Molecular basis and hematologic characterization of δβ-thalassemia and hereditary persistence of fetal hemoglobin in Thailand

Background and Objectives. Hereditary persistence of fetal hemoglobin (HPFH) and δβ-thalassemia are heterogeneous disorders characterized by increased levels of Hb F in adult life. The distinction between these two conditions is not always possible from routine hematologic analyses. This study investigated the hematologic and molecular characteristics of high Hb F determinants in Thailand, and describes a rapid DNA-based assay to facilitate diagnosis in a routine laboratory.

Design and Methods. A multiplex allele-specific polymerase chain reaction (PCR) system for rapid detection of three common DNA deletions causing (δβ)0-thalassemia and HPFH in South-east Asians was developed and used to examine the molecular basis for the high Hb F phenotypes in 273 unrelated Thai individuals. Hematologic data were recorded and correlated to the molecular findings.

Results. The multiplex PCR system was validated and results were completely concordant with those of other established methods. DNA analysis identified (γδβ)0-thalassemia in 148 cases (54.2%), deletional HPFH-6 in 83 (30.4%) and the deletion-inversion (γδβ)0-thalassemia in 22 (8.1%) cases, while another 20 (7.3%) subjects remained uncharacterized. Genotype-phenotype relationships are discussed.

Interpretation and Conclusions. These data emphasize the high frequencies of δβ-thalassemia and HPFH in Thailand and the need for differential diagnostic methods since the hematologic parameters associated with the conditions are very similar and overlap. The multiplex allele-specific PCR approach should prove useful in complementing routine Hb analysis for the differential diagnosis of these three common causes of high Hb F determinants and should facilitate a program of hemoglobinopathy screening in the region.

Key words: δβ thalassemia, HPFH-6, deletion-inversion, multiplex PCR.
Molecular diagnosis could be established in 253 patients (92.7%) whose hematologic phenotypes are presented and compared.

**Design and Methods**

**Subjects and hematologic analysis**

DNA samples with known (δβ)δ-thalassemia, deletion-inversion γ(γδβ)γ-thalassemia and HPFH-6 mutations from our earlier studies3-5 were used to establish a multiplex allele-specific PCR. Additional blood specimens, anticoagulated with EDTA, were taken from 273 unrelated Thai individuals whose Hb analysis suggested the possibility of their being δβ-thalassemia or HPFH carriers (Hb A:FA pattern). These selectively recruited, after informed consent, from our ongoing thalassemia screening program. They were in good general health and had developed normally. Fifty-eight normal individuals served as controls. Hb concentration and erythrocyte indices were determined using the Coulter STKS automated blood cell counter (Coulter Electronics, Hialeah, Fla., USA). Hb analysis was carried out using an automated high performance liquid chromatography (HPLC) Hb analyzer (VARIANT®; Bio-Rad Laboratories, Hercules, CA, USA). Globin chain analysis was performed using Triton urea gel electrophoresis as described previously. Hematologic data were compared statistically with the non-parametric Kruskal-Wallis test using Minitab statistical software (Minitab Inc., State College, PA, USA).

**DNA analysis and development of a multiplex system to identify (δβ)δ-thalassemia and HPFH**

Genomic DNA was prepared from peripheral blood leukocytes using the standard method. Individual globin gene deletions causing (δβ)δ-thalassemia, γ(γδβ)γ-thalassemia and HPFH-6, previously found in Thai patients, were identified using PCR methodology as described elsewhere.26 α-globin genotyping was also examined using previously described PCR methodology.9,10 Direct DNA sequencing of the amplified DNA was carried out using an ABI Prism 377 automated DNA sequencer (Perkin-Elmer Biosystem, Norwalk, Conn., USA). In order to provide a more rapid characterization of these high Hb F determinants, we also developed a multiplex PCR system as shown in Figure 1. With this simultaneous detection system, primers (T1; 5′ GTGGACGCGCATGCTAAAGA 3′ and T2; 5′ATCCACTGAACACCGAAGC 3′), (SF6; 5′ GCCCTAAATGCGAGATTG 3′ and F13; 5′GATAGGCATG 3′), (T1 & T3) and (SF6 and F13) were used to produce the 1,027 bp, 1,925 bp and 793 bp specific for the HPFH-6, the (δβ)δ-thalassemia and the γ(γδβ)γ-thalassemia deletions, respectively. With this system, the 1,145 bp fragment generated from primers δ1 and F13 can be used as an internal control of the PCR amplification. The multiplex PCR reaction mixture (50 µL) contains 50-200 ng genomic DNA, a specific amount of each primer (i.e. 45 pmol of δ1 and δ2, 30 pmol of T1 and T3 and 15 pmol of SF6 and F13), 200 µM dNTPs and 1 unit Taq DNA polymerase (Promega Co., Madison, WI, USA) in 10 mM Tris-HCl (pH 8.3), 50
The amplification reaction was carried out in a DNA Thermal Cycler 480 (Perkin-Elmer, Wellesley, MA, USA). After initial heating at 94°C for 3 minutes, 35 cycles were performed under the following PCR conditions: 93°C for 1 min, 60°C for 1 min and 72°C for 1.5 min with an additional final extension at 72°C for 10 min. The amplified product was analyzed on 1.5% agarose gel electrophoresis and visualized under UV light after ethidium bromide staining.

Results

Figure 1 demonstrates a successful application of the multiplex allele-specific PCR for simultaneous detection of the three high Hb F determinants previously found in Thailand i.e. the $\delta\beta$-thalassemia caused by a 12.5 kb deletion, the HPFH-6 caused by a 101 kb deletion, and the $\gamma(A\gamma\delta\beta)$-thalassemia caused by a deletion-inversion. As shown in Figure 1 lanes 1-4, while the 1,415 bp normal fragment generated from primers (81 and F13) was detected in all subjects tested, the fragments of 1,925 bp, 1,027 bp and 793 bp were specifically observed for the three determinants, respectively. The detection system was applied to a Thai family in which the father (lane 5), the mother (lane 6) and the sister (lane 7) and the proband (lane 8) were known to be HPFH-6 carriers, deletion-inversion $\gamma(A\gamma\delta\beta)$-thalassemia carriers and to have compound HPFH-6/deletion-inversion $\gamma(A\gamma\delta\beta)$-thalassemia, respectively. As shown in Figure 1, lanes 5-8, the multiplex allele-specific PCR approach genotyped these individuals correctly.

The multiplex PCR system was then used to characterize the high Hb F determinants in 273 additional Thai carriers and 58 normal control subjects. Among the 273 subjects examined, the molecular defects could be identified in 253 (92.7%) cases whose hematologic data are presented in Table 1. All normal control subjects were negative in the multiplex PCR test. Twenty samples (7.3%) remain uncharacterized. The results of this analysis were completely concordant with those obtained using the established methods based on the multiplex PCR described previously. The most common high Hb F determinant was found to be $\delta\beta$-thalassemia, which was detected in 148 (58.6%) of the cases, followed by the HPFH-6 and the deletion-inversion $\gamma(A\gamma\delta\beta)$-thalassemia, which were detected in 83 (32.8%) and 22 (8.6%) subjects, respectively. The hematologic data in Table 1 demonstrate that in their heterozygous forms, all the three high Hb F determinants are associated with hematologically mild phenotypes. All subjects had high Hb F levels but normal levels of Hb A2. The highest Hb F levels were observed among the HPFH-6 heterozygotes (24.6±4.4%) whereas in $\delta\beta$-thalassemia and deletion-inversion $\gamma(A\gamma\delta\beta)$-thalassemia heterozygotes the Hb F levels were 20.6±5.6% and 19.6±3.0%, respectively. Globin chain analysis demonstrated, as expected, that the Hb F subunits in $\delta\beta$-thalassemia consisted of both $\gamma\gamma$ and $\alpha\alpha$ whereas in HPFH-6 and deletion-inversion $\gamma(A\gamma\delta\beta)$-thalassemia, the $\gamma\gamma$ globin was predominant. There were statistically significant differences in MCV, MCH, RDW-CV and the level of Hb F between the

<table>
<thead>
<tr>
<th>Parameters</th>
<th>$(\delta\beta)$-thal</th>
<th>HPFH-6</th>
<th>Deletion- Inversion</th>
<th>Difference</th>
<th>Normal</th>
</tr>
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<tbody>
<tr>
<td>N. (%)</td>
<td>148 (58.6)</td>
<td>83 (32.8)</td>
<td>22 (8.6)</td>
<td>–</td>
<td>58</td>
</tr>
<tr>
<td>Sex (male/female)</td>
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<td>16/67</td>
<td>2/20</td>
<td>–</td>
<td>21/37</td>
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<td>Age (year)</td>
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<td>1.3–61</td>
<td>4–61</td>
<td>–</td>
<td>20–33</td>
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<tr>
<td>Rbc (x10^6/mm³)</td>
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<td>4.7±0.6</td>
<td>4.9±0.6</td>
<td>–</td>
<td>4.5±0.4</td>
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<tr>
<td>Hb (g/dL)</td>
<td>11.5±1.5</td>
<td>12.3±1.4</td>
<td>12.1±1.3</td>
<td>–</td>
<td>13.3±1.4</td>
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<tr>
<td>Hct (%)</td>
<td>35.7±4.5</td>
<td>37.5±4.4</td>
<td>37.3±3.6</td>
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<td>39.8±4.0</td>
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<td>MCV (FL)</td>
<td>75.4±7.0</td>
<td>79.9±6.8</td>
<td>76.4±5.6</td>
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<td>87.9±3.6</td>
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<td>MCH (pg)</td>
<td>24.4±2.0</td>
<td>25.7±2.2</td>
<td>24.3±1.9</td>
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<td>29.5±1.6</td>
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<td>MCHC (g/dL)</td>
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<td>RDW-CV (%)</td>
<td>20.5±2.4</td>
<td>16.6±2.7</td>
<td>16.8±2.4</td>
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<td>Hb A₂ (%)</td>
<td>2.2±0.4</td>
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<td>2.3±0.3</td>
<td>–</td>
<td>2.5±0.4</td>
</tr>
<tr>
<td>Hb F (%)</td>
<td>20.6±5.6</td>
<td>24.6±4.4</td>
<td>19.6±3.0</td>
<td>&lt; 0.001</td>
<td>&lt; 1.0</td>
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<tr>
<td>Hb F subunit</td>
<td>$\gamma$ &amp; $\gamma$</td>
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<td>$\gamma$</td>
<td>–</td>
<td>–</td>
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</table>
three groups \((p < 0.001)\). The MCV and MCH values in the HPFH–6 carriers were closer to normal levels than were those of the other two forms of thalassemia. However, it is difficult to distinguish the different conditions based on hematologic data as all values overlapped. This result indicates the need for another differential diagnostic method and the usefulness of the molecular diagnostic approach used in this study.

**Discussion**

The distinction between HPFH and \(\delta\beta\)-thalassemia is subtle and is made on clinical and hematologic grounds. HPFH is usually characterized in heterozygotes by higher levels of Hb F (up to 30\%) with normal red cell indices while heterozygotes for \(\delta\beta\)-thalassemia tend to have a modest elevation of Hb F (5–20\%) with hypochromic microcytic red cell indices. Individuals with these disorders exhibit milder clinical symptoms than those with typical \(\beta\)-thalassemia, due to the beneficial effect of Hb F on red blood cell production and survival.\(^{1,11,13}\) Two types of the determinants, namely deletional and non-deletional forms, have been classified on the basis of molecular studies.\(^{10,15}\)

In this study, we examined the molecular basis and the frequencies of high Hb F determinants in Thai population using a multiplex allele-specific PCR for three types of DNA deletion. As shown in Figure 1 and Table 1, this approach was able to define the molecular defects in most of the cases examined (253 of 273 subjects; 92.7\%). The most common defect was the \(\delta\beta\)-thalassemia which was detected in 148 cases (58.6\%). Further DNA sequence analysis revealed that this mutation was caused by a 12.5 kb deletion extending from IVSII of the \(\delta\)-globin gene to the L1 repeat element located on 3' of the \(\beta\)-globin gene, but leaving intact \(\gamma\) and \(\gamma\)globin genes, thus removing part of the \(\delta\)-globin gene and the entire \(\beta\)-globin gene, exactly as described previously.\(^{16}\) Similar DNA deletions have been described, based on restriction endonuclease mapping, for other Thai,\(^{17}\) Malaysian\(^{18}\) and Cantonese\(^{19}\) families with the \((\gamma\delta\beta)\)-thalassemia phenotypes. Sequencing of the breakpoint junctions in these families will be necessary to confirm the assumption that all these deletions are identical to those detected in this study. There has been doubt as to whether these deletions should be considered as a \((\gamma\delta\beta)\)-thalassemia or as an HPFH determinant. The higher Hb F levels than in other forms of \((\gamma\delta\beta)\)-thalassemia and the pancellular distribution of Hb F found in these families add weight to the latter hypothesis. The hematologic phenotypes of Thai carriers of this deletion, with near normal red blood cell indices as shown in Table 1, indirectly confirm that this DNA deletion is indeed responsible for a HPFH phenotype rather than \((\gamma\delta\beta)\)-thalassemia. In addition, an enhancer-like sequence located 521 bp downstream of the 3' breakpoint of this deletion was identified and led to a new description of this form of deletion as HPFH–6.\(^{12}\) The deletion enables the 3' enhancer element to be juxtaposed in close proximity with the \(\gamma\)globin gene; thereby increasing expression.

The last mutation, observed in 22 individuals (8.6\%), was the deletion inversion type \((\gamma\delta\beta)\)-thalassemia. The lesion was caused by a complex rearrangement within the \(\beta\)-globin cluster, involving the deletion of 0.9 kb of DNA around the \(\gamma\)globin gene, the loss of 7.5 kb DNA in and between the \(\delta\)- and \(\beta\)-globin genes, and inversion of the DNA remaining between the \(\gamma\) and \(\delta\)-globin genes. Hematologic data in Table 1 demonstrate that, in the heterozygous form, this mutation is associated with a phenotype similar to that of the 12.5 kb deletional \((\delta\beta)\)-thalassemia except that the predominant Hb F subunit is the \(\gamma\)globin. This complex double deletion–inversion rearrangement \((\gamma\delta\beta)\)-thalassemia appears to be a common form of \(\delta\beta\)-thalassemia found in central Asia, including in India,\(^{20,21}\) Kuwait,\(^{22}\) and Iran.\(^{11}\) Identification of this mutation in Thailand suggests that this \((\gamma\delta\beta)\)-thalassemia may have originated in India or South Asia and then become widespread in the Middle East and South-east Asia.

Co-inheritance of \(\delta\beta\)-thalassemia or HPFH with increased production of \(\gamma\)-chains has an ameliorating effect on the clinical picture of \(\beta\)-thalassemia disease.\(^{12}\) The differential diagnosis of these conditions is therefore important for providing appropriate treatment and genetic counseling to the patient. This diagnosis usually requires both a family study and hematologic analysis. However, as shown in Table 1, there is some degree of overlap in hematologic data between \(\delta\beta\)-thalassemia and HPFH and the level of Hb F alone cannot differentiate the two conditions. Direct DNA analysis of the molecular defect is there-
fore preferable. The multiplex PCR approach used in this study will prove useful to complement routine hemoglobin analysis in determining the genotype properly and will facilitate prevention and control programs of thalassemia and hemoglobinopathies in the region.

SP, SF, GF and KS were involved in the hematologic and DNA analysis of the patients, and setting up the multiplex PCR for detection of the high Hb F determinants observed. They were also involved in the statistical analysis and reading the manuscript. SS is the laboratory hematologist involved in the recognition and initial hematologic analysis of the subjects.

References