CHAPTER IV
RESULTS

1. The amounts of circulating DNA in maternal plasma samples

A TaqMan Real-Time PCR assay for the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) (Figure 12) was used to determined the level of maternal plasma DNA from pregnancies with and without Hb Bart's hydrops fetalis. Each sample was analyzed in duplicate and the mean cycle threshold was used for further speculation of the total plasma DNA concentration.

In this study, 76 plasma samples were obtained from pregnant women who attended the thalassemia-screening unit at Khon Kaen University because of their risks of having fetuses with severe thalassemia syndromes. As shown in Tables 7-8, genotype analysis by PCR of each couple was examined and fetal DNA analysis by CVS or amniotic analyis were used to diagnosis of Hb Bart's hydrop fetalis. With this fetal analysis, it was found that among 76 pregnancies, 21 were found to carry fetuses with homozygous α^0-thalassemia (Tables 7-8). Comparative C_T values of samples from pregnancies with and without Hb Bart's hydrops fetalis were plotted on Figure 19. The maximum and minimum C_T values were found to be 36.07 & 28.85 in pregnancies with non affected fetuses and to be 38.32 & 30.45 in pregnancies with affected fetuses. As lower C_T values describe to high concentration of plasma DNA than higher C_T values, pregnancies with Hb Bart's hydrops fetalis had a trend toward higher amounts of total DNA in maternal plasma than the normal group, although apparently overlapping and may not be statistically significance.

Table 7 Summary of the detection of GAPDH specific sequence by TaqMan real time PCR from maternal plasma specimens of 55 pregnancies with non-Hb Bart's hydrop fetalis fetuses

No.	code	GA (weeks)	Mother	Father	Fetus	CT
1	M551	-	A_2A (--/αα)	A_2A (--/αα)	CVS: (--/αα)	32.72
2	M626	16	EA (--/αα)	A_2A (--/αα)	AF: (αα/αα)	34.57
3	M708	14	A_2A (--/αα)	A_2A (--/αα)	CVS: (--/αα)	34.28
4	M724	15	A_2A (--/αα)	EE (--/αα)	AF: (--/αα)	33.06
5	M795	-	A_2A (--/αα)	A_2A(--/αα)	FB: (--/αα)	34.11
6	M803	17	A_2A (--/αα)	EA (--/αα)	FB: (--/αα)	33.94
7	M829	12	EA (--/αα)	A_2A (--/αα)	CVS: (--/αα)	31.90
8	M1437	10	EE (--/αα)	EA (--/αα)	CVS: (--/αα)	35.03
9	M1449	15	EA (--/αα)	A_2A (--/αα)	CVS: (--/αα)	34.72
10	M1467	10	EA (--/αα)	A_2A (--/αα)	CVS: (--/αα)	32.51
11	M1471	20	A_2A (--/αα)	EA (--/αα)	FB: (--/αα)	35.87
12	M1495	19	A_2A (--/αα)	A_2ABH(--/-$\alpha^{3.7}$)	FB: (--/αα)	35.13
13	M1526	-	EFA (αα/αα)	EA (αα/αα)	-	35.00
14	M1547	12	A_2A (--/αα)	EA (--/αα)	CVS: (--/αα)	34.12
15	M^8	20	A_2A (--/αα)	A_2A(--/αα)	FB: (--/αα)	32.88
16	M^82	17	EA (--/αα)	A_2A (--/αα)	AF: (--/αα)	31.26
17	M^92	13	A_2A (--/αα)	A_2ABH(--/-$\alpha^{3.7}$)	CVS: (--/αα)	33.06
18	M^99	19	A_2A (--/αα)	A_2A(--/αα)	FB: (--/αα)	34.17
19	M^126	19	A_2A (--/αα)	A_2A (--/αα)	AF: (--/αα)	33.97
20	M^134	19	EA (--/αα)	EA (--/αα)	AF: (--/αα)	37.77
21	M^229	13	EA (--/αα)	EA (--/αα)	CVS: (--/αα)	34.83
22	M^230	18	A_2A (--/αα)	A_2A (--/αα)	AF: (αα/αα)	33.93
23	M^278	-	A_2A (αα/αα)	EA (αα/αα)	-	35.58
24	M^289	21	CSEA (αα/α^{CS}α)	EA (αα/αα)	-	35.55
25	M^292	13	A_2A (--/αα)	A_2A (αα/αα)	-	35.01
26	M^294	12	A_2A (--/αα)	A_2A (--/αα)	CVS: (αα/αα)	33.84
27	M^318	12	A_2A (αα/αα)	EA (αα/αα)	-	35.43
28	M^319	20	A_2A (--/αα)	EA (--/αα)	FB: (--/αα)	34.75
29	M^326	17	A_2A (αα/αα)	EA (αα/αα)	-	35.63
30	M^353	23	(--/αα)	(--/αα)	FB: (αα/αα)	32.71
31	M^363	23	CSA_2ABH (--/α^{CS}α)	(--/αα)	FB: (--/-$\alpha^{3.7}$)	34.97
32	M^368	12	EA (αα/αα)	A_2A (αα/αα)	-	35.53
33	M^369	10	EA (αα/αα)	EA (αα/αα)	-	33.64
34	M^391	11	A_2A (αα/αα)	A_2A (αα/αα)	-	34.10
35	M^406	12	A_2A (--/αα)	A_2ABH(--/-$\alpha^{3.7}$)	CVS: (--/αα)	35.32
36	M^416	13	A_2ABH(--/-$\alpha^{3.7}$)	A_2A (--/αα)	CVS: (--/-$\alpha^{3.7}$)	37.74
37	M^452	20	A_2A (--/αα)	A_2ABH(--/-$\alpha^{3.7}$)	FB: (--/-$\alpha^{3.7}$)	34.92
38	M^453	19	A_2A (--/αα)	EA (--/αα)	FB: (--/αα)	32.36
39	M^458	23	EA (--/αα)	A_2A (--/αα)	AF: (--/αα)	34.85
40	M^466	22	A_2A (--/αα)	A_2A (--/αα)	FB: (--/αα)	38.32

Table 7 Summary of the detection of GAPDH specific sequence by TaqMan real time PCR from maternal plasma specimens of 55 pregnancies with non-Hb Bart's hydrop fetalis fetuses (Cont.)

No.	code	GA (weeks)	Mother	Father	Fetus	CT
41	M^478	12	A_2A (--/αα)	A_2A (--/αα)	FB: (αα/αα)	30.45
42	M^507	20	A_2A (--/αα)	EA (--/αα)	FB: (αα/αα)	34.04
43	M^508	19	A_2A (--/αα)	EA (--/αα)	FB: (--/αα)	34.65
44	M^509	12	EA (αα/αα)	EF (αα/αα)	-	35.18
45	M^517	26	EA ($-\alpha^{3.7}/-\alpha^{3.7}$)	EA (αα/αα)	-	34.62
46	M^520	12	A_2A (αα/αα)	A_2A (αα/αα)	-	34.48
47	M^526	17	EA (αα/$-\alpha^{3.7}$)	EA (--/αα)	-	32.22
48	M^528	20	EE (αα/αα)	A_2A (αα/αα)	-	33.91
49	M^530	17	EA (αα/αα)	EA ($-\alpha^{3.7}/-\alpha^{3.7}$)	-	34.15
50	M^531	21	A2A (--/αα)	EA (αα/αα)	-	33.58
51	M^592	20	A_2A (αα/αα)	EA (αα/αα)	-	33.17
52	M^622	17	A_2A (αα/αα)	EA (αα/$\alpha^{CS}\alpha$)	-	33.22
53	M^659	13	CSEA ($-\alpha^{4.2}/\alpha^{CS}\alpha$)	A2A (--/αα)	-	34.95
54	M^680	19	EA (--/αα)	A2A (--/αα)	FB: (--/αα)	34.65
55	M^693	15	EA (--/αα)	A2A (--/αα)	FB: (--/αα)	34.50
	Average GA = 16				Average CT = 34.31	

(CVS: Chorionic Villus Sampling, AF: Amniotic Fluid, FB: Fetal Blood)

Table 8 Summary of the detection of GAPDH specific sequence by TaqMan real time PCR from maternal plasma specimens of 21 pregnancies with Hb Bart's hydrop fetalis fetuses

No.	code	GA (weeks)	Mother	Father	Fetus	CT
1	M510	20	A2A (--/αα)	EA (--/αα)	FB: (--/--)	33.35
2	M636	16	A2A (--/αα)	A2ABH (--/-$\alpha^{3.7}$)	FB: (--/--)	35.49
3	M654	22	A2A (--/αα)	EA (--/αα)	FB: (--/--)	32.59
4	M699	17	A2A (--/αα)	CSA2ABH (--/$\alpha^{CS}\alpha$)	FB: (--/--)	34.56
5	M722	12	A2A (--/αα)	CSEABH (--/$\alpha^{CS}\alpha$)	CVS: (--/--)	32.99
6	M805	21	EE (--/αα)	EE (--/-$\alpha^{3.7}$)	AF: (--/--)	34.72
7	M827	12	EA (--/αα)	A2A (--/αα)	CVS: (--/--)	33.70
8	M1008	23	A2ABH (--/-$\alpha^{3.7}$)	A2A (--/αα)	FB: (--/--)	29.63
9	M1401	19	EA (--/αα)	A2A (--/αα)	FB: (--/--)	33.71
10	M1435	15	EE (--/αα)	A2A (--/αα)	CVS: (--/--)	33.62
11	M1466	14	EA (--/αα)	A2ABH (--/-$\alpha^{3.7}$)	AF: (--/--)	32.30
12	M1527	10	A2A (--/αα)	A2AB (--/-$\alpha^{3.7}$)	AF: (--/--)	34.12
13	M1596	11	A2A (--/αα)	A2A (--/αα)	CVS: (--/--)	28.85
14	M^36	14	EA (--/αα)	EA (--/$\alpha^{CS}\alpha$)	CVS: (--/--)	31.30
15	M^125	12	EA (--/αα)	A2A (--/αα)	CVS: (--/--)	36.07
16	M^127	14	A2A (--/αα)	A2A (--/αα)	CVS: (--/--)	35.41
17	M^287	32	A2A (--/αα)	A2A (--/αα)	FB: (--/--)	29.08
18	M^371	22	EA (--/αα)	A2A (--/αα)	FB: (--/--)	34.85
19	M^653	12	EA (--/αα)	A2A (--/αα)	CVS: (--/--)	34.38
20	M^671	19	A2A (--/αα)	A2A (--/αα)	FB: (--/--)	33.94
21	M^739	22	EA (--/αα)	EA/EF (--/αα)	FB: (--/--)	31.84
	Average GA=17				Average CT =33.17	

(CVS: Chorionic Villus Sampling, AF: Amniotic Fluid, FB: Fetal Blood)

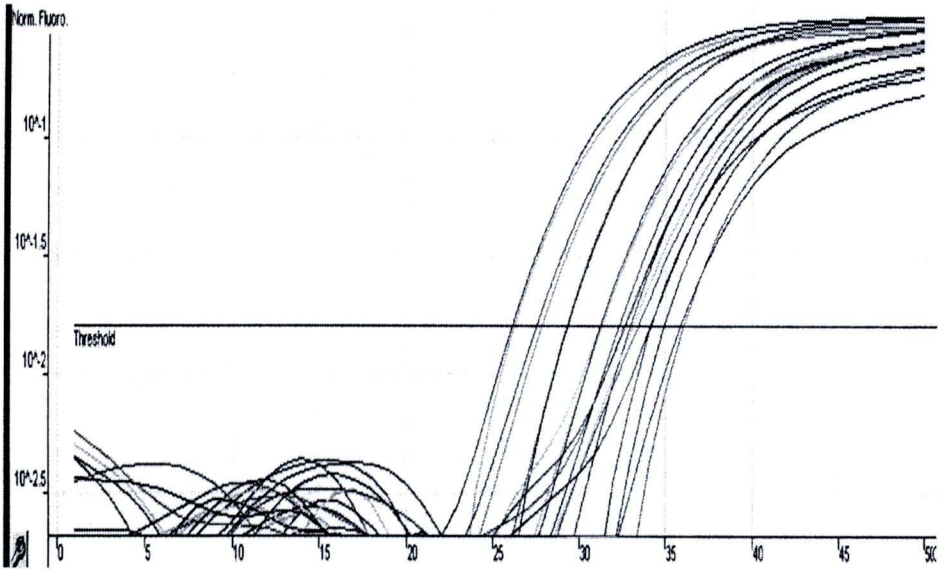


Figure 12 The TaqMan real time PCR for detection of GAPDH gene, C_T values are the amplification plot crosses the threshold for describe to concentration of plasma DNA.

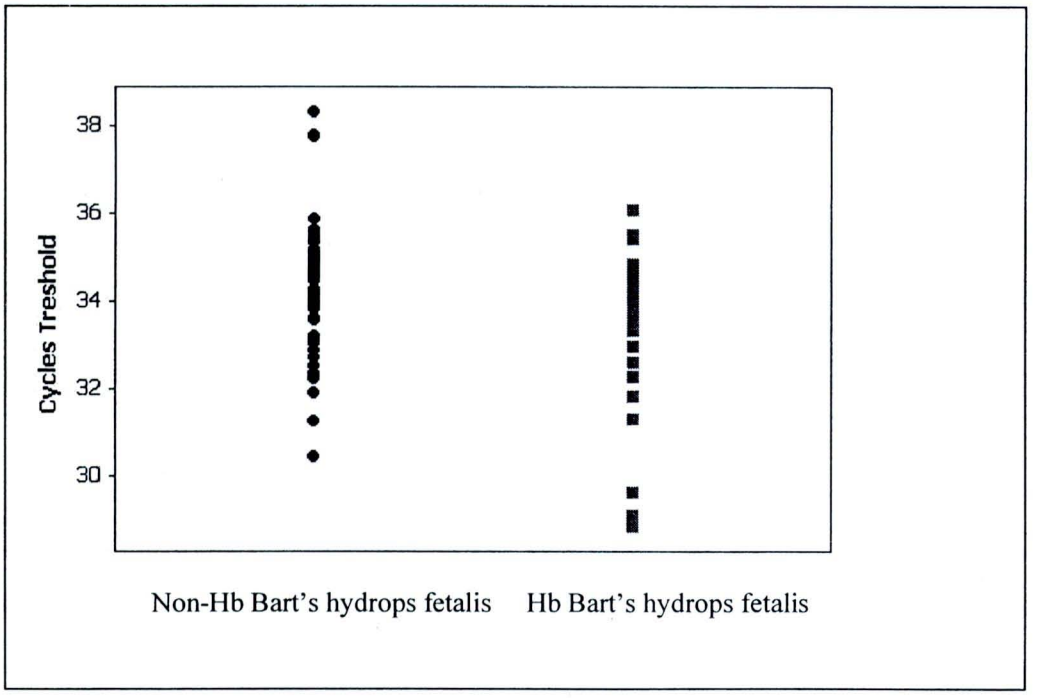


Figure 13 Scatter diagram indicating values of threshold cycle (C_T) of total DNA in maternal plasma from pregnancies with and without Hb Bart's hydrops fetalis fetuses.

2. Identification of known Hb variants

Table 9 summarized 13 known Hb variants detected at the Faculty of Associated Medical Sciences, Khon Kaen University during the past 7 years. Among 337 samples encountered, 276 samples were found to carry known Hb variants. These included 56 Hb Q-Thailand, 7 Hb Siam, 3 Hb Queens, 4 Hb Beijing, 1 Hb Hekinan, 17 Hb S, 27 Hb D-Punjab, 49 Hb Tak, 13 Hb Korle-Bu, 45 Hb Hope, 24 Hb Pyrgos, 31 Hb J-Bankkok and 15 Hb C. Among them, Hb Q-Thailand, Hb Hope and Hb Tak are most common Hb variants found in our laboratory. Therefore, interaction of these Hb variant with several forms of thalassemia and hemoglobinopathies were further investigated. In addition, three previously undescribed conditions of compound heterozygous for Hb Tak and $(\delta\beta)^0$-thalassemia, and association of homozygous Hb Tak with α^+-thalassemia. and compound heterozygous Hb Korle-Bu / Hb E with α^0-thalassemia were also studied in details.

Table 9 Number of subjects with known and unknown Hb variants encountered at the thalassemia service unit, CMDL, Khon Kaen University during Jan 2003- May 2010

	α-globin gene variants					β-globin gene variants								Un-identified	Total
	Hb Q-Thailand	Hb Siam	Hb Queens	Hb Heikinan	Hb Beijing	Hb S	Hb Tak	Hb D-Punjab	Hb Korle-Bu	Hb Hope	Hb J-Bangkok	Hb Pyrgos	Hb C		
2003	9	2	-	-	-	7	3	4	2	2	5	1	-	5	40
2004	5	-	-	-	4	2	3	2	1	4	3	4	6	7	41
2005	1	-	2	-	-	2	6	2	-	3	1	1	1	5	24
2006	2	-	-	-	-	2	4	1	1	5	4	-	-	4	23
2007	8	-	-	-	-	1	2	6	-	4	5	2	3	8	38
2008	20	2	-	-	-	2	11	6	5	9	6	8	3	13	75
2009	7	1	1	-	-	1	13	4	4	11	5	6	-	16	69
2010 (Jan-May)	4	2	-	1	-	-	6	2	-	6	2	2	2	3	27
Total	56	7	3	1	4	17	49	27	13	45	31	24	15	61	337

2.1 Interactions of Hb Q-Thailand with various hemoglobinopathies

56 subjects with Hb Q-Thailand are divided into 4 groups according to genotypes. DNA analysis by multiplex PCR identified the Hb Q-Thailand mutation (α74: GAC-CAC) and the linked 4.2 kb deletion α^+-thalassemia ($-\alpha^{4.2}$) in all cases (Figure 10). Twenty eight subjects with heterozygous ($-\alpha^{QT}/\alpha\alpha$) and a homozygous ($-\alpha^{QT}/-\alpha^{QT}$) Hb Q-Thailand were presented in group I where as those with interactions with other thalassemia genes including α^+-thalassemia ($-\alpha^{3.7}$), α^0-thalassemia ($--^{SEA}$), Hb Constant Spring ($\alpha^{CS}\alpha$), Hb E (β^E) and β^0-thalassemia (β^0) were categorized into groups II–IV. Hematological phenotypes of these subjects are presented in Table 10. As shown in the table, heterozygous and homozygous Hb Q-Thailand (Group I) had very mild hypochromic microcytic anemia. The levels of Hb F were within the normal range in all cases. In heterozygotes, the level of Hb Q-Thailand ($\alpha^{QT}{}_2\beta^A{}_2$) was at 29.8 ± 8.0 % and Hb A_2 ($\alpha^A{}_2\delta_2$) was within normal range (2.6 ± 1.0 %). In contrast, a major peak of Hb Q-Thailand (82.3 %) was observed in a homozygous subject and no Hb A_2 was detected as the patient had no α^A globin chain. We observed no Hb H (β_4) and Hb Bart's (γ_4) in this case. It was found that on HPLC analysis, Hb Q-Thailand and its derivative, the Hb QA_2 ($\alpha^{QT}{}_2\delta_2$), were not distinctly separated (Fig. 14A) but they were clearly identified on the capillary electrophoresis system (Fig. 14D).

Among the 9 subjects in group II with interactions of Hb Q-Thailand and other α-thalassemias, the most common was the interaction with α0-thalassemia (SEA deletion), causing the Hb Q-H disease ($--^{QT}/--^{SEA}$), which was found in 6 subjects. The patients had mild to moderate hypochromic microcytic anemia, characteristics of Hb H disease. Hb analysis showed a major peak of Hb Q-Thailand (79.4 ± 8.3 %) in addition to Hb H and Bart's. As shown in Table 10, this Hb Q-H disease appeared to have a similar hematological phenotype with that of the deletional Hb H disease commonly encountered in our routine practice [165]. Of the 3 remaining cases in this group, two were double heterozygotes for Hb Q-Thailand and Hb Constant Spring ($-\alpha^{QT}/\alpha^{CS}\alpha$) and 1 was a double heterozygote for Hb Q-Thailand and α^+-thalassemia ($-\alpha^{QT}/-\alpha^{3.7}$). All of them had mild hypochromic microcytic anemia but no Hb H and Hb Bart's was detected. The levels of Hb Q-Thailand were found to be 49.2% and 49.3% in the former and 34.7% in the latter genotypes, respectively. Group III

included those with Hb Q-Thailand found in association with Hb E in various combinations including 9 double heterozygotes for Hb Q-Thailand/Hb E ($-\alpha^{QT}/\alpha\alpha$, β^{E}/β^{A}), 3 double Hb Q-Thailand/Hb E and α^{+}-thalassemia ($-\alpha^{QT}/-\alpha^{3.7}$, β^{E}/β^{A}), 3 homozygous HbE/Hb Q-Thailand ($-\alpha^{QT}/\alpha\alpha$, $\beta^{E}\beta^{E}$) and a homozygous Hb E with compound Hb Q-Thailand/Hb Constant Spring ($-\alpha^{QT}/\alpha^{CS}\alpha$, β^{E}/β^{E}). In this group, in addition to Hb E ($\alpha^{A}\beta^{E}{}_{2}$) and Hb Q-Thailand ($\alpha^{QT}{}_{2}\beta^{A}{}_{2}$) fractions, a small peak of the Hb QE resulted from the ($\alpha^{QT}{}_{2}\beta^{E}{}_{2}$) tetrameric assembly with slower separation times was observed on both HPLC and capillary electrophoresis (Fig. 14B and E). However, as shown in the figures, capillary electrophoresis provided better separation of the Hb Q-Thailand and its Hb QE derivative. As expected, this Hb QE rather than Hb Q-Thailand was detected in all 3 homozygous Hb E/Hb Q-Thailand and a homozygous Hb E with compound Hb Q-Thailand/Hb Constant Spring since all these patients had no β^{A}-globin chain, required for the formation of Hb Q-Thailand. The remaining two cases in group IV were found to be double heterozygotes for Hb Q-Thailand and β^{0}-thalassemia. DNA analysis of β-globin gene identified the 4 bp deletions between codons 41/42, in both cases, the most common β-thalassemia mutation in our region [166]. In these cases, Hb analysis demonstrated, in addition to Hb Q-Thailand and elevated Hb A2 ($\alpha^{A}{}_{2}\delta_{2}$), 1.0 % and 2.6 % of the Hb QA_2 derivative ($\alpha^{QT}{}_{2}\delta_{2}$) was observed (Fig. 14C). It is noteworthy that diagnosis of β-thalassemia in these two cases was not altered due to the co-inheritance of Hb Q-Thailand, as Hb A_2 levels (4.9 % and 5.3 %) were still higher than normal. Other hematological parameters were as usually observed for a β-thalassemia carrier.

Table 11 demonstrated the α-globin gene haplotypes associated with normal α-globin gene, Hb Q-Thailand and the ($-\alpha^{4.2}$) α-thalassemia determinants in Thai population. Among 56 subjects studied, complete segregation could be obtained from 14 Hb Q-Thailand alleles. All of them are associated with a single haplotype; (+ − S + 0 − −). As shown in the table, this haplotype is exactly the same with that of the 4 ($-\alpha^{4.2}$) α-thalassemia determinant and is one of the common haplotypes observed for normal α-globin genes in Thai population.

Table 10 Hematological parameters and globin genotypes of the Hb Q-Thailand related disorders. Percentage of Hbs A2/E, F, QT and QE were based on HPLC analyzer. Hb QA2 was recorded on the capillary electrophoresis. Values are presented as mean±SD, range or as raw data where appropriate.

Gr. (n)	Genotype (n)	Rbc ($x10^{12}$/l)	Hb (g/dl)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (%)	A_2/E (%)	F (%)	Hb QT (%)	Hb QE (%)	Hb QA2 (%)
I (29)	Hb Q-T heterozygotes (28)	5.2±0.6	12.8±1.7	38.2±5.0	74.9±6.9	24.8±2.6	33.6±1.6	14.4±2.4	2.6±1.0	0.8+0.5	29.8±8.0	none	none
	Homozygous Hb Q-T (1)	5.5	12.8	40	72.2	23.1	32	16.4	none	<0.5	82.3	none	none
II (9)	Compound Hb Q-T /α^+-thalassemia($-\alpha^{3.7}$) (1)	4.5	9.7	31	70	22.2	31	15.7	none	none	34.7	none	none
	Hb Q-T/CS disease (2)	4.3, 4.6	9.7, 9.9	29.5, 31.6	69.1, 69.5	21.4, 23.2	30.7, 33.6	16.3, 15.9	1.5, 2.0	0.5	49.2, 49.3	none	none
	Hb Q-T/H disease (6)	4.4±0.5	8.0±1.2	28.2±2.3	70.2±3.6	19.7±4.5	29.4±1.6	23.8±1.8	none	none	79.4±8.3	none	<0.1
III (16)	Double heterozygotes for Hb Q-T /Hb E (9)	5.2±0.4	12.6±2.0	38.1±6.8	78.2±4.3	25.8±1.3	33.3±0.9	14.0±1.4	19.5±1.5	0.7±0.3	19.6±3.8	4.9±3.3	none
	Hb Q-T/α^+-thalassemia ($-\alpha^{3.7}$) /Hb E heterozygote (3)	4.9-6.7	13.3 - 15.6	37.5 - 46.6	62.0 – 69.0	22.3 - 23.1	33.4 – 36.0	12.3- 15.5	9.1 - 15.6	0.2	29.8 – 33.7	8.0-11.1	none
	Heterozygous Hb Q-T /Homozygous Hb E (3)	5.8±0.9	12.5±1.5	37.9±4.6	66.1±3.0	21.7±0.7	32.9±0.5	15.9±0.6	75.4±4.3	<0.1	none	14.4±0.7	none
	Heterozygous Hb Q-T /Homozygous Hb E/Hb CS (1)	5.5	11.7	37.6	68.2	21.2	31.5	15.2	44.8	none	none	50.1	none
IV (2)	Hb Q-T/β^0-thalassemia (2)	4.8, 4.6	10.0, 11.0	31.3, 31.6	65.3, 68.0	20.8, 23.2	31.9, 32.0	15.6, 15.3	4.9 , 5.3	0.4	13.8 ,16.6	none	1.0 ,2.6

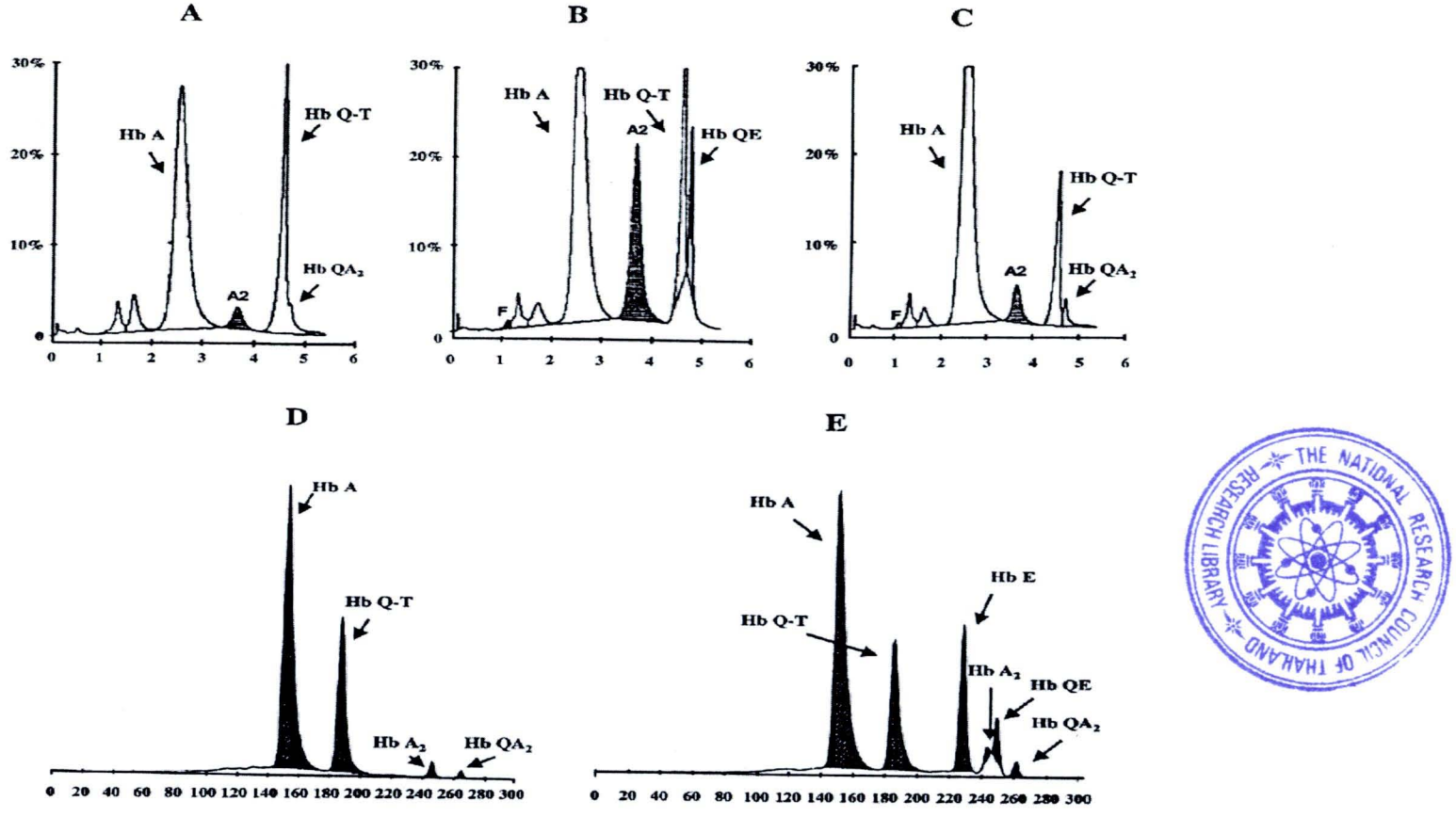
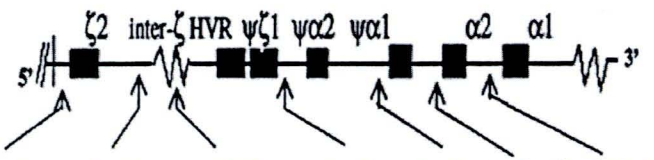


Figure 14 Representative Hb analysis demonstrating Hb Q-Thailand variant using automated HPLC (A, B and C) and capillary electrophoresis system (D and E). A and D, Hb Q-Thailand heterozygote. B and E, double heterozygote for Hb Q-Thailand and Hb E. C, double Hb Q-Thailand/β^0-thalassemia. Hb A, Hb Q-Thailand, Hb QE and Hb QA_2 are indicated by arrows.

Table 11 α-Globin gene haplotypes associated with $\alpha^{Q\text{-Thailand}}$, α^{+}-thalassemia (4.2 kb deletion) and normal α-globin genes in Thai population. Numbers indicate number of alleles associated with each specific haplotype. + and – indicate the presence and absence of each polymorphic site where as 0 indicates deletion. nd= not done.

Globin alleles								Number of allele
αα*	+	–	S	+	+	–	nd	10
	+	–	S	+	–	–	nd	21
	+	–	S	+	–	+	nd	1
	+	–	S	–	–	–	nd	1
$-\alpha^{4.2}$	+	–	S	+	0	–	–	4
$-\alpha^{Q\text{-Thailand}}$	+	–	S	+	0	–	–	14

* Data from the previous report [164]

2.2 Interactions of Hb Hope with various hemoglobinopathies

Hematological data and results of DNA analyses of 45 unrelated Thai subjects with Hb Hope are listed in Table 12, which are divided into 4 groups according to genotypes. Group I includes 27 heterozygous Hb Hope. Their abnormal Hb levels were 42.2 ± 8.2 % with slightly decrease of Hb level (10.8 ± 2.3 g/dl), but normal MCV and MCH values. Group II, double heterozygotes of Hb Hope and other forms of α-thalassemia including 3 double heterozygous Hb Hope/α^0-thalassemia, 2 heterozygous Hb Hope/α^+-thalssemia, 1 heterozygous Hb Hope/Hb CS and 2 heterozygous Hb Hope/Hb H-disease. The average levels of Hb Hope were 14.3 - 29.6 %, 48.5 %, 43.1 % and 18.7 & 21.6 %, respectively. Minute amounts of Hb Bart's but not Hb H was observed in a patient with Hb Hope/Hb H-disease. In this latter condition lower Hb Hope levels (18.7 % & 21.6 %) were observed. This genotype presented with severe anemia and hypochromic microcytosis with Hb of 7.7 & 10.4 g/dl, MCV of 50.0 & 57.6 fl and MCH of 16.5 & 16.7 pg. As shown in Figure 21, Hb analysis showed a peak of Hb Hope that moved slightly faster than Hb A, in addition to observed minute or absent of Hb Bart's but not observed Hb H in both cases. Group III included 2 compound heterozygous Hb Hope/β^0-thalassemia, 1 compound Hb Hope /β^0-thalassemia with α^+-thalassemia and 1 compound Hb Hope/β^0-thalassemia with α^0-thalassemia. These three genotypes presented with the amount of 90.5 %, 73.1 % and 78.5 % Hb Hope, respectively. Slight hypochromic microcytic anemia with Hb 10.2 and 11.7 g/dl, MCV of 62.0 and 73.1 fl, and MCH of 20.0 and 23.3 pg, were observed. Elevated amount of 5.4 and 6.0 % Hb A_2 was noted. DNA analysis of β-globin gene identified the $\beta^{71/72}$ and β^{17} in 2 Hb Hope/β^0-thalassemia, Hope/β^{17}-thalassemia with α^+-thalassemia and Hope/$\beta^{41/42}$-thalassemia with α^0-thalassemia. Group IV included 6 compound heterozygous Hb Hope/ Hb E with the amounts of 66.1 ± 1.7 % Hb Hope and 27.8 ± 1.8 % Hb E. This genotype presented with slight anemia and hypochromic microcytosis. The concentration of Hb, MCV and MCH are presented in Table 12. β-Globin gene haplotype analysis revealed a single β-globin gene haplotype [+ - - - - + +] for these Thai subjects, consistent with

previous studied [132, 134]. This indicated a single origin of this variant in Thai population.

Table 12 Hematological parameters and globin genotypes of the Hb Hope related disorders.

Gr. (n)	Genotypes (n)	Rbc ($x10^{12}$/l)	Hb (g/dl)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (%)	Hb A2/E (%)	Hb F (%)	Hb Hope (%)
I (27)	Heterozygous Hb Hope (27)	4.4 ± 0.8	10.8 ±2.3	34.2 ±8.9	84.6 ± 11.6	26.8 ± 2.0	31.9 ± 2.6	14.5 ± 4.0	3.5 ± 0.9	2.2 ± 1.8	42.2± 8.2
II (8)	Double heterozygous Hb Hope/α^{SEA} (3)	NA	NA	NA	NA	NA	NA	NA	2.5-3.5	NA	14.3-29.6
	Double heterozygous Hb Hope/$\alpha^{3.7}$ (2)	NA	NA	NA	NA	NA	NA	NA	2.4, 2.6	NA	38.3, 48.5
	Double heterozygous HbHope/CS (1)	NA	NA	NA	NA	NA	NA	NA	2.5	NA	43.1
	Double heterozygous Hb Hope/Hb H-disease($\alpha^{SEA}/\alpha^{3.7}$) (2)	6.3	7.7, 10.4	23.1,36.4	50.0,57.6	16.5, 16.7	28.6, 33.3	21.3	1.6, 2.8	3.4, 3.5	18.7, 21.6
III (4)	Compound heterozygous Hb Hope/β^0 (2)	5.17	10.2	32	62	20	32	17	5.4, 6.0	0.7, 0.9	90.5
	Compound heterozygous Hb Hope/$\beta^0/\alpha^{3.7}$ (1)	NA	NA	NA	NA	NA	NA	NA	5.5	5.5	73.1
	Compound heterozygous Hb Hope/β^0/α^{SEA} (1)	NA	11.7	36.7	73.1	23.3	31.9	14.6	4.5	NA	78.5
IV (6)	Compound heterozygous HbHope/Hb E (6)	3.97	9.3-10.1	28.9-32.0	60.2-80.0	19.4-26.0	31.9-32.2	14.8-18.3	27.8 ± 1.8	2.4±1.9	66.1±1.7

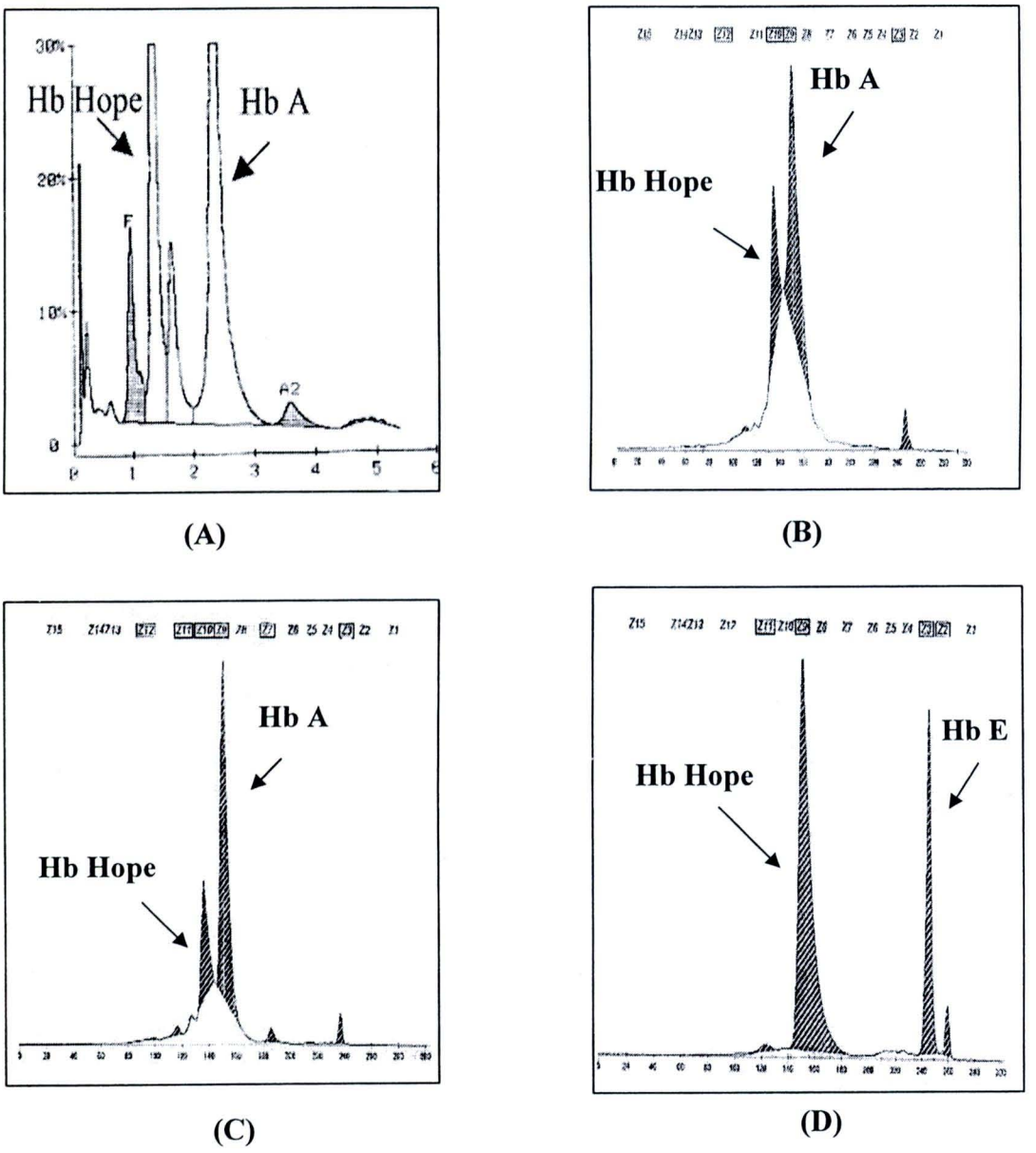


Figure 15 Representative Hb analysis demonstrating Hb Hope variant using automated HPLC (A) and capillary electrophoresis (B, C and D). A and B, Hb Hope heterozygotes. C, double heterozygous for Hb Hope and α^0-thalassemia. D, Compound heterozygous Hb Hope/Hb E. Hb A, Hb Hope and Hb E are indicated by arrows.

2.3 Interactions of Hb Tak with various hemoglobinopathies

Hematological data and results of DNA analyses of 49 unrelated Thai subjects with Hb Tak are listed in Table 13, which are divided into 4 groups according to genotypes. Twenty three with heterozygous Hb Tak were presented in group I. Interactions which orther thalassemia genes including α^{+}-thalassemia, α^{0}-thalassemia, Hb E and $(\delta\beta)^{0}$-thalassemia were categorized into groups II-IV. In this study two previously undescribed in routine laboratory, association of Hb Tak with $(\delta\beta)^{0}$-thalassemia and homozygous Hb Tak with α^{+}-thalassemia were also observed.

The patient with of Hb Tak/$(\delta\beta)^{0}$-thalassemia was a 48 years old man with no history of blood disorder. He was essentially in good health but was plethoric in appearance. As shown in Table 13, he had marked erythrocytosis and microcytosis with RBCs 8.7x 1012/l, Hb 19.5 g/dl, hematocrit (Hct) 59.2 %, mean corpuscular volume (MCV) 68.0 fl, mean corpuscular Hb (MCH) 22.4 pg and mean corpuscular Hb concentration (MCHC) 32.9 g/dl. Hb analyses using the LPLC Hb Analyzer (Hb Gold) showed Hb A_2 (72.5 %) and Hb F (30.0 %) without Hb A, whereas capillary electrophoresis revealed 2.3% Hb A_2, a major peak of Hb F (91.2 %) but no Hb A (Figure 17). These data indicated that he carried a β-globin chain variant that was coeluted with Hb A_2 on liquid chromatography but was comigrating with Hb F on capillary electrophoresis and another high Hb F determinant. DNA analysis using multiplex PCR for identification of Hb S, D-Punjab and Tak and multiplex PCR for identifying common high Hb F determinants in Thailand identified the Hb Tak mutation in trans to the 12.5-kb deletional $(\delta\beta)^{0}$-thalassemia determinant (Figure 16). No common α-thalassemia including α^{0}-thalassemia (SEA and THAI types), α^{+}-thalassemia (3.7- and 4.2-kb deletions), $\alpha^{\text{Constant Spring}}$ and $\alpha^{\text{Pakse'}}$ was detected. Therefore, he was a compound heterozygote for Hb Tak/$(\delta\beta)^{0}$-thalassemia, a hitherto undescribed condition.

The hematological data of the patient were compared with those observed in a Thai boy with a homozygous Hb Tak with α^{+}-thalassemia (3.7 kb), another undescribed condition encountered at our routine setting, and with those of 7 pure carriers of Hb Tak and 142 carriers of $(\delta\beta)^{0}$-thalassemia in our series (Table 13). We observed a similar phenotype for this boy with homozygous Hb Tak and α^{+}-

thalassemia. He had marked erythrocytosis and microcytosis with RBCs 8.6 x 10^{12}/l, Hb 18.9 g/dl, Hct 60.4 %, MCV 69.5 fl, MCH 21.8 pg and MCHC 31.3 g/dl. Hb analyses demonstrated a major peak of Hb Tak (91.9 %) and increased Hb A_2 (5.4 %) but no Hb A. Screening for β-thalassemia mutations commonly found in our region yielded a negative result. DNA analysis by allele-specific PCR revealed homozygosity for AC insertion at the termination codon of the β-globingene (Figure 16). In addition, family analysis revealed that his father was a compound Hb Tak/Hb E whereas his mother was a double heterozygote for Hb Tak and α^+-thalassemia (3.7-kb deletion). Therefore, the patient obtained Hb Tak from his father and inherited both Hb Tak and α^+-thalassemia from his mother. Although plethoric in appearance and with recurrent mild headaches, he was otherwise well and had normal growth and development. In contrast, all 21 carriers of Hb Tak and RBC and Hb levels within normal ranges. Hb A_2 was at borderline level (3.1 $\pm$ 1.3 %) and no microcytosis was observed. The percentage of Hb Tak was 29.1$\pm$ 5.5%. Carriers of $(\delta\beta)^0$-thalassemia were associated with hematologically mild phenotypes. All of them had high Hb F (20.7 $\pm$ 5.6 %) and normal Hb A2 (2.2 $\pm$ 0.4 %) levels. Reduced MCV (75.1 $\pm$ 7.8 fl) and MCH (24.6 $\pm$ 2.2 pg) values were noted, but RBC and Hb levels were within normal ranges.

Table 13 Hematological parameters and globin genotypes of the Hb Tak disorders.

Gr. (n)	Genotypes (n)	Rbc ($x10^{12}$/l)	Hb (g/dl)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (%)	Hb A_2/E (%)	Hb F (%)	Hb Tak (%)
I (23)	Heterozygous Hb Tak (23)	4.5±0.2	13.6±1.8	38.2±5.6	81.3±9.2	29.2±2.9	34.4±1.7	14.4±2.8	3.1±13	NA	29.1±5.5
II (9)	Double heterozygous Hb Tak/$\alpha^{3.7}$ (5)	NA	13.2-16.5	38.8-49.0	78.7-81.0	26.3-27.6	33.3-34.0	16	4.0±1.2	0.8±0.4	42.2±3.2
	Double heterozygous Hb Tak/α^{SEA} (1)	NA	NA	NA	NA	NA	NA	NA	NA	NA	40.6
	Double heterozygous Hb Tak/homozygous α^{+}-thalassemia (2)	NA	NA	NA	NA	NA	NA	NA	4.3	0.8	15.5, 13.9
	Double heterozygous Hb Tak/α^{+}-thalassemia (1)	8.6	18.9	60.4	69.5	21.8	13.3	NA	5.4	NA	91.9
III (16)	Compound heterozygousHb Tak/ HbE (15)	6.8±1.1	16.9±4.2	50.6±10.6	75.9±7.3	25.5±3.4	33.3±4.6	15.7±3.0	32.7±3.2	2.9±2.8	57.9±7.2
	Compound heterozygous Hb Tak/ HbE/$\alpha^{3.7}$ (1)	NA	NA	NA	NA	NA	NA	NA	39.6	1.4	51.6
IV (1)	Compound heterozygous Hb Tak/$\delta\beta^{0}$ (1)	NA	19.5	59.2	68	22.4	32.9	27.5	2.3	NA	91.2
	$\delta\beta^{0}$-thalassemia carrier (142)	4.9±0.7	12.3±7.6	35.7±4.7	75.1±7.8	24.6±2.2	32.3±1.0	NA	2.2±0.4	20.7±5.6	NA

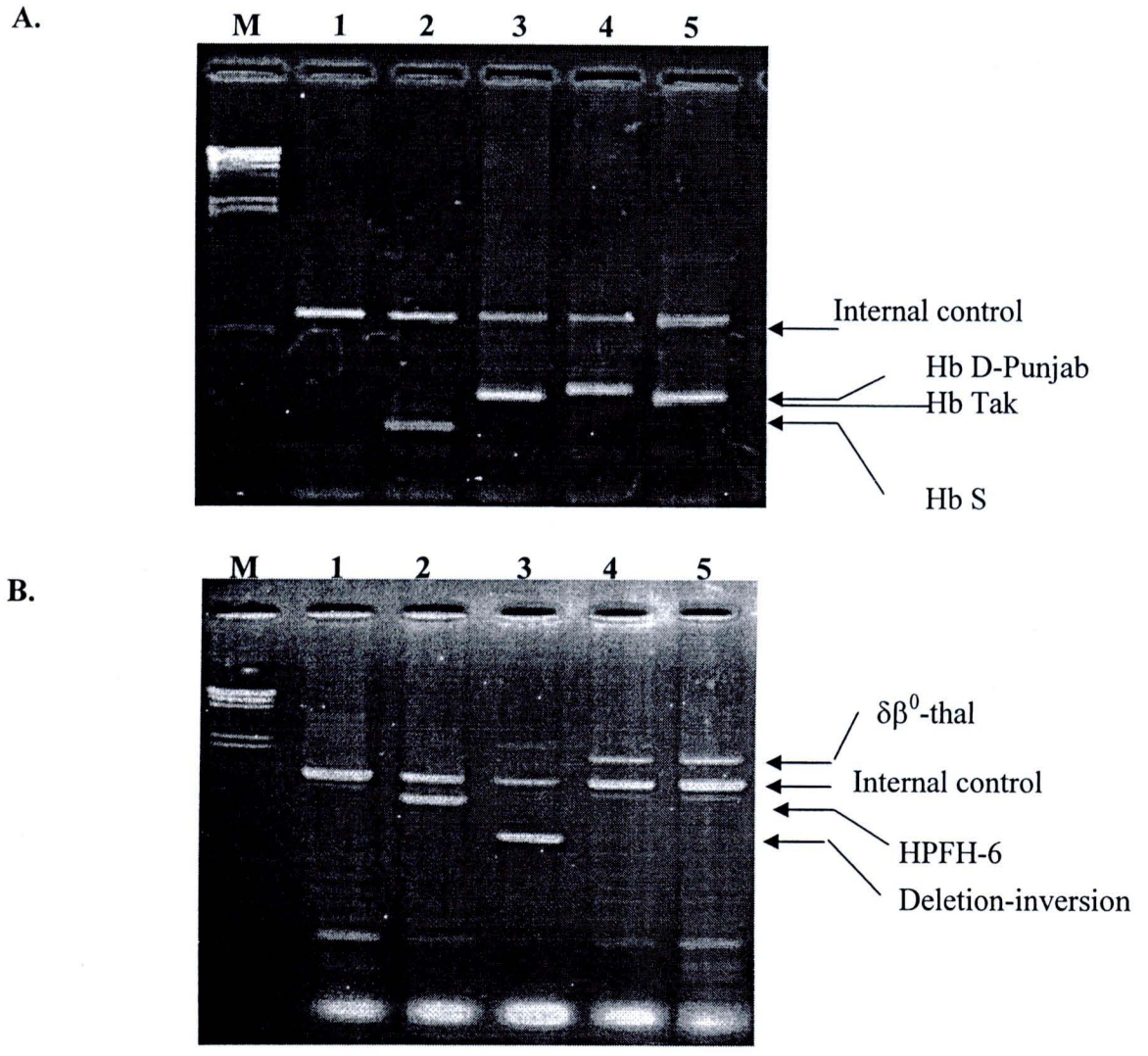
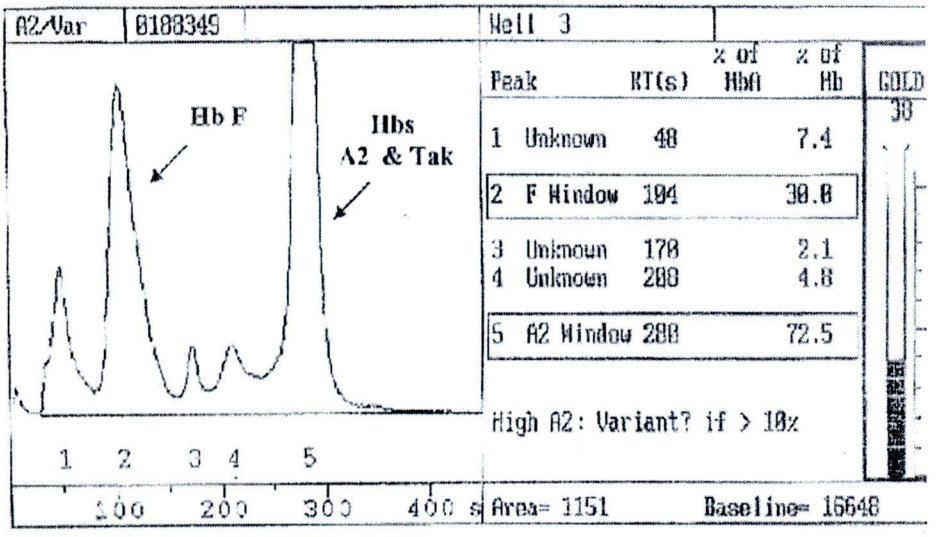


Figure 16 A representative agarose gel electrophoresis of the multiplex allele specific PCR analysis

A: Agarose gel electrophoresis of the multiplex allele specific PCR for identification of Hb Tak, Hb S and Hb D-Punjab mutations. Lane 1: normal control; lane 2: Hb S carrier; lane 3: Hb Tak carrier; lane 4: Hb D-Punjab carrier and lane 5: the proband. M represents the λ/*Hind* III size markers.

B: Multiplex PCR analysis for detection of common high Hb F determinants in Thailand. Lane 1: normal control; lane 2: HPFH-6 carrier; lane 3: deletion-inversion $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thalassemia carrier; lane 4: $\delta\beta^{0}$-thalassemia carrier and lane 5: the proband. M represents the λ/*Hind* III size markers.



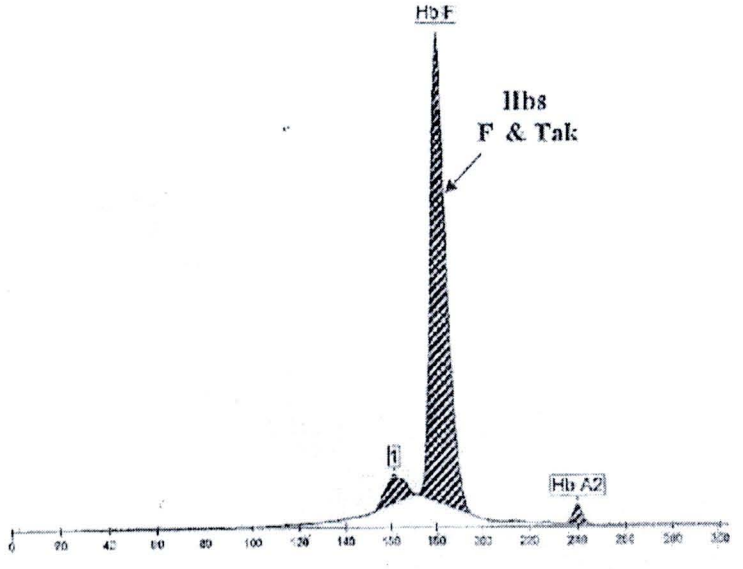


Figure 17 Representative Hb analysis demonstrating Hb Tak variant using LPLC in which it co-migrates with Hb A_2 and capillary electrophoresis in which it co-separated with Hb F.

2.4 Association of Hb Korle-Bu / Hb E with α^0-thalassemia

The proband was a 26-year-old pregnant woman who presented at our ongoing thalassemia screening program at the Health Promoting Centre Region 8, Nakornsawan, Thailand, with mild hypochromic microcytic anemia. She had positive results with both osmotic fragility and dichlorophenolindophenol (DCIP) tests, a combined screening test for thalassemia and hemoglobinopathies generally used in the prevention and control program of thalassemia in Thailand. Blood specimens from her family members including the father, mother and older sister were also available for testing. As shown in Table 14, the proband had the following values: Hb 10.3 g/dL, PCV 0.298 L/L, MCV 66.1 fL, MCH 22.8 pg, MCHC 34.6 g/dL and RDW 14.9%. Peripheral blood film examination showed slight anisocytosis and hypochromic red cells with target cells. Hemoglobin analysis using automated HPLC revealed no Hb A but a single peak of an abnormal Hb at the D window with the amount of 88.2% (Figure 18). She was therefore initially diagnosed as a homozygous Hb D-Punjab [β121(GH4)Glu→Gln, *GAA>CAA*] or Hb Tak [β147 (+AC)], the two variants commonly found in Thai population or other β chain variants [117, 167]. Inconsistencies with this diagnosis were a positive result obtained with a DCIP test for Hb E screening and a result of family analysis which identified the same variant only in the father but not in the mother. In addition, a negative result with a multiplex allele-specific polymerase chain reaction (PCR) for Hb D-Punjab, Hb Tak and Hb S [β6(A3)Glu→Val, G*A*G>G*T*G] mutations was obtained [157]. Further Hb analyses of the proband using capillary zone electrophoresis, however, clearly demonstrated Hb A_2 (3.6%), Hb E (16.4%) and an abnormal Hb (80.0%) migrating separately between Hb A and Hb E (Figure 24). This Hb analysis system can report Hb A_2 in the presence of Hb E. This abnormal Hb (45.9%) and Hb A_2 (2.2%) were also observed in addition to the Hb A (51.9%) in her father, whereas her mother was found to be a heterozygous Hb E. As with her father, this Hb analysis identified that her sister was heterozygous for the same variant. As shown in Table 14, the proband and her mother had relatively lower Hb E levels (16.4 and 23.5%, respectively) when compared to those described for Hb E heterozygotes, the data indicating a possibility of co-inheritance of α-thalassemia [168]. Therefore, α-globin genotyping by PCR was carried out for all

family members. With this analysis, we identified the Southeast Asian deletional α-thal-1 in the proband, her father and her sister but not in her mother who was found to carry the Hb CS mutation. Further DNA analysis using allele-specific PCR for abnormal Hbs found in Thailand identified that the Hb variant segregating in this family was Hb Korle-Bu caused by the G>A mutation at codon 73 of the β-globin gene that leads to a substitution of asparagine for aspartic acid [2]. In the present family, as expected, the Hb Korle-Bu mutation was identified in the proband, her father and her sister but not in her mother (Figure 19). Therefore, with these analyses we were able to conclude that the proband carried Hb E, Hb Korle-Bu and α-thal-1, whereas her father and her sister had Hb Korle-Bu and α-thal-1, both of which are novel conditions. Her mother was a double heterozygote for Hb E and Hb CS. In Table 14, the hematological parameters of the proband and her family members were compared with those of another pregnant Thai woman with Hb E/Hb Korle-Bu/α-thal-2 (3.7 kb deletion) described previously [2]. As shown in Table 17, the same β-globin haplotype, [− + − + + − +], was found to be associated with the $\beta^{\text{Korle-Bu}}$ in the family studied here.

Table 14 Hematological Data and Genotypes of the Proband, Her Family and the Previous Case [2]

Parameters	Father	Mother	Sister	Proband	Previous case
Sex-Age (years)	M-67	F-63	F-32	F-26	F-19
Osmotic fragility	positive	negative	positive	positive	positive
DCIP	negative	positive	negative	positive	positive
Hb (g/dL)	11.2	11.8	12.3	10.3	12.2
PCV (L/L)	0.335	0.362	0.376	0.298	0.360
MCV (fL)	76.5	80.1	63.6	66.1	73.2
MCH (pg)	25.6	25.9	20.8	22.8	24.8
MCHC (g/dL)	33.4	32.3	32.7	34.6	33.9
RDW (%)	19.3	13.7	15.7	14.9	15.9
Hb A (%)	51.9	73.5	51.9	–	–
Hb Korle-Bu (%)	45.9	–	45.4	80.0	69.3
Hb E (%)	–	23.5	–	16.4	22.4[a]
Hb A_2 (%)	2.2	3.0	2.7	3.6	–
α Genotype	$\alpha\alpha/--^{SEA}$	$\alpha\alpha/\alpha^{CS}\alpha$	$\alpha\alpha/--^{SEA}$	$\alpha\alpha/--^{SEA}$	$\alpha\alpha/-\alpha^{3.7}$
β Genotype	$\beta^A/\beta^{Korle\text{-}Bu}$	β^A/β^E	$\beta^A/\beta^{Korle\text{-}Bu}$	$\beta^E/\beta^{Korle\text{-}Bu}$	$\beta^E/\beta^{Korle\text{-}Bu}$
β Haplotype[b]	β^A [+ – – – – + –] $\beta^{Korle\text{-}Bu}$ [– + – + + – +]	β^A [– + – + + – +] β^E [– + – + + + –]	β^A [– + – + + – +] $\beta^{Korle\text{-}Bu}$ [– + – + + – +]	β^E [– + – + + + –] $\beta^{Korle\text{-}Bu}$ [– + – + + – +]	β^E [– + – + + + –] $\beta^{Korle\text{-}Bu}$ [– + – + + – +]

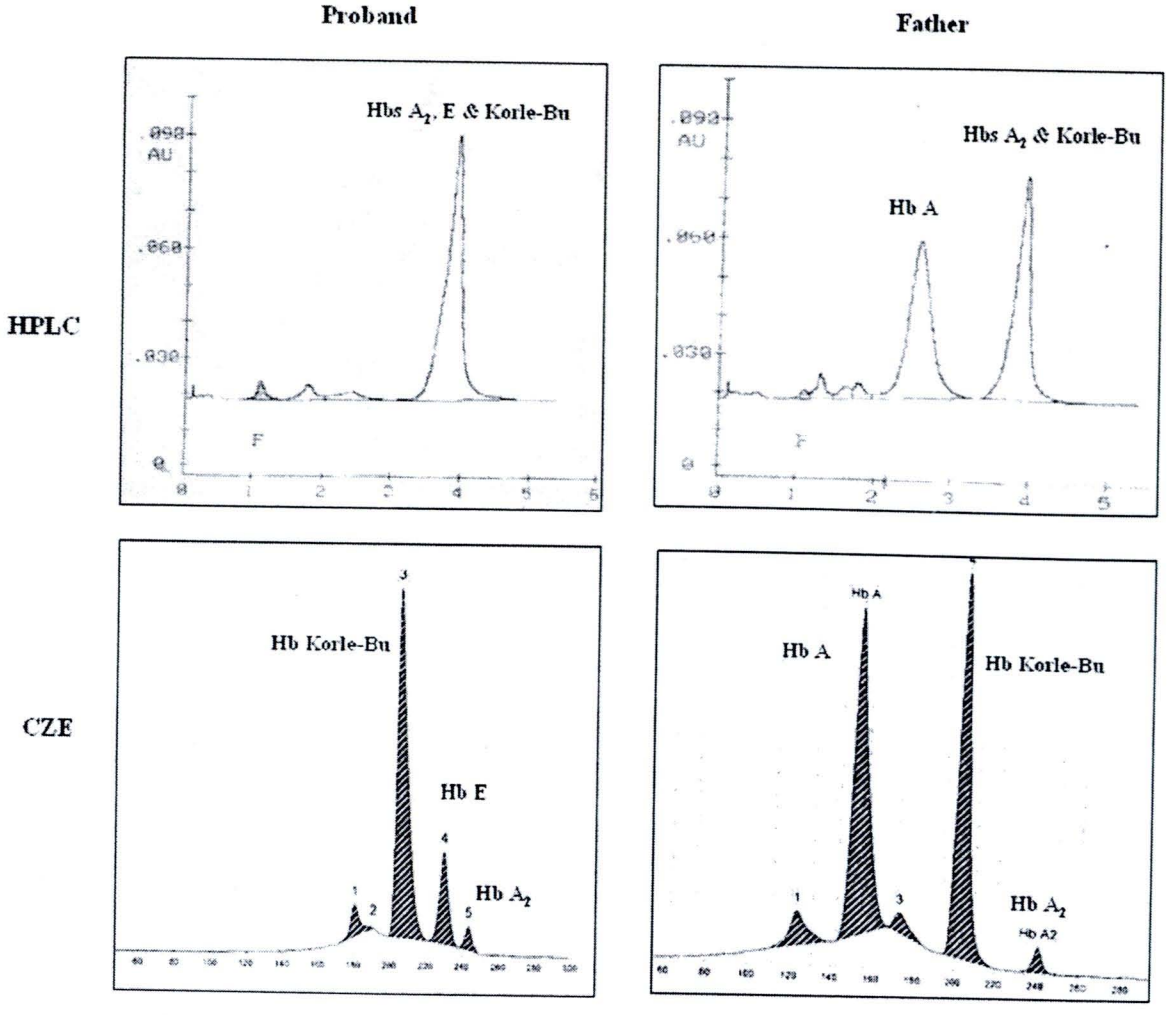


Figure 18 Hemoglobin analyses of the proband and her father using automated HPLC and capillary zone electrophoresis (CZE). The separating profiles of Hb A2, Hb E and Hb Korle-Bu are indicated.

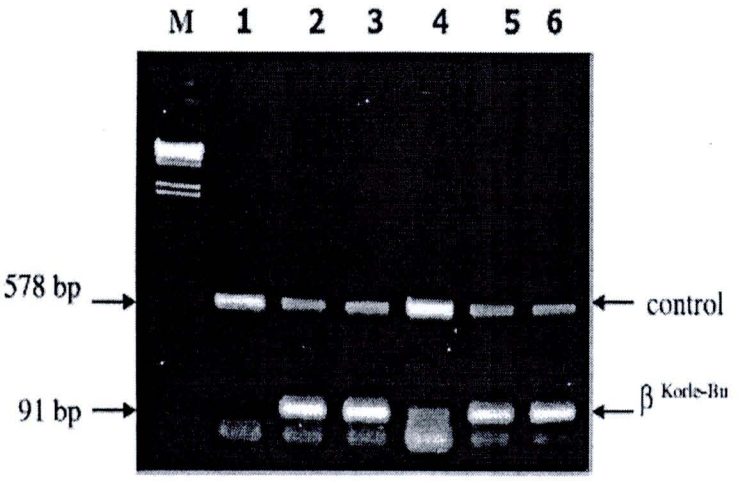


Figure 19 An allele specific PCR for detection of Hb Korle-Bu mutation.

A representative agarose gel electrophoresis of an allele specific PCR analysis. Lanes 1 and 2: normal and positive controls for Hb Korle-Bu; lanes 3–6 are the father, the mother, the sister and the proband, respectively. M represents the λ/*Hind* III size markers.

3. Identification of a novel β-globin chain variant, the Hb Phimai

Hematological data of the proband, her husband and her sister are listed in Table 15. The proband and her sister were generally healthy but her husband had thalassemia intermedia characteristics. Except for the positive results with DCIP screening test for Hb E, the proband and her sister had otherwise normal hematological parameters. Hb analysis using HPLC showed a major peak of Hb A, a smaller peak of an unknown variant (amounting to 33.5 % & 38.7 %) and Hb A_2 level of 3.5 - 3.6 % in both cases (Figures 20A & 20B). Analysis performed with capillary electrophoresis system (Capillarys 2, Sebia, Lisses, France) showed similar patterns in which the Hb variant was identified at zone 10 of the electrophoregram (Figures 20C & 20D). A small peak of Hb Constant Spring was also observed in the proband but not in her sister, consistent with the result of DNA analysis for the Hb Constant Spring mutation [154] which revealed the heterozygosity for Hb Constant Spring mutation of the proband. This result indicated that the proband and her sister are heterozygous for an unknown β-globin chain variant. As the HPLC mobility and capillary electrophoregram of this Hb variant are quite similar to that of the Hb Hope [β136(H14)Gly→Asp] occasionally found in Thai population, identification of the β^{Hope} mutation by allele specific PCR [158] was therefore carried out, a negative result was obtained. Identification of Hb Pyrgos [β83(EF7)Gly→Asp] and Hb J-Bangkok [β56(D7)Gly→Asp] mutations by allele specific PCR [159] also yielded negative result in both cases (data not shown). Further DNA sequence analysis of β-globin gene of the proband revealed a G→C transversion at the codon 72 (A<u>G</u>T→A<u>C</u>T), resulting in a substitution of threonine for serine (Figure 21), a previously undescribed Hb variant. This novel variant was named Hb Phimai after the name of a district in Nakhon Ratchasima province where the proband was inhabited.

In order to confirm the presence of G-C mutation in codon 72 and to establish a rapid differential diagnosis of Hb Phimai and Hb Hope, we have developed a multiplex allele specific PCR system as shown in Figure 9. As shown in the figure, the Hb Phimai mutation was detected in the proband and her sister (Figure 22, lanes 3 & 5) but not in her husband (Figure 22, lane 4). Hb Hope could also be confirmed in the same PCR assay (Figure 22 lane 2). The results indicate that this multiplex DNA

assay can be used as a confirmatory test and for differential diagnosis of Hb Phimai and Hb Hope in routine setting. Application of this multiplex PCR system on another 26 subjects with unknown variant resembling Hb Phimai & Hb Hope in our series yielded negative result. This data indicates that this Hb Phimai is relatively rare in Thai population. Further haplotype analysis of β-globin gene cluster including 7 polymorphic sites as described in the Materials and Methods section, demonstrated that both the proband and her sister had the same polymorphic pattern; (+/-, +/-, -/-, +/-, +/-, -/- , +/+). Unfortunately, due to the lack of specimens from other family members, the β^{Phimai} associated haplotype could not be accurately segregated.

From these Hb and DNA analyses, it could be concluded that the proband was a double heterozygous for Hb Phimai / Hb Constant Spring whereas her sister was a pure Hb Phimai heterozygote. As shown in Table 15, Hb and DNA analyses of her husband confirmed that he was suffered from a thalassemia intermedia associated with the AEBart's disease commonly encountered in northeast Thai population [165]. Appropriate explanation and genetic counseling were provided for the couple.

Table 15 Hematological data and genotypes of the proband, her husband and her sister. M; male , F; female, α^{CS}; Hb Constant Spring gene.

Parameters	**Proband**	**Husband**	**Sister**
Sex / Age (years)	F / 39	M / 42	F / 47
DCIP	Positive	Positive	Positive
Rbc (x 10^{12})/ l	4.6	4.5	5.4
Hb (g/dl)	12.8	7.3	15.9
Hct (%)	40.0	27.0	49.0
MCV (fl)	87.0	60.0	90.0
MCH (pg)	27.9	16.2	29.3
MCHC (g/dl)	32.2	27.0	32.6
RDW (%)	10.5	18.4	11.1
Hb F (%)	0.4	3.2	0.6
Hb Phimai (%)	33.5	-	38.7
Hb E / A_2 (%)	3.5	14.7	3.6
α-genotype	αα / $\alpha^{CS}\alpha$	$-\alpha^{3.7}$ / $--^{SEA}$	αα / αα
β-genotype	β^A / β^{Phimai}	β^A / β^E	β^A / β^{Phimai}

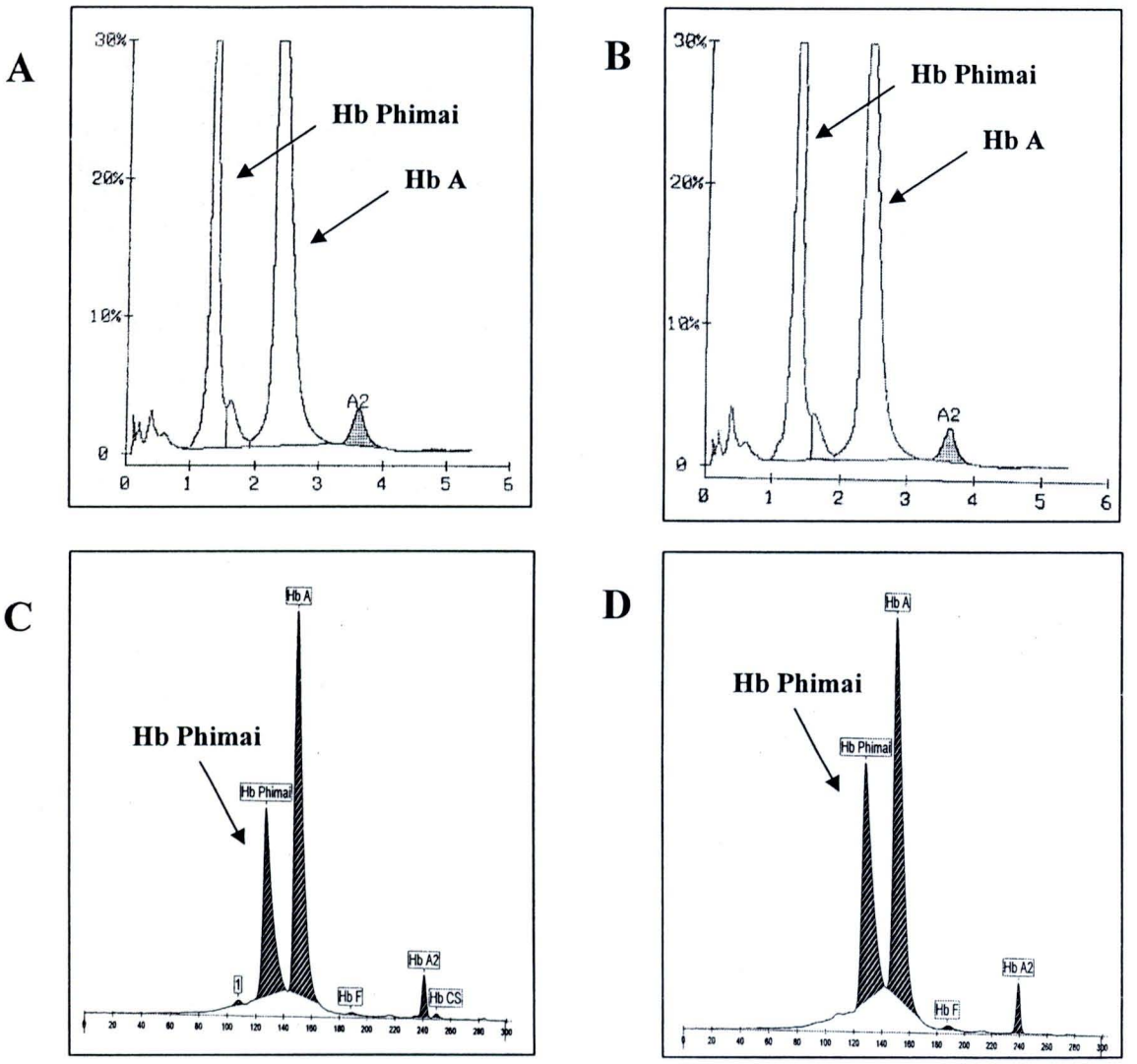


Figure 20 Hb analyses of the proband (A & C) and her sister (B & D) using automated HPLC (A & B) and capillary zone electrophoresis (C & D). The separating profiles of Hb Phimai and Hb A are indicated.

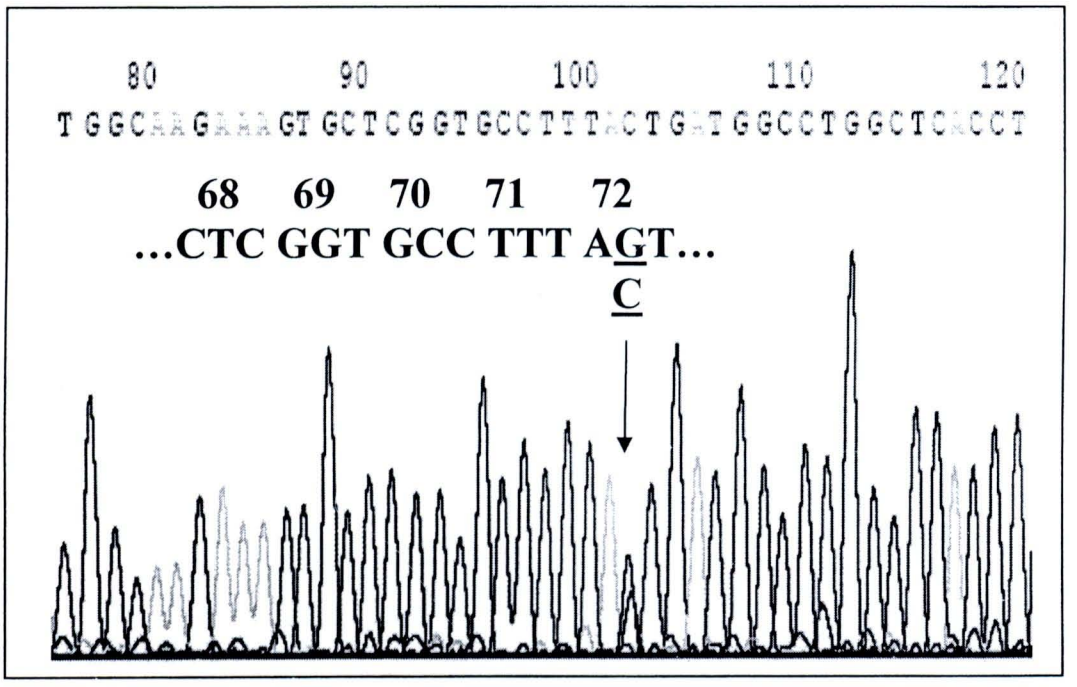


Figure 21 Direct DNA sequencing of β-globin gene demonstrating the heterozygosity for the G→C transversion at the codon 72 identified in the proband.

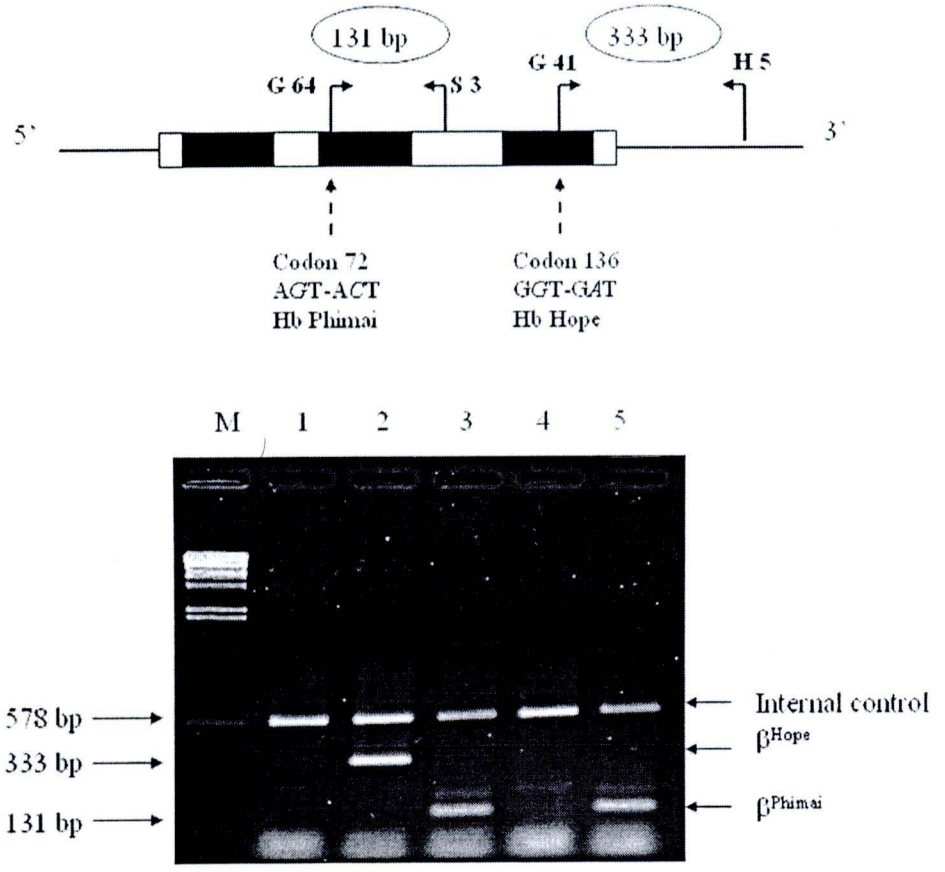


Figure 22 A multiplex allele specific PCR for identification of Hb Phimai and Hb Hope mutations. The locations and orientations of primers (G64 & S3) and (G41 & H5) that produce fragments of 131 bp and 333 bp specific for Hb Phimai and Hb Hope are indicated. The 578 bp band is an internal control fragment of the $^{G}\gamma$-globin gene promoter. Lane 1 = Normal control; lane 2 = Hb Hope carrier; lanes 3, 4 & 5 are the proband, her husband and her sister, respectively. M represents the λ /*Hind* III size markers. The Hb Phimai mutation was identified in the proband and her sister but not in her husband.