A Reliable Screening Protocol for Thalassemia and Hemoglobinopathies in Pregnancy

An Alternative Approach to Electronic Blood Cell Counting

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Abstract

Primary screening for thalassemia and hemoglobinopathies usually involves an accurate blood count using an expensive electronic blood cell counter. A cheaper alternative method was tested by using a modified osmotic fragility (OF) test and a modified dichlorophenolindophenol (DCIP) test. Altogether 423 pregnant Thai women participated in this project. Hemoglobin patterns and globin genotypes were determined using an automated high-performance liquid chromatography analyzer and polymerase chain reaction analysis of α- and β-globin genes. Among the 423 subjects, 264 (62.4%) carried thalassemia genes. The combined OF and DCIP tests detected all pregnant carriers of the 3 clinically important thalassemias, ie, α^0-thalassemia, β-thalassemia, and hemoglobin E with a sensitivity of 100.0%, specificity of 87.1%, positive predictive value of 84.5%, and negative predictive value of 100.0%, which show more effectiveness than these values for the standard method based on RBC counts. A combination of modified OF and DCIP tests should prove useful and applicable to prenatal screening programs for thalassemia and hemoglobinopathies in communities with limited facilities and economic resources.

Thalassemia and hemoglobinopathies, the most common inherited disorders of hemoglobin (Hb) synthesis, are among the major public health problems in many areas of the world, including Southeast Asia.[1,2] Although gene-gene interactions in this population can lead to several thalassemia syndromes, 3 targeted for prevention and control measures are homozygous α^0-thalassemia causing the Hb Bart hydrops fetalis, homozygous β-thalassemia, and β-thalassemia/Hb E.[3] Therefore, in a prevention and control program, rapid, accurate, and inexpensive screening protocols to identify carriers of α^0-thalassemia, β-thalassemia, and Hb E, especially in a prenatal population at risk for Hb disorders, are essential.

Conventionally, the primary screening method for all forms of thalassemia relies on hematologic index cutoffs, which involves an accurate blood count using an electronic cell counter. Individuals with mean corpuscular volume (MCV) values less than 80 μm^3 (<80 fL) and mean corpuscular hemoglobin (MCH) values less than 27 pg should be examined further to confirm or exclude the diagnoses of α-thalassemia and β-thalassemia.[4,5] This, however, requires an expensive electronic blood cell counting apparatus and cannot be applied in rural areas where laboratory facilities and economic resources are limited.

It has been demonstrated that a single-tube osmotic fragility (OF) test with 0.36% saline solution might be an attractive alternative to identify carriers of α- and β-thalassemias.[6-8] Recently it was demonstrated that the use of 0.34% instead of 0.36% saline solutions could greatly improve the specificity of the OF test for α^0-thalassemia and β-thalassemia, but an Hb E carrier would be missed.[9] A combined modified OF test and modified dichlorophenolindophenol (DCIP) test for Hb E[10] has been proposed for screening in

rural communities of Southeast Asia.[11] However, this screening protocol might not be appropriate for pregnant women because anemia might be more prevalent and severe owing to physiologic changes and/or iron deficiency. Therefore in this study, the effectiveness of the combined modified OF test and modified DCIP test for the identification of α^0-thalassemia, β-thalassemia, and Hb E in pregnancy was tested and compared with other standard screening protocols involving measurement of RBC indices.

Materials and Methods

Subjects

Study participants included 423 apparently healthy, pregnant Thai women consecutively attending an antenatal care service between January and December 2003 at Nampong and Chumpae district hospitals of Khon Kaen province in northeast Thailand. The study was approved by the institutional ethical committee of Khon Kaen University, Khon Kaen, Thailand. Only women in the first or second trimester were recruited. The mean ± SD age and gestational age of the subjects were 25.5 ± 6.0 years and 12.7 ± 3.7 weeks, respectively. After informed consent was obtained at the first visit to the antenatal care service, EDTA-anticoagulated blood samples were obtained and transferred on ice within 2 hours to the Faculty of Associated Medical Sciences, Khon Kaen University, where all laboratory investigations were performed.

Screening and Hematologic Analysis

Screening for thalassemia was performed with a modified OF test and for Hb E with a modified DCIP test using the KKU-OF and the KKU-DCIP-Clear reagent kits (PCL Holding, Bangkok, Thailand) and following the manufacturer's protocols as described.[11] Briefly, in the OF test, a sample of 20 μL of whole blood was mixed with 2 mL of 0.34% buffered saline solution in the test tube and left at room temperature for 15 minutes before being interpreted. For the DCIP precipitation test, 20 μL of whole blood was added to 2 mL of a modified DCIP reagent, and the mixture was incubated at 37°C for 15 minutes before the addition of 20 μL of stopping reagent supplied by the manufacturer to eliminate and decolorize the excess DCIP dye.

Both tests were interpreted by visualization as negative or positive. Negative samples are characterized by a clear solution and positive samples by a cloudy appearance. RBC indices were determined using the Coulter GenS automated blood cell counter (Coulter Electronics, Hialeah, FL). Hb patterns and levels were determined using an automated high-performance liquid chromatography system (Variant, Bio-Rad Laboratories, Hercules, CA).

DNA Analysis

Genomic DNA was extracted from peripheral blood WBCs by using a standard method. All common α-thalassemia mutations, including α^0-thalassemia (SEA type), α^+-thalassemia (3.7- and 4.2-kilobase deletions), Hb Constant Spring (Hb CS) and Hb Paksé (Hb Ps) were identified by the polymerase chain reaction (PCR) and related methods.[12-15] Common β-thalassemia mutations in Thailand also were examined in samples with Hb A_2 levels exceeding 3.5% using the allele-specific PCR routinely run in our laboratory.[16,17]

Statistical Analysis

Descriptive statistics, including mean and SD, were used to describe hematologic features of the subjects. To compare the effectiveness of the standard method based on RBC indices with that of the combined modified OF and DCIP tests for thalassemia and Hb E screening, we calculated sensitivity, specificity, positive predictive value, and negative predictive value. The results of Hb analysis with high-performance liquid chromatography and PCR analysis of α- and β-thalassemias were used as "gold standards."

Results

The results of thalassemia genotyping and hematologic characteristics of 423 pregnant women are summarized in **Table 1**. Among 423 pregnant women studied, 264 (62.4%) were found to carry thalassemias or hemoglobinopathies. No thalassemia gene was detected in the remaining 159 subjects (37.6%). In the former group, 22 thalassemia genotypes were observed. As expected, the most common genotype was Hb E heterozygote, which was identified in 94 subjects. Interactions of Hb E with several forms of α-thalassemia also were found. α-Thalassemias, including α^0- and α^+-thalassemia, Hb CS, and Hb Ps, were identified as pure heterozygotes or in association with other abnormal genes. Three β-thalassemia heterozygotes were detected. Two as yet undescribed conditions of homozygous Hb E with Hb Q-Thailand and a compound Hb Q-Thailand/Hb CS also were observed, which will be reported elsewhere. Compared with the nonthalassemia group, all forms of thalassemia differed in the RBC indices, particularly the MCV and MCH values. Changes in these parameters clearly were observed in women with α^0- and β-thalassemias and in those who carried 2 or more abnormal genes.

To compare the effectiveness of the screening strategies, the data collected as shown in Table 1 were separated into 2 groups based on genotypes, ie, clinically important and nonclinically important genotypes. The former included α^0-thalassemia, β-thalassemia, and Hb E, which are the target of thalassemia screening.

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Table 1

Thalassemia Genotypes Observed in 423 Pregnant Women and the Corresponding Hematologic Characteristics*

Thalassemia Genotype (No. of Cases)	RBC ($\times 10^{12}$/L)	Hb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	Hb Type	HbA_2/E (%)
Heterozygous Hb E (94)	4.6 ± 0.5	11.7 ± 1.1	35.3 ± 3.6	77.5 ± 4.7	25.7 ± 1.8	33.1 ± 1.0	14.3 ± 1.7	EA	29.3 ± 1.7
With α^+-thalassemia (26)†	4.3 ± 0.4	11.6 ± 1.0	35.1 ± 3.1	81.5 ± 3.7	27.1 ± 1.9	33.1 ± 1.1	13.9 ± 1.1	EA	28.0 ± 2.1
With Hb CS (7)	4.1 ± 0.5	11.1 ± 1.1	33.4 ± 3.1	81.9 ± 2.4	27.3 ± 0.9	33.2 ± 0.3	13.7 ± 0.7	EA/CSEA	27.0 ± 1.6
With Hb Ps (3)	5.1 ± 1.0	12.2 ± 1.3	37.9 ± 4.6	74.8 ± 7.7	24.2 ± 2.7	32.4 ± 0.5	15.5 ± 2.5	EA/PsEA	25.7 ± 1.1
With homozygous α^+-thalassemia (2)	5.1, 4.7	12.2, 10.1	37.5, 31.2	72.6, 66.4	23.6, 21.5	32.5, 32.3	15.3, 20.2	EA	21.1, 20.5
With α^0-thalassemia (6)	4.8 ± 0.3	10.5 ± 0.7	32.7 ± 2.0	68.5 ± 4.6	21.9 ± 1.6	32.0 ± 0.7	16.5 ± 1.9	EA	19.6 ± 1.3
With α^+-thalassemia/Hb Ps (1)	5.01	11.2	34.9	69.6	22.3	32	14.1	EA	20.4
With α^0-thalassemia/α^+-thalassemia (1)	4.86	7.7	24.7	50.8	15.8	31.1	22.6	EA	15.9
With α^0-thalassemia/Hb Ps (1)	4.92	7.6	28	56.8	15.5	27.3	23.6	EA	12.6
Homozygous Hb E (16)	4.7 ± 0.5	10.1 ± 0.8	31.4 ± 2.5	66.8 ± 2.6	21.5 ± 0.8	32.2 ± 0.4	16.9 ± 1.0	EE	89.9 ± 4.2
With α^+-thalassemia (3)	4.8 ± 0.5	10.4 ± 0.1	32.1 ± 0.9	67.2 ± 6.4	21.7 ± 1.9	32.4 ± 0.9	16.6 ± 2.4	EE	91.8 ± 4.0
With Hb Q (1)	4.89	10.8	32.7	66.8	22.1	33.1	15.3	EE with Q	79.4
Heterozygous α^0-thalassemia (10)	5.1 ± 0.6	11.0 ± 1.3	34.6 ± 3.9	68.0 ± 2.5	21.6 ± 1.6	31.8 ± 1.5	15.3 ± 1.0	A_2A	2.3 ± 0.3
Heterozygous α^+-thalassemia (53)‡	4.4 ± 0.4	12.1 ± 0.8	36.3 ± 2.6	82.3 ± 3.5	27.4 ± 1.5	33.3 ± 0.9	13.7 ± 1.0	A_2A	2.6 ± 0.3
Heterozygous Hb CS (26)	4.4 ± 0.3	11.6 ± 0.9	35.5 ± 2.9	81.5 ± 5.1	26.7 ± 1.6	32.7 ± 0.8	13.5 ± 0.9	A_2A/CSA_2A	2.6 ± 0.5
Heterozygous Hb Ps (3)	4.4 ± 0.4	12.1 ± 1.1	36.5 ± 4.1	83.4 ± 4.1	27.7 ± 0.7	33.2 ± 0.7	13.3 ± 0.2	A_2A/PsA_2A	2.6 ± 0.2
Homozygous α^+-thalassemia (4)	5.1 ± 0.7	11.7 ± 1.6	35.8 ± 3.4	70.4 ± 2.5	22.8 ± 0.3	32.4 ± 1.4	17.0 ± 2.6	A_2A	2.7 ± 0.5
Compound heterozygous									
α^0-thalassemia/Hb CS (1)	3.77	7.7	27.8	73.8	20.5	27.8	21.9	CSA_2A Bart Hb	1.0
α^+-thalassemia/Hb Ps (1)	4.81	11.1	35.4	73.6	23.1	31.4	13.9	A_2A	2.1
α^+-thalassemia/Hb CS (1)	5.53	11.9	37.2	67.2	21.5	32	14.5	A_2A	3.1
Hb Q/Hb CS (1)	4.55	9.7	31.6	69.5	21.4	30.7	16.3	A_2A Q	1.5
Heterozygous β-thalassemia (3)	5.1 ± 0.3	10.7 ± 0.9	33.3 ± 2.0	65.5 ± 0.5	21.1 ± 0.5	32.2 ± 0.5	15.7 ± 1.1	A_2A	5.2 ± 0.4
Nonthalassemia (159)	4.1 ± 0.4	12.2 ± 1.1	35.9 ± 4.0	87.9 ± 5.0	29.9 ± 2.1	34.0 ± 1.2	13.7 ± 1.4	A_2A	2.7 ± 0.3

CS, Constant Spring; Hb, hemoglobin; Hct, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; Ps, Paksé; RDW, red cell distribution width.

* Values are given as mean ± SD except when n = 1 or n = 2. Values for the RBC count and MCV are given as Système International (SI) units; conversions to conventional units are as follows: RBC count ($\times 10^6$/L), divide by 1.0; MCV (μm^3), divide by 1.0. Other values are given as conventional units; conversions to SI units are as follows: Hb (g/L), multiply by 10.0; Hct (proportion of 1.0), multiply by 0.01; MCH (units are the same); MCHC (g/L), multiply by 10.

† Of the cases, 23 were the $-\alpha^{3.7}$ type, and 3 were the $-\alpha^{4.2}$ type.

‡ Of the cases, 51 were the $-\alpha^{3.7}$ type, and 2 were the $-\alpha^{4.2}$ type.

Table 2 lists the number of women with various genotypes who had positive or negative results based on each screening protocol using standard RBC indices and a combination of modified OF and DCIP tests. Five screening methods were compared, ie, MCV alone (cutoff, 80 μm^3 [80 fL]); MCH alone (cutoff, 27 pg); a combined MCV and MCH; a combined MCV, MCH, and DCIP test; and combined OF and DCIP tests. As shown in Table 2, a total of 126, 138, and 143 women with clinically important thalassemias had positive results for MCV alone, MCH alone, and the combined MCV and MCH, respectively. False-positive and false-negative rates for these 3 screening protocols were 23.6% (39/165) and 19.0% (49/258) for MCV alone, 27.4% (52/190) and 15.9% (37/233) for MCH alone, and 28.5% (57/200) and 14.3% (32/223) for the combined MCV and MCH, respectively.

In contrast with the preceding 3 methods, the combined MCV, MCH, and DCIP protocol and the combined OF and DCIP protocol detected all 175 subjects with clinically important thalassemias. False-positive rates for these 2 screening protocols were 25.2% (59/234) and 15.5% (32/207), respectively. No false-negative results were encountered.

Based on these results, the effectiveness of each screening method for the 423 pregnant Thai women was determined and values compared **Table 3**. Excellent sensitivities were obtained with the last 2 protocols (combined MCV, MCH, and DCIP and combined OF and DCIP tests). The combined OF-DCIP method resulted in better specificity, positive predictive value, and negative predictive value than the other methods.

Discussion

The aim of screening for thalassemia and hemoglobinopathies in Southeast Asia is to offer carrier testing to the population before they have children.[2,3] In the present study, we compared the effectiveness of various carrier screening protocols, including existing protocols and a recently established

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Table 2
Number of Pregnancies With Positive and Negative Screening Based on RBC Indices and Combined OF/DCIP Tests Among Various Thalassemia Genotypes

	MCV*		MCH†		MCV or MCH‡		MCV or MCH or DCIP§		OF/DCIP			
Thalassemia Genotype	+	–	+	–	+	–	+	–	+/–	+/+	–/+	–/–
Clinically important												
Hb H disease (α^0/α^{CS})	1	0	1	0	1	0	1	0	0	1	0	0
Heterozygous α^0-thalassemia	10	0	10	0	10	0	10	0	10	0	0	0
Heterozygous β-thalassemia	3	0	3	0	3	0	3	0	3	0	0	0
Heterozygous Hb E												
Without α-thalassemia gene	68	25	74	19	79	14	93	0	0	45	48	0
Coinherited with												
α^0-thalassemia	6	0	6	0	6	0	6	0	0	6	0	0
α^+-thalassemia	8	18	11	15	11	15	26	0	0	16	10	0
Hb CS	2	5	4	3	4	3	7	0	0	1	6	0
Hb Ps	2	1	3	0	3	0	3	0	0	2	1	0
Homozygous α^+-thalassemia	2	0	2	0	2	0	2	0	0	2	0	0
α^+/α^{Ps}	1	0	1	0	1	0	1	0	0	1	0	0
α^0/α^+	1	0	1	0	1	0	1	0	0	1	0	0
α^0/α^{Ps}	1	0	1	0	1	0	1	0	0	1	0	0
Homozygous Hb E												
Without α-thalassemia	17	0	17	0	17	0	17	0	0	17	0	0
Coinherited with α^+-thalassemia	3	0	3	0	3	0	3	0	0	3	0	0
Coinherited with Hb Q	1	0	1	0	1	0	1	0	0	1	0	0
Total	126	49	138	37	143	32	175	0	13	97	65	0
Nonclinically important												
Nonthalassemia	8	151	11	148	13	146	14	145	8	0	2	149
Heterozygous												
α^+-thalassemia	16	37	23	30	25	28	25	28	9	0	1	43
Hb CS	7	19	11	15	11	15	12	14	3	1	1	21
Hb Ps	1	2	0	3	1	2	1	2	0	0	0	3
Homozygous α^+-thalassemia	4	0	4	0	4	0	4	0	4	0	0	0
Compound heterozygous α^+-thalassemia and												
Hb CS	1	0	1	0	1	0	1	0	1	0	0	0
Hb Ps	1	0	1	0	1	0	1	0	1	0	0	0
Heterozygous Hb CS with Hb Q	1	0	1	0	1	0	1	0	1	0	0	0
Total	39	209	52	196	57	191	59	189	27	1	4	216

CS, Constant Spring; DCIP, dichlorophenolindophenol; Hb, hemoglobin; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; OF, osmotic fragility; Ps, Paksé; +, positive; –, negative; +/–, positive for OF test but negative for DCIP test; +/+, positive for both tests; –/+, negative for OF test but positive for DCIP test; –/–, negative for both tests.
* Cutoff value, 80 μm^3 (80 fL).
† Cutoff value, 27 pg.
‡ Positive results were MCV <80 μm^3 (<80 fL) or MCH <27 pg.
§ Positive results were MCV <80 μm^3 (<80 fL) or MCH <27 pg or positive DCIP test result.

Table 3
Comparison of the Effectiveness of Thalassemia and Hemoglobinopathy Screening Among 423 Pregnant Women Using Standard RBC Indices and a Combination of a Modified OF Test and a Modified DCIP Test*

	MCV <80 fL	MCH <27 pg	MCV <80 fL or MCH <27 pg	MCV <80 fL or MCH <27 pg or Positive DCIP Test	OF/DCIP
Sensitivity	72.0	78.9	81.7	100.0	100.0
Specificity	84.3	79.0	77.0	76.2	87.1
Positive predictive value	76.4	72.6	71.5	74.8	84.5
Negative predictive value	81.0	84.1	85.6	100.0	100.0

DCIP, dichlorophenolindophenol; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; OF, osmotic fragility.
* Conventional units for MCV are <80 μm^3; conventional units for MCH are the same as the Système International units given.

protocol, in 423 pregnant Thai women. The identification of 22 types of thalassemia in this group of Thai women (Table 1) confirms a high prevalence and a diverse heterogeneity of this genetic disorder in Southeast Asia and underlines the need for an appropriate screening strategy.

As expected, Hb E, which is prevalent among Southeast Asian populations,[18] was the most common hemoglobinopathy encountered in this group of subjects; it was identified in 161 (38.1%) of 423 subjects. The β-thalassemia clearly was less common; it was found in only 3 (0.7%) of 423 subjects.

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The α-thalassemias, including α^0, α^+, Hb CS, and Hb Ps, also were prevalent and were detected at different frequencies. The high prevalence of Hb E and α-thalassemia in this group of the Thai population was supported by the observation of various interactions of Hb E with several forms of α-thalassemia (Table 1).

In this group of pregnant Thai women, we observed similar hematologic features associated with α^0-thalassemia, β-thalassemia, homozygous Hb E, pure Hb E heterozygote, and double heterozygote for Hb E/α-thalassemia to those reported in nonpregnant subjects.[14-19] Marked reductions in MCV and MCH values with higher numbers of RBCs were observed in women with α^0-thalassemia, β-thalassemia, and homozygous Hb E. All of these forms of thalassemia, therefore, tested positive when MCV or MCH was used as the primary screening tool (Table 2). It is noteworthy that the combined MCV and MCH method provided better sensitivity than that obtained using the MCV or MCH alone. For Hb E heterozygotes, however, many had normal RBC indices and, therefore, had negative results in MCV and MCH screening (Table 2). The false-negative result with MCV and MCH screening for Hb E carrier is unacceptable, especially when a population screened is known to have a high prevalence of Hb E, as is true for Southeast Asian populations. A reduction in MCV and MCH values also was noted in women with other mild forms of thalassemia, ie, α^+-thalassemia, Hb CS, and Hb Ps, compared with the 159 women without thalassemia. Other investigators found similar results in a Chinese population.[20,21]

The results of the present study indicate that during pregnancy, even mild thalassemia might influence hematologic parameters, leading to a false-positive screening result, when the RBC index is used as the primary screening method. The high false-positive and false-negative rates led to disappointing sensitivity and specificity values for the MCV and MCH screening methods. However, as shown in Table 3, the sensitivity of the MCV and MCH screening protocol was improved greatly (to 100.0%) when it was used in combination with a modified DCIP test because all subjects with Hb E had positive results with this test. Based on the results of our study, we strongly recommend the use of a modified DCIP test in addition to electronic blood cell counting for screening Southeast Asian populations to be able to identify and give proper advice to Hb E carriers.

It is interesting that the best screening result with 100.0% sensitivity, 87.1% specificity, 84.5% positive predictive value, and 100.0% negative predictive value was obtained with a combined OF-DCIP protocol (Table 3). According to these data, no false-negative results for the 3 clinically important forms of thalassemia, ie, α^0-thalassemia, β-thalassemia, and Hb E, could be found with this screening method, but approximately 15% of the positive cases would be false-positives. However, this is not important because the major concern of screening is to avoid false-negative results.[22] A screening method consisting of a combination of modified OF and DCIP tests, therefore, is an attractive screening alternative to an expensive electronic blood cell count. The protocol is simple, reliable, cost-effective, and practical enough to be carried out in a primary health care setting. Indeed, the protocol recently has been applied successfully for prevention and control of severe thalassemia in a provincial hospital in Thailand.[23] Using this method in screening for thalassemia and hemoglobinopathies in pregnant women in Southeast Asian communities should facilitate a prevention and control program of this common genetic disorder[24] in the region.

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