Novel interactions of two α-Hb variants with SEA deletion α⁰-thalassemia: hematological and molecular analyses

Hataichanok Srivorakun, Kritsada Singha, Goonnapa Fucharoen and Supan Fucharoen

Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand

Abstract

Objectives: To report the hematological and molecular features as well as diagnostic aspects of the hitherto un-described interactions of two rare α-globin chain variants with α⁰-thalassemia commonly found among Southeast Asian populations.

Methods: The study was done on two adult Thai patients (P1 and P2) who had hypochromic microcytic anemia. Hb analysis was carried out using high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Mutations were identified by PCR and related techniques.

Results: Hb analysis of P1 using HPLC showed a normal Hb pattern, but CE demonstrated an abnormal peak at zone 7. DNA sequencing identified a CCG-CTG mutation at codon 95 of the α2 globin gene corresponding to the Hb G-Georgia [α95(G2)Pro → Leu(α2)] previously undescribed in the Thai population. In contrast, Hb analysis of P2 demonstrated an abnormal peak not fully separated from Hb A on HPLC, but not on CE. DNA analysis identified the rarely described Hb Nakhon Ratchasima [α63(E12)Ala → Val(α2)] mutation. Routine DNA analysis detected the SEA deletion α⁰-thalassemia in trans to the Hb variants in both cases. Hematological parameters were compared with those of patients with compound heterozygote for other α-globin variants and α⁰-thalassemia previously documented.

Conclusions: Identification of the patients confirmed that interaction of these rare Hb variants with α⁰-thalassemia does not lead to the Hb H disease. Differentiation of these two Hb variants from other clinically relevant hemoglobinopathies in a routine setting is, however, necessary. This can be accomplished using a combined Hb-HPLC and CE analysis followed by PCR-RFLP assays.

Introduction

Thalassemia and hemoglobinopathies are common and heterogeneous in Southeast Asia. In Thailand, for example, the prevalence of α-thalassemia is about 20–30% and that of β-thalassemia is 3.0–9.0%. The frequency of hemoglobin (Hb) E [26(B6)Glu → Lys, GAG > AAG] is about 30–40%; that of Hb Constant Spring or Hb Pakse is 1.0–8.0% [1]. In addition, more than 30 Hb variants have been documented [2]. With this variety of hemoglobinopathic alleles, interactions between them are commonly encountered. These can lead to complex thalassemia syndromes with clinical manifestations or clinically harmless but complicated routine thalassemia diagnostics [3–6]. Interaction of Hb Constant Spring or Hb Pakse or α⁰-thalassemia with α⁰-thalassemia leads to severe Hb H disease, commonly encountered in the region [7]. In contrast, the interaction of α⁰-thalassemia with other α-globin chain variants has rarely been documented. Here, we report, for the first time, the interaction of two rare α-globin chain variants, the Hb G-Georgia [α95(G2)Pro → Leu(α2)(HBA2c:287 C > T)] and Hb Nakhon Ratchasima [α63(E12)Ala → Val(α2)] with α⁰-thalassemia commonly found among the Southeast Asian population. Hematological features, Hb analytical profiles on HPLC and capillary electrophoresis (CE) of this Hb variant, as well as simple molecular diagnostics based on PCR-restriction fragment length polymorphism (RFLP) are also presented.

Materials and methods

Subject and hematological analysis

Ethical approval of the study protocol was obtained from our Institutional Review Board (IRB) at Khon Kaen University, Thailand (HE552035). Blood specimens of two Thai subjects were referred to our routine diagnostic laboratory at Khon Kaen University, Thailand for thalassemia investigation. Hematological parameters were recorded on a standard blood cell counter. Hb analysis was done using HPLC (Variant II; Bio-Rad Laboratories, Hercules, CA, U.S.A.) and CE system (Capilarys2 Flex Piercing; Sebia, Lisses, France) (Figure 1).

Routine DNA analysis

Identifications of α⁰-thalassemia (SEA/THAI deletions), α⁰-thalassemia (−α⁰3.7 and −α⁰4.2), αConstant Spring and...
αPaksé are routinely performed in our laboratory using PCR and related techniques as has been described [8]. Identification of Hb Tak (β146+AC, Ter→Thr) and Hb Q-Thailand (α74GAC-CAC, Asp→His) with similar electrophoretic mobilities with those of Hb variants herein described were done using allele-specific PCR assays described elsewhere [9,10]. Direct DNA sequencing of the amplified α-globin gene was done using an ABI PRISM™ 3130 XL analyzer (Applied Biosystems Foster City, CA, U.S.A.).

Identification of the αG-Georgia and αNakhon Ratchasima mutations

To establish rapid methods for the identification of Hb G-Georgia and Hb Nakhon Ratchasima in a routine setting, methods based on PCR-RFLP assay were developed. Selective amplification of an α2-globin gene was done using specific primers αG39 (5’-ACTCTTCTGTCGCCACAGAC-3’) and C3 (5’-CCA TTGTGGCACCATTCCCG-3’) [11]. The amplified α2-globin gene fragment (883 bp) was then digested to completion with Msp I (5’-C*CCGG-3’) and Sal I (5’-G*TCGAC-3’) (New England Biolabs Inc., Beverly, MA, U.S.A.) for Hb G-Georgia and Hb Nakhon Ratchasima mutations, respectively. The expected fragments were (327, 258 and 158 instead of 327, 231 and 158 bps) for Hb G-Georgia and (539 and 344 instead of 883 bp) for Hb Nakhon Ratchasima, as shown in Figure 2.

Results

P1 was an adult male who had mild hypochromic microcytic anemia with Hb 11.4 g/dl, MCV 57.8 fl and MCH 18.2 pg, compatible with being a thalassemia carrier commonly found in the region. As shown in Figure 1, initial Hb-HPLC analysis revealed a normal Hb pattern with 2.0% Hb A2. However, analysis using a CE system identified, in addition to Hb A (52.3%) and Hb A2 (1.9%), an abnormal Hb separation at zone 7 (44.9%). This indicated that he was a carrier of an unknown Hb variant co-separating with Hb A on HPLC. Routine DNA analysis of α-thalassemia revealed as expected that he was also a carrier of α°-thalassemia (SEA deletion). Identification of Hbs Tak and Q-
Thailand, the two common Hb variants detected at zone 7 of CE by PCR [9,10] yielded negative results. Since the amount of abnormal Hb found in P1 pointed to a β-globin chain variant, DNA sequencing of the β-globin gene was carried out. No mutation was detected (data not shown). However, further DNA sequencing of an α2-globin gene [11] revealed unexpectedly a CCG (Pro) → CTG (Leu) transition at codon 95, corresponding to the Hb G-Georgia [12]. As this mutation eliminates an MspI restriction site (5' - CCG - 3') on the α2-globin gene, it could be confirmed by PCR-RFLP assay using MspI digestion as shown in Figure 2. These DNA analyses confirmed that P1 was a compound heterozygote for a previously undescribed condition of α0-thalassemia/Hb G-Georgia.

The analysis of P2 indicated that he was suffering from severe anemia with Hb 5.3 g/dl, MCV 69.0 fl and MCH 22.7 pg. However, a relatively low number of Rbc and reduced Hb and Hct values indicated that this could be due to other underlying defects. Hb analysis identified an abnormal Hb not fully separated from Hb A on HPLC but co-migrating with Hb A on CE (Figure 1). Hb A2 level was normal in both analyzers. PCR-RFLP analysis using SalI indicated that he was a carrier of Hb Nakhon Ratchasima [α63(E12)Ala → Val (α2)], an α2-globin chain variant previously documented in Thailand [13]. Routine α-thalassemia screening identified that he also carried an α0-thalassemia (SEA deletion). Accordingly, he was finally diagnosed as a compound heterozygote for α0-thalassemia/Hb Nakhon Ratchasima, another undescribed condition.

As for other α-globin chain variants including Hb Thailand [α56(ES)Lys → Thr(α1)], Hb Phnom Penh [α117(GH5)-Ile-α118(H1)(α1)] [5] and Hb Hekinan [α27(B8)Glu → Asp(α2)] [11] found in our laboratory, the association of Hb G-Georgia and Hb Nakhon Ratchasima with α0-thalassemia in the two patients did not lead to Hb H disease. Table 1 lists hematological findings of these Thai patients with multiple globin gene interactions comparatively.

**Discussion**

Hb variants are common in Thailand where thalassemia is prevalent and heterogeneous. Interactions between
them, resulting in complex syndromes with complicated laboratory diagnostics, are occasionally encountered in routine practices. Accurate diagnosis of the cases usually requires multiple testing. Using a combination of Hb-HPLC, Hb-CE followed by DNA analysis, we have now reported the hitherto undescribed conditions caused by interactions of two α-globin chain variants with δα-thalassemia (SEA deletion) in two Thai individuals.

In P1, we detected the interaction of Hb G-Georgia and δα-thalassemia. Hb G-Georgia is a rare variant found in heterozygote and double heterozygote with Hb C and Hb S [14,15]. The globin gene interaction observed in P1 is the first time this Hb variant has been found in association with δα-thalassemia. As shown in Figure 1, HPLC could not identify this Hb variant with a substitution of Leucine for Prolein at amino acid number 95 of the α-globin chain. However, CE could demonstrate it as an unknown Hb variant excellently, separating at zone 7 before Hb A in zone 9. It has been demonstrated that Hb G-Georgia moved slightly faster than Hb S on alkaline cellulose acetate electrophoresis [15]. However, with the amount of 44.9% Hb G-Georgia with no Hb A2 variant (ααG2δβ), Hb G-Georgia could be misinterpreted as Hb F or another β-globin chain variant. The level of Hb G-Georgia in the heterozygote state has been noted to be 23.4%, approximately one-fourth of the total Hb [12]. A relatively higher proportion of Hb G-Georgia (44.9%) in P1 indicates as for Hb Hekinan, as previously described [11], that with the limited availability of α-globin chain in δα-thalassemia, the formation of Hb G-Georgia is preferable. It is noteworthy that a Thai patient with Hb G-Georgia/δα-thalassemia did not develop Hb H disease [16], the data indicating that this Hb G Georgia is not an α-thalassemic allele. However, a positive result for a dichlorophenolindophenol screening test for Hb E [17] indicated instability of the Hb G-Georgia molecule (data not shown). This could be due to an alteration of α1β2 contact of the Hb molecule, though this should not have much effect on the Hb function. The hypochromic microcytosis observed in the patient could be explained by the δα-thalassemia allele. A Thai individual with the Hb St. Luke’s—Thailand [α95(G2)Pro-Arg], another Hb variant with a mutation at the same position, with Hb G-Georgia also presented with no clinical symptoms [18].

Hb Nakhon Ratchasima found in association with δα-thalassemia in P2 is another rare α-globin chain variant. It has only been reported in combination with Hb E and αα-thalassemia in another Thai patient with mild anemia, but with otherwise normal Rbc indices [13]. Hb Nakhon Ratchasima is likely a non-pathological α-globin chain variant. An amino acid substitution of Valine to Alanine at codon 63 of the α-globin chain of this Hb variant is located at the external surface, and appears to have no influence on the function of the Hb molecule. Accordingly, as for Hb G-Georgia, the association of Hb Nakhon Ratchasima with δα-thalassemia in P2 did not lead to the Hb H disease. Although P2 did have low MCV and MCH characteristics of an δα-thalassemia carrier, a relatively low number of Rbc and dramatically reduced Hb and Hct values pointed to other underlying defects, rather than the interaction of Hb Nakhon Ratchasima and δα-thalassemia. It is noteworthy, as shown in Figure 1, that unlike Hb G-Georgia, Hb Nakhon Ratchasima co-migrates with Hb A on CE, but can be separated from Hb A on HPLC. However, the best way to confirm this is PCR-RFLP using Sal I digestion, as shown in Figure 2.

Although both Hb G-Georgia and Hb Nakhon Rachasima are rarely encountered and should be considered as benign abnormalities in both heterozygote and compound heterozygote forms, differentiation from other clinically relevant Hb variants in a routine setting is necessary. We demonstrated that a

### Table 1. Hematological data and globin genotypes of Thai patients with Hb G-Georgia/αα-thalassemia and Hb Nakhon Rachasima/δα-thalassemia as compared to other α-globin chain variants with similar genotypes including Hbs Thailand [5], Phnom Penh [5] and Hekinan [11] previously found in our laboratory. GG = Hb G-Georgia, NR = Hb Nakhon Rachasima.

| Parameters | P1: Hb G-Georgia (This study) | P2: Hb Nakhon Rachasima (This study) | Hb Thailand [5] (n = 1) | Hb Phnom Penh [5] (n = 1) | Hb Hekinan [11] (n = 1) |
|------------|-------------------------------|--------------------------------------|--------------------------|---------------------------|-------------------------|
| CBC        |                               |                                      |                          |                           |                         |
| Rbc (×10^12/l) | 6.2                          | 2.3                                  | 6.8                      | 5.0                       | 5.9 ± 0.4               |
| Hb (g/dl)  | 11.4                          | 5.3                                  | 15.2                     | 9.9                       | 12.6 ± 0.4              |
| Hct (%)    | 36.1                          | 16.0                                  | 49.0                     | 30.0                      | 38.9 ± 1.0              |
| MCV (fl)   | 57.8                          | 69.0                                  | 72.0                     | 60.7                      | 65.6 ± 3.4              |
| MCH (pg)   | 18.2                          | 22.7                                  | 22.4                     | 19.8                      | 21.3 ± 4.4              |
| MCHC (g/dl)| 31.6                          | 33.1                                  | 31.1                     | 32.6                      | 32.5 ± 1.0              |
| RDW (%)    | 19.1                          | na                                    | 15.0                     | 16.3                      | na                      |
| Hb analysis|                               |                                       |                          |                           |                         |
| HbA2 (%)   | 1.9α                          | 1.6β                                  | 1.4                      | 1.6                       | 2.2 ± 0.2               |
| Hb variant (%) | 44.9α                        | 33.4β                                 | 40.5                     | 98.2                      | 97.2 ± 0.7α             |
| Hb A2-variant (%) | un-detected                 |                                       | 0.7                      | 0.2                       | na                      |
| Globin genotype | αα-genotype                  |                                       |                          |                           |                         |
| β-genotype | ααG2δβ-SEA                   | ααM2δβ-SEA                            | ααThailandδβ-SEA         | ααPhnom Penhδβ-SEA       | ααHekinanδβ-SEA         |
|            | ββ/βA                         | ββ/βA                                 | ββ/βA                    | ββ/βA                     | ββ/βA                   |

*a* Determined using CE.

*b* Determined using HPLC.

na: not available.
combination of Hb analysis using both HPLC and CE followed by PCR-RFLP assays is helpful in the identification of cases with the two Hb variants. This should facilitate genetic counseling and a prevention and control program for hemoglobinopathy in the region.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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