

ORIGINAL ARTICLE

Usefulness of Red Blood Cell Size Factor (RSf) in Screening Genetic Variants of Alpha Thalassaemia Thalassaemia Trait Regardless of Iron Status

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ABSTRACT

Introduction: Screening for alpha (α) thalassaemia trait (TT) is challenging especially in the presence of iron deficiency (ID). Red blood cell size factor (RSf) is a parameter introduced by Beckman Coulter capable of detecting acute and chronic changes to cellular haemoglobin status and iron supply. The research aimed to investigate the clinical usefulness of RSf as screening parameter for α TT and the effect of concomitant ID to RSf mean values (m.v) among respondents with α TT. **Methods:** A cross-sectional retrospective laboratory analysis involved 55 respondents' data selected from January 2014 to December 2015 in Pathology Department, Hospital Tuanku Ja'afar Seremban, Negeri Sembilan. The significant difference at $p < 0.05$ in the RSf m.v. among respondents with α^0 TT, α^+ TT, and ID groups and the effect concomitant ID to RSf m.v. were determined using statistical test, one-way analysis of variance (ANOVA). **Results:** Significant differences were detected in RSf m.v. i) between α^0 TT, α^+ TT and ID, $F(2, 52) = 18.99$, $p = 0.001$. ii) between α TT without ID, α TT with ID and ID cohorts for both a) α^0 TT [$F(2, 33) = 23.77$, $p = 0.001$] and b) α^+ TT [$F(2, 28) = 5.37$, $p = 0.011$]. iii) between α TT and ID group regardless of the presence of ID for both a) α^0 TT and b) α^+ TT. **Conclusion:** RSf is a potential screening parameter in evaluating patients with hypochromic microcytosis in identifying possible cases of alpha TT regardless of iron status.

Keywords: Thalassaemia trait, Iron deficiency, Thalassaemia screening, Red blood cell indices

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INTRODUCTION

Iron deficiency is a common cause of microcytic anaemia affecting the global population particularly children and pregnant women (1). Being microcytic, thalassaemia trait (TT) usually resembles iron deficiency (ID) with or without anaemia in morphology. However, the underlying pathogenesis, treatment and prognosis of iron deficiency anaemia (IDA) and thalassaemia are entirely different.

Since the early 1970s, automated full blood count (FBC) analysers have been used as initial screening tool to discriminate IDA and TT, partly because of its economical and practicality (2). Red blood cell (RBC) indices including mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and haemoglobin (Hb) concentration, are useful for thalassaemia screening. Unfortunately, none of these parameters is sufficiently reliable to identify the carrier

status due to highly inaccurate predictors of globin genotype. Multiple cut off points have been reported to be useful for thalassaemia trait screening between 60-70 fL for MCV and 19-23 pg for MCH in α -TT detection, whereas only slight reduction is usually observed in α -TT (3).

Quantification of HbA2 is recognised as an appropriate approach for screening and identifying classical beta thalassaemia trait. A Hb A2 cut-off level $> 4.0\%$ with MCV and MCH of < 75 fl and < 27 pg, respectively, were used as benchmark for screening and presumptive identification of classical beta thalassaemia carriers prior to deoxyribonucleic acid (DNA) studies (4). However, normal Hb A2 level does not exclude alpha (α) thalassaemia. A definitive diagnosis of a thalassaemia requires DNA analysis (3), however, is limited to research laboratories or specialist referral centres. Without conclusive diagnosis, some cases may be underdiagnosed, and unnecessary iron treatment may be given. Another important reason is to assist in the process of genetic counselling to family members and couples who are planning to be married.

Realising the importance of differentiating the two

conditions, many efforts had focused on the new red cell parameters. One of these applies reticulocytes measurement which is postulated to give a better estimate of erythropoietic bone marrow activity. The more immature reticulocytes are larger and contain more ribonucleic acid (RNA) (5,6). Hence, reticulocyte immaturity is directly proportional to the increase in light scatter or cell volume.

Red blood cell size factor (RSf) is a new parameter on the Beckman Coulter DxH800 that combines the information of both mean mature RBC volume and mean reticulocyte volume (MRV) by the formula $RSf = \sqrt{MCV \times MRV}$. By considering both dimensions of precursors and mature red cells, RSf indirectly reflects the cellular Hb content of both cell populations (7). Previous attempted studies on RSf were limited and showed variable results.

Urrechaga, who was the pioneer of this subject had demonstrated that RSf mean values and standard deviation (SD) for patients with IDA was higher than α TT even though this study did not particularly intend to differentiate TT and IDA. With the cut-off value of 87.7, receiver operating characteristic (ROC) analysis provided area under the curve (AUC) 0.983, 99.4% sensitivity and 90.1% specificity for identifying restricted erythropoiesis (IDA and α TT) (7). Interestingly, all α TT cases in this study were correctly diagnosed when this cut-off value was applied.

The aim of this study is to determine the significant difference in the RSf mean values between the different groups of alpha thalassaemia trait and iron deficiency anaemia and the effect of iron deficiency to the RSf mean values.

MATERIALS AND METHODS

Study design and sampling

This is a retrospective, cross-sectional study conducted in the Pathology Department, Hospital Tuanku Ja'afar, Seremban (HTJS), Negeri Sembilan from January 2014 to December 2015. Sample size was calculated using sample size calculator (OpenEpi) for comparing two means by utilising mean and SD for both TT and ID from previous study (7, 8). To achieve the power of study of 80% and 95% confidence interval, a minimum of 24 subjects are required. A total of 72 respondents were recruited by random selection from the list of patients who were suspected to have α thalassaemia and had their samples sent for DNA analysis.

Inclusion criteria were suspected cases of thalassaemia trait from FBC parameters, suspected of Hb Constant Spring from high performance liquid chromatography (HPLC) and family history of thalassaemia (cascade screening). Exclusion criteria were FBC results performed by other than Beckman Coulter DXH800 analyser, absence of iron studies, normal findings of both

molecular studies for α thalassaemia and biochemical tests for iron deficiency, concomitant α thalassaemia or any Hb variant, thalassaemia major and thalassaemia intermedia (HbH disease), underlying history of chronic illness or inflammation, RSf of > 100 (since RSf mean value for healthy individual is 100.9 meanwhile for anaemia of chronic disease (ACD) and chronic kidney disease (CKD) patients, the RSf mean values are 108.9 and 110.8, respectively) (7) and any patient age less than 6 months old.

Laboratory investigations

Molecular findings from DNA analysis of α globin gene were traced manually from the Record Unit, Haematopathology Department, Hospital Tuanku Ja'afar Seremban. All information regarding patients' age, gender, ethnicity and other related investigation results such as RBC parameters including RSf, serum iron, serum total iron binding capacity (TIBC), serum ferritin and HPLC findings were obtained from the integrated Laboratory Information System.

FBC parameters including RSf were measured by Beckman Coulter DXH800. Serum iron and TIBC were measured by Siemens Dimension RXL analyser. Serum ferritin was quantified by Cantaur XP analyser. HPLC on the Beta Thalassaemia Short Program of the Variant II (Bio-Rad, Hercules, CA) was the method used for identification of variant Hb and HbA2 quantification. DNA analysis utilised Multiplex Gap Polymerase Chain Reaction (PCR) and Multiplex Amplification Refractory Mutation System (ARMS) PCR.

Socio-demographic characteristics included were age, gender and ethnicity. Genetic variants of α thalassaemia trait were classified into α^+ thalassaemia and α^0 thalassaemia trait. α^+ thalassaemia is divided into deletional and non-deletional types. Evidence of iron deficiency (ID) is defined by either serum ferritin $< 50 \mu\text{g/L}$ and/or transferrin saturation: $\text{Iron/TIBC} \times 100 < 20\%$ (7).

Ethics

Ethical clearance to conduct the study was obtained from Medical Research and Ethics Committee, Ministry of Health Malaysia and Universiti Putra Malaysia's Research Ethics Committee.

Statistical analysis

Statistical software package IBM SPSS version 23.0 for windows was applied for statistical analysis. Descriptive analysis was used for describing socio-demographic criteria using number and percentage (%). Mean and standard deviation (SD) for RSf values were also determined for all respondents. Analysis of variance using statistical analysis one way-ANOVA followed by Post-Hoc test comparison were performed to determine significant difference in the RSf mean values among respondents between different groups. Group differences

were considered statistically significant at $p < 0.05$.

RESULTS

A total of 72 respondents were initially recruited for the study. However, 17 (23.6%) respondents were excluded due to incomplete data.

Majority of the subjects were aged more than 15 years old (85.5%) and females (87.3%). Malay was the largest population (69.1%) followed by Chinese (20%) and Indian (10.9%). This finding reflects the ethnic mixture of the local area served by the hospital and consistent with the ethnic distribution in Malaysia according to 2018 census (9). Nineteen cases (34.5%) were α^+ thalassaemia trait and 24 cases (43.6%) were α^0 thalassaemia trait. The remaining 12 cases (21.8%) had no abnormalities of the α gene tested by the same PCR method.

Within the Malay population, majority (31/38) were confirmed to have α thalassaemia alleles. The most common α globin gene abnormality among Malay respondents was the -- SEA (Southeast Asian double gene deletion) with 14 (36.8%) cases reported. Similar to Malay population, -- SEA deletion allele was also the most common (81.8%) abnormality detected among Chinese respondents. In contrast, majority of Indian subjects were iron deficient individuals. However, within this small Indian population, two cases of α TT (one α -3.7 allele and one α -4.2 allele) were identified.

There was a significant difference in the RSf mean values between α^0 thalassaemia trait, α^+ thalassaemia trait and iron deficiency group $F(2, 52) = 18.99$, $p=0.001$. Table I shows Post Hoc comparison results using the Tukey HSD test indicated that RSf mean values for both types of α TT (α^0 and α^+ thalassaemia trait were 79.9 ± 4.1 fL and 83.7 ± 4.4 fL, respectively) were significantly different from the ID group (89.4 ± 5.0 fL).

Effect of iron deficiency on RSf mean values of α TT (α^+ and α^0) was examined separately as depicted in Table

Table I: RSf mean value among respondents with α^0 thalassaemia trait, α^+ thalassaemia trait and evidence of iron deficiency

Variable	n	Mean (SD)	F(df)	p value
RSf			18.99 (2,52)	0.001
α^0 thalassaemia trait ^{1,2}	24	79.9 (4.1)		
α^+ thalassaemia trait ^{2,3}	19	83.7 (4.4)		
Iron deficiency ^{1,3}	12	89.4 (5.0)		

¹ Significant difference of RSf mean between α^0 thalassaemia trait and iron deficiency at $p=0.001$

² Significant difference of RSf mean between α^+ thalassaemia trait and α^0 thalassaemia trait at $p=0.016$

³ Significant difference of RSf mean between iron deficiency and α^+ thalassaemia trait at $p=0.003$

II and table III. For α^0 TT group, significant difference was observed in the RSf mean values between the three groups (α^0 TT without ID, α^0 TT with ID and ID only) $F(2, 33) = 23.77$, $p=0.001$. Table II shows Post Hoc comparison results using the Tukey HSD test indicated that RSf mean values for both types of α^0 TT (with and without ID were 77.9 ± 4.4 fL and 81.8 ± 2.8 fL respectively) were significantly different with the ID group (89.4 ± 5.0 fL). Lower RSf mean value in α^0 TT with ID group compared to α^0 TT without ID group was found. This, however, was not statistically significant.

Table II: Effect of iron deficiency to the RSf mean value of α^0 thalassaemia trait

Variable	n	Mean (SD)	F(df)	p value
RSf			23.77 (2,33)	0.001
α^0 TT without ID ¹	12	81.8 (2.8)		
Iron deficiency ^{1,2}	12	89.4 (5.0)		
α^0 TT with ID ²	12	77.9 (4.4)		

¹ Significant difference of RSf mean between α^0 TT without ID and iron deficiency at $p=0.001$

² Significant difference of RSf mean between iron deficiency and α^0 TT with ID at $p=0.001$

Table III: Effect of iron deficiency to the RSf mean value of α^+ thalassaemia trait

Variable	n	Mean (SD)	F(df)	P value
RSf			5.37 (2,28)	0.011
α^+ TT without ID ¹	9	83.6 (3.9)		
Iron deficiency ^{1,2}	12	89.4 (5.0)		
α^+ TT with ID ²	10	77.9 (5.0)		

¹ Significant difference of RSf mean between α^+ TT without ID and iron deficiency at $p=0.023$

² Significant difference of RSf mean between iron deficiency and α^+ TT with ID at $p=0.028$

Effect of iron deficiency on RSf mean value of α^+ thalassaemia trait was also observed. There was a significant difference in the RSf mean values for the three groups (α^+ TT without ID, α^+ TT with ID and ID only). $F(2, 28) = 5.37$, $p=0.011$ as shown by Table III. Post Hoc comparison results using the Tukey HSD test indicated that the RSf mean values for both types of α^+ TT (with and without ID were 77.9 ± 5.0 fL and 83.6 ± 3.9 fL respectively) were significantly different with the ID group (89.4 ± 5.0 fL). Lower RSf mean value in α^+ TT with ID group compared to α^+ TT without ID group was noted. This however was not statistically significant.

DISCUSSION

The main aim of this study is to determine whether RSf value is able to distinguish α thalassaemia trait from iron deficient cases. Demographic profile and the genetic variants of respondents in this study did not represent

the actual population prevalence of α thalassaemia variants in Negeri Sembilan. This is because the population sampling was purposive with tight inclusion and exclusion criteria. This led to nearly 24% drop outs from a total number of 72 randomly selected cases resulting in a final total of 55 respondents. Presence of concomitant Hb E detected from HPLC was also one of the common reasons of exclusion. A few subjects of infants less than six months old were also excluded from the study to minimise the effect of higher MCV, especially in newborns.

Significant differences in RSf mean values ($p < 0.05$) were detected in this study when groups with thalassaemia trait (α^0 and α^+ thalassaemia trait) were compared with iron deficiency patients. These findings support previous evidence that has recommended RSf as a useful parameter in differentiating thalassaemia traits and IDA patients (10). In this study, Ng et al., 2015 showed that IDA group had significantly higher RSf values than the TT group among Hong Kong population. However, due to significant overlap between the values in two groups, the performance of this single parameter is still questionable. One of the major limitations of Ng's study was the method used in classifying α TT, considered to be less reliable. Diagnosis of α TT in his study were made by occurrence of very occasional ($< 0.1\%$) HbH inclusion bodies with no molecular confirmation (10). In contrast, diagnosis of α TT in our study was confirmed by DNA analysis.

RSf combines the information of both mean mature RBC volume and mean reticulocyte volume (MRV) by the formula $RSf = \sqrt{MCV \times MRV}$. MRV is a real time measurement of the cell dimension of recently produced erythrocytes i.e. within the period of less than 48 hours and represents iron availability during haemoglobin synthesis. MCV is a measurement of the average size or volume of mature red blood cells. Adding a square root in the formula will average out the volume of both populations of erythrocytes.

RSf in IDA patients are postulated to be higher than thalassaemia trait individuals because of the presence of both normocytic and microcytic RBC population in iron-deficient states reflecting a progressive decrease in iron stores during continuous marrow erythropoiesis. In contrast, lower RSf mean values in thalassaemia traits are the result of constantly uniform population of microcytic erythrocytes due to globin gene mutation but normal iron stores (11).

Interestingly, RSf mean value for α^0 TT in this study was also significantly lower than α^+ thalassaemia trait individuals ($p = 0.016$). Thalassaemia trait individuals with two α gene deletions may be wrongly suspected of iron deficiency particularly when the levels of haemoglobin and RBC were mildly reduced to borderline low-normal but showed lower MCV and MCH values. The RBC

parameters in such cases may resemble iron deficiency but it is already known from previous studies that RBC morphology and its indices are not specific enough to accurately differentiate these two conditions (12, 18)

To the best of our knowledge, there is absence of report on reticulocyte parameter, particularly, RSf in characterising the different α gene mutations in thalassaemia trait. However, previous work that studied erythrocyte parameters suggested that lower MCV and MCH values correlated with the severity of the genotype findings (13). This is consistent with lower RSf mean values obtained in our study among TT with greater number of gene deletions. It is particularly important to recognize this observation which may be potentially useful in the future.

Diagnosis of thalassaemia trait in patients with coexisting iron deficiency is even more challenging. It has been demonstrated that lower haemoglobin levels and reduced red cells indices such as MCV and MCH and near normal RBC occurs in such cases (14). Quantification of HbA2 may be misleading as iron deficiency can cause reduction in HbA2 level and diagnosis of thalassaemia trait, particularly beta thalassaemia trait, can be missed (15). Several studies have reported evidence of iron deficiency among TT patients (14,16). Further reduction of Hb level in this group is thought to result from lack of nutrients for haemoglobinisation in addition to an imbalance in globin chain synthesis. TT and ID were previously distinguished by Mentzer index, a quantitative method that consider the ratio of MCV and RBC. Mentzer index of more than 13 was shown to be predictive of ID, meanwhile a value less than 13 favours thalassaemia trait (17). Unfortunately, this is not practical since in most instances the value is borderline and not clinically useful. Other methods of different sensitivity and specificity have also been described (12, 18).

In this study, RSf mean values of both types (α^0 and α^+) of thalassaemia traits with and without ID displayed significantly lower values than those with iron deficiency only cohorts. Haematological parameters of mild α thalassaemia trait mimic iron deficiency or close to normal population This can lead to misdiagnosis of mild α thalassaemia trait or unnecessary molecular study being done to patient with ID only. The differences of RSf values in our findings highlight the potential clinical utility of this parameter in the near future. The findings in this study suggest that the RSf is capable of identifying not only alpha thalassaemia trait cases but also alpha thalassaemia trait individuals with concomitant iron deficiency. However, further evaluation of this parameter on a larger sample to determine the local cut-off is required for confirmation.

One of the important limitations in this study is the reliability of the research data which used retrospective laboratory analysis. Similar to any laboratory parameters,

RSf values may be affected by the preanalytical factors involved during sample collection, sample storage, time frame from sample collection to sample processing and so forth. We therefore attempted to minimise the storage effect by excluding cases that have reported storage changes on the red blood cell morphology.

Another limitation is the lack of respondents' clinical data obtained from the request forms that might affect RSf values. Relevant clinical information is very important for RSf interpretation such as history of acute or chronic inflammation, pregnancy status, medication history and vitamin B12 and folate status. Although iron deficiency was defined using biochemical test results (ferritin and transferrin saturation), this however can be argued whether the results were captured at latent phase or whether the patients were on treatment.

CONCLUSION

The present study observed significant difference in RSf mean values ($p < 0.05$) between thalassaemia trait (α^0 and α^+ thalassaemia trait) and iron deficiency cohorts. RSf may also be useful to suggest the presence of globin gene mutation despite presence of biochemical evidence of concomitant iron deficiency. Thus, RSf is a potential parameter to be used as a first line screening tool in identifying likelihood cases of mild alpha TT which have either normal red blood cell indices or indices that mimic iron deficiency. These findings require validation with a larger subject population, using a prospective study design to achieve better selection criteria.

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