ORIGINAL ARTICLE

Haematologic Profile of Haemoglobin Constant Spring and Its Co-inheritance With Alpha and Beta Thalassaemia Among Form Four Students in Negeri Sembilan

Subithira Nahanthiran[1,2], Nik Hafidzah Nik Mustapha[1], Subashini Chellappah Thambiah[2], Malina Osman[3], Faridah Idris[2], Sabariah Md Noor[2]

[1] Haematology Unit, Department of Pathology, Hospital Tuanku Jaafar Seremban, Jalan Rasah, Bukit Rasah, 70300, Seremban, Negeri Sembilan
[2] Department of Pathology, University Putra Malaysia, Faculty of Medicine and Health Sciences, 43400, Serdang, Selangor
[3] Department of Medical Microbiology, University Putra Malaysia, Faculty of Medicine and Health Sciences, 43400, Serdang, Selangor

ABSTRACT

Introduction: In Malaysia, the commonest non-deletional alpha (α) thalassaemia is Haemoglobin Constant Spring (Hb CS) which occurs due to a mutation at the termination codon of α2 globin gene (TAA>CAA). Presence of an abnormal peak at Zone 2 on CE or presence of a small peak at c-window on HPLC can be suggestive of Hb CS. The objective of this study was to determine the proportion of form four students diagnosed with Hb CS and to study the haematologic profile of Hb CS and its co-inheritance with α or beta (β) thalassaemia. **Methods:** This was a cross-sectional study carried out at Hospital Tuanku Ja'afar Seremban (HTJS), involving 15-16 year old secondary school students screened for thalassaemia. The proportion of Hb CS and Hb CS with α or β thalassaemia co-inheritance was calculated and the correlation between the full blood count (FBC) parameters with CE and HPLC results were determined. **Results:** A total of 3121 students were diagnosed to have thalassaemia and the proportion of Hb CS was 3.24%. Hb CS with α thalassaemia co-inheritance had significantly lower mean corpuscular volume (MCV) compared to Hb CS without co-inheritance and Hb CS with β thalassaemia co-inheritance, $t(2)=4.16$, $p=0.02$. This study also has shown that the mean corpuscular haemoglobin (MCH) was significantly lower in Hb CS with α thalassaemia co-inheritance $t(2)=9.89$, $p<0.01$. **Conclusion:** The combination of both, FBC parameters and Hb analysis can be used in screening and in making a presumed diagnosis of Hb CS or co-inheritance with alpha thalassaemia.
Malaysian Journal of Medicine and Health Sciences (2022) 18(SUPP21): 23-29. doi:10.47836/mjmhs18.s21.5

Keywords: Hb Constant Spring, Co-inheritance with alpha and beta thalassaemia

Corresponding Author:
Sabariah Md Noor, MPath
Email: md_sabariah@upm.edu.my
Tel: +603-97692761

INTRODUCTION

Thalassaemia is a globin gene disorder first described by Cooley and Lee in 1925 (1). About 20% of the world population have alpha+ (α+) thalassaemia and 5.2% of the population carries beta (β) thalassaemia and α0 thalassaemia (2). In Malaysia, thalassaemia is one of the commonest inherited blood disorders, where about 4.5% of the population are carriers of either β or α thalassaemia (3). The common thalassaemia syndromes among Malaysians are β thalassaemia major, Eβ thalassaemia, Hb H disease, and Hb Barts hydrops foetalis (3).

In Malaysia, the commonest non-deletional α thalassaemia is Haemoglobin Constant Spring (Hb CS) (5). It is also the most prevalent non-deletional α thalassaemia among the South East Asia (SEA) population (6). It is noted to be more common among the Chinese population (7). Hb CS occurs due to a mutation at the termination codon of α2 globin gene (TAA>CAA) which leads to a reduction in α chain synthesis(7). Patients with Hb CS are usually asymptomatic but some may have mild anaemia. In a study done by R.Z Azma et al, homozygous Hb CS patients were found to have hypochromic microcytic red cells with anisopoikilocytosis, leucoerythroblastic picture and basophilic stippling. These findings are not observed among those who are heterozygotes (6). The nature of Hb CS is unstable and hence its detection can be commonly missed (7). Patients with heterozygous Hb CS have almost normal red cell parameters (7). This mutational haemoglobinopathy is usually suspected by presence of HbC/CS zone on capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) (6,8)(Figure 1). The mutant allele for Hb CS can be detected via DNA analysis using real time Polymerase Chain Reaction (PCR) (5).

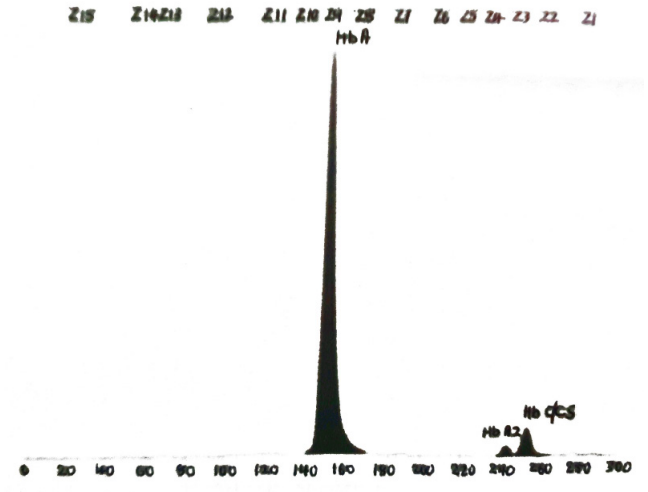

Figure 1: The Capillary electrophoresis electrophoregram of patients with Hb CS

Interaction between the different determinants of α thalassaemia and Hb CS produces a wide spectrum of clinical and haematological phenotypes, ranging from normal to intermediate thalassaemias (5). For example, the co-inheritance of Hb CS and a single α-3.7 deletion results in a clinical phenotype that is very similar to two α gene deletions (5). On the other hand, co-inheritance of Hb CS and Hb Adana, which is another mutational type can produce more severe anaemia (9). In SEA countries, it is frequent to find α and β thalassaemia co-inheritance as thalassaemia is highly prevalent in this region (10). The co-inheritance of α and β thalassaemia improves the phenotype and clinical symptoms of the patient due to alleviation of imbalance between α and non-α chains (5).

The Malaysian Government has set up a screening program to screen all form four students who are between the ages of 15 and 16 year old (11). The aim of this screening program is to determine the thalassaemia carrier rate among students in a secondary school and to educate these healthy carriers on the risks and options for preventing the birth of children with severe thalassaemia syndromes (11).

This study aimed to determine the proportion of Hb CS; a subtype of α thalassaemia among form four students who were diagnosed to have thalassaemia / haemoglobinopathies in Negeri Sembilan. Besides that, we also aimed to study the co-inheritance of Hb CS with α and β thalassaemia. This was to evaluate the possible different screening and diagnostic methods available in detecting Hb CS. We also aimed to determine the association within all the haematological parameters available, with the hope that the findings obtained would suggest a preferable or useful method for Hb CS screening and confirmation besides the commonly used molecular technique.

MATERIALS AND METHODS

Study location
This research was conducted in Haematopathology Unit, Department of Pathology, Hospital Tuanku Ja'afar Seremban (HTJS).

Study ethics
The Medical Research Ethics Committee (MREC), MREC Ref. No: NMRR-18-2602-43223 and Jawatankuasa Etika Universiti Untuk Penyelidikan Melibatkan Manusia (JKEUPM), JKEUPM Ref. No: JKEUPM-2020-207 reviewed and approved this study. It was carried out in accordance with the guidelines of the Malaysian Good Clinical Practice (GCP).

Study design and duration
This was a cross-sectional study involving form four students screened for thalassaemia under the National Thalassaemia Screening Program in Negeri Sembilan for a duration of 12 months, from January 2018 to December 2018.

Study population
The study population included students aged between 15-16 year old who were screened for Thalassaemia under the National Thalassaemia Screening Program in Negeri Sembilan and diagnosed to have thalassaemia / haemoglobinopathies.

Selection eligibility

Inclusion criteria
Form four students (aged 15-16) who were under the National Thalassaemia Screening Program from January 2018 to December 2018, with

b) MCH of ≤ 27 pg and complete Hb analysis (CE and HPLC) results

c) Complete DNA analysis results with Hb CS / Hb CS with common α and β thalassaemia co-inheritance

Exclusion criteria
Data with students known to have other medical problem (diabetes mellitus, chronic renal failure, autoimmune disease, malignancy) and incomplete data.

RESULTS

Background of study results
In the year 2018, HTJS received a total of 5155 samples to be screened for thalassaemia / haemoglobinopathies. Out of this, 3655 samples were from students aged 15-16 years, with hypochromic red cells (MCH < 27pg). Among these students who were screened for thalassaemia / haemoglobinopathies, 3121 were diagnosed to have either thalassaemia / haemoglobinopathies. 115 of them had Hb CS. However, only 101 samples were analysed in this study. The remaining 14 samples were rejected

due to incomplete data such as no HPLC performed due to long storage time (>72 hours), and no DNA analysis was sent to IMR for confirmation of results. Out of this 101 samples, 7 of them had co-inheritance with α thalassaemia and 2 had co-inheritance with β thalassaemia.

Proportion of Hb CS and its co-inheritance with α and β thalassaemia among Form 4 students who were tested to have thalassaemia / haemoglobinopathies

Total of 3121 students were diagnosed to have thalassaemia / haemoglobinopathies. Out of these cases, 3.24% were noted to have presumed heterozygous Hb CS (n=101). Among all the cases of Hb CS, 6.9% had co-inheritance with α thalassaemia. Heterozygous α+ thalassaemia 3.7 gene deletion was seen in four cases, one case of heterozygous α+ thalassaemia 4.2 gene deletion, one case of heterozygous α+ thalassaemia 3 gene deletion (Hb H / HbCS) and one had co-inheritance with Hb Adana. While, 2% had co-inheritance with β thalassaemia (one student was heterozygous for β+ thalassaemia and one had co-inheritance with D Punjab). In 91.1% of these cases, no associated co-inheritance was detected. The co-inheritance of alpha and beta thalassaemia were confirmed by DNA analysis.

Sociodemographic characteristics of students with Hb CS

There was almost an equal distribution of the disease among males (n = 52, 51.5%) and females (n = 49, 48.5%). The majority were Malays (n = 100, 99%), followed by Chinese (n = 1, 1%) and none among the Indians and other races. Among the students with co-inheritance, all of them were Malays.

Difference of means scores of MCV, MCH, Hb A2, Hb A, and Hb CS level by HPLC and CE in Hb CS with co-inheritance and Hb CS without co-inheritance

This study found that Hb CS with co-inheritance of α thalassaemia had significantly lower MCV, with mean of 73.79 fl compared to Hb CS without co-inheritance and Hb CS with β thalassaemia co-inheritance, t(2)=4.16, p=0.02. Besides that, the MCH was also significantly lower in Hb CS with α thalassaemia co-inheritance with mean of 22.77 pg compared to Hb CS without co-inheritance and with β thalassaemia co-inheritance, t(2)=9.89, p<0.01.

In Hb analysis, it was noted that the Hb A2 levels in HPLC was significantly lower in the Hb CS with α thalassaemia co-inheritance group with mean of 2.36 (0.29) % compared to Hb CS without co-inheritance and with β thalassaemia co-inheritance, t(2)= 4.28 with p value of 0.02. On the other hand, Hb A2 levels on CE was highest among the β thalassaemia co-inheritance group with mean of 3.10 (0.28) % compared to those without co-inheritance and with α thalassaemia co-inheritance, t(2)=6.45, p=0.04. Hb A in HPLC was observed to have a significantly highest value among the group with α thalassaemia co-inheritance [mean:87.91 (0.82) %] compared to those without co-inheritance or with β thalassaemia co-inheritance, with z value of 10.40(2), p=0.01. Zone 2 was highest in the group with α thalassaemia co-inheritance with mean of 1.11 (0.38) % compared to those without co-inheritance or those with β thalassaemia co-inheritance, z(2)=20.07, p<0.01. The difference in mean value for c-window was not statistically significant among these groups, (p=0.34) (Table I).

Table I:The haematological parameters of patients with Hb CS (with and without other alpha or beta co-inheritance)

Variable	Mean (SD)/Median (IQR)*			T statistics (df) / z statistics*	p value
	Presumed Heterozygous Hb CS	Co-inheritance with common alpha gene deletion	Co-inheritance with beta thalassaemia		
FBC					
Haemoglobin (g/dL)	13.62 (1.21)	12.76 (2.62)	14.650 (0.21)	2.02 (2)	0.14
RBC (x10^{12}/L)	5.47 (0.52)	5.62 (1.18)	5.94 (0.32)	0.78 (2)	0.46
Haematocrit (L/L)	0.42 (0.04)	0.41 (0.07)	0.46 (0.00)	1.46 (2)	0.24
MCV (fl)	77.62 (3.25)	73.79 (5.05)	77.85 (4.31)	4.16 (2)	0.02
MCHC(g/L)	321.24 (10.78)	309.57 (25.72)	317.50 (3.54)	3.04(2)	0.05
MCH (pg)	24.93 (1.21)	22.77 (1.56)	24.70 (1.70)	9.89(2)	<0.01
RDW (%)*	13.83 (1.67)	16.23 (6.01)	13.35 (0.64)	3.04(2)	0.22
Hb analysis					
HPLC					
Hb A_2 (%)	2.68 (0.23)	2.36 (0.29)	2.40 (1.98)	4.28(2)	0.02
C-window (%)	1.57 (0.62)	1.71 (0.49)	1.00 (0.00)	1.08(2)	0.34
Hb A (%) *	87.04 (1.02)	87.91 (0.82)	71.10 (22.06)	10.40 (2)	0.01
Hb F (%) *	0.41 (0.48)	0.74 (1.11)	0.00 (0.00)	1.17(2)	0.56
Other window (%)*	1.00 (0.00)	1.14 (0.38)	2.00 (1.41)	31.13(2)	<0.01
CE					
Hb A_2 (%)	2.18 (0.28)	1.83 (0.29)	3.10 (0.28)	16.41(2)	<0.01
Hb A (%)*	96.95 (0.74)	96.44 (1.22)	77.60 (26.45)	6.45(2)	0.04
Hb F (%)*	0.13 (0.41)	0.47 (0.88)	0.00 (0.50)	4.89(2)	0.09
Zone 2 (%)*	0.69 (0.58)	1.11 (0.38)	0.35 (0.07)	20.07(2)	<0.01
Other zones (%)*	1.03 (0.23)	1.43 (1.13)	3.00 (2.83)	14.55(2)	<0.01

* Non parametric variables
Statistically significant parameter (p<0.05)

DISCUSSION

Proportion of Hb CS

Malaysia and the neighbouring SEA countries have a high prevalence of α thalassaemia (4). The gene frequency of α thalassaemia in Malaysia is 4.1%, while

it is 16% in Southern Thailand, 5% in Philippines and about 4.3% in Brunei (4). Studies have shown that the frequencies and types of α thalassaemia defects are geographically specific(7). Hb CS is not uncommonly found in combination with other thalassaemias among the SEA population (7).

In this study, a total of 3121 form four students were diagnosed with either α / β / compound thalassaemia and amongst this, 3.24% had Hb CS. Hb CS with α thalassaemia co-inheritance was seen in 6.9% and 2% had Hb CS with β-thalassaemia co-inheritance. The proportion of Hb CS obtained in this study was similar to a study done by Ahmad et al in 2013 where the incidence of Hb CS was 3.2% among those who were detected to have α thalassaemia (4). A pilot study involving form four students from Penang, Melaka and Sabah carried out by Ahmad et al in 2012 showed that the prevalence rate of α thalassaemia was 4.08%, with 0.23% being carriers for Hb CS and only one student was compound heterozygote with $-\alpha^{3.7}/\alpha^{CS}\alpha$ (12). Ethnicity analysis in this study found that almost all the form four students with Hb CS were Malays (99%) followed by Chinese (1%) and there were none among Indians and other races. This was in accordance with the data collected from IMR in 2013, which showed that Hb CS among Malays was 4.3%, Chinese 0.70% and there were none among Indians. The highest incidence of Hb CS was reported among the Orang Asli, 11.5% (4). Among SEA countries, Hb CS had the highest frequency of almost 25% in central Vietnam and an incidence of 1-8% in the general Thai population and the Lao-speaking population (7).

In this study, it was noted that all the students who had Hb CS with α or β co-inheritance were Malays. Among those with co-inheritance, most of them had co-inheritance with -α3.7 deletion. According to the ethnicity in Malaysia, the commonest α thalassaemia detected among Malays is -α3.7 deletion followed by SEA deletion and Constant Spring mutation(12). Among the Chinese, SEA deletion was the most common, followed by -α3.7 deletion and Constant Spring mutation (12). In this study, only one student was Chinese and this student had Hb CS, with no co-inheritance. -α3.7 deletion was reported to be the commonest α thalassaemia determinant among the Indians (12).

Full blood count as an indicator to differentiate the different phenotype

The classification of thalassaemia depends on the defective globin chain and the underlying molecular defects. Thalassaemia carriers are easily recognised by basic haematological tests such as red cell indices, morphology of peripheral blood, and separation and measurement of Hb fractions (13). In this study, the Beckman Coulter DxH 800 haematology analyser was used to read the FBC. The FBC parameters that were analysed are the Hb, MCV, MCH, MCHC, RDW and Hct.

Generally, in thalassaemia the Hb level varies, depending on the severity of the disorder. α thalassaemia carriers with two dysfunctional α globin genes usually have mild anaemia with slight hypochromic and microcytic red cells. However, α thalassaemia carriers with one dysfunctional α globin gene may have normal red cells or minimally microcytic red cells (13). The more severe forms of α thalassaemia are associated with mild to moderate microcytic anaemia (13).

The mean Hb levels for the students with Hb CS and those with β thalassaemia co-inheritance were within normal range. However, the mean Hb for those with α thalassaemia co-inheritance was lower (mean = 12.7 g/dL). In this group, it was noted that two students who had co-inheritance with Hb Adana and co-inheritance with α3.7 deletion thalassaemia had Hb of 7.8 g/dL and 10.7 g/dL respectively. Hb CS and Hb Adana are highly unstable Hb variants, thus co-inheritance of these may result in a more severe phenotype or thalassaemia intermedia, which is associated with considerable clinical presentation, from carriers who are asymptomatic to those being transfusion dependent (9,14). A case series reported by Alauddin et al in 2014 has proven that co-inheritance of Hb Adana and Hb CS had lower Hb levels, and these patients were clinically more severe with significant extramedullary haemopoiesis compared to heterozygous Hb Adana stand alone (15).

Besides Hb, MCV and MCH are also able to provide information about the subtype of α thalassaemia. MCV and MCH values are known to be lower in those with two functional α globin genes compared to those with only one mutated α globin gene (16). A study by Ahmad et al in 2013 has observed the mean MCV among silent α carriers were 75.8 fL, while the mean MCV for α+ thalassaemia trait was 69.8 fL and α0 thalassaemia was 67.8 fL (4). The mean MCH among silent carriers were 24.2 pg and this value was approximately 1.5 pg lower among those with α thalassaemia traits (4). Looking into these values, MCV and MCH can be used as a reference in differentiating silent carriers from α thalassaemia traits. To take note on this, subjects with co-inheritance with β thalassaemia or other haemoglobinopathies were not excluded in this study (4). The British Committee for Standards in Haematology recommended testing individuals with MCH of less than 27 pg for thalassaemia. However, this does not constitute all the cases of α thalassaemia and therefore it isn't a reliable diagnostic marker. Heterozygous Hb CS is also known to have normal red cell indices due to hyperhydration status of the red cells, and could potentially be missed during routine screening (5). This statement is supported by the study done by Ahmad et al in 2013 where 19.7% of those diagnosed with α thalassaemia had MCV of ≥80 fL and 4.4% patients had MCH ≥ 27 pg (4). The higher MCV and MCH values here could probably be

due to presence of concomitant folate deficiency, which is frequently reported among thalassaemia patients due to chronic erythroid hyperplasia (4). This has also been previously reported among individuals with thalassaemia trait (17).

The current study noted that Hb CS with α thalassaemia co-inheritance had a significantly lower MCV and MCH compared to those without co-inheritance or with β thalassaemia co-inheritance. These findings were similar to a study by Uaprasert et al where they found that the levels of Hb, MCV, MCH, MCHC were significantly lower among Hb CS / α+ thalassaemia compared to Hb CS heterozygotes (13). This is because in co-inheritance with α gene deletion, the α globin gene defects are more severe, thus causing reduced α globin synthesis. There will also be an imbalance between the α globin and β globin chain (18). In Hb H-CS, the degree of anaemia is more severe, with reduced MCHC, higher MCV and MCH(1). The reduction of MCHC reflects decreased Hb synthesis and increased cellular hydration due to damage from the cell membrane(1).

Significance of Hb A2, and Hb CS levels by HPLC and CE

Hb CS can be detected on cellulose acetate electrophoresis at alkaline pH, especially if a heavy application is used and it moves between carbonic anhydrase enzyme and Hb A2, while in HPLC it appears in c-window (5). In this study, all the students showed a peak at Zone 2 on CE but 49 students did not show any peak in c-window using HPLC. Both students with β thalassaemia co-inheritance and two students with α gene deletion (α4.2 and α3.0 gene deletion) did not show a peak at c-window on HPLC. For those who had a small peak at c-window, the peak was at retention time 4.9 to 5.0 minutes in average. These findings were consistent with the study reported by Weneesorn et al who concluded that CE was more superior to HPLC in detecting Hb CS (19). Another study by Li et al (2014) also concluded that almost all Hb CS carriers can be detected by CE (20). However, a study conducted by Li et al (2013) has proven that Hb CS trait could not be identified by Sebia Capillarys2 (Sebia) when combined with β thalassaemia(36). This is because the amount of Hb CS (α2CSβ2) may be too small in compound heterozygotes where the β chain expression is reduced (21). The mean value for Hb CS levels by HPLC and CE in the current study has shown that Hb CS with co-inheritance with α thalassaemia has the highest mean compared to those without co-inheritance or with β thalassaemia co-inheritance.

A study by Wisedpanichkij et al concluded that HPLC has a sensitivity of 93.98% and specificity of 99.80% in diagnosing Hb CS (22). This was not evident in our study as some patients did not show any significant Hb CS peaks in HPLC. This could be due to probable longer storage time of sample. Misdiagnosis of Hb CS by HPLC can occur after 3 days of storage (22). It is suggested that Hb analysis should be done within 72 hours from time of sample collection, although 80% of samples had shown to have Hb CS peaks upon storage for more than 3 days (22).

Hb A2 values were significantly higher among those with β thalassaemia co-inheritance compared to those without co-inheritance or with α thalassaemia co-inheritance. This could be due to the relative lack of β globin in β thalassaemia, thus allowing more δ chains to be incorporated into the Hb forming increased Hb A2. Tan et al reported a patient with compound heterozygous β thalassaemia and Hb H-CS with increased Hb A2 of 9.7% (23).

Differentiating heterozygous Hb CS and homozygous Hb CS

The main confirmatory test done to diagnose Hb CS is DNA analysis by multiplex ARMS PCR. However, this method is not available in most laboratories in Malaysia. This method also needs a duplicated run to differentiate between heterozygous and homozygous Hb CS. DNA analysis with real time PCR using Taqman SNP genotyping assays are able to distinguish between homozygous and heterozygous Hb CS (6). However, this molecular technique is laborious and expensive. Hence, we need to be able to differentiate between heterozygous and homozygous Hb CS by analysing the FBC and Hb analysis parameters. Due to some limitations, which are discussed below, the current study was unable to conclude regarding the best cut off values in differentiating these two conditions.

According to previous studies, heterozygous Hb CS usually have normal Hb and MCV levels with slightly reduced MCH (7). These findings do not hold true for the case reported by Azma et al, where the heterozygous Hb CS subject had reduced MCV and MCH levels (6). Another heterozygous Hb CS case reported by Azma et al showed normal MCV and MCH levels, with presence of c-window on HPLC (6). With these findings, the carrier states of Hb CS can easily be missed if the screening is solely based on RBC indices.

A study on haplotypic heterogeneities and phylogenetic analysis amongst individuals with Hb CS conducted by Jomoui et al has demonstrated that patients with homozygous Hb CS have mild anaemia, with MCV ranging between 82.8 ± 8.5 fL and lower Hb A2 value compared to those who are heterozygous for Hb CS (7). In a study done by Waneesorn et al, a peak at retention time of 5.25 min was found in almost 90% patients with Hb H-CS and only 26.32% heterozygotes and 42.86% homozygotes were detected to have a similar peak in HPLC (19). Homozygous Hb CS tend to have higher Hb CS levels in CE and HPLC (6). Heterozygous Hb CS mostly had Hb CS levels of less than 2%. This is due to the unstable gene product of Hb CS (7,19).

CONCLUSION

Hb CS is a common disorder among form four students. Although the affected person can be asymptomatic, proper diagnostic approach is essential to avoid co-inheritance of more severe forms of thalassaemia. Although DNA analysis is the confirmatory method to diagnose Hb CS with or without co-inheritance, a more cost effective screening method includes FBC and CE. Certain indices in both FBC and Hb analysis such as MCV, MCH, Hb A2 and Hb CS level by HPLC and CE can aid the pathologist in making a presumed diagnosis of Hb CS and Hb CS with co-inheritance of α or β thalassaemia; whereby the mean levels of MCV, MCH and HbA2 is lower in Hb CS co-inheritance with alpha thalassaemia compared to those with only Hb CS or Hb CS with β thalassaemia co-inheritance.

REFERENCES

1. Bain, B.J (2020). Haemoglobinopathy Diagnosis. UK. Wiley Blackwell.
2. Li, C.-K. (2017). New trend in the epidemiology of thalassaemia. Best Practice & Research in Clinical Obstetrics & Gynaecology, 39, 16–26. doi:10.1016/j.bpobgyn.2016.10.013
3. Jameela, S., Sabirah, S. O. S., Babam, J., Phan, C. L., Visalachy, P., Chang, K. M., … Rahimah, A. (2011). Thalassaemia screening among students in a secondary school in Ampang, Malaysia. The Medical Journal of Malaysia, 66(5), 522. https://pubmed.ncbi.nlm.nih.gov/22390120/
4. Ahmad, R., Saleem, M., Aloysious, N. S., Yelumalai, P., Mohamed, N., & Hassan, S. (2013). Distribution of Alpha Thalassaemia Gene Variants in Diverse Ethnic Populations in Malaysia: Data from the Institute for Medical Research. International Journal of Molecular Sciences, 14(9), 18599–18614.doi:10.3390/ijms140918599
5. Azma, R. Z., Othman, A., Azman, N., Alauddin, H., Ithnin, A., Yusof, N., … Hussin, N. H. (2012). Co-inheritance of compound heterozygous Hb constant spring and a single -α 3.7 gene deletion with heterozygous δβ thalassaemia: A diagnostic challenge. The Malaysian Journal of Pathology, 34(1), 57–62. https://pubmed.ncbi.nlm.nih.gov/22870600/
6. Azma, R.-Z., M-Gaus, K., A-Aziz, S., Alauddin, H., Ithnin, A., Razak, N.-F., … Alias, H. (2016). Detection of Homozygous Haemoglobin Constant Spring by Capillary Electrophoresis Method. ARC Journal of Hematology, 1(1), 28–32. https://www.arcjournals.org/pdfs/ajh/v1-i1/6.pdf
7. Jomoui, W., Fucharoen, G., Sanchaisuriya, K., Nguyen, V. H. & Fucharoen, S. (2015). Hemoglobin Constant Spring among Southeast Asian populations: haplotypic heterogeneities and phylogenetic analysis. PLoS ONE 10, e0145230. doi:10.1371/journal.pone.0145230
8. Singsanan, S., Fucharoen, G., Savongsy, O., Sanchaisuriya, K., & Fucharoen, S.(2007). Molecular characterization and origins of Hb Constant Spring and Hb Paksй in Southeast Asian populations. Annals of Hematology, 86(9), 665–669. doi:10.1007/s00277-007-0310-x
9. Lam, J. C. M., Soh, S. Y., & Law, H. Y. (2014). Clinical and haematological features of Non-Deletional alpha thalassaemia mutations in singapore. Pathology, 46. doi: 10.1097/01.PAT.0000443666.03931.9e
10. Wee, Y. C., Tan, K. L., Kuldip, K., Tai, K. S., George, E., Tan, P. C., … Tan, J. A. M. A. (2008). Alpha-Thalassaemia in Association with Beta-Thalassaemia Patients in Malaysia: A Study on the Co-Inheritance of Both Disorders. Public Health Genomics, 11(3), 129–134. doi: 10.1159/000113874
11. National thalassaemia screening program, Ministry of Health; https://www.slideshare.net/ravindersan/national-thalassaemia-screening-program-malaysia
12. Ahmad, R., Sabrina, N., Bahrin, S., Hassan, R., Yelumalai, P., Hassan, S., … Negara, P. D. (2012). Distribution of alpha thalassaemia in 16 year old Malaysian Students in Penang, Melaka and Sabah. The Medical Journal of Malaysia, 67(6), 565. https://pubmed.ncbi.nlm.nih.gov/23770946/
13. Brancaleoni, V., Pierro, E. D., Motta, I., & Cappellini, M. D. (2016). Laboratory diagnosis of thalassemia. International Journal of Laboratory Hematology, 38, 32–40. doi: 10.1111/ijlh.12527
14. Lam, J. C. M., Soh, S. Y., & Law, H. Y. (2014). Clinical and haematological features of Non-Deletional alpha thalassaemia mutations in singapore. Pathology, 46. doi:10.1097/01.PAT.0000443666.03931.9e
15. Alauddin, H., Jaapar, N.-A., Azma, R. Z., Ithnin, A., Razak, N.-F. A., Loh, C.-K., … Othman, A. (2014). A case series of α-thalassemia intermedia due to compound heterozygosity for Hb Adana [HBA2: c179G>A (or HBA1); p.Gly60Asp] with other α-thalassemias in Malay families. Hemoglobin, 38(4), 277–281. doi:10.3109/03630269.2014.916720.
16. Akhavan-Niaki, H., Kamangari, R. Y., Banihashemi, A., Oskooei, V. K., Azizi, M., Tamaddoni, A., … Shabani, S. (2012). Hematologic features of alpha thalassemia carriers. International Journal of Molecular and Cellular Medicine, 1(3), 162–167. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3920506/
17. Lardhi, A., Ali, R. A., Ali, R., & Mohammed, T. (2018). Thalassemia minor presenting with vitamin B12 deficiency, paraparesis, and microcytosis. Journal of blood medicine, 9, 141–144. doi:10.2147/JBM.S163722
18. Uaprasert, Noppacharn, et al. (2011). "Hematological Characteristics and Effective

Screening for Compound Heterozygosity for Hb Constant Spring and Deletional A+-Thalassemia†." American Journal of Hematology, 86 (7), 615–617. https://doi.org /10.1002/ajh.22033. Epub 2011 Apr 20.

19. Waneesorn, J., Panyasai, S., Kongthai, K., Singboottra, P., & Pornprasert, S. (2011). Comparison between capillary electrophoresis and high performance liquid chromatography for detection and quantification of Hb constant spring [Hb CS; α142, Term→Gln (TAA>CAA IN α2)]. Hemoglobin, 35(4), 338–345. doi: 10.3109/03630269.2011.588140
20. Li, J., Xie, X.-M., Liao, C., & Li, D.-Z. (2014). Co-inheritance of α-thalassaemia and β-thalassaemia in a prenatal screening population in mainland China. Journal of Medical Screening, 21(4), 167–171. doi: 10.1177/0969141314548203
21. Li, Y.-Q., Li, R., & Li, D.-Z. (2013). Detection of Hb Constant Spring [α142, Term→Gln, TAA>CAA (α2)] in heterozygotes combined with β-thalassemia. Hemoglobin,37(2),197–200. doi: 10.3109/03630269.2013.768532.
22. Wisedpanichkij, R., Jindadamrongwech, S., & Butthep, P. (2015). Identification of Hb Constant Spring (HBA2: c.427T > C) by an Automated High Performance Liquid Chromatography Method. Hemoglobin, 39(3), 190–195. doi:10.3109/03630269.2015.1027828
23. Tan, J. A. M. A., Kok, J. L., Tan, K. L., Wee, Y. C., & George, E. (2009). Thalassemia intermedia in HbH-CS disease with compound heterozygosity for β-thalassemia: Challenges in hemoglobin analysis and clinical diagnosis. Genes & Genetic Systems, 84(1), 67–71. https://doi.org /10.1266/ggs.84.67.
24. Kabir, Amin & Dipta, Tashmim & Khatun, Hajera & Rahman, Mohammad & Haq, Mahfuz & Uddin, Mohammad & Begum, Masuda. (2015). A Screening Test for Iron Deficiency Anaemia and Thalassaemia Traits. Journal of Bangladesh College of Physicians and Surgeons. 32. 190. doi:10.3329/jbcps.v32i4.26063.