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Clinical phenotypes and molecular characterization of Hb H-Paksé disease

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Background and Objectives. Hemoglobin Constant Spring (Hb CS), caused by a termination codon mutation (TAA→CAA) in the α2 gene, is the most common non-deletional type of α thalassemia in Southeast Asia. This mutation can most easily be detected by loss of an *MseI*-restriction site (T/TAA) spanning the termination codon. Recently, we sequenced the α globin genes from patients with α thalassemia in whom this *MseI* site was absent. This revealed, a previously described termination codon mutation (TAA→TAT) associated with Hb Paksé. This prompted us to re-evaluate the molecular basis of α thalassaemia in other Thai patients with non-deletional types of Hb H disease.

Design and Methods. DNA samples from 30 patients, previously diagnosed as having Hb H-CS disease, were characterized by direct genomic sequencing and by using a mismatched polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Clinical and hematologic data were assessed.

Results. Hemoglobin electrophoresis in almost all 30 unrelated patients with non-deletional α thalassemia revealed a slow migrating band resembling Hb CS. Five of these patients were found to have Hb H-Paksé disease and the remainder had Hb H-CS disease. Comparing the hematology in patients with these two genotypes, no significant differences were found except that the proportion of Hb H was higher in patients with Hb H-Paksé disease.

Interpretation and Conclusions. These results suggest that termination codon mutations may have been previously misidentified in many cases of non-deletional Hb H disease. Findings from six unrelated families described in this study suggest that the proportion of patients with the Hb Paksé mutation might be underestimated and that this mutation could be prevalent in Southeast Asia. Analysis of mismatched-PCR-RFLP, described here, was shown to provide an unequivocal diagnosis and will be applicable in population screening programs.

Key words: α thalassemia, hemoglobin H-Paksé disease, hemoglobin H-CS disease, termination codon mutations.

Hemoglobin H disease, characterized by readily detectable amounts of hemoglobin H (Hb H, (β_4) in the peripheral blood, is the most severe viable form of α thalassemia. The molecular basis of Hb H disease can be divided into two types. The first results from deletions removing three copies of the alpha α globin genes, (--/-α) and is found in patients with compound heterozygosity for α^0 thalassemia (--) and α^+ thalassemia (-α). The second type results from an interaction between α^0 thalassemia and mutations, which cause only minor changes in the structure of the alpha globin genes. These are known as *non- deletional types of α thalassemia* (--/$\alpha^T\alpha$ or --/$\alpha\alpha^T$).[1,2] Most non-deletional mutations involve the α2 gene which is normally expressed 2-3 fold higher than its linked α1 gene.[2] Patients with Hb H disease have a wide spectrum of clinical phenotypes ranging from asymptomatic to severe and transfusion-dependent, although the latter is uncommon. It has been shown that the clinical phenotypes of Hb H disease caused by non-deletional types of α thalassemia, (--/$\alpha^T\alpha$) are usually more severe than those caused by deletional types of α^+ thalassemia (--/-α).[2]

The distribution of non-deletional types of α thalassemia varies from one geographic area to another. In South East Asia (SEA) and Southern China, the most common non-deletional type of α thalassemia mutation results from a mutation in the terminal codon mutation of the α2 gene (TAA→CAA) and is known as hemoglobin Constant Spring (Hb CS).[3-6] This mutation can be detected by the absence of an *Mse* I restriction site (T/TAA) spanning the termination codon of the α2 globin gene. Recently, this approach has been frequently used for the molecular diagnosis of this mutation.[7] In Thailand, Hb CS is thought to account for the majority of cases of non-deletional forms of Hb H disease.[4] Here, we report on six unrelated patients from central Thailand with Hb H disease caused by compound heterozygosity for α^0 thalassemia ($--^{SEA}$) and Hb H-Paksé, a form of non-deletional α thalassemia which is also caused by a mutation at the termination codon of the α2 gene (TAA→TAT). Interestingly, 5 of the 6 patients were previously diagnosed as having Hb H-CS disease based on their hemoglobin electrophoresis and the absence of an *Mse* I recognition site at the termination codon. To our knowledge, this is a first report of such patients from Thailand. Clinical phenotypes and the molecular characterization of Hb H-Paksé, including a mismatch PCR assay which can specifically identify both termination codon mutations, are described.

Design and Methods

Subjects

We studied 30 patients, previously diagnosed as having Hb H-CS disease, at the Department of Pediatrics, Faculty of Medicine, Siriraj Hospital, Bangkok, Thailand. Their ages ranged from 3 to 20 years. Eight patients received occasional blood transfusions to correct their anemia and all were splenectomized after which they no longer required transfusions. In the other 22 cases, the symptoms of anemia were moderate and the patients did not require transfusion. The patients' height and weight were within the normal range according to the standard growth and development chart of Thai children. The mean follow-up time was 10.5 years (range, 4 months–19 years).

Hematologic studies

Routine hematologic studies including detection of Hb H inclusion bodies by staining with brilliant cresyl blue were carried out on peripheral blood samples collected using EDTA as anticoagulant. Red blood cell indices were analyzed using an automatic red blood cell counter (Sysmex F280, Japan). Standard hemoglobin electrophoresis and chromatography were performed and quantified by cellulose acetate chromatography, iso-electric focusing (IEF) and the LPLC automated hemoglobin analyzer (HB Gold, Drew Scientific Ltd., Cumbria, UK). Starch gel electrophoresis was performed and stained with ortho-dianisidine to detect any abnormal hemoglobin bands running anodic to Hb A_2 and resembling the position of Hb CS. Hb F was assessed by alkali denaturation.[8]

Molecular analysis of the α globin genes by direct genomic sequencing of selectively amplified PCR products

Genomic DNA was extracted from peripheral blood leukocytes obtained from all patients in this study using EDTA as anticoagulant. Standard α globin genotypes were analyzed by Southern blotting as previously described.[9] Independently, multiplex PCRs specific for common α^0 and α^+ thalassemias were performed[10] to establish genotypes. The α2 and α1 genes were amplified by PCR and sequenced. In brief, *Hotstart* amplification was performed using 100 ng of genomic DNA, 25 pmol of forward and reverse primers, 1 mM $MgCl_2$, 200 μM of each dNTP, 2.5 U Taq polymerase (Roche Diagnostic, Mannheim, Germany) in 1X dimethyl sulfoxide (DMSO) buffer (32 mM$(NH_4)_2SO_4$, 134 mM Tris-HCl pH 8.8, 20% DMSO and 20 mM β-mercaptoethanol) with 0.7M betaine in a final reaction volume of 50 μL. Reactions were carried out in an MJ DNA Engine thermocycler (MJ Research, MA, USA) with an initial denaturation step of 95°C for 15 minutes and then 30 cycles of 95°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute and 30 seconds with a final extension at 72°C for 10 minutes. Amplification and sequencing primers are shown in Table 1. The α2 (1326 bp) and α1 (1390 bp) gene fragments were generated by PCR and sequenced using fluorescent labeled dideoxyterminators (Perkin Elmer Biosystems Co., Norwalk, USA) and analyzed on an ABI 377-automated sequencer (Applied Biosystems, Foster City, USA). Two sequencing primers were used; 5'-GCG GGT TGC GGG AGG T-3' and 5' TTA TTC AAA GAC CAG GAA GGG CCG-3'.

Mismatched-polymerase chain reaction-restriction fragment length polymorphism (mismatched-PCR-RFLP) for Hb CS and Hb Paksé

An approach for rapid diagnosis of Hb CS and Hb Paksé was developed as shown in Figure 1 and described in the legend. Both mutations occur at the termination codon (TAA) of the α2 gene and

Table 1. PCR conditions and amplification primers (according to the Genbank accession number AE005175) and expected PCR fragments.

PCR conditions	Forward primers	Reverse primers	Annealing temp. (C°)	Product size (bp)
1.α2 gene	5'-GGG GTG CAC GAG CCG ACA GC-3' (162569→162588)	5'-CTC TCA GGA CAG GGG ATG GTT CAG-3 (163895→163872)	58	1326
2.α1 gene	5'-GGG GTG CAC GAG CCG ACA GC-3' (166373→166392)	5'-AAC CTG CAT TGA ATC TGA AAA GTC-3' (167755→167778)	58	1390
3.Mismatched HbCS	5'-ACC GTG CTG ACC TCC AAA TAA CGT-3' (163573→163595)	5'-CTC TCA GGA CAG GGG ATG GTT CAG-3' (163895→163872)	60	323
4.Mismatched HbPaksé	5'-CCC GCC CGG ACC CAC A-3' (163044→163059)	5'-AAC GGC TAC CGA GGC TCA AGC3' (163620→163601)	60	577

both abolish an *MseI* restriction site (5'-T/TAA-3'). We designed and optimized a set of PCR reactions which can distinguish between the Hb CS and Hb Paksé mutations. The PCR reactions were primed with oligonucleotides containing specific mismatches (3F and 4R in Figure 1) compared with the normal sequences. Using the mismatched oligo 3F and perfect match oligo 3R, the amplification product across the termination codon (TAA) produced a new restriction site (*AclI*: 5'-AACGTT-3'). When the first nucleotide of termination codon is mutated in Hb CS, this restriction site is abolished (5'-AACGTC-3'). Similarly, using the mismatched oligo 4R and matched oligo 4F, the amplification product from the normal allele produced a new restriction site (*Hind* III: 3'-TTCGAA-5') which is abolished (3'-TACGAA-5') if the termination codon is mutated to TAT in Hb Paksé.

The PCR mixture for Hb CS-mismatched-PCR-RFLP consisted of 1.5 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, Tris-HCl pH 8.8, 200 μM of each dNTP, 25 pmol of each forward and reverse primer and 2.5 U of Taq polymerase (Roche Diagnostic, Mannheim, Germany) and 100 ng of genomic DNA in a final volume of 50 μL. For Hb Paksé-mismatched-PCR-RFLP, the reaction was performed using the same conditions as those used for the selective amplification of the α2 and α1 gene, described above. The primers, optimal temperatures for annealing, co-ordinate numbers and expected PCR fragment sizes are shown in Table 1.

Ten microliters of the PCR products were incubated for 4 hours at 37° C with 3 U of *AclI* for Hb CS and 10 U of *Hind*III for Hb Paksé-mismatched-PCR-RFLP, respectively, under the manufacturer's recommended condition (NE Biolabs, Beverly, MA, USA). The digested products were electrophoresed on 3% agarose gels followed by ethidium bromide staining and visualized on a UV transilluminator.

Table 2. Hematologic data and globin genotypes in 6 cases of Hb H- Paksé.

Pts.	Sex	Age (yrs)	Hb (g/dL)	Hct (%)	RBC (10^{12}/L)	MCV (fL)	MCH (pg)	MCHC (g/dL)	HbH Inclus. Bodies (%)	Genotypes
1.PC	M	5	8.9	30.9	4.86	63.5	18.3	28.8	22	$--^{SEA}/\alpha^{Paksé}\alpha$
2.YP	M	20	9.9	35.7	5.05	70.6	20.8	28.7	18	$--^{SEA}/\alpha^{Paksé}\alpha$
3.PP	M	15	7.8	37.2	4.25	87.5	18.4	21	8	$--^{SEA}/\alpha^{Paksé}\alpha$
4.PN	M	15.7	8.6	36	4.96	67.5	17.3	23.9	13	$--^{SEA}/\alpha^{Paksé}\alpha$
5.HO	F	10	5.8	24	2.89	76.9	20.1	24.2	10	$--^{SEA}/\alpha^{Paksé}\alpha$
6.KP	F	12	4.7	21.3	2.47	86.2	19.0	22.1	10	$--^{SEA}/\alpha^{Paksé}\alpha$

	Hemoglobin electophoresis					
	Bart's (%)	HbH (%)	HbF (%)	HbA (%)	HbA2	[2]Hb×
1.PC	3.5	21.4	1.1	70.2	0.7*	–
2.YP	5.8	12.7	1.5	79.2	0.8*	–
3.PP	12.2	14.7	–	68.2	4.9	+
4.PN	4.4	18	2.7	73.8	3.8	+
5.HO	4.5	14	4.3	77.8	3.7	+
6.KP	6.9	10	3.7	74.8	8.3	+

*[1]Percentages of abnormal hemoglobin A_2 + Hb X where Hb X is Hb Paksé or Hb CS, neither of which can separated from A_2 sufficiently for accurate quantitation under the conditions used. *indicates Hb A_2 alone because no slow-migrating species were observed. [2]The presence of Hb X refers to Hb Paksé (studied by electrophoresis and chromatography).*

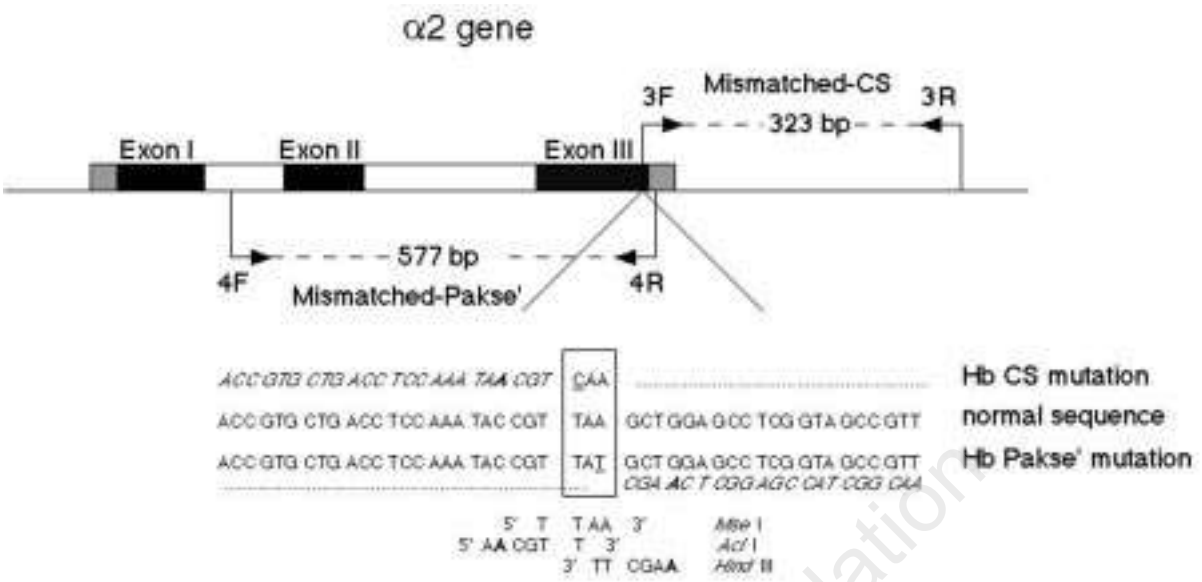

Figure 1. Schematic representation of the mismatched-PCR-RFLP strategy for rapid diagnosis of Hb CS (TAA→CAA) and Hb Paksé mutation (TAA→TAT). Mutations in termination codons are underlined (C and T). Sequences of primers used in both PCR conditions are shown (in italics) and mismatched nucleotides creating the restriction site of *Acl* I (cutting site loss in Hb CS allele) and Hind III (cutting site loss in Hb Paksé allele) are marked (bold).

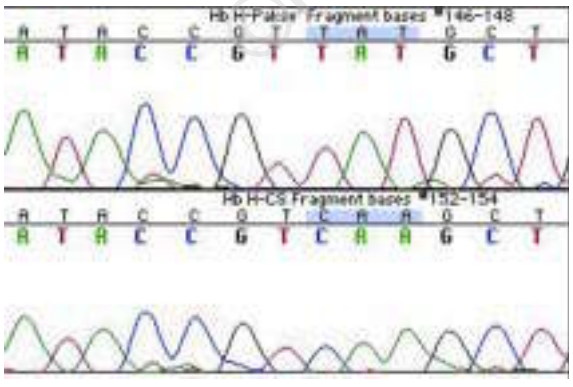

Figure 2. Direct genomic sequencing results comparing both termination codon mutations. The stop codon signal changes from TAA→CAA and TAT in Hb CS and Hb Paksé mutation, respectively. Both sequences showed only C or T changes since the other alleles were lost by co-inheritance of the SEA deletion (--^{SEA}).

Results

Clinical data

The propositus (P.C. in Table 2), a 5-year old boy from central Thailand, presented with chronic anemia at the age of 1 year and had since had two episodes of severe anemia following intercurrent infections. However, he had never received a blood transfusion and had no history of neonatal jaundice or hemoglobinuria. On physical examination, he did not have thalassemic facies but was pale with mild splenomegaly (2 cm below the costal margin). His parents and older brother appeared to be normal. Hematologic analysis revealed that he had hypochromic, microcytic anemia (Table 2) with positive Hb H inclusion bodies consistent with Hb H disease. The α globin genotype by Southern blotting and multiplex PCR showed that he is a compound heterozygote with the --/$\alpha^T\alpha$ genotype. Repeated hemoglobin electrophoresis using different techniques (see methods) failed to demonstrate any abnormal hemoglobin band(s) in his peripheral blood.

Molecular characterization of Hb Paksé (TAA→ TAT) by direct genomic sequencing and mismatched-PCR-RFLP

Subsequent analysis of the α2 and α1 genes of this patient by direct genomic sequencing was performed and revealed a mutation at the termination codon of the α2 gene (TAA→TAT) which has previously been described as Hb Paksé[11] (Figure 2). Therefore, this proband is a compound heterozygote for α^0 thalassemia and the Paksé mutation (--/$\alpha^{Paksé}\alpha$).

To confirm this diagnosis we designed two sets of mismatched-PCR-RFLP to distinguish between this termination codon mutation (TAA→TAT, Hb Paksé) and the termination codon mutation (TAA→CAA, Hb CS) associated with Hb CS. As shown in Figure 3, in Hb CS-mismatched-PCR-RFLP, mutation of the first nucleotide of the termination codon causing Hb CS abolishes an *Acl* I restriction site and the PCR amplification product from this mutant allele is resistant to digestion (remaining 323 bp band), whereas in the normal allele (αα) and Hb Paksé allele ($\alpha^{Paksé}\alpha$), the PCR products were digested to a fragment of 302 bp. In Hb Paksé-mismatched-PCR-RFLP, the A→T mutation at the termination codon was resistant to digestion by *Hind*III. The amplified PCR product from the Paksé allele remained undigested and revealed a band of 321 bp. The 256 bp fragment resulting from digestion of an internal *Hind*III site serves as a control for complete digestion. Therefore, normal (αα) and Hb CS ($\alpha^{CS}\alpha$) alleles are digested to produce fragments of 299 and 256 bp (and a 22 bp one which is not seen). In Southeast Asia, at least, where the Hb CS is highly prevalent, Hb Paksé-mismatched-PCR-RFLP may be applied to distinguish Hb Paksé from other termination codon mutations.

Identification of Hb H-Paksé disease as a common cause of Hb H disease in Thailand

The results of this case prompted us to re-evaluate the molecular basis of disease in 30 patients previously diagnosed as having Hb H-CS. Anecdotally, the hemoglobin electrophoresis in all except one (YP in Table 2) revealed an abnormal slow migrating band, anodic to Hb A_2 in starch gel electrophoresis, resembling Hb CS (Figure 4). α globin genotypes were studied by Southern blotting and

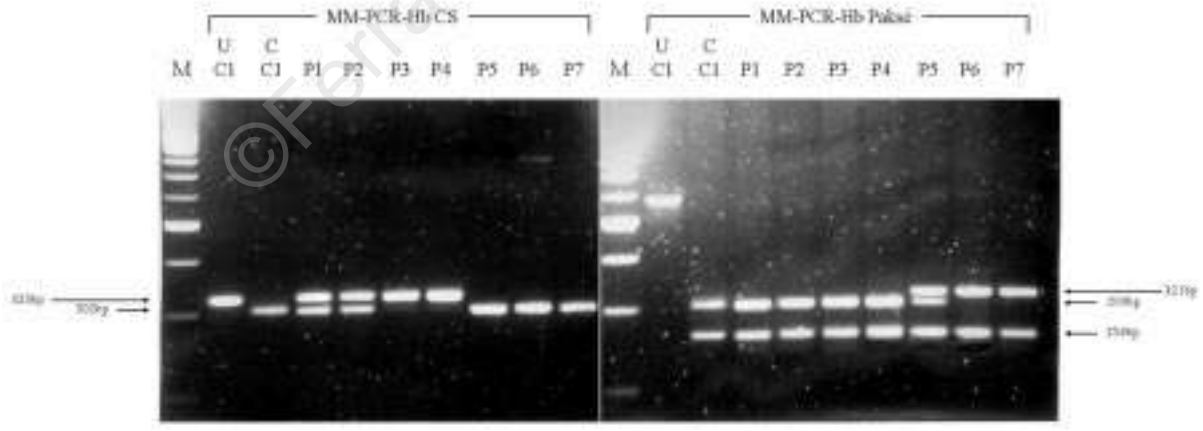

Figure 3. A 3% ethidium bromide-stained agarose gel showing the PCR products after digestion. Left panel: RFLP profile after *Acl*I enzyme digestion for detection of Hb CS allele. C1 (U: uncut and C: cut) represents a normal control (αα/αα) before and after digestion. *Acl*I digested normal allele from 323 bp to 302 bp. P1 and P2 are two heterozygotes of Hb CS mutation (αα/$\alpha^{CS}\alpha$) and revealed both digested and undigested alleles. P3 and P4 are two cases of Hb H-CS disease (--^{SEA}/$\alpha^{CS}\alpha$), only undigested bands present. P5 is a heterozygote of Hb Paksé (αα/$\alpha^{Pakse'}\alpha$), P6 and P7 are Hb H-Paksé (--^{SEA}/$\alpha^{Paksé}\alpha$); all of these samples show only normal digested bands. Right panel: RFLP profile after *Hind* III digestion for detection of Hb Paksé allele. In a normal control, PCR product was cut down from 577 bp to 299 and 256 bp (and 22 bp not seen). Only in samples with Hb Paksé mutation (P5, P6 and P7), were undigested bands of 321 bp revealed with the internal control cutting band of 256 bp.

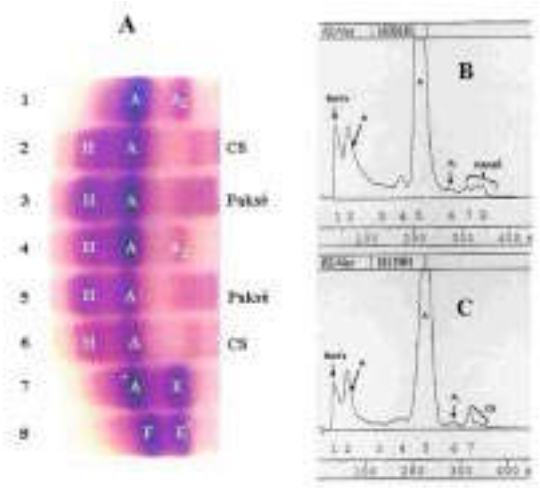

Figure 4. Hemoglobin electrophoresis and cation exchange chromatography in Hb H-like diseases A. Starch gel electrophoresis stained with ortho-dianisidine showing slow migrating bands located anodic to Hb A_2 in Hb H-CS disease patients (2 and 6) and Hb H-Paksé disease patients (3 and 5). Patient #1 has β thalassemia trait (increased Hb A_2), patient #4 has Hb H disease with a positive Hb H band ($--/-\alpha^{3.7}$), patient #7 has Hb E trait with an E band at the same position of Hb A_2 and patient #8 has Hb E/β thalassemia with Hb F and E bands. B and C are chromatograms showing a late eluted peak after Hb A_2 of Hb Paksé and Hb CS, respectively.

multiplex gap-PCR. All patients carried α^0 thalassemia ($--^{SEA}$) on one allele. On the other allele, specific PCR amplification of the α2 gene and subsequent digestion of the product with *Mse* I was performed in these patients as described elsewhere.[7] All patients carried a termination codon (TAA) mutation as shown by the absence of an *Mse* I cutting site (*data not shown*). However, this assay does not distinguish between the two different stop codon mutations. In Southeast Asia, Hb CS is the most common α2 termination codon mutation with a slow migrating band close to Hb A_2. Therefore, previously all patients were presumed to have Hb H-CS disease ($--^{SEA}/\alpha^{CS}\alpha$).

Molecular analysis of the termination codon mutation of the α2 gene was performed in these patients by direct genomic sequencing and/or mismatched-PCR-RFLP as described above. Surprisingly, another 5 cases (~20%) were found to have Hb H-Paksé disease ($--^{SEA}/\alpha^{Paksé}\alpha$). The hematologic parameters and globin genotypes of all six patients are shown in Table 2.

Previously, Hb Paksé was described in four cases, one with Hb H-Paksé disease ($--^{SEA}/\alpha^{Paksé}\alpha$),[11] two compound heterozygotes for Hb Paksé and α^+ thalassemia ($\alpha^{Paksé}\alpha/\alpha^{3.7}$) and Hb CS ($\alpha^{Paksé}\alpha/\alpha^{CS}\alpha$)[12] and one heterozygote ($\alpha^{Paksé}\alpha/\alpha\alpha$).[11] In these previous reports, Hb Paksé was not identified using a variety of different hemoglobin electrophoresis techniques. Of the six cases reported here, in one (P.C.), no abnormal hemoglobin band could be identified by any means. Another patient (Y.P.) had a barely detectable band, whereas the remaining 4 patients had an abnormal, slow-moving band resembling Hb CS. Since this band is indistinguishable from that associated with Hb CS it could contribute to a misdiagnosis of Hb H-CS rather than Hb H-Paksé disease.

It is noteworthy that patients with Hb H-Paksé disease had higher percentages of Hb H (mean 15.1±4%) than those found in the deletional type of Hb H disease, $--/-\alpha$ (mean 7.3±3.6%), but not different from those found in association with non-deletional α thalassemia (mean 15.9±8%).[6]

Table 3. Hematologic data in patients with Hb H-CS and Hb H-Paksé. *$p<0.05$.

Parameters	*Hb H-CS* ($--^{SEA}/\alpha^{CS}\alpha$) *n = 25*	*Hb H-Paksé* ($--^{SEA}/\alpha$Paksé α) *n = 6*	*Student t test p value*
Age (yrs)	10.5±3.7 (3-18)	12.7±5.2 (5-20)	0.356
Hb (g/dL)	6.7±1.5 (4.4-10.3)	7.6±1.9 (4.7-9.9)	0.363
Hct (%)	26.7±6.2 (14.2-36.6)	30.6±6.6 (21.3-37.2)	0.231
RBC (× 10^{12}/L)	3.7±0.8 (2.6-5.2)	4.1±1.1 (2.5-5.1)	0.531
MCV (fL)	72.5±7.5 (54.0-81.3)	75.6±9.7 (63.5-87.5)	0.498
MCH (pg)	18.7±2.6 (15.8-23.7)	18.9±1.3 (17.3-20.8)	0.728
MCHC(g/L)	25.1±4.2 (20.0-28.1)	24.7±3.3 (21.0-28.8)	0.856
Hb H (%)	9.1±3.9 (4.1-18.8)	15.1±4 (10.0-21.4)	0.01*
Hb Bart's (%)	8.8±3.1 (3.2-14.7)	6.2±3.1 (3.5-12.2)	0.111
Hb A_2 + X (%)	4.1±1.3 (1.2-6.4)	3.9±2.5 (0.7-8.3)	0.886

Comparative study of clinical and hematologic phenotypes in patients with Hb H-CS and Hb H-Paksé disease

Hematologic parameters, including hemoglobin data were compared between twenty-five Hb H-CS patients and six Hb H-Paksé patients (Table 3). There were no statistically significant differences between any red blood cell indices or the proportions of abnormal hemoglobin (Hb X%, representing Hb CS or Hb Paksé). The levels of Hb H% were significantly higher in patients with Hb H-Paksé disease than in those with Hb H-CS disease.

In terms of clinical severity of Hb H disease, it should be noted here that 5 of 6 patients with Hb H-Paksé had mild to moderate disease according to previously described criteria (*e.g.* onset of anemia, maintaining Hb levels without transfusion, growth and skeletal development and splenomegaly).[13] Of these 5 patients, all except one (YP, who required a single blood transfusion after an episode of acute hemolysis at 3 years of age), never received any transfusion during the period of follow up (4-19 years) and tolerated their baseline anemia (Hb range from 5.8-10.2 g/dL) achieving normal growth and development. Only one patient (KP) who had the lowest baseline Hb (4.8 g/dL) compared to others, required frequent transfusions (total 13 episodes in 2 years from 2-4 years of age) and had a splenectomy at the age of 4.5 years. After the operation, Hb levels in this patient remained between 9.5-11 g/dL without transfusion. Of the Hb H-CS patients, 7 of 25 can be classified as having a severe disease form since they all required frequent transfusions and eventually underwent splenectomy.

Discussion

Hemoglobin Constant Spring (Hb CS: TAA→CAA) is the most common non-deletional type of α thalassemia world-wide.[2] In Southeast Asia, where the α^0 thalassemias and Hb CS are both highly prevalent, co-inheritance of both determinants gives rise to Hb H-CS disease (--/$\alpha^{CS}\alpha$), which is the most common form of non-deletional Hb H disease in this region.[14-15]

Here we describe a group of six Thai patients with HbH-Paksé disease (--^{SEA}/$\alpha^{Paksé}\alpha$). Hb Paksé (TAA→TAT) was first described in a Laotian patient living in Canada who had Hb H disease.[11] Another family of Laotian origin has also been described.[12,16,17] In this family, Hb Paksé was shown to interact with either α^+ thalassemia (-$\alpha^{3.7}$) or Hb CS ($\alpha^{CS}\alpha$) to cause considerable microcytosis and hypochromia without Hb H. In both families, Hb Paksé could not be demonstrated by hemoglobin electrophoresis and chromatography. It was thought that this α globin variant ($\alpha^{Paksé}$) might be unstable and possibly degraded before it could form a tetramer with β globin chains and/or that the abnormal hemoglobin tetramers may be unstable.[11]

Since both mutations occur at the normal stop codon 142 (TAA) of the α2 gene, this allows translation of mRNA up to the next in-phase stop codon causing an extension of the α globin chain by 31 amino acids from the normal C-terminal, 141 arginine.[18] It has been shown, in cases of Hb CS, that the elongated mRNA molecules are unstable and corresponding α^{CS} peptides may also have decreased stability.[19] This results in a small proportion of Hb CS relative to total hemoglobin and the slowly migrating faint band of Hb CS may not be detected by Hb electrophoresis, even in homozygotes for this mutation ($\alpha^{CS}\alpha/\alpha^{CS}\alpha$).[20] It is possible that the mutation causing Hb Paksé also reduces the stability of the mRNA transcripts and the $\alpha^{Paksé}$ chain in a similar way. This is consistent with the failure to observe the abnormal band in the first two reported cases and two of our patients (PC and YP). However, some patients with HbH- Paksé disease did reveal an abnormal slow-migrating hemoglobin band in electrophoresis resembling that of Hb CS (Figure 4). This suggests that HbH-Paksé disease in Southeast Asia may be underestimated due to the misdiagnosis of some cases as Hb H-CS disease. The α^{CS} and $\alpha^{Paksé}$ chains differ by only a single amino acid (142 glutamine in the α^{CS} chains and 142 lysine in the $\alpha^{Paksé}$ chains) consistent with the observation that the abnormal hemoglobins migrate to the same position in Hb electrophoresis.

Both mutations occur at the termination codon (TAA) of the α2 gene and abolish an *Mse*I recognition site. Amplification of the α2 gene and subsequent digestion with *Mse* I provides a rapid screen for termination codon mutations.[7] This approach has been widely used[21] even in prenatal diagnostic testing for Hb H-CS disease.[22] Our report suggests that the frequency of the Hb Paksé mutation (TAA→TAT) might be higher in Thailand than previously thought, and possibly represents the second most common non-deletional α thalassemia in Southeast Asia. Hb Paksé is more accurately diagnosed by direct genomic sequencing or mismatched-PCR-RFLP, which distinguishes this mutation from other termination codon mutations and can be used to ascertain the actual frequency of Hb Paksé in Southeast Asia.

Many studies have shown that Hb CS has a more deleterious effect than would be expected from

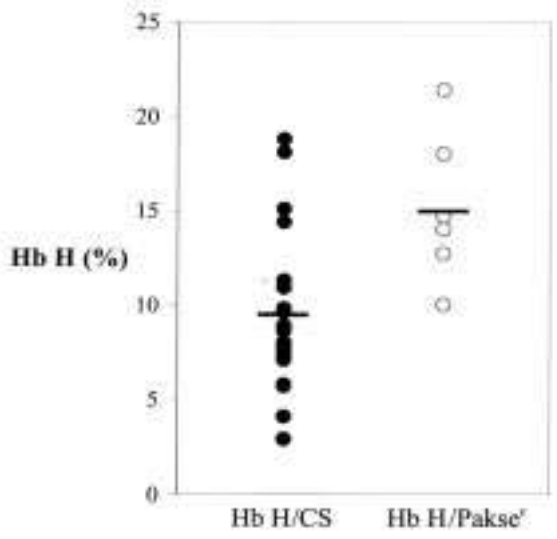

Figure 5. Distribution of Hb H levels in patients with Hb H-CS and Hb H-Paksé (mean Hb H (%) in patients with $--/-\alpha$ = 7.3±3.6%, bars represent mean Hb H).

the downregulation of α-globin expression and reduced hemoglobin production alone.[23-25] Ineffective erythropoiesis and decreased red blood cell survival also play an important role in the pathophysiology of Hb H-CS disease. Red blood cells from these patients show increased membrane rigidity, decreased deformability and altered cell hydration.[23-4] These changes are the result of perturbations induced by the association of oxidised α^{CS} chains with the red cell membrane and its skeletal proteins.[25] These cellular changes may explain the more severe phenotype found in Hb CS than that of the more common deletional type of α^+ thalassemias ($--/-\alpha$).

It will be of interest to determine whether the Hb Paksé mutation gives rise to similar effects. Our preliminary results show that the hematologic parameters in patients with $--/\alpha^{CS}\alpha$ and $--/\alpha^{Paksé}\alpha$ are not significantly different, except for the slightly higher levels of Hb H observed in Hb H-Paksé disease. However, the high proportion of Hb H cannot be used as a predictor for this condition since patients with Hb H-CS disease may have similarly high levels of Hb H (Figure 5). The levels of Hb H in Hb H disease were thought to reflect the imbalance between α and β globin synthesis and to correlate with clinical severity.[13,26,27] Moreover, it has been proposed that Hb H by itself has a negative influence on tissue oxygenation because Hb H, as a high affinity Hb, lacks the ability to carry oxygen to the tissues.[27] Therefore the clinical picture in Hb H-Paksé could be somewhat more severe than that of Hb H-CS. However, our preliminary results suggest that Hb H-Paksé patients are not significantly different from those with Hb H-CS and the molecular basis underlying higher levels of Hb H in patients with Hb Paksé remains unclear. In the future, a study of the natural history and disease progression of patients with these genotypes will provide some insight into the nature of these two termination codon mutations and their effects upon erythropoiesis.

Contributions and Acknowledgments

VV was the main investigator contributing to the design and concept of the study, all data and statistical analysis, performing molecular studies, design and setting up mismatched PCR-RFLP, interpretation of the results, drafting and article revision. VST was responsible for taking care of the patients, clinical data collection, concept of the study and editing the draft. PPA, SP and LS were involved in the hematologic analyses, data collection and preparing DNA samples. CF designed primers and PCR conditions for specific $\alpha 2$ and $\alpha 1$ gene amplification. DRH is the last author and participated in the design and concept of this study and editing the final version of manuscript. We thank our patients and their families for their participation in this study. The authors are grateful to Kevin Clark and Alun McCarthy for their excellent technical assistance, and Darika Seeleom and Narisara Viprakasit for helpful secretarial work including data processing. We thank David Garrick, Cristina Tufarelli and Tarra McDowell for their critical comments on the manuscript.

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Disclosures

Conflict of interest: none

Redundant publications: no substantial overlapping with previous papers.

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Peer Review Outcomes

What is already known on this topic

There has been only one previous report of HbH/Paksé disease.

What this study adds

The study indicates that the frequency of Hb H/Paksé may be higher than previously thought. The study provides a new method PCR-based technique to distinguish the termination codon mutations of Hb CS and Hb Paksé which can be used as an alternative to direct sequencing.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Prof. Carlo Brugnara, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Prof. Brugnara and the Editors. Manuscript received November 5, 2001; accepted December 3, 2001.

Potential implications for clinical practice

Due to the similar patterns in Hb electrophoresis, chromatography and molecular diagnosis (abolition of MSe restriction site) many previously diagnosed case of Hb H/Constant Spring could indeed be Hb H/Paksé disease. Clinically, the two diseases are very similar, with the only difference being a higher level of Hb H in HbH/Paksé disease.

Carlo Brugnara, Deputy Editor