

Thalassemia and erythroid transcription factor *KLF1* mutations associated with borderline hemoglobin A₂ in the Thai population

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Abstract

Introduction: Elevated hemoglobin (Hb) A₂ is an important diagnostic marker for β -thalassemia carriers. However, diagnosis of cases with borderline Hb A₂ may be problematic. We described the molecular characteristics found in a large cohort of Thai subjects with borderline Hb A₂.

Material and methods: Examination was done on 21,657 Thai subjects investigated for thalassemia at Khon Kaen University, Thailand. A total of 202 subjects with borderline Hb A₂ (3.5–4.0%) were selectively recruited and hematological parameters were recorded. DNA variants in α -, β -, δ -globin, and Krüppel-like factor 1 (*KLF1*) genes were examined using PCR.

Results: Among 202 subjects, DNA analysis identified carriers of α^+ -thalassemia ($n = 48$; 23.8%), β -thalassemia ($n = 22$; 10.9%) and *KLF1* mutations ($n = 48$; 23.8%). No molecular defect was observed in the remaining 84 (41.5%) subjects. Interaction of *KLF1* and α -thalassemia was observed in 10 cases. Of the 22 β -thalassemia carriers, five β^+ -thalassemia mutations were identified with lower MCV and higher Hb A₂. Seven *KLF1* mutations were detected in 10 genotypes in subjects with higher MCV and Hb F. No β^0 -thalassemia, α -globin gene triplication or δ -globin gene mutation was detected.

Conclusions: A large proportion of subjects with borderline Hb A₂ are not β -thalassemia carriers and for those with β -thalassemia, only mild β^+ -thalassemia mutations were detected. Evaluation of the patients using Hb A₂, Hb F and MCV values will help in selecting cases for further molecular analysis. The results should explain the unusual phenotype of the cases and facilitate a thalassemia screening program in the region.

Key words: borderline hemoglobin A₂, *KLF1*, thalassemia screening, globin gene defects.

Introduction

The level of hemoglobin (Hb) A₂ ($\alpha_2\delta_2$) in a normal adult is about 2.0–3.2% of total Hb. An elevated Hb A₂ between 4.0% and 8.0% is an important diagnostic marker for β -thalassemia carriers [1–3]. A study in a large cohort of β -thalassemia heterozygotes in Thailand detected Hb A₂ corresponding to β -thalassemia mutations in the range of $5.0 \pm 0.6\%$ to $7.0 \pm 0.8\%$. Interaction of β -thalassemia with α -thalassemia could alter the level of Hb A₂ but this does not affect the diagnosis of the cases [4].

In some cases, the levels of Hb A₂ are higher than normal but are not within the diagnostic range for a typical β -thalassemia carrier, making it difficult to make the diagnosis. This so-called borderline Hb A₂ usually ranges between the levels found in normal and classic β -thalassemia carriers, which are 3.2–3.9% [5, 6]. Therefore, accurate diagnoses of these cases are usually made through molecular identification of the β -thalassemia mutations [7]. However, this is not practical in a large population screening in an area where thalassemia is common and molecular testing is not available. Borderline Hb A₂ is prevalently reported in many populations. Several causes of reduced Hb A₂ have been noted including reduced δ -globin chain synthesis in δ -thalassemia or δ -chain variant, reduced Hb A₂ tetramer assembly in α -thalassemia, a silent β -thalassemia mutation, α -globin gene triplication and some acquired conditions such as iron deficiency, lead poisoning, sideroblastic anemia, and myeloproliferative disorders [8]. These causes could generally explain only a small fraction of the cases with borderline Hb A₂ in the populations [2, 7–10]. This indicates the involvement of other genetic factors. Among these factors is an erythroid-specific transcription factor, Krüppel-like factor 1 (*KLF1*), which plays an important role in erythropoiesis and Hb switching. *KLF1* mutations have been found to be associated with several hematological disorders, including hereditary persistence of fetal hemoglobin (HPFH), congenital dyserythropoietic anemia type IV, and transfusion-dependent hemolytic anemia. It has been found that *KLF1* mutations could also ameliorate the clinical severity of β -thalassemia disease [11–13]. Association of *KLF1* mutations with borderline Hb A₂ have been noted in multiple populations including Italian, Sardinian, Chinese and Saudi [7, 9, 10, 14]. We have also reported the association of *KLF1* mutations with higher phenotypic expression of Hb A₂ and Hb F in Hb EE syndrome [15, 16]. In this study, we examined globin genes and *KLF1* mutations to determine the molecular features underlying borderline Hb A₂ in a large cohort of the Thai population.

Material and methods

Subjects and hematological analysis

Ethical approval of the study protocol was obtained from the Institutional Review Board (IRB) of Khon Kaen University, Thailand (HE562059). A retrospective review of laboratory findings was carried out on 21,657 Thai subjects encountered at our ongoing thalassemia screening program at the Centre for Research and Development of Medical Diagnostic Laboratories, Khon Kaen University, Thailand. A total of 202 subjects was

selectively recruited based on Hb analysis with Hb A₂A pattern and Hb A₂ levels between 3.5 and 4.0%. Subjects with known heterozygous β -thalassemia and elevated Hb A₂ (> 4.0%), thalassemia diseases or Hb variants and iron deficiency anemia were excluded. Routine hematological parameters were recorded using a standard blood cell counter. Hemoglobin analysis and quantification were performed using Hb high-performance liquid chromatography (Variant II analyzer, Bio-Rad Laboratories, Hercules, CA, USA) or capillary electrophoresis (Capillarys 2 Flex Piercing; Sebia, Lisses, France).

Molecular analysis

Genomic DNA was prepared from peripheral blood leukocytes using the standard method. Identifications of β -thalassemia mutations, α^0 -thalassemia (SEA & THAI deletions) and α^+ -thalassemia (3.7 and 4.2 kb deletions), Hb Constant Spring and Hb Paksé mutations are performed routinely in our laboratory using PCR and related methods described elsewhere [17, 18]. Screening for α -globin gene triplication ($\alpha\alpha\alpha$ haplotype) and δ -globin gene variants previously documented in Thailand was done using PCR assays as described previously [19, 20].

For the *KLF1* gene, four common *KLF1* mutations found previously in the Thai population – G176AfsX179, -154(C-T), R328H and T334R – were examined using allele-specific PCR as described [15, 16]. Other rarer mutations including Q217X, S270W and G335R were identified using allele-specific PCR assays developed in this study as shown in Figure 1. These mutations were then confirmed by DNA sequencing (Figure 2). The Q217X and S270W mutations were detected in a multiplex PCR manner. With this system, the Q217X and S270W specific primers, G144 (5'-GAGCCCGGAAGAGCTA-3') and G145 (5'-CAAGCGAGGCCGACGTTG-3'), were used with primers G125 (5'-GCTCCCGACGCCTTCGT-3') and G146 (5'-TGTA-CTACAGCGGGCGCC-3'), respectively, to produce fragments of 237 bp and 177 bp specific for the two mutations. The 539 bp fragment was an internal control generated from primers G125 and G146. The PCR reaction (50 μ l) contained 50 ng of DNA, 0.6 pmol of primers G144 and G146, 1.2 pmol of primer G125, 0.33 pmol of primer G145, 200 mM dNTPs, 100 mM Tris HCl pH 8.3, 50 mM KCl, 3 mM MgCl₂, 5% DMSO, 1 M Betaine and 1 unit of *Taq* DNA polymerase (New England Biolabs Inc., MA, USA). PCR was performed with an initiation heating step at 94°C for 3 min followed by 35 cycles of 94°C for 30 s and 67°C for 1.30 min. In contrast, the G335R mutation was detected under a monoplex allele-specific PCR with the specific primer G147 (5'-GCCACTACCGGAAACACACGA-3')

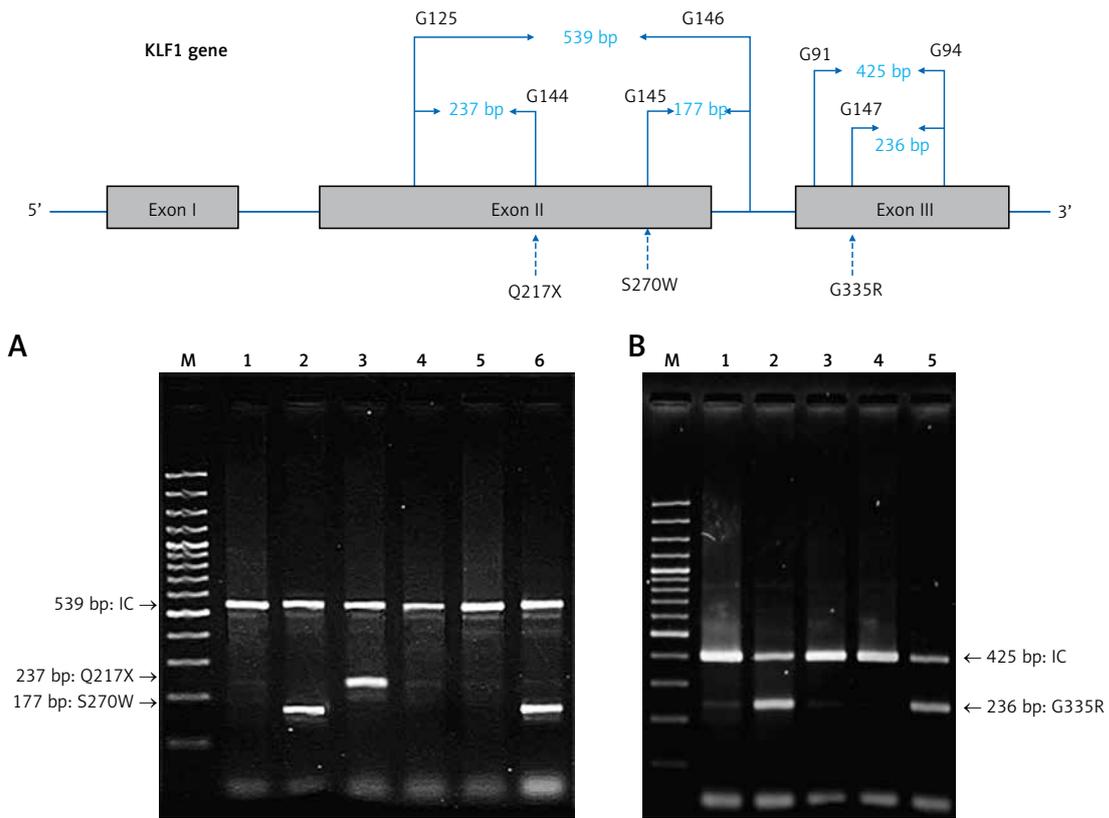


Figure 1. Identification of three *KLF1* mutations, namely Q217X, S270W and G335R, by allele specific PCR assays. The mutations, locations and orientations of all primers used as well as the sizes of amplified fragments are depicted. In both representative gel electrophoresis, M is 100 bp DNA ladder. **A** – 1: normal control, 2 and 6: carriers of S270W mutation, 3: carrier of Q217X mutation, 4 and 5: negative for S270W and Q217X mutations. **B** – 1: normal control, 2 and 5: carriers of G335R mutation, 3 and 4: negative for G335R mutation

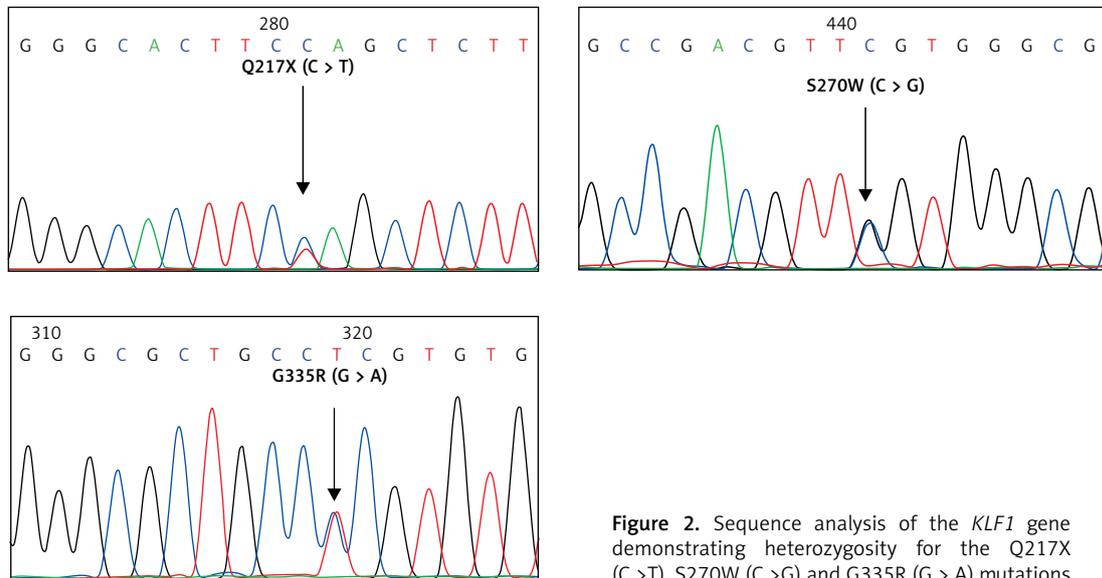


Figure 2. Sequence analysis of the *KLF1* gene demonstrating heterozygosity for the Q217X (C > T), S270W (C > G) and G335R (G > A) mutations

and the common primer G94 (5'-TCCAGGAGAGG-GTCCATTCGT-3') which produces a 236 bp specific fragment. An internal control of 425 bp was generated from primers G91 (5'-CAGCCCAGGCTGAGTA-AAGGG-3') and G94. The reaction mixture (50 μ l) contained 50 ng DNA, 0.72 pmol of primers G147,

0.6 pmol of primer G91 and G94, 200 mM dNTPs, 100 mM Tris HCl pH 8.3, 50 mM KCl, 3 mM MgCl₂ and 1 unit of *Taq* DNA polymerase (New England Biolabs Inc., MA, USA). PCR was performed with an initiation heating step at 94°C for 3 min followed by 35 cycles of (94°C for 30 s and 69°C for

1.30 min). The amplification was performed using the TProfessional Standard Thermocycler (Biometra, GmbH, Goettingen, Germany). The amplified product was separated on 2% agarose gel electrophoresis, stained with ethidium bromide (0.5 µg/ml) and visualized under UV light, shown representatively in Figure 1.

Statistical analysis

Mean and standard deviation were used to describe the hematological parameters of the subjects. The difference in the medians of MCV, Hb A₂ and Hb F was analyzed using the Kruskal-Wallis test and the Mann-Whitney *U* test. A *p*-value of < 0.05 was considered statistically significant. Box plot and statistical analysis were done using Minitab version 14 (Minitab Inc., USA).

Results

Among 202 subjects with borderline Hb A₂, DNA analysis revealed that 70 (34.7%) were positive for molecular defects in the α -globin gene (*n* = 48) and β -globin gene (*n* = 22) as shown in Table I. Forty eight subjects (23.8%) were found to carry *KLF1* mutations including 30 pure heterozygotes, 8 compound heterozygotes and 10 double heterozygotes for *KLF1* mutations and α -globin gene defects. No genetic interaction between *KLF1* and β -thalassemia was observed. In the remaining 84 (41.5%) subjects, no molecular defects in the α - and β -globin genes or in the *KLF1* gene were found.

As shown in Table I, among 48 subjects with α -thalassemia, 34 were heterozygous for α^+ -thalassemia with 3.7 kb deletion ($-\alpha^{3.7}/\alpha\alpha$), 12 were heterozygous for Hb Constant Spring ($\alpha^{CS}\alpha/\alpha\alpha$) and 2 were Hb Paksé carriers ($\alpha^{PS}\alpha/\alpha\alpha$). Interestingly, among 22 β -thalassemia carriers, only mild β^+ -thalassemia mutations were identified including Hb Malay or CD19 (HBB: c.59 A > G) in 10 cases, NT -31 (HBB: c.-81 A > G) in 5 cases, Hb Dhonburi or CD126 (HBB: c.380 T > G) in 3 cases, NT -28 (HBB: c.-78 A > G) in 3 cases and NT -50 (HBB: c.-100 G > A) in 1 case. Double heterozygosity for β^+ -thalassemia and α^+ -thalassemia ($-\alpha^{3.7}/\alpha\alpha$), Hb Constant Spring ($\alpha^{CS}\alpha/\alpha\alpha$), α^0 -thalassemia ($-^{SEA}/\alpha\alpha$) and Hb H-CS disease ($-^{SEA}/\alpha^{CS}\alpha$) were identified in two, one, one and one subjects, respectively. Thirty-eight of 202 (18.8%) subjects had the *KLF1* mutations alone including 30 pure heterozygotes and 8 compound heterozygotes. Double *KLF1* and α -thalassemia mutations were identified in 10 (5.0%) subjects including 7 with $-\alpha^{3.7}/\alpha\alpha$, 1 with $\alpha\alpha/\alpha^{CS}\alpha$, 1 with α^0 -thalassemia with SEA deletion ($-^{SEA}/\alpha^{CS}\alpha$) and 1 compound heterozygous for two *KLF1* mutations and α^+ -thalassemia ($-\alpha^{3.7}/\alpha\alpha$).

Table II summarizes the hematological findings of 48 subjects with *KLF1* mutations according to α -globin genotypes. We detected 7 different *KLF1* mutations among these 48 subjects. The most common *KLF1* mutation found in 17 of the 48 (35.4%) subjects was the G176AfsX179 frameshift mutation. Four missense mutations – T334R, S270W, R328H and G335R – were found in 7 (14.6%), 6 (12.5%), 4 (8.3%) and 2 (4.2%) cases, respectively. The remaining mutations detected were a nonsense mutation Q217X (*n* = 1; 2.1%) and the -154 C>T promoter mutation (*n* = 2; 4.2%). Nine subjects were found to be compound heterozygotes for two different *KLF1* mutations: -154 (C>T)/ Q217X (*n* = 6), G176AfsX179/ S270W (*n* = 2) and T334R/ R328H (*n* = 1). As expected, most of the subjects with α -thalassemia were found to have lower MCV & MCH values as compared to those without α -thalassemia. However, it is apparent that co-inheritance of *KLF1* mutation does not worsen hematological features of these α -thalassemia patients.

In order to visualize the effect of *KLF1* mutations on the levels of MCV, Hb A₂ and Hb F, box plots of MCV, Hb A₂ and Hb F were presented comparatively in Figure 3 for gr. 1, *KLF1* carriers; gr. 2, compound heterozygotes for *KLF1* mutations; gr. 3, α^+ -thalassemia trait; gr. 4, β^+ -thalassemia trait and gr. 5, subjects with no mutation. Significantly, the lowest MCV value was noted for gr. 4, β^+ -thalassemia trait (Figure 3 A). It is also noteworthy in Figure 3 B that although all subjects had similarly borderline Hb A₂ due to the selection criteria, a significantly higher level of Hb A₂ was again observed in gr. 4, β^+ -thalassemia trait (*p*-value < 0.05 with the Kruskal-Wallis and the Mann-Whitney *U* tests). For Hb F, subjects with *KLF1* mutations in gr. 1 and gr. 2 had significantly higher levels as compared to those of the remaining three groups without *KLF1* mutations (Figure 3 C).

Discussion

Elevated Hb A₂ is an important marker for diagnosis of β -thalassemia carrier. In countries with a high prevalence of thalassemia, individuals with normal or elevated levels of Hb A₂ are generally not problematic in routine diagnostics. However, those with borderline Hb A₂ are difficult to diagnose. Previous studies in multiple populations have demonstrated different molecular defects in α -, β - and δ -globin genes as well as in the *KLF1* gene [5, 7–10]. However, the results of these studies cannot be compared directly because of the differences in the Hb analyzers used in quantification of Hb A₂, the molecular basis of β -thalassemia in the population, and the definitions of borderline Hb A₂. For example, the definitions of borderline Hb A₂ were 3.1–3.9% in the studies

Table 1. Hematological parameters, *KLF1* mutations and globin genotypes found in 202 Thai subjects with borderline Hb A₂

Mutations	n	%	Hb Typing		RBC [10 ¹² /l]	Hb [g/dl]	Hct [%]	MCV [fl]	MCH [pg]	MCHC [g/dl]
			Hb A ₂ (%)	Hb F (%)						
No mutation	84	41.5	3.7 ±0.2	1.1 ±1.8	4.6 ±0.8	12.4 ±1.9	37.6 ±5.9	81.9 ±9.9	27.1 ±3.6	33.1 ±1.6
Globin gene mutations:										
α-thalassemia carriers:										
-α ^{3.7} /αα	34	16.9	3.6 ±0.4	0.4 ±0.4	4.9 ±0.8	12.4 ±1.6	37.8 ±5.1	77.9 ±4.7	25.6 ±1.7	32.6 ±1.3
α ^{CS} α/αα	12	5.9	3.7 ±0.2	1.3 ±1.6	4.8 ±0.6	12.1 ±1.9	38.2 ±5.4	79.2 ±4.7	25.2 ±2.0	31.7 ±1.1
α ^{PS} α/αα	2	1.0	4.0, 3.5	0, 0	4.6	11.4	34.2	70, 75.2	22, 25.1	33.3
β ⁺ -thalassemia carriers:										
β ⁺ -thal trait	17	8.4	3.8 ±0.1	2.4 ±3.4	4.9 ±0.9	11.7 ±2.0	35.1 ±5.3	72.4 ±11.8	23.8 ±5.1	32.6 ±2.0
β ⁺ -thal trait with -α ^{3.7} /αα	2	1.0	3.8, 3.7	0, 0.6	na	na	na	80.7	26.9	na
β ⁺ -thal trait with α ^{CS} α/αα	1	0.5	4.0	0.9	4.5	11.4	35.3	78	25.1	32.2
β ⁺ -thal trait with --SEA/αα	1	0.5	3.9	na	5.5	12.6	38	69.2	22.8	33
β ⁺ -thal trait with --SEA/α ^{CS} α	1	0.5	3.5	1.9	4.0	6.9	24.2	61	17.5	28.8
KLF1 mutations:										
<i>KLF1</i> trait	30	14.8	3.7 ±0.2	2.3 ±2.1	5.0 ±0.7	13.3 ±1.5	40.8 ±5.1	80.9 ±8.2	26.5 ±2.5	32.5 ±1.6
compound heterozygous <i>KLF1</i>	8	4.0	3.7 ±0.2	2.7 ±1.9	5.1 ±0.9	12.4 ±2.3	36.8 ±6.0	74.9 ±9.7	24.8 ±3.9	33.9 ±1.1
KLF1 mutations with globin gene mutations:										
<i>KLF1</i> trait with -α ^{3.7} /αα	7	3.5	3.7 ±0.2	1.1 ±0.9	5.9 ±0.3	13.9 ±1.4	43.5 ±3.8	73.3 ±5.1	23.3 ±2.1	30.4 ±2.7
<i>KLF1</i> trait with α ^{CS} α/αα	1	0.5	3.5	0	na	na	na	na	na	na
<i>KLF1</i> trait with --SEA/αα	1	0.5	3.6	4.2	6.3	12.3	41	65	19.6	30.3
Compound heterozygous <i>KLF1</i> with -α ^{3.7} /αα	1	0.5	3.6	0	5.4	12.6	38.4	71	23.4	34

Values are presented as N, mean ± SD or raw data where appropriate. *β⁺-thalassemia mutation; CD19 (A>G) (n = 10), NT -31 (A>G) (n = 5), NT -28 (A>G) (n = 3), CD126 (T>G) (n = 3), NT -50 (G>A) (n = 1), na – not available.

Table II. Hematologic parameters of 48 subjects with *KLF1* mutations and different α -globin genotypes

N	<i>KLF1</i> mutations (n)	α -genotype	Hb analysis		RBC [10 ¹² /l]	Hb [g/dl]	Hct [%]	MCV [fl]	MCH [pg]	MCHC [g/dl]
			Hb A ₂ (%)	Hb F (%)						
17	G176AfsX179 (13)	$\alpha\alpha/\alpha\alpha$	3.6 ±0.2	3.3 ±2.5	5.4 ±0.7	13.4 ±1.2	41.5 ±4.6	77.7 ±6.8	25.4 ±2.7	32.2 ±1.2
	G176AfsX179 (4)	$-\alpha^{3.7}/\alpha\alpha$ or $\alpha^{c5}/\alpha\alpha$	3.7 ±0.2	1.4 ±0.7	5.5 ±0.2	13.4 ±1.7	41.9 ±4.0	73.6 ±6.6	23.3 ±2.9	29.4 ±3.4
7	T334R (4)	$\alpha\alpha/\alpha\alpha$	3.8 ±0.2	2.8 ±1.2	5.7, 4.1	15.0, 11.9	46.4, 34.3	82.4 ±0.7	27.3 ±1.1	33.0, 34.6
	T334R (2)	$-\alpha^{3.7}/\alpha\alpha$	3.6, 3.9	0.1, 0.1	6.1, na	14.9, na	47.7, na	78.7, 72.2	24.6, 22.0	31.2, 30.5
	T334R (1)	$--^{56}/\alpha\alpha$	3.6	4.2	6.3	12.3	41	65	19.6	30.3
6	S270W	$\alpha\alpha/\alpha\alpha$	3.7 ±0.2	1.2 ±1.2	4.4 ±0.3	12.5 ±2.0	39.2 ±6.8	88.0 ±11.7	28.1 ±2.8	31.8 ±2.2
	R328H (3)	$\alpha\alpha/\alpha\alpha$	3.7	2.2, 3.2	5.3, -	14.9	42.8	92.1, 81.3	28.3	34.8
4	R328H (1)	$-\alpha^{3.7}/\alpha\alpha$	3.6	0	6.1	15.5	47.8	79.0	25.7	32.4
	-154 (C-T)	$\alpha\alpha/\alpha\alpha$	3.9, 3.5	0, 0	5.3	13.5	42.2	73.4, 80.1	25.6	32
2	G335R (1)	$\alpha\alpha/\alpha\alpha$	3.6	1.2	na	na	na	73.4	26.2	28.5
	G335R (1)	$-\alpha^{3.7}/\alpha\alpha$	3.8	0	6.3	14.4	44.0	70.0	23.0	32.7
1	Q217X	$\alpha\alpha/\alpha\alpha$	3.7	0.5	na	na	na	na	na	na
6	-154 (C-T) / Q217X	$\alpha\alpha/\alpha\alpha$	3.6 ±0.2	2.7 ±1.4	5.3 ±0.9	12.5 ±2.5	37.5 ±6.4	72.6 ±10.2	23.8 ±4.0	33.6 ±0.8
	G176AfsX179 / S270W (1)	$\alpha\alpha/\alpha\alpha$	3.6	0	na	na	na	83.6	26.8	31.2
2	G176AfsX179 / S270W (1)	$-\alpha^{3.7}/\alpha\alpha$	3.6	na	5.4	12.6	38.4	71.0	23.4	34.0
	T334R/R328H	$\alpha\alpha/\alpha\alpha$	4.0	5.4	4.2	11.9	33.4	80.0	28.5	35.6

Values are presented as mean ± SD or as raw data where appropriate. N – total number of subjects for each *KLF1* mutation, (n) – number of subjects for each *KLF1* mutation and corresponding α - and β -globin genotypes. na – not available.

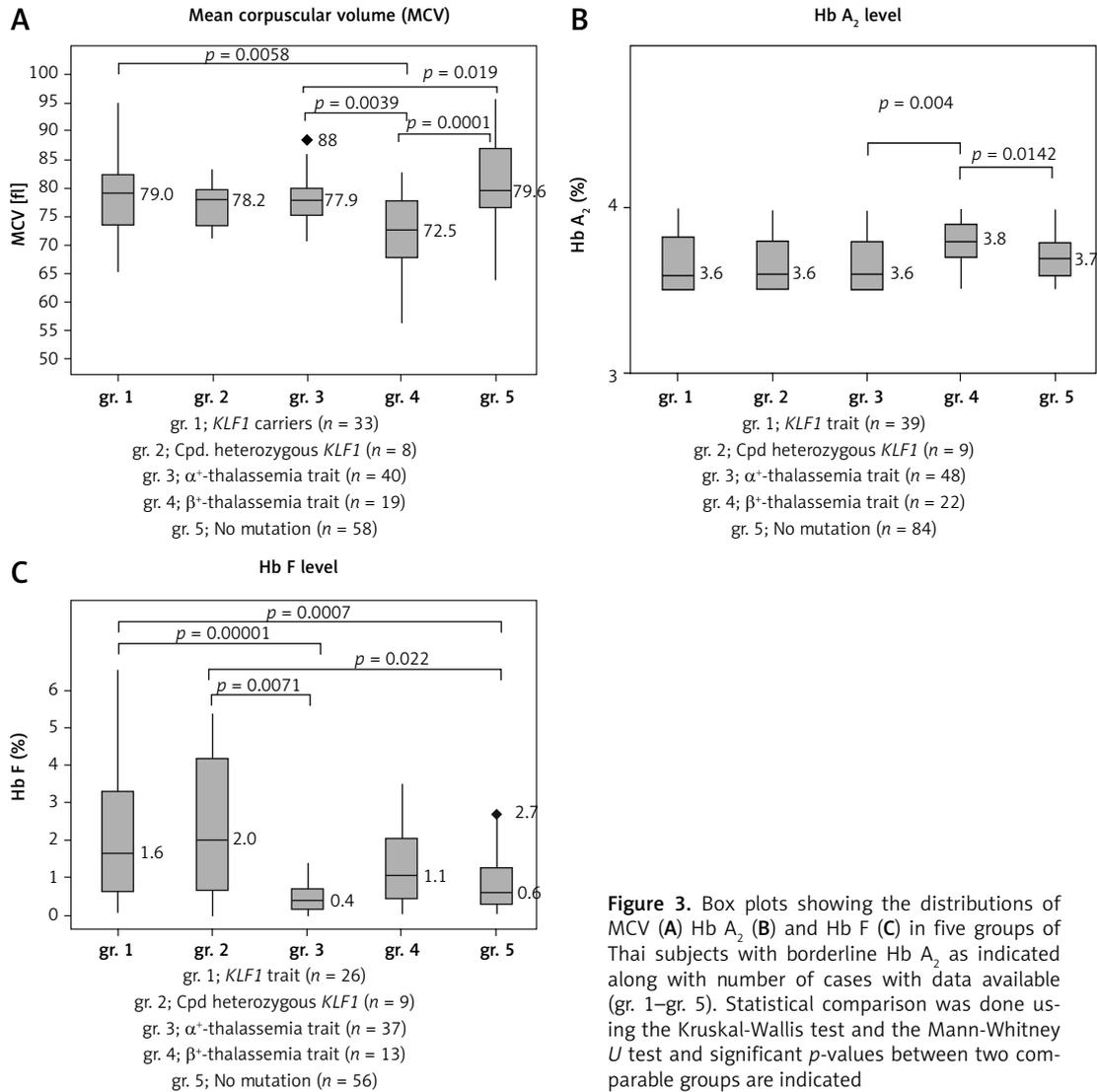


Figure 3. Box plots showing the distributions of MCV (A) Hb A₂ (B) and Hb F (C) in five groups of Thai subjects with borderline Hb A₂ as indicated along with number of cases with data available (gr. 1–gr. 5). Statistical comparison was done using the Kruskal-Wallis test and the Mann-Whitney U test and significant p-values between two comparable groups are indicated

of Italian [5] and Saudi [10] subjects, 3.3–4.1% in the Sardinian population [9] and 3.3–4.0% in the Chinese [7] population. In Thailand, the value of Hb A₂ > 4.0% has been used for diagnosis of β -thalassemia carriers [21]. Recently, Thailand's Ministry of Public Health changed this cut-off value to > 3.5% in the Clinical Practice Guideline (CPG) for laboratory diagnosis of thalassemia. We have noted in our ongoing thalassemia screening program that this change has increased the number of referral cases for molecular analysis of β -thalassemia and a large number of cases with no β -thalassemia mutation. This so-called false positive has rendered genetic counseling difficult and affected the prevention and control program in the country. Since no information on the molecular characteristics of borderline Hb A₂ in the Thai population is known, we looked at this matter in this study.

The level of Hb A₂ between 3.5 and 4.0% was defined as borderline Hb A₂ in this study and a total

of 202 subjects were recruited. We found that approximately 62.0% of the cases had MCV < 80 fl and 78.8% had MCH < 27 pg (Table I), which are quite similar to those of β -thalassemia carriers identified in the region [4]. However, as many as 84 out of 202 (41.5%) subjects were found to carry no mutations in α -, β - and δ -globin genes and the *KLF1* gene. When compared to other groups with mutations, these 84 subjects had higher MCV (81.9 \pm 9.9 fl) and MCH (27.1 \pm 3.6 pg) values. It is therefore recommended to consider cases with borderline Hb A₂ in combination with red blood cell indices before referring such cases for further molecular testing of thalassemia. This should lead to a significant reduction in the number of referral cases and workload of molecular testing.

Thalassemia mutations were found in 70 out of 202 (34.7%) subjects including 48 subjects with mild α^+ -thalassemia and 22 carriers of mild β^+ -thalassemia. Both are not the prime targets of thalassemia screening in Thailand, which focuses

mainly on α^0 -thalassemia, β^0 -thalassemia, and Hb E [17, 21]. This finding is also the case for the Italian, Chinese and Sardinian studies, in which most of the β -thalassemia mutations identified are β^+ -thalassemia [5, 7, 9]. In our study, neither α -globin gene triplication nor a δ -globin gene defect was detected. All these 70 cases could be considered as false positives in thalassemia screening. This should have further implications for the prevention and control program since it is not necessary to do mutation analysis for this group of subjects. This should also lead to a reduction in the number of referral cases for molecular study. However, we still recommend doing a molecular investigation in cases whose partners are β^0 -thalassemia or α^0 -thalassemia carriers in order to evaluate the risk of having fetuses with severe β -thalassemia major associated with compound β^0/β^+ -thalassemia and Hb H disease caused by the interaction of α^0 - and α^+ -thalassemias. It is also noteworthy, as shown in Figures 3 A and B, that subjects with β^+ -thalassemia had significantly lower MCV ($p = 0.0001$) and higher Hb A₂ ($p = 0.0142$) values as compared to those of subjects with no mutations. This characteristic should help in recognizing cases that require further molecular investigation.

Of interest is the finding of a high prevalence of *KLF1* mutations (48 of 202; 23.8%) in this group of Thai subjects with borderline Hb A₂. As many as 7 mutations, including both pure and compound heterozygotes, were encountered (Table II). We noted that this prevalence is higher than those described in the Chinese population [7] and in Thai subjects with Hb E disorder [15, 16]. This might indirectly support the association of *KLF1* mutation with a borderline Hb A₂ phenotype in the Thai population. We have shown previously in Hb EE disease that subjects with *KLF1* mutations had significantly higher Hb A₂ and Hb F as compared to those without *KLF1* mutations [15, 16]. An expression study also demonstrated an increased ratio of $\delta/\delta+\beta$ expression in *KLF1* mutation carriers and an increase in Hb A₂ level [14]. As for the Chinese population, the most common *KLF1* mutation observed in Thailand is G176AfsX179 [8]. This mutation has also been found to be associated with increased Hb F in many populations including Korean, Vietnamese and Chinese [22, 23]. This has now been confirmed in the Thai population as shown in Figure 3 C, i.e. those with *KLF1* mutations had significantly higher Hb F as compared to other groups. They also had higher MCV levels and lower Hb A₂ values as compared to those of β^+ -thalassemia carriers (Figures 3 A, B). However, we did not observe much difference in hematological parameters among heterozygous and compound heterozygotes for different *KLF1* mutations in Thai subjects (Table II). None of them had

any clinical symptoms either. The result indicated a broad spectrum of *KLF1* variants [24–26]. However, as reported previously [27], we did observe more hypochromic microcytosis in individuals with concomitant inheritance of *KLF1* and α -thalassemia mutations (MCV 73.0 \pm 4.9 fl, MCH 23.3 \pm 1.9 pg) as compared to those with *KLF1* mutations alone (MCV 77.9 \pm 4.7 fl, MCH 25.4 \pm 1.8 pg).

In conclusion, we demonstrated for the first time the molecular characteristics of borderline Hb A₂ in the Thai population in about half of the cases. Only a small proportion of them are β^+ -thalassemia carriers. Other genetic and non-genetic factors may be involved with borderline Hb A₂ phenotype in the remaining subjects such as HB-S1L-MYB and BCL11A variants [28], HIV-infected patients treated with zidovudine [29], and the coefficient of variation of the method used to measure Hb A₂ [1, 30]. Nonetheless, our study confirmed that most of the Thai individuals with borderline Hb A₂ would not be at risk of having fetuses with the three severe thalassemia syndromes targeted in a prevention and control program. Screening for multiple causative mutations corresponding to borderline Hb A₂ in the general population may not be cost-effective. However, careful evaluation of these individuals using combined Hb A₂, Hb F, and MCV values should help in effective selection of a small number of cases for further molecular study. This should lead to a significant reduction of workload for molecular testing and facilitate a prevention and control program for thalassemia in regions such as Thailand where thalassemia and hemoglobinopathies are exceptionally prevalent and heterogeneous.

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Conflict of interest

The authors declare no conflict of interest.

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