



Thalassemia Mutations in Lower Northern Part of Thailand

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Abstract

Thalassemia is one of the most serious genetically transmitted diseases creating health problems in many parts of Thailand including the lower North. Therefore, screening test for both alpha and beta globin gene mutations will be useful for a program of prevention and control of thalassemic disease. The screening test includes osmotic fragility (OF) test, dichlorophenol-indophenol (DCIP) test, quantitation of HbA₂, HbE screening and PCR analysis. Five hundred blood samples were collected from a population living in Uttaradit, Phetchabun, and Phitsanulok. The results showed a high frequency of thalassemic disorders (40.4%) in this population and one major thalassemic disease (compound heterozygous HbE/ β -thal). Among them, heterozygous Hemoglobin E (HbE trait) was found to be the most common in 133 subjects (26.6%) and alpha thalassemia 1, Southeast Asian type carriers (α -thal 1, SEA) (heterozygous α -thalassemia 1) in 39 subjects (7.8%). Homozygous HbE (Homo HbE) was found in 19 subjects (3.8%), whereas heterozygous beta thalassemia (β -thal trait) was found in 11 subjects (2.2%). In addition to HbE/ β -thal, 6 more subjects with compound heterozygote were also found, 4 of α -thal 1/HbE, and 2 of α -thal 1/ β -thal. By using PCR, HbE mutation was confirmed and β -thal traits were analyzed for 5 different mutations. Among 11 β -thal traits, mutation in codon 41/42 (4 bp deletion) was the most frequent in 9 samples (1.8%), codon 17 (A-T) mutation was detected in one sample (0.2%), IVS-1-5 (G-C) was detected in one sample (0.2%), whereas mutation in codon 71/72 (+A) and IVS-1-1 (G-T) was not detected. The data will be useful for setting a strategy for prevention and control of thalassemia in this area.

Keywords: thalassemia carrier, osmotic fragility test, dichlorophenol-indophenol test, polymerase chain reaction, hemoglobin E, Southeast Asian type

Introduction

Thalassemia is the most common genetic disorder in Thailand. One percent of 60 million people are affected and more than 20 million were thalassemia carriers (Greenberg et al., 2001). Thalassemia is, therefore, one of the major health problems in Thailand. There are two common types of thalassemia; α - and β -thal. The α -thal is mainly caused by a large deletion of the α -globin gene. In contrast, β -thal is generally caused by point mutations affecting the β -globin gene leading to a reduction (β^+) or absence (β^0) of β -globin gene production.

In Thailand, hemoglobin E (codon 26 G-A) which is the hallmark of Southeast Asia attains a frequency of 8-60% (Fucharoen and Winichagoon, 1992). The gene frequencies of β -thal reach 30-40% in Northern Thailand (Fucharoen and Winichagoon, 1992; Nopparatana, 1998). The most common type of α -thal 1 in the Thai population is the Southeast Asian type (SEA) (Fucharoen et al., 1995). The frequencies of β -thal vary between 1 to 9% (Nopparatana, 1998). Most of the β -thal genes are β^0 -thal mutations. The 4 bp deletion at codon 41/42 (-TTCT) was the most frequent (Laosombat et al., 2001), followed by the splice site mutation IVS-1-1 (G-T) (Sanguansermisri et al., 2001), the nonsense mutation at codon 17 (A-T) (Laosombat et al., 2001), a G-C at IVS-1-5 (Nopparatana et al., 1995), and "A" addition in codon 71/72 (Fukumaki et al., 1992).

These abnormal genes in different combinations lead to over 60 different thalassemia syndromes. The four major thalassemia diseases are Hb Bart's hydrops fetalis (homozygous α -thal 1), homozygous β -thal, β -thal/HbE and HbH disease. The strategies for thalassemia control in Thailand consist of offering the best treatment to patients and prevention of the birth of new cases. However, there would still be a large number of thalassemia carriers (Pansatiankul and Saisorn, 2003). The ultimate objective of thalassemia prevention is to screen for carriers, in order to offer prenatal diagnosis of the high risk fetus.

The objective of this study was to screen α - and β -thal carriers in 500 people who were served by Naresuan University Mobile Unit for people's health and occupational development. The data will be useful for setting a strategy for prevention and control of thalassemia in this area.

Preliminary screening was done using a combination of the one-tube osmotic fragility test (OF) together with the dichlorophenol-indophenol precipitation test (DCIP) which were proved by many groups to be an effective method for screening hemoglobin disorders (Indaratna, 1997; Paritpoken et al., 1999; Panyasai et al., 2002; Wiwanitkit et al., 2002) The samples that were positive to OF test will be screened for β -thal and HbE by HbA₂ quantitation and screened for α -thal 1 (SEA type) by PCR technique. The samples that were negative to OF test but positive to DCIP test would be screened further for HbE by DEAE-Sephadex microcolumn chromatography. The β -thal trait or HbE trait samples will be confirmed and identified mutations by PCR.

Materials and Methods

The protocol of this study has been approved by Naresuan University Ethics Committee (code 46 02 04 0012).

Subjects

Two ml of peripheral blood anticoagulated with EDTA was obtained from 500 people, living in Uttaradit, Phetchabun and Phitsanulok, who came for health to check up with Naresuan University Mobile Unit for people's health and occupational development between August 2003 and January 2004. Eighty two subjects (16.4%) were male and 418 subjects (83.6%) were female aged between 19 to 50 years. Hematocrit concentration was 21 - 49% with an average of 41.11%.

Primers

The DNA samples were analyzed for known α - and β -thal mutations commonly found in Thailand using amplification refractory mutation system (ARMS). The mutations were screened by allele specific primers on the PCR amplified DNA. Seventeen oligonucleotide primers were synthesized by Thaicam Biotechnology, USA. Sequences of primers used for α -thal 1 allele detection and allele-specific primers used for β -thal mutations were listed in Tables 1 and 2, respectively.

A one tube osmotic fragility test (OF)

A one-tube OF test was performed in all subjects (Chamrasratanokorn et al., 1998). A sample of 20 μ l whole blood was well mixed with 5 ml of 0.36% buffered saline solution (NaCl, Na₂HPO₄, NaH₂PO₄) and allowed to stand at room temperature for 5 min. The tests were evaluated by visualization as negative or positive. Negative samples were characterized by a clear red hemoglobin solution indicating complete hemolysis of the red cells whereas positive samples were identified by a cloudy appearance because of incomplete hemolysis of the red cells. In this report, we considered suspicious samples with a very fine cloudiness as positive.

Dichlorophenol indophenol precipitation test (DCIP test)

Five hundred subjects were screened for HbE and unstable hemoglobin by DCIP test using KKU-DCIP-clear reagent kit (Fucharoen S, Khon Khan University). A sample of 30 μ l whole blood was well mixed with 2 ml of DCIP solution, then incubated at 37°C for 15 min. After adding 30 μ l of clearing solution, mixed well, then let it stand in room temperature for 2-3 min. The tests were evaluated by visualization as negative or positive. If solution is clear means negative while cloudy appearance solution means positive.

Table 1 Oligonucleotide primers used for α -thal 1 mutation detection (Winichagoon, 1995)

primers	sequences (5'—3')
A4	GGGGGCGGCCTTGGGAGGTTC
A1B	ATATATGGGTCTGGAATGTATC
A9	GTTCCCTGAGCCCCGACACG

Table 2 Allele specific oligonucleotide primers using for detection of 6 β -thal mutations (N = Normal, M = Mutant ARMS primers) (Fucharoen et al., 1995)

Name	Sequences (5' ->3')	Type	Mutation	Size
G13	AAA GTG CTC GGT GCC TTT AG	N		
G14	AAA GTG CTC GGT GCC TTT AAA	M	+A codon 71/72	130 bp
G15	GAT GAA GTT GGT GGT GAG GCC CTG GGC AGG	N		
G16	GAT GAA GTT GGT GGT GAG GCC CTG GGC AGT	M	C->T IVS -1-1	345 bp
G17	ACT TCA TCC ACG TTC ACC TT	N		
G18	ACT TCA TCC ACG TTC ACC TA	M	A->T codon 17	243 bp
G19	CTG TCT TGT AAC CTT GAT AG	N		
G20	CTG TCT TGT AAC CTT GAT AC	M	G->C IVS-1- 5	286 bp
G21	GAG TGG ACA GAT CCC CAA AGG ACT CAA AGA	N		
G22	GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT	M	-4 bp codon 41/42	445 bp
G23	CGT GGA TGA AGT TGG TGG C	N		
G24	CGT GGA TGA AGT TGG TGG T	M	HbE, codon 26	276 bp
S1	TGT CAT CAC TTA GAC CTC AC	N	common	
S3	TCC CAT AGA CTC ACC CTG AA	N	common	
γ 4	GGC CTA AAA CCA CAG AGA GT	N	internal primer	
γ 5	CCA GAA GCG AGT GTG TGG GA	N	internal primer	578 bp

Quantitation of HbA₂

Two hundred and thirty four subjects with OF or DCIP-positive results were measured percentage of HbA₂ by DEAE-Sephadex microcolumn chromatography (Sanguansermisri et al., 2001). One ml of hemolysate (2 μ l of packed red cell in 0.05M Tris-KCN buffer pH8.5) was applied into column packing with DEAE Sephadex A-50 in 0.05M Tris-KCN buffer pH8.5. After 20 min, 10 ml of elution buffer, 0.05 M Tris-KCN buffer pH8.2, was applied. Then another 10 ml of elution buffer was applied again and started filtrate collecting for absorbance 415 (OD415) measurement. The percentage of Hemoglobin A2 was calculated comparing between OD415 of filtrate collecting from chromatography and OD 415 of 1:50 hemolysate in 0.05M Tris-KCN buffer pH8.5.

Screening for hemoglobin E

Fifty five subjects with OF-negative but DCIP-positive results were screened for HbE by DEAE-Sephadex microcolumn chromatography (Sanguansermisri et al., 2001). One ml of hemolysate (2 μ l of packed red cell in 0.05M Tris-KCN buffer pH8.5) was applied into column packing with DEAE Sephadex A-50 in 0.05M Tris-KCN buffer pH8.5. After 20 min, 10 ml of elution buffer, 0.05 M Tris-KCN buffer pH8.2, was applied. Since hemoglobin E could move down faster than hemoglobin A, the positive sample that contained hemoglobin E could be observed as red-colored moved more than a half of a column.

DNA isolation

Genomic DNA was extracted and purified from whole blood by using GFX™ Genomic Blood DNA Purification Kit (Amersham pharmacia biotech). Red blood cells were lysed by adding RBC lysis solution (10mM KHCO₃, 155mM NH₄Cl, 0.1mM EDTA). Then a chaotropic agent

was added to facilitate the lysis of nucleated cells and the binding of genomic DNA to a glass fiber matrix prepacked in a MicroSpin column. Wash solution (Tris-EDTA buffer in absolute ethanol) was applied into the columns. The purified DNA is eluted in a low ionic strength buffer ready for direct use or store at 4 °C.

DNA analysis

α -Thal 1 gene (SEA) screening

Two hundred thirty four DNA samples with OF positive were amplified using three primers; A4, A1B and A9. A4 was adjacent to the 5'-breakpoint of the α -thal 1 allele. A1B corresponded to nt 194 of the normal DNA sequence 3' to the first base of the primer A4. A9 located at nt 550 of the 5' to the breakpoint of α -thal 1 (Winichagoon et al, 1995).

Amplification was performed in a 25 μ l mixture of 50-100 ng genomic DNA, 50mM KCl, 10mM Tris-HCl, 1.5mM MgCl₂, 0.2mM of dNTP, 0.5 unit of Taq Polymerase (QIAGEN™) and 0.4 pmol of each primer. A 35 cycle protocol was used with denaturation at 95 °C for 1 min, annealing at 63 °C for 2 min and extension at 72 °C for 2 min, using GeneAmp 9700 DNA thermocycler (Perkin Elmer, USA).

β -Thal mutations screening

Eleven DNA samples containing HbA₂ between 3.5-10 were amplified for screening five different β -thal mutations: codon 71/72 (+A), IVS-1-1 (G-T), codon 17 (A-T), IVS-1-5 (G-C), codon 41/42 (4bp deletion) (Fucharoen et al., 1995). One hundred fifty two DNA samples containing HbA₂ more than 10 or HbE screening was positive were amplified for HbE mutation [codon 26 (G-A)]. Each mutation was screened by pair of allele-specific primers in both normal and mutation as represented in Table 2 and as diagram in Figure 1.

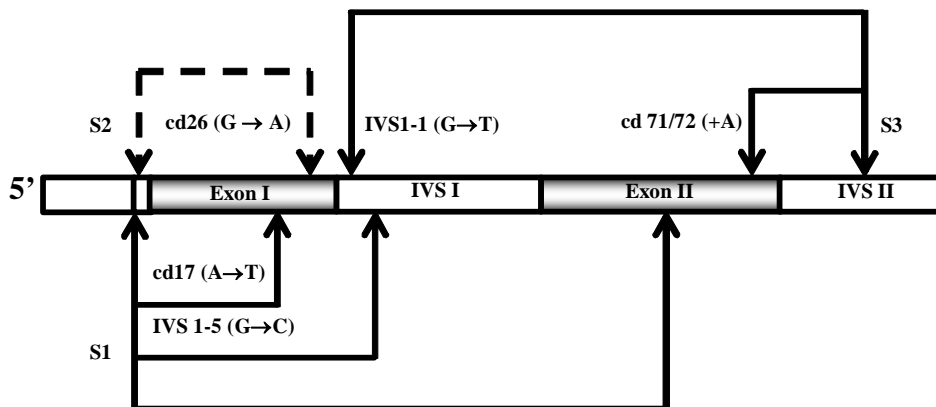


Figure 1 Diagram of β -globin gene showing the position of 6 ARMS primers to detect 6 β -thal mutations: codon 17 (A-T), IVS-1-1 (G-T), IVS-1-5 (G-C), HbE (codon 26 (G-A), codon 41/42 (4 bp deletion) and codon 71/72 (+A) (modified from Fucharoen et al., 1995).

Amplification was performed in a 25 μ l mixture of 50-100 ng genomic DNA, 20 mM Tris-Cl pH 8, 1.5 mM MgCl₂, 100 mM EDTA, 50% glycerol, 0.2 mM of dNTP, 0.25 unit of *Hotstart*Taq Polymerase (QIAGEN™) and 0.3 μ mol of each of four primers (two internal primers, antisense primer and ARMS primer). The reaction was performed at different conditions as shown in Table 3, using GeneAmp 9700 DNA thermocycler (Perkin Elmer, USA).

Table 3 The PCR condition for detection of β -thal mutations (Fucharoen et al., 1995)

Mutation	Primers	Thermal cyler
A codon 71/72	S1+G13	(95 C 4 min) 1 cycle
	S1+G14	(95 C 1 min/62 C 2 min/72 C 2 min) 30 cycles (72 C 3 min) 1 min
IVS-1-1 G->T	S1+G15	(95 C 4 min) 1 cycle
	S1+G16	(95 C 1 min/62 C 2 min/72 C 2 min) 30 cycles (72 C 3 min) 1 min
codon 17	S1+G17	(95 C 4 min) 1 cycle
	S1+G18	(95 C 1 min/64 C 2 min/72 C 2 min) 30 cycles (72 C 3 min) 1 min
IVS-1- 5 G->C	S1+G19	(95 C 4 min) 1 cycle
	S1+G20	(95 C 1 min/60 C 2 min/72 C 2 min) 30 cycles (72 C 3 min) 1 min
codon 41/42	S1+G21	(95 C 4 min) 1 cycle
	S1+G22	(95 C 1 min/62 C 2 min/72 C 2 min) 30 cycles (72 C 3 min) 1 min

Determination of PCR products

Following amplification, electrophoresis was carried out. Ten μ l of PCR product was electrophoresed in 2% agarose gel in 1xTAE buffer. Electricity was supplied at 100 volt for 30 min. Then the gel was stained with ethidium bromide and visualized on a UV transilluminator.

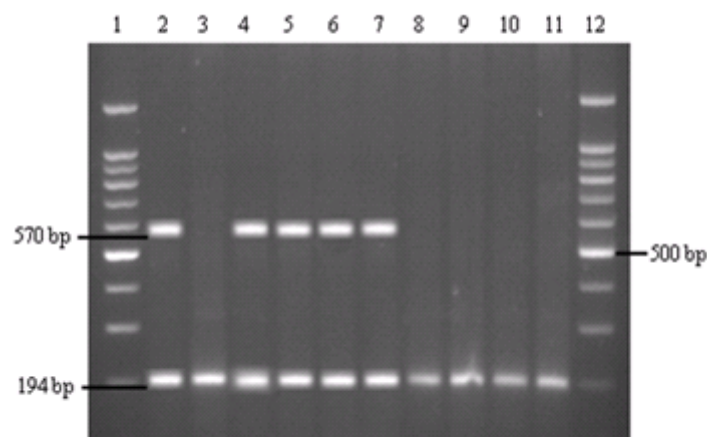
Results

The preliminary screening for thalassemia carriers were obtained using OF and DCIP test. Ninety eight samples were positive for both OF test and DCIP test, 136 samples were only OF positive, 57 samples were positive only for DCIP test, and 209 samples were negative for both OF test and DCIP test (as shown in Table 4). Two hundred ninety one out of 500 samples with OF or DCIP positive were analyzed. Two hundred thirty four samples that positive for OF test (including both negative and positive DCIP) were amplified for detection of α -thal 1 gene deletion. By PCR technique using 3 primers, A4, A1B and A9, two patterns of PCR product were seen as shown in Figure 2. The only one band of 194 bp DNA fragment was amplified from normal α -globin allele, while two bands of 194 bp and 570 bp DNA fragment was obtained from heterozygous α -thal 1 carrier. In this study, 39 from 234 samples were shown two bands of 194 bp and 570 bp as represented in Figure 2, corresponding to 7.8% α -thal 1 carriers found in this population.

Moreover, six different mutations of β -globin gene were also studied. 234 samples that were positive for OF test were analyzed by measuring HbA₂ concentration for finding β -thal trait, homozygous HbE, and heterozygous HbE. The elevated level of HbA₂ was the main diagnostic feature of β -thal carriers; 3.5-10 is β -thal trait, 10-30 is heterozygous HbE and over 60 is homozygous HbE. The results showed that 11 subjects (2.2%) were affected with β -thal trait, 97 subjects (19.4%) with heterozygous HbE and 19 subjects (3.8%) with homozygous HbE. In addition, 57 subjects positive only for DCIP test were screened further by HbE screening. The result showed 36 samples were HbE trait (7.2%). From 500 subjects, 39 alleles were β -thal 1, represented for 7.8% α -thal 1 trait, 11 subjects were β -thal trait (2.2%), 133 subjects were HbE trait (26.6%) and 36 subjects were Homo HbE (7.2%).

Table 4 Results of OF, DCIP, HbA₂ quantitation, HbE screening and PCR for α -thal 1 detection in 500 people living in Uttaradit, Pethchabun and Phitsanulok

OF	DCIP	No of Subjects	PCR		HbA ₂ quantitation		HbE screening	
			Heterozygous α -thal 1	β -thal trait	HbE trait	Homo HbE	HbE trait	
+	+	98	5	9	92	19	-	-
+	-	136	34	2	5	-	-	-
-	+	57	-	-	-	-	-	36
-	-	209	-	-	-	-	-	-
500			39 (7.8%)	11 (2.2%)	97 (19.4%)	19 (3.8%)	36 (7.2%)	
			202 (40.4%)					

**Figure 2** Agarose gel electrophoresis of amplified α -thal 1 PCR products. Two patterns of PCR products were shown. The pattern of two bands of 570 bp DNA fragment together with 194 bp DNA fragment was corresponded for α -thal 1 carriers, a pattern of only one band of 194 bp DNA fragment was corresponded for normal subject. The two bands of 570 and 194 bp were obtained from 39 subjects as represented in lane 4-7. The other subject were obtained only one band of 194 bp DNA fragment as represented in lane 8-11. Lanes 1 and 12 were 100 bp DNA ladder marker. The pattern of PCR product for α -thal 1 gene positive control was in lane 2, while for normal subject or negative control was in lane 3.

Eleven β -thal trait subjects were identified for β -thal mutation by ARMS technique. Five ARMS primers specific to codon 17 (A-T), IVS-1-1 (G-T), IVS-1-5 (G-C), codon 71/72 (+A) and codon 41/42 (4bp deletion) were used. The mutation analysis was shown in Figure 3. Nine of all β -thal trait subjects (81.8%) were codon 41/42 (4 bp deletion) trait, one (9.1%) was codon 17 (A-T) trait and one (9.1%) was IVS-1-5 (G-C). No new mutation was found in the presented study.

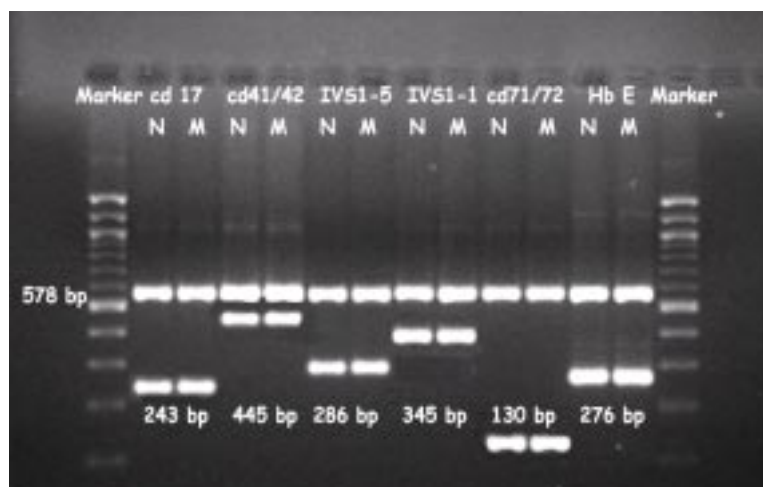


Figure 3 Agarose gel electrophoresis of amplified β -thal PCR products. Six different mutations were amplified from pairs of normal (N) and mutant (M) allele specific primers. The 578 bp DNA fragments in all lanes were obtained from internal primers. The six different patterns of PCR product were obtained from 6 different normal gene and mutations; 243 bp DNA fragment from codon 17, 445 bp from codon 41/42, 286 bp from IVS-1-5, 345 bp from IVS-1-1, 130 bp from codon 71/72 and 276 bp from codon 26 (HbE). DNA marker used was 100 bp ladder marker.

Discussion and Conclusion

Thalassemia is one of the most common health problems in Thailand. The high prevalences of the α -thal, β -thal and HbE gene in our population were 14%, 3-9%, and 13%, respectively (Fucharoen and Winichagoon, 1987; Lemmens-Zygluska et al., 1996). This leads to many births of children with severe thalassemia, i.e., homozygous α -thal, β -thal/HbE, and Hb Bart's hydrops fetalis. Prevention programs are devoted to control these diseases. The aims of thalassemia prevention are population screening for carriers, genetic counseling, and offering prenatal diagnosis of the high risk fetus, providing choices for the couples to consider. Although α - and β -thal are widespread in Thailand, their distribution throughout the country is variable. The most prevalence is found in the North as 32% α -thal, 9% β -thal, 10% HbE and 4-6% HbCS. In the Northeast, the prevalence of α -thal, β -thal and HbE are 20%, 2-6% and 20-30%, respectively; whereas 20-25% α -thal, 3% β -thal and 13-17% of HbE were reported in the Central. In the South, slightly lower prevalence was reported: 16% of α -thal, 2-4% of β -thal and 9-11% of HbE (Flatz et al., 1965; Fucharoen and Winichagoon, 1987; Winichagoon et al., 1992; Kitsirisakul et al., 1996; Lemmens-Zygluska et al., 1996; Sanugansermisri et al., 1998).

From our study, we found that 40.4% of population living in the lower northern part of Thailand was carriers which correlated to the report of the whole nation that approximately 40% of the population was carriers (Tanphaichitr, 1999). The prevalence of the α -thal, β -thal, and HbE gene in population living in Uttaradit, Phetchabun, and Phitsanlok is 7.8%, 2.2%, and 30.4% respectively. The prevalence of α -thal is similar to Chiang Mai (7%) (Sanguansermisri et al., 1998) and Phitsanulok (5%)(Choopayak et al., 2003). The prevalence of β -thal is lower in Chiang Mai (5.6%) (Sanguansermisri et al., 1998).

HbE is the most common in Thailand, the prevalence is especially high in the northeastern part (around 32.7%) (Na-Nakorn et al., 1956; Na-Nakorn and Wasi, 1978; Pravatuang et al., 1988). This study presents the high incidence of HbE (30.4%) similar to Phitsanulok (25.05%) which is the highest incidence reported in the north (Pravatuang et al., 1995).

In Thailand, the prevalence of β -thal gene carriers is rather high and varies from 3.0 to 9.0% (Wasi et al., 1980). So far, at least 19 β -globin gene mutations have been identified in the Thai population (Sutcharitchan et al., 1995). Due to the high diversity of mutations in the β -thal gene, mutations in one population may be different from the others. Even in Thailand, the previous studies have shown that mutations found in the northern part were different from those found in the southern or northeastern parts of the country (Laig et al., 1989; Fukumaki et al., 1992; Laosombat et al., 1992). Therefore, it is necessary to identify the mutation prevalence in each population in order to determine the common mutations of an individual population.

There are several methods for determining mutations including dot-blot analysis, reverse dot-blot, ARMS and restriction enzyme digestion. In the present study we used the ARMS technique to determine the 6 common β -thal mutations, codon 71/72 (+A), IVS 1nt1 (G-T), codon 17 (A-T), IVS 1nt5 (G-C), codon 41/42 (4bp deletion) and HbE, in 11 β -thal trait subjects. We were able to detect all the mutations on 22 alleles. Three different mutations have been found in the present study, codon 41/42 (-TTCT) (81.8%), codon 17 (A-T) (9.1%) and IVS-1-5, (G-C) (9.1%), respectively.

The most common was the codon 41/42 mutation, and this was the same finding observed in every part of Thailand (Laig et al., 1989; Fukumaki et al., 1992; Laosombat et al. 1992; Nopparatana et al., 1995), in China (Chan et al., 1987) and in Southeast Asia (Kazazian et al., 1986). However, the second most common mutation was codon 17, similar to north (Sirichotiyakul et al., 2003) and northeast Thailand (Fucharoen et al., 1989), whereas IVS-1-5 and IVS-II-654 were the second most common mutations observed in the south (Laosombat et al., 1992) and central (Thein et al., 1990) parts of Thailand, respectively. Furthermore, IVS1-5, accounting for 9.1% in the present study, is similar in the central (4%) and south (19%), while it was less common in other parts of north Thailand (2%) (Sirichotiyakul et al., 2003). This finding also showed that the spectrum of β -globin gene mutations in the lower northern part is similar to the northeast, central and southern than to the north of Thailand. This was probably due to the geographic position and migration.

The combination of OF test plus DCIP test is the preferred method for screening abnormal hemoglobin (Wiwanitkit et al., 2002). It is convenient, easy and inexpensive method compared to standard hemoglobin electrophoresis. If the OF test was positive, the level of HbA₂ was measured in order to screen for β -thal trait, and the PCR technique would be used for α -thal 1 screening. If the OF test was negative, the HbE was screened for hemoglobin E traits. By using OF plus DCIP, we could screen out around half of the subjects for further investigations. Noticeably, there were also some HbE traits (7.2%) that were positive only to DCIP, not to OF, and a few samples of β -thal trait and HbE trait that were negative to DCIP (as shown in Table 4). Hence, we recommended using a combination of OF and DCIP for screening thalassemia disorders, and then randomly amplifying the α -thal 1 gene from some of the OF-negative samples. This produced only 194 bp PCR product, no 570 bp DNA fragment was obtained.

In conclusion, the distribution of the β -globin gene mutations obtained here will be useful for planning a prenatal diagnosis program especially for this region in Thailand. To improve the efficacy of prevention of the new cases, all pregnant women in the high prevalence areas should be tested for the carrier status. To achieve this purpose, the carrier detection policy should be extended to the premarriage or preconceptional testing and early antenatal care must be encouraged.

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