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Molecular Study in Identifying Genotypes to Phenotypes Relations of Transfusion-Dependent Thalassemia Patients in Cirebon, West Java

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Abstract. Molecular characterization is important prior to clinical management as it can provide additional information for the clinical management of patients. This study aims to characterize the most common mutation and identify genotype-to-phenotype relations in transfusion-dependent thalassemia patients. A total of 30 transfusion-dependent patients were recruited who had never undergone thalassemia detection. Peripheral blood samples were collected and analyzed for hematological parameters, hemoglobin component, and *HBA* and *HBB* gene mutation analysis. The most common mutation in the *HBB* gene was IVS1-5 (41.7%) and CD26/HbE (23.3%), with homozygous IVS1-5 (23.3%) and IVS1-5/HbE (30%) as the most common genotype. The study revealed a genotype and phenotype correlation of the most common thalassemia mutations in Cirebon, West Java, Indonesia, with four alleles dominating the genotype, covering 88.4% of the population. A significant difference in HbA2+HbE and HbF levels was observed between homozygous β-thalassemia and β-thalassemia/HbE. Homozygous β-thalassemia with α-thalassemia trait has better hematological parameters compared to homozygous β-thalassemia and β-thalassemia/HbE but does not translate to a better severity index. Characterizing the most common thalassemia mutations in the Indonesian population can streamline the subsequent diagnostic approaches by focusing on the small range of predominant alleles instead of a wide range of alleles, which can provide critical data for better patient management.

Keywords: Genotype-phenotype; HBA; HBB; Mahidol severity index; Thalassemia

1. Introduction

Thalassemias are autosomal recessive disorders marked by quantitative defects in globin chain synthesis. This disease can be further classified based on the globin chain(s) affected by defects in synthesis, whereby defects in the α-globin chain cause α-thalassemia while defects in the β-globin chain cause β-thalassemia (Piel & Weatherall, 2014; Nienhuis & Nathan, 2012).

Thalassemia is one of the most common inherited hemoglobin disorders globally (Taher et al., 2018) and is currently a public health concern affecting Indonesia's multi-

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ethnic population. Studies have shown that 3-8% of the 264 million population are carriers of the thalassemia gene, with an estimated 6000 individuals born each year with transfusion-dependent Thalassemia (Fucharoen & Weatherall, 2016).

In thalassemia, emphasis should be given to the severity of the clinical appearance and the complexity of the genetic background associated with the disease. More than 100 types of α-thalassemia with over 800 types of mutations and structural variants of the α-globin gene have been identified. And 300 types of β-thalassemia with over 900 types of mutations and structural variants of the β-globin gene have been reported that lead to various clinical states because of the varying arrangements of compound heterozygous alleles (Farashi & Harteveld, 2018; Thein, 2018; Giardine et al., 2014). Clinical features of these conditions reflect a wide range of transfusion requirements ranging from transfusion-independent to lifelong, regular blood transfusions (Viprakasit & Ekwattanakit, 2018).

Thalassemia screening in Indonesia relies on general practitioners to promptly identify the possibility of thalassemia or hemoglobinopathy. And the traits while ruling out iron deficiency as the first differential diagnosis. Identifying microcytic anemia using blood analyzers and utilizing the Mentzer index were used as early screening. Once referred to specialist centers, the diagnosis is usually followed up with hemoglobin analysis and DNA analysis, although both services are only available in larger cities and remain scarce in smaller cities and rural areas (Wahidiyat et al., 2020). Diagnosed patients receive blood transfusion according to their hemoglobin levels (usually <7.0 g/dL) on an average of every 2-4 weeks, depending on the need to maintain hemoglobin levels at 9-10.5 g/dL. Patients also receive iron chelation therapy to treat iron overload and prevent cardiac hemosiderosis.

Since the diversity of the clinical spectrum is closely related to the variety of mutations, it is important to perform molecular characterization of the genotype to gain information on disease severity, expected transfusion requirement, and chelation therapy before clinical management for thalassemia. In current existing clinical setups in Indonesia, most diagnoses of thalassemia are based on data obtained from clinical findings, erythrocyte morphology, and hemoglobin levels rather than molecular characterization.

Therefore, this study aims to investigate the genotype-to-phenotype relationship of these mutations by studying the hematological parameters, hemoglobin typing, and disease severity. This study will show the most common mutations, allelic and genotypic frequency, and genotype-phenotype correlation of the mutations as disease severity of transfusion-dependent patients enrolled at Cirebon, West Java, Indonesia.

2. Methods

2.1. Patient Selection

Thirty transfusion-dependent thalassemia patients were enrolled at the Hematology, Oncology, and Thalassemia Clinic, Gunung Jati Regional Public Hospital in Cirebon. None of the patients have previously undergone molecular analysis for thalassemia detection. Written informed consent was obtained from all participants prior to enrolment. A written consent was obtained from their parents or guardians for underaged patients. The Institutional Review Board of Faculty of Medicine Universitas Swadaya Gunung Jati, Cirebon, Indonesia, approved the study protocol and followed the ethical principles of the Declaration of Helsinki of 1975 and its revision. All patients were previously classified as thalassemic based on hemoglobin levels and clinical appearance. There were 12 males and 18 females, with an average age of 12.77 years. Patient clinical history was recorded along with the age of onset, age at first blood transfusion, transfusion requirements, spleen size, height, and weight.

2.2. Hematological and Biochemical Analysis

Hematological analysis was carried out using Sysmex XN-1000 Automated Hematology Analyzer (Sysmex Corporation, Kobe, Japan). Hemoglobin component analysis was determined using the BioRad Variant II HPLC system (Bio-Rad Laboratories Inc, Hercules, CA, USA) according to the manufacturer's instructions.

2.3. Genetic Analysis

Peripheral blood samples were collected in EDTA. Genomic DNA extraction was carried out using the spin-column method. Mutations in the *HBA* gene were analyzed using multiplex GAP-PCR which detects $-\alpha^{3.7}$ (NG_000006.1: g.34164_37967del3804), $-\alpha^{4.2}$, $--^{SEA}$ (NG_000006.1:g.26264_4556 4del19301), $--^{THAI}$ (NG_ 000006.1:g.10664_44164del33501) mutations as previously described (Chong et al., 2000a; Chong et al., 2000b). The mutations in the *HBB* gene were analyzed using the Reverse Dot Blot method to simultaneously detect the 10 most common Thai *HBB* gene mutations [-28 (A>G), CD17 (HBB:c.52A>T); CD19 (HBB:c.59A>G), CD26 (HbE) (HBB:c.79G>A), IVS1-1 (G>T) (HBB:c.92 + 1G>T), IVS1-5 (G>C) (HBB:c.92 + 5G>C); CD35 (HBB:c.35C>A), CD41/42 (HBB:c.127_130delCTTT), CD71-72 (HBB:c.216_217insA), and IVS2-654 (HBB:c.316-197C>T)] (Sutcharitchan et al., 1995).

2.4. Disease Severity

The disease severity was classified into mild, moderate, or severe using the Mahidol Severity Index by measuring the hemoglobin level at steady state, age at thalassemia presentation, age at receiving a first blood transfusion, requirement for blood transfusion, size of the spleen, and growth and development (Sripichai et al., 2008).

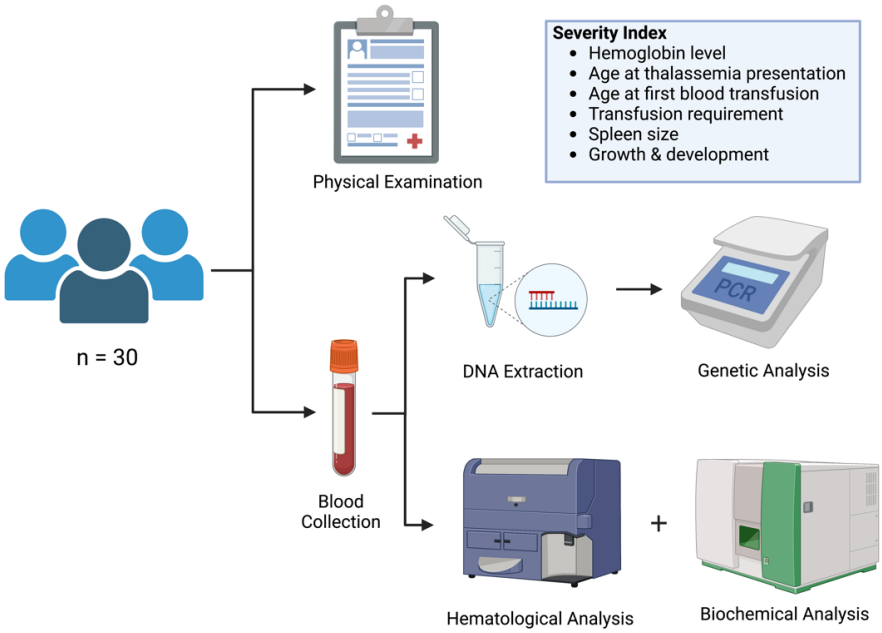

Figure 1 Workflow schematic

3. Results and Discussion

3.1. Patient Characteristics

The thalassemia patients in this study have an average age of 12.77 years old 20 of them have an age of onset below three years old (67%), and 23 subjects have an age of first blood transfusion below three years old (77%) as shown in Table 1.

Table 1 Characteristics of transfusion-dependent thalassemia patients in Cirebon

		Frequency	%
Average age (years)	12.77 (± 6.99)		
Sex	Male	12	40%
	Female	18	60%
Age at onset	<3 years old	20	67%
	3-10 years old	9	30%
	>10 years old	1	3%
Age at first blood transfusion	<5 years old	23	77%
	5-10 years old	6	20%
	>10 years old	1	3%
Transfusion requirement	>2x/year	30	100%
	1-2x/year	0	0%
	<1x/year	0	0%
Spleen size	<3 cm	15	50%
	3-10cm	14	47%
	>10cm	1	3%
	Splenectomized	0	0%
Severity Index	Normal	0	0%
	Moderate	8	26.7%
	Severe	22	73.3%

All the patients underwent a hyper-transfusion regime, receiving more than two blood transfusions in a month. The transfusion regime aims to keep hemoglobin levels above ten g/dL. The hyper-transfusion regime, however, has a long-term side effect of iron overload. Iron is required for viability and utilized in heme formation and is central in functioning hemoglobins, the essential oxygen-carrying molecule in all vertebrates. In contrast, free iron is extremely cytotoxic as it can form reactive superoxide radical (O^{2-}) or hydrogen peroxide, generating the hydroxyl (•OH) radical. Which can oxidize biological macromolecules. Including lipids, proteins, and DNA leading to cellular damage (Leecharoenkiat et al., 2016). Humans carry around 3-5 g of iron in various forms, and since iron has low bioavailability, humans have evolved to be highly efficient in conserving iron with no mechanism of excreting excess iron under conditions of iron overload (Lawen & Lane, 2012). Humans only excrete 1-2 mg of iron daily, which is less than 0.1% of the body's total iron, which is replenished through dietary sources. Meanwhile, a unit of transfused red blood cells contains around 200mg of iron, and in the absence of iron chelation, the iron will continuously accumulate and result in iron overload (Nienhuis & Nathan, 2012). Iron overload, in turn, will lead to irreversible organ damage, such as cirrhosis, diabetes, heart disease, and hypogonadism (Leecharoenkiat et al., 2016).

To counter the effect of iron overload, thalassemia patients rely heavily on the continuous use of iron chelators to aid the excretion of excess iron. There are three significant iron chelators mainly used in Indonesia, namely deferoxamine (DFO), deferiprone (DFP), and deferasirox (DFX) (Wahidiyat et al., 2020). Though these drugs are widely available, the distribution and continuous supply of these drugs remain a problem in smaller cities.

In terms of disease severity, most patients fall under the severe phenotype base in the Mahidol Severity Index (73.3%), followed by moderate severity (26.7%), with no patients

having the normal phenotype. This clinically means that all subjects have to be monitored for their iron chelation therapy, growth retardation, and spleen and liver enlargement to ensure a higher quality of life.

3.2. Genotypic Data

The *HBA* mutation analysis (Table 2) showed 28 normal *HBA* with two patients with -$\alpha^{3.7}$ mutation, making -$\alpha^{3.7}$ the only mutation detected in the patient population. The *HBB* mutation analysis (Table 3) yielded eight alleles and nine genotypes. The most common allele was IVS1-5 (46.7%), a mutation in the consensus splice site in the first intron causing reduced splicing at the mutated donor site (Malik & Tisdale, 2017), followed by hemoglobin E, a mutation in codon 26 of *HBB* (31.7%), CD41/42 (5%), and CD15 (5%) respectively. This finding is similar to previous studies conducted in Indonesia for *HBB* mutations, where IVS1-5 and HbE were generally found throughout Indonesia (Susanto et al., 2020; Wahidiyat et al., 2020; Rujito et al., 2015; Hernanda et al., 2012), while CD41/42 and CD15 found in this study were mutations specific to Cirebon ethnicity.

Table 2 Allele frequency and genotype distribution for α-thalassemia gene defects

Mutation types		Frequency	%
HBA Alleles	-$\alpha^{3.7}$	2	3,3%
	αα (normal)	58	96.7%
HBA Genotypes	-α3.7/αα	2	6.7%
	αα/αα (normal)	28	93.3%

Table 3 Allele frequency and genotype distribution for β-thalassemia mutations

Mutation types		Frequency	%
HBB Alleles	IVS1-5	28	46.7%
	CD26 (HbE)	19	31.7%
	CD41/42	3	5%
	CD15	3	5%
	IVS1-1	1	1.7%
	IVS1-1 (G>A)	1	1.7%
	IVS2-654	1	1.7%
	Unknown	4	6.7%
HBB Genotypes	IVS1-5/CD26	13	43.3%
	IVS1-5/IVS1-5	7	23.3%
	CD41/42/CD26	3	10%
	IVS2-654/CD26	1	3.3%
	CD15/CD15	1	3.3%
	IVS1-5/IVS1-1	1	3.3%
	IVS1-1(G>A)/CD26	1	3.3%
	CD15/CD26	1	3.3%
	Unknown	2	6.7%

From the *HBB* alleles, the most common *HBB* genotypes were IVS1-5/CD26 (43.3%), followed by IVS1-5/IVS1-5 (23.3%), and CD41/42/CD26 (10%), and there were 2 subjects with unknown *HBB* mutations. This finding can be used to develop a more tailored method for screening and diagnosis, focusing on the most commonly occurring alleles as opposed to rarely occurring alleles (Hassan et al., 2013; Bhardwaj et al., 2005; Varawalla et al., 1991). These four major *HBB* mutations cover 88.4% of the subjects; developing a quadruplex PCR to screen and diagnose thalassemia can simplify and hasten the diagnostic approach while being cost-effective.

3.3. Genotypic and Phenotypic Correlation

From the *HBA* and *HBB* mutation analysis, the genotypes can be categorized into four main groups (Table 4), with β-thalassemia/hemoglobin E genotypes as the dominant

genotype (60%), followed by the homozygous β-thalassemia (26.6%), and two unknown mutations. To establish the genotype-phenotype relations in transfusion-dependent thalassemia patients in Cirebon the red blood cell indices between the thalassemia genotypes were compared unknown excluded (Table 5). The difference between hemoglobin levels, MCV, MCH, RDW, and hemoglobin A, and disease severity were not statistically significant between the homozygous β-thalassemia and β-thalassemia/hemoglobin E genotypes. In contrast, the HbA2+HbE and HbF levels between the homozygous β-thalassemia and β-thalassemia/hemoglobin E genotypes were significant. The β-thalassemia/hemoglobin E has a higher HbA2+HbE percentage (20.28 ± 16.05 vs. 9.91 ± 10.23) compared to their homozygous β-thalassemia counterparts. Hemoglobin E is a common structural hemoglobin variant arising from a mutation in the HBB gene and can be detected with hemoglobin electrophoresis (Fucharoen & Weatherall, 2012). This abnormal hemoglobin is not present in the homozygous β-thalassemia and can be used as a diagnostic tool to differentiate between the two genotypes.

Table 4 Thalassemia genotypes of the transfusion-dependent thalassemia patients

Thalassemia genotype	Frequency	%
Homozygous β-thalassemia	8	26.6%
β-thalassemia/CD26 (HbE)	18	60%
Homozygous β-thalassemia with α-thalassemia trait	2	6.7%
Unknown	2	6.7%

Table 5 Red Blood Indices for homozygous β-thalassemia and Blood Indices β-thalassemia/CD26 (HbE)

Parameter	Homozygous β-thalassemia (n=8)	Homozygous β-thalassemia with α-thalassemia trait (n=2)	β-thalassemia/CD26 (HbE) (n=18)
Hb Level (g/dL)	9.27 ± 2.21	10.30 ± 0.42*	9.32 ± 1.48
MCV (fL)	77.30 ± 6.65	80.50 ± 2.12*	73.89 ± 6.82
MCH (pg)	24.90 ± 3.14	25.50 ± 0.71	23.06 ± 2.88
RDW (%)	18.21 ± 7.48	15.15 ± 3.75	20.36 ± 6.55
HbA	74.35 ± 10.89	80.35 ± 2.62*	61.81 ± 20.81
HbA2+HbE (%)⁺	9.91 ± 10.23*	5.05 ± 3.04*	20.28 ± 16.05*
HbF (%)	4.48 ± 3.49*	2.00 ± 0.42*	8.72 ± 9.16*
Mahidol Severity Index	8.0 ± 0.82	8.5 ± 0.71	7.8 ± 0.77

Hb = Hemoglobin, **MCV** = Mean Corpuscular Volume, **MCH** = Mean Corpuscular Hemoglobin, **RDW** = Red-cell Distribution Width

⁺ Hemoglobin A2 and Hemoglobin E on the same value in the HPLC system (Variant II)

* $p < 0.05$

The HbF levels between the homozygous β-thalassemia and β-thalassemia/hemoglobin E genotypes were also significant. The HbF level of the β-thalassemia/hemoglobin E is higher than the homozygous β-thalassemia (8.72 ± 9.16 vs. 4.48 ± 3.49). The β-thalassemia/hemoglobin E genotype is often associated with higher levels of HbF and can reduce the severity of the disease. This is due to the results of XmnI +/+ polymorphism genotype that is often co-inherited with the β-thalassemia/hemoglobin E genotype (Kesornsit et al., 2018; Rujito et al., 2016). To date, several drugs, such as thalidomide/lenalidomide, sirolimus, ruxolitinib/pacritinib (JAK2 inhibitors), and luspatercept/sotatercept (activin receptor-II trap ligands), have been used to boost the production of HbF in thalassemia patients with hope to alleviate severity. Hydroxyurea was the first drug to be approved by the FDA and has been shown to increase the expression of HbF by 2- to 9-fold, while luspatercept, a recently FDA-approved drug, inhibits over-

activated SMAD signaling proteins in the erythroid precursors (Oikonomidou & Rivella, 2018; Taher et al., 2018; Soni, 2017).

An interesting finding in genotype-phenotype was observed in the co-inheritance of homozygous β-thalassemia with the α-thalassemia trait. The RBC indices showed better parameters and borderline normal indices when compared to the homozygous β-thalassemia and β-thalassemia/hemoglobin E genotypes. It had higher hemoglobin values, higher MCV and MCH, lower RDW, higher HbA, and lower HbA2+HbE levels akin to normal phenotype. Co-inheritance with α-thalassemia often improves the phenotypes due to reduced unpaired α-globin to match the reduced or absent production of β-globin (Taher et al., 2018). However, the alpha trait in this study did not appear to alleviate the severity, a similar observation that had also been reported previously (Svasti et al., 2002).

Among the three groups, the results from this study showed a genotype-to-phenotype correlation, especially between the homozygous β-thalassemia and β-thalassemia/HbE. This information can be used as a tool for early prediction of the clinical severity of the disease, assisting in early intervention in children born with the disease, and promoting prevention through genetic counseling. Newborn screening for specific alleles of Thalassemia in Indonesia can help control the number of newborns with thalassemia in the Indonesian population.

4. Conclusions

The study revealed a genotype and phenotype correlation of the most common thalassemia mutations in Cirebon, West Java, Indonesia, with four alleles dominating the genotype, covering 88.4% of the population. A significant difference in HbA2+HbE and HbF levels was observed between homozygous β-thalassemia and β-thalassemia/HbE. Homozygous β-thalassemia with α-thalassemia trait has better hematological parameters compared to homozygous β-thalassemia and β-thalassemia/HbE but does not translate to a better severity index. The hematological data and clinical appearance are closely linked to the type of mutation. Molecular analysis can provide a multitude of information regarding the disease, which can help in the clinical management of patients, including fine-tuning the blood transfusion requirements and reducing iron overload. Identification of mutation alleles in specific regions can be used to predict clinical manifestation and severity better and develop a more efficient screening and diagnostic method based on the most common alleles that cover most of the mutations found.

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