Molecular and haematological characterization of compound Hb E/Hb Pyrgos and Hb E/Hb J-Bangkok in Thai patients

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Summary
We describe haematological and DNA characterization of haemoglobinopathies in Thai adolescents caused by compound heterozygosities for Hb E [β26(B8) Glu-Lys] and two other β-globin chain variants, Hb Pyrgos [β83(EF7) Gly-Asp] and Hb J Bangkok [β56(D7) Gly-Asp]. Hb analysis demonstrated that although these two β-chain variants have separated elution profiles on liquid chromatography-based Hb analysis, they have similar alkaline electrophoretic mobilities on cellulose acetate electrophoresis. Haematological data associated with these two previously undescribed conditions were compared with those of pure carriers of the variants found in other unrelated Thai individuals. β-Globin gene haplotypes linked to these two β-chain variants and a simple DNA testing based on multiplex allele-specific polymerized chain reaction for differential diagnosis are presented.

Keywords
Hb E, Hb Pyrgos, Hb J Bangkok, Multiplex-allele-specific PCR, β-globin haplotype

Introduction
Many Hb variants have been characterized worldwide (Weatherall & Clegg, 2001). In Thailand and other Asian countries, both thalassaemia and Hb variants are very common. α-Thalassaemia has a frequency of 20–30% whereas that of β-thalassaemia ranges from 3 to 9%. The average frequency of Hb E is approximately 13% (but rises to nearly 50% in the north-east) and that of the Hb Constant Spring varies between 1 and 8% (Fucharoen & Winichagoon, 1987). Other abnormal Hbs caused by both α-chain and β-chain variants are occasionally reported (Svasti et al., 1999). Therefore, patients with complex phenotypes caused by these gene to gene interactions in the same individuals are not uncommon. While most variants can usually be identified by Hb electrophoresis or high-performance liquid chromatography (HPLC), other uncommon ones should be confirmed by DNA analysis. Using a combination of Hb electrophoresis, automated liquid chromatography and DNA analysis, we have characterized two cases of Thai patients with compound heterozygosities for Hb E [β26(B8) Glu-Lys] and two other β-chain variants with the same amino acid substitution, i.e. Hb Pyrgos [β83(EF7) Gly-Asp] (Tatsis, Sofroniadou & Stergiopoulos, 1976) and Hb J Bangkok [β56(D7) Gly-Asp] (Pootrakul, Gray & Dixon, 1970). Haematological features of the patients were compared with those of pure carriers of the variants and other combinations found in unrelated Thai subjects. β-Globin gene haplotypes associated with these variants and a rapid DNA test for their differential diagnosis are also described.

Materials and methods

Subjects and haematological analysis
The two probands (P1 and P2) were pregnant Thai women who were found in our ongoing thalassaemia and haemoglobinopathy screening programme as they carried Hb E (Fucharoen et al., 2004). Both of them were in good general health. Haematological data was collected using an automated blood cell counter (Coulter STKS; Beckman-Coulter Co., Fullerton, CA, USA). Hb analysis was carried out by standard cellulose acetate electrophoresis at pH 8.6
and using a low-pressure liquid chromatography (LPLC) automated Hb analyser (Hb Gold; Drew Scientific, Cumbria, UK) as shown in Figure 1.

**DNA analysis**

Genomic DNA was prepared from peripheral blood leucocytes. β-Globin gene analysis was performed by the PCR and direct DNA sequencing as has been described (Fucharoen et al., 1989). α-Globin genotyping was carried out with PCR methods described elsewhere (Fucharoen & Fucharoen, 1994; Fucharoen et al., 2002, 2003). Seven polymorphic restriction sites in the β-globin gene cluster, including the HincII 5′ to the e-globin gene, the HindIII sites in the Cγ and Aγ-globin genes, the HincII sites in the ψβ-globin gene and its 3′ region, and the AatII and BamHI sites in the β-globin gene and its 3′ region, were determined by PCR analysis followed by restriction digests as has been described (Fucharoen et al., 2001). The multiplex allele-specific PCR approach for differential diagnosis of Hb Pyrgos and Hb J-Bangkok was developed as shown in Figure 2. With this simultaneous detection system, primers G32 (5′-CAC CTG GAC AAC CTC AAG GA-3′) and G36 (5′-ACT CCT GAT GCT GTT ATG GA-3′) located respectively at codons 83 and 56 of the β-globin gene were used as allele-specific primers for the βPyrgos (codon 83; GGC → GAC) and the βJ-Bangkok (codon 56; GGC → GAC) mutations to produce specific fragments of 89 bp and 179 bp with a common primer S3 (5′-TCC CAT AGA CTC ACC CTG AA-3′). In each reaction tube, as an internal control for amplification, two additional primers with the sequences (5′–GGC CTA AAA CCA CAG AGA GT-3′) and (5′-CCA GAA CGG AGT GTG TGG AA-3′), which produce the 578 bp fragment of the Cγ-globin gene promoter were also included (Fucharoen, Shimizu & Fukumaki, 1990). The multiplex PCR reaction mixture (50 µl) contains 50–200 ng genomic DNA, 15 pmol of each primer, 200 µm dNTPs and 1-unit Taq DNA polymerase (Promega Co., Madison, WI, USA) in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.01% gelatine and 3 mM MgCl2. The amplification reaction was carried out in a DNA Thermal Cycler 480 (Perkin-Elmer, Wellesley, MA, USA). A total of 30 cycles after initial heating at 94 °C for 3 min was performed under the following PCR conditions: 94 °C for 1 min and 65 °C for 1.5 min. The amplified product was analysed on 2% agarose gel electrophoresis and visualized under UV light after ethidium bromide staining.

**Results**

The two probands, P1 and P2, tested positive in our ongoing screening for thalassaemia and haemoglobinopathy using a combination of a one-tube osmotic fragility...
OF) test and a modified dichlorophenolindophenol (DCIP) precipitation test (Fucharoen et al., 2004). Further investigations were therefore carried out. Haematological data is listed in Table 1. P1 presented with slight anaemia and hypochromic microcytosis with Hb 9.5 g/dl, MCV 76.5 fl and MCH 23.3 pg whereas P2 had nearly normal haematological data with Hb 12.3 g/dl, MCV 79.7 fl and MCH 26.9 pg. The higher red blood cell distribution width (RDW) value of P1 (21.2%) likely reflected a pregnancy-associated iron depletion. Unfortunately, serum ferritin and other iron parameters are not available. Further Hb analysis with cellulose acetate electrophoresis at pH 8.6 demonstrated no Hb A but in addition to Hb E, similar Hb variants migrated more anodic to that of Hb A (Figure 1a, lane 2) in both patients. As no other abnormal band was observed, these abnormal Hbs were most likely β-globin

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Figure 2. A simultaneous multiplex allele-specific polymerase chain reaction (PCR) for differentiation of Hb Pyrgos and Hb J Bangkok and a representative gel electrophoresis. The locations and orientations of primers (G32 and S3) and (G36 and S3) that produce fragments of 89 bp and 179 bp, specific for Hb Pyrgos and Hb J Bangkok are indicated. The 578 bp fragment of δ-γ-globin gene promoter is an internal control for the PCR amplification. Lanes 1 and 2, normal controls; lanes 3 and 4, Hb J Bangkok carriers; lane 5, Hb Pyrgos carrier. M represents the λ/Hind III size markers.

Table 1. Haematological data of Thai subjects with compound heterozygosities for Hb E/Hb Pyrgos and Hb E/Hb J-Bangkok as compared to carriers of Hb Pyrgos and Hb J-Bangkok and other combinations

<table>
<thead>
<tr>
<th></th>
<th>P1</th>
<th>P2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>1</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Sex/Age</td>
<td>F/21</td>
<td>F/18</td>
<td>F/25</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>9.5</td>
<td>14.0 ± 1.2</td>
<td>11.1</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>31.5</td>
<td>43.6 ± 5.7</td>
<td>35.0</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>76.5</td>
<td>88.1 ± 8.3</td>
<td>68.4</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>23.3</td>
<td>28.4 ± 1.9</td>
<td>32.0</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>30.4</td>
<td>32.3 ± 2.4</td>
<td>–</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>21.2</td>
<td>13.1 ± 0.7</td>
<td>–</td>
</tr>
<tr>
<td>Hb E (%)</td>
<td>26.5</td>
<td>none</td>
<td>19.4</td>
</tr>
<tr>
<td>Abnormal Hb (%)</td>
<td>71.2</td>
<td>53.3 ± 5.0</td>
<td>73.9</td>
</tr>
<tr>
<td>α-genotype</td>
<td>γA/γA</td>
<td>γA/γA</td>
<td>γA/γA</td>
</tr>
<tr>
<td>β-genotype</td>
<td>ββ/βPyrgos</td>
<td>ββ/βPyrgos</td>
<td>ββ/βJ-BKK</td>
</tr>
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</table>

Values are presented as raw data or as mean ± SD where appropriate.
F, female; M, male. Data are from *Sawangareetrakul et al. (2002) and Jetsrisuparb et al. (2002).
chain variants. The amounts of Hb E of the two probands are, however, different, i.e. 26.5% in P1 and 32.3% in P2 respectively. To define the molecular defects causing these Hb variants, β-globin genes of the two probands were characterized by PCR and direct DNA sequencing. With this analysis, in addition to the β6 mutation (codon 26; GAG → AAG) in both cases, we detected the GGC → GAC mutation at codon 83 in P1 and the GGC → GAC at codon 56 in P2, both of which lead to the substitution of Asp for Gly, previously described for the Hb Pyrgos (Tatsis et al., 1976) and the Hb J Bangkok (Pootrakul et al., 1970) respectively. P1 and P2 were therefore compound heterozygotes for Hb E/Hb Pyrgos and Hb E/Hb J Bangkok, respectively. Although these two β-chain variants have the same electrophoretic mobilities, they indeed have different elution profiles when analysed on an automated LPLC Hb analyser (Figure 1b). Further DNA polymorphic analyses in the β-globin gene clusters of the two probands, including the seven restriction sites described earlier, demonstrated that βPyrgos and βBangkok detected in these Thai individuals were associated with haplotypes (+ - - - - -+) and (+ - - - + +), respectively. No α-thalassaemia gene was identified in both cases.

To facilitate the diagnoses of the two variants, we developed a multiplex allele-specific PCR shown in Figure 2. allele-specific primers, G32 and G36 located respectively at codons 83 and 56 of the β-globin gene were used with a common primer S3 to produce the 89 bp βPyrgos and the 179 bp βBangkok specific fragments. As shown in Figure 2, although the 578 bp internal control was found in all samples tested, the two specific fragments were observed only in samples with the two variants. The results indicated that this simple DNA assay can be used as a confirmatory test and for differential diagnosis of Hb Pyrgos and Hb J Bangkok in a routine laboratory. With this technique, we were able to further identify six carriers of Hb Pyrgos and eight carriers of Hb J Bangkok in other unrelated Thai individuals whose haematological findings are also presented in Table 1.

Discussion

The most common β-thalassaemic haemoglobinopathy is Hb E [β26(B8) Glu-Lys] in South-east Asia whereas Hb J Bangkok [β56(D7) Gly-Asp] and Hb Pyrgos [β83(EF7) Gly-Asp] are relatively rare nonpathological β-chain variants reported in a diverse ethnic group. Hb Pyrgos was first reported in a 3-year-old Greek boy with Hb S [β6(A3)Glu-Val] (Tatsis et al., 1976), and later found in Japanese (Yamada et al., 1977), Malian (Wajcman et al., 1978), Sicilian (Schilliro et al., 1991), Chinese (Qin et al., 1994) and Thai (Fucharoen et al., 1997). Hb J Bangkok (also known as Hb J-Meinung) was first described in a Chinese–Canadian newborn (Pootrakul et al., 1970) and found occasionally in Black Americans (Honig et al., 1982), Japanese (Iuchi et al., 1982), Taiwanese (Chang et al., 2002) and Thai (Fucharoen et al., 2001). In heterozygous forms, these two β-chain variants are not associated with disease but homozygous or compound heterozygous states with other haemoglobinopathies and thalassaemias can cause clinical conditions (Fucharoen et al., 1997, 2001; Chang et al., 2002; Jetsrisuparb et al., 2002; Sawangareetrakul et al., 2002). It is therefore important to distinguish these Hb variants from other α and β-chain variants with less or no clinical significance.

We have described the hitherto undescribed conditions in which these two β-chain variants were found in combination with Hb E in two unrelated Thai individuals. Obviously, association of these two variants with Hb E does not lead to serious clinical conditions. As shown in Table 1, P1 who had compound heterozygosity for Hb Pyrgos/Hb E presented with only mild hypochromic microcytosis with slightly reduced MCV and MCH values whereas P2, a compound Hb J Bangkok/Hb E was apparently normal. The major components of Hb Pyrgos (71.2%) and Hb J Bangkok (67.7%) in both cases correlate with other Hb E traits in which Hb E is a minor fraction. Both βPyrgos and βBangkok are relatively more negative than the β6 chain and thus combine more readily with a relatively positive α-chain on the αβ-subunit formation. Difference in the levels of Hb E of the two probands (26.5% in P1 vs. 32.3% in P2), however, indicates that although both variants are caused by the same amino acid substitution, the effects on Hb subunit formation of the two mutations differ. As shown in Table 1, further reduction in the levels of Hb E (19.4–25.5%) were noted previously when the compound Hb E/Hb Pyrgos was found in association with α-thalassaemia and Hb Constant Spring, the conditions with limited availability of α-chain (Jetsrisuparb et al., 2002; Sawangareetrakul et al., 2002). The higher level of Hb E in P2 (32.3%) when compared with P1 (26.5%) probably indicates that the positive α-chain combines more effectively with the βPyrgos than the βBangkok. The levels of the two Hb in heterozygous states in Table 1 (53.3 ± 5.0% for Hb Pyrgos and 44.5 ± 4.7% for Hb J Bangkok) indirectly support this.

Although both Hb Pyrgos and Hb J Bangkok have been found in various populations, the data on their origins remain to be elucidated. The findings of βPyrgos with a single haplotype (+ - - - - - +) and βBangkok with haplotype (+ - - - + +) in this study and in previous reports for other Thai individuals (Fucharoen et al., 1997, 2001) indicate...
the same origin of these two variants in the Thai population. It is not certain, however, that these Thai β-chain variants have the same origins as those reported in other populations because haplotype information is not available in those population groups.

It is conceivable that both Hb Pyrgos and Hb J Bangkok may not be uncommon in populations and their interactions with other haemoglobinopathies may lead to complex phenotypes. However, as shown in Figure 1, laboratory diagnosis of these two Hb variants may be problematic in routine investigation as they have similar mobilities on a routine alkaline cellulose acetate electrophoresis. Carriers of these variants also have normal haematological phenotypes as shown in Table 1. Direct detection of the mutations causing these Hb variants by DNA analysis would be another diagnostic alternative. The multiplex allele-specific PCR approach shown in Figure 2 will prove useful in complementing routine HB analysis for a definitive diagnosis of these variants. Our result also supports that when possible, haemoglobinopathies could be easily diagnosed by the PCR-based method without the need for studies at the protein level which would greatly facilitate a programme of haemoglobinopathy screening in the region.

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References


