Received: 20 March 2023 | Revised: 2 June 2023 | Accepted: 20 June 2023

DOI: 10.1002/jha2.750

eJHaem

RESEARCH ARTICLE

Clinical and haematological characteristics of 38 individuals with Hb G-Makassar in Malaysia

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Abstract

Haemoglobin (Hb) G-Makassar is a rare Hb variant. It presents a diagnostic challenge as it imitates sickle Hb (Hb S) in standard electrophoresis and high-performance liquid chromatography assays requiring DNA analysis to confirm diagnosis. Both have point mutations in codon 6, exon 1 in the β-globin (*HBB*) gene with different pathogenicities. This study describes the clinical phenotype, haematology and genotype of Hb G-Makassar. Clinical and laboratory data of 38 cases of Hb G-Makassar over 8 years were analysed. Hb G-Makassar was confirmed by a direct sequencing of *HBB* gene and co-inheritance of α-thalassaemia determined through multiplex gap-PCR and multiplex Amplification Refractory Mutation System polymerase chain reaction. All cases were Malays, predominantly from Terengganu ($n = 20$, 52.6%). There were 14 (36.8%) males and 24 (63.2%) females with median age of 25 years. Majority ($n = 33$, 86.8%) had features of thalassaemia trait with mean ± SD for Hb, mean cell volume (MCV) and mean cell haemoglobin (MCH) as 13.21 g/dL ± 1.69, 73.06 ± 4.48 fL and 24.71 ± 1.82 pg, respectively. None had evidence of haemolysis or thromboembolic complications. Six genotypes were identified; $ß^{G\text{-}Makassar}/ß,\alpha\alpha/\alpha\alpha$ ($n = 19$, 50.0%), $ß^{G\text{-}Makassar}/ß^{E},\alpha\alpha/\alpha\alpha$ ($n = 4$, 10.5%), $ß^{G\text{-}Makassar}/ß^{NewYork},\alpha\alpha/\alpha\alpha$ ($n = 1$, 2.6%), $ß^{G\text{-}Makassar}/ß,\alpha\alpha/\text{-}\alpha$ ($n = 11$, 28.9%), $ß^{G\text{-}Makassar}/ß,\alpha\alpha/\alpha^{Adana}\alpha$ ($n = 2$, 5.3%) and $ß^{G\text{-}Makassar}/ß,\alpha\alpha/-^{SEA}$ ($n = 1$, 2.6%). The $ß^{G\text{-}Makassar}/ß,\alpha\alpha/\alpha\alpha$ showed that features of thalassaemia trait with mean ± SD for Hb, MCV and MCH were 13.74 g/dL ± 2.40, 76.18 ± 6.02 fL and 25.79 ± 2.41 pg, respectively. This is the largest study reporting a significant number of Hb G-Makassar in Malaysia. Although the mutation is similar to Hb S, the phenotype is benign.

KEYWORDS
clinical phenotype, genotype, haematological characteristics, Hb G-Makassar

1 | INTRODUCTION

Haemoglobin (Hb) G-Makassar (NG_000007.3:g.[70614A > C]) is a rare β-chain variant in Southeast Asia. It was named after a city in Sulawesi, Indonesia, called Makassar, where it was first discovered in 1969 [1]. The following incidence was then identified in Thailand, Malaysia and the United Kingdom, but higher incidence was observed in Southeast Asia than in Europe [2–7].

Hb G-Makassar exhibits identical characteristics to Hb S (NG_000007.3:g.[70614A > T]) in a few standard methods of Hb analysis. The Hb fraction is eluted at a similar retention time interval on cation-exchange high-performance liquid chromatography (HPLC), forming a peak that resembles S-window. The variant is not possible to be distinguished in agarose gel and cellulose acetate Hb electrophoresis. The mobility rate is as slow as sickle Hb (Hb S) in alkaline phase and migrates to the S position in acid phase [2, 6]. Moreover, it appears in zone 5 with comparable values to Hb S in capillary electrophoresis (CE) and co-migrates with Hb S in isoelectric focusing [3]. Although these screening tests can highly be misinterpreted as Hb S, it should be pointed out that these tests can only provide a presumptive diagnosis; thus, DNA analysis is necessary for a definitive identification.

The similarities found in Hb analysis for Hb G-Makassar and Hb S are postulated to happen because the molecular pathogenesis of the variants is very much alike [2]. The point mutations of these variants occur at the same spot in codon 6 of exon 1 in *HBB* gene of chromosome 11. Normally, the nucleotide sequence at this codon is GAG, which forms glutamic acid. However, in Hb G-Makassar, the normal A nucleotide is replaced by C, resulting in the substitution of glutamic acid to alanine (HBB:c.20A > C; p.Glu7Ala). In Hb S, instead of A, it is replaced by T nucleotide, resulting in the production of valine (HBB:c.20A > T; p.Glu7Val) [3, 8].

The pathogenicity of Hb G-Makassar and Hb S is reported to be markedly different. Hb G-Makassar is considered a benign Hb variant. The patients may present with no symptom or mild anaemia, be it for those with Hb G-Makassar heterozygote (NG_000007.3:g.[70614A > C];[70614A =]) or homozygote (NG_000007.3:g.[70614A > C];[70614A > C]); or those with a compound Hb G-Makassar/β-globin variant [1–6]. In contrast, Hb S has a spectrum of clinical presentation according to the interaction of the variant with different genotypes in *HBB* allele. Sickle cell disease (SCD) such as homozygous Hb S (NG_000007.3:g.[70614A > T];[70614A > T]), compound heterozygous Hb S/C (NG_000007.3:g.[70614A > T];[70613G > A]) and compound Hb S/β-thalassaemia are typically presented with moderate-to-severe chronic haemolytic anaemia with recurrent painful vaso-occlusive crisis, which sometimes can be fatal. Unlike SCD, heterozygous Hb S or sickle cell trait does not have crises, but it may not be completely benign. Sickle cell trait may develop complications following exposure to conditions that favour sickling such as severe hypoxia, dehydration, increase in sympathetic outflow, hypothermia/hyperthermia, high 2,3-DPG levels and release of inflammatory cells. These conditions could cause ischaemia in multiple organs, and repeated attacks may lead to organ damage [9].

This study describes the clinical phenotype, haematological characteristic and genotype of 38 Hb G-Makassar cases identified within 8-year duration. The data are expected to provide evidence-based information for a better understanding of the variant in the context of Malaysia.

2 | METHOD

The study is designed as a retrospective cross-sectional study on 13,140 genotyping data of β-globin variant cases recorded from January 2014 to August 2021. The cases were part of data available in Institute for Medical Research (IMR), Malaysia. The blood samples of the cases were sent to IMR from various hospitals for the confirmation of the presumptive diagnosis of an Hb variant. Out of 13,140 cases, 10,541 (80.2%) were confirmed to have β-globin variant mutations and 38 were identified as Hb G-Makassar for this study. These cases were recorded to occur between October 2014 and January 2021. The clinical information and thalassaemia screening data were collected from the medical records. The patients' status of alive versus death was reviewed from the Malaysian National Registration Department, and related laboratory test results were traced through the hospital information system. Some follow-ups to gather information on the current health condition and the clinical complications were also performed through phone calls. The questionnaire with the following questions were asked: (1) Have you ever experienced acute pain crisis? (2) Have you ever had a blood clot in your body that needed to be treated with blood thinning medication [10]? Have you ever had a stroke? (4) Have you ever had a heart attack? (5) Have you ever had a problem with bleeding that required you to see a doctor? (6) Do you have any problem with your kidneys?

2.1 | Thalassaemia screening tests and DNA analysis

The National Thalassaemia Screening Program in Malaysia has been carried out on the voluntary basis by target and cascade screening approach. The target screening is mainly performed for high school students, and it is also being performed during antenatal and premarital screening. The cascade screening is carried out for those with

family history of thalassaemia/haemoglobinopathy. One of the activities in this national programme is the laboratory test which includes thalassaemia screening tests and DNA analysis.

The thalassaemia screening tests 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 such as peripheral blood film (PBF), CE (SEBIA), HPLC (Bio-Rad Laboratories) and Hb electrophoresis (SEBIA). The laboratory information gathered from these screening tests are used to make a presumptive diagnosis of an Hb variant.

The definitive diagnosis was made through DNA analysis. In this test, the DNA was first extracted from peripheral blood using QIA symphony SP (Qiagen, GmbH). The concentration and quality of the extracted DNA were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc). Subsequently, a few genotyping tests were performed on the DNA to identify the specific mutations which include Amplification Refractory Mutation System polymerase chain reaction (ARMS-PCR) for Hb S mutation; followed by a direct DNA sequencing analysis of the *HBB* gene when the Hb S mutation was negative on ARMS-PCR. However, as we gained more experience, direct DNA sequencing was performed straight away without performing ARMS-PCR for Hb S, as the former method was capable of detecting many β-globin variants, including Hb G-Makassar and Hb S simultaneously. In this analysis, the amplicon of 2020 base pairs in size was initially obtained from the PCR assay consisting of the *HBB* gene and 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 a BigDye Terminator v3.1 cycle sequencing kit. Five primers targeting the *HBB* gene were used in cycle sequencing. The sequences were then read through ABI 3730XL DNA Analyser (Applied Biosystems) before they were analysed by CLC Main Workbench (CLC Bio).

In addition, multiplex gap-PCR and multiplex ARMS-PCR were performed to detect the common α-thalassaemia deletions and mutations using previously described methods as α-thalassaemia is quite prevalent in Malaysia [11, 12]. 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 $--(\alpha)^{20.5}$. The multiplex ARMS-PCR was used to detect the non-deletional α-thalassaemia such as codon 142/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.

2.2 | Statistical analysis

Statistical Package for the Social Sciences (IBM-SPSS) version 27 was used for data analysis. The quantitative variables were analysed through descriptive statistics, either mean ± standard deviation (SD) or median, while the qualitative variables employed frequency and percentage (*n*, %) reporting. The mean's significances for blood count parameters were compared using one-way ANOVA for the first three genotypes (Table 1). However, the mean's significance for Hb subtype fractions were compared using a Student's *t*-test between the first (heterozygous Hb G-Makassar) and second group (heterozygous Hb G-Makassar with co-inherited heterozygous α^{+}-thalassaemia). The third genotype group (compound heterozygous Hb G-Makassar/Hb E) was excluded for analysis because both CE and HPLC are able to identify the concomitant Hb E mutation in the third group. The data were considered statistically significant when the *p*-value was less than 0.05.

2.3 | Ethics approval

This study was conducted according to the Declaration of Helsinki and approved by Medical Research and Ethics Committee, Ministry of Health (NMRR-21-1408-60675, IIR), 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 were entirely kept anonymous, and confidentiality was maintained through the suitable use of coding.

3 | RESULTS

All 38 Hb G-Makassar cases were of Malay ethnicity. Their age ranged from 1- to 49-year old with the median of 25-year old. There were 14/38 (36.8%) males and 24/38 (63.2%) females. The majority (n = 20/38, 52.6%) of them were referred from Terengganu, one of the states in the east coast of Peninsular Malaysia. The indication for thalassaemia screening was mostly for high school students (n = 23/38, 60.5%), whereas the rest were for family (n = 6/38, 15.8%), premarital (n = 6/38, 15.8%) and antenatal (n = 1/38, 2.6%) screening. Two (n = 2/38, 5.3%) hospitalised cases were screened for thalassaemia as part of anaemia workup when they presented with low Hb and hypochromic microcytic indices.

3.1 | Genotype groups

There were six genotype groups as shown in Table 1. The genotype group of heterozygous Hb G-Makassar had the highest incidence (n = 19/38, 50.0%). This genotype was assumed to have no mutations in the α-globin gene (homozygous wild-type) as it was negative for the common deletional and non-deletional α-thalassaemia. The heterozygous Hb G-Makassar with co-inherited heterozygous α^{+}-thalassaemia in the next group is consisted of 10 heterozygous Hb G-Makassar with co-inherited $\alpha\alpha/-\alpha^{3.7}$ (NG_000007.3:g.[70614A > C];[70614A =],NG_000006.1:g.[34164_37967del];[34164_37967 =]) and one heterozygous Hb G-Makassar with co-inherited $\alpha\alpha/-\alpha^{4.2}$ (NG_000007.3:g.[70614A > C];[70614A =],NG_000006.1:g.[30682_34939del];[30682_34939 =]) Both types of single deletional α-thalassaemia were grouped together for statistical analysis as they are phenotypically indistinguishable.

TABLE 1 Haematological parameters and haemoglobin (Hb) subtype profiles of Hb G-Makassar spectrum.

Genotype Type of mutation	$ß^{G-M}/ß$ αα/αα Het Hb G-Makassar	$ß^{G-M}/ß$ αα/$α^{+}$ Het Hb G-Makassar with co-inherited het $α^{+}$-thal	$ß^{G-M}/ß^{E}$ αα/αα Compound Hb G-Makassar/Hb E	$ß^{G-M}/ß$ αα/$α^{A}α$ Het HbG-Makassar with co-inherited het Hb Adana	$ß^{G-M}/ß$ αα/$–^{SEA}$ Het Hb G-Makassar with co-inherited het $–^{SEA}$-thal	$ß^{G-M}/ß^{NY}$ αα/αα Compound Hb G-Makassar/Hb New York	Normal reference range (adult)	
							Male	Female
n (%)	19 (50.0)	11 (28.9)	4 (10.5)	2 (5.3)	1 (2.6)	1 (2.6)	–	–
	mean (SD)			median (value)	–	–		
RBC ($\times 10^{12}$/L)	5.32 (0.72)	5.15 (0.46)	5.50 (0.42)	5.98 (5.76, 6.19)	6.50	6.20	3.40–5.20	3.10–4.80
Hb (g/dL)	13.74 (2.40)	12.96 (1.28)	13.08 (2.03)	14.35 (13.80, 14.90)	14.20	16.20	13.00–17.00	11.10–14.80
MCV (fL)	76.18 (6.02)	74.93 (3.23)	67.90 (6.03)	72.25 (70.00, 74.00)	65.40	77.30	83.30–98.00	
MCH (pg)	25.79 (2.41)	25.18 (1.31)	23.73 (2.83)	24.05 (24.00, 24.10)	21.80	26.10	27.70–33.20	
MCHC (g/dL)	33.84 (1.58)	33.63 (1.34)	34.90 (1.10)	33.25 (32.30, 34.20)	33.40	33.80	32.30–35.90	31.70–35.10
RDW (%)	14.85 (1.50)	13.40 (0.75)	15.10 (2.03)	13.20 (12.60, 13.80)	13.10	15.50	12.20–14.70	
RDW-SD (fL)	37.61 (9.79)	38.71 (5.31)	41.30 (6.56)	37.5 (37.50, –)	–	35.00	39.00–46.00	
CE (%)								
Hb A	54.11 (1.93)	56.15 (0.94)	1.53 (2.65)	57.05 (56.20, 57.90)	60.70	0.00	>97.00	
Hb A_2	3.10 (0.95)	2.97 (0.39)	4.75 (0.34)	3.00 (2.80, 3.20)	2.80	2.90	2.70–3.2	
Hb F	0.34 (0.63)	0.61 (0.55)	3.08 (2.40)	1.20 (0.40, 2.00)	0.40	3.00	0.03	
Hb G-Makassar[a]	42.38 (1.00)	40.25 (0.71)	66.55 (3.71)	38.75 (38.50, 39.00)	36.10	45.50	–	
Hb E	–	–	24.40 (1.25)	–	–	–	–	
Hb New York[b]	–	–	–	–	–	48.60	–	
HPLC (%)								
Hb A	47.60 (2.13)	49.70 (3.39)	5.37 (0.29)	52.4 (52.4, –)	53.30	43.30	>97.00	
Hb A_2	3.37 (0.22)	3.37 (0.98)	27.03 (1.86)[c]	4.20 (3.40, 5.00)	3.60	3.80	2.88–3.2	
Hb F	0.32 (0.14)	0.52 (0.75)	1.47 (0.95)	0.55 (0.40, 0.70)	0.30	1.00	0.58	
Hb G-Makassar[a]	40.98 (1.04)	37.17 (2.25)	63.20 (0.60)	36.20 (35.40, 37.00)	35.00	46.70	–	

Abbreviations/Remarks: $α^{+}$, single-alpha gene deletion (-$α^{3.7}$ and -$α^{4.2}$); $α^{A}$, Hb Adana; $ß^{E}$, Hb E; $ß^{G-M}$, Hb G-Makassar; $ß^{NY}$, Hb New York; CE, capillary electrophoresis; het, heterozygous; HPLC, high-performance liquid chromatography; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; MCV, mean cell volume; *n*, number of cases; RBC, red blood cells; RDW, red cell distribution width; $–^{SEA}$, alpha –SEA deletion; thal, thalassaemia.

[a]CE and HPLC had detected the variant-peak in zone 5 and S-window, where the Hb S-peak typically appears. We name the variant-peak in this table as Hb G-Makassar as DNA analysis had detected this variant.

[b]The Hb New York co-elutes with Hb A in HPLC but is separated in CE, as it appears in zone 11, whereas no Hb A appears in zone 9.

[c]Hb E co-elutes with Hb A2 in HPLC.

3.2 | Clinical phenotype

The review of medical records showed that every case in this study was asymptomatic during the screening although there were two cases screened while hospitalised as part of an anaemia workup. However, the medical history questionnaire response was available in 97.4% (37/38) of the cases. All of them indicated that they were well, free of anaemic symptoms and had no history of thromboembolic event such as acute pain crisis, thrombus, stroke, heart attack or kidney disease. Nevertheless, there were three cases with history of anaemia, of whom two stated they had history of bleeding.

The first case was from a 45-year-old male with a history of anaemia secondary to bleeding haemorrhoid as previously described by Mohamad et al. [6]. Because of persistent hypochromic microcytic indices (Hb 13.9 g/dL, mean cell volume [MCV], 73.90 fL and mean cell haemoglobin [MCH], 26.80 pg) in spite of iron treatment, Hb analysis was performed and incidentally detected a finding suggestive of compound heterozygous Hb S/Hb E NG_000007.3:g.[70614A > T];[70673G > A]. But, genotyping of the β-globin gene identified a compound heterozygous Hb G-Makassar/Hb E (NG_000007.3:g.[70614A > C];[70673G > A]).

The second case was from a 1-year-old boy who was hospitalised for fever and was eventually managed for anaemia caused by congenital hypothyroidism. He had mild hypochromic microcytic anaemia (Hb, 10.7 g/dL, MCV, 61.00 fL and MCH, 20.60 pg) and was presumptively diagnosed as having compound Hb S/Hb E by Hb analysis, but DNA analysis has shown that it was compound Hb G-Makassar/Hb E.

The third case was a 28-year-old female who happened to be the second case's mother. Although she had past medical history of anaemia as a result of dysfunctional uterine bleeding, she was well during the family screening. Her Hb was normal (13 g/dL) with normochromic normocytic indices (MCV, 85 fL and MCH, 28.10 pg). Her Hb analysis revealed heterozygous Hb S, but the DNA analysis had detected heterozygous Hb G-Makassar.

3.3 | Haematological characteristics

Majority (81.6%, $n = 31/38$) of the cases had normal Hb level (Table 1), whereas four (10.5%, $n = 4/38$) had low Hb level and three (7.9%, $n = 3/38$) had high Hb level (Table 2). In those with low Hb: Case A was the same person who had congenital hypothyroidism as described before; case B was a pregnant woman and had co-inherited α^{+}-thalassaemia; case C was a high school student and case D was a non-pregnant woman, who came for family screening. In those with high Hb: Case E was a male, who came for pre-marital screening and he was a non-smoker; case F was a high school student, of whom secondary polycythaemia needed to be ruled out as she had a high haematocrit level (59.5%) and very packed red blood cells (RBC) in her PBF. However, she did not turn up for further investigation. Case G was also a high school student with no remarkable clinical history.

The overall blood indices for all genotype groups showed low MCV and MCH, raised RBC, and normal red cell distribution width (RDW) and mean cell haemoglobin concentration. The mean for indices was not statistically significant to discriminate the first three groups except for MCV, where there was a significant difference between heterozygous Hb G-Makassar and compound Hb G-Makassar/Hb E ($p = 0.023$).

Morphologically, there was no sickle cell seen in the PBF. The RBC appear hypochromic microcytic in 33 ($n = 33/38$, 86.8%) with mean ± SD for Hb, MCV and MCH 13.21 g/dL ± 1.69, 73.06 ± 4.48 fL and 24.71 ± 1.82 pg. The remaining five ($n = 5/38$, 13.2%) cases that exhibited normochromic normocytic features were corresponding to the normal MCV (83.92 ± 3.29 fL) and MCH (28.52 ± 1.10 pg) values. Four of these cases were screened for thalassaemia because they had a history of thalassaemia in family, whereas one case was tested for pre-marital screening. The genotype for these five cases was heterozygous Hb G-Makassar.

Hb analysis had detected Hb S/Hb G-Makassar peak in 243 ($n = 243/10{,}541$, 2.3%) cases; in which 203 ($n = 38/10{,}541$, 1.9%) cases were confirmed to have Hb S and only 38 ($n = 38/10{,}541$, 04%) cases were confirmed to have Hb G-Makassar by DNA analysis. Amongst the Hb G-Makassar cases, CE and HPLC were done in 17/38 (44.7%), CE alone in 11/38 (28.9%), CE and Hb electrophoresis in 5/38 (13.2%), HPLC alone in 3/38 (7.9%) and all three methods in 2/38 (5.3%). The Hb subtype profiles generated from CE and HPLC are shown in Table 1. The Hb electrophoresis of the seven cases consistently showed the presence of S band in alkaline and acid phase. From these profiles, most ($n = 29/38$, 76.3%) were mistakenly interpreted as suggestive of Hb S trait ($n = 25$, 65.8%), 3/38 (7.9%) as compound Hb S/Hb E and one ($n = 1/38$, 7.9%) as compound Hb S/β-variant Hb. Other cases were presumptively reported to have β-variant Hb trait ($n = 5/38$, 13.2%) and only a minority ($n = 4$, 10.5%) had considered trait for either Hb S or Hb G-Makassar. The mean values for Hb subtypes obtained from CE and HPLC analyses between heterozygous Hb G-Makassar and heterozygous Hb G-Makassar co-inherited with α^{+}-thalassaemia ($\alpha^{3.7}$ and $\alpha^{4.2}$-thalassaemia) were not significant ($p = 0.30$–1.00).

4 | DISCUSSION

Thalassaemia is the most common monogenic disorder that causes major public health problem in Malaysia. In 2018, 8681 patients were registered in Malaysian Thalassaemia Registry of which 7984 were reported alive. Out of this number, 56.73% were on regular blood transfusions and 61.72% were on chelation therapy. The common thalassaemia syndromes are Hb E/β-thalassaemia ($n = 2744$, 34.4%), β-thalassaemia major ($n = 2676$, 33.5%) and Hb H disease ($n = 1458$, 18.3%) [13]. The cumulative numbers of the disease are high as 1 of 15 people carries the genetic mutation (the carrier rate is 6.8%) [14]. The carrier rate for each α- and β-thalassaemia is 4.5%–5.0% and Hb E is the most common (3.0%–40.0%) β-thalassaemia variant [15, 16].

Hb G-Makassar is considered a rare β-variant Hb in Malaysia, because within 8 years of analysis (from 2014 to 2021), only 38 ($n = 38/11{,}541$, 0.33%) cases were detected to have Hb G-Makassar. These data include the cases reported in 2017 and 2018, as IMR is a

TABLE 2 Haematological parameters of Hb G-Makassar cases with abnormal haemoglobin (Hb) level.

Hb level	Low				High			Normal reference range (adult)	
Gender	M	F	F	F	M	F	F	M	F
Case	A	B	C	D	E	F	G	–	–
Age	1[a]	27	16	30	26	16	16		
RBC ($\times 10^{12}$/L)	5.20	4.29	5.21	4.28	5.78	7.21	6.20	3.40–5.20	3.10–4.80
Hb (g/dL)	10.70	11.00	10.90	10.40	17.50	19.4	16.20	13.00–17.00	11.10–14.80
MCV (fL)	61.00	73.20	67.60	69.40	79.40	82.50	77.30	83.30–98.00	
MCH (pg)	20.60	25.60	20.90	24.30	30.30	26.90	26.10	27.70–33.20	
MCHC (g/dL)	33.80	35.00	31.00	35.00	38.10	32.60	33.80	32.30–35.90	31.70–35.10
RDW (%)	15.60	13.40	16.10	17.20	15.00	15.20	15.50	12.20–14.70	
RDW-SD (fL)	33.20	34.40	–	42.20	39.30	–	35.00	39.00–46.00	
CE (%)									
Hb A	–	57.20	55.30	55.00	54.00	55.90	0.00	>97.00	
Hb A_2	4.80	2.60	2.30	2.60	2.90	2.50	2.90	2.70–3.2	
Hb F	6.60	0.50	0.00	1.10	0.00	0.00	3.00	0.03	
Hb G-Makassar[b]	63.80	39.70	42.40	41.30	43.10	41.60	45.50	–	
Hb E	24.50	–	–	–	–	–	–	–	
Hb New York[c]	–	–	–	–	–	–	48.60	–	
HPLC (%)									
Hb A	5.70	57.2	–	50.80	49.20	49.70	43.30	>97.00	
Hb A_2	25.50[d]	3.40	3.10	3.10	3.30	3.50	3.80	2.88–3.2	
Hb F	2.40	0.6	0.10	0.60	0.30	0.30	1.00	0.58	
Hb G-Makassar[b]	63.80	36.40	41.10	40.90	42.00	39.10	46.70	–	
Genotype	$ß^{G-M}/ß^{E}$ $\alpha\alpha/\alpha\alpha$	$ß^{G-M}/ß$ $\alpha\alpha/\alpha^{+}$	$ß^{G-M}/ß$ $\alpha\alpha/\alpha\alpha$	$ß^{G-M}/ß$ $\alpha\alpha/\alpha\alpha$	$ß^{G-M}/ß$ $\alpha\alpha/\alpha\alpha$	$ß^{G-M}/ß$ $\alpha\alpha/\alpha\alpha$	$ß^{G-M}/ß^{NY}$ $\alpha\alpha/\alpha\alpha$	–	

Notes: Types of mutation for (i) case A: compound Hb G-Makassar/Hb E; (ii) case B: heterozygous Hb G-Makassar with co-inherited heterozygous α^{+}-thalassaemia; (iii) cases C–F: heterozygous Hb G-Makassar; and (iv) case G: compound Hb G-Makassar/Hb New York.

Abbreviations: CE, capillary electrophoresis; HPLC, high-performance liquid chromatography; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; MCV, mean cell volume; RBC, red blood cells; RDW, red cell distribution width.

[a]Normal reference range for 1-year old children: RBC 3.9–5.3 $\times$ 10^{12}/L, Hb 11.5–14.0 g/dL, MCV 72–84 fL, MCH 25.0–29.0 pg, MCHC 32.0–36.0 g/L, RDW 12.3%–15.6%.

[b]CE and HPLC had detected the variant-peak in zone 5 and S-window, where the Hb S-peak typically appears. We name the variant-peak in this table as Hb G-Makassar since DNA analysis had detected this variant.

[c]The Hb New York co-elutes with Hb A in HPLC but is separated in CE, as it appears in zone 11, whereas no Hb A appears in zone 9.

[d]Hb E co-elutes with Hb A_2 in HPLC.

sole facility in Malaysia that provides the diagnostic service for rare thalassaemia variant [5, 6]. Sixty-one per cent (n = 23/38) of the cases were detected among high school students, which accounted for less than 1% of a total 689,460 students who were screened for thalassaemia. However, this is likely an underestimate of the true incidence of Hb G-Makassar as only individuals with abnormal screening blood indices would be sent for Hb analysis. In our study, we found five (n = 5/38, 13.2%) cases with normal blood indices, and the Hb analyses were indicated for family and premarital screening. Incidental finding of the Hb variant from this screening test required confirmation by DNA analysis; which turned up as heterozygous Hb G-Makassar. The inaccuracy of the incidence for Hb G-Makassar is also affected by the underrepresented cases of β-thalassaemia in our registry. This is because not all β-thalassaemia carriers in Malaysia need a definitive analysis. Moreover, DNA analysis for some of the β-thalassaemia mutations has been offered by other centre beginning from year 2020 to certain states in Malaysia, whereas we are focusing on analysis of the rarer mutations. Therefore, lesser cases were referred to IMR.

Our cohort of cases is larger than the reported cases in Thailand [2–4]. The variant was exclusively found among the Malays, with the majority of them from Terengganu (n = 20/38, 52.6%). The cases found in Songkhla, Thailand might share the same ancestral origin with our cases. Songkhla is a province of Southern Thailand, near the border of Malaysia. A large proportion of Siamese Muslim people in Songkhla speak Malay-related language, called Yawi language. The nearer Malaysian states to Southern Thailand are Perlis, Kedah, Perak

and Kelantan; but in this study, the variant was only found in Kelantan and it was only one case ($n = 1/38$). To the best of our knowledge, no prevalence study on Hb G-Makassar was done in Thailand. Hence, the origin of the variant cannot be determined.

Our findings are in accordance with other studies on the benign state of Hb G-Makassar [1–4]. The anaemia in two hospitalised cases in our study was secondary to an identified co-morbidity which was resolved after specific treatment. There were no anaemia symptoms and development of thromboembolic complications noted on follow-up assessments. The findings are consistent with the in vitro study that demonstrated that the alanine-type Hb behaved as functional Hb. It did not polymerise when was exposed to a low oxygen threshold condition, where valine-type Hb of Hb S readily polymerises. The polymerisation of Hb is responsible for causing haemolysis, vaso-occlusive crises and multi-organ damage in Hb S [17–20]. The approach on changing the Hb S to Hb G-Makassar is potentially encouraging to treat patients with SCD that could be explored in future clinical trials [21, 22].

The Hb levels for most of the Hb G-Makassar variants in this study were normal (Table 1), corresponding to other cases reported in Thailand [2–4]. Although all of them were asymptomatic, seven had abnormal Hb level (Table 2). Two of them (case A and F) had known contributing factors, whereas the other five might be caused by the following conditions: concomitant iron deficiency in three female cases (cases B–D) with low Hb, and pre-analytical error in two cases (cases E and G) with high Hb. The cause of mild-to-moderate anaemia in three females is likely due to concomitant iron deficiency, as this condition is quite common in Malaysia [22, 23]. The pre-analytical error might occur when the EDTA tube is overfilled with patient's blood sample. It can lead to inadequate sample mixing and cause inaccurate measurement of Hb parameter by an automated analyser which results in pseudopolycythaemia. This cause is quite common, especially when the Hb level is slightly higher than normal.

The other haematological indices of the variants in this study were mostly ($n = 33/38$, 86.8%) like thalassaemia trait; and this is in agreement with some cases found in Thailand [3, 4]. As expected, the Hb G-Makassar individuals who had another defect in *HBB* allele had lower MCV and MCH values than Hb G-Makassar carrier who had another unaffected *HBB* allele. These characteristics were seen in two genotype groups: compound heterozygous Hb G-Makassar/Hb E and compound heterozygous Hb G-Makassar/Hb New York (NG_000007.3:g.[70614A > C];[71915T > A]). However, only the MCV value was proven to be significantly different between heterozygous Hb G-Makassar and compound heterozygous Hb G-Makassar/Hb E, with mean ± SD of 76.18 ± 6.02 fL and 67.9 ± 6.03 pg respectively. Lower indices are suspected if the Hb G-Makassar individuals are also acquired β^+ and β^0 genotype, as seen in one case reported by Viprakasit et al. who acquired an IVS-1-1 (G > T) deletion, a β^0 genotype [3]. We found minority ($n = 5/38$, 13.2%) of those with heterozygous Hb G-Makassar genotype showed silent features of β-thalassaemia with normal mean ± SD for MCV and MCH (83.92 ± 3.29 fL and 28.52 ± 1.10 pg respectively) which is consistent with other study who had the same genotype [3]. The normochromic normocytic indices were also observed in the homozygous state [2], but more cases are needed to confirm this finding.

It is not uncommon to find different Hb variants co-elute at the same position on a certain method of Hb analyses. Therefore, it is always necessary to perform more than one method to solve the problem. The most common example in our population is Hb E and Hb Lepore, which are co-eluted with Hb A_2 in HPLC, producing a false high percentage value of Hb A_2; but in CE, they are separated in different zone [24]. In this study, the Hb New York was eluted in Hb A area in HPLC but clearly appeared in a different zone in CE. The same issue is also encountered in Hb electrophoresis; some haemoglobins migrate at the same rate in alkaline phase but separate in acid phase. Even in CE, we may see multiple variant-peaks appear in the same zone, but those variants can be differentiated in other methods [25]. The strategy of performing at least two different methods, however, is not constantly the solution, especially if it is a rare Hb variant. In this study, all screening methods consistently showed identical findings of Hb S. But surprisingly, the ARMS-PCR for Hb S was negative on repeated procedure. It was rather challenging at first because some quality control measures were taken to rule out the possibility of false result. DNA sequencing analysis of *HBB* gene had somehow overcome the matter when we found it was Hb G-Makassar.

The percentage of Hb G-Makassar measured by HPLC in heterozygote state was in concordance to a study done by Viprakasit et al., ranging from 39% to 41%. There was a slight increase (48.60%) when it interacted with Hb New York, a β-variant Hb known to be phenotypically mild. The percentage increased significantly (63.20%) when it interacted with Hb E and increased further (84.00%) with no Hb A fraction at all when it interacted with β^0-thalassaemia (IVS 1-1 G > T) [3]. The highest (93.20%) percentage of Hb G-Makassar was reported to occur in a homozygous state, found in a 27-year-old pregnant Thai woman [2]. In our study, we found the percentage of Hb subtypes measured by CE and HPLC were statistically not significant between heterozygous Hb G-Makassar and heterozygous Hb G-Makassar co-inherited with α^+-thalassaemia.

The mean Hb A_2 percentage by HPLC in heterozygous Hb G-Makassar either with/without deletional α-thalassaemia co-inheritance in our study was higher (3.37%–3.59%) than normal control (2.88%–3.20%). This finding is in agreement with another study, although ours was lower than theirs (3.70%–4.70%) [3]. Similar observation is found in heterozygous Hb S with/without co-inheritance of alpha thalassaemia. It is believed that the elevation of the Hb A_2 value occurs when the affinity of β^S-chain towards α-chain is reduced; therefore, more α-chains are available to combine with δ-chains forming Hb A_2 [26]. The same concept was applied to Hb G-Makassar.

5 | CONCLUSION

This is the largest study reporting a significant number of individuals with Hb G-Makassar in Malaysia. Our cases might have the same ancestry as those in Thailand, and the findings suggest that this variant is prevalent in Malay people. The haematological characteristics and

gene mutation co-ordinate of the variant is in common with Hb S. However, the clinical phenotype is absolutely different. Hb G-Makassar is a benign Hb variant, in contrary to Hb S.

AUTHOR CONTRIBUTIONS

Faidatul Syazlin Abdul Hamid, Roszymah Hamzah and Ahmad Sabry Mohamad collected the data. Ezalia Esa, Roszymah Hamzah, Guo Chen and Ahmad Sabry Mohamad wrote the manuscript. Ezalia Esa, Roszymah Hamzah, Ahmad Sabry Mohamad, Norafiza Mohd Yasin, Nur Aisyah Aziz, Veena Sevaratnam and Guo Chen reviewed and revised the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We express our appreciation to Director General of Health, Ministry of Health, Malaysia for his permission to publish this article. We thank the Deputy Director General of Health (Research and Technical Support, Ministry of Health) and Director of Institute for Medical Research (IMR) Malaysia for their continuous supports. The authors are grateful to Dr. Zaihawa Hamad from Department of Pathology, Putrajaya Hospital, Putrajaya, Dr. Norhafizatul Aida Amir Khalid from Medical Department, Hospital Sultanah Nur Zahirah Terengganu, Dr. Tan Sen Mui, Head of Haematology Department, Ampang Hospital and Dr. Mohammad Azannee Saad from International Islamic University Malaysia. We also thank all patients and staff from various states for the support and for participating in this study.

CONFLICT OF INTEREST STATEMENT

Guo Chen is an employee and stockholder of Beam Therapeutics Inc.

FUNDING INFORMATION

This study was funded by Beam Therapeutics Inc.

PATIENT CONSENT STATEMENT

Written informed consent for clinical information and molecular genotyping was obtained from the cases prior to blood taking. The collected data were entirely kept anonymous, and confidentiality was maintained through the suitable use of coding.

CLINICAL TRIAL REGISTRATION (INCLUDING TRIAL NUMBER)

The authors have confirmed that clinical trial registration is not needed for this submission.

DATA AVAILABILITY STATEMENT

The data used for this research are available upon request made to the corresponding author with reasonable reason. The data are not publicly available due to privacy or ethical restrictions.

ETHICS STATEMENT

This study was conducted according to the Declaration of Helsinki and approved by Medical Research and Ethics Committee, Ministry of Health (NMRR-21-1408-60675, IIR), the regional ethical board in Malaysia.

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How to cite this article: Esa E, Mohamad AS, Hamzah R, Hamid FSA, Aziz NA, Sevaratnam V, et al. Clinical and haematological characteristics of 38 individuals with Hb G-Makassar in Malaysia. eJHaem. 2023;4:940–948. https://doi.org/10.1002/jha2.750