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Haematological and Molecular Characteristics of Hb Singapore [*HBA2*: c.425G>C] Unique Among the Malays from Kelantan, Malaysia

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Abstract

Background: Haemoglobin (Hb) Singapore is a rare alpha variant that typically involved alpha-2 gene. Misidentification can occur without molecular diagnosis as the characteristics in Hb analysis is typical of Hb J variant. **Objective**: This study aims to describe the haematological parameters, phenotype and genotype characterisation of Hb Singapore along with a proposed classification of the variant based on American Collage of Molecular Genetics and Genomics (ACMG) guideline. **Methods**: Analysis involved nineteen confirmed cases of Hb Singapore retrieved from our databases. Cases referred to Institute for Medical Research (IMR) from 2018 to 2022. The clinical information and haematological parameters provided by the hospitals were evaluated. The cases were subjected to direct sequencing of *HBA* gene for variant detection. **Results**: Interestingly, all recorded cases involved Malay patients, predominantly from Kelantan (n=16, 80%). There were (n=7, 36.8%) males and (n=12, 63.2%) females. The median age of the cases was 26 years old with age ranges between 2 to 56 years old. All individuals were asymptomatic during the screening with most of them being screened as part of the National Thalassaemia Screening Programme and (n=3,15.8%) were suspected of Hb variant after an abnormal HbA1c finding as part of routine monitoring for diabetes mellitus follow-up. All of them had haematological features of thalassaemia trait with the mean ± SD for Hb, MCV and MCH were 14.9g/dl ± 2.0, 81.1 ± 7.7 fL and 26.5 ± 3.2 pg respectively. Three genotypes were identified; ααHb Singapore/αα (n=17, 89.5%), ααHb Singapore/αα, βCD 26/β (n=1, 5.3%), ααHb Singapore/αα, Gγ(Aγδβ)°-thal, Asian-Indian inversion/deletion (n=1, 5.3%). **Conclusion**: Interestingly, two cases were associated with two different mutations which are co-inherited with HbE and Gγ(Aγδβ)°-thal, Asian-Indian inversion/deletion without apparent effect on the variant expression. This is the largest reported case of Hb Singapore in the literature and the unique ethnicity affected by this variant suggested that it is higher among Malays from Kelantan. Based on our data, we propose to classify this variant as a benign or likely benign variant (B/LB).

Keywords: Hb J, thalassaemia, alpha variant, molecular analysis, unique variant among Malays

Introduction

Haemoglobinopathies and thalassemia syndromes are a diverse group of inherited disorders of haemoglobin synthesis that result from qualitative defects (haemoglobinopathies) or quantitative defects (thalassemia syndromes) in globin synthesis. As a group, they are the most common and clinically significant single gene disorder in the world and pose a serious health problem in many countries (Mohd Ibrahim *et al*., 2020, Alwi and Syed-Hassan, 2022). Mutations that produce structurally abnormal globin proteins are called Hb variants. These variants alter haemoglobin structure and biochemical properties with physiological effects ranging from clinically silent to severe phenotype (Thom *et al*., 2013). The location of the amino acid changed by the mutation can often be correlated with the resultant phenotype. Most Hb variants are listed on the Globin Gene database (Giardine *et al*., 2014) (https://globin.bx.psu.edu/hbvar). Classically, these variants are named after the geographic

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origin of the affected individual (Thom *et al.*, 2013, and Clegg *et al.*, 1969).

In our centre, identification of Hb variants is made based on detection of abnormal peak in Hb analysis typing, either high performance liquid chromatography (HPLC) or capillary electrophoresis (CE). Many amino acid substitutions alter surface charge and are thus detected by both techniques. Hb Singapore exhibits similar electrophoretic mobility with more common Hb J variants (a fast moving haemoglobin) with characteristic fast anodal movement compared to Hb A on gel electrophoresis (Srinivas, Mahapatra and Pati, 2007, Shrestha *et al.*, 2022). The Hb fraction is eluted at P3 or P2 of HPLC and zone 12 in CE similar to Hb J variants. The variant can be the result of substitution of either α, β and γ globin genes and DNA analysis is required for a definitive diagnosis (Srinivas, Mahapatra and Pati, 2007).

Haemoglobin (Hb) Singapore (*HBA2*: c.425G>C) is a rare alpha variant that typically involves alpha-2 gene. To date, very limited literature describes this rare variant. This variant was first discovered in asymptomatic Malay family in 1969 in Singapore. It is described by Clegg et al. as an acidic haemoglobin variant that comprised about 25% of total haemoglobin with electrophoretic activity, similar to Hb A3 (Clegg *et al.*, 1969). The missense mutation of this variant occurs at codon 141 of *HBA2* gene that replaced a normal G nucleotide to C nucleotide. This led to a substitution of arginine to proline that might cause either unstable or functionally abnormal Hb variant like in the cases of Hb Genova, Hb Dhofar, Hb Santa Ana and Hb Bibba (Clegg *et al.*, 1969). However, Hb Singapore and Hb Chiapas does not cause significant haematological and clinical symptoms (Clegg *et al.*, 1969,Richard T. Jones, 1967). Subsequently, in 2015, this variant was reported in one analysis study from United Kingdom through direct DNA sequencing method, however no details on haematological parameters and ethnicity were provided in the study (Henderson *et al.*, 2015).

This study aims to describe the haematological parameters, phenotype, and genotype characteristics of 19 cases of Hb Singapore identified within a five year's duration. An attempt should be made to classify this variant according to the standard for accurate and reliable diagnostic genetic services in the context of the Malaysian population.

Materials and Methods

This is a retrospective cross-sectional study on 12,925 genotyping data of a rare α-globin variants cases recorded from January 2018 to November 2022. The data retrieved from Institute for Medical Research (IMR) database. The blood samples of the cases were sent to IMR from various hospitals from whole country for confirmation of the presumptive diagnosis of alpha thalassaemia or variant. The inclusion criteria include molecularly confirmed Hb Singapore by Sanger sequencing method, either heterozygous, compound heterozygous, or co-inheritance with other thalassaemia variants. Out of 12,925 samples, 19 cases were molecularly confirmed to have Hb Singapore. The haematological parameters and clinical phenotype were retrieved from the request form provided.

Presumptive Diagnosis and DNA analysis

The presumptive diagnosis of thalassaemia consisted of full blood count (FBC) and Hb analysis. The FBC was done using an automated haematology analyser and the Hb analysis was performed according to a set of tests i.e., peripheral blood film (PBF), CE (SEBIA, France), HPLC (Bio-Rad Laboratories, USA) and Hb electrophoresis (SEBIA, France). The laboratory information gathered from these screening tests are used to make a presumptive diagnosis of Hb variant. Based on Hb analysis findings, most cases (n= 10, 52.6%) have a presumptive diagnosis of Hb J variant, alpha thalassaemia variant (n=5, 26.3%) and Hb Singapore (n=4, 21.0%). The definitive diagnosis was made through DNA analysis. In this test, the DNA was extracted from peripheral blood leukocytes using QIA symphony SP (Qiagen, GmbH, Germany). The concentration and quality of the extracted DNA was measured using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc, Wilmington, DE, USA). Direct DNA sequencing of *HBA2* and *HBA2* genes were done. All DNA samples underwent PCR to selectively amplify the α1 and α2 genes using the primer sequences in Table 1. Four sequencing reactions were set up for each sample, one for the α1 gene (with primers 1 and 3), one for the α2 gene (primers 1 and 2). The PCR product was checked using gel electrophoresis (1.2% w/v agarose gel). After the PCR product purification, cycle sequencing analysis was done using BigDye® Terminator v3.1 cycle sequencing kit. The sequences were then read through ABI 3730XL DNA Analyser (Applied Biosystems, Foster City, CA, USA) before they were analysed by CLC Main Workbench (CLC Bio, Aarhus, Denmark).

To complete routine molecular investigation, multiplex gap-PCR was performed to detect the common α-thalassaemia deletions using previously described methods to exclude compound heterozygous with common deletional alpha thalassaemia (Arnold S.-C. Tan, Thuan C. Quah, Poh S. Low, 2001). The multiplex gap-PCR

was carried out to detect two single gene deletions -$\alpha^{3.7}$ and -$\alpha^{4.2}$, and five double gene deletions, --^{SEA}, --^{MED}, --^{FIL}, --^{THAI} and –$(\alpha)^{20.5}$. Randomly, five cases were selected for delta sequencing for samples with low HbA2 level ranging from 1.4-1.9% by CE or HPLC with normal range between 2.0-3.3% to rule out co-inheritance with delta variant or delta thalassaemia (details in supplementary data).

Statistical analysis

The demographic data including the age, states, and ethnicity of Hb Singapore were analyzed by descriptive analysis. All statistical analyzed were performed using the SPSS software (Ver. 22, SPSS Inc., Chicago, USA). Means were reported with standard deviation (SD) and medians with interquartile range (IQR). Data and results were presented in the form of figures and table.

Ethics approval

This study was conducted according to the Declaration of Helsinki and approved by Medical Research and Ethics Committee, Ministry of Health, the regional ethical board in Malaysia. Written informed consent for clinical information and molecular genotyping was obtained from the cases prior to blood taking. The collected data was entirely kept anonymous, and confidentiality was maintained through the suitable use of coding.

Results

All cases of Hb Singapore were of Malay ethnicity and interestingly majority of them (n=15, 78.9%) were from Kelantan province, a state that located at north-eastern corner of the Peninsular Malaysia. Other samples were reported from Kuala Lumpur (n=3, 15.8%) and Kedah (n=1, 5.3%). Their age ranged between 2 to 56 years old years old with the median of 26 years old. There were 7/19 (36.8%) males and 12/19 (63.2%) females. All individuals were asymptomatic during the screening with most of them being screened as part of the National Thalassaemia Screening Programme and (n=3,15.8%) were suspected of Hb variant after an abnormal HbA1c finding as part of routine monitoring for diabetes mellitus follow-up.

There were three genotype groups as described in Table 2. The heterozygous Hb Singapore had the highest incidence (n=17/19, 89.5.%). All the samples were negative for common deletional alpha thalassaemia, namely -α3.7, -α4.2, --SEA, --MED, --FIL, --THAI and –(α)20.5. The levels of Hb were within the normal range across the three groups (Table 2). The mean Hb, RDW, MCV, MCH, RBC for heterozygous Hb Singapore were 14.9+2.0, 14.6+1.5, 81.1+7.7, 26.5+3.2 and 5.7+0.8 respectively. Interestingly, two cases of Hb Singapore with co-inherited heterozygous Hb E and Gγ(Aγδβ) °-thal, Asian-Indian inversion/deletion had no apparent effect on the variant expression. All of them were asymptomatic with mild hypochromic microcytosis indices from full blood count (FBC). Twelve heterozygous Hb Singapore cases presented with MCH < 27 pg or in the borderline range with a mean of 26.5 + 3.2. Based on our current cut off for MCH value for National Thalassaemia Screening, which is MCH of < 27pg, most of them will require further Hb analysis.

Unexpectedly, as described in Table 2, the HbA2 value in majority of heterozygous cases was low and consistently all the samples have a small peak at zone 5 with mean of 0.6 $\pm$ 0.2 in CE. The small peak found in CE is representing $\alpha^{Singapore}\delta$ variant or HbA2 variant (figure 2). The level of HbA2 percentage is not explained by the single alpha plus mutation. Five cases with low HbA2 levels were randomly selected for delta (δ) sequencing to exclude the possibility of δ-variant or thalassaemia and showed negative results (supplementary data). The true value of HbA2 in all the samples should be a sum of the HbA2 + HbA2 variant, making normal HbA2 value.

The Hb subtypes profiles generated from CE and HPLC are shown in Table 2. The Hb electrophoresis of the 12 cases consistently showed presence of fast band in agarose gel electrophoresis at alkaline phase (P1-P12 in table 2/ supplementary data). From these profiles, most (n=10/19, 52.6%) were misinterpreted as suggestive of Hb J (alpha variant) trait, (n=4/19, 21.1%) as Hb Singapore and (n=5/19, 26.3%) as an alpha variant. The mean for Hb subtypes obtained from HPLC and CE analysis were 30.0% $\pm$ 1.6 and 30.2% $\pm$ 0.7 that eluted at P2/P3 and Zone 5 respectively.

The electropherogram for the Hb Singapore shown in Figure 1. The incident of Hb Singapore in our study was 0.15%. The mean Hb for heterozygous Hb Singapore was similar with the Hb level for both cases of Hb Singapore co-inheritance with Hb E and Gγ(Aγδβ) °-thal, Asian-Indian inversion/deletion.

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Table 1. Primer used for sequencing of α1 and α2-globin gene.

	PRIMER NAME	PRIMER SEQUENCE (5' > 3')
1	BE10 F (α1 and α2 gene)	TGG AGG GTG GAG ACG TCC TG
2	BE17 R (α2 gene)	CCA TTG TTG GCA CAT TCC GGG A
3	BE12 R (α1 gene)	CCA TGC CTG GCA CGT TTG CTG AGG

Table 2. Hematological parameters and Hb profiles of 17 individuals with Heterozygous Hb Singapore and 2 individuals with Hb Singapore with co-inherited heterozygous Hb E and Gγ(Aγδβ) ^{o}-thal, Asian-Indian inversion/deletion

Patient	Age	Gender	Genotype	RBC (10^6/ul)	Hb (g/dl)	RDW (%)	MCV (fL)	MCH (pg)	HPLC				CE			
									HbA (%)	HbF (%)	HbA2 (%)	P2/P3 (%)	HbA (%)	HbA2 (%)	Z5 (%)	Z12 (%)
P1	35	M	$\alpha^{cd\ 141}$ α/αα	6.1	18.8	14.2	93.8	31.1	62.5	0.6	2.1	29.8	67.7	1.7	0.6	30.0
P2	2	M	$\alpha^{cd\ 141}$ α/αα	6.0	12.3	18.7	65.7	20.5	62.4	0.9	1.7	29.9*	68.4	1.4	**	30.2
P3	56	F	$\alpha^{cd\ 141}$ α/αα	5.0	14.3	12.6	84.6	28.6	59.7	1.4	2.0	-	68.0	1.8	0.7	29.5
P4	40	F	$\alpha^{cd\ 141}$ α/αα	5.1	14.7	14.2	89.4	28.8	61.5	0.7	2.0	30.2	66.4	1.7	0.5	31.4
P5	31	F	$\alpha^{cd\ 141}$ α/αα	4.0	10.7	16.2	88.7	27.0	63.5	1.1	2.0	30.0	67.3	1.7	0.6	30.0
P6	66	M	$\alpha^{cd\ 141}$ α/αα	5.0	16.5	14.6	83.7	32.7	61.7	1.2	2.2	26.4	67.5	2.0	0.7	30.5
P7	16	M	$\alpha^{cd\ 141}$ α/αα	6.1	15.3	13.4	81.0	25.2	63.1	0.8	1.9	30.0	67.7	1.4	0.5	30.4
P8	16	M	$\alpha^{cd\ 141}$ α/αα	6.0	16.0	13.8	84.8	26.7	62.8	0.9	2.3	26.0	67.3	1.8	0.7	30.2
P9	42	F	$\alpha^{cd\ 141}$ α/αα	5.4	14.3	15.7	83.2	26.4	61.5	0.7	2.0	30.2	66.4	1.7	0.5	31.4
P10	26	F	$\alpha^{cd\ 141}$ α/αα	6.2	17.0	12.8	81.8	27.4	60.6	1.5	2.0	30.1	67.2	1.7	0.6	30.5
P11	16	M	$\alpha^{cd\ 141}$ α/αα	7.7	16.6	14.9	69.7	21.5	58.6	2.1	3.3	24.9*	66.3	2.9	1.2	29.2
P12	17	F	$\alpha^{cd\ 141}$ α/αα	5.6	13.1	15.6	70.7	23.6	61.6	1.1	1.8	30.0	67.7	1.6	0.6	30.1
P13	16	F	$\alpha^{cd\ 141}$ α/αα	5.1	13.9	15.6	85.0	27.3	63.3	1.1	2.2	30.1	67.7	1.7	**	30.0
P14	55	F	$\alpha^{cd\ 141}$ α/αα	5.5	15.8	12.7	85.8	28.6	60.4	1.4	1.8	30.3	98.0	1.6	0.5	29.9
P15	43	F	$\alpha^{cd\ 141}$ α/αα	5.9	13.7	14.1	71.5	23.1	62.4	0.7	2.0	30.6	67.6	1.6	**	30.3

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P16	17	F	$\alpha^{cd\ 141}$ α/αα	5.6	13.6	15.4	78.2	24.5	63.3	1.2	2.1	28.9	67.5	1.6	0.6	30.3
P17	16	M	$\alpha^{cd\ 141}$ α/αα	6.3	16.6	13.6	80.5	26.6	63.3	0.9	2.0	27.9	69.0	1.8	0.6	28.6
N=17	26.0 ±26.5[b]	-	$\alpha^{cd\ 141}$ α/αα	5.7 ± 0.8[a]	14.9 ± 2.0[a]	14.6 ± 1.5[a]	81.1 ± 7.7[a]	26.5 ± 3.2[a]	61.9 ± 1.4[a]	2.0 ± 0.2[a]	1.1 ± 0.4[b]	30.0± 1.6[b]	67.6 ±0.6[b]	1.7 ± 0.2[b]	0.6 ± 0.2[b]	30.2 ± 0.7[a]
P18	16	F	$\alpha^{cd\ 141}$ α/αα, β^{E}/ β	5.79	14.0	17.7	75.3	24.2	42.2	1.8	20.6	21.5	49.0	3.0	17.8[Ψ]	23.6 and zone 6 (6.6)
P19	17	F	$\alpha^{cd\ 141}$ α/αα, Gγ(Aγ δβ)°/ β	5.83	13.4	22.3	70.8	23.0	57.6	10.9	1.6	27.6	62.5	1.2	9.1[#]	27.2

[a]Normal distribution data and data presented as mean ± SD
[b]Non-normal distribution data and data presented as median ± IQR
Abbreviation: RBC, red blood cells; Hb, haemoglobin; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW, red cell distribution width; CE, capillary electrophoresis; HPLC, high performance liquid chromatography.
*Value for P3
** Presence of small peak at zone 5 (not quantified by the software)
Ψ HbE value (zone 4)
HbF value
P2 window HPLC (RT Range 1.39 to 1.42%)
P3 window HPLC (RT Range 1.45 to 1.54%)

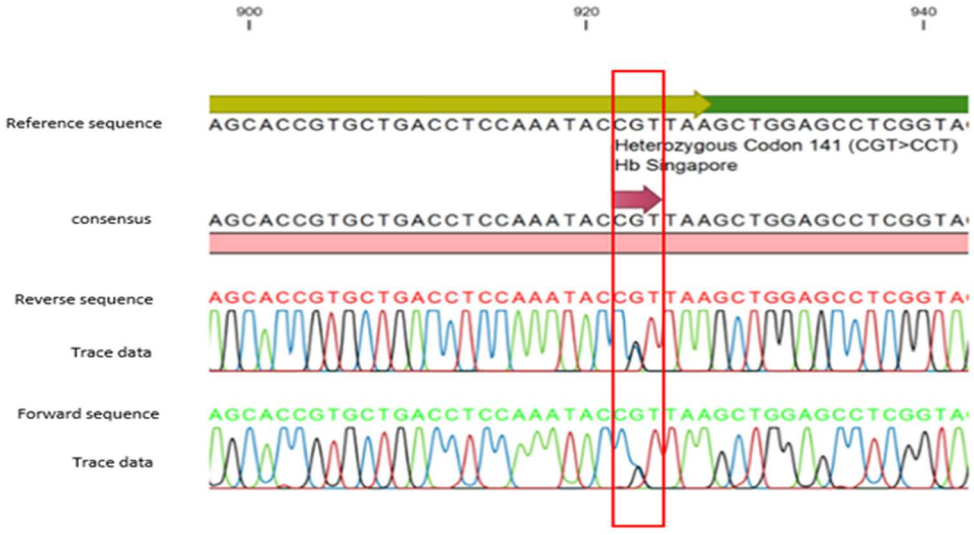

Figure 1. The chromatogram showed a nucleotide substitution of G to C at codon 141 of exon 3 *HBA2* gene indicating a heterozygous state of Hb Singapore; showed in presence of overlapping peaks (black and blue as highlighted by the red box). This results in production of arginine to proline HBA2:c.425G>C (p.Arg142Pro).

(a), reference; (b) consensus data; (c) data generated from reverse primer extension; (d) data generated from forward primer extension. The colours of the peaks represent the type of the nucleotides; A, green; C, blue; G, black and T, red.

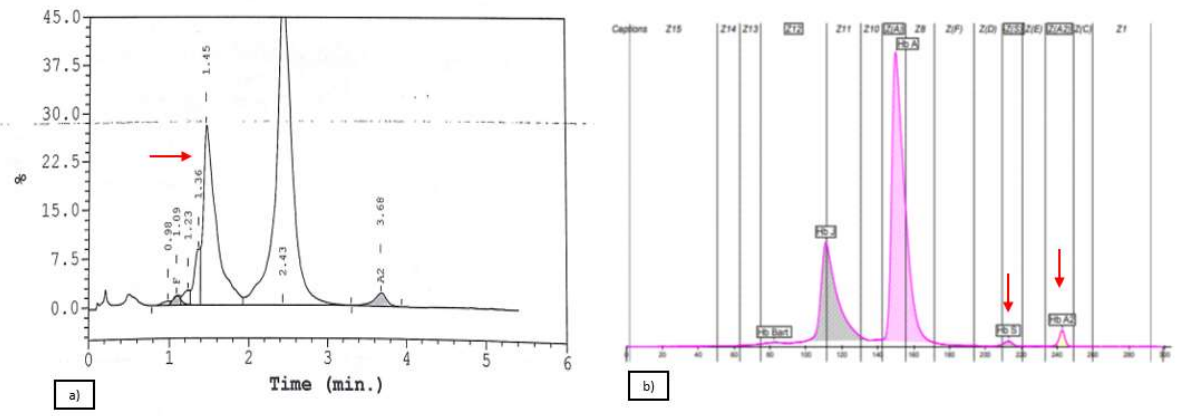

Figure 2. (a,b) A representative result of Hb Singapore by HPLC and CE analyses. The red arrow represents the Hb Singapore peak at RT 1.45, (27.9%), Hb A2 at RT 3.68, (2.0%) and CE at zone 12, (28.7%), HbA2' at zone 5 (0.7%).

Discussion

Thalassaemia and haemoglobinopathies are heterogeneous at the molecular level and are regionally specific. Some of the thalassaemias and variants have a unique representation representing each population. In the Malaysian population, limited variants have been classified as unique to specific ethnicity. To the best of our knowledge, Hb Singapore is the first variant reported unique among Malay ethnicity. Hb Singapore is considered a rare α-variant Hb in our country, because within 5 years of analysis (from 2018 to 2022), only 19 cases were detected to have Hb Singapore. The data presented in this study is representative of the nationwide incident as IMR is a referral centre for rare alpha thalassaemia variants and haemoglobinopathies in the country. However, this variant could be missed in a proportion of cases as it could have unremarkable blood indices, hence not likely to be sent for Hb analysis.

Our study is the largest reported series of Hb Singapore in 19 unrelated individuals compared to previous literature (Clegg *et al.*, 1969, Henderson *et al.*, 2015). To the best of our knowledge, no prevalence study on Hb Singapore was done in Singapore. Based on published data, only a few thalassaemia or haemoglobinopathy have been reported commonly among Malays, namely Hb Constant Spring (HBA2:c.427T>C)(Ahmad *et al.*, 2013, Raja Sabudin *et al.*, 2014), -α3.7 deletion(Ahmad *et al.*, 2012, Raja Sabudin *et al.*, 2014, and Yap *et al.*, 2005), Hb Arya (HBA1:c.142G>A)(Neiza *et al.*, 2018), and Hb G-Makassar (HBB:c.20A>C) (unpublished IMR data). Another types of β-thalassaemia mutations commonly found in the Malays group were codon 19 (A>G) (or Hb Malay (HBB: c.59A>G), IVS-I-1 (G>T) (HBB:c.92+1G>T), IVS-I-5 (G>C) (HBB:c.92+5G>C) and polyadenylated signal (polyA) (AATAAA>AATAGA) (HBB:c.*112A>G) and the structural variant, Hb E (HBB:c.79G>A) (Elizabeth and Ann, 2010, Alwi and Syed-Hassan, 2022). To the best of our knowledge, our study is the first reported largest cohort of Hb Singapore involving Malays from Kelantan population.

It is always necessary to perform more than one method to solve the problem of variants that co-eluate at the same position in a certain method of Hb analysis. The same issue is also encountered in Hb electrophoresis; some haemoglobins migrate at the same rate in the alkaline phase but separate in the acid phase. Even in CE, we may see multiple variant-peaks appear in the same zone, but those variants can be differentiated by other methods (Angastiniotis M *et al.*, 2013). The strategy of performing at least two different methods, however, is not constantly be the solution, especially if genotyping is feasible. In this study, Hb Singapore has electrophoretic mobility similar to Hb J and both cannot be differentiated based on Hb analysis findings. However, DNA sequencing analysis of the HBA gene revealed the diagnosis of Hb Singapore.

The percentage of Hb Singapore measured by either CE or HPLC in a heterozygote state was slightly higher in our study, with a mean of 30.0 $\pm$ 1.6 and 30.2 $\pm$ 0.7 respectively, as compared to previous study by Clegg et al., with a mean of 25% (Clegg *et al.*, 1969, Kountouris *et al.*, 2014 and George, 2013). The Hb A2 percentage in heterozygous Hb Singapore was lower than expected with median of 1.1 $\pm$ 0.4 and 1.7 $\pm$ 0.2 by HPLC and CE respectively. Based on our study, all the cases had a small peak at zone 5 by CE with a median of 0.6 $\pm$ 0.2. The true HbA2 value should be

summed to approximate the total amount of α-δ tetramers present (Oleske *et al.*, 2014, Greene *et al.*, 2012). Thus, by adding the value of HbA2 and the HbA2 variant make the level of HbA2 normal (figure 2) (Mandrile *et al.*, 2022). However, our study does not show any small peak eluted at S window from HPLC as the small peak at S window may represent either alpha, delta variant or HbA2'. It could be explained by the more sensitive method by using CE for the detection of HbA2' (Oleske *et al.*, 2014).

Based on our reported cases, all of them have a silent phenotype. Hb Singapore heterozygotes are asymptomatic and haematologically normal. This variant has been reported as Likely Pathogenic/ Pathogenic (LP/P) variant (Kountouris *et al.*, 2014). However, based on our study data considering the clinical phenotype of 19 individuals, together with haematological parameters and previous literatures, we propose to classify this variant as likely benign or benign variant (Kountouris *et al.*, 2022). The classification is important for precise genetic counselling.

Conclusion

The majority of haemoglobin variants, particularly alpha variants fortuitously discovered, are of minimal clinical interest; however, identification of this variant is important to avoid misdiagnosis of a carrier. An attempt to classify those variants is important for precise genetic counselling and issues on the possibility of interaction with other thalassaemic syndrome in the future should be addressed. Accurate and consistent classification of variants is vital to the delivery of safe and reliable diagnostic genetic services. Every laboratory should make an effort to classify the variants found based on standard classification to reduce discordance.

Limitation

This analysis had a few significant limitations. The incident reported here would not accurately correspond to the population prevalence of Hb Singapore. In particular, the denominator used for the calculation is based on the total number of samples sent to IMR to rule out alpha variants without considering other common variants detected in the first line test i.e. codon142/termination codon (TAA→CAA) Hb Constant Spring, codon 125 (CTG→CCG) Hb Quong Sze, codon 59 (GGC→GAC) Hb Adana, initiation codon mutation (ATG→A–G), codon 30 mutation (ΔGAC) and codon 35 mutation (TCC→CCC) Hb Evora. Secondly, as the samples were submitted only for *α* thalassaemia screening, other causes of microcytic anaemia such as iron deficiency were not determined.

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Disclosure of Conflict of Interests

The authors report no conflict of interests.

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