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Borderline hemoglobin A_2 levels in northern Thai population: *HBB* genotypes and effects of coinherited alpha-thalassemia

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ABSTRACT

Introduction: Identification of beta-thalassemia carrier in prenatal screening relies on the elevated Hb A_2 level. Borderline Hb A_2 levels pose a diagnostic challenge. We determined the *HBB* genotypes in subjects with borderline Hb A_2 in northern Thailand and studied the effects of coinherited alpha^0-thalassemia on Hb A_2 levels.
Methods: Blood samples with Hb A_2 3.1–10.0% from 2193 samples submitted for prenatal thalassemia screening were selected. Information on *HBB* genotypes and coinherited alpha^0-thalassemia were collected. All samples with unknown *HBB* genotypes underwent an automated DNA sequencing. The Hb A_2 levels were compared according to the coinherited alpha^0-thalassemia.
Results: *HBB* mutations were found in 298 (98.7%) of 302 samples with Hb A_2 4.0–10.0%. In the 106 samples with Hb A_2 3.1–3.9%, six had *HBB* mutations; four Hb Dhonburi [codon 126 (T > G)], one CAP site mutation [CAP + 1 (A > C)] and one beta^0-thalassemia [codon 41/42 (-TTCT)] with a coinherited *HBD* mutation [nt-77 (T > C)]. The Hb A_2 levels in beta-thalassemia carriers with and without coinherited alpha^0-thalassemia were not significantly different.
Conclusions: *HBB* mutations in northern Thais with borderline Hb A_2 levels comprise an unstable variant Hb Dhonburi and CAP + 1 (A > C) mutation. Coinherited *HBD* mutation lowers Hb A_2 and can cause a misidentification of a beta-thalassemia carrier.

1. Introduction

Thalassemia is an inherited hemolytic anemia that is highly prevalent in malaria-endemic area including Thailand and Southeast Asia. The prevalence of thalassemia carriers in Thailand is 20–30% for alpha-thalassemia, 3–9% for beta-thalassemia and 10–53% for hemoglobin (Hb) E [1]. Prenatal screening program for couples at risk of fetal severe thalassemia and fetal diagnosis has been universally established in Thailand [2]. To identify the couple at risk for severe beta-thalassemia diseases including homozygous beta-thalassemia and Hb E/beta-thalassemia, beta-thalassemia carriers are screened by using the mean corpuscular volume (MCV) of 80 fl or lower and further identified by Hb fractionation and molecular analysis [2].

Beta-thalassemia carriers are diagnosed by Hb analysis showing Hb AA_2 pattern with elevated Hb A_2 level. The diagnosis is then confirmed by a molecular identification of the mutation. The recommended cutoff Hb A_2 level varies from 3.5–4.0% [2–5]. Several factors including type of beta-globin gene (*HBB*) mutation, co-inherited alpha-thalassemia or delta-thalassemia and iron deficiency have been shown to lower the Hb A_2 level and affect the identification of beta-thalassemia carriers [6–9]. On the other hand, *KLF1* gene mutations and alpha-globin gene triplication have been shown to be associated with elevated Hb A_2 level in subjects without *HBB* mutations [9,10]. Therefore, the borderline Hb A_2 levels pose a challenge in making the diagnosis of beta-thalassemia carriers.

Herein we studied the *HBB* genotypes of the subjects with borderline Hb A_2 levels of 3.1–3.9% in northern Thailand to determine the genotypes of beta-thalassemia carriers. The effect of co-inherited alpha^0-thalassemia, which is highly prevalent in the region, on the Hb A_2 levels was also studied.

2. Materials and methods

The institutional ethics committee approved the study. Results of

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hematologic parameters and Hb analysis of blood samples from prenatal thalassemia screening program at Chiang Mai University Hospital between July–December 2016 were reviewed. The program received both new samples and samples which were previously screened by low MCV and/or positive for Hb E screening tests from Chiang Mai University Hospital and other hospitals within northern Thailand. The Hb analysis was done by high pressure liquid column chromatography (HPLC) using the Variant II HPLC system (Bio-Rad Laboratories, CA, USA) according to the manufacturer's recommendation. The leftover samples with Hb AA_2 pattern with Hb A_2 of 3.1–10.0% were selected for further study. Borderline Hb A_2 levels were defined as Hb A_2 levels of 3.1–3.9%.

In the prenatal thalassemia screening program at Chiang Mai University, *HBB* gene analysis was done by a high-resolution melting analysis (HRM) method in samples from beta-thalassemia carriers which were defined by Hb A_2 levels of 3.6–10.0%. The HRM method for *HBB* analysis which could detect 17 common point mutations and the 3.4 kb deletion was performed as previously described [11]. In case that *HBB* mutations were not identified, the samples were further tested with an automated DNA sequencing method of *HBB* [12]. The results of *HBB* gene analysis from the program were retrospectively collected.

In the groups with Hb A_2 levels of 3.1–3.5%, *HBB* analysis was performed by automated DNA sequencing method and gap-PCR for the 3.4 kb deletion [11,12]. A selected case of molecularly confirmed beta-thalassemia carrier with Hb A_2 of 3.3% was further tested by automated DNA sequencing of the delta-globin gene (*HBD*) as per a previously described method [13].

The gap-PCR results of alpha^0-thalassemia (Southeast Asian and Thai deletions) which was done when the mean corpuscular volume (MCV) ≤80 fl were collected. The gap-PCR method for alpha^0-thalassemia were performed as previously described [14].

The frequencies of *HBB* mutations identified in each Hb A_2 level group were reported as number and percentage. The Hb A_2 levels were presented as mean ± SD and were compared among groups with and without alpha^0-thalassemia by Student's *t*-test. The statistical analysis was performed using SPSS Statistics for Windows, version 22.0 (IBM Corporation). The *p*-value < 0.05 was considered significant.

3. Results

Among 2193 prenatal screening results available for review, there were 1531 samples with Hb AA_2 pattern and Hb $A_2 \leq 10.0\%$. The distribution of samples according to the Hb A_2 level was as shown in Fig. 1. There were 106 samples with borderline Hb A_2 levels of 3.1–3.9%, and 302 samples with Hb A_2 levels of 4.0–10.0%.

Among the 302 samples with Hb A_2 levels of 4.0–10.0%, *HBB* mutations were identified in 298 (98.7%). Thirteen mutations causing beta-thalassemia and two causing Hb variant were identified. The codon 17 (A > T) and codon 41/42 (-TTCT) mutations were the most common mutations.

Among the 106 samples with Hb A_2 levels of 3.1–3.9%, *HBB* mutations were identified in six (5.7%) samples; four were carriers of Hb Dhonburi [codon 126 (T > G)], one case had CAP site mutation [CAP + 1 (A > C)] and one case had beta-thalassemia carrier with the frameshift mutation [codon 41/42 (-TTCT)]. All samples with Hb Dhonburi and the CAP site mutation had Hb $A_2 \geq 3.7\%$ and MCV ≤80 fl. The beta^0-thalassemia carrier had a Hb A_2 level of 3.3%. Further investigation by *HBD* sequencing revealed a co-inherited *HBD* mutation [nt-77 (T > C), c.127 T > C]. The frequencies and Hb A_2 levels according to each *HBB* mutation were summarized in Table 1. The hematologic parameters of the six subjects with identified *HBB* mutations were shown in Table 2.

Co-inherited heterozygous alpha^0-thalassemia were seen in 25 (9.1%) of 276 samples with molecularly confirmed beta-thalassemia carrier. The Hb A_2 levels in groups with and without co-inherited alpha^0-thalassemia were 5.35 ± 0.63% and 5.46 ± 0.52% respectively. The levels were not significantly different between the two groups ($p = 0.34$).

4. Discussion

The *HBB* mutations identified in northern Thais with borderline Hb A_2 levels consisted of the Hb Dhonburi and CAP site mutations. An unexpected case of beta^0-thalassemia carrier with a normal Hb A_2 level was identified. The coinherited *HBD* mutation explained the finding of a lowered Hb A_2 level.

Elevated Hb A_2 levels has been a well-established criterion for an identification of beta-thalassemia carriers. However, the Hb A_2 levels in the borderline range remains a challenge for the diagnosis. From the distribution curve of Hb A_2 levels in Fig. 1, there are two distinct groups of samples and the Hb A_2 levels of 3.5–4.0% seems to be the overlapping zone.

From our study, *HBB* mutations were found in 298 of 302 (98.7%) samples with Hb A_2 4.0–10.0%. Fifteen *HBB* mutations were identified and four mutations, codon 17 (A > T), codon 41/42 (-TTCT), IVS-I-1(G > T) and codon 71/72 (+A), comprised approximately 80%.

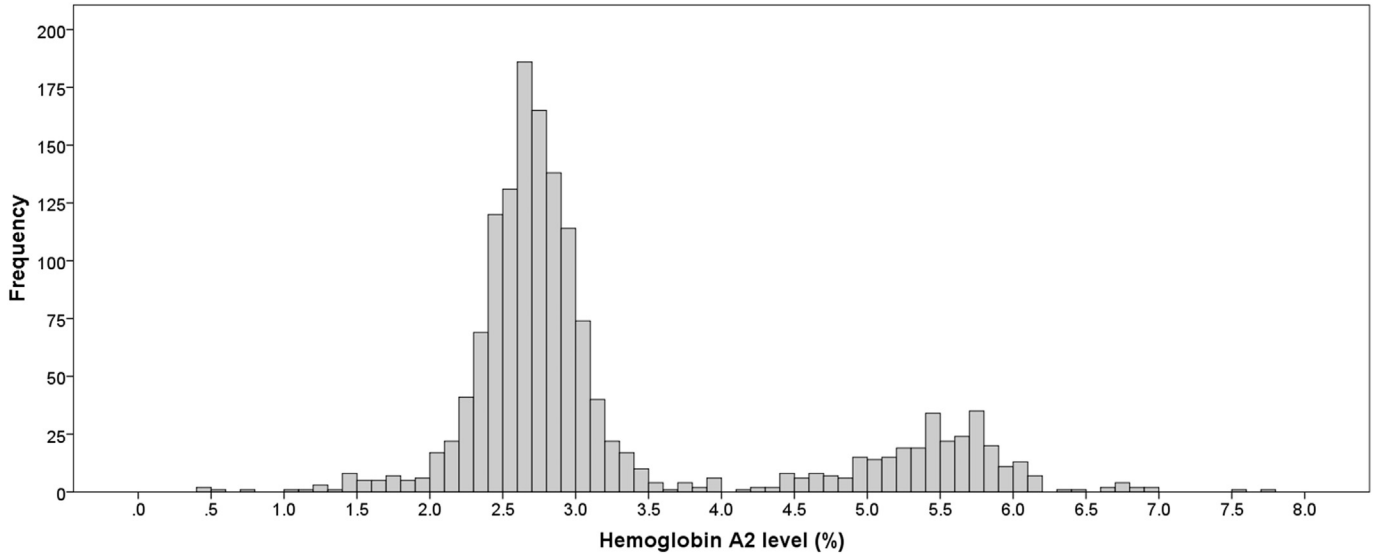

Fig. 1. The distribution of samples according to the Hb A_2 levels in the 1531 samples with Hb AA_2 pattern and Hb A_2 levels ≤ 10%.

Table 1
Frequencies of beta-globin gene (*HBB*) mutations and Hb A_2 levels according to the mutations.

HBB mutations	HGVS nomenclature	Frequencies (N)	Percent	Hb A_2 levels (%)		
				Mean ± SD	Min	Max
codon 17 (A > T)	c.52A > T	111	36.5	5.5 ± 0.5	4.5	7.7
codons 41/42 (-TTCT)	c.126_129delTTCT	98	32.2	5.5 ± 0.5	3.3	6.9
IVS-I-1 (G > T)	c.92 + 1G > T	26	8.6	5.2 ± 0.4	4.4	5.8
codons 71/72 (+A)	c.216_217insA	13	4.3	5.4 ± 0.4	4.5	6.0
nt-28 (A > G)	c.−78A > G	13	4.3	5.5 ± 0.3	4.7	6.0
3.4 kb deletion	–	9	3.0	6.2 ± 0.7	5.1	7.5
nt-31 (A > G)	c.−81A > G	8	2.6	4.5 ± 0.2	4.2	4.9
codon 126 (T > G)	c.380 T > G	7	2.3	4.1 ± 0.5	3.7	5.0
codons 27/28 (+C)	c.84_85insC	6	2.0	5.3 ± 0.4	4.9	5.7
codon 19 (A > G)	c.59A > G	5	1.7	4.8 ± 0.5	4.3	5.6
nt-87 (C > A)	c.−137C > A	3	1.0	5.4 ± 0.4	5.0	5.7
CAP+1 (A > C)	c.−50A > C	1	0.3	3.9		
codon 30 (G > C)	c.92G > C	1	0.3	4.3		
codon 35 (C > A)	c.108C > A	1	0.3	4.9		
codon 41 (−C)	c.126delC	1	0.3	5.8		
codon 43 (G > T)	c.130G > T	1	0.3	4.6		
Total		304	100	5.4 ± 0.6	3.3	7.7

Table 2
The hematologic parameters of subjects with borderline Hb A_2 levels and identified *HBB* mutations.

Number of cases	*HBB* mutations	*HBD* mutations	Alpha^0-thalassemia (SEA, Thai deletions)	MCV (fl)	MCH (pg)	Hb A_2 levels (%)
1	Codons 41/42 (-TTCT)	nt-77 (T > C)	Negative	63.4	19.9	3.3
1	CAP+1 (A > C)	Not done	Negative	78.8	25.4	3.9
4	Codon 126 (T > G)	Not done	Negative	77.2 ± 1.0	25.3 ± 0.5	3.8 ± 0.1

Of the 106 samples with Hb A_2 3.1–3.9%, six were identified with *HBB* mutations; four Hb Dhonburi, one CAP site mutation and one beta^0-thalassemia. All cases of Hb Dhonburi and CAP site mutations had Hb A_2 level of 3.7% or higher. Hb Dhonburi or Neapolis results from a nucleotide substitution (T > G) at codon 126 of *HBB* which results in valine to glycine substitution causing an unstable Hb variant [15]. The variant is electrophoretically silent [15–17]. Hb Dhonburi or Neapolis have been reported in diverse populations and have been identified to be of independent origins [18]. The heterozygous mutation causes mild microcytosis and mildly increased Hb A_2 [15–17]. Hb Dhonburi or Hb Neapolis in combination with beta-thalassemia almost exclusively results in thalassemia intermedia, although a case of compound heterozygous Hb Neapolis and IVS-II-1 was transfusion dependent [16,19].

CAP + 1 (A > C) mutation is a silent *HBB* mutation firstly reported in an Asian Indian subject who had homozygous mutation with low MCV of 71 fl, increased Hb A_2 level of 4.1% and Hb F level of < 2% [20]. The carriers of the CAP + 1 (A > C) mutation have been shown to have borderline hematologic profiles [21,22]. A study in Northern Indians with the CAP + 1 (A > C) mutation showed that the carriers with normal iron status had average MCV of 81.2 fl and Hb A_2 level of 3.6%. About half of the carriers had normal hematologic profiles [21]. This same study showed that compound heterozygosity of the CAP + 1 (A > C) mutation and beta-thalassemia resulted in variable phenotype from non-transfusion dependent to transfusion-dependent thalassemia. Eighteen out of 30 subjects with compound heterozygous CAP + 1 (A > C) mutation and beta-thalassemia were transfusion-dependent [21].

The case of beta^0-thalassemia carrier with normal Hb A_2 of 3.3% was explained by a co-inherited *HBD* mutation. The nt-77 (T > C) mutation was the most common *HBD* mutation seen in Chinese subjects with lower than expected Hb A_2 levels. As the co-inherited *HBD* mutation can decrease Hb A_2 of a beta-thalassemia carrier to normal levels, it was recommended that detailed molecular analysis is necessary in a partner with low MCV of a definite beta-thalassemia carrier [23].

Co-inherited alpha-thalassemia has been shown to lower Hb A_2 level [8,24,25]. However the interfering effect on identification of beta-thalassemia carriers is controversial. Our study showed that co-inherited alpha^0-thalassemia did not alter Hb A_2 levels in beta-thalassemia carrier.

Iron deficiency is another important factor that may cause decreased Hb A_2 level which may cause a misdiagnosis of beta-thalassemia carriers [26,27]. However, other studies show conflicting results and it was observed that the decreased Hb A_2 level may result from other causes such as the type of beta-globin mutations [27–29]. A large cohort of beta-thalassemia carriers showed that iron deficiency resulted in a small decrease of Hb A_2 level, but not to the level causing a misdiagnosis [30]. Therefore it remains controversial whether iron deficiency alone effects the identification of beta-thalassemia carriers. Iron deficiency was not searched for in our study.

The mean Hb A_2 level in samples with *HBB* mutation was 5.4 ± 0.6%. The levels were comparable among mutations, except for the nt-31 promoter mutation, Hb Dhonburi and Hb Malay that had lower mean Hb A_2 level and the 3.4 kb deletion that had higher mean Hb A_2 level. Hb Malay [(codon 19, A > G), *HBB*:c.59A > G] mutation creates an alternative splicing site and results in beta$^+$-thalassemia phenotype. Hb Malay carriers have borderline increased Hb A_2 levels and when presents with beta-thalassemia may result in both non transfusion-dependent and severe transfusion-dependent thalassemia [5,31]. The 3.4 kb deletion of beta-globin was first reported in a northern Thai family in 1990 [32]. Carriers of deletional beta-thalassemia are known to have highly elevated Hb A_2 level. This can be explained by the deletion of *HBB* promoter region which results in an enhanced delta-globin synthesis [33].

The findings of six subjects with *HBB* mutations out of 106 northern Thais with borderline Hb A_2 from our study was similar to the results from the Chinese individuals where only four out of 165 subjects with borderline Hb A_2 of 3.3–4.0% had *HBB* mutations; two nt-50 (G > A), and one each of codon 17 (A > T) and nt-31 (A > C) [34]. The *HBB* mutations prevalent in northern Thais are similar to mutations in the Chinese population, which mostly comprise beta^0 or beta$^+$ thalassemia mutations that result in elevated Hb A_2 levels in the typical heterozygous range [34]. The silent mutations are rare. On the other hand,

when comparing to the results from a large study of 410 subjects from Italy with borderline Hb A_2 of 3.1–3.9%, the findings were different as 94 subjects (22.9%) in the study had mutations in either *HBB, HBA* or *HBD*. This was explained by the higher prevalence of milder *HBB* mutations such as IVS-I-6 (T > C) and silent promoter mutations [9]. Another study in Italian subjects demonstrated similar HBB mutations that the nt-101 (C > T) and IVS-I-6 (T > C) were predominantly seen [24].

Most of the cases of borderline Hb A_2 in our report (100 of 106, 94.3%) and four of 302 (1.3%) of cases with Hb A_2 levels of 4.0–10.0% had no identified *HBB* mutations. Their causes for the borderline Hb A_2 remained unresolved. Other major possible causes that were not searched for in our study included the mutations in the *KLF1* or Krüppel-like factor 1 gene and *HBA* triplications. KLF1 zinc finger protein regulates the switching form Hb F to Hb A by binding to the *HBB* promoter and *BCL11A* which is a repressor of gamma-globin genes. As KLF1 protein does not bind to *HBD*, *KLF1* mutations may cause increased Hb A_2 and Hb F [35–37].

Mutations in *KLF1* were reported to cause borderline increased Hb A_2 levels of 3.3–3.9% with normal or slightly decreased MCV in a study in Sardinia [10]. In a study in Italy, *KLF1* mutations were identified in 52 of 145 subjects with borderline Hb A_2 of 3.3–4.1% [38]. Liu et al. reported that *KLF1* mutations was prevalent in a thalassemia endemic region in China and ameliorated the severity of beta-thalassemia. Subjects with heterozygous *KLF1* mutations in the study had borderline increased Hb A_2 levels of 3.28 ± 0.45% [39]. In the previous report of 165 Chinese subjects with borderline Hb A_2 levels, eight *KLF1* mutations were identified in 20 subjects [34]. Further study is needed to characterize the *KLF1* mutations in our population with borderline Hb A_2 level.

HBA triplications were identified in three of 165 subjects' borderline Hb A_2 levels and 15 of 410 subjects from the study in Italy [9,34]. From these two studies, all except one subject had normal MCV. *HBA* triplications should be searched for as a cause of borderline Hb A_2 level especially in subjects with normal MCV.

Although all beta^0-thalassemia has Hb A_2 levels of 4.0% or more, as a compound heterozygosity of beta^0-thalassemia and the CAP site mutation or Hb Dhonburi may result in transfusion-dependent thalassemia, our study supports the cutoff Hb A_2 levels of 3.6–10.0% for identification of beta-thalassemia carrier. There is limited data on the prevalence of beta^0-thalassemia carrier with *HBD* mutation that results in normal Hb A_2 level. In case of an identified beta-thalassemia carrier in prenatal screening program, upfront molecular identification of both common and silent *HBB* mutations in the partner who has low MCV regardless of Hb A_2 level should increase the sensitivity of beta-thalassemia carrier identification.

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Conflicts of interest

All authors declare no conflicts of interest.

Contribution

P.M.C. and P.C. conceived and designed the study, interpreted the results, wrote the paper and gave critical comments. A.P. performed the laboratory assay. P.M.C. collected the data. All authors were involved in the final revision of the article, gave contribution to the final analysis of the data and final approval.

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