CHAPTER III

EXPERIMENTAL RESULTS

3.1 Detection of β-thalassemia mutations by Mutagenically Separated Polymerase Chain Reaction (MS-PCR)

As the MS-PCR was firstly employed for the detection of β-thalassemia mutation in the present study, it is essential to ensure that the results obtained from the MS-PCR were accurate and reliable; i.e. be able to determine correct mutation. For this reason, nucleotide sequencing of the region covering the mutations of interest was primarily performed. The results of the MS-PCR and nucleotide sequencing for each mutation were compared.

The results of MS-PCR and the corresponding nucleotide sequencing for the detection of β-thalassemia mutations including the 4 bp (-TTCT) deletion at codons 41/42 is shown in figure 3.1B, A-T substitution at codon 17 in figure 3.2B, adenine addition at codons 71/72 in figure 3.3B, A-G substitution at nucleotide –28 of β-globin promoter in figure 3.4B, G-A substitution at codon 26 in Figure 3.5B and C-T substitution at IVS2 nt 654 in figure 3.6B.

The types of all β-thalassemia mutations determined by the MS-PCR were completely consistent with those obtained from the direct nucleotide sequencing.

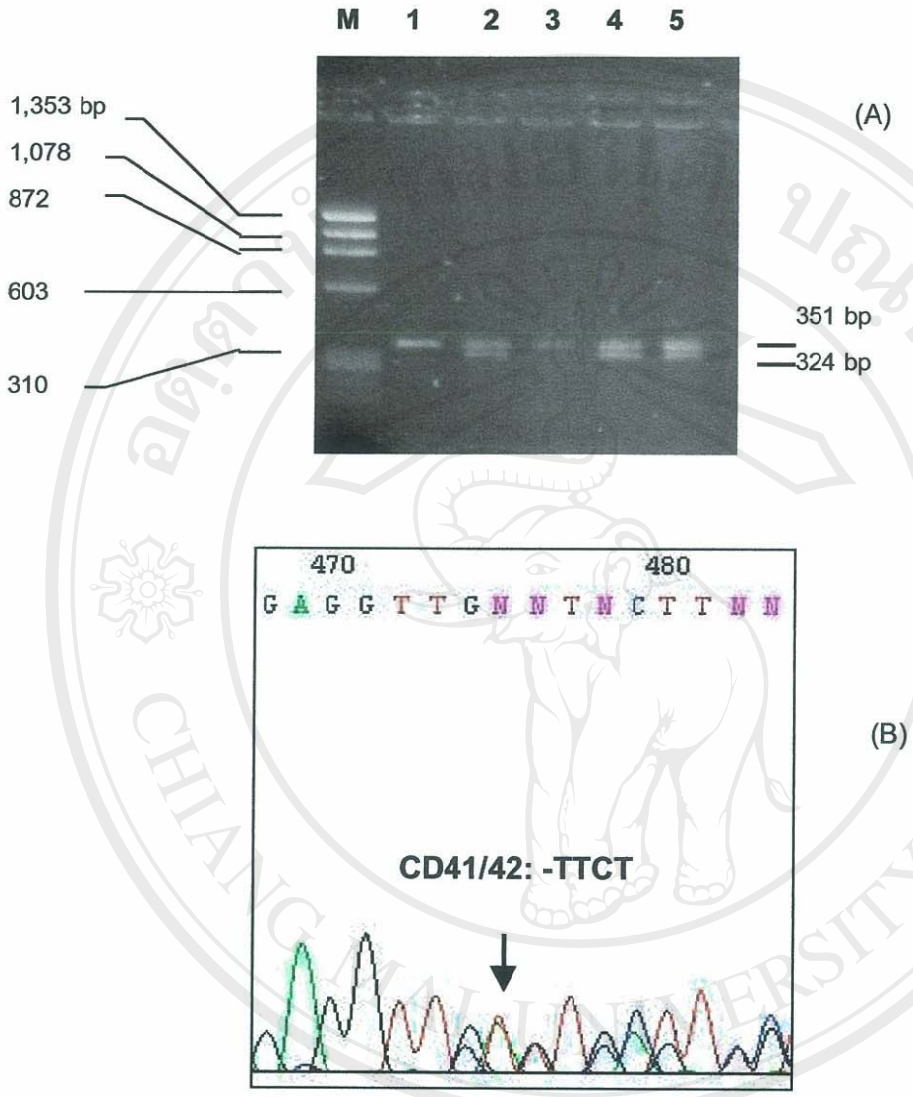

Figure 3.1 (A) The result of MS-PCR for the detection of the 4bp (-TTCT) deletion at codons 41/42 [codons 41/42 (-TTCT)]. Lane M indicates the ϕX 174 Hae III digested DNA size marker. Lanes 1 and 3 represent those negative for codons 41/42 (-TTCT). Lanes 2,4 and 5 were heterozygote codons 41/42 (-TTCT). The corresponding nucleotide sequences are covering this region of individual # 5 is shown in "B".

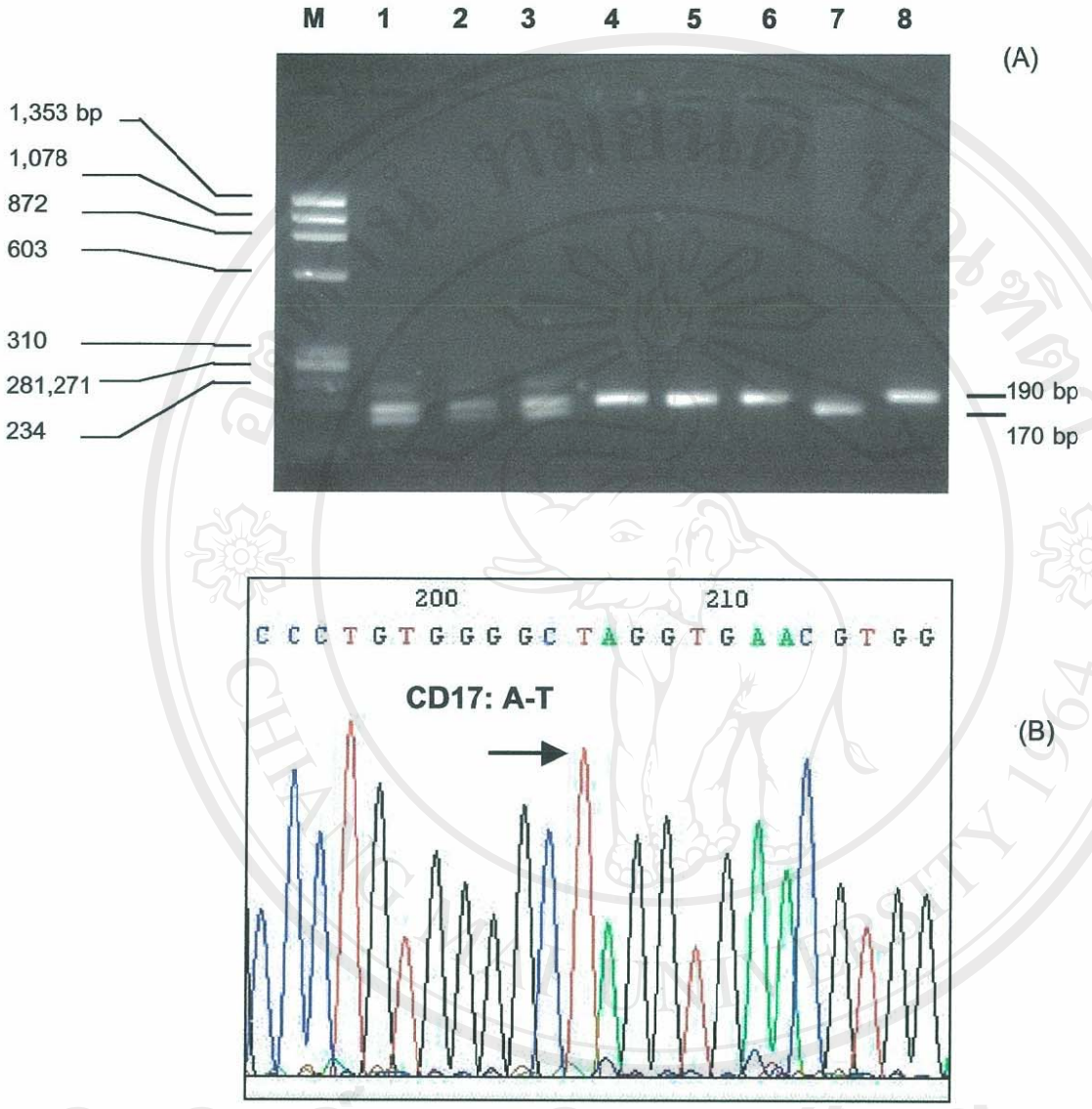

Figure 3.2 (A) The result of MS-PCR for the detection at Codon 17 [Codon 17 (A-T)]. Lane M indicates the ϕX 174 Hae III digested DNA size marker. Lanes 4,5,6 and 8 represents negative individuals, producing only 190-bp fragments. Lanes 1, 2 and 3 are heterozygote with 190-bp and 170-bp amplified fragments. Lane 7 is homozygous individual with only 170-bp amplified fragment was generated, of which the corresponding nucleotide sequencing is shown in "B".

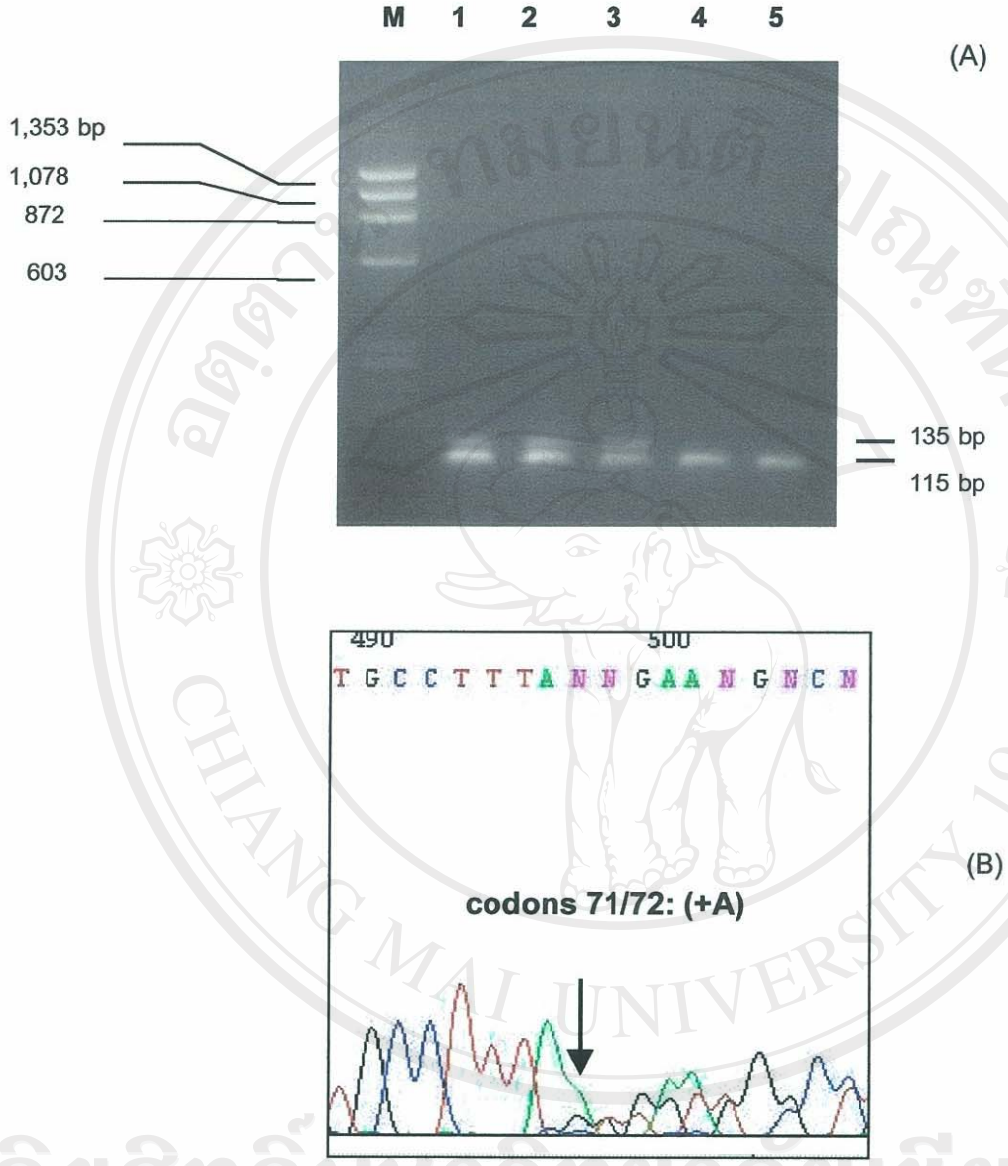

Figure 3.3 (A) The result of MS-PCR for the determination of the alanine addition at codons 71/72 [codons 71/72 (+A)]. Lane M indicates the ϕX 174 Hae III digested DNA size marker. Lanes 1, 2 and 3 represent heterozygous state with 135-bp and 115-bp amplified fragments. The 115-bp amplified fragment were seen in lanes 4 and 5 who are homozygous for this mutation. The corresponding nucleotide sequencing of this mutation of lane 3 are shown in "B".

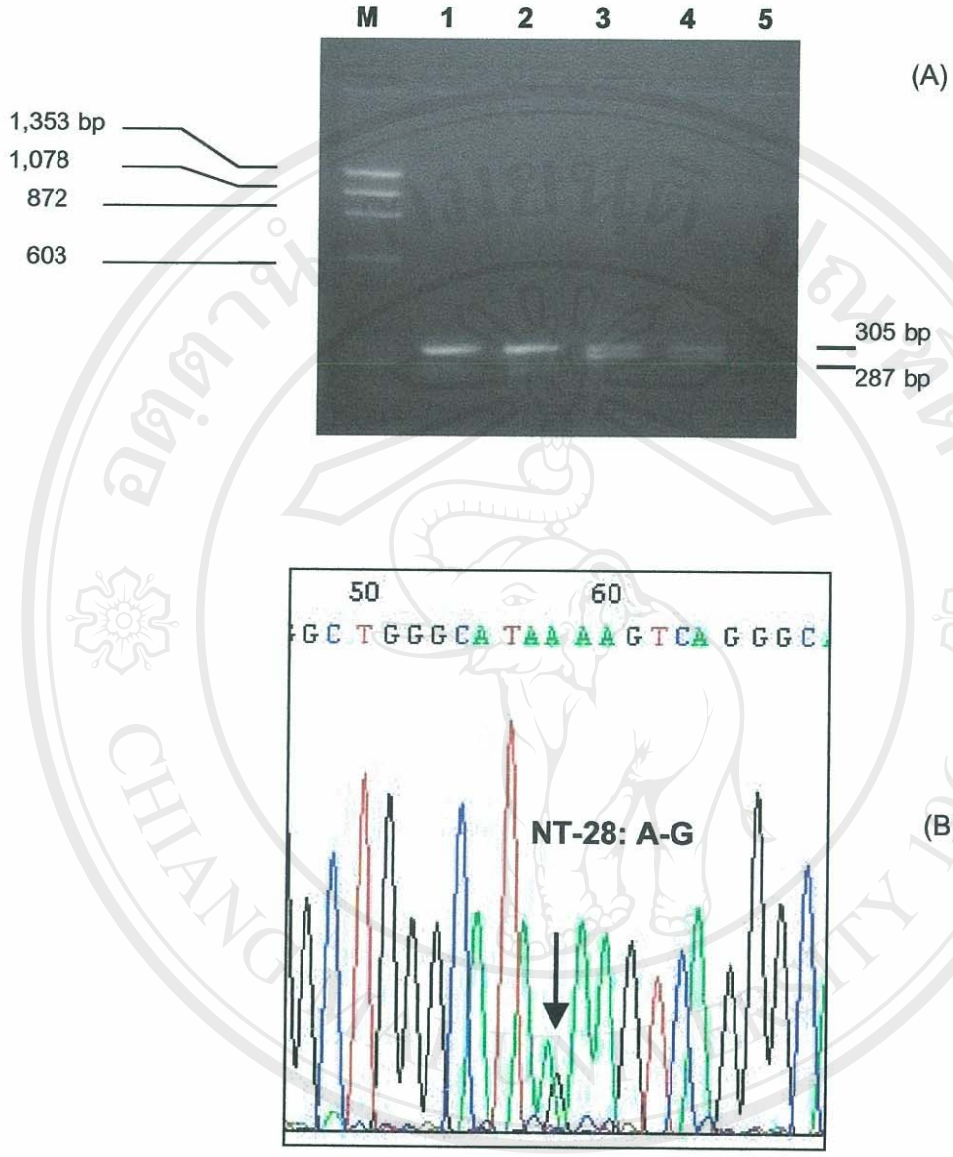

Figure 3.4 (A) The result of MS-PCR for for the determination of the A-G substitution at nucleotide-28 of the β-globin gene promoter [NT-28 (A-G)]. Lane M indicates the ϕX 174 Hae III digested DNA size marker. Lanes 1 and 2 represent negative individuals with 305-bp fragment. Lanes 3 and 4 represent heterozygous state with 305-bp and 287-bp fragments. The corresponding nucleotide sequencing of this mutation of lane 3 is shown in "B".

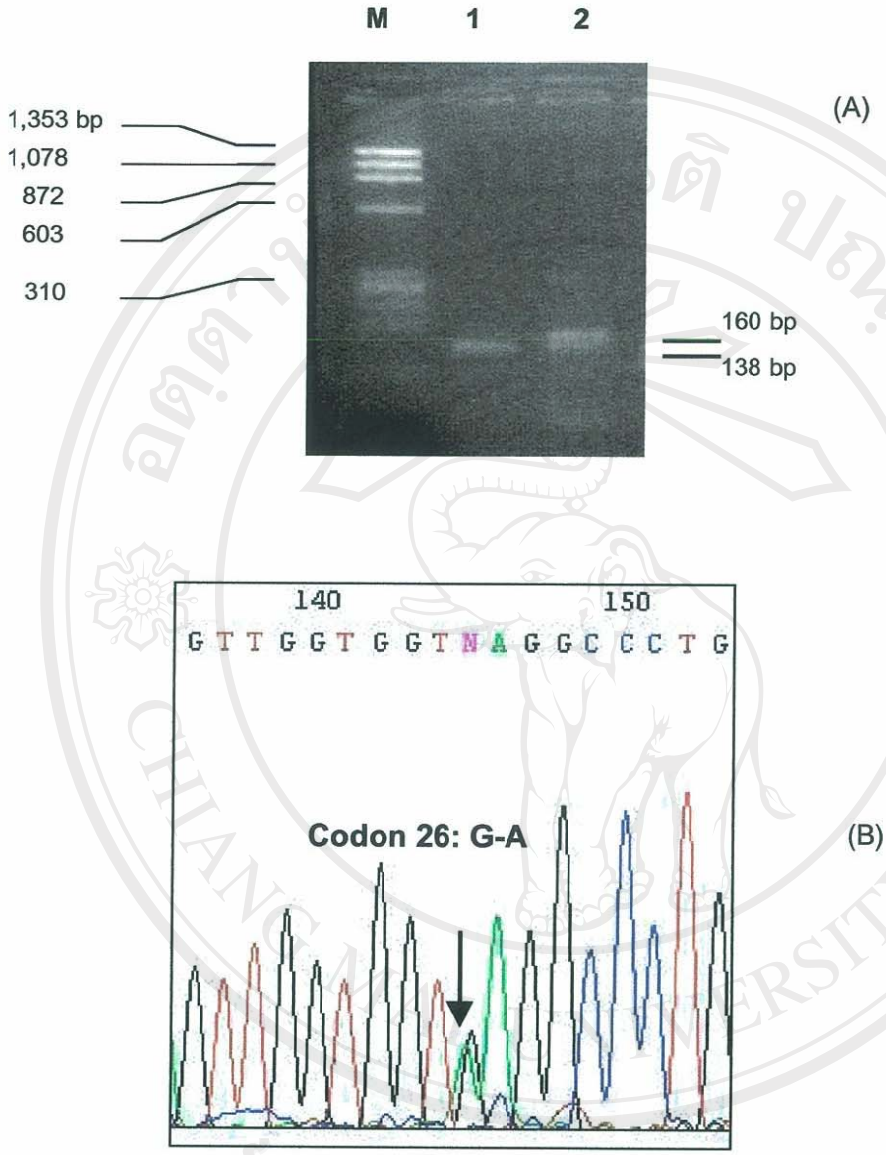

Figure 3.5 (A) The result of MS-PCR for the determination of the G-A substitution at codon 26 (HbE). Lane M indicates the ϕX 174 Hae III digested DNA size marker. Lane 1 were those negative for HbE allele. Lane 2 represent heterozygote state for this mutation. The corresponding nucleotide sequencing of this mutation individual #2 is shown in "B".

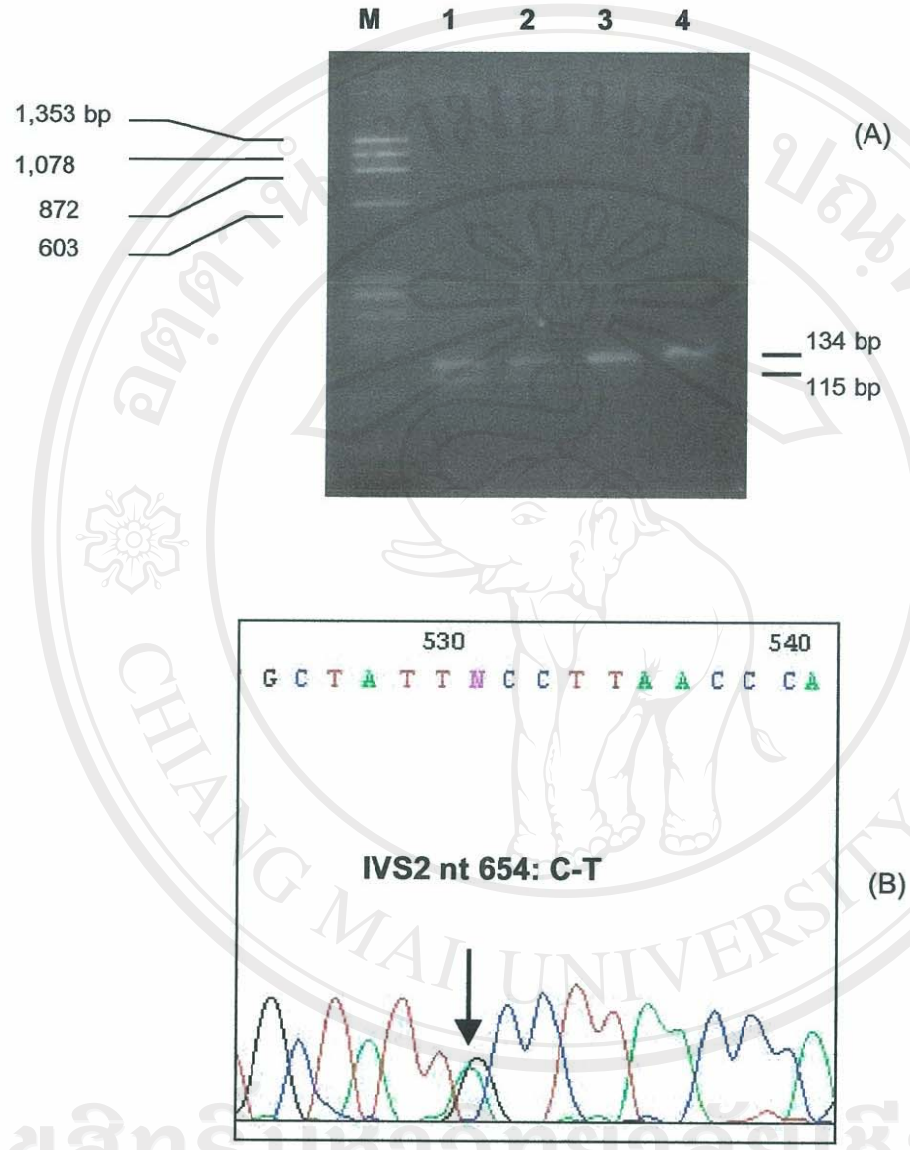

Figure 3.6 The result of MS-PCR for the determination of the C-T substitution at nucleotide 654 within IVS2 (IVS2 nt 654). Lane M indicates the ϕX 174 Hae III digested DNA size marker. Lanes 1 and 2 represent heterozygotes with 134-bp and 115-bp amplified fragments. Lanes 3 and 4 are individuals negative for this particular mutation. The corresponding nucleotide sequencing of this mutation in individual #1 is shown in "B".

3.2 Detection of uncharacterized β-thalassemia mutations

From this study, uncharacterized samples were determined by sequencing analysis. The β-mutations detected were NT-87; C-A (Figure 3.7), G-T at codon 43 (Figure 3.8), IVS1 nt 1; G-T (Figure 3.9).

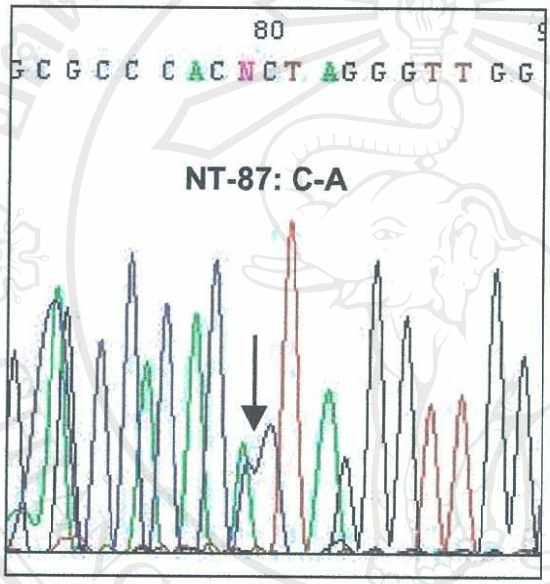

Figure 3.7 Exon1-2 sequencing analysis shows the C-A substitution at nucleotide –87 relative to cap site of β-globin gene.

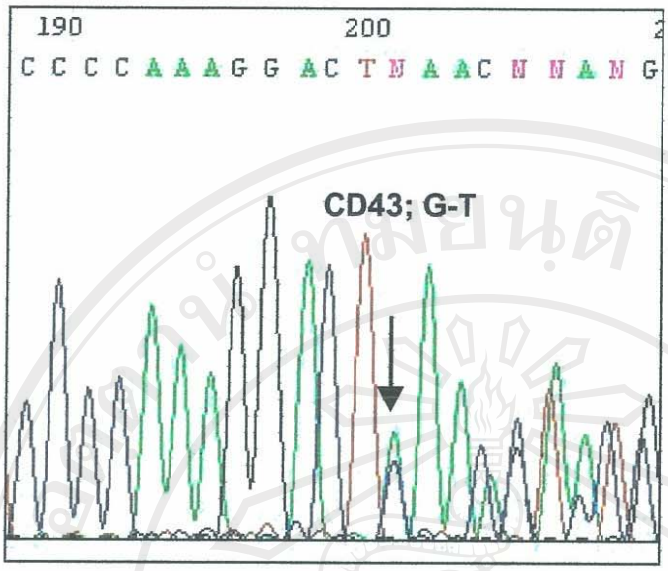

Figure 3.8 Exon1-2 sequencing analysis shows mutation at codon 43 (G-T); the patient was compound heterozygote for G-T substitution at codon 43 and 4bp deletion (-TTCT) at codons 41/42.

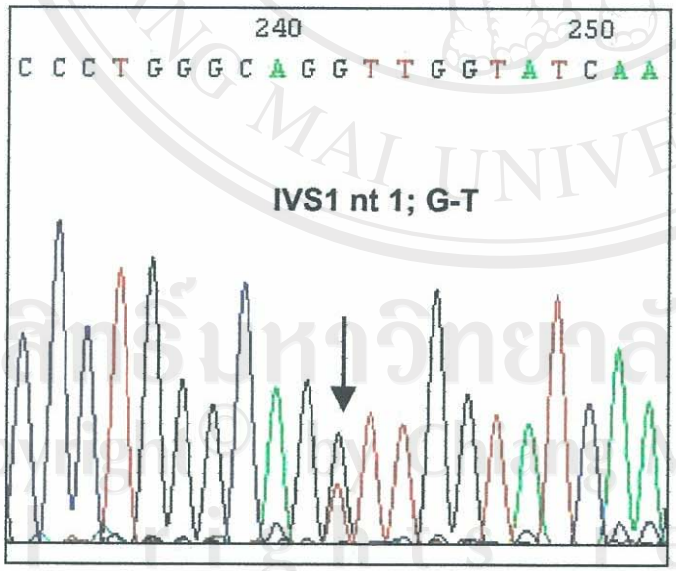

Figure 3.9 Exon1-2 sequencing analysis shows G-T substitution at IVS1 nt 1 .

3.3 Summary of the patients

Among 60 patients studied, the mean age at presentation in 32 patients of β-thalassemia major was 19 months and that of β-thalassemia intermedia was 42.9 months. In the β-thalassemia major group, requirement for blood transfusion ranged from 5 to 15 times per year. In the thalassemia intermedia group, majority of patients required infrequent transfusions; of which 14 out of 28 cases (50%) did not required any transfusions; while the rest 14 cases only required their first transfusions between 6 and 15 years of age. Splenectomy was performed in 4 individuals in β-thalassemia major group. Finally, patients in the β-thalassemia intermedia group were appropriately 12 years older than those in the β-thalassemia major group (10 years old). Table 3.1 describes the clinical classification of the 60 β-thalassemia patients, in which 32 cases were categorized as β-thalassemia major and 28 cases as β-thalassemia intermedia (Appexdicies-D and -E).

Table 3.1 Summary of the studied β-thalassemia patients

Clinical diagnosis	**β-thalassemia major** n (%)	**β-thalassemia intermedia** n (%)
1. Homozygous or compound heterozygous β-thalassemia	25 (83.3)	5 (16.6)
2. β-thalassemia/HbE	7 (23.3)	23 (76.6)
Total	32	28

3.4 Hematological parameters

Four hematogical parameters including hemoglobin levels (Hb), HbF levels, F cell (FC) levels as well as the reticulocyte count were analyzed. It was found that the

patients with β-thalassemia major had significantly lower Hb levels and FC levels than the β-thalassemia intermedia group ($p<0.005$). However, no differences were observed when the HbF levels and reticulocyte count were compared between the two groups (Table 3.2).

Table 3.2 Hematological data of the studied subjects

Hematological parameter	**β-thalassemia major** (mean ± SD)	**β-thalassemia intermedia** (mean ± SD)	***p*- value**[1]
Hemoglobin	6.1 ± 0.6	7.1 ± 0.6	$p = 0.002$
% HbF	25.7 ± 12.4	27.1 ± 6.7	NS[2]
Reticulocyte count	4.0 ± 1.4	3.8 ± 1.3	NS
F cells	50.0 ± 13.4	77.7 ± 12.0	$p = 0.003$
n	32	28	

[1]Student's unpaired *t* –test

[2]Not significant

3.5 Frequencies of the three genetic elements among studies subjects

The three genetic elements including the *Xmn*I-$^{G}\gamma$ polymorphism, the α-thalassemia 1 (SEA type) and the β-thalassemia mutations were studied. The results are shown in Table 3.3. The majority of patients did not possess the *Xmn*I-$^{G}\gamma$ polymorphism as well as the α-globin gene deletion of SEA type. The β-thalassemia mutations predominantly observed in the present study included the codons 41/42 (-TTCT) followed by HbE (codon 26, G-A) and codon 17 (A-T), respectively (Table 3.3).

Table 3.3 Frequencies of the genetic elements analyzed in the present study

Genetic elements	Allele	Alleles (%)
*Xmn*I $^{G}\gamma$ polymorphism	+	35(29.1)
	-	85(70.8)
SEA-type	+	5(4.1)
	-	115(95.8)
β-thalassemia mutations	$\beta^{41/42}$	51(42.5)
	β^{17}	21(17.5)
	$\beta^{27/28}$	1(0.83)
	β^{NT-87}	1(0.83)
	β^{43}	1(0.83)
	β^{NT-28}	8(6.6)
	β^{IVS1}	7(5.8)
	β^{E}	30(25)

Moreover, when the β-thalassemia mutations were grouped into β^{0}, β^{+} and β^{E}, the allele frequency of β^{0}-thalassemia mutations were mostly observed, as demonstrated in Table 3.4.

Table 3.4 Allele frequencies of the β-thalassemia mutations among the studied subjects grouped according to the degree of severity of the defects

β-mutations	Alleles (%)
β^{0}	82 (68.3)
β^{+}	8 (6.6)
β^{E}	30 (25)

3.6 The comparison of β-thalassemia mutations between β-thalassemia major and β-thalassemia intermedia

Eight β-thalassemia mutations were identified among 120 chromosomes shown in Table 3.3. Genetic analysis revealed 7 different interactions of β-thalassemia mutations in the β-thalassemia intermedia and 10 in the β-thalassemia major groups. Among these mutations, 97.4 % of the chromosomes had five common mutations including $\beta^{41/42}$, β^{17}, β^{NT-28}, β^{IVSI} and β^{E}, where the $\beta^{41/42}$ accounted predominantly (42.5%) followed by the β^{E} (25%).

The distribution of the interaction of β-thalassemia mutations (homozygotes or compound heterozygotes) between the β-thalassemia major and β-thalassemia intermedia groups is shown in Table 3.5 and, after summarised according to the severity, in Table 3.6. As shown in both tables, the homozygotes or compound heterozygotes of severe β-thalassemia mutations were mostly found in the β-thalassemia major individuals, whereas compound heterozygotes of β-thalassemia mutations and HbE allele clustered in the intermedia groups. The similar phenomena is still seen even when the β-thalassemia allele was compared between these two groups of β-thalassemia (Table 3.7) where β^{0}allele accounted significantly larger in β-thalassemia major than β-thalassemia intermedia. Additionally, HbE allele frequency was still significantly higher in β-thalassemia intermedia than β-thalassemia major, while β^{+}-allele frequency was identical. Finally, as no distinction was clearly observed in the distribution of the β-thalassemia mutations between the two β-thalassemia groups, it would suggest the existence of other modifying factor(s) that modulated the clinical phenotype of these studied β-thalassemia patients.

Table 3.5 Numbers of homozygous or compound heterozygous β-thalassemia individuals observed in β-thalassemia major and β-thalassemia intermedia patients

β-thalassemia mutations	β-thalassemia major (n)	β-thalassemia Intermedia (n)
1. $\beta^{41/42}/\beta^{41/42}$	7	0
2. $\beta^{41/42}/\beta^{17}$	8	2
3. $\beta^{41/42}/\beta^{27/28}$	1	0
4. $\beta^{41/42}/\beta^{NT-87}$	0	1
5. $\beta^{41/42}/\beta^{43}$	1	0
6. $\beta^{41/42}/\beta^{NT-28}$	1	1
7. β^{17}/β^{17}	2	0
8. β^{17}/β^{NT-28}	2	1
9. $\beta^{IVS1}/\beta^{IVS1}$	3	0
10. $\beta^{41/42}/\beta^{E}$	6	18
11. β^{17}/β^{E}	1	3
12. β^{NT-28}/β^{E}	0	2
Total	32	28

Table 3.6 Comparison of numbers and frequencies of β-thalassemia genotypes observed in β-thalassemia major and β-thalassemia intermedia patients

β-thalassemia mutations	β-thalassemia major n (%)	β-thalassemia Intermedia n (%)	*p*-value*
1. β^{0}/β^{0}	19 (59.3)	2 (7.1)	$p = 0.002$
2. β^{0}/β^{+}	3 (9.3)	3 (10.7)	$p = 0.002$
3. β^{++}/β^{++}	3 (9.3)	0 (0)	$p = 0.002$
4. β^{0}/β^{E}	7 (21.8)	21 (75)	$p = 0.009$
5. β^{+}/β^{E}	0 (0)	2 (7.1)	$p = 0.002$
Total	32	28	

*Z-score for comparison of the proportion

Table 3.7 Comparison of frequencies of β-thalassemia alleles between β-thalassemia major and β-thalassemia intermedia patients

	β-thalassemia Major n (%)	**β-thalassemia Intermedia** n (%)	***p*-value***
β^0	48(75)	28(50)	$p = 0.002$
β^+	9(14)	5(8.9)	$p = 0.002$
β^E	7(10.9)	23(41)	$p = 0.028$
Total	64	56	

*Z-score for comparison of the proportion

3.7 Comparison of α-thalassemia-1 (SEA type)

The α-globin gene status were examined in 60 patients. The α-thalassemia deletion in this study was Southeast Asian type; the most common α-globin gene deletion in Northern of Thailand. Five heterozygous α-thalassemia 1 (SEA) type (Table 3.8) were only observed in the β-thalassemia major group (15.6%); 2 cases in β^0/β^0, 1 case in β^0/β^+ and 2 cases in β^0/β^E as shown in table 3.11.

Table 3.8 Numbers and frequencies of α-thalassemia gene (SEA-type) in β-thalassemia major and β-thalassemia intermedia patients

Genotype	**β-thalassemia major** n (%)	**β-thalassemia Intermedia** n (%)
αα/αα	27 (84.3)	28 (100)
$--^{SEA}/\alpha\alpha$	5 (15.6)	0 (0)
Total	32	28

3.8 Comparison of *Xmn*I-$^{G}\gamma$ polymorphism

The presence of the *Xmn*I-$^{G}\gamma$ site was detected in 31 of 60 patients studied, 27 heterozygous and 4 homozygous for the presence of the *Xmn*I-$^{G}\gamma$ polymorphism. The homozygous state for the presence of the -158 (C-T) substitution in the $^{G}\gamma$ -promoter region (*Xmn*I, +/+) was more frequently observed in β-thalassemia major than in the β-thalassemia intermedia. The same phenomenon was seen for the homozygous state for the absence of the *Xmn*I-$^{G}\gamma$ site. In contrast, heterozygous state of the *Xmn*I-$^{G}\gamma$ polymorphism (*Xmn*I-$^{G}\gamma$, +/-) was commonly observed in the thalassemia intermedia group as compared to the β-thalassemia major cases. The differences in the frequencies of *Xmn*I-$^{G}\gamma$ genotypes between the two types of β-thalassemia were shown. Table 3.9 details the numerical presentation of this analysis.

The allele frequencies of the *Xmn*I-$^{G}\gamma$ polymorphism were also compared between the β-thalassemia major and the β-thalassemia intermedia groups. The Z-score statistics analyzed for the comparison of the proportion of the *Xmn*I-$^{G}\gamma$ allele between the two group was carried out. It was found that the presence of the *Xmn*I-$^{G}\gamma$ polymorphism was significantly confined in the β-thalassemia intermedia group, while the absence of the *Xmn*I-$^{G}\gamma$ polymorphism was significantly more commonly observed in the β-thalassemia major than the β-thalassemia intermedia groups (Table 3.10).

Table 3.9 Comparison of frequencies of *Xmn*I-$^{G}\gamma$ genotypes between β-thalassemia major and β-thalassemia intermedia

Clinical diagnosis	***Xmn*I-$^{G}\gamma$ polymorphism (%)**		
	-/- n (%)	**+/-** n (%)	**+/+** n (%)
β-thalassemia Major	20 (62.5)	9 (28.1)	3 (9.3)
β-thalassemia Intermedia	9 (32.1)	18 (64.2)	1 (3.6)
Total	29	27	4

-/- = homozygote for the absence of *Xmn*I-$^{G}\gamma$ site

+/- = heterozygote for the *Xmn*I-$^{G}\gamma$ site

+/+ = homozygote for the presence of *Xmn*I-$^{G}\gamma$ site

Table 3.10 Comparision of *Xmn*I-$^{G}\gamma$ allele frequencies between β-thalassemia major and β-thalassemia intermedia

***Xmn*I allele**	**β-thalassemia major** n (%)	**β-thalassemia intermedia** n (%)	***p*-value***
+	15(23.4)	20(35.7)	p =0.002
-	49(76.6)	36(64.3)	p =0.002
Total	64(100)	56(100)	

*Z-score for comparison of the proportion

3.9 Interaction of three genetic elements in β-thalassemia major and β-thalassemia intermedia

As it was clearly shown that an individual analyzed genetic element (β-thalassemia mutations, α-thalassemia gene (SEA type) or *Xmn*I-$^{G}\gamma$ polymorphism) seems not to serve as a major clinical alleviating determinant in the β-thalassemia patients, the combinations of these genetic factors were analyzed to see how different there would be between these two groups of patients. Four groups of the patients were observed according to their β-thalassemia genotypes, which comprised 21 in β^0/β^0, 6 in β^0/β^+, 3 in β^+/β^+, 28 in β^+/β^E and 2 in β^+/β^E groups. Both clinical phenotypes were observed for all of these β-thalassemia genotypes, except the β^+/β^+ that generated only β-thalassemia major. None of individuals in β-thalassemia intermedia group had α-thalassemia gene (SEA type) which was only seen in the five cases of β-thalassemia major. The presence of the *Xmn*I-$^{G}\gamma$ polymorphism (in heterozygous or homozygous form) was observed in both groups. Among 21 individuals carrying β^0/β^0 genotypes, most of them were clinically classified as the β-thalassemia major regardless of the presence of the α-thalassemia and the *Xmn*I-$^{G}\gamma$ site. The same figure was also demonstrated for those having the β^0/β^+ genotype, except equal proportion of the patients were categorised into both groups. Interestingly, those patients carrying β^E gene were commonly seen in the β-thalassemia intermedia group and most of them possessed the *Xmn*I-$^{G}\gamma$ polymorphism. The detail of the results are shown in Table 3.11. Table 3.12 describes the three genetic factors observed in β-thalassemia/HbE disease in which most of the patients clusters in the β-thalassemia intermedia.

Table 3.11 Interaction of β-thalassemia mutations, α-thalassemia gene (SEA type) and *Xmn*I-$^{G}\gamma$ in β-thalassemia major and β-thalassemia intermedia

β-thalassemia genotype	*Xmn*I genotype	β-thalassemia major		β-thalassemia intermedia	
		non-SEA[b]	SEA[c]	non-SEA	SEA
β^0/β^0	+/+	1	-	-	-
n=21	+/-	2	-	2	-
	-/-	14	2	-	-
β^0/β^+	+/+	-	-	-	-
n=6	+/-	1	-	2	-
	-/-	1	1	1	-
β^+/β^{+a}	+/+	2	-	-	-
n=3	+/-	-	-	-	-
	-/-	1	-	-	-
β^0/β^E	+/+	-	-	1	-
n=28	+/-	4	2	12	-
	-/-	1	-	8	-
β^+/β^E	+/+	-	-	-	-
n=2	+/-	-	-	2	-
	-/-	-	-	-	-

[a] severe form of β^+-thalassemia alleles

[b] absence of α-thalassemia 1 (SEA type) on both chromosome

[c] heterozygote for α-thalassemia 1 (SEA type)

Table 3.12 Three genetic factors observed in β-thalassemia / HbE patient

Clinical diagnosis	β-thalassemia mutations			SEA type		*Xmn*I genotype		
	$\beta^{41/42}/\beta^{E}$ n(%)	β^{17}/β^{E} n(%)	β^{NT-28}/β^{E} n(%)	Non-SEA n(%)	SEA n(%)	+/+ n(%)	+/- n(%)	-/- n(%)
β-thalassemia major	6 (25)	1 (25)	0	6 (20.6)	1 (100)	0	6 (30)	1 (11.1)
β-thalassemia intermedia	18 (75)	3 (75)	2 (100)	23 (79.3)	0	1 (100)	14 (70)	8 (88.8)
total	24	4	2	29	1	1	20	9