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ORIGINAL ARTICLE

Molecular Landscape of Cases with Borderline HbA2 Levels in a Malaysian Tertiary Medical Centre

LAILATUL HADZIYAH MOHD PAUZY[1], RAJA ZAHRATUL AZMA RAJA SABUDIN[2], AZLIN ITHNIN[2], RINI ALBERT[2], HAFIZA ALAUDDIN[2]*

[1]Department Laboratory Diagnostic Services, Hospital Canselor Tuanku Muhriz, Universiti Kebangsaan Malaysia, 56000 Cheras, Kuala Lumpur, Malaysia
[2]Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia, 56000 Cheras, Kuala Lumpur, Malaysia

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ABSTRAK

Talasemia merupakan penyakit genetik yang lazim di Malaysia dan menjejaskan sehingga 6% daripada populasi. Peningkatan paras hemoglobin A2 (HbA2) melebihi 4% merupakan salah satu parameter penting dalam mengenal pasti pembawa talasemia β. Namun begitu, terdapat juga kes dengan paras HbA2 pada julat sempadan antara 3.0% hingga 3.9% yang dikesan mempunyai keabnormalan pada gen α-, β-globin dan/atau Krüppel-like factor 1 (KLF1). Kajian ini melaporkan hasil penyiasatan molekul terhadap sekumpulan subjek dengan paras HbA2 sempadan. Seramai 45 subjek dengan paras HbA2 sempadan di Hospital Canselor Tuanku Muhriz (HCTM) dikenal pasti melalui sistem maklumat hospital. Data kiraan darah penuh menggunakan Sysmex XN-1000 dan kuantifikasi hemoglobin menggunakan Sebia Capillarys 2 dikumpulkan. DNA subjek dianalisis bagi mengesan mutasi patologi gen β-globin yang lazim di Malaysia, penghapusan gen α-globin serta mutasi gen KLF1. Kajian ini mendapati bahawa 44% daripada kes HbA2 sempadan di HCTM menunjukkan keabnormalan genetik di peringkat molekul yang merangkumi mutasi gen β-globin (29%), kecacatan gen α-globin (9%), keabnormalan gen KLF1 (4%) dan pewarisan bersama kecacatan gen α dan β (2%). Berdasarkan penemuan ini, kami mencadangkan agar kaedah molekul yang melibatkan kajian gen β- dan α-globin dipertimbangkan dalam penyiasatan hemoglobinopati bagi pesakit dengan HbA2 sempadan. Kajian lanjut mengenai mutasi gen KLF1 juga disarankan untuk mencirikan mutasi lazim gen tersebut dengan lebih terperinci.
Kata kunci: *Gen KLF1; julat sempadan HbA2; thalassaemia*

ABSTRACT

Thalassaemia is a prevalent genetic disease in Malaysia affecting up to 6% of the population. A rise in haemoglobin A2 (HbA2) of more than 4% is among the most important parameters for identification of β-thalassaemia carriers. However, some cases with the HbA2 levels in the borderline range between 3.0% to 3.9% has been shown to harbour α-, β-globin and/or Krüppel-like factor 1 (*KLF1*) gene abnormalities too. Here, we reported the results of molecular investigations on a group of subjects with borderline HbA2. A total of 45 subjects with borderline HbA2 levels in *Hospital Canselor Tuanku Muhriz* (HCTM)

Correspondence: Hafiza Alauddin. Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Yaacob Latiff, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia. Tel: +603-91455373 Email: drhafiza@hctm.ukm.edu.my

were identified from the hospital information system. Data of their full blood count using Sysmex XN-1000, and haemoglobin quantitation using Sebia Capillary Variant II were collected. Their DNA were investigated for the presence of locally common pathological mutations in the β-globin gene, deletions of the α-globin gene as well as screened for KLF1 gene mutation. This study found that 44% of the cases with borderline HbA2 in HCTM showed genetic abnormality at the molecular level comprising of β-globin gene mutation (29%), α-gene defect (9%), KLF1 gene abnormalities (4%) and coinheritance of α- and β-gene defect (2%). On the basis of our findings, we strongly suggest molecular methods consisting of β- and α-globin genes study to be considered in the investigation for haemoglobinopathies in the borderline HbA2 group of patients. Further study on KLF1 gene mutation test in our population is recommended to further characterise the common mutations of the gene.
Keywords: Borderline HbA2; KLF1 gene; thalassaemia

INTRODUCTION

Thalassaemia is a heterogeneous group of disorders affecting haemoglobin synthesis which results in abnormal production of α- or β-globin chains of haemoglobin. This disease exerts a significant health burden in Asian regions with high prevalence rate especially in Southeast Asia (Zhang et al. 2024). In Malaysia, thalassaemia is the commonest inherited blood disorder with 3-6% carrier rate and it poses a major public health issue. The disease incurs large cost burden to the health ministry as treatment involves repeated blood transfusions and long-term monitoring for treatment side effect (Malaysian Thalassaemia Registry Report 2019).

The defective genes for thalassaemia can be inherited autosomal recessively from either parent (Hoffbrand 2024). Clinically, it can manifest as non-transfusion-dependent-thalassaemia or transfusion dependent thalassaemia; also known as thalassaemia major. When a carrier of thalassaemia has a child with another carrier, every pregnancy will have a 25% risk of producing a child with thalassaemia major and 50% risk of producing a carrier of thalassaemia. As most carriers of this syndrome are asymptomatic and are not aware of their genetic status, screening program plays a major role in controlling the disease.

According to the Malaysian Thalassaemia Registry Report (2019), the Thalassaemia Prevention and Control Programme was established in 2004 to eliminate new carrier of the disease by the year 2040 through early detection via screening followed by genetic counselling. Screening for the disease was initially targeted at pregnant women attending government antenatal health clinics and has since been expanded and currently includes screening of all young adults aged 16 years old.

Investigation starts with testing of full blood count. A mean corpuscular heamoglobin (MCH) levels below <27 pg/L with normal haemoglobin (Hb) or a persistent low Hb levels despite adequate iron therapy warrants further investigation. The next line of investigation consists of Hb quantitation using either high performance liquid chromatography or capillary electrophoresis as per recommended by The International Council for the Standardisation of Haematology (ICSH) Board (Stephens et al. 2011). These methods allow for quantification of the types of haemoglobin in a sample.

Identification of classical β-thalassaemia trait and most Hb variants can usually be detected by Hb quantitation. Patients with classical β-thalassaemia trait will have a high HbA_2 (>4.0%) and patients with haemoglobinopathies will have variant Hb detected by this analysis. However, not all clinically significant thalassaemia traits can be detected by these methods. α-thalassaemia traits and some β-thalassaaemia carrier states for example, may not be apparent from a Hb quantitation test alone.

A particular example where diagnosis may be missed from Hb quantitation alone is in cases of

borderline levels of HbA_2. Normal HbA_2 level is often referred to as 2.0-3.3%. Levels lower than this is often related to iron deficiency anaemia or α-thalassaemia (Denic et al. 2013; Mosca et al. 2009). Nonetheless thalassaemia phenotype is highly heterogenous and unsurprisingly several studies have indicated that a significant proportion of patients with normal to borderline levels of HbA_2 (3.0-3.9%) harbours either α- or β-gene defect (Denic et al. 2013; Lou et al. 2014; Rosnah et al. 2017). HbA_2 levels between 3.0 to 3.9% may not always raise suspicion of disease, leading to these patient's carrier states being missed during screening.

Observation by Mosca et al. (2009) showed that the occurrence of borderline HbA_2, may range from 2 to 4%. Other reports have also found that the incidence may go up to 16.7% of population especially in population in area where thalassaemia is endemic.

Fortunately, molecular methods of DNA analysis for α and β-gene are more sensitive and accurate for detection of thalassemia genes abnormalities (Lee et al. 2021). The application of molecular method in this group of patients may prove to be useful and even necessary especially when dealing with at risk couples.

In Malaysia the most common β-mutations are Cd 41/42 (-TTCT), Cd 26 (A-G) HbE, IVS 1-1 (G-T) and IVS 1-5 (G-C) among the Malays. Whereas Cd 41/42 (-TTCT) and IVS 2-654 (C-T) were most common among the Chinese. In Indians IVS 1-5 (G-C), Cd 8/9 (+G), Cd 15 (G-A), Cd 43 (G-T) mutation are commonest (Hassan et al. 2013). The commonest α-gene deletions in our population includes -(SEA)deletion, -α(3.7) rightward deletion and -α(4.2) deletion and Hb Constant Spring mutation (Azma et al. 2014).

Although the general molecular landscape for classical β- and common α-gene defects in Malaysia has been established, the specific molecular abnormality for patients with borderline HbA_2 in this country is yet to be explored. A published study by Rosnah et al. in 2017 from Kelantan on cases with borderline HbA_2 level found mutations, either in α- or β-globin in 38.5% of the sample.

More recently, more studies have shown that aside from the mutations in globin genes, KLF1 gene mutation may also cause borderline HbA_2 level (Liu et al. 2014; Perseu et al. 2011; Waye & Eng 2015; Yu et al. 2015) with or without reduction in the MCV and/or MCH. KLF1 gene is one of the regulatory genes for the globin gene expression. It is proposed that mutation in the KLF1 causes impaired looping of the locus control region of the β-globin gene that results in lower number of β-globin chain and concomittent increased expression of the competing δ-globin gene, causing a raise in HbA_2 levels.

A study in Sardinia by Satta et al. in 2017 found that 35.9% of borderline HbA_2 levels in their cohort is caused by a mutation in the KLF1 gene. Out of these cases, 13.3% (26 cases) also have concurrent α-thalassaemia traits. Yu et al. (2015) then shows that in patients with α-thalassaemia, other than bringing the HbA_2 in the high normal or borderline range, coinheritance of KLF1 gene mutation also significantly affects red cell phenotypes – causing lower MCV and MCH values. These studies show that in the borderline population, KLF1 gene mutation, either alone or with coinheritance of α is commonly found.

Several studies in China also outlined the common KLF1 mutations in Asian population the commonest being a frameshift mutation of c.519_525dup (Liu et al. 2014; Yu et al. 2015). A study in Thailand replicated this finding (Tepakhan et al. 2016). These studies also proposed that coinheritance of β-thalassaemia/variants with KLF1 gene mutation often ameliorates disease phenotypes.

Despite the frequency and significance of the KLF1 gene mutation, especially in the borderline HbA_2 cases, hitherto, no published study has been recorded on this gene mutation in the Malaysia where the thalassaemia molecular landscape is significantly different from other regions within the thalassaemia belt.

This study aimed to describe the molecular characterisation including α-, β- and KLF1 gene abnormalities in cases with borderline levels of HbA_2 in Hospital Canselor Tuanku Muhriz, a tertiary Hospital at the center of Malaysian capital

city of Kuala Lumpur.

MATERIALS AND METHODS

This study was approved by the Universiti Kebangsaan Malaysia Ethics Committee (JEP-2020-078). All patient with borderline HbA_2 from the year 2014 to 2020, defined as HbA_2 values between 3.0% and 3.9% based on Sebia Capillary variant II analyser (Sebia S.A., Lisses, France), were traced through hospital information system. The analyser evaluation was conducted prior to routine use in the diagnostic laboratory of Hospital Canselor Tunku Muhriz – an ISO 15189 accredited medical testing laboratory. This process included evaluation of imprecision and random error by performing precision study on the analyser as per CLSI guideline (Clinical and Laboratory Standards Institute 2014). Both normal and pathological samples showed a 'within run' CV of of <4.00%, and 'between run' CV of <5.1%, as per manufacturer's claim thus the precision of the analyser was deemed acceptable and thus was fit for routine diagnostic use.

Cases with iron deficiency anaemia were excluded based on complete iron profile panel test (serum iron, total iron binding capacity and ferritin level). Cases with detection of any haemoglobin variants (eg: Haemoglobin E, Haemoglobin D or Haemoglobin S) were excluded as these conditions may confound HbA_2 level.

Sample size estimation was conducted using the population proportion formula, as outlined by Kish (1968). Prior data suggested that the proportion of borderline HbA_2 ranged from 2.2% to 16.7%, while observations from data collected in the Hospital Canselor Tuanku Muhriz (HCTM) lab indicated an occurrence of borderline HbA_2 at 3%. With an expected prevalence of 3% and a desired accuracy level of 0.05, the required sample size for the study was 45.

The information regarding their full blood count done using Sysmex XN-1000 (Sysmex Corp., Kobe, Japan) automated blood cell counter were collected. All cases gave informed consent for further DNA analysis. Genomic DNA extraction was prepared from peripheral blood using QIA symphony SP (QIAGEN N.V., Hilden, Germany).

Alpha-thalassaemia genotypes were tested for 9 different abnormalities commonly found in Malaysia and those reported from the Southeast Asian region. The panel included one double α-gene deletion (--SEA), two single gene deletions ($\alpha^{3.7}$ rightward deletion and $-\alpha^{4.2}$ leftward deletion) and six non-deletion α2-globin gene mutations namely initiation codon (ATG > A-G), codon 30 (ΔGAG), codon 35 (TCC > CCC), codon 59 (GGC > GAC), codon 125/HbQuangZhe (CTG > CCG) and termination codon/Hb Constant Spring (TAA > CAA). Multiplexed Gap-PCR and multiplexed amplification refractory mutation system (M-ARMS) methods were used in parallel for gene deletion and mutation testing, respectively.

For β-thalassaemia genotyping, 20 different mutations were tested: 19 by M-ARMS and one by simple amplification refractory mutation system (ARMS) technique. In the first M-ARMS-A reaction, allele specific primers for four mutations IVS 1-5 (G > C), Cd 41/42 (TTCT), Cd 17 (A > T) and Cd 26 (G > A) were multiplexed, while IVS 1-1 (G > T), Cd 8/9 (+G), 28 (A > G) and Cd 71/72 (+A) mutations were amplified in M-ARMS-B reaction. In the third M-ARMS-C reaction, alleles IVS 1-1 (G > A), Cd 43 (G > T), Cd 16 (C), and Poly A (A > G) were multiplexed, while M-ARMS-D had allele-specific primers targeted at 88 (C > T), initiation codon (ATG > AGG), Cd 15 (G > A) and 29 (A > G) mutations. Finally, allele specific primers used in M-ARMS-E reaction were used to screen for mutations 86 (C > G), Cd 19 (A > G) and Cap + 1 (A > C) [13]. IVS 2-654 (C > T) in a separate ARMS reaction.

The samples were also screened for KLF1 gene mutation using high resolution melting point (HRM) assay. The forward primer sequence of (5' to 3') CGAGACTCTGGGCGCATA and reverse primer sequence of GGAAGTGCCCTTGGTACTGA, as previously used by Liu et al. (2014) were used to amplify KLF1 gene at Exon 2.2, where the commonest mutation in Asian population (c.519_525dup)

was located. All samples were the analysed using HRM method to assess their melt curve and compared against wild type control curve. For confirmation, Sanger sequencing was performed on selected sample that had a different melt curve from the wild type.

All continuous independent and dependent variables were assessed for normality using histogram and bell-shaped curved, complemented with Shapiro-Wilk test. Independent t-test were used to compare means between the groups. All statistical analysis were performed using SPSS software version 22 (IBM Corp, Armonk, NY)

RESULTS

A total of 45 cases were identified for this study. The age range for our sample was from 3 to 78 years old with a median age of 20 years old. There were 62% (n=28) females and the rest were males. The majority of the sample were of Malay race (87%, n=39), some were Chinese (11%, n=5) and one Indian patient (2%) were included in our sample.

All our sample had HbA_2 level between 3.0-3.9% with a mean level of 3.25% (SD 0.31%). The mean Hb concentration was 11.97 g/L (SD 1.49 g/L), the average MCV was 75.94 fl (SD 9.0 fl), and the average MCH was 24.76 pg (SD 3.45 pg).

β-gene (HBB) Mutation

A total of 31 samples were found to have no β-gene defects and 31% of sample (n=14) harboured β-gene mutations. The abnormal β-genotype with highest occurrence within cases with borderline HbA_2 level was β^N/β^{PolyA} with 7 samples found to have this mutation. Other mutations found from our study include $\beta^N/\beta^{IVS\ 1\text{-}5\ (G>C)}$, $\beta^N/\beta^{IVS\ 2\text{-}654}$ and $\beta^N/\beta^{Cap\ +1\ (A>C)}$. Two samples were found to have $\beta^N/\beta^{IVS\ 2\text{-}16\ (G>C)}$ after further DNA sequencing were performed. Figure 1 summarised the type of β-gene mutation in relation to HbA_2 levels.

The lowest HbA_2 showed β-gene defect was HbA_2 of 3.1% and all samples with HbA_2 of 3.9% harboured a β-gene mutation (n=4). Table 1 summarised the means of the haematological

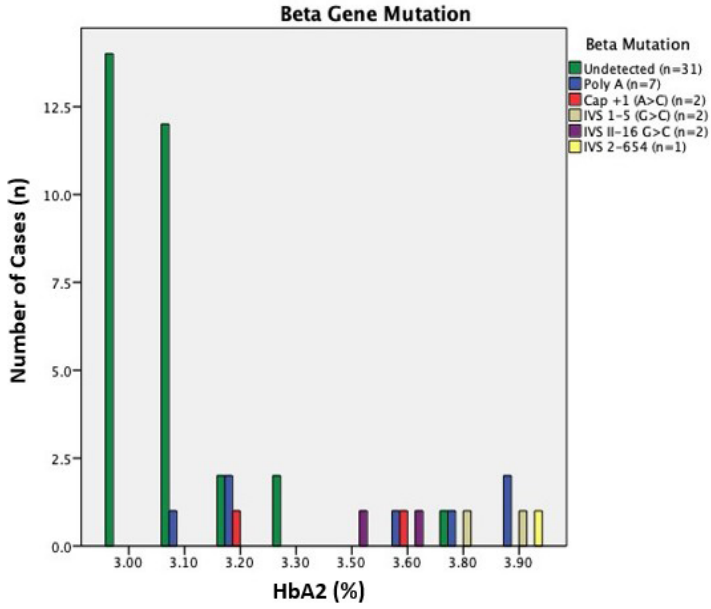

FIGURE 1: Beta gene abnormality in relation to HbA2 levels

TABLE 1: Summary of β-globin genotypes with the means of their respective hematological parameters

Beta genotype	n	HbA2 (%)	HbF (%)	RBC ($x10^{12}$/L)	Hb (g/dL)	MCV (fl)	MCH (pg)
Normal							
β^N/β^N	31	3.10 (±0.03)	0.82 (±1.61)	4.68 (±0.73)	12.12 (±1.62)	79.08 (±8.12)	26.05 (±2.92)
Abnormal							
$\beta^N/\beta^{Poly\ A}$, $\beta^N/\beta^{IVS\ 1\text{-}5\ (G>C)}$, $\beta^N/\beta^{Cap+1\ (A>C)}$, $\beta^N/\beta^{IVS\ 2\text{-}16\ (G>C)}$, $\beta^N/\beta^{IVS\ 2\text{-}654}$	14	3.59* (±0.30)	0.54 (±0.78)	5.37* (±0.51)	11.65 (±1.13)	69.00* (±6.97)	21.91* (±2.84)
p-value		0.001	0.551	0.003	0.334	0.001	0.001

Independent T-test were used to compare means.
*denoted statistical significance (p<0.05)

parameters (RBC, Hb, MCV, MCH, HbF and HbA_2) for each type of β-mutation. The HbA_2, red blood cell (RBC), MCV and MCH were significantly different (p<0.05, 95% CI) between the cases with and without β-gene mutations. The difference in Hb and HbF parameters were not significant (p > 0.05, 95% CI).

Alpha-Gene Deletion / Mutation

For α-gene globin, five samples (11%) were found to have α-gene defect while the rest were found to have normal α-genotype. The lowest HbA_2 which showed α-gene defect was HbA_2 of 3.0%. Figure 2 summarised the type of α-gene mutation in relation to HbA_2 levels.

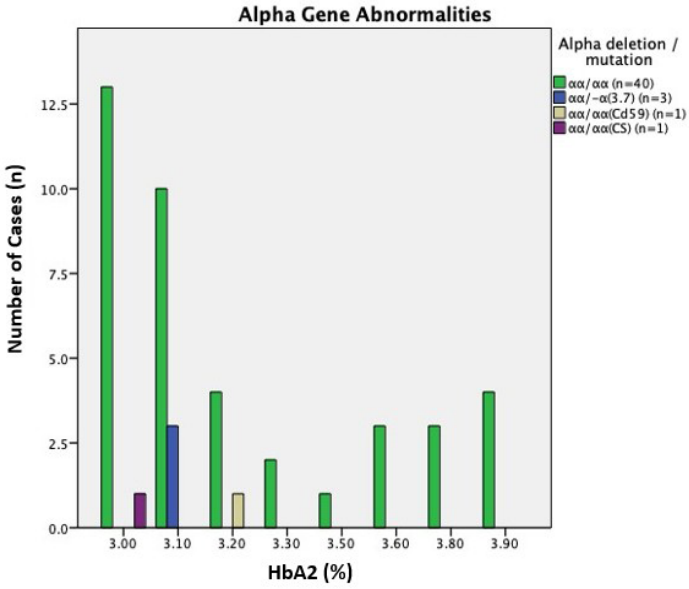

FIGURE 2: Alpha gene abnormality in relation to HbA2 levels

The means of the haematological parameters (RBC, Hb, MCV, MCH, HbF and HbA_2) for cases with and without α-mutation were summarised in Table 2. The difference in HbA_2, HbF, RBC, Hb, MCV and MCH were not significant between the cases with and without α-gene mutations ($p > 0.05$, 95% CI).

One case was found to have concomitant β- and α-gene defects. This case was found to be double heterozygous for Poly A (A > G) *HBB* mutation and codon 59 (GGC > GAC) mutation in the *HBB*. Her HbA_2 level was 3.2% and she had thalassaemic indiced with MCV of 77 fl and MCH of 24.3 pg.

KLF1 Gene Abnormalities

For KLF1 gene, the high resolution melting (HRM) assay followed by sequencing revealed no case with the commonest reported variant in Asian population c.519_525dup. However, one variant was detected in two of our sample. Both of these sample had no abnormalities detected in the β- and α-gene analysis. The variant, c.633 G>C, was further illustrated in Figure 3.

The mean HbA_2 level for cases with KLF1 variant was 3.05% (SD 0.07%) and HbF was 0%. The mean haemoglobin concentration was 12.45 g/L (SD 2.62 g/L), mean MCV was 86.454 fl (SD 4.74 fl), and mean MCH was 29.35 pg (SD 0.49 pg). Statistical analysis showed no significant difference in all hematological parameters (Hb, MCV, MCH, RBC, HbF and HbA_2) between cases with wild type and variant in KLF1 gene.

To summarise, 44% of the cases with borderline HbA_2 in HCTM showed genetic abnormality at the molecular level. *HBB* mutation was only found in 29% (n = 13) of the sample, α-gene defect was only seen in 9% (n=4) of the sample, KLF1 gene abnormality was only seen in 4% (n = 2) of the sample and 2% (n=1) of the sample population harboured coinheritance of α- and β-gene defect. The other 25 cases with borderline HbA_2 levels showed no abnormality in any of the three genes analysed in this study.

DISCUSSION

HbA_2 level is characteristically raised in a carrier of β-thalassaemia. A reduction in β-globin chain production is a result of one mutated β-allele leads to an increased availability of free α-globin chain, favouring binding with δ-chain which in turn causes a raise in HbA_2 level. Thus, this parameter has been used as a basis for diagnosing B-thalassaemia carriers. However, cases with borderline HbA_2 levels are not uncommon especially in areas where thalassaemia is endemic such as Malaysia.

In HCTM, 44% of the sample with borderline HbA_2 has a defect at molecular level; either in β-, α- or KLF1 gene. This is higher as compared

TABLE 2: Summary of α-globin genotypes with the means of their respective hematological parameters

Alpha genotype	n	HbA2 (%)	HbF (%)	RBC ($x10^{12}$/L)	Hb (g/dL)	MCV (fl)	MCH (pg)
Normal							
αα/αα	40	3.26 (±0.32)	0.75 (±1.46)	4.86 (±0.74)	11.95 (±1.41)	76.31 (±9.36)	24.95 (±3.59)
Abnormal							
$αα/αα^{-(3.7)}$, $αα/αα^{(CS)}$, $αα/αα^{(Cd59)}$	5	3.10 (±0.07)	0.62 (±0.83)	5.21 (±0.69)	12.12 (±2.22)	72.98 (±5.61)	23.2 (±1.61)
p-value		0.258	0.853	0.318	0.818	0.443	0.302

Independent T-test were used to compare means.
No statistical significance ($p<0.05$) was noted between cases with normal and abnormal alpha genotypes.

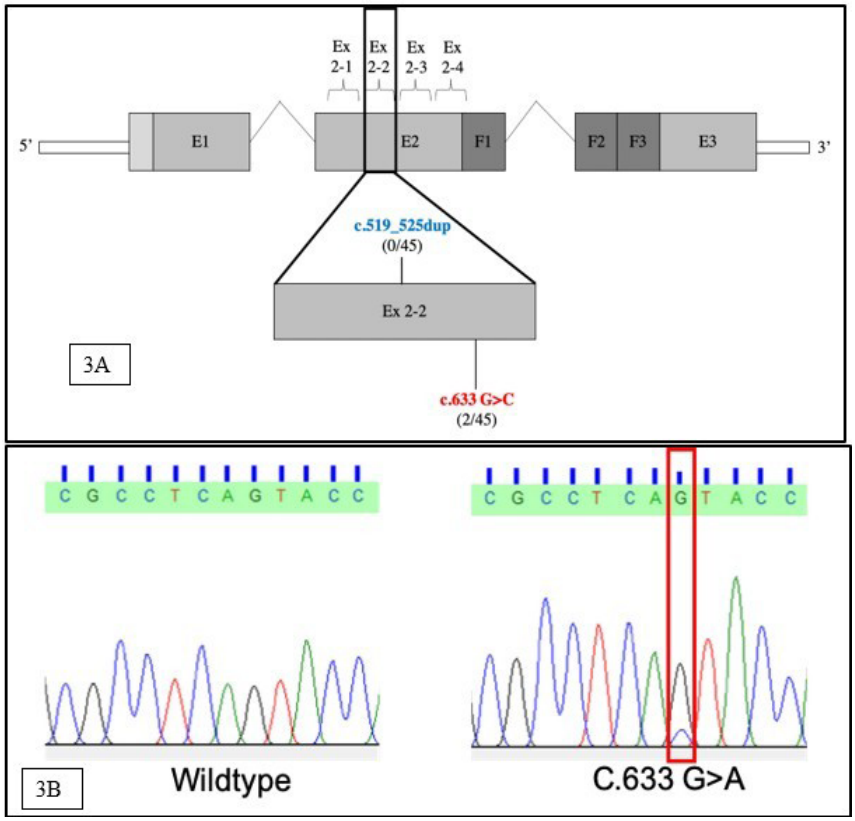

FIGURE 3: (A) Schematic representation of the KLF1 gene illustrating the positions for the variant identified in this study (in red) together with the frequency, as well as the previously described commonest mutation among Asian population (in blue) within the gene at exon 2.2. E1, E2 and E3 indicated exons 1, 2 and 3, respectively. F1, F2 and F3 were the three zinc finger domains; (B) Illustrates showed the sequencing result for the novel mutation found; c.633 G>A. Our sample was heterozygous for this mutation (Nucleotide numbering was based on NM_006563.5 in NCBI database)

to most recent literature by Colaco and Nadkarni (2021) that summarises the available molecular data on borderline HbA_2 cases worldwide. Their study reported that on average, 36% of sample harbours genetic alterations either in α, β, δ and/or KLF1 gene. The high frequency of mutation found in our study population is indeed crucial and should serve as a guide in making a diagnostic algorithm in this specific group of patients.

The commonest defect seen in our borderline HbA_2 group is Poly A (A > G) mutation in the HBB gene. A previous study in the state of Kelantan shows the commonest mutation in their borderline HbA_2 population as Cd 19 (A > G) mutation and Poly (A>G) mutation is only the third commonest mutation seen. This variation may be attributed to differences in local ethnic diversity in the respective studies. (Malaysian Thalassemia Registry Committee 2019).

Poly A (A>G) mutation (HGVS name HBB:c.*111A>G) is classified as a β^{++} mutation to reflect very minimal deficit in β-chain synthesis (Thein 2018). It is also known as a 'silent' mutation in which red cell indices are only mildly affected and HbA_2 levels are not typically raised. Thus the high frequency of this mutation in our study is not surprising. More importantly, when inherited in compound heterozygosiy with a severe β-allele

patients may present with intermedia phenotype, requiring intermittent transfusion, making identification of this mutation exceptionally important.

Generally, in β-thalassaemia carriers, levels of HbA_2 (α2δ2) may also be lower than expected as a consequence of coinheritance of α-gene defect. When there is insufficient α-chain production, α-β dimerisation to form HbA is prioritised over α-δ dimerisation for HbA_2 production leading to lower HbA_2 levels. In 2018, a study by Zamri et al. (2018) involving a larger Malaysian cohort of 299 cases with borderline HbA_2 levels shows 11% of cases to have such mutation. However, in our current study, coinheritance of β- and α-mutation was only detected in 2% of cases. This is comparable to the findings from Kelantan group (Rosnah et al 2017) which found 1% of cases with defects in both α and β gene (n=117). A small sample size may have been the limiting factor that causes underestimation of coinheritance of α- and β-mutation among the borderline HbA_2 in our study. Table 3 summarises the findings from this study in comparison to two previously published Malaysian study regarding borderline HbA_2.

Another key molecular determinant in causing low HbA_2 levels is when there is reduced δ globin chain synthesis for example in the case of δ- and β-mutation. However, this test was not performed in our study as such mutation will usually cause high HbF- which is not prominent in our sample population. The presence of α-triplication alone or in coinheritance with β-gene abnormalities has also been implicated to cause borderline HbA2 level however this aspect was also not explored in our study due to the rarity of the mutation in a population like Malaysia (Yap et al. 2013).

The role of KLF1 mutation in causing borderline levels of HbA_2 has piqued more interests and taken the centre stage in the recent years. To date, our study is the first to explore the presence of mutation in this gene in the Malaysian borderline HbA_2 cohort. Fascinatingly, we demonstrated a

TABLE 3: Comparison between current study with two published Malaysian studies on borderline HbA_2 levels

	Zamri et al. (2018)	**Rosnah et al. (2017)**	**Present study**	**Total**
Parameters not investigated	KLF1, δ gene, α mutation, α-triplication	KLF1, δ gene, α mutation, α-triplication	δ gene, α-triplication	
Total cases investigated	299	117	45	461
β thal mutation	174 (58%)	36 (31%)	13 (29%)	223 (48%)
Commonest mutation detected	NA	CD 19 (A>G) (15%) IVS 1-1 G>A (8%) Poly A (4%)	Poly A (16%) IVS 1-5 (G>C) (4%) Cap +1 (A>C) (4%)	
α thal	15 (5%)	8 (7%)	4 (9%)	27 (6%)
Commonest abnormality detected	NA	-α 3.7 (6%) -α 4.2 (1%)	$-\alpha^{3.7\ (6\%)}$ $\alpha\alpha^{CD\ 59}$ $\alpha\alpha^{CS}$	
β + α thal	33 (11%)	1 (1%)	1 (2%)	35 (8%)
Abnormality detected	NA	NA	Poly A with $\alpha\alpha^{CD\ 59}$	
KLF1 mutation	NA	NA	2 (4%)	2 (1%)

*borderline HbA_2 was defined as 3.3-3.9% by Zamri et al. (2018) and 3.0-3.9% by Rosnah et al. (2017)
NA: Not available

new mutation found in 2% of our sample. This mutation, c.633G>C will cause an amino acid change of glutamine to histidine at protein position number 211 of KLF1 gene. Although this mutation has not been described before, a mutation at the neighbouring nucleotide, (c.632 A>G, HGVS ID NM_006563.4:c.632A>G) which causes glutamine to serine substitution instead, has been reported. National Center for Biotechnology Information and Varsome database classified this change in protein number 211 of KLF1 gene to be of uncertain clinical significance thus possibly, similar assumption can be made for the novel mutation found in this study.

More interestingly, both cases with KLF1 mutation have normal hematological parameters with borderline HbA_2 levels. This is consistent with findings made by several studies (Liu et al. 2014; Perseu et al. 2011; Waye & Eng 2015). Perseu et al. (2011) further explores the mechanism behind elevation of HbA_2 level into borderline ranges in these individuals. They found that the mild increase in HbA_2 in KLF1 mutation carriers was associated with an increased ratio of δ : δ+β production. KLF1 is a known transcription factor for β-globin gene expression and mutation in the gene causes decreased β-globin production and lead to higher-than-normal HbA_2 levels, without affecting other red cell indices.

We also found 56% of cases with borderline HbA_2 without any molecular abnormalities detected from our investigation. Notwithstanding the mutations not included in our study, several factors are also known to affect HbA_2 levels. For example, B12/folate deficiency, antiretroviral drugs and untreated hypothyroid cases have been reported to cause mild HbA_2 elevation in individuals without globin gene defects (Colaco and Nadkarni 2021). Finally, awareness on these cases may simply be outlier values of the normal distribution curve of HbA_2 level in the non-carrier population without harbouring underlying genetic defects also needs to be appreciated.

Borderline HbA_2 levels is not uncommon in populations with high thalassaemic load such as ours and will be encountered often in a laboratory setting. To date, only two published studies have briefly described the molecular characterisation of this unique group of patients (Table 3). While our study assessed multiple genes that may play a key role in causing borderline HbA_2 level, our limitations include a small sample size and the limited exploration of the KLF1 gene- with only exon 2.2 of the gene was scrutinised. More large-scale studies to characterise the underlying causes of borderline HbA_2 levels are needed to prove the importance of further investigation for the underlying genetic defect and allow for a development of a specific testing algorithm for this group of patients. Data from the studies will enable better phenotypic prediction which is especially useful in genetic counselling for couples with borderline HbA_2.

CONCLUSION

In conclusion, our study demonstrated a high frequency of genetic defect particularly in β-globin gene among the borderline HbA_2 population in HCTM, with Poly A mutation having highest frequency. We therefore strongly suggest for further molecular analysis to be performed; preferably including common β-mutation and α-deletions or at least a β-mutation panel including Poly A mutation for cases with borderline HbA_2 levels, especially when the individual has a partner who is a β-thalassaemia carrier. We also pioneered the investigation of KLF1 mutation in this country and excitingly, discovered one new mutation in our cohort. We recommend further research in this area to further characterise the common type of KLF1 mutations in our population.

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