ABSTRACT

BACKGROUND
Thalassemia, a monogenic genetic disease of red blood cells, is spread widely throughout the world. Glutathione S transferase (GST) enzymes have an antioxidant role in detoxification processes of toxic substances. This study aimed to determine the role of the genetic modifier genes GSTT1 and GSTM1, and the catalase (CAT) gene in clinical degrees of hemoglobin (Hb)E/β thalassemia.

METHODS
Sixty HbE/β Thalassemia patients were examined to determine their clinical pictures. Clinical score was based on age when thalassemia symptoms appeared, time of diagnosis, time of first blood transfusion, pre-transfusion hemoglobin concentration, frequency of transfusions, and enlargement of spleen. Ferritin concentration was also obtained from medical records. Gene polymorphisms of GSTT1, GSTM1, and CAT were measured using PCR and PCR-RFLP methods. Clinical scores were categorized into mild (0-3.5), moderate (4-7), and severe (7.5-10) degrees, while ferritin level was expressed in mg/dL. One way Anova was used to analyze the data.

RESULTS
The clinical appearance showed that severe, moderate, and mild degrees accounted for 42%, 45%, and 13%, respectively. The majority had a high ferritin level of more than 5000 mg/dL (67%). GSTT1 null, GSTM1 null, and CAT minor allele genotypes were 21.7%, 33.3%, and 12.1%, respectively. GSTT1, GSTM1, and CAT genotypes had no impact on the severity of thalassemia patients (p=0.091, p=0.082, and p=0.141, respectively).

CONCLUSION
GSTT1, GSTM1, CAT gene polymorphisms tend to be a minor aspect of severity of clinical outcome for HbE/β thalassemia patients and should be not considered a routine laboratory check.

Keywords: GSTT1, GSTM1, CAT, HbE/β thalassemia, clinical score
INTRODUCTION

Clinical pictures of hemoglobin E (HbE)/β thalassemia have been reported widely, ranging from the type with a healthy appearance needing no transfusion to the regular transfusion-dependent type. The phenotype modification of HbE/β thalassemia has been described extensively concerning the types of beta gene mutations, and the presence of the secondary modifiers XMN1, BCL11A, and HBS1L-Myb. The other findings reported that genes in the redox system are thought to play a role in the clinical conditions of thalassemia patients. It relates to the system that regulates the oxidant-antioxidant reaction especially against free radicals from routine transfusions and lack of iron chelation. Glutathione-S-transferase mu (GSTM1) and glutathione-S-transferase theta (GSTT1), the genes responsible for the production of glutathione S-transferase, affect the degree of cardiac siderosis as one of the factors related to the death of thalassemia patients.

On the other hand, GSTT1 and GSTM1 also have an effect on iron overload in thalassemia patients. The “null” genotype of the GST genes results in increased susceptibility to oxidative reactions, with the increased risk of tumors of the prostate and cardiac disorders. Likewise, with catalase (CAT) genes, the polymorphism of exon nine at the rs769217 locus was allegedly related to low catalase activity resulting in reducing the effect of the catalase enzyme. Research on GSTM and GSTT1 against many antioxidant factors has been widely reviewed in various articles. In general, the data shows that GSTM and GSTT1 affect the molecular level. However, there is less knowledge in the literature on the internet especially on the clinical aspects of thalassemia. The present study was intended to add data about the effect of GSTM, GSTT1 and CAT gene polymorphisms in clinical issues, especially HbE/thalassemia.

METHODS

Research design

The study was of cross-sectional design and used secondary clinical data from a previous study that were retrieved through the medical records of Banyumas Hospital. Genotyping was carried out in the Research Laboratory of the Faculty of Medicine, Universitas Jenderal Soedirman, in the 2017-2018 period.

Research subjects

Using the formula of Slovin for a total population of 350, 60 patients aged 10-12 years were recruited to participate in this study. The subjects who came from the three categories of mild, moderate, and severe types, underwent genotyping for the genes of interest. HbE/β thalassemia status was acquired from the previous genotyping study.

Genotyping

Genotyping for each gene was carried out according to the following description: GSTT1 and GSTM1 were obtained by the polymerase chain reaction (PCR) technique, whereas CAT was determined using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Table 1 contains the details of primers and enzymes used in this study.

The PCR cocktails containing 50 ng template DNA, PCR mix Kit (Invitrogen Corp., Carlsbad, CA, USA), H_2O, and primers were subjected to a cycle at 94°C for 10 minutes, then to 30 cycles each at 9°C for 55 seconds, 69.5°C for 55 seconds, and 72°C for 90 seconds. Ten IU of restriction enzyme in RFLP application was incubated overnight at 37°C with 15 μl of PCR product. We then used gel documentation procedure for band visualization.

Clinical score

The Mahidol score was used to determine the clinical appearances of the HbE/β thalassemia patients as described elsewhere. This score included the age at which thalassemia symptoms
The gel documentation system showed PCR and RFLP visualization. Bands were presenting the genotyping of GSTT, GSTM1, and CAT genes on the agarose gel labeled with ethidium bromide (Figure 1).

The genotype distribution of the GSTT, GSTM1, and CAT gene polymorphisms and the correlation picture for clinical scores are depicted in Tables 3 and 4. The normal GSTM1 and the GSTM1 null alleles both had a frequency of 33.3% (20/60), which was higher than that of GSTT1 null((21.7%)(13/60)), while in the CAT gene, the T/T minor genotype accounted for 12.1%.

**DISCUSSION**

HbE/β-Thalassemia is a variant of thalassemia which has a phenotype spread from mild to severe. Mild conditions, in general, are manifestations of the mild combination of HbE and Beta thalassemia genotypes. Hemoglobin E itself in the heterozygous and homozygous states gives rise to mild clinical conditions, and generally without the need for transfusion. However, some patients may require transfusion since HbE mutations are a kind of cryptic mutations.

Clinical symptoms in HbE/β-Thalassemia patients appear in extensive variations, stretching in the age range 1-15 years. Similar to the data in the present study, generally, transfusion in HbE/β-Thalassemia patients follows the type of mutant in β-Thalassemia. The more severe the mutant type, the more frequent transfusions must be carried out, varying from 2 weeks to sporadic in months. The present study obtained the same data.

**Statistical analysis**

One way Anova was used to determine the differences in mean clinical score for each genotype. A p<0.05 was considered to indicate statistical significance.

**Ethical clearance**

The Medical Ethics Committee of Universitas Jenderal Soedirman approved the ethics of the study protocol for the research, under registry number 1328/KEPK/III/2018.

**RESULTS**

The majority of the severe patients were diagnosed with anemia at the age of 1-5 years. In the less severe types, usually the symptoms appeared at an older age. Because of the limitations of the previously documented data, we obtained data for pre-transfusion hemoglobin for three months, where the concentration was between 7 to 9 g/dL. Some patients had been splenectomized, but most of the spleens were only mildly enlarged. Ferritin level was also higher in the severe type of patients (Table 2).

The gel documentation system showed PCR and RFLP visualization. Bands were presenting the genotyping of GSTT, GSTM1, and CAT genes on the agarose gel labeled with ethidium bromide (Figure 1).

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**DISCUSSION**

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### Table 1. Primers and enzymes used for genotyping the genes

| Gene   | Primers                             | Enzymes (RFLP) | PCR Product | interpretation |
|--------|-------------------------------------|----------------|-------------|----------------|
| GSTM1  | 5' AGGAACTCCTGAAAAGCTAAA 3' 5'TGGGCTCAAATATACGGTGAG 3' | -              | 215 bp      | + = presence   |
| GSTT1  | 5' CCTACTGTCCTCCTACATCTCTTT 3' 5'AGC TCA CCGGAT CATGGC 3' | -              | 480 bp      | - = null       |
| CAT    | 5' GCCGCTTCTTTTGCTATCCCT-3' 5'TCCCGCCCATCTGCTCCAC-3' | BstXI          | 202 bp      | C = remaining 202 bp |
| CYP*   | 5' GAACTGCCACCTACGCTTGCTC-3' 5'TCACCCGATCATGGCCAGCA-3' | -              | 312 bp      | T = digested 108, 94 bp |

*CYP gene was used as a control of multiplex PCR*
as the previous study. There are patients with moderate and severe symptoms with initial transfusions occurring at the age of 10 years, which may possibly be due to delayed diagnosis or increased blood needs such as during menstruation in female patients.\(^{(10)}\) The iron levels represented by ferritin also showed significant differences between groups of patients. The number of transfusions in each group correlated with iron levels stored in the body. In general, there were clear differences in the time of clinical appearance between groups (Table 2).

Genetic modification is one of the essential topics to be studied considering the diversity of clinical symptoms that appear in patients. The primary modifier is a mutation in the beta genes, whereas the subsequent modification is the involvement of hemoglobin F coding genes and co-inheritance with alpha genes mutations.\(^{(11)}\) Repeated transfusion has been known as the cause of redox reaction disturbance due to iron overload. GSTM1 and GSTT1 genes are among the possible factors that play a role in the clinical modification process of thalassemia patients.

This study revealed that the genotype of GSTM1 null had the highest percentage of patients with thalassemia major, as high as 33.3%, while the frequency of GSTT1 null was 21.7% (Table 4). It was in an equilibrium state according to previous data on the SNP database. According to the literature review, the GSTM1 null frequency reaches a range of 23-62% in different

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**Table 2. Clinical appearance of HbE/β thalassemia**

| Clinical parameters         | Mild (n=20) | Moderate (n=20) | Severe (n=20) | p value |
|-----------------------------|------------|----------------|--------------|---------|
| First diagnosis (age in years) | 15.50±3.25 | 5.25±4.75     | 1.50±1.50    | 0.032   |
| First transfusion (age in years) | 17.25±2.05 | 6.50±2.05    | 1.25±1.50    | 0.026   |
| Pretransfusion Hb (g/dL)    | 9.15±0.25  | 8±1.45        | 7±1.75       | 0.052   |
| Splenomegaly (cm)           | No         | 4.68±3.25     | 10.75±4.25   | 0.001   |
| Ferritin (mg/dL)            | 358.75±153.50 | 5752.00±134.75 | 9370.75±1250.50 | 0.024   |
| Clinical score              | 3.05±0.25  | 7.25±1.65     | 8.75±1.00    | 0.042   |

*CYP gene was used as a control of multiplex PCR

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**Figure 1. Genotyping of GSTM1, GSTT1, and CAT genes**

Lane 2 is GSTT1 and GSTM1 positive (480 bp and 215 bp). Lane 3 and Lane 4 are GSTT1+/GSTM- and GSTT-/GSTM1+ respectively. Lanes 5 and 7 are the CC genotype in the CAT gene (uncut 202 bp), whereas lane 6 depicts the CT heterozygote (202, 108, 94 bp). The TT alleles of the CAT gene are depicted in lane 8 (108, 94 bp), while the 312 bp lane is a control for the PCR process.
populations worldwide. Meanwhile, GSTT1 null has been found to be 15-30% in Caucasians and more than 50% in Mongoloid races.\(^{12}\) The present study also showed that GSTT1 and GSTM1 alleles did not determine the clinical severity of the groups. Each group has this type of allele gene polymorphism. This demographic distribution, combined with the previous spreading data illustrate that GSTM1 and GSTT1 alleles are types of genes that have uniform distribution among world populations so that the polymorphisms of these genes become random and cannot be a reference for the determination of certain ethnic groups.

Our study indicated that there was no significant relationship between GSTT1 and GSTM1 gene polymorphisms against the clinical degree of HbE/Thalassemia β patients in Indonesia (Table 3). The data has used both comprehensive outcome data, namely Mahidol score and using ferritin levels only. Ferritin is used as a protein marker of iron in thalassemia patients who undergo regular transfusions. The ferritin level increases with the number of transfusions and lack of compliance with iron chelation medications.\(^{13}\) Ferritin in the GSTT1 and GSTM1 null groups showed higher mean levels than the normal group, but this difference was not significant. Likewise in the CAT gene, groups containing allele mutants also showed higher values than those in the wild-type group, but did not show data significance (Table 4).

| Genotype       | Frequency | Mild (n=20) | Moderate (n=20) | Severe (n=20) | p value | Disease severity |
|----------------|-----------|-------------|-----------------|--------------|---------|-----------------|
| GSTT1          |           |             |                 |              |         |                 |
| Null           | 4         | 4           | 5               |              | 0.906   |                 |
| Non-null       | 16        | 16          | 15              |              |         |                 |
| GSTM1          |           |             |                 |              |         |                 |
| Null           | 7         | 8           | 5               |              | 0.591   |                 |
| Non-null       | 13        | 12          | 15              |              |         |                 |
| GSTT1+GSTM1    |           |             |                 |              |         |                 |
| Null           | 2         | 2           | 3               |              | 0.850   |                 |
| Non-null       | 18        | 18          | 17              |              |         |                 |
| CAT genes      |           |             |                 |              |         |                 |
| C/C            | 13        | 15          | 13              |              |         |                 |
| C/T            | 4         | 3           | 5               |              | 0.912   |                 |
| T/T            | 3         | 2           | 2               |              |         |                 |

Table 3. Frequency distribution of GST gene polymorphisms among HbE/β thalassemia patients

| Genotype          | Frequency | Mild (n=20) | Moderate (n=20) | Severe (n=20) | p value | Clinical Score | Ferritin level (mg/dL) | p value |
|-------------------|-----------|-------------|-----------------|--------------|---------|----------------|------------------------|---------|
| **GSTT and GSTM** |           |             |                 |              |         |                |                        |         |
| genes             |           |             |                 |              |         |                |                        |         |
| Normal            | 7         | 6           | 7               | 20 (33.3)    | 6.27±1.19| 4726.42±71.67 | 0.08                   |         |
| GSTT1 null and    | 2         | 2           | 3               | 7 (11.7)     | 5.14±1.31| 5954.57±54.46 | 0.08                   |         |
| GSTM1 null        | 7         | 8           | 5               | 20 (33.3)    | 6.15±0.74| 5723.50±140.72| 0.08                   |         |
| GSTT1 null        | 4         | 4           | 5               | 13 (21.7)    | 5.8±1.52 | 5224.85±98.24 | 0.08                   |         |
| **CAT gene**      |           |             |                 |              |         |                |                        |         |
| C/C               | 13        | 15          | 13              | 41 (70.7)    | 5.4±1.40 | 5473±102.92   | 0.08                   |         |
| C/T               | 4         | 3           | 5               | 12 (17.21)   | 5.9±0.6  | 5002±48.38    | 0.10                   |         |
| T/T               | 3         | 2           | 2               | 7 (12.1)     | 8.5±1.15 | 5974±218.46   | 0.10                   |         |

Table 4. Genotype frequencies of GSTT, GSTM1, and CAT genes and the genotype-phenotype relationship with severity of disease and the ferritin level
data were reported previously by Sharma and colleagues who concluded that GSTT1 and GSTM1 genotype deletions worsened the clinical outcome in major thalassemia patients characterized by elevated serum iron and serum ferritin levels compared with major thalassemia patients with normal GST genotypes.\textsuperscript{14} For major thalassemia, these data did not explain in detail the type of mutation involved. Differences in primary mutants may cause different results considering that there are more than 300 variations recorded in the human beta gene variation database.\textsuperscript{13} This fact may explain why there are differences between studies.

Observation of ferritin levels in these patients showed no different levels in various types of genotypes. This indicates that ferritin iron levels are not affected by GSTT1 and GSTM1 alleles, although on average the minor allele group shows an increase over other groups. The Sharma\textsuperscript{14} study was based on iron parameters only whereas the present study used the general clinical outcome and also the serum ferritin level. In some hematological diseases, these genes may constitute independent risk factors, such as in acute myeloid leukemia (AML), with no impact on prognosis.\textsuperscript{16} The GSTT and GSTM1 null also have an impact on the pro-hepcidin level on iron overload and organ dysfunction. However, these mutants were not as good a biomarker as ferritin for the iron stores. Another report in sickle cell patients was also following this issue, especially in iron overload status.\textsuperscript{17,18} Glutathione S transferase expression levels are an essential factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals, but it does not appear to be a vital element in influencing the clinical state of thalassemia patients.\textsuperscript{19} However, comparison of GSTM1 and GSTT1 in healthy and affected individuals shows that deletion in both GST genes influences GST enzyme activity which was demonstrated by low GST levels in the group of individuals with double deletions in both genes. The results is a deficiency of the enzyme in favor of free radical reduction processes due to excessive iron deposits.\textsuperscript{3,20}

Studies of polymorphisms in exon nine at the locus rs769217 CAT genes in thalassemia are limited. However, in some other chronic diseases such as diabetes mellitus, it is reported that the minor alleles CC and C have decreased activity of catalase and hemoglobin A1c and allow the risk of complications to increase.\textsuperscript{21} In other chronic diseases such as chronic hepatitis and hepatocarcinoma, the polymorphism of the CAT gene was also reported to influence disease severity.\textsuperscript{22} We found a report in the PubMed database that was allegedly associated with low catalase activity and reduced catalase protection against free radicals in the body.\textsuperscript{5,23} However, the data of the present study did not show a significant association between the CAT gene and the clinical condition of the HbE/thalassemia $\beta$ patients. In the case of minor thalassemia, there is also a change in oxidative reactions, which are thought to be associated with hypochromic microcytic anemia, similar to iron deficiency anemia. However, other previous findings of catalase activity in hypochromic microcytic anemia reported conflicting results.\textsuperscript{24,25}

Many factors influence the resulting non-significant relationship in the present study. The type of mutation in the globin gene may play a role in the clinical state of major thalassemia patients. Additionally, the presence of XmnI+ site polymorphisms in the $\beta$ globin gene cluster, and the secondary modifier BCL11A and HBS1L-MYB in patients with thalassemia major has been reported to be advantageous since it may reduce clinical manifestations although belonging to thalassemia major.\textsuperscript{1,26}

This study used HbE/\(\beta\)-Thalassemia patients with a long transfusion history. Routine transfusion bias can obscure the patient’s clinical history. It is necessary to use patients who are in an early stage of diagnosis and follow the next clinical developments to ensure a causative relationship. The study showed that the GSTT1, GSTM1, and CAT genes were not important modifiers of HbE/Thalassemia, so that they cannot be a routine checking procedure. This study needs further exploration to find modifiers.
related to clinical variation, other than primary mutations of beta genes.

CONCLUSION

Polymorphisms of GSTT1, GSTM1, COLIA1, and CAT genes did not modify outcome pictures of HbE/Thalassemia β.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

CONTRIBUTORS

LR contributed to the research design, conceptual framework, laboratory experiment, data analysis, and manuscript development. YRW and GS contributed to laboratory experiment and data analysis. QS and ATH contributed to the research design, data analysis, and manuscript development. All authors have read and approved the final manuscript.

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