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THE ROLE OF COMPLETE BLOOD COUNT IN THE DIAGNOSIS OF HEMOGLOBIN E IN PSEUDO HIGH LEVEL OF HEMOGLOBIN A_2

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ABSTRACT

Aim: *To evaluate the usefulness of complete blood count (hemoglobin concentration, MCV: mean corpuscular volume, and MCH: mean corpuscular hemoglobin), in diagnostic of hemoglobin E (HbE) when hemoglobin analysis showed pseudo high level of HbA_2.*

Methods: *Secondary data were obtained from laboratory records of patients who were referred to the GenNeka Genetic Clinic, Eijkman Institute for Molecular Biology, Jakarta, for thalassemia screening. The patients employed were heterozygous HbE, compound heterozygous HbE with other β-thalassemia mutations, homozygous HbE, and co-inheritance with α-thalassemia, that was confirmed by molecular analysis. Complete blood count (CBC) was examined using an automated blood cell analyzer. HbA_2 level was measured by HPLC. Molecular analysis was performed by PCR-RFLP method. Hemoglobin concentration, MCV, and MCH values will be presented as a mean or median, and will be compared between heterozygous HbE and compound heterozygous HbE. A comparison was also done between males and females in the heterozygous HbE group. Homozygous HbE and co-inheritance with α-thalassemia will be described as an extrapolation.*

Results: *The hemoglobin concentration, MCV, and MCH values were significantly different between heterozygous HbE and compound heterozygous HbE. Only the hemoglobin concentration and the MCV values were significantly different between males and females in the heterozygous HbE group.*

Conclusion: *CBC is useful in assisting the differentiation of HbE types in misleadingly high HbA_2 levels. The sex of the patient has to be considered in the interpretation of CBC results.*

INTRODUCTION

Beta (β)-thalassemia is a common inherited disease around the world. Throughout history, it is concentrated in malaria endemic areas including Indonesia. However, nowadays it is spreading all over the world because of diaspora and migrations of the world population [1]. The underlying genetic abnormality is a mutation in the β-globin gene causing a decreased or absence

of the β-globin chain that results in a low level of hemoglobin concentration [2]. Hemoglobin E (HbE) is the most common hemoglobin variant in Southeast Asia, including Indonesia, caused by mutation of codon 26 ($GAG^{Glu}\rightarrow AAG^{Lys}$; HBB:c.79 G>A) of β-globin gene that to a certain level will affect the mRNA splicing process [3, 4]. Clinically it can be manifested as a β-thalassemia, in particular in homozygous conditions or compound heterozygous with other β-globin gene mutations [5]. The clinical and hematological manifestations vary greatly, from normal to mild anemia in heterozygote and mild to severe anemia in homozygote and compound heterozygote. The variation of severity of the clinical symptoms affected the management of the disease, including diagnosis and treatment decisions of HbE.

Based on the algorithm proposed by Dacie and Lewis [6], we used complete blood count (CBC) to obtain hemoglobin level, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH) values, and hemoglobin analysis for quantification of the hemoglobin A_2 (HbA_2) level, as the first line tests for the laboratory diagnosis of β-thalassemia. By employing this algorithm combined with the HbA_2 level, it is possible to differentiate β-thalassemia with Hb variants.

At the GenNeka genetic clinic, Eijkman Institute for Molecular Biology, Jakarta, quantification of HbA_2 was carried out using a high performance liquid chromatography (HPLC) method, by which the HbA_2 and HbE will be co-eluted and the HbA_2 level cannot be determined. According to previous reports, total HbA_2 plus HbE level was 24.5 to 31.7% [7, 8]. In our experience, if the total area under the curve of HbA_2 of the chromatogram is around those values, most likely the area consists of HbA_2 and HbE, since HbE is the most prevalent hemoglobin variant in Indonesia. However, there is a possibility that it is not HbE which is co-eluted with HbA_2; other Hb variants, such as Hb Lepore, Hb D-Iran, and Hb Osu-Christianborg could also contribute to the same area under the curve [9]. Depending on the type of Hb variant, the clinical manifestation may vary from mild to severe. Specifically for HbE heterozygote, reports have mentioned that the manifestations are mild [8]. Thus it is important to interpret the HPLC result with additional considerations of CBC, red blood cell morphology, and clinical features.

The aim of this report is to evaluate the usefulness of the hemoglobin concentration, MCV and MCH values, as a simple routine hematology test in the screening of HbE carriers in adults. Separate analysis of the data will also be conducted in each sex. Although the hematological features in HbE have been well documented, this evaluation of the parameters will hopefully give direction in handling of the patients with pseudo high level of HbA_2, since many laboratories in Indonesia use the HPLC method for hemoglobin analysis, though not all laboratories are equipped with facilities to conduct such molecular diagnoses.

MATERIALS AND METHODS

The data of CBC and HbA_2 levels of heterozygote HbE, homozygote HbE, compound heterozygote HbE with other β-thalassemia mutations, and co-inheritance HbE and α-thalassemia were collected as secondary data from laboratory records of the GenNeka genetic clinic, Eijkman Institute for Molecular Biology, Jakarta. They were obtained from adult (≥16 years old) patients who were referred to our clinic for thalassemia screening, from January 2011 to December 2013. The CBC was examined using an automated blood cell analyzer (Cell-Dyn® 1700, Abbot Diagnostic, IL, USA) and confirmed by red blood cell morphology assessment on blood smears using light microscope with 400x magnification. HbA_2 level was measured by HPLC using the VARIANT™ Hemoglobin Testing System (β-thalassemia Short Program, Bio-Rad, Hercules, CA, USA). Only data from patients with a high level of HbA_2 (around or more than 24%) will be included, since it is evidence of the possible occurrence of HbE component in the hemoglobin analysis using this method [10].

A molecular analysis by PCR-RFLP method was performed to detect codon 26 of β-globin gene mutation ($GAG^{Glu}\rightarrow AAG^{Lys}$) as a definite identification of HbE. PCR was done using a pair of primers (forward: ACCTCACCC TGTGGAGCCAC; reverse: CTATTGGTCTCCTTAAACCTGTCTTGTAACC TTGCTA) to amplify 293 base pairs (bp) of the β-globin gene segment that is flanking codon 26. Digestion of the amplicon by *MnlI* restriction enzyme, gives one band of 122 bp in the presence

of β^E mutation or two bands of 60 and 62 bp in the absence of β^E mutation [11]. Other mutations of β-or α-globin gene were detected by methods previously published [11-14]. Particularly, for detection of Cd35 (-C) mutation, β-globin gene was amplified by PCR (primer forward: CTCTTGTTTCCCAAAACCTAATAAGTA; reverse: GGGCCTATGATAGGGTAATAAGACAG) and analyzed by direct sequencing using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

The data were analyzed using RStudio software version 0.98.490© 2009-2013 (RStudio, Inc.). The MCV value and HbA_2 level of heterozygous HbE and compound heterozygote will be presented as a range and mean ±1 standard deviation or median with 50% interquartile. Other cases will be described as an illustration. Comparison of the parameters will be done between heterozygous and compound heterozygous HbE, and males and females of heterozygous HbE.

RESULTS

Data was obtained from the laboratory record of 55 patients (24 males and 31 females, aged 16 to 62 years-old) that showed unusual high levels of (HbA_2 + possible HbE) by HPLC hemoglobin analysis. We divided the patients into 4 HbE types: 41 were confirmed as heterozygous HbE by molecular method to detect the mutation at codon 26 of β-globin gene, 2 homozygotes, 10 compound heterozygotes with other β-globin mutations (IVS1-nt5, IVS2-nt654, Cd35 (-C), Cd 41-42, and Hb Malay), and 2 combined with α-thalassemia ($\alpha^{-3.7}$, α^{-SEA}) (Table 1).

Data of heterozygous HbE (n = 41) and compound heterozygote (n = 10) were further analyzed to deduce the central tendency values. Accordingly, the hemoglobin concentration and the MCH value is presented by mean ± standard deviation, and the MCV is presented by median and 50% interquartile (Table 2). The range of hemoglobin concentration was 9.6-16.8 g/dl, mean 13.0 g/dl in heterozygote, and 4.0-12.1 g/dl, mean 8.1 g/dl in compound heterozygote. The range of MCV was 54.4-84.0 fl (median 74.0 fl) in heterozygote, while in compound heterozygote the range was 56.4-75.6 fl (median 66.3 fl). The range of MCH was 18.4-29.0 pg (mean 24.2 pg) in heterozygote and 17.6-26.1pg (mean 21.9 pg) in compound heterozygote. All parameters are significantly different between heterozygote and compound heterozygote ($p < 0.05$).

Table 1. The composition of patients with hemoglobin E.

Hemoglobin E	Number of patients	Mutation(s)
Heterozygote	41	β^E/β
Homozygote	2	β^E/β^E
Compound heterozygote	10	β^E/β^{thal*}
C-inherited with α-mutation	2	β^E/β, α^{thal**}/α
Total	55	

*β-thalassemia type of mutations: IVS1-nt5 (HBB:c.92+5 G>C), IVS2-nt654 (HBB:c.316-197 C>T), Cd35 –C (HBB:c.108delC), Cd 41-42 (HBB:c.126_129delCTTT), and Hb Malay (HBB:c.59 A>G)

** α-thalassemia type of mutations: $\alpha^{-3.7}$, α^{-SEA}

Table 2. Comparison of hemoglobin concentration, MCV and MCH of heterozygous HbE and compound heterozygous HbE/β-thalassemia.

Parameter	*Reference value	Heterozygote (n=41)		Compound heterozygote (n=10)		p
		Range	Mean/ Median	Range	Mean/ Median	
Hb (g/dl)	Male 14-17.4 Female 12-16	9.6-16.8	13.0 ± 1.8	4.0-12.1	8.1 ± 2.3	< 0.001
MCV (fl)	80-100	54.4-84.0	74.0 (70.8-75.4)	56.4-75.6	66.3 (63.4-70.0)	0.003
MCH (pg)	28-34	18.4-29.0	24.2 ± 1.9	17.6-26.1	21.9 ± 2.9	0.03

Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin.

*Hematology reference values adapted from Mckenzie and Williams [15]

The hemoglobin concentration and MCV between sexes of the heterozygous HbE are significantly different: females were lower than males, as shown in table 3. There is no significant difference in MCH values of both sexes.

Table 3. Comparison of hemoglobin concentration, MCV and MCH of males and females in heterozygous HbE.

Parameter	*Reference value	Males		Females		p
		Range	Mean/ Median	Range	Mean/ Median	
Hb (g/dl)	Male 14-17.4 Female 12-16	10.4-16.8	14.1 ± 2.0	9.6-14.1	12.5 ± 1.0	< 0.01
MCV (fl)	80-100	66.1-84.0	75.2 (74.7 - 76.3)	54.4-82.0	71.9 (70.2 - 74.4)	0.01
MCH (pg)	28-34	21.1-28.0	24.7 ± 1.6	18.4-29.0	23.8 ± 2.1	0.1

Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin.
*Hematology reference values adapted from Mckenzie and Williams [15]

Two cases of homozygous HbE, both female, exhibit a low concentration of hemoglobin (10.0 and 11.0 g/dl), low MCV (61.5 and 63.3 fl) and low MCH (20.2 and 21.0 pg). The levels of HbA_2 are very high at 92.6 and 87.8%.

Two cases of heterozygous HbE that was co-inheritance with α-thalassemia mutation ($\alpha^{-3.7}$, α^{-SEA}) have hemoglobin concentration of 12.0 and 11.6 g/dl, very low MCV (58.7 and 59.4 fl), and low MCH (18.9 and 19.7pg). The levels of HbA_2 are 19.1 and 19.6%. Both are female.

DISCUSSION

Hemoglobin E (HbE) is one of the hemoglobinopathies very commonly found in Indonesia, mostly expressed as a mild to intermediate clinical symptom. In combination with other mutations of β-globin gene, it can be seen as a thalassemia major, a severe clinical manifestation that needs routine transfusions laden with many problems such as cost, transmission of infectious diseases, and iron overload.

Heterozygotes or carriers are clinically normal. Hemoglobin concentration is slightly decreased or even normal at the upper limit of reference value, but the MCV and MCH in most cases are decreased, correlating with the clinical manifestation. The MCV can be used as an index for clinical severity [8]. The microcytic red blood cells are typical for hemoglobinopathies. However, other abnormalities of red blood cells, such as iron deficiency also show similar microcytic morphology [16]. Unfortunately, iron deficiency is also common in Indonesia, producing more problems in thalassemia screening, especially in rural area where the facilities for laboratory diagnostics are negligible. In our clinic a complete panel of β-thalassemia screening consists of CBC, hemoglobin analysis by HPLC, and blood smear evaluation. If necessary, we send the sample to another laboratory for an iron status assessment. Using HPLC for hemoglobin analysis, the HbA_2 and HbE will be eluted at the same fraction. It is important to understand that even though most of the time the HbE will be within that fraction, the results do not guarantee that it is actually HbE.

To verify that it is indeed HbE, several approaches can be applied. A dichlorophenolindophenol (DCIP) test is a simple method that was used by many laboratories to confirm the presence of HbE. Besides the difficulty in reading the result of the reaction, false positive or equivocal results were reported in 14% of individuals with other disorders of globin chain synthesis [17]. Based on our fifteen years of experience in using the VARIANT™ β-thalassemia (Bio-Rad, Hercules, CA, USA), we have noticed that almost all of the samples having pseudo high level of HbA_2 were due to HbE, as proven by PCR-RFLP detection of HbE mutation at codon 26 of β-globin gene ($GAG^{Glu} \rightarrow AAG^{Lys}$). Presently, when the result of HPLC hemoglobin analysis showed unusual high level of HbA_2, we are able to estimate whether it is HbE or other hemoglobin variants, by assessing the CBC results and the blood smear.

The hemoglobin analysis by HPLC that shows an unusual high level of HbA_2 did not agree with β-thalassemia. As we explained before, this is due to co-elution of HbA_2 and other hemoglobin variants, in particular, HbE for the Indonesian population. This evaluation of the usefulness of CBC in the prediction of the type of HbE showed that the hemoglobin concentration, MCV and MCH, are all higher in HbE hetrozygote compare to compound heterozygote. The most significant difference is the hemoglobin concentration ($p<0.001$). In heterozygous HbE the mean

hemoglobin concentration appeared to be within the normal range, although some of the patients showed lower values. This low hemoglobin concentration was commonly observed in patients with infection or other causes associated with the increased reactive oxygen species [5]. On the other hand, in compound heterozygous HbE patients, the hemoglobin level is lower than reference value. The patients were proven to carry β^0- or severe β^+- thalassemia mutation that cause absence or decrease in hemoglobin production.

Both heterozygous and compound heterozygous HbE showed low level of MCV. However, the MCV of HbE heterozygote was significantly higher than compound heterozygote (74.0 vs 66.3fl; p = 0.003). It has been reported by other investigators that the MCV of heterozygous HbE were ranged between 79 to 89 fl [8]. Our patients' MCVs were within this range, although some of them have MCV less than this value. It is well documented that the MCV values varied among the heterozygotes and also in compound heterozygotes.

The MCH values of heterozygous and compound heterozygous HbE were also significantly different, although the differences are less significant compare to the other two parameters. The mean of MCH value in heterozygous HbE were within the reference range, while the compound heterozygotes were slightly below.

Based on our results, of these three parameters, the hemoglobin level is the most differentiated value between heterozygote and compound heterozygote. Nevertheless, it is well known that there are lots of other factors influencing anemia as indicated by low levels of hemoglobin [18]. The hemoglobin level also does not correlate with the size of the cells, whether they are microcytic or normocytic. Therefore, hemoglobin alone cannot be used as an indicator to discriminate heterozygous HbE from compound heterozygote. On the other hand, the MCV value reflects not only anemia status, but also the cell size. Thus it is more reliable to be used as indicator of heterozygous versus compound heterozygous HbE.

When the hemoglobin level, MCV and MCH in the heterozygous HbE group were compared between male and female patients, it was clearly observed that female patients had lower values in all parameters, except the MCH. The possible explanation for these differences were, among others, the menstrual cycle experienced each month.

Homozygous HbE are very clearly different from heterozygous ones as they show very high level of HbA_2, which indeed are actually composed of the HbE. They exhibit lower concentrations of hemoglobin compared to the heterozygote, and much lower MCV.

In two female cases of co-inheritance of HbE and α-thalassemia, the levels of HbA_2+HbE were lower than HbE heterozygote. The hemoglobin concentrations were at the borderline value, with very low MCV and MCH. The results illustrate that they have very mild clinical manifestation, but the MCV was remarkably low.

We are aware that our study has limitations. We know that similar observations have been reported in other populations before. However, this report has an added value in that it describes the importance of the routine and simple CBC interpretation in assisting the differentiation of HbE types in misleadingly high HbA_2 levels. This ability to interpret is a necessity in clinical laboratories where molecular diagnostic capability is not readily available.

CONCLUSION

The three parameters of CBC, which are hemoglobin level, MCV and MCH, were found to be useful in assisting HbE type determination in pseudo high HbA_2 level. The sex of the patient has to be considered in the interpretation of CBC results.

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CONFLICT OF INTEREST

None.

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