RESEARCH ARTICLE

Erythrocyte Indices MCV and/or MCH as First Round Screening Followed by Hb-analysis for β-thalassemia Carrier State

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Abstract

BACKGROUND: Being located in the global thalassemia belt area, Indonesia is estimated harboring about 10% thalassemia carriers; however, screening program is still diversely scattered across the country. Numerous erythrocyte indices have been introduced to help identifying thalassemia carriers with contradictory results. Therefore, this study had compared the use of mean corpuscular volume (MCV) and/or mean corpuscular hemoglobin (MCH) values and the most erythrocyte indices used in Indonesia which were Mentzer Index (MI) and Shine & Lal Index (SLI), as a first attempt in a mass screening for β-thalassemia carrier.

METHODS: This was a retrospective study, evaluating laboratory data from family members of thalassemia major subjects. The sensitivity and specificity of MI and SLI were calculated. HbA2 >3.5% was used as a golden standard for β-thalassemia carrier and DNA examination was conducted to confirm β-globin mutation.

RESULTS: Out of 160, 28.8% of the subjects had low Hb concentration. Interestingly, 79.4% of the subjects had low MCV and/or MCH with or without low Hb concentration. In this study, specificity and sensitivity of MI were 82.2% and 83.8%, whereas of SLI were 96% and 40.5%, respectively. Low MCV and/or MCH had covered IVS1nt5 and Cd26 mutation at β-globin gene; whereas MI and SLI had missed some samples, leading to false negative of thalassemia carrier results, when using MI or SLI only.

CONCLUSION: MCV<80 fl and/or MCH<27 pg is the best first round mass screening method for β-thalassemia carrier in a limited facility area. However, Hb electrophoresis should be gradually installed regionally in various places wherever possible, as well as DNA analysis to confirm the mutation for an optimal carrier diagnosis.

KEYWORDS: HbA2, HbE, iron deficiency anemia, Mentzer Index, Shine and Lal Index

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Introduction

Thalassemia, a condition of imbalance globin-chain synthesis that might affects the hemoglobin production, is highly prevalent in countries across Mediterranean, Africa, the Middle East, and stretch out to the Indian subcontinent and South-East Asia, including Indonesia.(1,2) There are around 10% thalassemia carriers among Indonesian population, however, national wide prevention program is still diversly scattered due to limited facilities in this country.(3) β-thalassemia is defined as a quantitative defect in production of β-globin chains due to mutation on β-globin (HBB) gene.(4,5) In attempt to simplify mass screening procedure, several indices using blood cell count parameters have been introduced to discriminate microcytic anemia

due to β-thalassemia carrier (BTC) or iron deficiency (6) since iron deficiency anemia (IDA) is also common in Indonesia (7).

Although it has been known for years that mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) are the best parameters for initial screening of β-thalassemia, various indices have been developed to better predict thalassemia carriers. Those erythrocyte indices use parameters including MCV, MCH, red blood cells (RBC) count, and hemoglobin (Hb) concentration that are commonly examined using a very simple blood counter.(8) Over forty erythrocyte indices have been published, including Mentzer Index (MI), Srivastava Index (SI), Shine & Lal Index (SLI), England & Fraser Index (EFI).(9) The best performance has been shown by formula developed by Green & King (MCV2xRDW(Hbx100)), however, this formula is dependent on the red cell distribution width (RDW), a parameter that is not provided in every automatic blood counter. Moreover, the specificity and sensitivity values, as well as the positive and negative predictive values among erythrocyte indices have been documented in many previous studies, yet with different results among population. In Thailand, formula of RDW/RBC has been proved to be the most reliable index (10), whereas MI has a substantial concordance with the HbA2 result in western part of Indonesia (11). Furthermore, new modified cut-offs have been proposed for a better discrimination between IDA and BTC.(12) Even more, the latest erythrocyte index has been introduced from reticulocyte hemoglobin (Ret-He) level, suggesting as a potential marker for diagnosis of IDA and BTC.(13)

Another recent technology has developed in Hb analysis, including fractions of HbA2, HbA, or HbF that may confirm or exclude BTC. HbE fraction can be detected separately, thus recommending Hb analysis for further confirmation results of HbE carrier screening.(14) Since thalassemia is a monogenic disease with more than hundreds different mutations and deletion in α- or β-globin gene, it is necessary to confirm and to map the genetic spectrum of the population, especially in the area where common mutation or deletions are not yet known.

Hb analysis is scarce and expensive in Indonesia, and even more, the molecular examination to confirm the mutation in α- or β-globin gene. To only rely on blood examination by calculating the erythrocyte indices commonly used in Indonesia such as MI and SLI is becoming challenging. Therefore, we have explored the use of MCV and/or MCH values, MI and SLI as a first attempt in thalassemia carrier screening in general population.

Methods

Study Design

This was a retrospective descriptive cross-sectional study, evaluating laboratory data from previous study, included sibling and extended family members of thalassemia major subjects during a thalassemia carrier screening program in Bandung.(15) The initial study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Padjadjaran (No. 966/UN6.C.10/PN/2017) and for this current study of secondary data, clearance was exempted.

Subjects Recruitment Criteria

Only data of sibling and family members aged >5 years old were analysed in this study. Anemia was defined based on age classification in children according to WHO criteria, that was Hb <11.5 g/dL for age 5-12 years old and Hb <12 g/dL for females or Hb <13 g/dL for males aged >12 years old.(16) MI (MCV/RBC) and SLI ($MCV^2 \times MCH/100$) were calculated to discriminate iron deficiency anemia (MI >13 or SLI >1530) or thalassemia carrier (MI <13 or SLI <1530). The indices were then compared with the data of MCV<80 fl and/or MCH <27pg values, that was used as first screening according to protocol from Ministry of Health Republic of Indonesia.(3)

HbA2 Measurement

Data on Hb analysis were classified based on HbA2 fraction values according to the protocol of the manufacture, and >3.5% was indicated as β-thalassemia carriers; 2.0–3.5% as normal value, and <2.0%, suggesting for α-thalassemia carriers. HbE peak shown by Hb analyzer (Minicap Sebia, Paris, France) indicated HbE carriers.

Globin B Gene Sequencing

Data on sequencing of globin B gene (HBB; NM_000518) in a subset of samples with HbA2 >3.5% or with a HbE peak were then assessed. HBB gene was amplified using the forward and reverse primer sets as previously described (15) using PCR condition as followed; initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 68°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. The polymerase chain reaction (PCR) result was visualized before further sequenced.

Statistical Analysis

Specificity and sensitivity of erythrocyte indices were calculated against HbA2 result, using SPSS version 22.0

(IBM Corporation, Armonk, NY, USA). The distribution of erythrocyte indices MI, SLI and MCV and/or MCH, as well as Hb Analysis and DNA examination were presented in percentage.

Results

In total, data of 160 family members of thalassemia major patients were retrieved, including 67 male (41.9%) and 93 female (58.1%), with median age of 28 years old (5-74 years). The respondents were grouped as unmarried group (43.7%), consisting of children aged 5-11 years old (13.8%), adolescents aged 12-15 years old (9.4%) and >15-24 years old (20.6%).

Iron Deficiency Anemia or β-thalassemia Carrier Screening Based on Erythrocyte Indices

Low Hb or anemia, corrected by gender, was only found in 46 family members (28.8%). Interestingly, 127 family members (79.4%) was found to have low MCV and/or low MCH, with or without anemia (Table 1). Based on workflow from the Ministry of Health Republic of Indonesia shown in Figure 1, respondents with low MCV and/or low MCH with no anemia were directly examined for Hb analysis; whereas those with anemia (n=33) were further explored whether anemia was due to IDA or BTC. In our current study, all respondent with low MCV and/or low MCH with or without anemia (n=127) were further examined in level 2 for Hb analysis. Of note, none were given iron supplementation as directed in the workflow. In the other calculation, BTC was suspected when MI <13 or SLI <1530, resulting in 53.7% (n=86) or 67.5% (n=108) of BTC, respectively (Table 1).

Table 1. Anemia status among family members of thalassemia major subjects aged >5 years old.

Parameter	n (%)		
	Not Anemia (n=114)	Anemia (n=46)	Total (n=160)
MCV and/or MCH			
Normal	31 (27.2%)	2 (4.4%)	33 (20.6%)
Low	83 (72.8%)	44 (95.6%)	127 (79.4%)
Mentzer Index			
≥13	61 (53.5%)[n]	13 (28.3%)[a]	74 (46.3%)
<13	53 (46.5%)[c]	33 (71.7%)[b]	86 (53.7%)
Shine & Lal Index			
≥1530	49 (43.0%)[n]	3 (6.5%)[a]	52 (32.5%)
<1530	65 (57.0%)[c]	43 (93.5%)[b]	108 (67.5%)

[a]suspect IDA; [b]suspect BTC; [c]suspect other hemoglobinopathies; [n]normal.

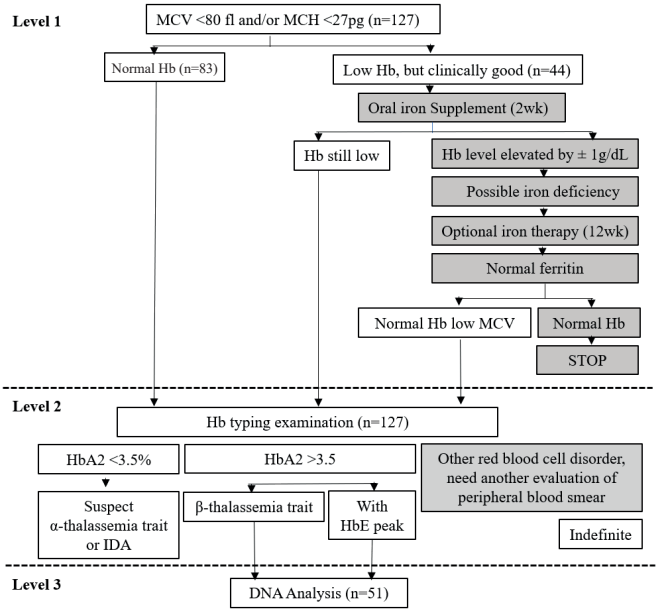

Figure 1. Flow chart of carrier thalassemia detection.(2) The grey area was not performed. Only HbA2 >3.5% were examined for the DNA analysis.

β-thalassemia Carrier Based on Hb Analysis and DNA Examination

The specificity and sensitivity were calculated with HbA2 as the golden standard for BTC. The SLI had the highest sensitivity (96%), however, the specificity was low (40.5%), whereas the specificity and sensitivity of MI were 82.2% and 83.8%, respectively. The sensitivity and specificity of low MCV and/or low MCH resulted in only 38.6% and 67.6%, respectively.

DNA examination was only performed in a subset (n=51) of samples with HbA2 >3.5% due to limited fund. Some of samples had very low HbA2 <2%, suggesting α- or δ- or other thalassemia mutation, that needed to be confirmed further. The distribution of samples based on level 1, level 2 and level 3 was depicted in Table 2. Avery high HbE peak with normal HbA2 peak, indicating HbE variant; whereas HbA2 >3.5% was confirmed to have mutation in Hb B gene such as IVS1nt5, Cd 8/9 and Cd 19.

Although low MCV and/or low MCH value had low sensitivity and specificity, BTC and HbE carriers were detected by DNA examination, whereas SLI missed some samples and MI even more (Table 3). Unfortunately, DNA analysis was not performed for HbA2 <2.0%. In our study, some respondents with MI ≥13 or SLI ≥1530 whose suggested to have iron deficiency anemia had HbA2

Table 2. Distribution of thalassemia carrier status based on Level 1 (complete blood count), Level 2 (Hb analyses) and Level 3 (DNA examination).

Level 2 (HbA2 Analysis)	Level 1 (Complete Blood Count)				Total (n=160)	Level 3 (DNA examination)
	Low MCV and/or Low MCH (n=127)		Normal MCV and MCH (n=33)			
	Normal Hb (n=83)	Low Hb (n=44)	Normal Hb (n=31)	Low Hb (n=2)		
>3.5[a]	14	12	-	-	26	**IVS1nt5**
	-	1	-	-	1	**Cd 8/9**
	1	-	-	-	1	**Cd 19**
	24	22	-	-	46	**n.e.**
2.0-3.5*	21	2	-	-	23	**Cd26**
2.0-3.5	22	7	31[c]	2[d]	62	**n.e.**
<2.0[b]	1	-	-	-	1	**n.e.**

n.e.: not examined; *High HbE peak; [a]suspect BTC; [b]suspect α-thalassemia carrier; [c]considered normal; [d]suspect IDA.

level >3.5%, suspecting BTC and some others had HbE peak, indicating HbE carrier, that was confirmed by DNA examination (Table 3).

Discussion

In Indonesia, the prevalence of anemia is high and often described as IDA, especially in children and young women. (7) However, since Indonesia is located in global thalassemia belt, the etiology of anemia in this area needs to be well defined. The WHO advocates prevention and reduction of the burden of thalassemia through voluntary genetic screening.(17,18) The DNA analysis in Indonesia is limited in big cities and mostly not accessible due to high cost in the examination. Therefore, screening strategies for BTC or HbB variant such as HbE in rural communities might be well directed to the first step by using MCV and/or MCH values, followed by level 2 of Hb analysis by referring to regional laboratory (Figure 1). Of note, this flow chart is not suitable for women already pregnant, since the possibility of prenatal diagnosis in advanced week of pregnancy may induce less acceptance of choosing termination if an affected fetus is detected.

Study in India has shown that MCH <27 pg is a more suitable cut-off value in that population.(19) However, various erythrocyte indices have been developed to help discriminating IDA and BTC of which MI (Hb/RBC) is more efficient in discriminating microcytic anemia due to BTC or iron deficiency.(20) Some other erythrocytes parameters and algorithm have been applied to improve the prediction whether microcytic/hypochromic anemia is due to iron deficiency or BTC.(17) In Indonesia itself, new cut-off erythrocyte index has been explored and showed a better validity for Green & King as well as for RDWI (21), however, for simple mass screening using simple blood counter equipment in the primary health care this can not be well performed.

Our previous study used SLI (MCVxMCVxMCH/100) to predict BTC.(15) However, after careful re-analysis in this current study, SLI has missed some of BTC, especially HbE carriers (Table 3). Moreover, this current study has shown that MI has also failed to predict even more respondents. Though erythrocyte indices are of essential use in area with limited resources, those erythrocyte indices cannot solely be examined and thus, Hb analysis is imperative. Carriership needs to be further examined thoroughly, as we cannot overlook those false negative cases. In the area with limited facilities as well as with low economic resources, a combination of osmotic fragility test and dichlorophenolindophenol might be useful (22), hence, the human skill is required which is not easily performed. Ideally, Hb analyser needs to be installed regionally to help confirming the carrier status.

Hb analysis plays an important role in distinguishing IDA and BTC. For example, anemia with HbA2 >3.5% indicates a BTC. Again, since thalassemia is a genetic disorder, DNA examination is needed to confirm the result. Mutations at IVS1nt5 (c.92G > C) and at Cd26 (c.79G > A) are common in our study site in western part of Indonesia (Table 2). Interestingly, elevated HbA2 is a more reliable diagnostic measurement for BTC, even in the presence of iron deficiency.(23) Currently, by using Hb analysis in our study, it has shown that high peak HbE were all confirmed

Table 3. Distribution of BTC status based on Level 1 stratified by MI and SLI.

Level 1	Level 2 HbA2 (%)	Level 3 (DNA Examination)				
		IVS1nt5 (n=26)	Cd 8/9 (n=1)	Cd 19 (n=1)	Cd 26 (n=23)	n.e. (n=76)
Mentzer Index (n=127)						
<13 (n=86)	>3.5	25	1	1	-	44[a]
	2.0 – 3.5*	-	-	-	11	-
	2.0 – 3.5	-	-	-	-	4[b]
≥13 (n=41)	>3.5	**1**[$]	-	-	-	**2**[a $]
	>3.5*	-	-	-	**1**[c $]	-
	2.0 – 3.5*	-	-	-	**11**[$]	-
	2.0 – 3.5	-	-	-	-	25[d]
	<2.0	-	-	-	-	**1**[c $]
Shine & Lal Index (n=127)						
<1530 (n=108)	>3.5	26	1	1	-	46[a]
	2.0 – 3.5*	-	-	-	19	-
	2.0 – 3.5	-	-	-	-	14[b]
	<2.0	-	-	-	-	1[c]
≥1530 (n=19)	>3.5*	-	-	-	**1**[$]	-
	2.0 – 3.5*	-	-	-	**3**[$]	-
	2.0 – 3.5	-	-	-	-	15[d]

Level 1 was designated as low Hb and/or MCV and/or MCH. n.e.: not examined; *normal HbA2 with high HbE peak; [a]suspect BTC; [b]suspect other hemoglobinopathies; [c]HbE/β-thalassemia; [d]suspect IDA; [$]false negative.

for c.79G > A mutation, indicating Cd26 heterozygous or HbE carrier (Table 2). The HbE high peak is clearly separated from HbA2, thus, with this newest development, HbE peak in Hb-analysis becomes an important parameter for HbE carrier identification. In contrary, some other machines show a very high value of HbA2, indicating the HbE variant, although the HbA2 itself is normal. Thus, HbE carriers would have been picked up by high-performance liquid chromatography (HPLC) alone and would not require DNA analysis.(14)

Furthermore, HbA2 <3.5% could have potentially been erroneously attributed to iron deficiency.(32) Low value of SLI or MI together with normal to borderline HbA2 levels might suggest an IDA or otherwise possible an α-thalassemia carrier. The possible α-thalassemia defect might be deletional or non-deletional α-globin, and therefore, need to be further analysed by molecular analysis. (17) Our previous study among pregnant women in the eastern part of Indonesia where iron deficiency anemia cases are prevalent, shows that α-thalassemia gene deletion is also common.(24) In addition, borderline HbA2 3.2-4% needs further investigation for β-gene mutation, depends on the cut-off point the machine used. Interestingly, the co-inheritance of α- and β-thalassemia carrier might be existed, leading to a reduced or abolished globin-chain synthesis or cause structurally abnormal hemoglobin (25), therefore, testing for α, δ- and other globin gene abnormalities should be pursued.

The national program to screen general population in Indonesia is not in place yet. The most ideal period for thalassemia carrier screening is in high school or young adult period, before marriage.(26) In some countries, thalassemia carrier screening is encouraged during premarital or otherwise in antenatal period through basic health care services.(27,28) Our study has shown that 43.7% of the siblings are not yet married, therefore, confirming their carrier status is essential since the consequence of such result will impact the future decision of marriage. Interestingly, study in Pakistan has shown that the most cost-effective and practical approach to identify thalassemia carrier is the immediate family members.(29) Cascade screening among family members of thalassemia major patients in area where funding is limited has proven to be more cost effective compared to massive mass screening.(29) However, some reluctancy might occur in the extended family members to screen the carriership of their own.(30) Depends on the country priority, therefore, a structured prevention program is a must in area where thalassemia carriers are prevalent.

With a good implementation of population screening and prevention strategies, thalassemia birth reductions can be achieved, aiming for zero growth.(26)

This study encountered several limitations, among others that DNA examination have been only performed in samples with HbA2 >4% and those with HbE peak. However, with only limited cases, our study shows that both MI and SLI have failed to confirm most of HbE that are prevalent in our population in western part of Indonesia, that may lead to false negative results when using MI or SLI only. Furthermore, blood smear and ferritin examination were not performed, neither iron supplementation were given. Although this study does not provide additional new information that is already known in the literature with various mathematical formulas of erythrocytes indices (31), however, the MCV and/or MCH values seems to be more acceptable in our area with limited facilities. Therefore, a careful diagnosis needs to be erected, especially for premarital screening, as a false negative result is unacceptable. Moreover, since the use of MCV and MCH indexes is targeted to detect thalassemia carriers, a larger cohort of young adults only need to be analysed separately from children and adolescents, as the latest group is probably having more iron deficiency, thus, making the analysis less accurate. Future study on various globin mutation is imperative to map the mutation spectrum among ethnicities in Indonesia.

Conclusion

To reduce thalassemia burden in Indonesia, screening thalassemia carrier state is urgently needed. We proposed MCV<80 fl and/or MCH<27 pg as the best first round mass screening method for BTC in a limited facility area. However, HbA2 analysis should be gradually installed regionally in various places wherever possible for a more conclusive diagnosis, although molecular diagnosis is the best to confirm point mutation or deletion in α- or β-thalassemia carrier.

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Authors Contribution

ES, AMM and LR were involved in concepting and planning the research. AMM and LR performed the data acquisition/collection. ES, AMM, and DP calculated the experimental data and performed the analysis. ES drafted the manuscript and designed the figures. ES, AMM, LR and DP aided in interpreting the results. All authors took parts in giving critical revision of the manuscript.

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