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Significance of Zone 2 Peak on Capillary Electrophoresis in the Detection of Hemoglobin Constant Spring

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ABSTRACT

Objectives: Hemoglobin constant spring (Hb CS) is a point mutational defect associated with α thalassemia. The aims of this study were to compare the hematological profiles between different Hb CS genotypes and to estimate the range for Zone 2 peak using capillary electrophoresis (CE) with different Hb CS genotypes. ***Methods***: For this cross-sectional study, patient blood samples that showed a positive peak in zone 2 of CE were selected. Hemoglobin and DNA of the samples were investigated to ascertain the presence and levels of non-deletional and deletional α thalassemia. The results were statistically analyzed. ***Results:*** Of the 137 samples investigated, 118 (86.1%) were positive for termination codon Hb CS mutation. Heterozygous Hb CS was found in 92 (67.2%), compound heterozygous Hb CS in 22 (16.1%), and homozygous Hb CS in four (2.9%) samples. The ranges of Hb CS level for heterozygous Hb CS, compound heterozygous Hb CS, and homozygous Hb CS were within 0.2–2.7%, 0.3–2.2%, and 4.5–5.5%, respectively. Significant hematological differences in the Hb level, mean cell volume, mean cell hemoglobin, red cell distribution width, red blood cell count, and Hb CS level were observed between heterozygous, homozygous, and compound heterozygous Hb CS. ***Conclusions:*** In view of the overlapping prevalence range of Hb CS level for heterozygous and compound heterozygous Hb CS, only Hb CS level within the range 4.5–5.5% was helpful in the diagnosis of homozygous Hb CS.

Hemoglobin constant spring (Hb CS) is a non-deletional alpha (α) thalassemia. Mutation of termination codon on α2-globin genes impairs RNA translation which leads to the production of unstable and elongated α-globin chains with 172 instead of 141 amino acid residues, as well as a decrease in the rate of normal α-globin chain synthesis.[1] Heterozygotes of Hb CS usually have normal clinical and hematological features but can be presented with mild hypochromic anemia. Meanwhile, homozygotes show clinical presentation similar to that of thalassemia intermedia: mild anemia, jaundice, and hepatosplenomegaly. Furthermore, the interaction of the Hb CS gene with 2 gene deletion α thalassemia is usually more severe than deletional hemoglobin H–constant spring (Hb HCS) disease which may render some patients dependent on blood transfusion.[2]

Screening for Hb CS is performed by capillary electrophoresis (CE), a widely used analytical separation technique.[3] The CE system can be used to separate and quantitate abnormal forms of Hb such as Hb A_2 and Hb F. CE will give peak at Zone 2 for Hb CS. Another common variant that shares the same peak as Hb CS (and with similar clinical presentation) is Hb Paksé, though not described in Malaysia previously. Other rare variants that can give similar peak in Zone 2 of CE are Hb C, Hb F Texas, Hb C-Harlem, and variant Hb A2 'Setif'.[4]

Since Hb CS is prevalent in this region and shows clinical heterogeneity, the reliable detection of Hb CS by CE is essential, especially in centers that do not have access to molecular studies. Therefore, this study aimed to determine the significance of Zone 2 peak on CE for the detection of Hb CS.

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METHODS

This was a cross-sectional study conducted during a one-year period at Hospital Raja Perempuan Zainab 2, a tertiary hospital which caters to the requirements for Hb analysis for patients from the adjoining districts. Based on the sample size calculation using single proportion formula with 0.06 prevalence of Hb CS[5] and 0.04 precision, the minimum required sample size was 135. The samples showing a peak in Zone 2 of CE were included in the study. Samples where iron deficiency anemia was a cause for low mean cell volume (MCV) and low Hb were excluded. Ethical approval was obtained from the ethics committee in National Medical Research Registry (NMRR) (NMRR-15-724-25404 IIR) and Hospital Universiti Sains Malaysia (HUSM) (USM/JEPem/15050169).

Full blood count was performed using Sysmex XN-3000 (Sysmex Corporation, USA) an automated six-part hematology analyzer. Hb separation and quantitation of Hb variants (e.g., HbA2, HbE, Hb CS, and others) were performed using CE; CAPILLARYS2 Flex Piercing System, Sebia, PN 1227, France.[4] Genomic DNA was extracted from leukocytes using QIAsymphony SP, Qiagen Company, Germany, according to the manufacturer's instructions.

Multiplex ARMS assay as described by Eng et al,[6] was used to genotype non-deletional α-gene mutations common in Malaysia: termination codon TAA→CAA mutation or Hb CS; the codon 125 CTG→CCG mutation or Hb Quong Sze; codon 59 mutation (GGC→GAC) or Hb Adana; and initiation codon mutation (ATG→A-G), codon 30 mutation (ΔGAC), and codon 35 mutation (TCC→CCC) for Hb Evora. The amplification was carried out with 0.5–1.0 μg of DNA in a 50 μL solution containing 2.5U HotStarTaq DNA Polymerase in the supplied buffer, 1.5mM $MgCl_2$, 200 μM each of deoxynucleoside triphosphates, 1X Q-solution and primers in the various concentrations. Reactions were conducted with an initial 15-minute activation or denaturation at 96 °C followed by 30 cycles of denaturation at 98 °C for 45 seconds, annealing at 62 °C for 60 seconds, extension at 72 °C for 135 seconds, and a final extension at 72 °C for five minutes. Following amplification, the polymerase chain reaction (PCR) products were electrophoresed through 1.5% agarose gel. The DNA bands then were visualized under UV light transilluminator.[5] Where the Multiplex ARMS showed negative for Hb CS mutation, further molecular analysis was conducted using ARMS PCR to detect presence of Hb Paksé, which is also found at Zone 2 of CE.[7]

Multiplex Gap PCR as described by Chong et al,[8] was used to genotype the common α-gene deletion; two single-gene deletions viz., $\alpha^{-3.7}$, $-\alpha^{4.2}$ and five two-gene deletions, viz., --SEA, --FIL, --MED, $--(\alpha)^{20.5}$, --THAI. Multiplex ARMS PCR for concomitant β-globin gene mutation was also carried out according to Hassan et al.[9]

RESULTS

A total of 137 samples taken from 109 (79.6%) female and 28 (20.4%) male patients were included in this study, 46.0% of whom were in the 21–30 years age group. The ethnicity of 132 (96.4%) patients was Malay, four (2.9%) were Siamese, and one (0.7%) was Chinese. Of the 137 samples, 118 (86.1%) were positive for termination codon mutation for Hb CS (TAA→ CAA). Out of these 118 positive samples, 92 were classified as heterozygous group, 22 were compound heterozygous, and four were homozygous Hb CS. No mutation was detected in 19 (13.9%) samples [Table 1].

The level of Zone 2 peak between different groups of Hb CS varied. The medians of Hb CS, Hb, MCV, mean cell hemoglobin (MCH), red cell distribution width coefficient of variation (RDW-CV%), and red blood cell (RBC) level of the three groups of Hb CS were significantly different ($p < 0.001$) [Table 2]. However, there were no significant differences between MCV of heterozygous and homozygous Hb CS, between MCH of heterozygous

Table 1: Distribution of samples according to types of hemoglobin constant spring (Hb CS) (N = 137).

Types and co-inheritance	n	%
Heterozygous Hb CS	92	67.2
Homozygous Hb CS	4	2.9
Compound heterozygous Hb CS		
Hb CS with $\alpha^{-3.7}$	12	8.8
Hb CS with $-\alpha^{4.2}$	1	0.7
Hb CS with $\alpha^{-3.7}$ and Hb E trait	1	0.7
Hb CS with β thalassemia trait	1	0.7
Hb CS with Hb E trait	4	2.9
Hb CS with $--^{SEA}$	2	1.5
Hb CS with $--^{THAI}$	1	0.7
No Hb CS mutation detected	19	13.9

Table 2: Ranges of Zone 2 peak on capillary electrophoresis for various types of Hb CS.

Group	No. of samples	Range of Zone 2 peak/ Hb CS (SD)	Median Hb CS (IqR)	Median Hb (IqR)	Median MCV (IqR)	Median MCH (IqR)	Median RDW-CV% (IqR)	Median RBC (IqR)
Heterozygous Hb CS	92	0.2–2.7 (2.7)	0.60 (0.20)	11.50 (1.70)	77.40 (0.60)	24.90 (1.90)	14.05 (1.50)	4.72 (0.79)
Homozygous Hb CS	4	4.5–5.5 (0.4)	5.30 (0.80)	8.95 (1.00)	80.65 (8.90)	23.80 (3.30)	16.40 (2.30)	3.77 (0.95)
Compound heterozygous Hb CS	22	0.3–2.2 (0.5)	1.00 (1.90)	10.85 (2.80)	74.65 (6.00)	22.95 (2.70)	14.35 (2.10)	5.12 (1.18)

IqR: interquartile range; Hb CS: hemoglobin constant spring; MCV: mean cell volume; MCH: mean cell hemoglobin; RDW-CV: red cell distribution width coefficient of variation.

Table 3: Hematological parameters of individuals (n = 22) with compound heterozygous Hb CS.

Co-inheritance (n)	Mean Hb ± SD (g/dL)	Mean MCH (pg) ± SD	Mean MCV (fl) ± SD	RBC level ± SD	Mean RDW-CV (%) ± SD	Hb CS level ± SD
Hb CS with $\alpha^{-3.7}$(12)	11.3 ± 1.7	22.4 ± 1.4	72.1 ± 4.8	5.0 ± 0.7	14.7 ± 1.5	1.05 ± 0.3
Hb CS with $-\alpha^{4.2}$ (1)	12.2	22.8	74.9	5.3	14.1	0.9
Hb CS with $\alpha^{-3.7}$ deletion and Hb E trait (1)	9.8	23.4	75.2	4.1	14.2	0.9
Hb CS with β thalassemia trait (1)	9.6	25.7	84.2	3.7	14.6	0.3
Hb CS with Hb E trait (4)	11.7 ± 1.9	24.2 ± 1.4	73.7 ± 2.9	4.8 ± 0.8	14.3 ± 1.3	0.6 ± 0.6
Hb CS with $--^{SEA}$ (2)	8.2 ± 1.8	21.8 ± 2.3	77.5 ± 7.3	3.8 ± 1.2	24.7 ± 2.4	1.8 ± 0.2
Hb CS with $--^{THAI}$ (1)	9.2	15.3	52.4	6.0	20.6	2.2

Hb CS: hemoglobin constant spring; MCH: mean cell hemoglobin; MCV: mean cell volume; RBC: red blood cell; RDW-CV: red cell distribution width coefficient of variation.

and homozygous Hb CS, between homozygous Hb CS and compound-heterozygous Hb CS, or between RBC heterozygous and compound heterozygous Hb CS.

While 22 individuals were found to have Hb CS with other co-inheritance, most had concurrence with single gene deletion of α thalassemia which was $\alpha^{-3.7}$. The hematological parameters were described according to co-inheritance with Hb CS as summarized in Table 3.

DISCUSSION

Upon analysis by CE, 118 of 137 (86.1%) samples which showed positive peak in Zone 2 of CE were positive for Hb CS. The remaining 13.9% were negative for Hb CS and Hb Paksé. (To date there is no published data on the prevalence of Hb Paksé in Malaysia.)

Among the 118 samples, Hb CS levels ranged 0.2–5.5%. When these were further classified as per the various Hb CS phenotypes, the prevalence of heterozygous Hb CS ranged 0.2–2.7%, homozygous Hb CS ranged from 4.5–5.5%, and compound heterozygous Hb CS was in the range of 0.3–2.2%. Liao et al,[2] found that the levels of Hb CS in heterozygotes ranged from 0.1–1.0%, demonstrating that the CE technique was capable of quantifying Hb CS at a level as low as 0.1%. In the current study, however, the lowest Hb CS level detected was 0.2%.

Nineteen of 137 samples with negative results for termination codon Hb CS showed Hb CS level in the range 0.2–1.2%. The possible causes of false positive peak on Zone 2 of CE may include plasma proteins from the sample, for instance, if the patient had low Hb with low RBCs per plasma ratio.[4] Another possible reason for Zone 2 peak on CE could be the presence of other Hb variants that share the same zone as Hb CS.

The significant differences in hematological parameters between heterozygous and compound heterozygous Hb CS were in the levels of Hb, MCV, MCH, RDW, and Hb CS. Meanwhile comparing between heterozygous and homozygous Hb CS, the significant hematological differences were Hb level, RDW, RBC count, and Hb CS level. For comparison between homozygous and compound heterozygous Hb CS, the significant parameters of difference

were Hb level, MCV, RDW, RBC count, and Hb CS level. The Hb level of homozygous group in this study ranged from 8.6–9.7 g/dL. The presence of the homozygotes is indicative of thalassemia intermedia with typical presenting symptoms of anemia, jaundice, and hepatosplenomegaly.[10]

In compound heterozygous Hb CS samples in the current study, Hb ranged 6.9–13.9 g/dL. One individual in the cohort with severe anemia with Hb of 6.9 g/dL had concurrent --SEA. This was similar to a previous study which had compared the severity of Hb H deletional with Hb H-CS, and found that the most common co-inheritance was with --SEA.[11] Its findings included moderate anemia, regular transfusion therapy in 24% of patients, and splenomegaly or prior splenectomy in one-third.[10] Hb H-CS and deletional Hb H disease are two different clinical entities and should be recognized as distinct thalassemia syndromes with a high risk of life-threatening anemia during the febrile stage.[12] Individuals with non-deletional Hb H commonly develop complications within the first decade of life, including recurrent transfusions, iron overload, and significant growth delay.[13]

The MCV levels in this study showed a significant difference between heterozygous and compound heterozygous groups as well as between homozygous and compound heterozygous groups. Nevertheless, no significant difference was noted between heterozygous and homozygous groups. Though, MCH < 27 pg had been chosen as the value for screening of thalassemia and hemoglobinopathy compared to MCV as the latter is susceptible to storage changes. In this study, the mean MCV of heterozygotes of Hb CS was found to be borderline normal with a level of 77.4 fl. Hence, this variant of Hb CS had an almost normal MCV, supported by a previous study which yielded comparable results.[14]

The median of MCH was significantly different between heterozygous and compound heterozygous groups which is similar to a previous study.[15] Meanwhile, the value of MCH in compound heterozygotes varied according to co-inheritance [Table 3]. This showed that co-inheritance with α+ thalassemia, β thalassemia, and heterozygous Hb E can lower the MCH level.[14] In this study, the mean MCH of compound heterozygous with co-inheritance 2 gene deletions which were --THAI and --SEA showed the lowest level compared to 1 gene deletion, $\alpha^{-3.7}$ and $-\alpha^{4.2}$ [Table 3].

The RBC counts as well as RDW-CV% were significantly different, as in previous findings among Southeast Asian populations.[15,16] In essence, most of the hematological parameters can be used as a guide to differentiate between these three different genotypes; heterozygous, compound heterozygous and homozygous Hb CS, and homozygous Hb CS. However, if the quantification of Hb CS level is at a low level which is < 0.6%, the molecular analysis should be performed to detect the presence of termination codon Hb CS mutation.

In this study, there were a few singular cases. One was a Malay individual with --THAI deletion, which is rare in Malaysians but common among the Thai people.[5] One female patient had --SEA with concurrent Hb CS. She had severe hypochromic anemia with Hb of 6.9 g/dL and MCH of 23.5 pg, but normal MCV of 82.7 fl. Another patient with similar co-inheritance had only mild hypochromic microcytic anemia with Hb of 9.5 g/dL, MCV of 72.3 fl, and MCH of 20.2 pg.

In this study, four cases coexisted with Hb E, a common hemoglobinopathy in Southeast Asia. Hb E is considered a hallmark of this region, reaching a prevalence of 50–60% at the junction of Thailand, Laos, and Cambodia, also called 'Hb E Triangle'.[17] In Malaysia, it occurs among the Malays with 5% frequency and higher among Orang Asli aborigines in Peninsular Malaysia.[18] As our study was conducted in the northern region of Kelantan which shares a border with Thailand, the high frequency of Hb E and its coexistence with Hb CS may be attributed to genetic intermixing. The levels of Hb E in these patients with compound heterozygotes with Hb CS ranged 19.8–23.2%. Typically for the manifestation of the Hb E trait, the level of Hb E should be > 30%. However, if it coexists with α thalassemia trait, the level of Hb E is much lowered. Thus, individuals with < 25% of Hb E need to be suspected to have coexisting α thalassemia trait.[19] A study regarding concurrent inheritance of deletional α thalassemia in Malays with Hb E trait found that Hb A2/ E levels were lower in Hb E carriers with α thalassemia. One proposed mechanism is that fewer unmatched α-globin chains lead to more balanced α/non α-globin ratio.[20] Another suggestion is that when α subunits are present in limited quantities as a consequence of α thalassemia, Hb A is formed more rapidly than Hb E whose level falls.[21]

One patient in the current study had β thalassemia trait with heterozygous Hb CS. She had a mild hypochromic anemia with the hematological parameters summarized in Table 3. However, she had normal MCV of 84.2 fl and Hb A2 level of 4.0%. This may be due to the normalizing effect of MCV by CS RBCs, a view supported by Schrier et al,[22] who found RBC containing Hb CS to be noticeably overhydrated than deletional α thalassemia variants. In addition, the derangement of volume regulation was fully expressed at the reticulocyte stage even though the cell hydration occurred early in erythroid maturation. Therefore, they proposed that increased RBC hydration was caused by damage to the potassium chloride co-transport pathway, and that the derangement was induced by membrane-associated oxidized β chains.[22]

In this study, the Hb CS level among patients with compound heterozygotes with co-inheritance of Hb E and β trait had lower Hb CS levels than if they had concurrent deletional α thalassemia. However, these findings could not be confirmed and need further exploration.

CONCLUSION

This study showed that presence of Zone 2 peak in CE had a significant value in determining the homozygotes of Hb CS. This might aid in diagnosis of Hb CS where the molecular technique is not available.

Disclosure

The authors declared no conflicts of interest. No funding was received for this study.

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