

Hemoglobin E Allele Screening in Adolescent Girls at Kepanjen and Gondanglegi Districts, Malang Regency, East Java-Indonesia

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Abstract— Woman in reproductive age have higher risk of iron deficiency anemia. However, there are other factors than iron deficiency that can cause anemia, such as genetic blood disorders or hemoglobinopathies. Hemoglobin E (HbE) is one of common blood disorders in Indonesia. HbE carriers have higher risk to have offspring inheriting HbE double mutation with other kinds of mild or severe β -thalassemia. This research has the purpose to determine allele frequency of HbE in adolescent girls in Kepanjen and Gondanglegi districts, Malang regency. The research started with hematological screening of venous blood samples from the participants. As many as 70 blood samples underwent DNA sequencing to detect the mutation. From the sequencing results, there were 8 individuals detected with HbE single mutation and one individual with HbE/ β^+ -thalassemia double mutation. The individual with HbE/ β^+ -thalassemia had a hemoglobin level of 7.5 g/dL and mean corpuscular volume (MCV) of 59 μm^3 indicating moderately severe HbE/ β^+ -thalassemia. The clinical picture is caused by double mutation, one at codon 26 (HbE) and the other one at IVS1-5 (β^+ -thalassemia). Both mutations cause disturbance in the pre-mRNA splicing process resulting in non-functional mRNA and a decrease in normal β -globin (β^N) production along with higher production rate of E-mutated β -globin (β^E). HbE allele frequency in adolescent girls in Kepanjen and Gondanglegi districts is 20%.

Keywords— β -thalassemia, hemoglobin E, HbE/ β^+ -thalassemia

I. INTRODUCTION

Anemias are body conditions that lack of functional red blood cells. Anemias can be caused by insufficient nutrition intake (malnutrition), genetic disorders, or infections. The most common type of malnutritional anemia found in Indonesia is iron deficiency anemia [1].

Women, especially at reproductive age, have higher risk of iron deficiency than men, because they need to replenish the iron loss caused by menstruation [2]. However, adequate iron intake may not be able to ameliorate other forms

of anemia, i.e., non-iron-deficiency anemias caused by infections, chronic diseases, or genetic disorders [3]. The most common anemias caused by genetic disorders are thalassemias with at least 1 out of 100.000 individuals around the world and a prevalence in Indonesia of 3-10% [4,5].

Thalassemia is a condition in which the human body cannot produce fully functional hemoglobin (Hb). There are many variations based on its gene defects, both in α - and β -thalassemias. A common type in South East Asia is hemoglobin E syndrome (β -thalassemia with codon 26 mutation). Hemoglobin E can be stated as a hemoglobin variation, but is also categorized into thalassemia minor [6]. Individuals who have hemoglobin E heterozygote or homozygote mutations usually have a microcytic condition, with or without anemia [3]. Therefore, most carriers of hemoglobin E mutations are asymptomatic, but their offspring have a high risk of fatal mutations, i.e., HbE combined with another type of thalassemia mutation [3], which will then show symptoms of moderate to severe thalassemia.

According to their clinical severity, thalassemias are classified into minor (or trait), intermediate, and major. Individuals with β -thalassemia major, especially in some developing countries, are not treated properly due to the lack of resources. Patients often have growth retardation, poor musculature, bone and joint deformities like *genu valgum*, hepatosplenomegaly, and acute or chronic anemic condition [4]. To manage those medical complications, estimated healthcare costs are around 200-300 million IDR/year [7]. Therefore, couples who wish to have children participate in thalassemia prevention programs such as thalassemia screening to minimize the risk of having thalassemia major offspring [7]. Moreover, prevention is much cheaper than healthcare. Because of the lack of thalassemia data in Indonesia, hemoglobinopathy screening should be conducted nation-wide to estimate the prevalence of HbE and thalassemia carriers.

This research has a purpose to determine the allele frequency of HbE in adolescent girls in Kepanjen and Gondanglegi, Malang District.

II. RESEARCH METHODS

A. Blood Sampling

Blood sampling was conducted in two randomly chosen subdistricts of Malang District, East Java, i.e., Kepanjen, 20 km south of Malang and Gondanglegi, located east of Kepanjen. Three high schools were also randomly chosen, SMAN 1 Kepanjen, SMA Islam Kepanjen, and SMAN 1 Gondanglegi. Inclusion criteria were: age between 15 and 19 years, living in Malang District, willing to participate in the sampling, healthy, not pregnant, not fasting, and not menstruating.

B. First and Second Stage of Screening

In blood sampling, two stages were differentiated. The first stage was screening the anemic status and collecting anthropometric data. Using the hypothesis test for two populations (anemic and non-anemic), minimum sample size for the screening was calculated 334 respondents to obtain minimum of 76 anemic respondents based on the anemia prevalence in Indonesia [8].

In the second stage venous blood (3 mL) was drawn from 116 respondents randomly taken from the screening list. Whole blood samples went through complete hematology and blood count test. Subsequently, whole blood samples were stored at 4°C until used for molecular analysis.

C. Beta-Globin Gene (HBB) Amplification

The genome was isolated from whole blood samples and β -globin genes (HBB) amplified with polymerase chain reaction (PCR). Designed primers for human subunit HBB gene were: forward primer 5'-tagcaatttgactgatgtaggg-3' and reverse primer 5'-ttcccaagggttgaactagctctt-3'. Reagent composition used for PCR is listed in Table 1 and PCR condition in Table 2. PCR was done with Thermal Cycler™ (Applied Biosystems), the results then analyzed with agarose gel electrophoresis, ethidium bromide (EtBr), and UV transilluminator for visualization process.

D. HBB Gene Band Confirmation

To separate and confirm the desired gene bands, agarose gel (1%) electrophoresis was applied. The samples were mixed with loading dye 6X. TAE electrophoresis buffer 1X was used. Electrophoresis were run at 100 volts for 60 minutes. For comparison of DNA molecular weight, Kapa™ Universal Ladder was used. Electrophoresis results were visualized with EtBr staining under UV light.

E. Sequence Analysis

Beta-globin gene amplification results went through sequence analysis to detect the existence of mutations at codon 26. We used DNA sequencing service from First Base Company, the results were then interpreted with a specific software for detection of the mutations.

TABLE 1 Reagent Composition for PCR

Composition	Initial Concentration	Final Concentration	Final Volume (μL)
Nuclease-free Deion. Water	-	-	9.5
Kapa 2G Fast PCR Mix	10X	1X	12.5
Forward primer	10μmol	1 μmol/mL	1
Reverse primer	10μmol	1 μmol/mL	1
Template DNA	< 250 ng		1
Total Volume (μL)			25

TABLE 2 PCR Condition for Amplification

Stages	Temperature (°C)	Duration
Denaturation	94	5'
Amplification 40 cycles	Denaturation	2'
	Annealing	30''
	Elongation	1'
Final Elongation	72	7'
Storage	4	∞

III. RESULTS AND DISCUSSION

A. Respondent Screening Result

This research is part of a thesis research entitled *Food-Based Recommendations Using Linear Programming Approach For Combating Anemia Among Adolescent Schoolgirls in Rural Malang District, East Java Province, Indonesia* focusing on the prevention of iron deficiency anemia due to an unhealthy diet [9].

In the first stage of screening, there were 72 anemic respondents (Hb levels < 12 g/dL). Only 43 anemic respondents agreed to participate in venous blood draw of the second stage. Apart from these, 53 samples from non-anemic respondents were drawn, a total of 96 samples for genomic isolation. From these, 70 samples were amplified and sequenced. The sequence result of 63 samples could be interpreted, the other 7 samples were too damaged. Based on the sequence results, there were 55 individuals having no HbE mutations, 8 HbE carriers, and one individual with double mutations, HbE/β⁺-thalassemia. Sequence results with mutant individuals are shown in Table 3.

B. Hemoglobin E

By definition HbE is a variation of hemoglobin structure and HbE carriers have diverse phenotypes, some individuals are asymptomatic, while others show β -thalassemia minor symptoms. Diversity is the result of single nucleotide polymorphism (SNP) [10] and the appearance of varying phenotypes may depend on the amounts of erythroid precursors [3]. Some HbE carriers may exert phenotypes similar to β -thalassemia minor with low mean corpuscular volume (MCV), low mean corpuscular hematocrit (MCH), and low threshold value of HbA₂ [3].

HbE mutations occur in HBB gene codon 26 where guanine is substituted by adenine (G→A). The mutation causes an alternative splicing site at codon 26 [11]. If mRNA splicing occurs at codon 26, it will produce a cryptic splicing site which generates non-functional mRNA. Cryptic splicing site at codon 26 will cause a part of exon 1 to be cut out in the splicing process resulting in unstable β -globin because of the absence of some amino acids in protein synthesis [12].

C. Beta-Thalassemia

There are two types of β -thalassemia, β^+ -thalassemia and β^0 -thalassemia. The former is a condition of decreased normal β -globin synthesis. By contrast, individuals with β^0 -thalassemia do not have normal β -globin in the affected allele [4]. We found one individual with HbE/ β^+ -thalassemia double mutation (Table 3); the individual inherited both heterozygotes from each mutation. The type of β^+ -thalassemia mutation detected in this individual is IVS1nt5, a mutation that occurs at intron 1. The cause of the mutation is a substitution between guanine and cytosine (G→C). This type of β^+ -thalassemia mutation can suppress the efficiency of the normal splicing process which results in appearance of a cryptic splicing site because of its position close to the splice junction. The IVS1nt5 mutation can reduce normal β -globin synthesis, consistently [3].

Heterozygote HbE mutation that interferes with heterozygote β^+ -thalassemia (IVS1nt5) mutation may result in a more visible phenotype compared to HbE carriers. It can be seen in Table 3 that the individual with HbE/ β^+ -thalassemia has low MCV, MCH, and hemoglobin values, which may be caused by hemolysis or ineffective erythropoiesis [3]. Hemolysis and ineffective erythropoiesis happen because of relative over-expression of α -globin chains. The latter precipitate as hemichrome (denatured hemoglobin), finally forming inclusion bodies [3]. In addition to forming inclusion bodies, precipitation of free α -globin chains can cause structural and functional alterations of the red cell membrane which effect stability, deformability, and hydration of the erythrocytes [3]. The alterations may result in rigid, inflexible and fragile erythrocyte membranes [13].

D. Hemoglobin E Allele Frequency

Based on the research data, the number of HbE carriers is 8 individuals out of 70 respondents. Using Hardy-Weinberg equilibrium method, heterozygote HbE allele frequency in adolescent girls in Kepanjen and Gondanglegi subdistricts is 20% while the prevalence of HbE carriers is 11.4% and the

prevalence of HbE/ β^+ -thalassemia in these adolescent girls is 1.4%.

TABLE 3 Sequence Result of Mutant Individuals

ID	Hb [g/dL]	RBC [10 ⁶ / μ L]	MCV [fL]	MCH [pg]	Status
0101016	11.2	-	-	-	Hb E
0101021	11.2	-	-	-	Hb E
0101076	12.7	4.74	80	26.8	Hb E
0101123	14.9	5.42	84	27.4	Hb E
0102005	14.5	4.79	87	30.3	Hb E
0102017	14.2	4.9	85	29.1	Hb E
0102032	11.9	4.28	88	27.8	Hb E
0203041	7.5	5.31	59	14.2	Hb E/ β^+ -Thalassemia
0203086	12.0	5.26	76	22.8	Hb E

IV. CONCLUSION

The number of HbE carriers, based on the screening results, is 8 from 70 people and the prevalence is 11.4% with allele frequency of 20%. There is one individual with HbE/ β^+ -thalassemia double mutation, i.e., prevalence of 1.4%.

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