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

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SITTHICHAI PANYASAI
SUPAN FUCHAROEN
SATJA SURAPOT
GOONNAPA FUCHAROEN
KANOKWAN SANCHAISURIYA

A B S T R A C T

**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 HbF 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 $(\delta\beta)^0$-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 $^{G}\gamma^{A}\gamma(\delta\beta)^0$-thalassemia in 148 cases (54.2%), deletional HPFH-6 in 83 (30.4%) and the deletion-inversion $^{G}\gamma(^{A}\gamma\delta\beta)^0$-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.

*From the Graduate School (SP), Department of Clinical Chemistry (SP, SF) and Department of Clinical Microscopy, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand (GF, KS), Department of Clinical Pathology, Maharaj Hospital, Nakornsrithammaraj, Thailand (SS), Center for Research and Development of Medical Diagnostic Laboratories, Khon Kaen University, Khon Kaen, Thailand (SP, SF, GF).*

*Correspondence: Dr. Supan Fucharoen, Department of Clinical Chemistry, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand. E-mail:supan@kku.ac.th*

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The level of fetal Hb (Hb F) normally declines during early childhood to less than 1% of total Hb but in some genetic conditions increased levels of Hb F are found in adult life.[1] Hereditary persistence of fetal Hb (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. Molecular analysis of these conditions has demonstrated many deletional and non-deletional types of the two conditions.[2] Although the deletional forms of HPFH and δβ-thalassemia are less common than the non-deletional forms, only deletional forms have recently been reported in Thailand. These include the 12.5 kb deletional δβ-thalassemia, the 101 kb deletional HPFH-6 and the Indian deletion-inversion $^{G}\gamma(^{A}\gamma\delta\beta)$-thalassemia, which have been found in pure heterozygotes or in association with Hb E or β-thalassemia.[3-6] As the clinical and hematologic phenotypes of these three different causes of high Hb F determinant in heterozygotes are quite similar, the diagnosis usually relies on molecular identification of the globin gene defects. In order to provide data on the frequency distributions of these high Hb F determinants and to correlate genotype-phenotype relationships, we extensively examined the molecular defects causing δβ-thalassemia and HPFH in the Thai population. A simple, multiplex polymerase chain reaction (PCR) assay for simultaneous detection of the three common high Hb F determinants was developed and used to screen a group of 273 unrelated Thai individuals with δβ-thalassemia or HPFH heterozygote phenotypes, the largest series to

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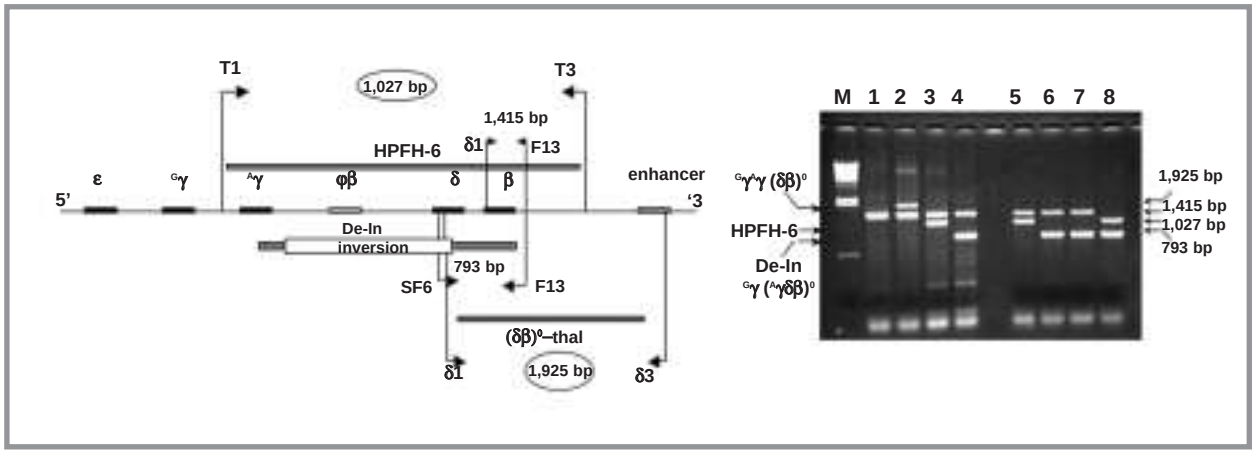


**Figure 1. A simultaneous multiplex allele-specific PCR for identifying $(\delta\beta)^0$-thalassemia, HPFH-6 and deletion-inversion $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia. A: the locations and orientations of primers (δ1 and δ3), (T1 & T3) and (SF6 and F13) that produce fragments of 1,925 bp, 1,027 bp and 793 bp, specific for $(\delta\beta)^0$-thalassemia, HPFH-6 and deletion-inversion $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia, respectively. The 1,415 bp fragment generated from primers (δ1 and F13) is an internal control for the PCR amplification. B: a representative agarose gel electrophoresis of the multiplex allele-specific PCR analysis. Lanes 1: normal control, lanes 2-4: $(\delta\beta)^0$-thalassemia carrier, HPFH-6 carrier and deletion -inversion $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia carrier, respectively. Lanes 5-8: results of DNA analysis of a Thai family[4] in which the father (lane 5), the mother and the sister (lanes 6 and 7) and the proband (lane 8) were a HPFH-6 carrier, deletion-inversion $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia carriers and a compound heterozygote for HPFH-6/deletion-inversion $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia, respectively. M represents the λ/*Hind* III size markers.**

date of these relatively uncommon disorders. 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 $(\delta\beta)^0$-thalassemia, deletion-inversion $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia and HPFH-6 mutations from our earlier studies[3-5] 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 $A_2$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.[7] 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 $(\delta\beta)^0$-thalassemia and HPFH*

Genomic DNA was prepared from peripheral blood leukocytes using the standard method.[8] Individual globin gene deletions causing $(\delta\beta)^0$-thalassemia, $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia and HPFH-6, previously found in Thai patients, were identified using PCR methodology as described elsewhere.[3-5] 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' TGTTGACGCATGCCTAAAGA 3' and T2; 5'AATCCACTGAACACCGAAGC 3'), (δ1; 5' TTGGGTTTCTGATAGGCATG 3' and δ3; 5' TAGATCCCTTTGCCATTATG 3'), (SF6; 5' GCCCTCAAGTGTGCAGATTG 3' and F13; 5'AATGTGGGAGGTCAGTGCATT 3') were used to produce the 1,027 bp, 1,925 bp and 793 bp specific for the HPFH-6, the $(\delta\beta)^0$-thalassemia and the $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia deletions, respectively. With this system, the 1,415 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

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**Table 1. Results of hematologic analyses of 253 adult Thai carriers of three different high Hb F determinants and 58 normal individuals. Values are expressed as mean ± standard deviation. The three determinants were statistically compared using the Kruskal-Wallis non-parametric test.**

| *Parameters* | *$(\delta\beta)^0$ -thal* | *HPFH-6* | *Deletion-Inversion* | *Difference* | *Normal* |
|---|---|---|---|---|---|
| N. (%) | 148 (58.6) | 83 (32.8) | 22 (8.6) | – | 58 |
| Sex (male/female) | 34/114 | 16/67 | 2/20 | – | 21/37 |
| Age (year) | 3–68 | 1.3–61 | 4–61 | – | 20–33 |
| Rbc ($\times 10^6$/mm$^3$) | 4.8±0.8 | 4.7±0.6 | 4.9±0.6 | – | 4.5±0.4 |
| Hb (g/dL) | 11.5±1.5 | 12.3±1.4 | 12.1±1.3 | – | 13.3±1.4 |
| Hct (%) | 35.7± 4.5 | 37.5±4.4 | 37.3±3.6 | – | 39.8±4.0 |
| MCV (fL) | 75.4± 7.0 | 79.9±6.8 | 76.4±5.6 | < 0.001 | 87.9±3.6 |
| MCH (pg) | 24.4±2.0 | 25.7±2.2 | 24.3±1.9 | < 0.001 | 29.5±1.6 |
| MCHC (g/dL) | 32.3±1.0 | 32.8±1.1 | 32.4±1.1 | – | 33.5±0.9 |
| RDW-CV (%) | 20.5±2.4 | 16.6±2.7 | 16.8±2.4 | < 0.001 | 13.3±0.8 |
| Hb-type | $A_2FA$ | $A_2FA$ | $A_2FA$ | – | $A_2A$ |
| Hb $A_2$ (%) | 2.2±0.4 | 2.0±0.4 | 2.3±0.3 | – | 2.5±0.4 |
| Hb F (%) | 20.6±5.6 | 24.6±4.4 | 19.6±3.0 | < 0.001 | < 1.0 |
| Hb F subunit | $^G\gamma$ & $^A\gamma$ | $^G\gamma$ | $^G\gamma$ | – | – |

mM KCl, 0.01% gelatin, 3 mM $MgCl_2$ and 0.375 M betaine. 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 $^G\gamma^A\gamma(\delta\beta)^0$-thalassemia caused by a 12.5 kb deletion,[3] the HPFH-6 caused by a 101 kb deletion[4,5] and the $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia caused by a deletion-inversion.[4] As shown in Figure 1 lanes 1-4, while the 1,415 bp normal fragment generated from primers (δ1 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 a HPFH-6 carrier, deletion-inversion $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia carriers and to have compound HPFH-6/deletion-inversion $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia, respectively.[4] 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 monoplex PCR described previously.[3-5] The most common high Hb F determinant was found to be $(\delta\beta)^0$-thalassemia, which was detected in 148 (58.6%) of the cases, followed by the HPFH-6 and the deletion-inversion $^G\gamma(^A\gamma\delta\beta)^0$-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 $A_2$. The highest Hb F levels were observed among the HPFH-6 heterozygotes (24.6±4.4%) whereas in $(\delta\beta)^0$-thalassemia and deletion-inversion $^G\gamma(^A\gamma\delta\beta)^0$-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 $(\delta\beta)^0$-thalassemia consisted of both $^G\gamma$ and $^A\gamma$ whereas in HPFH-6 and deletion-inversion $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia, the $^G\gamma$ globin was predominant. There were statistically significant differences in MCV, MCH, RDW-CV and the level of Hb F between the

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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 δβ-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 δβ-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 β-thalassemia, due to the beneficial effect of Hb F on red blood cell production and survival.[11,12] Two types of the determinants, namely deletional and non-deletional forms, have been classified on the basis of molecular studies.[13,15]

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 $(\delta\beta)^0$-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 δ-globin gene to the L1 repeat element located on 3′ of the β-globin gene, but leaving intact $^G\gamma$ and $^A\gamma$ globin genes, thus removing part of the δ-globin gene and the entire β-globin gene, exactly as reported previously.[3] Data in Table 1 clearly demonstrate that in the heterozygous form this $(\delta\beta)^0$-thalassemia is associated with hypochromic microcytic red blood cell indices. Identification of this mutation in a large number of Thai individuals confirms that this $(\delta\beta)^0$-thalassemia gene, which has also been described sporadically in Laos[16] and Vietnamese,[13] is the most common high Hb F determinant among South-east Asian populations.

Another common mutation, detected in 83 (32.8%) cases, was the HPFH-6 determinant which is caused by a DNA deletion involving the $^A\gamma$, ψβ, δ and β globin genes. DNA sequence analysis of the deletion breakpoint revealed, as expected, that the deletion is caused by an approximately 101 kb deletion with the 5′ breakpoint occurring 1,260 bp 3′ of the $^G\gamma$-globin gene and the 3′ end lying 74 kb downstream of the β-globin gene, exactly as described previously.[4-6] Similar DNA deletions have been described, based on restriction endonuclease mapping, for other Thai,[17] Malaysian[18] and Cantonese[19] families with the $(^A\gamma\delta\beta)^0$-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 $(^A\gamma\delta\beta)^0$-thalassemia or as an HPFH determinant. The higher Hb F levels than in other forms of $(^A\gamma\delta\beta)^0$-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 $(^A\gamma\delta\beta)^0$-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.[6] The deletion enables the 3′ enhancer element to be juxtaposed in close proximity with the $^G\gamma$-globin gene, thereby increasing expression.

The last mutation, observed in 22 individuals (8.6%), was the deletion inversion type $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia. The lesion was caused by a complex rearrangement within the β-globin cluster, involving the deletion of 0.9 kb of DNA around the $^A\gamma$-globin gene, the loss of 7.5 kb DNA in and between the δ- and β- globin genes, and inversion of the DNA remaining between the $^A\gamma$- and δ-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 $(\delta\beta)^0$-thalassemia except that the predominant Hb F subunit is the $^G\gamma$-globin. This complex double deletion-inversion rearrangement $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia appears to be a common form of δβ-thalassemia found in central Asia, including in India,[20,21] Kuwait,[22] and Iran.[13] Identification of this mutation in Thailand suggests that this $^G\gamma(^A\gamma\delta\beta)^0$-thalassemia may have originated in India or South Asia and then become widespread in the Middle East and South-east Asia.

Co-inheritance of δβ-thalassemia or HPFH with increased production of γ-chains has an ameliorating effect on the clinical picture of β-thalassemia disease.[1,2] 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 δβ-thalassemia and HPFH and the level of Hb F alone cannot differentiate the two conditions. Direct DNA analysis of the molecular defect is there-

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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.*

*SF was the main investigator contributing to the design and concept of the whole study, interpretation of the results, drafting and editing the final manuscript. We thank Dr. Ian Thomas for helpful comments on the manuscript.*

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