Two independent origins of Hb Dhonburi (Neapolis) [β 126 (H4) Val→Gly]:
An electrophoretically silent hemoglobin variant

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Received 23 September 2005; received in revised form 9 August 2006; accepted 15 August 2006
Available online 17 August 2006

Abstract

Background: A β-hemoglobin variant (β 126 (H4) Val→Gly) was reported from Thailand and Naples (Southern Italy) as Hb Dhonburi (1) and Hb Neapolis (2), respectively. This abnormal hemoglobin, resulting from a valine to glycine substitution in the contact region between α and β subunits, gives rise to instability at non-physiological conditions. However, it was difficult to distinguish this variant from Hb A using hemoglobin electrophoresis and cation exchange liquid chromatography. Hb Dhonburi was rarely reported, possibly due to a relatively milder phenotype in heterozygote with slightly decreased MCV. Thus several Hb Dhonburi carriers might have been under-diagnosed.

Methods: Combined molecular analyses by PCR-single strand conformation polymorphism (PCR-SSCP) and direct genomic sequencing of the β globin genes were carried out in 2 pediatric patients with mild thalassemia intermedia. A novel amplification refractory mutation system (ARMS-PCR) was developed and performed in five individuals with microcytosis and borderline Hb A2.

Results: Both patients were compound heterozygotes for Hb E and Hb Dhonburi. In addition, 5 Hb Dhonburi heterozygotes, including 3 identified through thalassemia carrier screening, were identified by ARMS-PCR. Linkage analysis of the affected families revealed that the haplotype of Hb Dhonburi in Thailand (VII) was different from that of Hb Neapolis (V) suggesting 2 independent mutational events.

Conclusions: The molecular strategy described provides a robust and economical measure, alternative to the whole β globin genes sequencing, to identify rare or unknown β globin mutations. To overcome its ‘silent’ nature on electrophoresis, we proposed a novel ARMS-PCR for a rapid diagnosis of Hb Dhonburi in future cases.

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Keywords: Hb Dhonburi; High performance liquid chromatography (HPLC); Single strand conformation polymorphism (SSCP); Amplification refractory mutation system polymerase chain reaction (ARMS-PCR); β globin haplotype

1. Introduction

Thalassemias and hemoglobinopathies are very common inherited disorders with worldwide distribution [3]. In particular, Thailand, where both types of hemoglobin disorder are extremely prevalent as it was estimated that nearly 30% of population in this country carry one of such disorders [4]. Moreover, the wide variety of mutations make up >60 different thalassemia and hemoglobinopathy syndromes contributing to the complexity in diagnosis and proper management [4]. A notable demographic transition following improvements in public health, nutrition, hygiene and the availability of medical services in recent years results in significant reductions in infant and childhood mortality in Thailand. Therefore thalassemia and hemoglobin disorders, which, in the past, affected young patients might have died before a definitive diagnosis, are increasingly important. It was expected that in several developing countries, from Indian subcontinent, Southeast Asia to Oceania, thalassemia and hemoglobin disorders would cause a major public health burden [5].

The majority of hemoglobinopathies or hemoglobin variants result from single base substitution in the coding sequences giving rise to amino acid replacement and generating either novel α or β globin chains with different biochemical properties
from the normal counterparts [3]. Standard hemoglobin studies using electrophoresis, iso-electric focusing and ion exchange chromatography, usually detect most of these variants. However, some may escape the tests and this increases the difficulty in definitive diagnosis of unknown hemoglobin variants.

2. Materials and methods

2.1. Hematological study

Routine hematological parameters including red blood cell indices were carried out by an automated red blood cell counter (Sysmex F280, Kobe, Japan) on peripheral blood samples collected using EDTA as anticoagulant. The reticulocyte count was performed by staining peripheral blood with methylene blue. Hemoglobin was analysed by iso-electric focusing (IEF), starch gel electrophoresis at pH 8.6 and cation exchange HPLC using the Variant system (Bio-Rad Laboratories, Richmond, CA). Hb F was additionally determined by alkaline denaturation.

2.2. Molecular characterisation of the β globin genes

2.2.1. Reverse dot blot hybridization

After informed consent, molecular studies were performed in peripheral leukocyte DNA after a standard-phenol chloroform extraction. At first, we determined affected β-thalassemia alleles in the index families using the reverse dot blot (RDB) hybridization technique as described elsewhere. This technique could concurrently detect 10 common β-thalassemia mutations described in Thailand [6].

2.2.2. Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) technique

To identify unknown or rare β thalassemia mutations, which escape detection by the RDB, we performed a molecular screening using a recently developed PCR-single strand conformation polymorphism (PCR-SSCP) analysis as described [7]. Briefly, 6 PCR fragments, encompassing the promoter, the entire coding regions and parts of the introns I and II of the β globin genes, were amplified. The PCR amplifications were performed in 25 μl consisting of 100 ng DNA, 10 mmol/l Tris- HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l MgCl2, 200 μmol/l of each dNTP, 25 pmol primers and 1 unit of Taq polymerase (Roche Diagnostics, Mannheim, Germany). The reaction was carried out with initial denaturation at 94 °C for 5 min and then 30 cycles of 94 °C 30 s, 55 °C 30 s and 72 °C 30 s and a final extension at 72 °C for 10 min in thermocycler. The PCR products were run in 2.5% ethidium bromide stained agarose gel and directly visualized under an UV light transilluminator. This technique was applied to detect 3 unrelated individuals with mild hypochromic microcytosis with higher Hb A2 (>3.5%) characteristic of β thalassemia trait.

2.3. Molecular characterisation of the α globin genes

The α globin genotypes were analysed using a multiplex gap-PCR technique to detect simultaneously seven common α thalassemia deletions [9]. Two common non-deletional α thalassemia due to termination codon mutations in Thailand, namely Hb Constant Spring (TAA→CAA) and Hb Pakse (TAA→TAT) were determined by a mismatched-PCR-RFLP as described [10].

3. Results

3.1. Case study

3.1.1. Family A

The first proband (P1) was a 2-year-old Thai boy who has been diagnosed with hypochromic–microcytic anemia at 8 months of age during an acute febrile illness. The patient was born as a full-term neonate with the gestational age of 40 weeks. His birth weight was 3.3 kg. The proband was referred to the Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok to evaluate his anemic symptom. Physical examination revealed that the proband had no organomegaly or other clinical symptoms suggesting severe thalassemia. The iron study was normal excluding iron deficiency anemia. Since the father was not available for the studies, we studied only the mother (M1) and revealed that she had the phenotype of β thalassemia trait (low MCV and MCH with high Hb A2) (Table 1). The patient (P1) had moderate anemia with the hemoglobin level of 9.2 g/dL and marked microcytosis (MCV 50 fL). Hemoglobin analysis in the patient revealed the presence of abnormal hemoglobin, which was eluted from the HPLC column at the same retention time as Hb E (data not shown). However, the amount of this abnormal hemoglobin was unusually higher (43.8%) than what normally observed in simple Hb E heterozygotes (27–30%) by cation exchange liquid chromatography casting some doubt on its true nature. It is possible, due to heterogeneity of several hemoglobin variants found in Thailand, that this abnormal hemoglobin may, in fact, be another variant. Using several electrophoretic techniques, we could not identify additional aberrant hemoglobin in the proband.
3.1.2. Family B

The second proband (P2) was a 3-year-old Thai boy who had history of several episodes of being more pallid during febrile illness. However the patient (P2) had never received transfusion and his anemic symptom was fully recovered after supportive therapy. His pre- and post-natal history was uneventful with the birth weight of 3.7 kg and the proband had no hepatosplenomegaly. Interestingly, the second proband also had hypochromic microcytic anemia with similar hemoglobin profile to the first proband (Hb E 44%, Table 1). The father (F2) had normal hematological parameters (MCV>80 fL) and normal level of Hb A2 while the mother (M2) had the phenotype of Hb E trait.

In family B, using the same molecular strategy, we identified a single nucleotide substitution (G → A) at codon 26 (GAG→AAG) mutation (data not shown). However the RDB technique failed to detect any of 10 common β globin mutations in Thailand in his mother (M1) suggesting that she might be a heterozygote of a rare β globin mutation. The unusual degree of severe microcytosis and higher level of Hb E than simple Hb E trait in the first proband prompted us to evaluate the β globin genes using a novel PCR-SSCP technique since it is plausible that the patient might be a compound heterozygote for Hb E and a rare β thalassemia allele inherited from the mother. Analysing six amplified fragments of the β globin genes in the polyacrylamide gel electrophoresis revealed the typical mobility shift of Hb E mutation in the β globin gene exon I in the patient (Fig. 1A). Interestingly, a novel mobility shift was also found in the exon III in the patient and the mother (P1 and M1 in Fig. 1B) suggesting that they both carried nucleotide mutation located within this exon. Indeed, the sequencing analysis of the exon III of the β globin genes from the mother and the patient demonstrated a single nucleotide substitution (GTG→GGG) at codon 126, resulting in a glycine replacement for valine at this position (V126G) (Fig. 1C). This mutation has previously been reported by Bardakdjian-Michau et al. as the underlying cause of a β globin variant namely Hb Dhonburi. Therefore in this family, the patient was a compound heterozygote for Hb E and Hb Dhonburi while the mother was Hb Dhonburi trait.

In family B, the presence of Hb E in the proband (P1) was subsequently confirmed at the molecular level by the RDB technique showing a heterozygous pattern of the codon 26 (GAG→AAG) mutation (data not shown). However the RDB technique failed to detect any of 10 common β globin mutations in Thailand in his mother (M1) suggesting that she might be a heterozygote of a rare β globin mutation. The unusual degree of severe microcytic anemia and higher level of Hb E than simple Hb E trait in the first proband prompted us to evaluate the β globin genes using a novel PCR-SSCP technique since it is plausible that the patient might be a compound heterozygote for Hb E and a rare β thalassemia allele inherited from the mother. Analysing six amplified fragments of the β globin genes in the polyacrylamide gel electrophoresis revealed the typical mobility shift of Hb E mutation in the β globin gene exon I in the patient (Fig. 1A). Interestingly, a novel mobility shift was also found in the exon III in the patient and the mother (P1 and M1 in Fig. 1B) suggesting that they both carried nucleotide mutation located within this exon. Indeed, the sequencing analysis of the exon III of the β globin genes from the mother and the patient demonstrated a single nucleotide substitution (GTG→GGG) at codon 126, resulting in a glycine replacement for valine at this position (V126G) (Fig. 1C). This mutation has previously been reported by Bardakdjian-Michau et al. as the underlying cause of a β globin variant namely Hb Dhonburi. Therefore in this family, the patient was a compound heterozygote for Hb E and Hb Dhonburi while the mother was Hb Dhonburi trait.

![Fig. 1. Molecular diagnosis of Hb Dhonburi by the PCR-SSCP analysis and direct sequencing of the β globin genes. Mobility shifts of single stranded DNA (black arrows) were observed in the β globin gene exons I and III compared to normal controls (lanes N1–N3 and N1, A).](image)

**Table 1.** Hematological data and hemoglobin analysis in 6 individuals with Hb Dhonburi and Hb E-Hb Dhonburi

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Age (year)</th>
<th>Hb (g/dl)</th>
<th>Hct (%)</th>
<th>MCV (fL)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dL)</th>
<th>Serum ferritin (µg/l)</th>
<th>Hb typing</th>
<th>α globin genotype</th>
<th>β globin genotype</th>
</tr>
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<tbody>
<tr>
<td>Family A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) M1</td>
<td>20</td>
<td>11.4</td>
<td>34</td>
<td>73.8</td>
<td>24.2</td>
<td>32.8</td>
<td>ND</td>
<td>96.5</td>
<td>αα/αα</td>
<td>β126 (T→G)/βN</td>
</tr>
<tr>
<td>(2) P1</td>
<td>2</td>
<td>9.2</td>
<td>28</td>
<td>58.1</td>
<td>18.4</td>
<td>32.3</td>
<td>125</td>
<td>50.0</td>
<td>αα</td>
<td>β126 (T→G)/βK</td>
</tr>
<tr>
<td>Family B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3) F2</td>
<td>31</td>
<td>14.6</td>
<td>43.2</td>
<td>81.4</td>
<td>27.5</td>
<td>33.8</td>
<td>ND</td>
<td>96.7</td>
<td>αα</td>
<td>β126 (T→G)/βN</td>
</tr>
<tr>
<td>(4) M2</td>
<td>30</td>
<td>11.9</td>
<td>34.4</td>
<td>78.0</td>
<td>27.0</td>
<td>34.6</td>
<td>ND</td>
<td>70.2</td>
<td>αα</td>
<td>β126 (T→G)/βN</td>
</tr>
<tr>
<td>(5) P2</td>
<td>3</td>
<td>10.4</td>
<td>31.6</td>
<td>61.0</td>
<td>20.1</td>
<td>32.9</td>
<td>89</td>
<td>55.1</td>
<td>αα</td>
<td>β126 (T→G)/βN</td>
</tr>
<tr>
<td>(6) T1 (female)</td>
<td>24</td>
<td>10.8</td>
<td>31.4</td>
<td>71.3</td>
<td>24.5</td>
<td>31.0</td>
<td>112</td>
<td>94.5</td>
<td>αα</td>
<td>β126 (T→G)/βN</td>
</tr>
<tr>
<td>(7) T2 (female)</td>
<td>30</td>
<td>11.0</td>
<td>33.2</td>
<td>82.4</td>
<td>24.0</td>
<td>32.0</td>
<td>56</td>
<td>96.0</td>
<td>αα</td>
<td>β126 (T→G)/βN</td>
</tr>
<tr>
<td>(8) T3 (male)</td>
<td>36</td>
<td>13.4</td>
<td>43.2</td>
<td>60.5</td>
<td>19.1</td>
<td>31.5</td>
<td>64</td>
<td>93.7</td>
<td>αα</td>
<td>β126 (T→G)/βN</td>
</tr>
</tbody>
</table>

* Marked microcytosis (MCV 60.5 fL) in this Hb Dhonburi heterozygote likely results from a co-inheritance of αβ thalassemia deletion (T–261). ND=not done. T = Hb Dhonburi trait.
was Hb Dhonburi heterozygote and the mother was Hb E trait. Summary of hematological analysis and the \( \alpha \) and \( \beta \) globin genotypes from both family was shown in Table 1.

3.2.1. ARMS-PCR

The presence of Hb Dhonburi was confirmed using the newly developed ARMS-PCR. In the normal PCR panel (using the normal forward primer (126-NF)), a 373 bp fragment representing the amplification from normal allele was presented in all samples studied (C, M1, P1, F2, M2 and P2, Fig. 2). However, using the mutant primer (126-MF), a 373 bp fragment in which is specific to the T\( \rightarrow \)G mutation in Hb Dhonburi has been amplified successfully in the patients (P1 and P2), the mother from family A (M1) and the father from family B (F2) but not in the control (C) and the mother from family B (M2). In both PCR panels, a 457-bp internal control PCR fragment of the UGT1A1 gene was successfully amplified. This technique has been applied to three individuals who have been identified through our thalassemia-screening programme in pregnant women at Siriraj hospital. All had hypochromasia and microcytosis with raised thalassemia. Moreover, they both had no organomegaly with normal growth and height development. In addition, no facial bony change, specific to thalassemia (thalassemic facie) was observed. This finding is consistent with our previous observation that the type of \( \beta \) thalassemia is a primary genetic factor significantly determining the clinical severity in patients with Hb E/\( \beta \) thalassemia; cases with Hb E/\( \beta \) thalassemia usually have a milder phenotype [17]. This information will be of importance as a guideline for appropriate genetic counselling and risk estimation in future cases and couples at risk.

Heterozygotes for Hb Dhonburi appeared to have mildly hypochromic microcytosis with, in most cases, slightly increased levels of Hb A2 (>3.5%), typical for \( \beta \) thalassemia trait. However a borderline level (3.0–3.3%) of Hb A2 was recently observed in Hb Dhonburi trait in an Iranian family. The variation in the Hb A2 levels, reflecting differences on the \( \delta \) globin output, may result from additional undetermined mutations or polymorphism(s) associated with a down-regulation of the \( \delta \) globin gene expression as suggested by Grosso et al. [15]. In their study, the Italian proband who had a relatively low level of Hb A2 was found to coinherit a \( \delta ^{+27(G\rightarrow T)} \) mutation in trans to the Hb Dhonburi mutation [15]. In our study, the variation in Hb A2 levels in Hb Dhonburi carriers was also observed ranging from 3.3% to 5.8% (Table 1). This warrants a further study on the \( \delta \) globin genes and its regulatory sequences in these individuals to resolve this obscure observation. It also highlights the possible pitfalls in genetic counselling in regions where globin gene disorders are prevalent since Hb Dhonburi carriers who have mild hypochromic–microcytic anaemia with borderline Hb A2 might have been misdiagnosed as being nutritional or iron deficiency anaemia. Therefore, the molecular variations might originate from either the East or the West origins or by a de novo mutation [12–16].

The valine to glycine substitution causing Hb Dhonburi does not result in any change in the net charge of the hemoglobin molecule and thus produces a novel \( \beta \) globin variant with biochemical properties similar to Hb A [1,2] Therefore, it was not possible to distinguish the Hb Dhonburi from Hb A on a standard electrophoresis, iso-electric focusing or HPLC. However this amino acid substitution was expected to introduce a difference in the \( \alpha \)-helical conformation and destabilise the whole structure of the \( \beta \) globin chain causing Hb Dhonburi instability [2]. Together with the unstable nature, Hb Dhonburi mutation might also activate a cryptic splice site in the sequence region from codons 123 to 126 (5′ACC CCA CCA G/TG 3′→5′ACC CCA CCA G/GG 3′) [2]. This should result in a reduction of normal \( \beta \) globin transcripts by similar mechanism to Hb E, Hb Malay and Hb Knossos [3]. Although the direct measurement of total and nascent mRNAs from individuals with Hb Dhonburi to confirm the proposed hypothesis has not yet been documented. By all these mechanisms, Hb Dhonburi was, thus far, classified as \( \beta ^{+} \) thalassemia.

Compound heterozygote for Hb E and Hb Dhonburi have a rather mild phenotype since both patients had relatively higher baseline hemoglobin compared to that of other patients with Hb E/\( \beta \) thalassemia. Moreover, they both had no organomegaly with normal growth and height development. In addition, no facial bony change, specific to thalassemia (thalassemic facie) was observed. This finding is consistent with our previous observation that the type of \( \beta \) thalassemia is a primary genetic factor significantly determining the clinical severity in patients with Hb E/\( \beta \) thalassemia; cases with Hb E/\( \beta ^{+} \) thalassemia usually have a milder phenotype [17]. This information will be of importance as a guideline for appropriate genetic counselling and risk estimation in future cases and couples at risk.
study is the only measure to identify this rare and ‘silent’ β globin variant.

As shown in this study, we demonstrated, as a further proof of the principle, that the PCR-SSCP could be applied to identify unknown or rare hemoglobin variants and also indicate the coding regions in which the mutation(s) is residing. The PCR-SSCP technique could minimise the cost required for direct genomic sequencing of the whole β globin genes to a smaller region(s) suggested by this method. This technique may also be used for family linkage analysis of unidentified β globin mutants segregated in the affected family. Application of combined molecular approach described in this report will be useful as a guideline for identification and characterization of future cases with abnormal hemoglobin variants especially in Southeast Asian population where both thalassemia and hemoglobinopathies are highly prevalent, before proper genetic counselling and risk estimation can be given.

Acknowledgement

Vip Viprakasit was supported by Thailand Research Fund (TRF-Research Scholar) and by the Siriraj Grant for Research Development and Medical Education, the Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand. We thank Emeritus Professor Voravarn S. Tanphaichitr for her long-term support and encouragement.

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