

Rare β -Globin Gene Mutations including a de novo Mutation of Hemoglobin Hammersmith in Southern Thailand

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Abstract:

Objective: The aim of this study was to characterize unknown β -globin gene mutations in individuals who attended Songklanagarind Hospital for thalassemia screening and genetic counseling.

Material and Methods: β -thalassemia mutations in individuals with hemoglobin (Hb) A2 levels >3.5% originating from various provinces in southern Thailand were characterized by reverse dot blot hybridization (RDB) and multiplex gap-polymerase chain reaction using a panel of 30 allele-specific probes for point mutations and 6 sets of specific primers for large deletions. Mutations which could not be identified by these two methods were further analyzed by direct deoxy-ribonucleic acid (DNA) sequencing.

Results: Nineteen subjects found to have uncharacterized β -globin gene mutations were analyzed by direct DNA sequencing. Nine different rare mutations were identified, four of which have not been to date described in Thailand: -30 (T>C), codon 5 (-CT), Hb Monroe (codon 30, G>C) and Hb Hammersmith (codon 42, T>C). An Hb Hammersmith mutation detected in one subject appeared to be a spontaneous mutation, unrelated to family history. The other five mutations have been reported previously within Thailand, but here they were identified in the southern part of Thailand for the first time: -31 (A>G), codon 15 (-T), codon 35 (C>A), codon 95 (+A) and Hb Dhonburi (codon 126, T>G). The presence of the mutations was confirmed by RDB.

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Conclusion: In addition to the already reported β -globin gene mutations, 9 other different types of mutations were identified. This information should be useful for planning genetic counseling and prenatal diagnosis programs for prevention and control of thalassemia diseases.

Keywords: hemoglobinopathy, rare mutation, southern Thailand, β -globin gene

Introduction

β -Thalassemia (β -thal) is a common inherited blood disorder in Thailand.^{1,2} It was estimated that with a population of 63 million in the millennium year 2007, there were about 5,749 infants born each year with severe thalassemia, including 1,017 with homozygous α^0 -thal, 779 with homozygous β -thal and 3,953 with hemoglobin (Hb) E/ β -thal disease.³ Previous studies have shown that the molecular basis of β -thal in Thailand is heterogeneous and there are at least 40 known mutations on the β -globin gene which cause β -thal disease. The mutations have different frequencies in different parts of the country with various ethnicities. In the south of Thailand, about 30 mutations have been detected. There are seven known common mutations including cod 41/42 (-CTTT) (HBB: c.126_129delCTTT), IVS I#5 (G>C) (HBB: c.92+5G>C), cod 19 (A>G) (HBB: c.59A>G), cod 17 (A>T) (HBB: c.52A>T), IVS I#1 (G>T) (HBB: c.92+1G>T), -28 (A>G) (HBB: c.-78A>G) and 3.5 kilobase (kb) deletion, which account for about 90.0% of the cases.⁴ However, because of the diversity of the mutations, unidentified-to-date mutations are occasionally found in routine analysis, identified as rare or unknown mutations. In order to provide an effective thalassemia control program, identification of each unknown mutation should be carried out. Therefore, the objective of this study was to characterize the unknown β -globin mutations found during the routine test.

Material and Methods

Ethical approval for the study was obtained from the Human Research Ethics Committee, Faculty of Medicine, Prince of Songkla University (REC: 57-331-05-8). The

study subjects were 19 unrelated individuals who had been referred to the Thalassemia Unit of Songklanagarind Hospital, the largest tertiary care center and referral hospital in southern Thailand, for molecular screening of β -globin mutations. Blood samples were taken from each subject for hematology and hemoglobin analysis to assess their thalassemia status. Hematological data were obtained using an automated blood cell counter (Sysmex XN 3000; Sysmex, Japan). Hemoglobin analysis was performed by either high-performance liquid chromatography (HPLC) [Variant™; Bio-Rad Laboratories, Hercules, California, the United States of America (USA)] or capillary electrophoresis (CE) (Capillarys 2; Sebia, Lisses, France).

Molecular analysis of β -globin mutations by reverse dot blot hybridization and multiplex gap-polymerase chain reaction

Genomic deoxyribonucleic acid (DNA) was extracted from the blood samples with a genomic DNA Mini Kit (Geneaid, Taiwan). The yield and purity of the DNA were determined by gel electrophoresis and spectrophotometer. The DNA samples were screened for common or prevalent β -globin mutations by reverse dot blot hybridization (RDB) and multiplex gap-polymerase chain reaction (PCR) using a panel of 30 allele-specific probes for point mutations and 6 sets of specific primers for large deletions.^{5,6} A total of 19 samples which were found to have unknown β -globin mutations were subjected to direct DNA sequencing using an automated DNA sequencing system (ABI 3500; ABI, USA). Two regions of the β -globin gene of the samples were analyzed by two sets of primers; R1 (5'-TCCCCAGTTAACCTCCTATT-3') and R2 (5'-

TCATTCGTCTGTTTCCCATTCTAAAC-3') designed for initial site, exon I, intron I and exon II; and R3 (5'-GTGTACACATATTGACCAAATC-3') and R4: (5'-CAGATTCGGGTCCTGTG-3') designed for intron II, exon III and polyA site. The presence of identified mutations was confirmed by RDB.

Results

Following DNA sequence analysis of the PCR-amplified fragments, 9 different mutations were identified in the 19 unrelated subjects. Three of these mutations were β^+ -thal involving two promoter mutations: 2 subjects with -30 (T>C) (HBB: c.-80T>C) and 2 subjects with -31 (A>G) (HBB: c.-81A>G); and one missense mutation: 3 subjects with Hb Dhonburi [cod 126, (T>G), HBB: c.380T>G]; while the other five mutations were β^0 -thal: three frameshift mutations, 1 subject with cod 5 (-CT) (HBB: c.17_18delCT), 5 subjects with cod 15 (-T) (HBB: c.46delT), and 1 subject with cod 95 (+A)/Hb E (HBB: c.287_288insA; 79G>A); one nonsense mutation: 2 subjects with cod 35 (C>A) (HBB: c.108C>A); and one missense mutation: 2 subjects with Hb Monroe [cod 30 (G>C), HBB: c.92G>C]. Also, one mutation causing an unstable Hb variant was found in 1 subject with Hb Hammersmith [cod 42 (T>C), HBB:c.128T>C] (Table 1). Eighteen subjects were heterozygous for β -thal alleles. One subject was compound heterozygous for β -thal and HbE (cod 95 (+A)/Hb E).

Among the 18 subjects with heterozygous β -thal, 8 subjects including 3 subjects with cod 15 (-T), 3 subjects with Hb Dhonburi, 1 subject with -30 (T>C) and 1 subject with -31 (A>G) showed no clinical phenotype, whereas 8 subjects who were pregnant at the time their blood was obtained had mild anemia, including 2 subjects with cod 15 (-T), 1 subject with -30 (T>C), 1 subject with -31 (A>G), 1 subject with cod 5 (-CT), 1 subject with Hb Monroe, and 2 subjects with cod 35 (C>A). These could have been complications relating to their pregnancies. The

other 2 non-pregnant subjects had moderate to severe anemia, including 1 subject with Hb Monroe and 1 subject with Hb Hammersmith. A subject with compound heterozygous β -thal (cod 95 (+A)/Hb E) had moderate anemia as shown in Table 1.

Sequencing chromatograms of the family with Hb Hammersmith are shown in Figure 1. The studies indicated that only the proband carried the mutation [cod 42 (T>C)]. The possibility of non-paternity in this family was tested by restriction fragment length polymorphism typing of variable-number tandem repeat loci D1S80.⁷ The result of the paternity test are shown in Figure 2.

Discussion

In this study, we report nine different β -globin mutations, including -30 (T>C), -31 (A>G), cod 126 (T>G), cod 5 (-CT), cod 15 (-T), cod 95 (+A), cod 35 (C>A), Hb Monroe [cod 30 (G>C)], and Hb Hammersmith [cod 42 (T>C)] found in thalassemia patients in southern Thailand. Among these, four mutations, -30 (T>C), cod 5 (-CT), Hb Monroe, and Hb Hammersmith, were firstly identified in Thailand

The -30 (T>C) mutation is located in the TATA box region leading to reduced transcription factor binding efficiency, ultimately resulting in decreased β -globin gene transcription and manifesting as β^+ -thal. This mutation was described originally in a Chinese male at prenatal diagnosis.⁸ There have been no other reports of this mutation until our two subjects. It is noteworthy that they had borderline mean corpuscular volume (MCV) values, 80.2 and 80.8 fL, suggesting that other -30 (C>T) carriers might have been misdiagnosed at thalassemia screenings using red blood cell indices with MCV cut-off values <80 fL.⁹ However, the mean corpuscular hemoglobin values were lower than normal (<27 pg) (Table 1), this should be helpful in the screening for this mutation.

Table 1 Hematological parameters of the 19 unrelated study subjects and their rare β-globin gene mutations. Values are presented as mean±S.D. or as raw data as appropriate

Mutation	HGVS nomenclature	n	Hb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	RDW (%)	Hb A ₂ (%)	Hb F (%)	Hb E (%)
codon 15 (-T)/N	HBB:c.46delT; [=]	5	12.0±1.4	35.6±4.7	63.9±6.8	20.6±2.0	17.8±1.8	5.7±0.6	0.0	0.0
codon 126 (T>G) ¹ /N	HBB:c.380T>G; [=]	3	13.8,13.2,	35.8, 40.3,	78.0, 60.7,	22.3,19.9,	16.8, 17.1,	3.8, 5.1,	0.0	0.0
-30 (T>C)/N	HBB:c.-80T>C; [=]	2	14.1	44.3	77.4	24.7	12.8	4.5		
-31 (A>G)/N	HBB:c.-81A>G; [=]	2	11.7, 14.6	36.1, 46.9	80.2, 80.8	26.0, 25.2	15.4, 13.1	4.1, 4.8	0.0	0.0
codon 30 (G>C) ² /N	HBB:c.92G>C; [=]	2	9.8, 13.1	29.8, 39.3	74.7, 73.0	24.6, NA	14.6, NA	5.9, NA	3.6, NA	0.0
codon 35 (C>A)/N	HBB:c.108C>A; [=]	2	9.2, 6.5	29.0, 21.1	68.2, 68.0	21.6, 21.0	16.0, 18.9	5.1, 5.6	0, 3.0	0.0
codon 5 (-CT)/N	HBB:c.17_18delCT; [=]	1	9.2, 11.3	28.0, 35.5	63.3, 62.5	20.8, 19.9	16.0, 18.1	5.6, 4.8	0, 5.6	0.0
codon 95 (+A)/HbE	HBB:c.287_288insA; 79G>A	1	8.0	25.2	65.6	20.8	21	5.7	0	0.0
codon 42 (T>C) ³ /N	HBB:c.128T>C; [=]	1	7.4	23.1	61.9	19.8	30.4	3.6	34.1	25.1
			4.6	16.4	100.6	28.2	27	4.1	0.0	0.0

¹Hb Dhonburi, ²Hb Monroe, ³Hb Hammersmith, n=number, N=normal, NA=not available, HGVS=human genome variation society, HBB=beta-globin gene, Hb=hemoglobin, Hct=hematocrit, MCV=mean corpuscular volume, MCH=mean corpuscular hemoglobin, RDW=red blood cell distribution width, T=thymine, C=cytosine, N=normal, G=guanine, A=adenine, g/dL=grams per deciliter, fL=femtoliter, pg=picogram (Note: A2, F and E refer to different types of hemoglobin)

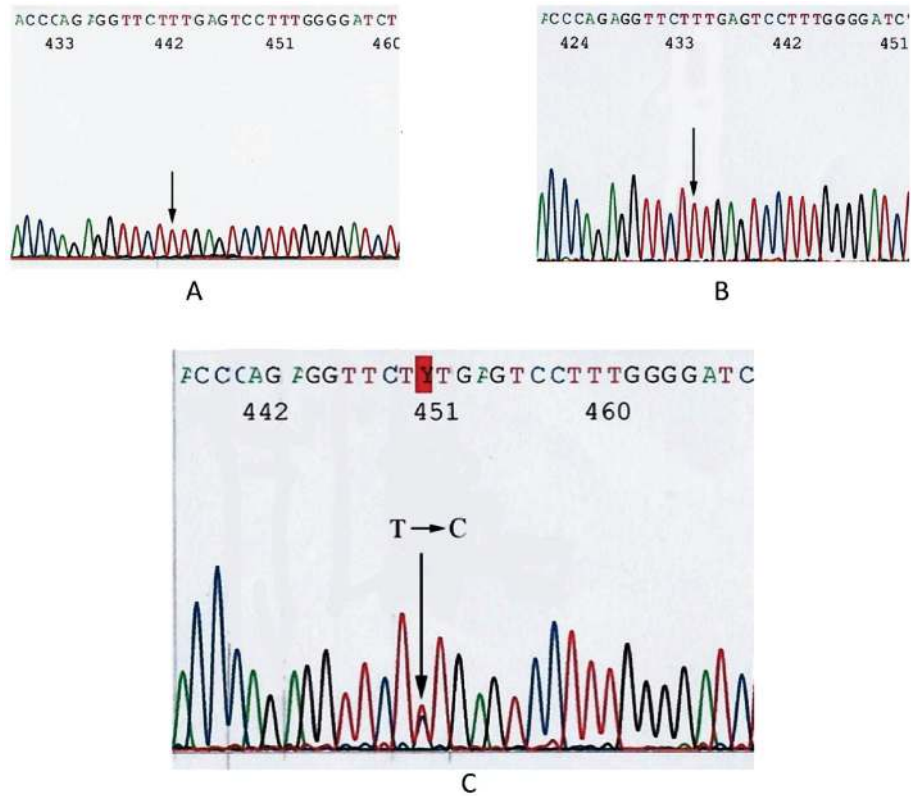


Figure 1 Sequencing chromatograms of the family with Hb Hammersmith: A, father, B, mother, and C, 6-year-old female patient. Arrows indicate the location of the mutation site.

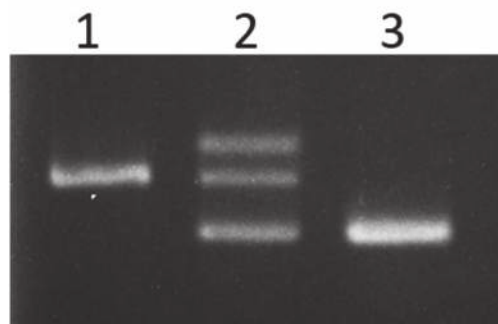


Figure 2 Agarose gel electrophoresis of VNTR loci D1S80 in the family of the patient with de novo Hb Hammersmith: Lane 1 shows the mother’s allele, Lane 3 shows the father’s allele, and Lane 2 shows the patient’s allele, as she received alleles from her mother and father.

The cod 5 (-CT) mutation, a frameshift mutation of Mediterranean origin, results in a termination of translation at cod 21, causing β^0 -thal. This mutation was observed originally in a Greek patient with transfusion-dependent anemia and has also been reported in Western and South Asian populations, specifically in Asian Indian, Iranian and Arab populations.¹⁰⁻¹³ Recently, it was also documented in Yunnan in Southwest China.¹⁴

Hb Monroe is an Hb variant resulting from a nucleotide substitution from G to C at a splice site area of cod 30. This mutation leads to the inhibition of normal splicing, causing β^0 -thal.¹⁵ The first description of this Hb variant was in an African-American girl in the USA, which she co-inherited with the -29 (A>G) mutation.¹⁶ The mutation has also been reported in Tunisian, Lebanese, United Arab Emirates, Asian Indian and Iranian populations.¹¹⁻¹³ The clinical phenotype of the heterozygote is mild anemia, although in combination with other mutations it can present as transfusion-dependent β -thal major.^{16,17} In this study, we also found one of two cases of the Hb Monroe heterozygote presented with severe anemia (Table 1), possibly involving other causes of anemia such as iron deficiency, which is highly prevalent in Thailand.¹⁸

Another unstable β -chain variant found by this study for the first time in Thailand was Hb Hammersmith. This Hb variant is caused by a T to C substitution in the second base of cod 42 resulting in an amino acid change from phenylalanine to serine. This replacement reduces the strength of the bond between heme and globin, causing denaturation and precipitation of the hemoglobin molecule on the RBC membrane which results in RBC destruction. Since this Hb is co-separated with Hb A, it is undetectable on routine Hb analysis by CE. However, increasing Hb A₂ levels indicates a β -thal carrier and further DNA analysis is needed to detect this mutation. Hb Hammersmith was first described in two unrelated children with hemolytic anemia in Britain.¹⁹ Many studies

have shown that this mutation usually presents as an autosomal dominant resulting in severe hemolytic anemia, which was also found in the patient of this study. The blood smear of this patient showed hypochromic microcytic RBCs with ovalocytes, rare target cells and a few schistocytes. In addition, basophilic stippling and Howell-Jolly bodies were seen. Hb Hammersmith has been found in several ethnic backgrounds to date, including English, Tunisian, Japanese, Caucasian, Indian, Chinese, African, American and Brazilian.²⁰⁻²⁵ Interestingly, all of the known cases to date have been female, as was the patient in this study. However, two recent studies have reported this mutation in a Korean male and a Chinese male with hemolytic anemia.^{26,27} Still, the higher frequency of female patients than male is not yet understood and further investigations are needed to explore the mechanism and molecular pathogenesis of this mutation. Many studies have shown that Hb Hammersmith in most patients is a spontaneous mutation^{20-24,26,27}, as it was in the patient in this study (Figure 1).

In addition, we identified five mutations reported previously in Thailand including, -31 (A>G), cod 15 (-T), cod 35 (C>A), cod 95 (+A) and Hb Dhonburi [cod 126 (T>G)] found in southern Thailand for the first time. The -31 (A>G) mutation, a point mutation in the TATA box of the β -globin promoter causing β^+ -thal, was reported firstly homozygously in a Japanese patient, and was subsequently found to have a high incidence in Japan.²⁸ In Thailand, carriers of this mutation have been found in many parts of the country, including the North and Northeast.^{29,30} Another β^+ -thal mutation was found in this study, Hb Dhonburi, also known as Hb Neapolis. This variant is caused by a T to G substitution at cod 126 resulting in the changing of valine to glycine and activation of a cryptic splice site in the exon. This induces instability of the Hb molecule but no change in its oxygen binding properties.³¹ It is noteworthy that Hb Dhonburi has been found to be a co-migrant with Hb A from CE and HPLC analysis.³² It could easily be missed in routine

analysis unless DNA analysis is performed. Hb Dhonburi was discovered originally in a Thai male who presented as the β -thal intermedia phenotype because of a combination with β^0 -thal.³¹ Hb Dhonburi has been subsequently found in various populations including northeastern and central Thais, and in Italy and Iran.^{30,32,33} In this study we also identified three β^0 -thal mutations described previously in Thailand, cod 15 (-T), cod 95 (+A) and cod 35 (C>A). The frameshift mutations at cod 15 (-T) and cod 95 (+A) lead to a premature termination codon at cod 18 and 101, respectively. Cod 15 (-T) was identified for the first time in a Malaysian family in combination with the polyA (-AT) (HBB: c.[*109_*110delAT]) mutation causing the β -thal major phenotype. Since then, cod 15 (-T) has been documented occasionally in Asia, including the Thai, Bangladeshi, Indonesian and Indian populations.³⁴⁻³⁷ Initially, cod 95 (+A) was found in combination with Hb E (HBB: c.79G>A) in a patient who presented as a β -thal intermedia phenotype from the central part of Thailand, and it has also recently been reported in northern Thailand.³⁴ In this study, we also observed this mutation co-inherited with Hb E in one patient with moderate anemia (Table 1). In addition to Thailand, this mutation has been frequently observed in Vietnamese individuals, in either the heterozygous stage or compound heterozygous with Hb E, known as the "Vietnamese mutation".³⁸ The nonsense mutation at cod 35 is caused by a C to A substitution which creates a premature stop codon (TAA) at cod 35. Originally, this mutation was discovered in combination with Hb E in a Thai patient from central Thailand, and has been reported only rarely.³⁹ Recently, the homozygous cod 35 (C>A) was identified in a Moroccan girl who presented with a thalassemia intermedia phenotype.⁴⁰

Conclusion

The results of this study, in combination with other studies, improves the knowledge of the spectrum of

thalassemia mutations in Thailand, which will be useful for planning genetic counseling and prenatal diagnosis programs for prevention and control of the disease.

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Conflict of interest

There are no potential conflicts of interest to declare.

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