Analysis of Common Beta-Thalassemia (\(\beta\)-Thalassemia) Mutations in East Java, Indonesia

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Background: The frequency of the beta-thalassemia (\(\beta\)-thalassemia) gene in Indonesia ranges from 3 to 10%. However, in the East Java province, there is still limited information on the prevalence of \(\beta\)-thalassemia mutations in clinically diagnosed beta-thalassemia patients of East Java. Therefore, this study aimed to characterize \(\beta\)-thalassemia mutations in selected patients in the East Java province of Indonesia.

Methods: This is an analytical observational study. Diagnosis of \(\beta\)-thalassemia was based on clinical presentation, complete blood count (CBC), and hemoglobin (Hb) electrophoresis. Blood specimens taken from each patient in three ethylenediaminetetraacetic acid (EDTA) tubes were analyzed for CBC and Hb electrophoresis and processed for DNA extraction and subsequent polymerase chain reaction (PCR). Detection of mutations in Hemoglobin Subunit Beta (HBβ) gene exons 1–3 of the \(\beta\)-thalassemia gene as the common mutation in Indonesia was done using PCR followed by Sanger sequencing.

Results: In total, 33 (n = 33) participants were involved in this study with ages ranging from 5 to 17 years comprising 19 women and 14 men. Their ethnic origins were Javanese (n = 30) and Chinese (n = 3). CBC results showed that mean ± standard deviation (SD) for Hb, red blood cell (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW)-CV were 81.2 ± 71.05 g/L; 3.40 ± 0.39 × 10^9/L; 71.05 ± 5.72 fl; 24.12 ± 2.45 pg; 33.91 ± 1.47 g/dl; 24.38 ± 6.02%, respectively. Hb electrophoresis revealed that 5 out of 33 participants had beta-thalassemia and 28 out of 33 participants had hemoglobinopathy (Hb) E/\(\beta\)-beta-thalassemia. Results of Sanger sequencing showed the following genotype variations in the samples: 12 (36.4%) with \(\beta^{CD26}\)/\(\beta^{IVS-I-5}\), 6 (18.2%) with \(\beta^{CD26}\)/\(\beta^{CD35}\), 3 (9.1%) with \(\beta^{CD26}\)/\(\beta^{IVS-I-2}\), 2 (6.1%) with \(\beta^{CD26}\)/\(\beta^{CD40}\), 2 (6.1%) with \(\beta^{IVS-I-1}/\beta^{CAP+1}\); and 1 (3%) sample, respectively, and 1 (3%) had no abnormality detected in sequencing even...
INTRODUCTION

Beta thalassemia (β-thalassemia) is a disorder in hemoglobin synthesis characterized by decreased or absent β-globin chain synthesis. There are two (2) groups of β-thalassemia based on the amount of β-globin chain synthesis, namely, β0 if the globin chain is not synthesized at all, and β+ thalassemia if the globin chain synthesis is reduced. β0 thalassemia is mainly caused by point mutations in the coding region or exon-intron junction of the β-globin gene that causes premature stop codons or causes abnormal β-globin mRNA (1).

More than 200 different mutations underlying β-thalassemia have been identified (2, 3). These mutations are divided into β-globin gene deletions and non-deletional mutations that affect the transcription, processing, or translation of the globin messenger. Point mutations include mutations at the Catabolite Gene Activator Protein (CAP) site, frameshift, initiation site, nonsense mutation, polyA addition site mutation, promoter, and splicing mutation (2).

Some of these mutations underlie the different clinical manifestations in the affected patients and are classified as transfusion-dependent (TD) and non-transfusion-dependent (NTD) thalassemia, based on the transfusion needs of a patient (3). Patients classified as NTD thalassemia (NTDT) may not require frequent blood transfusions for survival, whereas those with TD thalassemia (TDT) require life-long regular blood transfusions (4).

The epidemiology of thalassemia involves more than 150 countries in the world that includes the Mediterranean, certain parts of North and West Africa, the Middle East, the Indian subcontinent, Southern Far East, with Southeast Asia having the highest prevalence (1, 4). Indonesia is not spared, having a high frequency of those with thalassemia genes. This is evident from epidemiological studies in Indonesia, which found that the frequency of beta-thalassemia genes ranged from 3 to 10% (5). Every year about 300,000–500,000 newborns are accompanied by severe hemoglobin abnormalities and 50,000–100,000 children die from thalassemia; 80% of them reside in developing countries. Data obtained from all teaching hospitals only registered about 7,670 thalassemia major patients throughout Indonesia. This number is still much lower than the actual estimated number. This could be because the types of gene mutations that exist in Indonesia vary from very severe to mild, thus they do not require transfusion (asymptomatic), resulting in under-diagnosis (5).

The molecular basis of the thalassemia’s has been studied in many of the world’s population and studies pertaining to the molecular basis of thalassemia in Indonesia have been reported (6, 7), especially in East Java. However, in these earlier reports, there were a limited number of samples, and studies were conducted about 10 years ago. A preliminary study conducted on 17 patients with TDT revealed the presence of seven (7) genotypic variations of beta-thalassemia (6). Currently, in Indonesia, the number of patients reported having severe thalassemia is increasing annually, and these numbers have increased four-fold in the last 20 years (7).

One of the reasons for this increase in the number of patients with thalassemia is due to high population migration to and from East Java over the last 10 years resulting in an increase in inter-ethnic marriages (8, 9).

Dr. Hospital Soetomo is a referral and the largest hospital in eastern Indonesia, with a land area of 166,061 hectares and 1,444 beds. This hospital was established in 1950 and provides services, education, and research functions, obtaining the Joint Commission International (JCI) accreditation in 2018 (10). Regarding data on new patients with TDT, there were 51 patients in 2014, 69 patients in 2015, 39 patients in 2016, 47 patients in 2017, 41 patients in 2018, and 57 patients in 2019. Routine pediatric outpatient visits were around 187 per month (unpublished data, Hematology-Oncology Division-Department of Pediatrics, Dr. Soetomo General Academic Hospital, 2022).

In East Java province, there is limited information on the prevalence of β-thalassemia mutations in the affected population. Therefore, this study aimed to characterize β-thalassemia mutations in clinically diagnosed patients with beta-thalassemia TDT in the East Java province of Indonesia.

MATERIALS AND METHODS

Patients and Study Design
The design of this study was analytical and observational. Participating patients were recruited from the One Day Care Unit of the Department of Pediatrics, Dr. Soetomo General Hospital, Surabaya, Indonesia. Previously diagnosed beta-thalassemia patients (<18 years old) who have undergone treatment that included blood transfusions at this institution were recruited. This study was carried out between July 2021 and January 2022.
Ethical clearance was obtained from the Ethics Committee of RSUD Dr. Soetomo No.: 0224/KEPK/VII/2021, and all of the patient’s parents have agreed to give consent form.

**Laboratory Tests**

Blood samples were taken from all patients on their follow-up prior to their blood transfusion. A total of 6 ml of blood was divided into two ethylenediaminetetraacetic acid (EDTA) tubes. The first tube was analyzed for complete blood count (CBC) using a Sysmex XN 1000 Analyzer (Sysmex Corporation, Kobe, Japan) and run for mini capillary hemoglobin electrophoresis (Sebia 9 Hydragel K20 Hemoglobin; Capillarys®; Sebia, Lisses, France), while the other tube for DNA extraction using the QIAamp® DNA Blood Mini Kit lot no. kit. 166051764 (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. DNA samples were stored at −80°C, before subsequent analysis, i.e., performed polymerase chain reaction (PCR) and followed by the Sanger sequencing on these DNA samples. Ferritin, liver function tests, and renal function tests data were obtained from medical records based on the latest data. Molecular analysis was carried out at the Universitas Gajah Mada (UGM) Integrated Research and Testing Laboratory. Analytical statistics included the calculation of frequency distribution, mean, and standard deviation (SD).

**Genotype Determination**

Extracted DNA samples were subjected to Sanger sequencing. In this PCR reaction, the mixture contained 12.5 µl (Bioline My Taq HS Red Mix), 50–150 ng of genomic DNA, and two pairs of primers (Table 1) at concentrations of 0.4µM each. The PCR reactions were performed using Bio-Rad T100 (Bio-Rad Laboratories, Inc., USA). The PCR cycle reactions were as follows: an initial 2 min of denaturation at 95°C; 15 s of denaturation (35 cycles) at 95°C; 15 s of annealing at 54°C; 15 s of extension at 72°C; and 2 min of extension at 72°C. Subsequently, 5 µl of the amplified product was aliquoted prior to visualization using gel electrophoresis. The gel preparation was as follows: 1% agarose gel in 1× Tris-Borate-EDTA buffer. Electrophoresis was conducted for 25 min at 100 V and then viewed under a UV transilluminator prior to documentation (11).

**DNA Sequence Analysis**

The PCR products encompassing the β globin genes were amplified with double reaction PCR. The amplified fragments were subsequently sequenced by Sanger methods by DNA Sequencing Services (UGM Integrated Research and Testing Laboratory). The sequences were analyzed and aligned using the Benchling web service (https://www.benchling.com/) with the reference sequence from NCBI Reference Seq: NG_059281 to determine the genotype (Applied Biosystems, 3500 Genetic Analyzer, Hitachi Corp Tokyo, Japan).

**RESULTS**

There were thirty-three study participants (19 women and 14 men) wherein 29 were Javanese and 4 were Chinese involved in this study with ages ranging from 5 to 17 years. CBC results showed that mean ± SD for Hb, red blood count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin (MCHC), and red cell distribution width (RDW)-CV were 81.2 ± 7.0 g/L; 3.40 ± 0.39 × 10⁹/L; 71.05 ± 5.72 fl; 24.12 ± 2.45 pg; 33.91 ± 1.47 g/dl; and 22.91 ± 8.66%, respectively. The patients have hypochromic microcytic anemia with anisocytosis on the peripheral blood film, the results of transaminase were mildly increased, however, the mean result of renal function was still within the normal range (Table 2).

Hemoglobin electrophoresis revealed that five (5) out of 33 patients had β-thalassemia and 28 out of 33 participants had hemoglobin variant (Hb) E/β-thalassemia. Results of Sanger sequencing showed the following genotype variations: 12 (36.4%) with β^{CD26/IVS-1-5}; 6 (18.2%) with β^{CD26/IVS-1-2}; 3 (9.1%) with β^{CD26/IVS-1}; 2 (6.1%) with β^{CD27/28/CD40}; 2 (6.1%) with IVS-1-1/β{CAP+1}; and 2 (6.1%) with IVS-1-1/β{CAP+1} and β^{CD26/IVS-1-1}; IVS-1-5/β{CAP+1}; IVS-1-5/β{CD55}; β^{CD26/CD35}; β^{CD26/CD35}; β^{CD26/CD40}; and IVS-1-5/β{CD19} in each sample (3%), respectively, while one (1) sample (3%) had no mutation detected even though the Hb electrophoresis results showed abnormality in the migration pattern.

| Parameter | Value |
|-----------|-------|
| Hemoglobin (g/L) | 81.2 ± 7.0 (68.0–96.0) |
| RBC (×10⁹/L) | 3.40 ± 0.39 (2.62–4.30) |
| MCV (fl) | 71.05 ± 5.72 (60.00–86.30) |
| MCH (pg) | 24.12 ± 2.45 (19.50–29.00) |
| MCHC (g/dL) | 33.91 ± 1.47 (31.80–37.80) |
| RDW-CV | 24.38 ± 6.02 (14.60–37.60) |
| WBC (>2.0×10⁹/L) | 7.01 ± 1.98 (4.11–11.97) |
| PLT (<1.0×10⁹/L) | 294.82 ± 138.65 (84.00–765.00) |
| Ferritin (ng/ml) | 2564.39 ± 1816.40 (218.70–7109.30) |
| Serum glutamic oxaloacetic transaminase (SGOT) (IU/L) | 44.61 ± 24.26 (20.00–128.00) |
| Serum glutamic pyruvic transaminase (SGPT) (IU/L) | 39.52 ± 33.06 (12.00–149.00) |
| Blood urea nitrogen (BUN) (mmol/L) | 3.26 ± 1.08 (0.71–5.71) |
| Serum creatinine (µmol/L) | 33.59 ± 9.72 (8.84–53.04) |

**TABLE 1** | Sequence and size of the primers used for DNA amplification (17).

| Primers | Sequence | Size (bp) |
|---------|----------|-----------|
| Primer 1 | 5′ CCA AGG ACA GGT ACG GCT GTC ATC 3′ | 704 bp |
| Primer 5 | 5′ CCT TCC TAT GAC ATG AAT TCA ATC 3′ | |
| Primer 6 | 5′ CTT TCC ATG ATG TCT TTC TTT CAG G 3′ | 470 bp |
| Primer 9 | 5′ GGA ACA AAG GAA CCT TTA AAT G 3′ | |
There were 12 mutations detected, with $\beta^{CD26/IVS-1-5}$ being the most common identified, followed by $\beta^{CD26/CD35}$ and $\beta^{CD26/IVS-1-2}$, respectively (Table 3). All samples, which were identified to have the $\beta^{CD26/IVS-1-5}$ mutation, were noted to have Hb E/β-thalassemia on Hb electrophoresis. These samples were from 11 Javanese and 1 Chinese patient.

Patient nos. 17 and 18 were siblings and they had the same type of mutation but with different Hb electrophoresis results. Patient no. 18 who had HbE/β-thalassemia had lower Hb, MCV, MCH, and mean corpuscular hemoglobin concentration (MCHC) and higher RDW-CV as compared to patient no. 17 which showed six (6) $\beta$-globin gene mutations, which were homozygous $\text{IVS1nt5}$, IVS1nt5/IVS1nt5 (no paired mutation) 4.4%, heterozygous $\text{IVS1nt5/HbE}$ 9.9%, and heterozygous $\text{IVS1nt5/IVS1nt1}$ 5.4% (14). These results were slightly different from our study findings. This could be due to the different geographical locations of Java, which was West Java whereas our study was conducted in East Java.

A study conducted on 180 adolescent schoolgirls from East Java and West Java (15) found five (5) types of $\beta$-globin gene polymorphism that were $\text{CD2, CD26/HbE, IVS1nt5, IVS2nt16,}$ and $\text{IVS2nt74}$, which were quite similar in findings to our study; nevertheless $\text{CD2, IVS2nt16,}$ and $\text{IVS2nt74}$ were not found in our cohort.

In another recent study on 31 beta-thalassemia patients from East Kalimantan, which predominantly consisted of Javanese (64.5%), the results revealed seven (7) types mutant alleles, which were $\text{CD26/HbE}$ at 48.4%, $\text{IVS-1-5}$ at 14.5%, $\text{IVS-1-2}$ at 12.9%, $\text{CD35}$ at 8.1%, and $\text{IVS-1-1}$ at 6.5%. These were partially comparable to our results especially $\text{CD26/HbE, IVS-1-2,}$ and $\text{IVS-1-5}$ (16).

In comparison to other studies, our results revealed more mutations. It may reflect the variation of $\beta$-globin mutation in the East Java population, since Dr. Soetomo Hospital is the biggest hospital in East Java and has become a referral hospital in East Java and eastern Indonesia. The results are fairly similar because West Java, Central Java, East Java, and the Special Region of Yogyakarta are all on one island, Java. Although East Kalimantan is part of Borneo island, i.e., a different island, the research was conducted in an area where the population is immigrants from Java, thus the results are almost the same.

Interestingly, 2 patients in our study were siblings (Table 4), and the CBC results in the sibling with HbE/β-thalassemia were much reduced than the sibling who had only β-thalassemia. This may be due to the fact that those with compound heterozygotes $\beta$-thalassemia/HbE have more severe clinical manifestations than those having only $\beta$-thalassemia. While one of them had no HbE detected in the Hb electrophoresis, both genotypes are identical.

### Table 3

| No | Genotype | Number of patients | Frequency (%) |
|----|----------|--------------------|---------------|
| 1  | $\beta^{CD26/IVS-1-5}$ | 12 | 36.4 |
| 2  | $\beta^{CD26/CD35}$ | 6 | 16.2 |
| 3  | $\beta^{CD26/IVS-1-2}$ | 3 | 9.1 |
| 4  | $\beta^{CD26/IVS-1-1}$ | 1 | 3 |
| 5  | $\beta^{IVS-1-5/\beta^{CAP+1}}$ | 1 | 3 |
| 6  | $\beta^{IVS-1-5/\beta^{CD35}}$ | 1 | 3 |
| 7  | $\beta^{CD26/CD35}$ | 1 | 3 |
| 8  | $\beta^{CD26/CD35}$ | 1 | 3 |
| 9  | $\beta^{CD26/IVS-1-2}$ | 2 | 6.1 |
| 10 | $\beta^{CD26/IVS-1-1}$ | 1 | 3 |
| 11 | $\beta^{IVS-1-5/\beta^{CD19}}$ | 1 | 3 |
| 12 | $\beta^{IVS-1-5/\beta^{CAP+1}}$ | 2 | 6.1 |
M, male; F, female. GenBank accession number was obtained after depositing the nucleotide sequences into GenBank. The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession numbers can be found below: https://www.ncbi.nlm.nih.gov/genbank/, ON584430–ON584462.

Noteworthily, there was no mutation detected in one patient via Sanger sequencing. Nevertheless, this patient was transfusion dependent thus indicating a severe form of thalassemia. This finding highlights the limitation of this study as it only targeted exons 1–3 of the HBB gene. It is also not feasible to sequence all 20 exons of this gene via Sanger sequencing. Therefore, this scenario creates a need for a more effective and high throughput technology in the form of Next-Generation Sequencing (NGS) technology, which would greatly aid in unraveling the entire spectrum of beta-thalassemia mutations in the Indonesian population. Furthermore, a larger cohort would give a more accurate picture of the underlying molecular spectrum of mutations in patients with TD and NTD beta-thalassemia in Indonesia.

**CONCLUSION**

The underlying genetic variations are heterogeneous in patients with TDT of East Java where there were 12 variants found, the most common of which is $\beta^{CD26/IVS-1-5}$, found in all patients with Hb E/beta-thalassemia detected via Hb electrophoresis.
The results of this study provide data that may be useful for the prevention and control strategies of thalassemia in Indonesia.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession numbers can be found below: https://www.ncbi.nlm.nih.gov/genbank/: ON584430, ON584431, ON584432, ON584433, ON584434, ON584435, ON584436, ON584437, ON584438, ON584439, ON584440, ON584441, ON584442, ON584443, ON584444, ON584445, ON584446, ON584447, ON584448, ON584449, ON584450, ON584451, ON584452, ON584453, ON584454, ON584455, ON584456, ON584457, ON584458, ON584459, ON584460, ON584461, ON584462.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of RSUD Dr. Soetomo with No.: 0224/KEPK/VII/2021. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

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AUTHOR CONTRIBUTIONS

YH designed and wrote the manuscript. YS collected samples and processed until DNA extraction, then sent to UGM. YI assisted in handling Hb electrophoresis, complete blood count. PR wrote the proposal to obtain funding. MA arrange for recruitment of pediatric patients. NY proofread and assisted with corrections until final version of manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The authors thank the Faculty of Medicine Universitas Airlangga for funding the research, granted by Decree of the Chancellor of Airlangga University Number 212/UN3/2021.

ACKNOWLEDGMENTS

The authors would like to thank all the patients who participated and the hospital staffs who have rendered their assistance and contributions to this study.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
The handling editor ZAL declared a past co-authorship with the author NY.

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