients GSTM+/GSTT+ show normal levels of cardiac iron. Single deletion of only one gene *GSTT1* or *GSTM1* instead has no significant effect on cardiac overload. About cardiac iron accumulation in genotype GSTT null, we found: 16% light, 66% normal 16% severe; in patients with GSTM null: normal 87% light 6% e border line 6%; in patients with genotype double null 50% normal and 50% severe. The same can be established by observing liver MRI data. Serum ferritin value is not correlated with glutathione transferase gene deletion instead, and it cannot predict cardiac iron loading;[16,17] it was increased in 70% of patients. It did not correlate with the signal intensity of the liver and heart MRI (correlation index -0.046 and -0.22 respectively). We found that mean ferritin value in patients with GSTT1- genotype is on average lowest, but it is probably due to the fact that our statistical sample is not large enough. In conclusion in this study levels of serum ferritin did not differ significantly between GSTM1 or GSTT1 or both null patients, in fact we considered this parameter not to be predictive. In two cases with genotype GSTM1-/GSTT1- a decrease in heart and liver MRI value is observed, which is associated with cardiac and hepatic iron accumulation. Instead, GSTM1 null and GSTT1 null genotypes only show a slight heart MRI value decrease (value is, however, in the range of normality) and on average a more substantial decrease in liver MRI value (on average 4.2-4.3) that was associated with a slight accumulation of iron within this organ. Thus, the absence of one of these alleles did not significantly influenced the cardiac iron load, but rather the hepatic one, although not so heavily. Instead the loss of both alleles seems to strongly lead to accumulation on both heart and liver. This suggests that these enzymes have an important role, especially on the hepatic iron deposition. Indeed almost surely these enzymes are not solely responsible for iron-related complications development. A large prospective study of antioxidant polymorphism is needed to determine the role they play in the development of iron-related complications in patients with -thalassemia transfusion dependent.

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