

The Value of Red Blood Cells (Rbc) Indices and Osmotic Fragility Test as Screening Tests in Malay Pregnant Women with Alpha Thalassaemia

Rosline Hassan^{1*}, Nurul Ain Fathma Abdullah¹, Rosnah Bahar¹, Selamah Ghazali¹ and Nor Aliza Abdul Ghaffar²

¹Department of Haematology, Universiti Sains Malaysia, Kubang Kerian Kelantan, Malaysia

²Department of Obstetrics and Gynaecology, Universiti Sains, Malaysia, Kubang Kerian Kelantan, Malaysia

Abstract

Red blood cell (RBC) parameters obtained from hematology analyser has been very useful as the first line screening for thalassaemia. The changes are prominent in most type of β -thalassaemia but milder in α -thalassaemia. RBC parameters could have been normalized especially during pregnancy and in such situation screening these patients would require a different protocol. Blood samples from two hundred (200) Malay pregnant women attended obstetric clinic at Hospital Universiti Sains Malaysia (HUSM) were collected to screen for double α -gene deletion (-SEA, and -THAI), and the two single α -globin gene deletion ($-\alpha^{3.7}$ and $-\alpha^{4.2}$). Standard hematological analyses including red blood cell count and indices, and hemoglobin quantitation were performed on the blood samples. Of these, sixteen were excluded as they had HbA₂ levels more than 4% and were diagnosed as HbE or β -thalassaemia trait. Then, multiplex GAP polymerase chain reaction (PCR) analyses of α -globin gene were performed on the remaining 184 blood samples. Total of 17 from 184 subjects confirmed to have α -thalassaemia ($-\alpha^{3.7}/\alpha\alpha$ and $^{-SEA}/\alpha\alpha$ genotype). The RBC indices were compared between those with α -thalassaemia and normal pregnant women and they were significantly different. $-\alpha^{3.7}$ kb single gene deletion (8.1%) was the commonest type followed by double gene South East Asia ($^{-SEA}$) deletion (1.1%). We conclude that mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) with the cut off value of less than 86.3fl and 27.4 pg respectively are two useful RBC indices which can be used for screening of α -thalassaemia in pregnant women.

Keywords: Alpha thalassaemia; Multiplex GAP PCR; Osmotic fragility test (OFT); Pregnant women; Red blood cell (RBC) indices

Introduction

Thalassaemia is a group of genetic, inherited disorders of the blood. More specifically, it is a disorder of the globin gene synthesis. Thalassaemia is a major health problem worldwide and it is common in people of Asian descent and has emerged as public health problem in Malaysia [1].

There are two common types of thalassaemia which is α - and β -thalassaemia. The α -thalassaemia is mainly caused by a large deletion of the α -globin gene and occurs when one or more of the four alphas (α) chain genes fail to function. α -thalassaemia is the one of the most common inherited disorder of hemoglobin synthesis and commonly found in Southeast Asian, Mediterranean and Middle Eastern population [2]. While, β -thalassaemia generally caused by point mutations affecting the β -globin gene leading to a reduction (β^+) or absence (β^0) of β -globin gene production.

α -thalassaemia can be classified into two types; α -thalassaemia 1 (α^0 -thalassaemia), the deletion of both $\alpha 1$ - and $\alpha 2$ - globin genes; and α -thalassaemia 2 (α^+ -thalassaemia) where only one α -globin gene deletion has occurred [3]. An individual with only one α -globin deletion or heterozygous for α^+ -thalassaemia normally is a silent carrier ($\alpha \alpha/\alpha^-$) [4].

Many methods are currently used for screening and diagnosis of α -thalassaemia. Simple erythrocyte osmotic fragility (OF) test is commonly used for screening of α -thalassaemia disorder followed by high performance liquid chromatography (HPLC) and polymerase chain reaction (PCR) [5,6].

Identification of carrier state in early pregnancy using screening protocol for each population is important for the prevention and control of this disease. Therefore, the aim of this study is to identify the RBC indices and its cut off level as the screening tool for common

deletional types of α -thalassaemia in pregnant women in Hospital Universiti Sains Malaysia (HUSM).

Materials and Methods

A total of two hundred (200) Malay pregnant women, attending for antenatal check-up at Obstetrics & Gynecology Clinic in Hospital Universiti Sains Malaysia (HUSM) were recruited into this study. Written informed consent was taken from them. The study protocol was reviewed and approved by the Research and Ethics Committee, School of Medical Sciences, Universiti Sains Malaysia, Kelantan Health Campus. A total of 3ml whole blood was collected in ethylene diamine tetra acetic acid (EDTA) bottle and immediately screened for α -thalassaemia using the modified one tube osmotic fragility (OF) test following the manufacturer's protocols as described in earlier study [5]. Full blood count parameters were determined using an automated blood counter (SYSMEX[®] KX-21, Sysmex Corporation, Kobe, Japan) for all blood samples. The percentage of hemoglobin A₂ (HbA₂) and hemoglobin F (HbF) were measured for all samples by using Bio-Rad VARIANT Hemoglobin Testing System (Variant, Bio-Rad Laboratories, Hercules, CA). For subject who had an HbA₂ level <4%, molecular diagnostic techniques were used to detect the type of α -thalassaemia.

***Corresponding author:** Rosline Hassan, Department of Haematology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia, Tel: 609 7676191; Fax: 609 7673333; E-mail: roslin@kb.usm.my

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Genomic deoxyribonucleic acid (DNA) was extracted and purified from whole blood sample using the Gentra Puregene Blood Kit (Qiagen, Germany). Deletion analysis of the α -globin gene was carried out using multiplex GAP PCR with the primers designed to detect 2 double α -gene deletion ($-\text{SEA}$, and $-\text{THAI}$), and the 2 single α -globin gene deletion ($-\alpha^{3.7}$ and $-\alpha^{4.2}$).

A total volume of 25 μl of reaction mixture contained 200 ng/ μl of genomic DNA in final concentration of 1X PCR buffer (Qiagen, Germany), 0.2 mM of deoxyribonucleotide triphosphate (dNTP) mix (Applied Biosystems, California, USA), 1.5 mM magnesium chloride (MgCl_2) (Qiagen, Germany), 10-20 pmol of each specific primers (Sigma Prologo, Singapore), 2.5 U of Hot Star Taq DNA polymerase (Qiagen, Germany), 1X Q-Solution (Qiagen, Germany) and double distilled water (ddH_2O). The reaction amplification started with initial denaturation at 95°C for 15 minutes, followed by 30 cycles of denaturation at 97°C for 45 seconds, annealing at 60°C for 75 seconds, extension at 72°C for 150 seconds and final extension at 72°C for 5 minutes. Amplified products were electrophoreses in 1% agarose gel in 1X Tris-Borate-EDTA (TBE) buffer. The gel was stained with ethidium bromide (EtBr) placed on transluminator and photographed.

Statistical Analysis

Data entry and analysis was done using IBM Statistical Package for Social Sciences (SPSS) version 20 for windows (IBM Inc, USA). Means and standard deviations were calculated for numerical variables. Frequencies, percentages and chi-square were calculated for all categorical variables.

Results

A total of 200 Malay pregnant women were recruited into this study. They came for their first antenatal check-up at Obstetric clinic, Hospital Universiti Sains Malaysia, Malaysia. 73% were in their first trimester, 22% second and 5% third trimester. Their mean age was 31.5 ± 9.4 year old. Of these, 16 were later excluded because they were diagnosed as having HbE or β -thalassaemia trait. The remaining 184 women were available for molecular analysis. Out of 184 women, 17 were detected to have α -thalassaemia while 167 were normal.

Hematological parameters were compared between α -thalassaemia carrier and normal individuals. Analysis of hematologic data from the subjects showed significantly difference in MCV and MCH between normal and α -thalassaemia carriers ($p < 0.001$; Table 1).

Based on the receiver operating characteristic (ROC) curve for the MCV and MCH, the best cut-off point for predicting the presence of the α -thalassaemia carrier in pregnant women was 86.3 fL giving 77%

Hematological Parameters	α -thalassaemia trait	Normal	p-value
	Mean \pm SD N=17	Mean \pm SD N=167	
Hb (g/dL)	11.32 (0.87)	11.61 (1.06)	0.835
MCV (fL)	82.32(5.69)	88.60 (4.75)	0.000*
MCH (pg)	25.97(2.11)	28.60 (2.14)	0.000*
HbA2 (%)	2.98(0.31)	3.00 (0.34)	0.888
HbF (%)	0.34(0.37)	0.36 (0.49)	0.269

**independent t test showed significant at $p < 0.005$

* Alleles are represented as follows: Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; HbA₂, hemoglobin A₂; SD, standard deviation; fL, femtoliter; pg, picograms.

Table 1: Mean \pm SD of the difference hematological parameters in α -thalassaemia trait and normal subject.

of sensitivity and 71% of specificity (Figure 1) and 27.4 pg giving 76% of sensitivity and 71% of specificity, respectively (Figure 2).

A total of 16 subjects were positive by one-tube osmotic fragility test however 17 were confirmed as α -thalassaemia based on molecular testing. Among 17 who were carrier of α -thalassaemia, 4 (3 heterozygous of $-\alpha^{3.7}$ kb deletion and 1 $-\text{SEA}$ type deletion) were consistently positive with OF while the other 13 were negative. Among 184 pregnant women studied, 17 (9.2%) were found to carry α -thalassaemia gene (Table 2). In this study group, two α -thalassaemia genotypes were observed: $-\alpha^{3.7}/\alpha\alpha$ and $-\text{SEA}/\alpha\alpha$. The most common genotype was heterozygous $-\alpha^{3.7}$ rightward single gene deletion (8.1%) followed by $-\text{SEA}$ deletion (1.1%). $-\alpha^{4.2}$ rightward single gene deletion and $-\text{THAI}$ deletion were not detected.

Discussion

Thalassaemias are the most common inherited disorder worldwide and represent as a major health problem in many areas [7]. α -thalassaemia is the most prevalent and the diagnosis is challenging especially during pregnancy. A total of 200 Malay pregnant women who

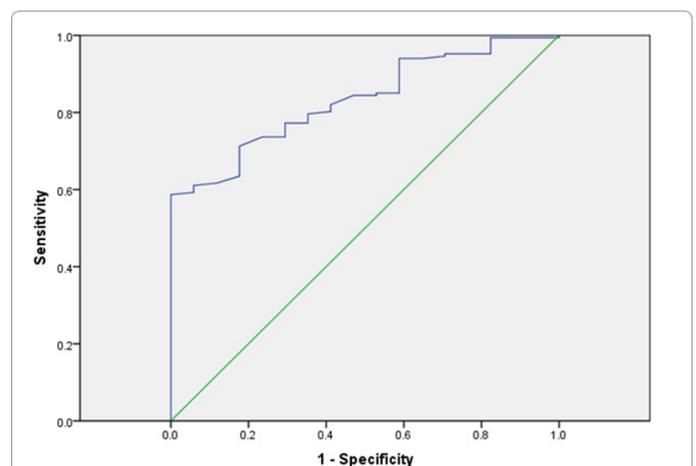


Figure 1: ROC curve for MCV predicting the presence of the α -thalassaemia carrier in pregnant women. (Area under the curve, 0.83; 95% confidence interval, 0.76-0.91).

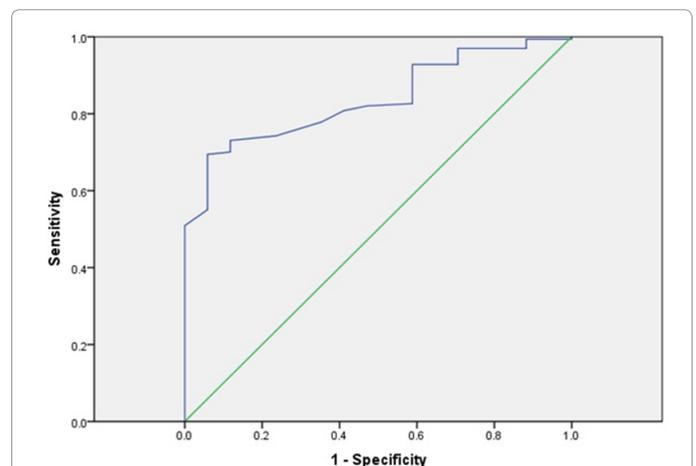


Figure 2: ROC curve for MCH predicting the presence of the α -thalassaemia carrier in pregnant women. (Area under the curve, 0.84; 95% confidence interval, 0.77-0.91).

Patient No	Parameter				α genotyping
	Hb	MCH	MCV	OFT	
1	12.6	27.8	88.1	negative	- α 3.7/ $\alpha\alpha$
2	11.4	27.3	86.6	negative	- α 3.7/ $\alpha\alpha$
3	12.3	26.9	86.2	negative	- α 3.7/ $\alpha\alpha$
4	11.1	27.2	86.5	negative	- α 3.7/ $\alpha\alpha$
5	11.2	26.9	84.9	negative	- α 3.7/ $\alpha\alpha$
6	10.8	28.6	87.6	negative	- α 3.7/ $\alpha\alpha$
7	11.5	27.5	85.2	negative	- α 3.7/ $\alpha\alpha$
8	11.9	24.7	79.5	negative	- α 3.7/ $\alpha\alpha$
9	11.2	27.5	87.5	negative	- α 3.7/ $\alpha\alpha$
10	10.7	24.4	79.7	negative	- α 3.7/ $\alpha\alpha$
11	11.8	24	73.6	negative	- α 3.7/ $\alpha\alpha$
12	13.2	27.4	85.1	negative	- α 3.7/ $\alpha\alpha$
13	10.4	25.7	80.4	positive	- α 3.7/ $\alpha\alpha$
14	10.4	25.7	80.5	positive	- α 3.7/ $\alpha\alpha$
15	11.8	27	85.4	positive	- α 3.7/ $\alpha\alpha$
16	10.1	20.9	69.2	negative	-- _{SEA} / $\alpha\alpha$
17	10.1	22.1	73.5	positive	-- _{SEA} / $\alpha\alpha$

^aData presented as raw data.

^bAlleles are represented as follows: - α ^{3.7}/ $\alpha\alpha$ =3.7 kb deletion, -_{SEA}/ $\alpha\alpha$ =SEA deletion. Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; OFT, Osmotic Fragility Test.

Table 2: Characteristic features of 17 α -thalassaemia carrier among 184 pregnant women at HUSM.

attended an antenatal checkup at Hospital Universiti Sains Malaysia were screened for α -thalassaemia. Of these, 16 were excluded as they were diagnosed as β -thalassaemia/HbE trait. Out of 184, 17 cases were identified with α -thalassaemia carrier.

Full blood count with red blood cell indices usually were used as the initial method for screening of α -thalassaemia [5]. The red blood cell indices used for screening included hemoglobin count (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), hemoglobin A₂ count (HbA₂) and hemoglobin F count (HbF).

MCV and MCH are two routinely parameters carried out worldwide during pregnancy. It is cheap, quiet and simple method especially in area with highest incidence. The importance of thalassaemia screening during antenatal follow-up is mainly to identify patients with two gene deletion who may be at risk to have hydrops baby. Further molecular testing may also required in highly suspicious case in order to avoid unnecessary supplementation of iron tablet.

The MCV cut-off values used for thalassaemia screening vary widely and cut-off value between 75 and 80 fL have been used in most institutions as previously reported [5,8,9]. Based on our ROC curve of MCV values, our study suggests the best cut-off point for predicting the present of α -thalassaemia in pregnant women were 86.3fL giving 77% sensitivity with a specificity of 71%.

In our study, MCV \leq 86.3fL were used to detect single gene deletion in Malaysian population. This finding was in agreement with previous study [5,10]. The range of MCV for - α ^{3.7}/ $\alpha\alpha$ genotype was 78-89 fL in studies by Fucharoen G et al. [5], Sanchaisuriya K et al. [8], and Sirichotiyakul S et al. [9].

Meanwhile MCV for two of our patients identified as two gene deletion (-_{SEA}/ $\alpha\alpha$ genotype) was 68 fL and 74fL (MCV \leq 80) respectively. They have lower value, a similar finding as previously reported in studies with α ⁰-thalassaemia [11,12].

It is important to screen for α -thalassaemia in women with child bearing age as the disease is prevalent in Malaysia and may cause some social economic burden to the country. Fetus may be at risk to

develop deletional Hb Barts hydrops foetalis syndrome or deletional HbH disease. Hydrops fetalis has been reported in Chinese-Malaysian with 0.3/1000 births [13]. The projected number of pregnancies at risk and contribute for deletional Hb Barts hydrops foetalis syndrome and deletional HbH disease each year in Malays was 30 and 120 [13]. In view of its high probability, it is important for us to identify them early and the best times to catch them are doing their antenatal check-up.

During pregnancy, the reference interval of MCV for α -thalassaemia in this present study was higher than reference interval used in normal adult (80 fL) and this could be due to the differences in folate and vitamin B₁₂ status. MCV is also a poor marker of iron deficiency in pregnancy because of the physiological increased in MCV during gestation counterbalances the microcytosis of iron deficiency [14]. Generally individuals with MCV <80 fL or MCH <27 pg, were investigated for an underlying thalassaemia by doing hemoglobin, HPLC or electrophoresis.

In addition serum ferritin should be ordered concurrently particularly in pregnant women, although in our study, serum ferritin was not performed due to budget limitation.

A modified one-tube OF test was used as a screening method in this study to identify individuals who may be a carrier of α -thalassaemia. This method has been proven to be useful in the prevention and control program for thalassaemia in Thailand [5,8,15]. But in this present study, we found OF test were indiscriminative for α -thalassaemia carrier especially α ⁺-thalassaemia. A total of 10 α ⁺-thalassaemia carrier was negative for OF test. This study also showed that there was no significance difference in OF test between α -thalassaemia and non- α -thalassaemia in pregnant women. Thus, we conclude that it is not a useful screening test during pregnancy.

There are several methods to confirm α -thalassaemia carrier including dot-blot analysis, reverse dot-blot analysis, amplification refractory mutation system (ARMS), restriction enzyme (RE) digestion, denaturing gradient gel and so on. In this study, we have performed multiplex GAP PCR to detect two common deletional types of

α -thalassaemia genotypes: α^0 -thalassaemia gene deletion ($--^{SEA}$ and $--^{THAI}$) and α^+ -thalassaemia gene deletion ($-\alpha^{3.7}$ deletion and $-\alpha^{4.2}$ deletion).

$-\alpha^{3.7}$ -globin gene was the most common cause of α -thalassaemia [16] and most dominant in African, Mediterranean and Asian population [2,17]. This study was performed in Kelantan, a state in Malaysia which comprised 80% of the population are Malays. Single gene deletion was found to be more prevalent with 8.1% of the patients confirmed as heterozygous $-\alpha^{3.7}/\alpha\alpha$ genotype.

The South-East Asian deletion ($--^{SEA}$) is the most common α^0 -thalassaemia variant in South-East Asian population [18]. However α^0 -thalassaemia with the $--^{SEA}$ deletion was detected in only 1.1% (2/184) of the Malay pregnant women studied. This finding may suggest that there is a risk having Hb Bart's hydrops fetalis baby among Malay and subsequent hematological screening and molecular analysis for α -thalassaemia should be performed in their spouses.

In conclusion, we still recommended using MCV and MCH as screening parameters for α -thalassaemia in pregnant women. However the cut-off value for MCV and MCH should be less than 86.3fl and 27.4pg respectively. This should be followed by screening for α -thalassaemia in spouse for further genetic counselling. In our hand, OF test has failed to predict the carrier status in pregnant women.

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