

## Molecular Analysis of Common Types of $\alpha$ -Thalassemia Associated with $\beta$ -Thalassemia in Northern Thailand

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We applied PCR strategies to detect the common types of  $\alpha$ -thalassemia determinants which were associated with  $\beta$ -thalassemia in northern Thailand. Two types of deletions in the  $\alpha$ -globin gene locus; the 18 kb deletion of Southeast Asian type ( $-\alpha^{SEA}$ ) and the 3.7 kb rightward deletion ( $-\alpha^{3.7}$ ), and the most prevalent non-deletion mutation, Hb Constant Spring ( $\alpha^{CS}$ , TAA to CAA at the codon 141) were investigated in 22 cases of  $\beta$ -thalassemia. Nine  $\beta$ -thalassemia patients were found to be associated with one or two of these defective  $\alpha$ -globin gene determinants and the mean hemoglobin concentration in these patients was  $6.3 \pm 1.1$  g/dl whereas it was  $5.6 \pm 0.8$  g/dl in 12  $\beta$ -thalassemia patients without  $\alpha$ -globin gene abnormalities; the difference is statistically insignificant ( $p = 0.08$ ). The level of anemia was severe in the  $\beta$ -thalassemia patients carrying a single  $\alpha$ -globin gene abnormality in the heterozygous compounds; whereas the  $\beta$ -thalassemias with  $\alpha$ -globin gene defects in both alleles showed less severe anemia. A patient carrying the  $\alpha^{CS}$  determinant in homozygous compound showed the highest hemoglobin level among these  $\beta$ -thalassemia patients.

**Key words:**  $\alpha$ -thalassemia,  $\beta$ -thalassemia, polymerase chain reaction, anemia

### Introduction

Clinical symptoms of  $\beta$ -thalassemia are primarily caused by the excessive accumulation of  $\alpha$ -globin molecules in erythroid cells giving rise to severe hemolysis and ineffective erythropoiesis in bone marrow<sup>1,2)</sup>. The association of defects in the  $\alpha$ -globin biosynthesis with  $\beta$ -thalassemia often results in the alleviation of the clinical symptoms of  $\beta$ -thalassemia patients<sup>1,2)</sup>.  $\alpha$ -Thalassemia which is characterized by the decreased level of the  $\alpha$ -

globin synthesis is due to deletion in the  $\alpha$ -globin gene locus or mutation causing the instability of  $\alpha$ -globin mRNA or  $\alpha$ -globin polypeptide chain<sup>3)</sup>. Of the two  $\alpha$ -globin genes on the chromosome 16, the protein product of the  $\alpha_2$  gene exceeds two to three-fold over that of the  $\alpha_1$  gene<sup>3)</sup>. Therefore, mutations affecting the  $\alpha_2$  gene rather than the  $\alpha_1$  gene result in severe phenotypic decrease in the  $\alpha$ -globin synthesis. Non-deletion mutation in the dominant  $\alpha_2$  gene is not always compensated by the expression of the remaining functional  $\alpha_1$  gene and in general the non-deletion  $\alpha^+$  thalassemia results in more severe reduction in the  $\alpha$ -globin synthesis than that caused by the deletion of the  $\alpha_2$  gene<sup>3)</sup>.

In Thailand,  $\alpha$ - and  $\beta$ -thalassemia, hemoglobin (Hb) E and Hb Constant Spring (CS) are prevalent<sup>3,6)</sup>. 30% to 40% of the population are affected with at least one of the abnormal globin genes<sup>6)</sup>. In addition, different combinations of the abnormal globin genes are expressed as more than 60 thalassemic syndromes<sup>5,6)</sup>. The aim of this work is to investigate the incidence of common  $\alpha$ -thalassemia determinants in  $\beta$ -thalassemia in northern Thailand<sup>7,8)</sup> and to learn whether different spectrums of molecular defects in  $\alpha$ -thalassemia associated with  $\beta$ -thalassemia are correlated to the clinical severity of the patients.

### Materials and Methods

#### *Patients and preparation of DNA samples*

$\beta$ -Thalassemia patients aged seven to seventeen-year-old were examined at Chiang Mai University Hospital. Hematologic data and treatments they received are summarized in Table 1. The diagnosis of  $\beta$ -thalassemia was made based on the clinical features including severe hemolysis, ineffective erythropoiesis, hepatosplenomegaly and characteristic bone deformity. The hemoglobin component analysis by electrophoresis was also used as a parameter for diagnosis. Peripheral blood samples were

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**Table 1.** Summary of the clinical features and molecular characterization of  $\alpha$ - and  $\beta$ -globin gene defects in 22  $\beta$ -thalassemia patients in northern Thailand.

Patient #	Age	4 bp-deletion in $\beta$ gene*	Assigned $\alpha$ -globin genotypes**	Notable Hb-type***	Hb A2 (%)	Hb (g/dl)	Hct (%)	Blood transfusion interval in months	Splenectomy
1	13					5	16	1	
2	14	+	$-\alpha^{\text{SEA}}/\alpha\alpha$	AF(F Barts)	2.4	5.5	16.5	1	+
3	12		$-\alpha^{\text{SEA}}/\alpha\alpha$	A/F	3	4.5	9.5	1	+
4	dead	+							
5	11	+	$-\alpha^{\text{SEA}}/\alpha^{\text{CS}}\alpha$	A/F	2.9	7	21	1	
6	17			F	2.6	6	20	1	+
7	10		$\alpha^{\text{CS}}\alpha/\alpha^{\text{CS}}\alpha$	F	1.6	8	24	2	
8	7	+		E/F	32.5	7	22	3	
9	13	+	$\alpha^{\text{CS}}\alpha/\alpha\alpha$	A/F	3	6	18.5	1	+
10	12	+				6.5	20	3	
11	11	+	$\alpha^{\text{CS}}\alpha/\alpha\alpha$	F	2.7	5.5	17	1	
12	7		$-\alpha^{\text{SEA}}/\alpha\alpha$	F	4.4	6.5	20	2	
13	16	+				5	16	1	+
14	10	+		A/F	2.5	5.5	18	1	+
15	14	+	$\alpha^{\text{CS}}\alpha/\alpha\alpha$	F	2.5	7	21	1	+
16	6	+		A/F	6.9	5	16	1	+
17	7			A/F	2.8	5	16	1	+
18	14	+		F	2.9	7	22	2	+
19	13	+		A/F		5	16	1	+
20	13	+				4.8	14	1	+
21	12	+		F		5	16	1	+
22	12		$-\alpha^{\text{SEA}}/\alpha\alpha$	F	4.8	7	21	1	+

\* the 4bp-deletion occurs in codon 41 through 42 of the  $\beta$ -globin gene.

\*\* refer to the text for the description of the each  $\alpha$ -globin gene determinant.

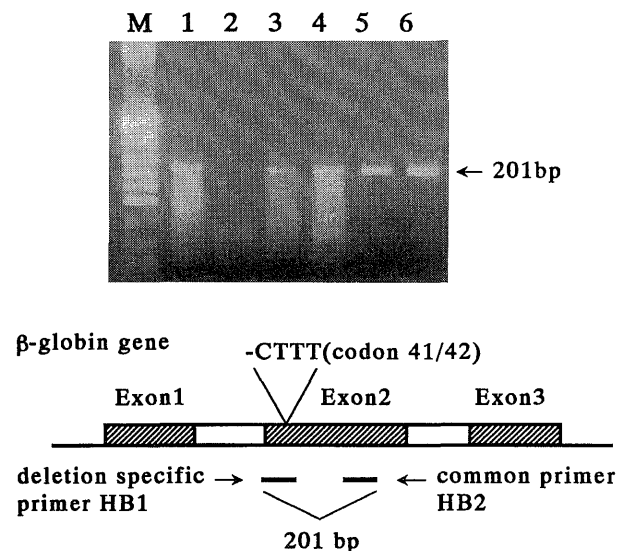
\*\*\* Abbreviations : A, F and E are hemoglobin A, F and E, respectively.

collected from 22 cases of  $\beta$ -thalassemia and DNA was isolated from the buffy coat of each blood sample as described previously<sup>9,10</sup> and stored at 4°C.

#### Oligonucleotide primers

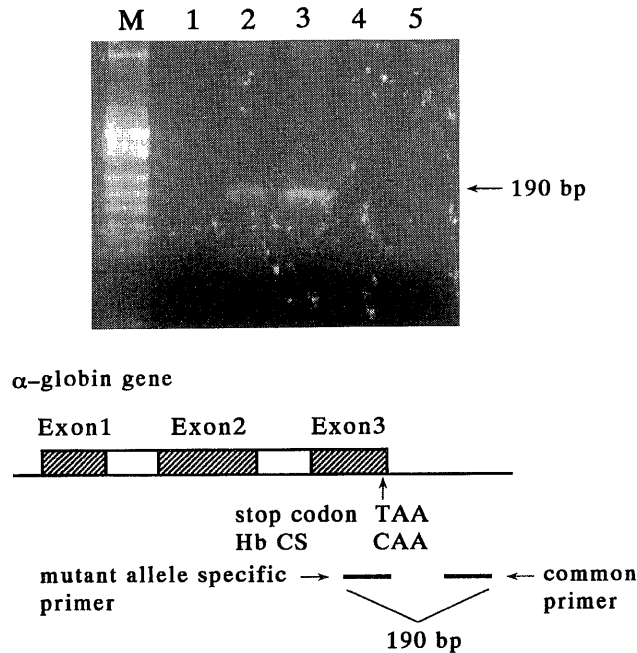
To detect the 4 bp-deletion in codon 41/42 of the  $\beta$ -globin gene which is frequently found in the northern Thailand population<sup>11</sup>, the sense primer HB 1 (5'-CCTTGGACCCAGAGGTTGAG-3') specific for the mutant allele and the anti-sense HB 2 (5'-CTGAAGTTCTCAGGATCCACGT-3') common for both normal and mutant alleles were synthesized. Using these primers, a 201 bp DNA fragment was specifically amplified in the DNA samples of  $\beta$ -thalassemia patients carrying the 4 bp-deletion (Figure 1). The primers, CS 1 and 2, for the selective amplification of the sequence between the intron 2 and the 3' side of the termination codon of the  $\alpha_2$ -globin gene were already described previously<sup>10</sup>. Allele specific primers for detection of the Hb Constant Spring gene were originally described by Fucharoen et al<sup>12</sup>, and an amplified 190 bp-DNA was recognized in the patients carrying the Hb CS determinant ( $\alpha^{\text{CS}}$ ) (Figure 2). To detect the 3.7 kb rightward deletion of the  $\alpha$ -globin locus ( $-\alpha^{37}$  determinant), the Z2 forward primer (5'-TCTCCCCTGTCCCTTCCCTA-3') which locates upstream to the  $\alpha_2$ -globin gene and the Z1 reverse primer (5'-GTTCTAGCCATGTGTGTTCCC-3') which is in the 3' side of the  $\alpha_1$ -globin gene were used. In a case affected with  $-\alpha^{37}$  determinant, the amplification of a DNA fragment of approximately 1.7 kb was expected by PCR using these primers<sup>13</sup>. Southeast Asian

type  $\alpha$ -thalassemia ( $-\alpha^{\text{SEA}}$  determinant) shows a 18 kb-deletion affecting the most of  $\alpha$ -type globin genes and two pseudo-genes<sup>3,14</sup>. A set of primers, 5' SEA and 3' SEA reported by Bowden et al<sup>14</sup>, were used to identify the 18 kb deletion and the expected size of 560 bp was amplified by PCR on the DNA samples of patients carrying the  $-\alpha^{\text{SEA}}$  determinant (Figure 3).

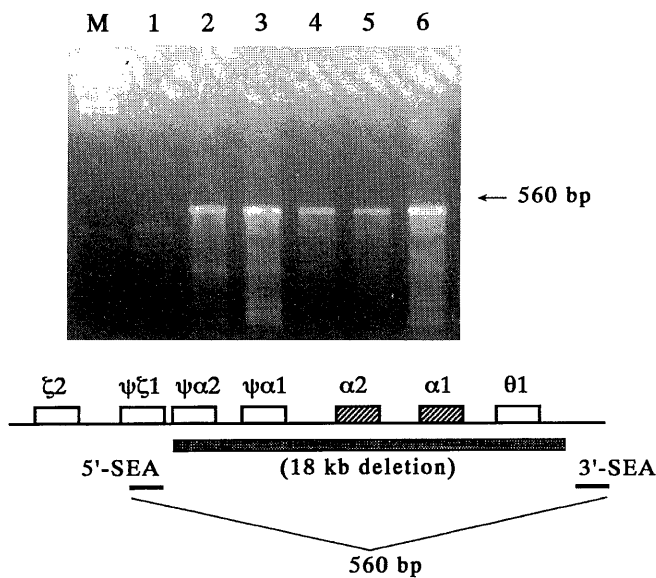


**Figure 1.** Detection of the 4bp-deletion in codon 41/42 of the  $\beta$ -globin gene by PCR.

Lane M : DNA size marker, 1 : Positive control, 2 : Negative control, 3 : Patient 2, 4 : Patient 4, 5 : Patient 11, 6 : Patient 15.



**Figure 2.** Identification of the  $\alpha^{CS}$  determinant (TAA to CAA at the codon 141) using the allele specific primers. Lane M: DNA size marker, 1: Negative control, 2: Positive control, 3: Patient 7, 4: Patient 1, 5: Patient 6. \*Primers were adopted following the method of Fucharoen S, Fucharoen G and Fukumaki Y<sup>12</sup>.



**Figure 3.** Detection of the  $-\alpha^{SEA}$  determinant (18kb-deletion) by PCR. Lane M: DNA size marker, 1: Negative control, 2: Patient 2, 3: Patient 3, 4: Patient 5, 5: Patient 12, 6: Patient 22. \*The PCR strategy was based on the method of Bowden DK, Vickers MA and Higgs DR<sup>10</sup>.

### Polymerase chain reaction

40  $\mu$ l of sample solution was mixed with 34  $\mu$ l of distilled water, 10  $\mu$ l of 10x PCR buffer (100 mM Tris-HCl pH 8.4, 500 mM KCl, 15 mM MgCl<sub>2</sub>, 1% Triton X100 and 0.1% gelatin), 2.5  $\mu$ l of each primer solution (0.1  $\mu$ g/ $\mu$ l), 10  $\mu$ l of 2 mM dNTP and 2.5 units of Tth DNA polymerase of *Thermus thermophilus* HB8 (Toyobo, Tokyo) in a 500  $\mu$ l microfuge tube. 80 to 100  $\mu$ l of mineral oil was overlaid to cover the surface of the reaction mixture. PCR was carried out using Minicycler TM (MJ Research, Watertown, MA).

The PCR amplification conditions for the each set of primers were similar to those reported by other groups<sup>10-14</sup>, however various modifications were tested to obtain the optimum results. A portion of the PCR product was electrophoresed on a gel consisting of 4% Nusieve agarose. The amplified DNA was detected by staining with Ethidium Bromide. In case of the Hb CS gene detection, a 276 bp DNA fragment amplified by using CS1 and CS2 primers was digested with restriction enzyme *Mse* I or *Tru* 9I (both obtained from Sigma Chemical Co., St. Louis, MO) and the digest was electrophoresed on an agarose gel<sup>10</sup>.

### Clinical Laboratory Data

Hematologic data were provided by Dr. Torpong Sanguanserm Sri at the Hematology Clinic of the Department of Pediatrics, Chiang Mai University, Chiang Mai, Thailand.

### Results and Discussion

#### Molecular characterization of $\beta$ -thalassemia

Molecular defects in the  $\beta$ -thalassemia patients in this study were not fully elucidated. Nevertheless, 15 of 22  $\beta$ -thalassemia patients were shown to have the 4bp-deletion at codon 41/42 in the  $\beta$ -globin gene by PCR using the primers HB1 and HB2 which were specific for the defective allele (Table 1).

#### Molecular types of $\alpha$ -thalassemia co-associated with $\beta$ -thalassemia

Deletion in the  $\alpha$ -globin gene locus is the most frequent cause of  $\alpha$ -thalassemia in Southeast Asia. In particular, the 18 kb SEA type deletion ( $-\alpha^{SEA}$ ), and the 3.7 kb rightward ( $-\alpha^{37}$ ) and the 4.2 kb leftward ( $-\alpha^{42}$ ) deletion are prevalent defects<sup>37</sup>. Hb CS comprises a significant cause of non-deletion  $\alpha$ -thalassemia<sup>37</sup>. In the present analysis,  $-\alpha^{SEA}$  and  $\alpha^{CS}$  determinants were recognized in 9

of the 22  $\beta$ -thalassemia patients (Table1). Among these  $\beta$ -thalassemia patients, seven cases showed defects in the  $\alpha$ -globin gene locus at either one of the two alleles ;  $\alpha$ -globin genotypes were  $-\alpha^{SEA}/\alpha$  in four and  $\alpha^{CS}\alpha/\alpha$  in three. Two cases of the  $\beta$ -thalassemia patients carried  $\alpha$ -globin gene abnormalities on both alleles :  $-\alpha^{SEA}/\alpha^{CS}\alpha$  and  $\alpha^{CS}\alpha/\alpha^{CS}\alpha$ . In this study, the 3.7 kb deletion was not detected presumably due to the difficulty in the allele specific PCR amplification of the  $\alpha$ -globin gene<sup>23</sup>.

*Clinical evaluation of the  $\beta$ -thalassemia patients with or without complication with  $\alpha$ -thalassemia*

The hemoglobin concentration in the peripheral blood of the  $\beta$ -thalassemia patients with or without complication of  $\alpha$ -thalassemia was compared (Figure 4). The mean hemoglobin concentration in the  $\beta$ -thalassemia patients being complicated with  $\alpha$ -thalassemia was  $6.3 \pm 1.1$  g/dl (n = 9) and in the patients without  $\alpha$ -globin abnormalities, it was  $5.6 \pm 0.8$  g/dl (n = 12). The difference is, however, statistically insignificant (p = 0.08). Among the  $\beta$ -thalassemia patients associated with  $\alpha$ -thalassemia, the hemoglobin concentration was lower in the patients carrying the defective  $\alpha$ -globin locus only in one chromosome. The mean value for hemoglobin in the  $\beta$ -thalassemia patients affected with the  $-\alpha^{SEA}/\alpha$  genotype was  $5.9 \pm 1.1$  g/dl (n = 4) and it was  $6.2 \pm 0.8$  g/dl (n = 3) in the  $\beta$ -thalassemias carrying the  $\alpha^{CS}\alpha/\alpha$  genotype. The level of hemoglobin concentration was slightly higher in a  $\beta$ -thalassemia having the  $-\alpha^{SEA}/\alpha^{CS}\alpha$  genotype (7.0 g/dl) and in a patient with homozygous  $\alpha^{CS}\alpha/\alpha^{CS}\alpha$  determinants (8.0 g/dl) (Figure 5).

In case of the SEA type deletion, two functional  $\alpha$ -genes on another chromosome compensate the  $\alpha$ -globin synthesis so that  $\alpha$ -globin can be produced to 90% of normal level. Mutations in the terminal codon of the  $\alpha_2$ -globin gene often result in severe reduction of the  $\alpha$ -globin synthesis since the  $\alpha^{CS}$  mRNA is highly unstable and the compensation by the remaining functional  $\alpha_1$ -globin gene is not effective<sup>16</sup>. It is expected that the  $\alpha^{CS}$  gene causes a more decreased level of the  $\alpha$ -globin synthesis than that observed in the carriers of deleted  $\alpha$ -globin genes. The severity in reduction of the  $\alpha$ -globin synthesis caused by mutations in the  $\alpha$ -globin genes in  $\beta$ -thalassemia patients in turn appears to alleviate the hemolysis and the suppression of erythropoiesis in bone marrow. This is supported by the report that the excessive accumulation of the denatured  $\alpha$ -globin molecules causes the apoptotic cell death of erythroid precursor cells in bone marrow of  $\beta$ -thalassemia<sup>16</sup>. The hemoglobin concentration in the  $\beta$ -thalassemia patients in this study appear to correlate reciprocally to the degree of reduction in the  $\alpha$ -globin synthesis expected by the nature of defects in the  $\alpha$ -globin genes. It is also noteworthy that splenectomy was

performed in 9 out of 12  $\beta$ -thalassemia patients without  $\alpha$ -globin gene defects meanwhile 5 out of 9  $\beta$ -thalassemia patients complicated with  $\alpha$ -thalassemia were treated with splenectomy.

The present study indicates that the different spectrums of molecular defects in the  $\alpha$ -globin genes are correlated to the severity and prognostic evaluation of  $\beta$ -thalassemia patients when they are complicated with  $\alpha$ -thalassemia. Accumulation of the knowledge of the molecular defects on both  $\alpha$ - and  $\beta$ -thalassemia and careful follow-up studies of the patients will enable us to understand the pathogenesis of the diseases and to establish more effective therapeutic means.

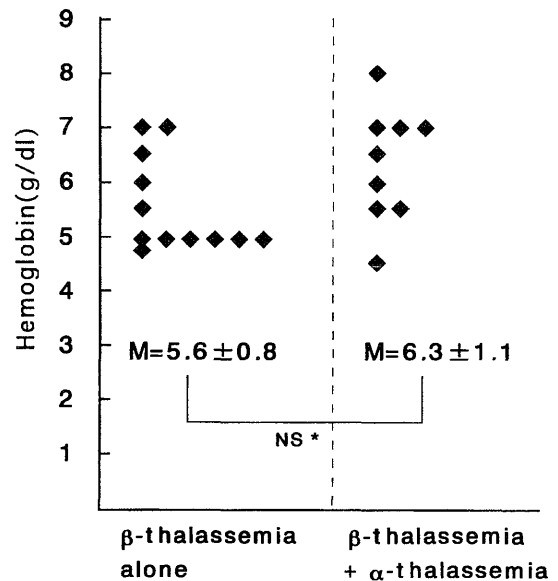


Figure 4. The hemoglobin concentration of the  $\beta$ -thalassemia patients with or without complication of  $\alpha$ -thalassemia. \*P = 0.08

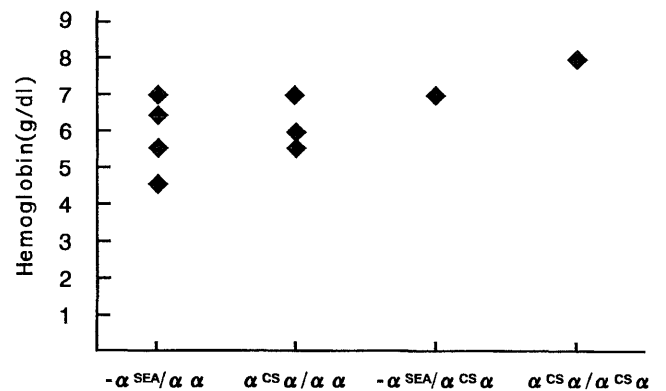


Figure 5. The molecular types of  $\alpha$ -thalassemia and the hemoglobin concentration in the  $\beta$ -thalassemia patients.

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