

# Genetic heterogeneity of thalassemia major patients in Rembang Regency, Central Java, Indonesia



<sup>1</sup>Department of Clinical Pathology,  
Faculty of Medicine, Universitas  
Diponegoro, Semarang, Indonesia;  
<sup>2</sup>Eijkman Research Center for Molecular  
Biology, National Research and  
Innovation Agency, Bogor, Indonesia;

<sup>3</sup>Department of Clinical Pathology,  
School of Medicine and Health Sciences,  
Universitas Katolik Indonesia Atma Jaya,  
Jakarta, Indonesia;

<sup>4</sup>Department of Child Health, Faculty  
of Medicine, Universitas Diponegoro,  
Semarang, Indonesia;

<sup>5</sup>dr. R Soetrasno Regional Public Hospital,  
Rembang, Indonesia;

<sup>6</sup>Department of Clinical Pathology  
and Laboratory Medicine, Faculty of  
Medicine, Public Health and Nursing,  
Universitas Gadjah Mada, Yogyakarta,  
Indonesia;

\*Corresponding author:  
Nyoman Suci Widyastiti;  
Department of Clinical Pathology, Faculty  
of Medicine, Universitas Diponegoro,  
Semarang, Indonesia;  
nyoman.suci@fk.undip.ac.id

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Nyoman Suci Widyastiti<sup>1\*</sup>, Ita Margaretha Nainggolan<sup>2,3</sup>, Edward Kurnia Setiawan Limijadi<sup>1</sup>,  
Meita Hendrianingtyas<sup>1</sup>, Dwi Retnoningrum<sup>1</sup>, Ariosta<sup>1</sup>, Yetty Movieta Nancy<sup>4</sup>,  
Mayasari Dewi<sup>5</sup>, Sutamti<sup>5</sup>, Tri Ratnaningsih<sup>6</sup>, Usi Sukorini<sup>6</sup>

## ABSTRACT

**Background:** Thalassemia and hemoglobinopathies are caused by mutations in the globin genes that reduce haemoglobin synthesis. In many Asian countries, including Indonesia, thalassemia is a common inherited disorder. Thalassemia has specific characteristics due to the wide variability of the mutations from various populations. Therefore, screening protocol should be designed on specific populations' regional gene mutation patterns. This study aims to investigate the mutational diversity of the globin genes in thalassemia major patients in Rembang Regency, Central Java, Indonesia.

**Methods:** Genetic mutations were carried out in all major thalassemia patients who were recorded and underwent routine blood transfusions at the Rembang District Hospital from 2018 – 2020. Mutation identification was carried out using a PCR-RFLP (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism) based method, the ACRS (Amplified Created Restriction Site) method, and genome sequencing.

**Results:** The most common genotypes are double heterozygous HbE and Cd 35 mutation (-C) (42.87%), HbE and IVS1-nt.5 mutations (G> C) (28.57%), HbE and IVS1- nt.2 mutations (T> C), HbE and codon 8/9 (insertion + G), codon 8/9 (insertion + G) and Hb Malay, as well as Hb Adana and Hb Constant Spring (1.74%).

**Conclusion:** The  $\beta$ -thalassemia and  $\alpha$ -thalassemia mutations most commonly found in Rembang Regency are Cd 35 (-C) and non-deletional mutations.

**Keywords:** Thalassemia, Mutation,  $\alpha$  and  $\beta$  Globin Genes.

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## INTRODUCTION

Thalassemia is an autosomal recessive genetic disorder characterized by reduced or absent globin gene expression and the synthesis of alpha ( $\alpha$ ) or beta ( $\beta$ ) chains from hemoglobin (Hb), abnormal maturation, and shortened lifespan of red blood cells. A carrier is asymptomatic or associated with mild anemia. However, patients with severe diseases require lifelong blood transfusions to survive.<sup>1</sup> The term “thalassemia” comes from the Ancient Greek “*thalassa*” (sea) and “*haima*” (blood) since the disorder was first discovered in the area around the Mediterranean Sea.<sup>2</sup>  $\alpha$  and  $\beta$  thalassemia, as well as hemoglobinopathies, are common in Southeast Asia. The clinical phenotype ranges from an asymptomatic carrier state to thalassemia major, a severe transfusion-dependent condition,

or Intra Uterine Fetal Death (IUFD) and stillbirth.<sup>1,3</sup>

Mutations or deletions of the  $\alpha$  and  $\beta$  globin genes lead to reduced or absent production of the corresponding chains. Two  $\alpha$  globin genes are located on chromosome 16, denoted as  $\alpha 1$  and  $\alpha 2$ . A type of deletion causes about 90% of  $\alpha$  globin gene mutations in one ( $-\alpha$ ) or both ( $-/-$ ) gene deletions. Furthermore,  $\alpha^+$  thalassemia occurs when one of the two  $\alpha$ -globin genes experiences deletion, while  $\alpha^0$  thalassemia occurs in the absence of  $\alpha$ -globin genes in one allele.<sup>3,4</sup> The  $\beta$  globin gene is located on chromosome 11, with one allele on each. Many gene mutations have been identified, and more than 300 have been reported.  $\beta^0$  thalassemia mutation causes no  $\beta$  globin production, while  $\beta^+$  can produce globin chain production but is significantly reduced.<sup>2-4</sup>

Thalassemia and hemoglobinopathies are major genetic problems in many Asian countries, including Indonesia. The carrier frequencies in various ethnic populations are 3-20%, 3%, and 1-33% for  $\alpha$ -thalassemia,  $\beta$ -thalassemia, and HbE, respectively. The World Health Organization (WHO) has recognized thalassemia as a hereditary genetic disorder mainly affecting low-income populations. There has been an alarming increase in this disease due to a lack of knowledge and proper health care. In 2015, thalassemia major patients were 7,029, which increased to 8,011 in 2017, and the high prevalence was recorded in the West and Central Java Provinces.<sup>5</sup>

Patients with homozygous  $\beta$ -thalassemia should receive regular transfusions every 2 to 4 weeks, putting them at risk of iron overload.

Complications from iron overload include cardiac complications, liver disease, and endocrine and musculoskeletal disorders. Approximately 10% (2.9%-20.9%) of thalassemia patients with repeated transfusions develop heart failure. Other significant complications reported include 25%-69% of pain, 25%-30% psychiatric disorder, and decreased quality of life. Even though there is an increase in survival, thalassemia patients still risk premature death. Mortality in patients with thalassemia major is 12.8%, where the primary comorbidity is osteoporosis of 16%-100%, followed by 30% heart disease, 22% hypogonadism, 12% hypothyroidism, and 2% depression.<sup>6,7</sup>

The average annual cost per person to treat patients with  $\beta$ -thalassemia who receive regular blood transfusions overseas is US\$128,062.<sup>7</sup> Health financing for managing patients in Indonesia ranks 5th among non-communicable diseases after heart complications, cancer, kidney impairment, and stroke. There is a significant annual increase in the financing of patients. The cost of treating patients in 2014 was 225 billion rupiahs, increasing to 452, 496, 532, and 397 billion rupiahs in 2015, 2016, 2017, and 2018.<sup>8</sup> For one patient with a thalassemia major, the Indonesian government has to pay around 400 million rupiahs annually. This expense does not include costs for routine monitoring of organ function and management of complications. The cost required for thalassemia screening is only around 400 thousand rupiahs. Therefore, the Indonesian government is intensifying screening to prevent thalassemia major.<sup>8</sup>

As a genetic disorder, thalassemia has specific characteristics due to the wide variability of mutations. More than 300 mutations of the  $\beta$ -globin gene have been identified in various regions worldwide and are relatively precise in a population and ethnicity.<sup>9</sup> HbE, the most common thalassemia mutation in Indonesia, occurs at 28.2%.<sup>4,10</sup> There are variations in the spectrum of these mutations in numerous regions of Indonesia.<sup>11</sup> The most common in Surabaya are c.79 G>A (HbE) (47.0%) and IVS-I-5 G>C (20.6%).<sup>12</sup> Furthermore, the most common  $\beta$ -thalassemia gene mutation in Sumatra is IVS-1 nt5 (G >

C) (Lanni, 2014), while IVS-I-5 (G>C) (43.5%) and c.79 G>A (HbE) (28.2%) are very common in Banyumas Regency.<sup>10</sup> The most common  $\beta$ -thalassemia gene mutations found in Pekanbaru (Riau) are codon 26 (G > A) and IVS-1 nt5 (G > C).<sup>13</sup>, while codon 2, IVS2nt16, and IVS2nt74 were reported in Malang and Sukabumi.<sup>14</sup> The prevalence of  $\beta$ -thalassemia gene mutation in Bandung is IVS1nt5 homozygous (47.4%) and IVS1nt5/HbE double heterozygosity (9.9%).<sup>11</sup>

Thalassemia is a disease that cannot be cured; hence, prevention is the most appropriate management effort WHO recommends.<sup>15</sup> The right strategy begins with assessing the problem before designing a prevention program. The magnitude of the thalassemia problem in each country can be assessed from the frequency and spectrum of the mutations and the severity of the disease. Prevalence does not necessarily reflect the magnitude of the problem because the mutations present a broad spectrum of clinical phenotypes.<sup>4,9</sup>

The spectrum mutation of thalassemia is relatively specific in certain populations and ethnicities, and the mutations result in different screening protocols in each region.<sup>2,4,9,16</sup> Data on the types of mutations specific to a particular population or ethnicity can be used to develop a thalassemia screening framework focused on the determinants of mutations. It depends on the population or ethnicity to develop an appropriate thalassemia mutation examination panel and reduce the time and costs incurred. Information on the spectrum is also needed for better patient management and appropriate genetic counseling.<sup>4,9,15</sup>

Data on the spectrum and frequency of thalassemia mutations based on population and ethnicity will provide a database as a basis/guideline for designing mutation panels in Indonesia's national thalassemia screening program. Several mutations have been reported by various previous research, but very few reported the spectrum of thalassemia mutations in remote areas. Therefore, this research aims to characterize the type and frequency of mutations in all major thalassemia patients in the Rembang Regency.

## METHODS

This research was a cross-sectional descriptive study of all major thalassemia patients undergoing transfusion therapy at Dr. R Soetrasno Regional Public Hospital, Rembang, Indonesia, during the 2018–2020 period. The sampling method used total sampling, and 14 thalassemia patients were found. All patients agreed to participate in this study and their parents signed informed consent. Complete blood count using Sysmex XN-1000 automatic hematology tool to obtain Hemoglobin (Hb), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), and Red Blood Cell (RBC) results. Hb analysis using the microcapillary Hb electrophoresis (CE) method (Sebia capillary zone electrophoresis). Accuracy and precision for both methods were assessed using control materials optimized for each device before sample analysis. Thalassemia gene mutations were examined at the Eijkman Research Center for Molecular Biology, Jakarta. The SPSS software version 20.0 for Windows was used to analyze data descriptively.

### DNA isolation

Genomic DNA was isolated from leukocytes using a modified Genra Puregene Kit (Qiagen Inc., Valencia, CA, USA) and used as a template for mutation detection based on the Polymerase Chain Reaction (PCR) method.

### $\alpha$ -thalassemia gene mutation

Detection of HbCS mutations used a PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) -based method, and the 25  $\mu$ L reaction mixture consisted of 20 mM Tris-HCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM KCl, 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100, pH 8.8, 200  $\mu$ MdNTPs, 0.4  $\mu$ M of each primer (E3a2: GCG GGT TGC GGG AGG T and GAA CGG CTA CCG AGG CTC CAG CTC), 0.625 units of Taq DNA polymerase (New England BioLabs Inc, Ipswich, MA, USA), and 100 ng of genomic DNA. The PCR reaction was performed on a 9700 thermal cycler (Applied Biosystems) using the following conditions: initial denaturation at 95°C

for 5 minutes; 35 cycles at 95°C for 30 seconds, 68°C for 30 seconds, 72°C for 1 minute; and final extension at 72°C for 5 minutes. Digestion uses the restriction enzyme Taq $\alpha$ -I according to the manufacturer's instructions. The normal RFLP DNA product size was not digested (222 kb), and heterozygous Hb Constant Spring (HbCS) produced 222 kb, 200 kb, and 22 kb DNA, while homozygous HbCS produced 200 kb and 22 kb DNA.<sup>17</sup> Subsequently, the digested products were electrophoresed in 2.0% agarose, and the gels were photographed using Gel Doc™ XR (Bio-Rad Laboratories).

Examining Hb Adana point mutation used the ACRS (amplified created restriction site) method to detect mutations in  $\alpha$ 2 codon 59.18 directly. The codon 59modF is a modified primer in which T (thymine) inserts the recognition sequence for the restriction enzyme Taq $\alpha$ -I in the mutant allele. The forward and reverse primers, codons 59modF (5'-GCT CTG CCC AGG TTA AGG GCC TCG-3') and 2R (5'-GGG AGG CCC ATC GGG CAG GAGGAA C-3'), were used for amplification of the 460 bp fragment (GenBank, NG\_000006). In addition, the PCR reaction mix of 25 $\mu$ l contained 100 ng DNA, 10  $\mu$ M of each primer, 200  $\mu$ M dNTPs, 1X PCR buffer, and 1.25 units of Taq DNA polymerase (New England Biolabs, Beverly, MA, USA). The reaction was conducted in a PE 9700 thermocycler (Perkin Elmer Applied Biosystems, Foster City, CA, USA) with initial denaturation of 5 minutes at 95°C followed by 35 cycles at 95°C for 30 seconds, at 68°C and 72°C for 1 minute, as well as 1 cycle at 72°C for 5 minutes. PCR products were digested using the restriction enzyme Taq $\alpha$ -I (New England Biolabs) and visualized under UV light (Gel Doc; Bio-Rad Laboratories).

### $\beta$ -thalassemia gene mutation

Examination of  $\beta$ -thalassemia gene mutation used PCR-based restriction fragment length polymorphism analysis (PCR-RFLP) developed by Pramoodjago.<sup>19</sup> The PCR amplifications use primer sets TLF62028-TLR62320 and TLF62392-TLR62703 producing a DNA product of 293 kb.<sup>19</sup>

The primers used were : TLF62028-5'ACCTCACCCCTGTGGAGCCAC3';

TLR62703-5'CTATTGGTCTCCTTAAACCTGTCTT GTAACCTTGCTA3'; TLF62392-5'TAT TTTCCCACCCTTAGGCTGCTGGTG GTCTACCCTT GGACCCAGAGGTC3'; TLR62703-5' CCCCTTCCTAT GACATGAACTTAA 3'.

## RESULTS

### Characteristics of research subjects

A total of 14 patients in 2018 - 2020 were willing to be research subjects. The mean age of patients was 12.1 years, with the youngest and oldest being 9 and 16 years, respectively. There were 6 and 8 male and female patients amounting to 42.9% and 57.1%, mostly Javanese and from Rembang Regency (Table 1).

### Hematological examination and Hb analysis results

The hematological examination results are shown in Table 1, and the average Hb level in all subjects was  $9.90 \pm 1.20$  g/dL. Most patients had moderate anemia of 85.8%; 1 male (1.7%) and 1 female (1.7%) had severe and mild anemia. The MCV examination results were less than 80 fL with a mean of  $71.90 \pm 7.90$  fL; a subject (1.7%) had an MCV of more than 80 fL (87 fL). Furthermore, the MCH examination results of all subjects were less than 27 pg with a mean of  $24.50 \pm 2.30$  pg (Table 1).

The Hb electrophoresis examination showed that 12 (85.7%) subjects had an HbE fraction. Based on clinical symptoms of thalassemia major and an increase in the fraction, the 12 subjects were presumptively diagnosed with  $\beta$ -thalassemia/HbE. One of the subjects had clinical symptoms of thalassemia major with an average HbA2 fraction of 3.5%. However, there was an increase in the HbF fraction, diagnosed presumptively with  $\beta$ -thalassemia major. The Hb analysis showed that 1 (1.7%) subject had an HbC zone fraction, leading to a presumptive diagnosis of thalassemia/hemoglobinopathy on HbCS. It is a hemoglobinopathy in the  $\alpha$  globin gene, and further molecular analysis is used on the  $\alpha$  globin gene.

The examination showed that 13 (92.86%) patients have  $\beta$ -thalassemia, and 1 (7.14%) is affected by  $\alpha$ -thalassemia (Table 1). The hematological examination and Hb analysis results in  $\beta$ -thalassemia

mutations are described in Table 2.

Of the 13 patients with mutations in the  $\beta$ -thalassemia gene, 6 (46.16%) had a double heterozygous HbE (Codon 26, GAG<sup>Glutamate</sup>>AAG<sup>Lysine</sup>) and a deletion mutation in Cd 35 (-C). Meanwhile, 4 (30.77%) had a double heterozygous HbE (Codon 26, GAG<sup>Glutamate</sup>>AAG<sup>Lysine</sup>) and IVS1-nt.5 mutations (G>C). The other three patients had double heterozygous HbE (Codon 26, GAG<sup>Glutamate</sup>>AAG<sup>Lysine</sup>) and the IVS1-nt2 mutations (T>C), double heterozygous HbE (Codon 26, GAG<sup>Glutamate</sup>>AAG<sup>Lysine</sup>) and codon 8/9 (insertion + G), and double heterozygous codon 8/9 (insertion + G) and Hb Malay (Codon 19, AAC<sup>Asparagine</sup>>AGC<sup>Serine</sup>).

The lowest mean MCV and MCH were found in the double heterozygous HbE (Codon 26, GAG<sup>Glutamate</sup>>AAG<sup>Lysine</sup>) and the deletion at Cd 35 (-C). The lowest levels of HbA2, HbF, and HbE were found in the double heterozygous HbE mutation (Codon 26, GAG<sup>Glutamate</sup>>AAG<sup>Lysine</sup>) and the deletion at Cd 35 (-C) (Table 3).

## DISCUSSION

The three frequent  $\beta$ -globin mutations that account for 73% of mutations in Malay are HbE, IVS 1-5 (G-C), and IVS 1-1 (G-T). The five common  $\beta$ -globin mutations that cause 90% of mutations in Chinese-Malaysians are CD 41-42 (-TCTT), IVS 654 (C to T), -28 (A to G), CD 17 (A to T), and CD 71/72 (+ A).<sup>20</sup> In Thailand, the prevalence of  $\beta$ -thalassemia and HbE  $\beta$ -thalassemia are 3-9% and 13%, respectively. HbE beta-thalassemia is common in Thailand, Indonesia, India, Bangladesh, and Sri Lanka. There are several mutations in the  $\beta$  globin chain, and the most common mutation was 41/42 frame-shift at 50.9%, whereas the IVS-1 nt5 mutation was only found in 5.2% of patients. IVS1-nt5 (G°C) was the most common mutation found in China, India, Malaysia, and Indonesia, with a prevalence of 48.3%, 22.5%, 48.8%, and 35.3%, respectively. Previous research found IVS1-nt5 (G°C) in 66.7% of major thalassemia patients at Dr. Cipto Mangunkusumo Hospital, Jakarta.<sup>16</sup>

Several mutations in Indonesia have been reported by previous research. Lie-Injo stated that in the case of Javanese ethnicity, the most common mutation



found was IVS-1-5 (G>C), Cd26/HbE (GAG>AAG), IVS-II-654 (C >T), and Cd41/42 (-TTCT) and Cd35 (-C) at 24%, 18%, 9.7%, and 1.4%, respectively. Hernanda reported IVS-1-5 (G > C) and Cd26/HbE (GAG > AAG) at 21% and 37%.<sup>12</sup> IVS1-5 (G > C) was the most common mutation in Southern Central Java at 43.5%, followed by Cd26 (GAG > AAG), IVS1-1 (G > A), and Cd15 (TGG > TAG) at 28%, 5%, and 3.8%.<sup>10</sup> Research in Riau found 4 mutant alleles in the HBB gene, including IVS1-nt-5 (G>C), Cd26/HbE (GAG>AAG), IVS1-nt1 (G>T), and IVS1-nt2 (T>C), with IVS1-nt5/Cd26 being the most common at 41.1%.<sup>13</sup> Susanto (2020) identified several mutations reported by previous research, such as Cd26/HbE (GAG > AAG) with the highest prevalence of 48.4%, followed by IVS-1-5 (G > C), IVS- 1-2 (T > C), Cd35 (-C), IVS-1-1 (G > T), as well as Cd30 (AGG > ACG) and Cd60 (GTG > GAG) at 14.5%, 12.9%, 8.1%, 6.5%, and 3.2%.<sup>15</sup>

The results of thalassemia mutations in Rembang Regency differ from findings in other regions. The most common

mutations found in the regency are double heterozygous HbE mutation (Codon 26, GAGGlutamate>AAGLysine) and deletion mutation at Cd 35 (-C) at 42.87%. The most common mutation found in Southeast Asia and most parts of Indonesia (IVS-1-5 (G > C)) was in second place with a frequency of 28.57%.

The interaction of HbE with  $\beta$ -thalassemia results in HbE  $\beta$ -thalassemia, a very heterogeneous clinical condition and is the most common form found in Southeast Asia. Furthermore, it is common in Malays and native people of Peninsular Malaysia.<sup>21</sup> Most patients with HbE  $\beta$ -thalassemia were diagnosed at 14.5 years (9 months-40 years). Some received blood transfusions at local hospitals before being diagnosed with an underlying condition, and HbE  $\beta$ -thalassemia has a diverse clinical phenotype. Hb levels range from 5.5 to 11.0 g/dL, where the severity of anemia results from ineffective erythropoiesis and peripheral hemolysis.<sup>21</sup> Fucharoen et al. stated that Hb levels in HbE  $\beta$ -thalassemia have a wide range between the different

phenotypes, from 3 g/dl or less to 11 g/dl.<sup>9</sup>

Patients with multiple heterozygous for E and  $\beta^0$ -thalassemia have HbE of 40-60%, while those who are homozygous for HbE have a percentage of 85-99%. However, HbF is 30-40% in HbE  $\beta^0$ -thalassemia and varies widely from 5% - 87%. Most of the HbE and  $\beta^0$  thalassemia patients (66.1%) had Hb levels below 5 g/dl at the time of diagnosis. The mean of HbA, HbE, and HbF levels was  $10.1\% \pm 13.4$ ,  $50.7\% \pm 14.7$ , and  $32.6\% \pm 11$ , ranging from 0.7 to 45.4%, 29 to 80.5%, and 10.8 to 54.3%. Furthermore, there was no significant correlation between HbF level and disease severity. All cases showed microcytic hypochromic features with a mean MCV, MCH, and MCHC of  $61.7 \text{ fL} \pm 6.3$ ,  $18.4 \text{ pcgm} \pm 2.3$ , and  $29.3 \text{ gm}\% \pm 3.8$ .<sup>9</sup>

The most common form of HbE  $\beta$ -thalassemia in Southeast Asia and Malaysia is HbE -  $\beta$  [IVS1-5 (G  $\rightarrow$  C)]. The IVS1-5 mutation (G  $\rightarrow$  C) has a  $\beta$  + - thalassemia phenotype and is severe because there is little synthesis at 2.7-5.8%. This group of patients had a mean Hb level of 6.5 g/dL, moderate to severe splenomegaly, and bone abnormalities. Early diagnosis should be made to identify mild, moderate, and severe HbE  $\beta$ -thalassemia types.<sup>21</sup>

HbE  $\beta$ -thalassemia patients with IVS1-nt5 (G $\rightarrow$ C) mutation had more severe clinical manifestations than other types. Research conducted at Dr. Cipto Mangunkusumo Hospital, Jakarta, the

**Table 1. Hematological examination results of research subjects.**

Parameter	Mean $\pm$ standard deviation		
	Male (N=6)	Female (N=8)	Total (N=14)
Hb (g/dL)	9.28 $\pm$ 0.89	10.36 $\pm$ 1.28	9.90 $\pm$ 1.20
Erythrocyte count ( $10^6$ /uL)	3.98 $\pm$ 0.30	4.35 $\pm$ 0.56	4.20 $\pm$ 0.50
MCV (fL)	72.63 $\pm$ 7.94	71.36 $\pm$ 8.37	71.90 $\pm$ 7.90
MCH (pg)	23.32 $\pm$ 7.94	24.00 $\pm$ 2.90	24.50 $\pm$ 2.30
MCHC	32.27 $\pm$ 2.29	33.56 $\pm$ 0.52	33.30 $\pm$ 1.60

**Table 2. Type and frequency of thalassemia mutations in all research subjects.**

Mutation type	Number	%
Beta thalassemia		
Double heterozygous HbE mutation (Codon 26, GAG <sup>Glutamate</sup> >AAG <sup>Lysine</sup> ) and deletion mutation at Cd 35 (-C)	6	42,87
Double heterozygous HbE mutation (Codon 26, GAG <sup>Glutamate</sup> >AAG <sup>Lysine</sup> ) and IVS1-nt.5 (G>C) mutation	4	28,57
Double heterozygous HbE mutation (Codon 26, GAG <sup>Glutamate</sup> >AAG <sup>Lysine</sup> ) and IVS1-nt.2 (T>C) mutation	1	7,14
Double heterozygous HbE mutation (Codon 26, GAG <sup>Glutamate</sup> >AAG <sup>Lysine</sup> ) and codon 8/9 (insertion + G)	1	7,14
Double heterozygous codon 8/9 (insertion + G) and Malay Hb (Codon 19, AAC <sup>Asparagine</sup> > AGC <sup>Serine</sup> )	1	7,14
Subtotal	13	92,86
Alpha thalassemia		
Double heterozygous mutation of Codon 59 $\alpha$ 2-globin gene (GGC <sup>Glicine</sup> *GAC <sup>Aspartate</sup> ) or Hb Adana and Hb Constant Spring (CD142 $\alpha$ 2-globin gene, TAA <sup>Stop</sup> *CAA <sup>Glutamine+30aa</sup> )	1	7,14
Subtotal	1	7,14
Total	14	100

**Table 3.** Hematological examination and Hb analysis results in  $\beta$ -thalassemia mutations.

Parameter	Mutation type				
	Double heterozygous HbE mutation (Codon 26, GAG <sup>Glutamate</sup> →AAG <sup>Lysine</sup> ) and deletion mutation at Cd 35 (-C)	Double heterozygous HbE mutation (Codon 26, GAG <sup>Glutamate</sup> →AAG <sup>Lysine</sup> ) and IVS1-nt.5 mutations (G>C)	Double heterozygous HbE mutation (Codon 26, GAG <sup>Glutamate</sup> →AAG <sup>Lysine</sup> ) and IVS1-nt.2 mutations (T>C)	Double heterozygous HbE mutation (Codon 26, GAG <sup>Glutamate</sup> →AAG <sup>Lysine</sup> ) and codon 8/9 (insertion + G)	Double heterozygous codon 8/9 (insertion + G) and Hb Malay (Codon 19, AAC <sup>Asparagine</sup> →AGC <sup>Serine</sup> )
Hb (g/dL)	10.25 ± 1.36	9.08 ± 0.83	10.40	9.50	11.80
Erythrocytes (10 <sup>6</sup> /uL)	4.54 ± 0.51	3.79 ± 0.12	4.24	3.85	4.52
MCV (fL)	67.38 ± 8.75	72.35 ± 3.04	73.60	74.50	77.70
MCH (pg)	22.75 ± 3.20	23.95 ± 1.59	24.50	24.70	26.10
HbA (%)	73.92 ± 11.98	72.10 ± 4.39	75.20	79.10	88.60
HbA <sub>2</sub> (%)	4.25 ± 1.93	3.40 ± 0.55	3.60	3.00	3.00
HbF (%)	9.48 ± 6.80	8.55 ± 5.38	5.60	8.30	8.40
HbE (%)	24.70 ± 24.09	15.95 ± 3.44	15.60	9.60	-

HbE  $\beta$ -thalassemia group with positive IVS1-nt5 mutations had lower mean pre-transfusion Hb levels, younger age for first blood transfusion, lower HbF and HbE levels, and higher total transfusions per year compared to the group without the mutation.<sup>16</sup>

The AAC → AGC mutation causes Hb Malay at codon 19 of the  $\beta$  globin gene resulting in the substitution of serine for asparagine. The mutation creates a cryptic RNA junction site in exon 1 of the  $\beta$  globin gene leading to abnormal processing. This mutation produces the Hb variant and mild  $\beta^+$  thalassemia phenotype.<sup>22</sup> A definite diagnosis of Hb Malay can only be made by molecular analysis. The HPLC technique and capillary zone electrophoresis (CZE) cannot differentiate between Hb A and Hb Malay because they migrate together. Previous research stated that there was an increase in HbF production between 12-32% in homozygous Hb Malay and double heterozygous HbE/Malay cases. The HbE/Malay patients were also asymptomatic, although the mean Hb was lower (10g/dL) than the classic HbE character (12.4g/dL). Meanwhile, the HbF levels in HbE/Malay were reported to be above 12%. This finding is under the Hb Malay phenotype, which resembles  $\beta^+$  thalassemia.<sup>22</sup>

A case of  $\alpha$ -thalassemia, which is a double heterozygous Codon 59  $\alpha$ 2-globin gene mutation (GGC<sup>Glycine</sup>→GAC<sup>Aspartate</sup>) or Hb Adana and HbCS (CD142 globin- $\alpha$ 2 gene, TAA<sup>Stop</sup>→CAA<sup>Glutamine+30aa</sup>) was also reported. Double heterozygosity of Hb Adana and HbCS is double heterozygous thalassemia with non-deletional  $\alpha$ -thalassemia mutations. There are several types of non-deletional  $\alpha$ -thalassemia gene mutations where more than 100 types are known. In Southeast Asia, non-deletional  $\alpha$ -thalassemia mutations include HbCS (codon 142), Hb Pakse (codon 142), Hb Quong Sze (codon 125), Hb Suan Dok (codon 109), Hb Adana (codon 59), and codon 30. The most common non-deletional  $\alpha$ -thalassemia mutations in Malaysia are HbCS and Hb Adana. Meanwhile, non-deletional  $\alpha$ -thalassemia mutations often found in Indonesia are in the  $\alpha$ 2 globin gene, namely at codon 59 (Hb Adana), codon 22 (GGC<sup>Glycine</sup>→GGT<sup>Glycine</sup>), and HbCS. Hb Adana is relatively rare in Malaysia at 0.14 to 3.7% but is quite

common in Indonesia at 16%. Muktiarti and Widyastiti reported the HbAdana case in Javanese ethnic patients.<sup>23,24</sup> Widyastiti also reported Hb Quong Sze patient in Chinese Indonesian proband.<sup>25</sup>

The clinical picture of  $\alpha$ -thalassemia depends on the number of  $\alpha$  chains present, the type of mutation that occurs, or the gene affected. Due to unstable globin chains, the clinical picture of patients with non-deletional mutations is typically more severe than that of patients with deletional gene mutations.<sup>23</sup> The MCV value in non-deletional  $\alpha$ -thalassemia but experiences more severe rates of anemia and reticulosis. This finding may explain the higher MCV value in non-deletional  $\alpha$ -thalassemia.<sup>26</sup>

Mutations at codon 59 (Hb Adana) result in converting the amino acid glycine to aspartate (GGC<sup>Glycine</sup>→GAC<sup>Aspartate</sup>) and a highly unstable haemoglobin variant. The clinical picture in Hb Adana ranges from thalassemia intermedia, thalassemia major, and hydrops fetalis.<sup>23</sup> Mutations at codon 59 (Hb Adana) result in the formation of molecules that are unstable and have no product to be visualized by routine screening examinations.<sup>26</sup> Therefore, the actual frequency of this molecular anomaly is unknown because the defect can only be ascertained by DNA analysis. Hb Adana carrier is asymptomatic or shows mild anemia with a red blood cell index similar to  $\alpha$ -thalassemia due to the deletion of a single  $\alpha$  globin gene deletion.<sup>26</sup> It is a hyper unstable (very unstable) variation of Hb; hence, it should be investigated in patients with a clinical presentation of thalassemia intermedia with harmful HbH inclusion objects and normal Hb electrophoresis results.<sup>26,27</sup> Heterozygous Hb Adana diagnosis will be easily missed when the clinical history is not considered.

HbCS is formed as a result of a mutation in the  $\alpha$ 2 globin stop codon gene (TAA > CAA), which leads to decreased synthesis of  $\alpha$  chain (1% of normal) with 31 different amino acids. HbCS is characterized by highly ineffective erythropoiesis and much more severe erythroid apoptosis than deletional  $\alpha$ -thalassemia. The HbCS level is lower (mean of 2 g/dL) than deletional  $\alpha$ -thalassemia, while MCV is higher because overhydration of red blood cells

containing HbCS increases the volume of red blood cells relative to the Hb content. This is due to the entry of oxidized  $\alpha$ CS globin chains into the red blood cell membrane and their cytoskeleton causing erythrocyte hyperhydration.<sup>22</sup>

Heterozygous HbCS have normal Hb levels with normal MCV and slightly low MCH. Furthermore, HbCS patients experience a less significant decrease in MCH value with normal mean MCV due to damage to red blood cell membranes by oxidized  $\alpha$  and  $\alpha$ CS globin chains. Therefore, the carrier status of HbCS can be easily missed if the screening is mainly based on the red blood cell index.<sup>22</sup>

The unstable nature of HbCS can lead to errors in detection by routine examination methods. Hb analysis using fresh blood samples with cellulose acetate electrophoresis at alkaline pH can quickly identify the HbCS band. The band moves between the carbonic anhydrase enzyme and HbA<sub>2</sub>, while HbCS is in window C when using HPLC. However, in the case of heterozygous HbCS, no peak is found in the region. In acid electrophoresis, HbCS moves together with HbS. HbC is detected in the HbC/CS zone using Capillary Electrophoresis (CE). The CE method is superior to HPLC for detecting HbCS and useful in screening heterozygous and homozygous CS and HbH-CS. Capillary Electrophoresis is the preferred method for HbCS screening.<sup>22</sup>

The results of  $\beta$ -thalassemia mutations are different from findings in other regions in Indonesia. In Rembang Regency, the most common mutations found are double heterozygous HbE mutation (Codon 26, GAG<sup>Glutamate</sup>>AAG<sup>Lysine</sup>) and deletion mutation on Cd 35 (-C) at 46.16%. The deletion mutation on Cd 35 (-C) is an allele of  $\beta$ -thalassemia mutation rarely found in Indonesia. The Eijkman Institute for Molecular Biology, as a reference center for the national thalassemia DNA examination service, uses the ARMS PCR method to detect  $\beta$ -thalassemia. It is commonly found in Indonesia, namely IVS1-nt.1 (G>T), IVS1-nt.1 (G>A), IVS1-nt.2 (T>C), IVS1-nt.5 (G>C), Hb Malay (Codon 19, AAC<sup>Asparagine</sup>>AGC<sup>Serine</sup>), HbE (Codon 26, GAG<sup>Glutamate</sup>>AAG<sup>Lysine</sup>), Codon 26, GAG<sup>Glutamate</sup>> AAG<sup>Stop</sup>, Codon 15 (TGG<sup>Tryptophan</sup>>TAG<sup>Stop</sup>), Codon 17 (AAG<sup>Lys</sup>> TAG<sup>Stop</sup>) and Codon 8/9 (insertion +

G) using the available primers. Detection of deletion mutations on Cd 35 (-C) was conducted using advanced DNA examination with sequencing techniques to read DNA bases. DNA sequencing can consider the selected (main) thalassemia DNA examination technique because the most common type of  $\beta$ -thalassemia mutation is a deletion mutation at Cd 35 (-C).

Research in Rembang Regency found 1 patient with double heterozygous in two types of non-deletional  $\alpha$ -thalassemia mutations (Hb Adana and HbCS). The involvement has resulted in a much more severe phenotype than the combination of non-deletional and deletional mutations. This is because non-deletional mutations cause more severe effects on red blood cells.<sup>22</sup> Routine laboratory screening programs using complete blood counts cannot readily facilitate the detection of non-deletional  $\alpha$ -thalassemia. This is because the HbH inclusion object examination results are negative on Hb Adana, and the Hb electrophoresis examination results are normal. Furthermore, HbCS is very unstable Hb and undetectable on analysis (HPLC, Hb electrophoresis). This indicates, in non-deletional  $\alpha$ -thalassemia mutations, the diagnosis of thalassemia is based on the DNA analysis results.

The limitation of this study is that the probands were thalassemia major patients undergoing transfusion therapy in a hospital (hospital-based subject), so the types of thalassemia mutations detected were the severe types of mutations. It is necessary to conduct population-based research in Rembang so that other types of thalassemia mutations can be detected, especially mild or non-severe types of thalassemia mutations.

## CONCLUSION

Spectrum (type and frequency) of thalassemia mutations are double heterozygous HbE mutation (Codon 26, GAG<sup>Glutamate</sup>>AAG<sup>Lysine</sup>) and deletion mutation at Cd 35 (-C) by 42.87%, double heterozygous HbE mutation (Codon 26, GAG<sup>Glutamate</sup>>AAG<sup>Lysine</sup>) and IVS1-nt.5 mutations (G>C) by 28.57%, double heterozygous HbE mutation (Codon 26, GAG<sup>Glutamate</sup>>AAG<sup>Lysine</sup>) and

IVS1-nt.2 mutations (T>C) by 1.74%, double heterozygous HbE mutation (Codon 26, GAG<sup>Glutamate</sup>>AAG<sup>Lysine</sup>) and codon 8/9 (insertion + G) by 1.74%, double heterozygous codon 8/9 (insertion + G), and Malay Hb (Codon 19, AAC<sup>Asparagine</sup>>AGC<sup>Serine</sup>) by 1.74%, and double heterozygous Codon 59  $\alpha$ 2-globin gene mutation (GGC<sup>Glycine</sup>\*GAC<sup>Aspartat</sup>) GAC<sup>Aspartate</sup>), or Hb Adana and HbCS (CD142  $\alpha$ 2-globin gene, TAA<sup>Stop</sup>\*CAA<sup>Glutamine+30aa</sup>) by 1.74%.

## SUGGESTION

The most common type of  $\beta$ -thalassemia mutation found in Rembang Regency is deletion at Cd 35 (-C). Therefore, examining  $\beta$ -thalassemia DNA in patients using the DNA sequencing method is recommended. The type of  $\alpha$ -thalassemia mutation found is a non-deletional mutation, and it is often undetermined as thalassemia during screening (complete blood count and Hb analysis). In clinical patients with thalassemia intermedia, a DNA examination is required. It is advisable to examine thalassemia mutations in various ethnicities and regions due to variations in frequency and types of mutations.

## CONFLICT OF INTEREST

The authors report no conflicts of interest in this research.

## RESEARCH ETHICS

Ethical clearance for this research was obtained from the Health Research Ethics Committee, Faculty of Medicine, Diponegoro University, with number 604/EC/FK-UNDIP/EC/2018.

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## AUTHOR CONTRIBUTION

All authors equally contribute to this study from the conceptual framework, data acquisition, and data analysis until reporting the study results through publication.

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