

Correlation Between Some Discrimination Functions and Hemoglobin E

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Abstract

The most widely used discriminant functions and red cell indices for differential diagnosis of thalassemia traits from iron deficiency anemia were evaluated for their abilities to identify HbE-containing blood samples. The functions were as follows : $F1 = 0.01 \times MCH \times (MCV)^2$; $F2 = RDW \times MCH \times (MCV)^2 / Hb \times 100$; $F3 = MCV/RBC$; and $F4 = MCH/RBC$. Other red cell parameters including RDW, hemoglobin content, mean cell volume, mean cell hemoglobin as well as red cell counts, were also evaluated to distinguish HbE from the normal population. Hemoglobin electrophoresis was used as a confirmatory test. The results showed that F1, F2 and F3 as well as other red cell parameters of HbE-containing samples were different from those of HbA₂A-containing red cells although there was no statistical significance. However, F4 and MCHC showed no difference between the two groups. It can be concluded from the present study that identification of hemoglobin E especially the heterozygous form by using parameters from an electronic cell counter is not easy. Discriminant functions and red cell indices might be used as an initial diagnosis. But confirmation is needed in all cases. Applying the MCV of 80 fl will miss 5 per cent of hemoglobin E carrier but will not miss the homozygous form.

Key word : Discriminant Function, HbE, Hematologic Parameters

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Thalassemias and hemoglobinopathies are hemolytic genetic diseases resulting from abnormal quantitative or qualitative synthesis of globin chains (1,2) which are commonly found in Southeast Asia (3-6). A high incidence of hemoglobin E is especially found in the northeastern part of Thailand

(3-7). Although homozygous hemoglobin E (EE) is not so severe, its combination with beta thalassemia can give rise to a pathologic condition as severe as homozygous beta-thalassemia(1). One of the most effective ways to reduce thalassemic disease in high-risk populations is the national programme based

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on carrier screening at the population level. Typical hemoglobinopathic carriers may present as a mild microcytic hypochromic anemia which have to be differentiated from iron deficiency anemia⁽⁸⁻¹³⁾. Discriminant functions (DFs) was first described by Fisher in 1936⁽¹⁴⁾. Several DFs based on routine complete blood count parameters have been proposed for an initial differential diagnosis⁽⁸⁻¹⁴⁾. Mean red cell volume (MCV)^(15,17,18), mean red cell hemoglobin (MCH)^(17,19), red cell distribution width (RDW)^(17,19-21) and also the combination of red cell parameters as a mathematic formula have been shown to be useful in a quick screening process^(16,17,22). It was the purpose of this study to re-evaluate the utility of DFs to initially identify hemoglobin E using hemoglobin typing as the reference diagnostic technique.

MATERIAL AND METHOD

Chemicals

Unless specified, all chemicals used in the present study were purchased from Sigma (St. Louis, MO, USA).

Specimens

EDTA blood samples were collected from routine hematologic laboratory at the Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University. Blood samples were then separated into 3 groups based on their mean cell volume (MCV) which were 80-98, 70-79 and < 70 fl. Blood cell parameters were statistically analysed using Microsoft Excel 7.0. The remainder of the same blood samples were further investigated for one-tube osmotic fragility test (OF), dichlorophenol indophenol precipitation test (DCIP) and hemoglobin identification by cellulose acetate electrophoresis.

Determination of discriminant factors

Discriminant functions (DFs) of each sample were calculated from blood parameters. The 4 discriminant functions were used in the present study. F1 represents $0.01 \times \text{MCH} \times (\text{MCV})^2$ ⁽²³⁾ while F2 represents $\text{RDW} \times \text{MCH} \times (\text{MCV})^2 / \text{Hb} \times 100$ ^(9,11,24,25) and F3, F4 were the ratio of MCV/RBC ⁽²⁶⁾ and MCH/RBC ,^(20,27) respectively.

One tube osmotic fragility

The osmotic fragility (OF) test was used to

determine the degree of red blood cell hemolysis produced by osmotic stress. Osmotic lysis is dependent upon cell volume, surface area, and functional integrity of cell membranes. The variation of cell lysis with stress reflects underlying cell subpopulations and their membranes' cytoskeletal functionality. The screening one tube method using 0.36 per cent buffered saline was performed as described^(28,29). Twenty microliters of whole blood was pipetted into 5 ml of 0.36 per cent buffered saline. Mixed well. After 5 min, the hemolysis was evaluated by visualisation. Negative and positive controls were done simultaneously.

DCIP precipitation

The blue dye, dichlorophenol-indophenol (DCIP), weakened the contact between $\alpha 1$ and $\beta 1$ globin chain causing precipitation and cloudy appearance. The method was performed as previously described^(30,31). Dye solution was prepared by dissolving 4.36 g of Trisma base, 2.68 g of EDTA, 0.0275 g of DCIP and 0.05 g of saponin in deionized water. The pH of this solution was adjusted to 7.5 and then the volume was made to 500 ml. Twenty microlitres of packed red cells was dissolved in 5 ml of the dye solution and left at room temperature for 10 min. The solution was further incubated without disturbing at 37°C for 1 h. Hemoglobin precipitation or cloudiness was read carefully.

Hemoglobin identification

Cellulose acetate hemoglobin electrophoresis at an alkaline pH was carried out using the Helena Laboratories equipment^(32,33). Packed red cells were prepared from EDTA blood and then lysed with hemolysing solution. The hemolysate was subjected to electrophoresis at 350 volts for 20 min. Ponceau S was used to stain the cellulose acetate after electrophoresis was done.

RESULTS

MCV and hemoglobin E genotype

As shown in Table 1, not more than 5 per cent of hemoglobin E trait (EA) had normal MCV (80-98 fl). None of the homozygous hemoglobin E (EE) had normal MCV. However, the interesting data showed further that the percentage of EA increased to 39 per cent when MCV decreased to 70-79 fl. The percentage of EE was gradually increased from 4 per cent to 13 per cent when MCV decreased

Table 1. Hemoglobin E genotype of blood samples at various MCV levels.

MCV (fl)	Total (cases)	A ₂ A (%)	EA (%)	EE (%)
80-98	103	95.2	4.8	0
70-80	105	57	39	4
< 70	100	65	22	13

from 70-79 fl to < 70 fl. It should be noted that the number of EA at MCV < 70 fl (22%) was lower than that at 70-79 fl (39%).

Hemoglobin E, OF and DCIP

DCIP is a specific test for identification of hemoglobin E. All cases of EA and EE were positive for DCIP while none of A₂A genotype were DCIP positive (Table 2). However, the present study found no correlation between the degree of dye precipitation and severity of gene mutation. Some cases of EA showed more precipitation than EE genotype while some cases of EE showed less precipitation than EA genotype (data not shown).

One-tube osmotic fragility test was not as specific as DCIP precipitation to identify hemoglobin E (Table 2). A small number of A₂A with normal MCV showed decreased OF (2.9%). However, the number of OF positive increased from 26.7 to

54 per cent when MCV decreased from 70-79 to < 70 fl, respectively. All cases of EE had decreased OF while EA showed variation of red cell fragility (100%, 83% and 95% at MCV 80-89, 70-79 and < 70 fl, respectively).

Hemoglobin E, hematologic parameters and discriminant functions

The correlation between other hematologic parameters and hemoglobin E genotype was also evaluated (Table 2). Trends of correlation between hematologic parameters and hemoglobin E could be seen when MCV was normal. Red cell number, hemoglobin content, MCV, MCH as well F1 of A₂A were higher while RDW, F2 and F3 were lower than those of EA. However, this correlation has no statistical significance ($P > 0.05$). It is interesting that MCHC and F4 of all samples were not related to the presence of hemoglobin E.

Similar correlation as mentioned above, except F2 and F3, could be seen in the group with MCV 70-79 fl only when comparison was made between A₂A and EA on one side with that of EE on the other side. There was no specific correlation between red cell parameters and hemoglobin E in the group that MCV < 70 fl (Table 2).

The data in Table 2 were re-analysed based primarily on hemoglobin typing and shown in Table 3. The present study investigated 223 of A₂A, 68

Table 2. Means and standard deviations of some hematologic parameters and discriminant functions of blood samples at various MCV levels. (F1 = $0.01 \times \text{MCH} \times (\text{MCV})^2$; F2 = $\text{RDW} \times \text{MCH} \times (\text{MCV})^2 / \text{Hb} \times 100$; F3 = MCV/RBC ; F4 = MCH/RBC).

MCV (fl)	80 - 89		70 - 79			< 70		
	A ₂ A	EA	A ₂ A	EA	EE	A ₂ A	EA	EE
Hb type (%)	95.2	4.8	57	39	4	65	22	13
DCIP+ (%)	0	4.8	0	39	4	0	22	13
OF+ (%)	2.9	4.8	26.7	32.4	4	54	21	13
RDW (%)	13±1.4	16±4.3	15±2.8	15±3.4	18±2.4	18±4.4	19±4.9	17±5.3
RBC ($\times 10^{12}/\text{L}$)	4.6±0.4	4.1±0.6	4.7±0.9	4.9±0.7	4.6±0.4	5.2±1.1	4.9±0.9	5.4±0.7
Hb (g/dl)	14±1.3	12±1.5	12±2.3	13±3.3	11±1.2	11±3.8	11±2	11±1.5
MCV (fl)	89±4	85±1.9	76±2.7	76±2.9	72±1	65±4.5	66±3	64±4
MCH (pg)	30±1.4	28±0.9	25±1.3	25±1.2	24±0.7	21±1.8	22±1.2	21±1.1
MCHC (g/dl)	33.7±0.9	33.5±0.6	33±1	33±1	32.6±0.6	32±1.4	32.6±0.9	32.6±1
F1	2395	2035	1459	1450	1223	893	959	870
	± 310	± 147	± 168	± 169	± 46	± 178	± 129	± 142
F2	2272	2864	2020	1712	2020	1518	1713	1267
	± 563	± 963	± 600	± 737	± 449	± 581	± 592	± 558
F3	20±2.5	21±3.9	17±4.1	16±3.3	16±1.6	13±3.4	14±3.1	12±1.9
F4	7±1	7.1±1.4	6±1	5.3±1.1	5.2±0.5	4±1	4.6±1.1	3.9±0.5

of EA and 17 cases of EE. All EA and EE gave positive DCIP while none of A₂A showed false positive. All cases of EE, 88 per cent of EA and 38 per cent of A₂A showed decreased OF. The means of RDW of A₂A < EA < EE while means of MCV, MCH, F1, F2, F3 and F4 of A₂A > EA > EE. However, the difference had no statistical significance ($P > 0.05$).

The means of red cell count and hemoglobin content of EA was not different from that of A₂A but both were different from those of EE although there was also no statistical significance. It is interesting that there was only no difference of MCHC among A₂A, EA and EE.

DISCUSSION

The widespread use of electronic cell counters has generated attempts to manipulate routine blood count parameters for a quick differential screening of iron deficiency anemia from β -thalassemic carriers and other hemoglobinopathies(34). Discriminant functions (DFs) obtained from mathematic formula of the parameters was first described by Fisher in 1936(14). Since then, various numerical functions have been widely developed. They are MCV/RBC (26), MCH/RBC (20,27), $(MCV)^2 \times MCH$ (23), $[(MCV-RBC) - (8.1 \times Hb) - 3.4]$ (24,25,34), $0.01 \times MCH \times (MCV)^2$ (23), $RDW \times MCH \times (MCV)^2 / Hb \times 100$ (9,11). Originally, the DFs were mostly applied to the differentiation of microcytic anemia due to iron deficiency anemia from heterozygous β -thalassemia(8-13). A few investigators used the DFs to screen for abnormal hemoglobin such as hemoglobin E, C and S traits(16,17,35). Hemoglobin E is found mostly in Southeast Asia, especially in the northeastern part of Thailand(4,5). Usually, hemoglobin E has a mild β -thalassemia phenotype. But the possible combination of HbE with β -thalassemia may result in a clinical disorder which can be as severe as that seen in homozygous β -thalassemia(4-6). However, population screening for HbE has not been widely reported compared to that of thalassemic carriers although the identification of hemoglobin E can indirectly help to control thalassemic disease. The present study tried to apply DFs for an initial diagnosis of hemoglobin E. The standard hemoglobin electrophoresis was used as a confirmatory test.

As shown in Table 1, 95.2 per cent of normal MCV had A₂A hemoglobin typing. This could be normal individuals, β -thalassemia 2 trait or

carriers of hemoglobin Constant Spring (HbCS)(3) since HbCS is unstable and may not be detected(1). It was found that 4.8 per cent of normal MCV had EA genotype while none had EE. This finding indicated that applying a discriminant value of 80 fl to the MCV in the population screening would miss around 5 per cent of the HbE traits. The result is consistent with the previous report(22). However, data shown in Table 1 is only preliminary since the lower MCV could include α -thalassemia trait and/or iron deficiency anemia. The differential diagnosis can be done by family study to rule out α -thalassemia trait and by study of iron status to rule out iron deficiency anemia.

When samples of moderately microcytosis (MCV = 70-79 fl) were analysed it was found that 57 per cent had A₂A while 39 per cent had EA and 4 per cent had EE (Table 1). It is possible that A₂A genotype may be real A₂A or high HbA₂ which could be β -thalassemia trait(3). The confirmation can be done by measurement of HbA₂ which was not done in the present study. However, cellulose acetate electrophoresis did not show any thicker HbA₂ bands. Iron deficiency anemia and α -thalassemia 1 trait could also be diagnosed(3). If hemoglobin content is less than 10 g/dl then iron deficiency can be a case while α -thalassemia 1 trait can be confirmed by determination of anti-3 globin(3).

The same interpretation could be made with the group of MCV < 70 fl. However, only 22 per cent of this group had EA typing while EE increased to 13 per cent indicating that microcytosis correlates with the severity of hemoglobin E mutations. The definite diagnosis of homozygous hemoglobin E can be made from EE typing while EA typing can be either heterozygous hemoglobin E or combination with α -thalassemia trait or iron deficiency(3). Therefore, other methods should be included for differential diagnosis.

As shown in Table 2, DCIP is a specific test for HbE identification. None of the HbE-containing samples showed negative DCIP, and none of the A₂A showed false positive DCIP. The present study found that the degree of precipitation by DCIP was not correlated with genotype severity. Some samples of EE gave milder precipitation than that of EA, while some samples of EA gave greater precipitation than that of EE. This finding is not consistent with the previous report which showed that Hb EE gave greater precipitation than Hb EA which in

turn was greater than other hemoglobins respectively⁽³¹⁾.

One tube osmotic fragility test is not as specific as DCIP for screening of HbE. Although all EE had decreased OF a small number of EA could have normal OF (Table 2). The present study found that 2.9 per cent of normal MCV with hemoglobin A₂A had false positive OF (Table 2). This was consistent with the previous report stating that over 96 per cent of normal persons showed hemolysis in this buffer⁽³⁾. The number of OF positive samples increased to 26.7 and 54 per cent when MCV decreased to 70-79 and < 70 fl, respectively. It is possible that these OF positives might have resulted from iron deficiency or a thalassemia carrier.

The present finding showed that some hematologic parameters and discriminant functions had correlation with severity of hemoglobin E mutation when MCV > 70 fl although there was no statistical significance ($P > 0.05$) (Table 2). The different numbers were seen between A₂A and EA in the group with normal MCV. However, a similar difference between A₂A and EA was not seen in the group with MCV 70-79 fl. The difference could only be seen when comparing A₂A and EA on one side with EE on the other side. MCHC was the only parameter that did not relate to hemoglobin E genotype. The correlation between hematologic values and hemoglobin genotype also could not be seen when MCV < 70 fl (Table 2).

The better apparent correlation could be seen when the data were re-analysed based primarily on hemoglobin genotype (Table 3). The results indicated that RDW, MCV, MCH, F1, F2, F3 and F4 could be used as an initial step for screening of EA and EE from A₂A. However, the mean difference of these parameters was not statistically significant. Red cell count, hemoglobin content and MCHC could not help in the differential diagnosis of EA and EE from A₂A.

It is clear from Table 2 and 3 that normal individuals have MCV more than 80 fl and hemoglobin content more than 10 g/dl. Both heterozygous and homozygous hemoglobin E have hemoglobin content more than 10 g/dl.

Yeo et al⁽²²⁾ used the value of MCV to screen beta thalassemia and HbE trait in antenatal patients and found that MCV for HbE trait was 75.7 ± 4.1 fl. The number was slightly higher than that found in the present study (74 ± 6.2 fl) (Table 3) but they were still within the same range.

The present finding confirmed a previous report⁽³⁶⁾ that applying the discriminant functions taken from traditional red cell indices for HbE diagnosis is difficult. Some discrimination functions that can be used to differentiate iron deficiency from β -thalassemia trait could not clearly identify HbE from the population (Table 2). Nevertheless, although there was non-significant correlation, some parameters such as RDW, MCV, MCH, F1, F2, and F3 showed promising results (Table 3). A larger sample size might reveal some more useful information. An alternate screening method composed of an initial discriminant factor and a confirmation by cellulose acetate electrophoresis might be sensitive and specific enough for routine work. Recently, the sensitivity of cellulose acetate electrophoresis for hemoglobin identification has been emphasized⁽³³⁾.

Table 3. Means and standard deviations of some hematologic parameters and discriminant functions of blood samples with different hemoglobin type (A₂A, EA, EE). (F1 = $0.01 \times \text{MCH} \times (\text{MCV})^2$; F2 = $\text{RDW} \times \text{MCH} \times (\text{MCV})^2 / \text{Hb} \times 100$; F3 = MCV/RBC ; F4 = MCH/RBC).

Hb typing	A ₂ A	EA	EE
No. (cases)	223	68	17
DCIP+ (%)	0	100	100
OF+ (%)	38	88	100
RDW (%)	15 ± 3.8	16 ± 4.4	17 ± 4.6
RBC ($\times 10^{12}/\text{L}$)	4.8 ± 0.9	4.8 ± 0.8	5.2 ± 0.7
Hb (g/dl)	12 ± 2.8	12 ± 3	11 ± 1.4
MCV (fl)	78 ± 12	74 ± 6.2	66 ± 4.9
MCH (pg)	26 ± 4.3	24 ± 2.3	21 ± 1.5
MCHC (g/dl)	33 ± 1.09	33 ± 0.84	33 ± 0.81
F1	1693 ± 702	1344 ± 347	953 ± 198
F2	1965 ± 663	1819 ± 776	1444 ± 616
F3	17 ± 4.3	16 ± 3.7	13 ± 2.4
F4	6 ± 1	5 ± 1	4 ± 1

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การประเมินค่าความสัมพันธ์ระหว่าง discriminant functions และ ฮีโมโกลบิน อี

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การศึกษานี้มีวัตถุประสงค์เพื่อประเมินค่า discriminant function 4 ค่า คือ $F1 = 0.01 \times MCH \times (MCV)^2$; $F2 = RDW \times MCH \times (MCV)^2 / Hb \times 100$; $F3 = MCV/RBC$; and $F4 = MCH/RBC$ และค่าดัชนีเม็ดเลือดแดงว่าสามารถนำมาตรวจคัดกรอง hemoglobin E ได้หรือไม่ ตามปกติแล้วค่าต่างๆเหล่านี้จะนำมาใช้ในการวินิจฉัยแยกพาหะของธาลัสซีเมียออกจากภาวะโลหิตจางเนื่องจากการขาดเหล็ก ผลการศึกษาพบว่าค่าที่ประเมินทุกค่ายกเว้น $F4$ และ MCHC ในเลือดที่มี hemoglobin E ต่างจากเลือดที่เป็น A_2A แต่ความแตกต่างนั้นไม่มีความสำคัญทางสถิติ หากใช้ค่า MCV ที่ 80 fl เป็นตัวคัดกรองดังที่มีรายงานมาก่อน พบว่าจะทำให้การคัดกรองพาหะของ hemoglobin E (EA) ผิดไปประมาณ 5% เนื่องจาก 5% ของ EA จะมีค่า MCV สูงกว่า 80 fl จึงสรุปได้ว่าการนำค่า discriminant function และ ดัชนีเม็ดเลือดแดงมาใช้คัดกรอง hemoglobin E ไม่ชัดเจนเหมือนกับผลที่ศึกษาในพาหะของธาลัสซีเมีย ดังนั้นจึงต้องระมัดระวังและต้องทำร่วมกับการทดสอบยืนยันเสมอ

คำสำคัญ : Discriminant Function, HbE, Hematologic Parameters

วนิดา อีรูรัตน์ และคณะ

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