

Interaction of hemoglobin E and several forms of α -thalassemia in Cambodian families

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Background and Objectives. This study aimed to describe hematologic and molecular characterization of the interaction of hemoglobin (Hb) E and several forms of α -thalassemia causing complex thalassemia syndromes in two Cambodian families as well as to establish a rapid polymerase chain reaction (PCR) assay for simultaneous detection of Hb Constant Spring (CS) and Hb Pakse' (PS).

Design and Methods. Using PCR and DNA sequencing, the α - and β -globin genotypes were examined. Clinical and hematologic data were assessed. A multiplex asymmetric allele-specific PCR for differential diagnosis of HbCS and HbPS was developed and validated.

Results. Eight genotypes including heterozygous HbCS, heterozygous HbPS, double heterozygous HbE/HbPS, double heterozygous HbE/ α -thalassemia 2, triple heterozygous HbE/ α -thalassemia /HbPS, homozygous HbE/ α -thalassemia 2, compound α -thalassemia 2/HbCS and a hitherto undescribed compound HbCS/HbPS were found in these two families. Genotype-phenotype relationships are discussed and successful application of a multiplex PCR system for differential diagnosis of HbCS and HbPS is described.

Interpretation and Conclusions. The interaction of several globin gene abnormalities in Cambodian families emphasizes the high frequencies of thalassemia and hemoglobinopathies. Identification of HbPS suggests that this mutation might be common and underestimated among South-east Asian populations. A simplified PCR assay for simultaneous detection of HbCS and HbPS would facilitate characterization of these genotypes in both the clinical setting and population screening programs in the region.

Key words: hemoglobin E, hemoglobin Constant Spring, hemoglobin Pakse', thalassemia syndrome, Cambodia.

Haematologica 2003; 88:1092-1098
http://www.haematologica.org/2003_10/1092.htm

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Thalassemia is a heterogeneous group of hereditary anemias characterized by reduced or absent production of globin chains. It is very common in South-east Asia, the region where another structural β -globin gene variant, hemoglobin E (HbE), is also prevalent.^{1,2} This abnormal hemoglobin results from a single amino acid substitution of glutamic acid to lysine, due to a point mutation (GAG→AAG) in codon 26 of the β -globin gene. Cambodia, a small country with a population of about 11.5 million, has one of the worst health systems in South-east Asia. There is a significant incidence of microcytic hypochromic anemias, which in the past were mostly assumed to be caused by iron deficiency. However, with a study based on phenotype analysis, it soon became apparent that thalassemia and hemoglobinopathies are major genetic disorders in this country.³ As for other South-east Asian countries, interactions of thalassemia and hemoglobinopathies can be complex and confusing. Moreover, there is a high incidence of consanguineous marriages so the occurrence of genetic disorders is likely to increase without proper diagnosis and genetic counseling. During routine investigation of anemias in the Siem Reap province, Cambodia, we encountered two families with such interactions. Extensive investigation, including hematologic and direct globin gene analysis, revealed interaction of Hb E with several forms of α -thalassemia including one previously undescribed in this population, the HbPS.⁴ Co-segregation of HbE with different α -thalassemia genes in these two Cambodian families allowed us to compare genotype-phenotype interactions of these complex thalassemia syndromes. A simple polymerase chain reaction (PCR) assay for differential diagnosis of HbCS and HbPS is also described.

Design and Methods

Subjects and hematologic analysis

A total of 11 EDTA-blood samples from Cambodian subjects were obtained from two unrelated families who attended the Angkor Hospital for Children, Siem Reap, Cambodia (Figures 1 and 2). Hb level and erythrocyte indices were immediately determined at Siem Reap using the Sysmex KX 21 electronic cell counter (TOA Medical Electronics Co., Kobe, Japan). Serum ferritin level was determined using a microparticle enzyme immunoassay⁵ with the AxSYM automated system (Abbott Laboratories, Abbott Park, Illinois, USA). The remaining blood specimens were sent to the Faculty of

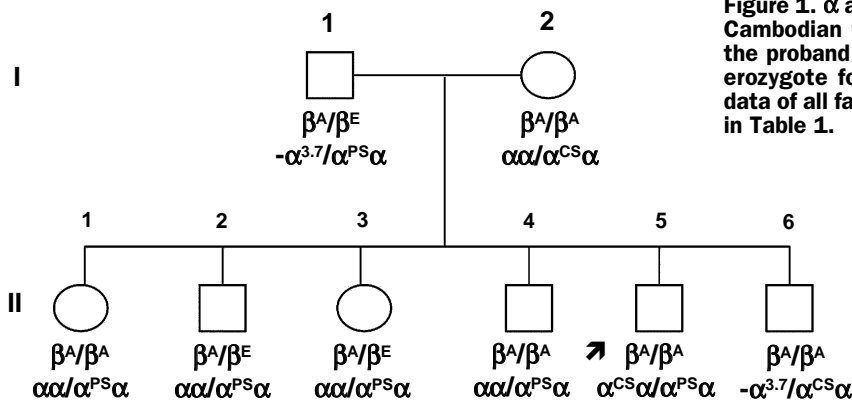


Figure 1. α and β – globin genotypes in the Cambodian family 1. The arrow indicates the proband who was a compound Hb heterozygote for HbCS/HbPS. Hematologic data of all family members are summarized in Table 1.

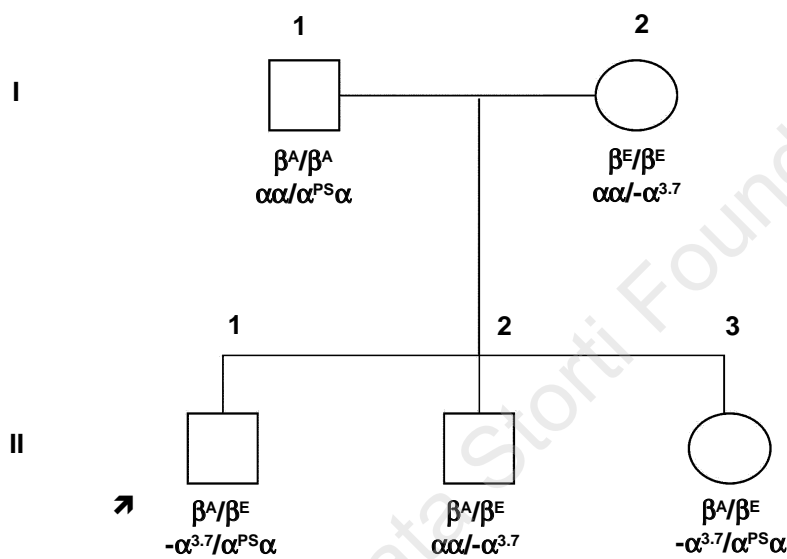


Figure 2. α and β – globin genotypes in the Cambodian family 2. The arrow indicates the proband of this family who was a triple heterozygote for HbE, HbPS and a deletional α -thalassemia 2. Hematologic data of the family members are summarized in Table 1.

Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand for further analysis. Hb types were determined using cellulose acetate electrophoresis and automated high-performance liquid chromatography (HPLC) (VARIANT™; Bio-Rad Laboratories, Hercules, CA, USA). HbA₂ and HbE were also quantified using a standard cellulose acetate electrophoresis and elution technique.⁶

DNA analysis

Genomic DNA was prepared from peripheral blood leukocytes using the standard method. Identification of α -thalassemia 1 (SEA-type) and α -thalassemia 2 (3.7 kb and 4.2 kb deletions) was performed using the PCR methodologies described elsewhere.⁷⁻⁹ The HbE, HbCS and HbPS mutations were identified using the allele-specific PCR described previously.¹⁰⁻¹² Direct DNA sequencing of the amplified DNA fragment was performed on an

ABI Prism 377 automated DNA sequencer, using the ABI Prism cycle sequencing dye primer-ready kit according to the manufacturer's instructions (Perkin-Elmer Biosystems Co.) (Figure 3). In order to provide a rapid differential DNA diagnosis of HbCS and HbPS, a multiplex asymmetric allele-specific PCR was also developed as shown in Figure 4. In this PCR system, two specific primer pairs; α G2 (5'-GCTGACCTCCAA ATACCGTC-3') and C3 (5'-CCATTGTTGGCACATTCCGG-3') and α G17 (5'-AGATGGCGCCTTCTCTCAGG-3') and α G18 (5'-ACGGCTACCGAGGCTCCAGCA-3') were used for detection of the α^{CS} and α^{PS} mutations, respectively. The 391 bp generated from primers α G17 and C3 was used as an internal control of PCR amplification. The multiplex PCR reaction mixture (50 μ L) contains 0.1 μ g DNA, 37.5 pmole of α G2, 30 pmole of C3, 3.75 pmole of α G17, 30 pmole of α G18, 200 μ M dNTPs, and 1 unit of *Taq* DNA polymerase (Pro-

Table 1. Hematologic values in the two Cambodian families with HbE and α -thalassemia.

| Patients | Age (yr) | Sex | RBC ($10^{12}/L$) | Hb (g/dL) | Hct (%) | MCV (fL) | MCH (pg) | MCHC (g/dL) | Hb type | HbA ₂ /E (%) |
|----------|----------|-----|------------------------|--------------|------------|-------------|-------------|----------------|---------------------|----------------------------|
| Family 1 | | | | | | | | | | |
| I-1 | 53 | M | 5.2 | 11.4 | 38 | 72 | 22 | 30.2 | EA | 20.2 |
| I-2 | 47 | F | 4.5 | 12.2 | 36 | 80 | 27 | 33.7 | A ₂ A | 2.3 |
| II-1 | 21 | F | 4.6 | 11.1 | 35 | 76 | 24 | 31.6 | A ₂ A | 2.1 |
| II-2 | 20 | M | 5.0 | 12.5 | 39 | 78 | 25 | 32.3 | EA | 26.4 |
| II-3 | 17 | F | 4.8 | 11.5 | 36 | 76 | 24 | 31.9 | EA | 27.3 |
| II-4 | 14 | M | 4.8 | 11.3 | 35 | 74 | 24 | 31.9 | A ₂ A | 2.4 |
| II-5 | 11 | M | 4.4 | 10.4 | 34 | 77 | 24 | 30.3 | CS A ₂ A | 2.0 |
| II-6 | 10 | M | 4.8 | 9.9 | 32 | 69 | 21 | 30.7 | CS A ₂ A | 2.3 |
| Family 2 | | | | | | | | | | |
| I-1 | 33 | M | 5.5 | 13.9 | 44 | 80 | 25 | 31.6 | A ₂ A | 3.3 |
| I-2 | 31 | F | 5.5 | 11.2 | 35 | 64 | 20 | 31.6 | EE | 86.7 |
| II-1 | 10 | M | 4.8 | 9.9 | 33 | 67 | 21 | 30.5 | A | 21.2 |
| II-2 | 8 | M | 4.6 | 11.4 | 35 | 77 | 25 | 32.3 | EA | 28.0 |
| II-3 | 3 | F | 5.6 | 10.7 | 36 | 66 | 19 | 29.4 | EA | 20.7 |

M: male; F: female.

mega, Madison, WI, USA) in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 4.8% DMSO, 1 M betaine and 3.0 mM MgCl₂. After initial heating at 94°C for 30 min, 30 cycles of the PCR process (94°C for 1 min and 65°C for 1 min 30 sec) were carried out on a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Norwalk, CT, USA). The amplified DNA product was analyzed by electrophoresis on a 2.0% agarose gel and visualized under UV-light after ethidium bromide staining.

Results

Hematologic and DNA analysis

The molecular data of the two families are shown in Figures 1 and 2 and their hematological data are listed in Table 1. In the first family, the proband (II-5), an 11-year old boy, had a thalassemia intermedia phenotype with an Hb level of 10.4 g/dL, and slight hypochromic microcytosis with MCV 77 fL and MCH 24 pg. Hemoglobin analysis revealed HbCS in addition to HbA₂ and HbA with a normal HbA₂ level (2.0%). Physical examination revealed mild pallor with no characteristic thalassemic facies. His spleen was normal but the liver was palpable 3 cm below the right costal margin. The peripheral blood film showed polychromasia, anisopoikilocytosis and marked basophilic stippling. His serum ferritin level was 92 µg/L (normal range: 20-180 µg/L). His younger brother (II-6), a 10-year-old boy had a similar phenotype but with lower Hb, MCV and MCH values. Phenotypically, they were both diagnosed as HbCS carriers. Hematologic

analysis of the family members identified that his father (I-1) was a carrier of HbE with low Hb, MCV and MCH values. The level of HbE in the father (20.2%) was, however, significantly lower than that usually observed in HbE carriers (25%-35%) and observed for II-2 and II-3 (26.4% and 27.3%, respectively). Hematologic findings in the mother (I-2) were normal. The II-1 and II-4 siblings had normal Hb patterns with mild hypochromic microcytosis. Since no HbCS was observed in the parents, direct DNA sequencing of the amplified DNA was used to confirm the existence of this Hb variant in the proband. As shown in Figure 3, we unexpectedly observed a compound heterozygosity for TAA to CAA and TAA to TAT mutations at the termination codon of an α 2-globin gene. The former is a well known HbCS specific mutation whereas the latter changes the normally present termination codon to a codon for tyrosine, with consequent synthesis of an elongated α -globin chain (172 instead of 141 residues) quite similar to HbCS. This mutation, recognized previously as a rare α -thalassemia 2 gene, namely HbPS, was described originally in one Laotian family with HbH disease⁴ and recently reported for several cases of Thai patients¹²⁻¹³ but not in other populations. The proband was therefore a compound heterozygote for HbCS and HbPS. Complete DNA analyses of α -thalassemia genes using PCR were therefore performed for all family members. With these analyses, it was found that in addition to HbE, the father (I-1) also carried an α -thalassemia 2 (3.7 kb deletion) determinant and the HbPS gene. He was

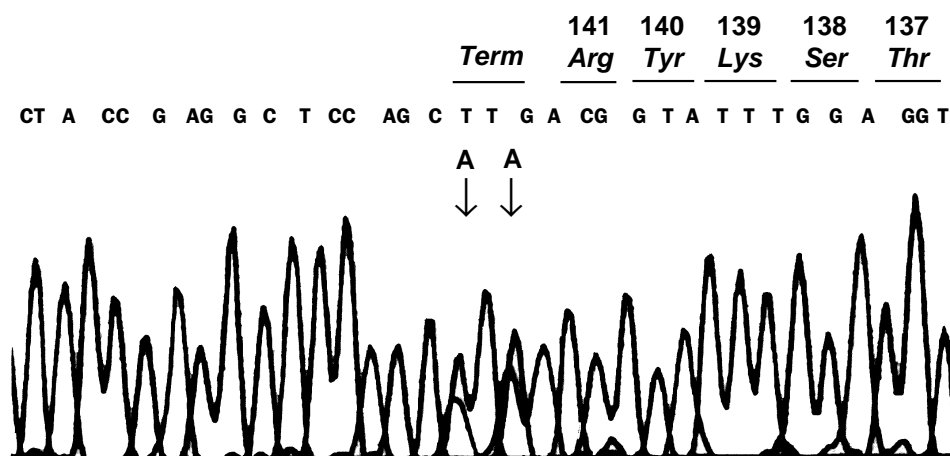


Figure 3. Direct DNA sequencing analysis (anti-sense strand) around the termination codon (Term) of the $\alpha 2$ -globin gene of proband 1 who was a compound heterozygote for HbCS and HbPS. The two vertical arrows indicate the double ladder patterns of HbCS and HbPS mutations at the termination codon of the gene.

therefore a triple heterozygote for HbE, HbPS and a deletional α -thalassemia 2 whereas the mother (I-2) was a pure Hb CS carrier though HbCS was not observed. As summarized in Figure 1, II-1 and II-4 were pure carriers of HbPS whereas II-2 and II-3 were double heterozygotes for HbE and HbPS. The last subject, II-6 was found to be a compound α -thalassemia 2/HbCS.

DNA analysis of family 2 (Figure 2) revealed that the proband (II-1) and his sister (II-3) were both triple heterozygotes for HbE/HbPS/ α -thalassemia 2 (3.7 kb deletion) as had been observed for the father of family 1. At the time of referral, the proband was suffering from iron deficiency anemia with a serum ferritin of 10 μ g/L, Hb 7.4 g/dL, Hct 27%, MCV 66 fL and MCH 15 pg. He had a normal spleen but the liver was palpable 3 cm below the right costal margin. After iron therapy, his Hb, Hct, MCV, MCH and MCHC values rose to 9.9 g/dL, 33%, 67 fL, 21 pg and 30.5 g/dL, respectively as shown in Table 1. The hematologic phenotypes of the proband and his sister (II-3) were quite similar to those of the father of family 1 i.e. the lower percentage of HbE (21.2% and 20.7%) and no Hb corresponding to HbCS or HbPS observable. His mother (I-2) was found to be homozygous for HbE with a deletional α -thalassemia 2 (3.7 kb deletion). Further DNA analyses revealed that his father (I-1) was a carrier of HbPS and his brother (II-2) was a double heterozygote for HbE and HbPS with 28.0% HbE. The father had a relatively normal hematologic phenotype while the brother had a similar phenotype to those of the II-2 and II-3 individuals of family 1.

Multiplex asymmetric allele-specific PCR for α^{CS} and α^{PS} gene detection

As mis-diagnosis of HbCS and HbPS is common in routine hematologic analyses, especially in individuals with HbE, we have developed a simultaneous system based on multiplex asymmetric allele-specific PCR for detection and differential diagnosis of these two Hb variants (Figure 4). As shown in the analysis of family 1 members, while the 391 bp control fragment was observed in all cases, the 253 bp α^{PS} -specific and the 180 bp α^{CS} specific fragments were observed only in individuals with these mutations. A compound HbCS/HbPS could also be diagnosed on a single PCR reaction (Figure 4B, lane 7). This result indicates that our simple and multiplex DNA testing can be used as a simultaneous DNA diagnosis for both HbCS and HbPS.

Discussion

The finding of interactions of several globin gene abnormalities in the two Cambodian families described in this study emphasizes, as for other south-east Asian countries,² the high frequencies of thalassemia and hemoglobinopathies in Cambodia. A previous study in Cambodian refugee children in California, USA revealed that 19% had HbE, 12% α -thalassemia and 3% β -thalassemia.¹⁴ A study conducted in France on Cambodian refugees calculated a gene frequency of 0.11 for deletional α -thalassemia 2.¹⁵ Further studies in a population from Cambodia's capital, Phnom Penh, confirmed the high gene frequencies for both HbE and deletional α -thalassemia in Cambodia.^{16,17} These gene frequencies are similar to those seen in Thai and oth-

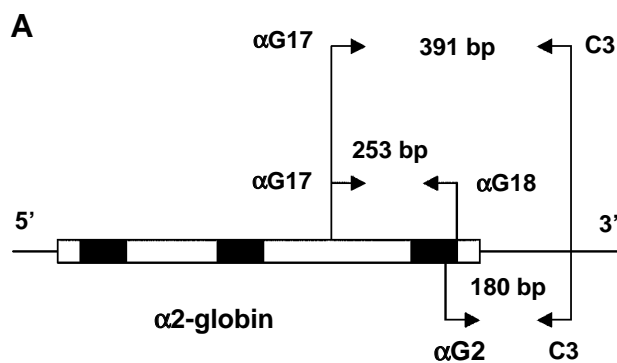
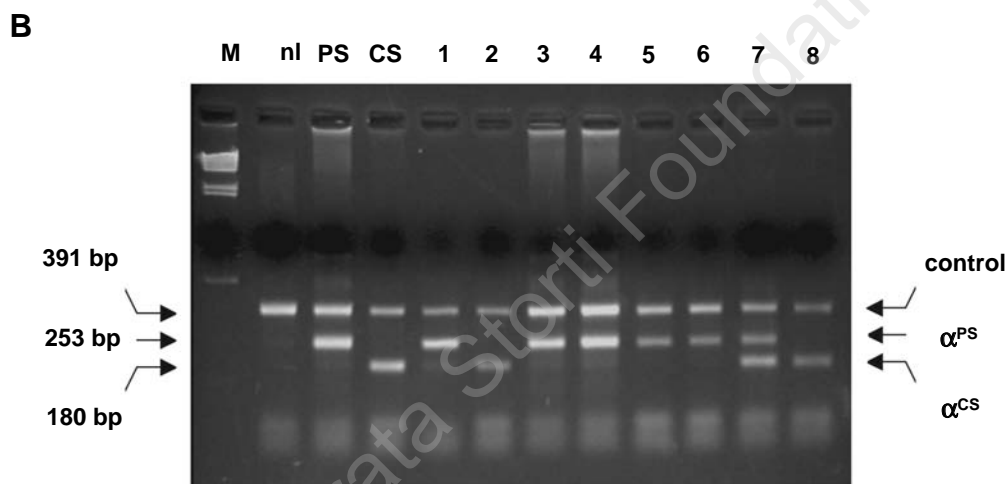


Figure 4. Identification of the HbCS and HbPS mutations by a multiplex asymmetric allele-specific PCR. **A:** The locations and orientations of amplification primers used in the multiplex system. The α G2 and α G18 are HbCS and HbPS-specific primers, respectively, whereas primers α G17 and C3 were used to generate the control fragment of 391 bp. The size of each specific amplified fragment is indicated in base pairs (bp). **B:** Electrophoresis on 2.0% agarose gel of the amplified DNA for detecting HbCS and HbPS mutations by the simultaneous PCR assay for 8 individuals of family 1. The 391 bp control fragment, the 253 bp for α^{PS} - mutation and the 180 bp for α^{CS} - mutation are indicated. Lanes 1-8 correspond to I-1, I-2, II-1, II-2, II-3, II-4, II-5 and II-6 of family 1, respectively. No HbCS or HbPS mutation was detected in the normal control (nl). PS and CS are positive controls for the two mutations. M represents the λ /*Hind* III size markers.



er South-east Asian populations. HbCS is known to be present throughout the region with carrier rates being approximately 4%.^{1,2} Complex thalassemia syndromes caused by these gene-gene interactions are presumably common in this population.¹⁸ The two families described in this study examples of different globin gene interactions. The presence of several forms of α -thalassemia and HbE in these families allows comparison of the phenotypes of the conditions.

The proband (II-5) in the first family, a compound heterozygote for HbCS and HbPS had a mild hypochromic microcytosis characteristic of thalassemia intermedia as usually observed with homozygous HbCS, although the latter rarely results in microcytosis. Splenomegaly was not observed but polychromasia on the peripheral blood film indicated reticu-

locytosis as usually observed with homozygous HbCS.^{19,20} Although different mutations, both HbCS and HbPS had similar phenotypic expression.^{4,12,13} Compared with his brother, II-6 who was a compound heterozygote for HbCS and a deletional α -thalassemia 2, the proband had a much higher MCV value (i.e. 77 fL v.s. 69 fL). It has been shown previously that overhydration of erythrocyte containing HbCS is responsible for the higher MCV value compared to that of other thalassemic erythrocytes.²¹ The compound HbCS/HbPS erythrocytes might behave similarly. Further study on cell density using a discontinuous density gradient and determination of the volume, Hb content and Hb concentration of individual RBC should provide additional information related to the hydrocytosis in HbPS.^{22,23}

As shown in Table 1, our study demonstrates that

interaction of Hb E heterozygosity with α -thalassemia may or may not influence the amount of HbE and other hematological parameters. Obviously, the co-inheritance of HbPS in HbE heterozygotes, observed in the two children of family 1, II-2 and II-3, does not lead to the lower proportion of HbE (i.e. 26.4% and 27.3%) as compared to that of the father (I-1; 20.2%) who was a triple heterozygote for HbE/HbPS/ α -thalassemia 2. Only minimal changes in hematologic data were observed. This indicates, as for the double heterozygosity for HbE/HbCS in our series,²⁴ that the interaction of HbE and HbPS is not associated with severe thalassemia. This result indirectly confirms a similar phenotypic expression of the HbCS and the HbPS mutations. HbPS is an α -thalassemia² determinant caused by a termination codon mutation of the $\alpha 2$ -globin gene (TAA→TAT) described originally for a Laos patient with HbH disease.⁴ Recently, a significant frequency of HbPS in the Thai population has been reported independently.^{12,13} Therefore, interaction of HbE and HbPS may not be uncommon among the Southeast Asian population. It is noteworthy that all these subjects were mis-diagnosed at routine Hb analysis as being pure HbE carriers, since neither HbCS or HbPS was identified. However, this is not unexpected since both HbCS and HbPS, the two α -globin chain variants with 31 extra amino acid residues, are relatively unstable and usually presented at very low levels. Therefore, neither can usually be detected by routine Hb electrophoresis, especially when there is another slow moving Hb such as HbE in the same sample. Dramatic changes in the level of HbE and hematologic parameters were obvious in the interaction of HbE heterozygotes with compound α -thalassemia 2/HbPS (Family 1, I-1 and Family 2, II-1 & II-3) who had significant lower MCV values (66-72 fl) and lower proportions of HbE (20.2-21.2%). These findings are similar to those observed for HbE heterozygote with homozygous α -thalassemia 2 or heterozygous α -thalassemia 1²⁴⁻²⁷ and indirectly indicate similar phenotypic expression of a compound α -thalassemia 2/HbPS with that of α -thalassemia 1 heterozygote. The low levels of HbE in these interactions likely represents the effect of a decreased availability of α chains on the $\alpha\beta^E$ subunit formation. The normal β^A subunit with a -2.5 charge combines more readily with α -subunit with a +2.4 charge than does the β^E subunit which is relatively more positive than the β^A . Consequently, when α -subunits are present in limited quantities as a result of α -thalassemia, HbA will be formed more rapidly than HbE and the HbE will therefore be represented in lower relative amounts.²⁶

It is noteworthy again that the HbPS was not detected in all cases with HbE. This result indicates as for HbCS, a possibility of mis-diagnosis of the HbPS in routine Hb analysis unless appropriate DNA characterization is performed. We therefore recom-

mend DNA analyses of both α - and β -globin genes in all cases of HbE heterozygotes with very low levels of HbE and reduced erythrocyte indices to obtain correct genotypes of the patients. It is very important to define these gene-gene interactions to be able to provide appropriate counseling to the affected members especially among Southeast Asian populations. In addition, a different origin of HbCS has recently been reported for Mediterranean patients.²⁹ It is conceivable therefore that the prevalence of HbPS might also be underestimated in other populations. Although several PCR related methods have been described for identification of HbCS^{9,11,30-33} and HbPS,^{12,13} the availability of a simple DNA test herein described for the simultaneous detection and differential diagnosis of the two Hb variants (Figure 4) is simpler, faster and does not require additional steps of enzymatic digestion or hybridization of specific probes. This method should prove useful in characterization and diagnosis of complex thalassemia syndromes in the regions where HbE, α -thalassemia and other hemoglobinopathies occur simultaneously. The method would also be particularly useful in epidemiological data acquisition in population screening programs.

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Pre-publication Report & Outcomes of Peer Review

Contributions

SF, KS, GF and SP were involved in the DNA analysis of the patients, design and setting up the multiplex PCR for detection of HbCS and HbPS and reading the manuscript. RD and LL are medical staff in Cambodia who played a role in recognition and initial hematologic analysis of the Cambodian families. 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.

Funding

This work was supported by grants from the National Science and Technology for Development Agency (NSTDA) and the Khon Kaen University, Khon Kaen, Thailand.

Disclosures

Conflict of interest: none

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received August 4, 2003; accepted August 25, 2003.

In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic

Thalassaemic syndromes and hemoglobinopathies are both highly prevalent in some regions of the world, including South-east Asia. Interactions between different globin gene defects are therefore very common.

What this study adds

This paper shows interactions of several globin gene abnormalities in two Cambodian families, emphasizing, as for other South-east Asian countries, the high frequencies of thalassaemia and hemoglobinopathies in Cambodia.