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Molecular Characterisation of Alpha Thalassaemia Diagnosed in Hospital Kuala Lumpur (HKL) with Comparison to Molecular Genetic of Alpha Thalassaemia in Southeast Asia

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Abstract

Alpha thalassaemia is the commonest inherited disorder of haemoglobin (Hb) synthesis. High performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are incapable to diagnose the carrier thus require molecular analysis. We analysed alpha thalassaemia cases referred for molecular analysis in Hospital Kuala Lumpur (HKL) and compare with the alpha thalassaemia genotypes in Southeast Asia. 5100 cases received by HKL for five months duration in year 2015 for alpha thalassemia molecular testing were analysed. Molecular diagnosis were done using Multiplex GAP and ARMS PCR, and 160 samples (MCH < 26 pg, normal finding by Multiplex PCR) were selected for MLPA analysis. The alpha thalassaemia genotypes in Southeast Asia review done by searched on the electronic databases from January 2010 to March 2021 using the terms based on the Medical Subject Headings (MeSH) related to molecular genetic of alpha thalassaemia in Southeast Asia. Molecular analysis confirmed alpha thalassemia in 2586 cases. Most of the cases were Malays (80.24%), Chinese (11.3%), Sabah & Sarawak Ethnics (3.4%) and Indians (2%). The alpha thalassaemia genotype were αα/-α3.7 (41%), αα/-SEA (26%), αα/ααCS (13.1%), -α3.7/-α3.7 (4.6%), -α3.7/-SEA (3.3%), αα/-α4.2 (3%), αα/ααCD59 (2.2%), -α3.7/ααCS (1.3%) and others (<1%). The alpha thalassaemia genotypes in Southeast Asia showed similar findings of α+-thalassaemia (-α3.7) and non-deletional α-thalassaemia (Hb Constant Spring) were the most prevalent form. Our study showed diverse range of alpha thalassaemia gene abnormalities similar with the Southeast Asia population. Therefore, regular update of the molecular genetics data and development of effective diagnostic strategies are obligatory anticipating future diagnostic difficulty.

Keywords: Alpha thalassaemia, Malaysia, Southeast Asian, molecular genetics analysis, multiplex gap PCR, MLPA

Introduction

Alpha (α) thalassaemia is the commonest worldwide inherited disorder of haemoglobin (Hb) which frequently found in Southeast Asia, Mediterranean and Middle East populations (Azma et al., 2014). Among the South-East Asia countries, Vietnam have the highest prevalence rate of alpha thalassaemia (51.5%), followed by Cambodia (39.5%), Laos, Malaysia and Thailand with almost similar prevalence ranging from 17.3% to 26.8% (Goh et al., 2020). It is one of the hereditary disease that can cause significant morbidity, mortality and financial burden to the affected countries, especially the low socioeconomic countries. In Malaysia, alpha thalassaemia is one of the public health concerns which can be effectively prevented to reduce the disease prevalence and burden.

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The alpha thalassaemia is characterized by deletion or mutation of the α-globin genes which resulting in reduction or total absence of the α-globin chains. Therefore, the resultant phenotype of a person affected by this inherited disease is diverse and depends on the number of deleted or mutated genes along with the type of deletion or mutation (Rosnah et al., 2012). The remaining number of functional α-globin gene inherited will determined the clinical syndromes of the patient. The most common forms of alpha thalassaemia are deletion of one or both α-globin genes which are expressed as (-α//αα) and (--/αα) respectively. The most severe form which is known incompatible to extra-uterine life is haemoglobin (Hb) Bart's hydrops foetalis, also known as α thalassaemia major (--/--). Thalassaemia intermedia, is an inheritance of a single functional α-globin gene resulting in Hb H disease (--/-α), as the result of the compound heterozygous state of $α^{°}$ thalassaemia with $α^{+}$ thalassaemia which give rise to the variable spectrum of chronic haemolytic anaemia. The cis or trans positional loss of two α genes will results in α thalassaemia minor or trait. Three functional α-globin genes will cause silent α thalassaemia carriers, asymptomatic or will manifests as mild hematological indices changes (Ahmad et al., 2013).

On the other hand, the non-deletional defects (point mutation) usually producing the α-globin chain variants of non-clinical significance unless if it involves critically positioned amino-acid residues which ultimately generate the highly unstable haemoglobin's which can give rise to the symptomatic alpha thalassaemia syndrome of variable severity (Ezalia, Tan et al., 2014). Alpha thalassaemia due to point mutations in either α2 ($α^{T}α$) or α1 ($αα^{T}$) gene is common in Southeast Asia compared to the rest of the world. Mutations of the α2 gene is more common compared to the α1 gene and affected patient usually showed higher degree of anaemia compared to mutation in α1 gene. This is due to production of α-globin genes mRNA in α2 gene is 2–3 times more than α1 gene (Alauddin et al., 2014).

In Malaysia which is a multi-ethnic country, the molecular pathology of alpha thalassaemia are heterogenous and common molecular defects that associated with the major ethnic in Malaysia have been identified as --^{SEA}/aa, -$a^{3.7}$/aa, -$a^{3.7}$/--^{SEA}, -$a^{3.7}$/- $a^{3.7}$ and aa/-$a^{4.2}$. There are the commonest alpha thalassaemia mutations, especially among Malays and Chinese. Among these mutations, (---^{SEA}) deletion is of concern as it can result in Hb Bart's hydrops fetalis whenever inherited in the homozygous state while compound heterozygosity, -$a^{3.7}$ or -$a^{4.2}$ with –^{SEA} deletion will cause HbH disease (Lee et al., 2016).

The laboratory diagnosis of alpha thalassemia requires various of tests including red blood cell indices, Hb and DNA analyses (Munkongdee et al., 2020). However, there are some limitations of the established method such as inability to detect individuals with normal or borderline red blood cell indices (Yilmaz et al., 2021). Automated haematology analyzer is usually used as a primary screening for thalassaemia as it detects the microcytosis and low Hb level of the red blood cells which are the usual hallmarks of thalassaemia syndrome but cannot discriminate the different types of thalassaemia and its clinical severity. Hb analysis such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) system are able to detect beta thalassaemia and some of haemoglobin variants. However for alpha thalassaemia, Hb analysis is able to detect the severe forms of such as Hb H, Hb Barts and some of alpha thalassaemia variant but not mild forms of α-thalassaemia, where production of β-globin chains (Hb H molecule) is not excess enough to be detected by the analyzer. Ultimately, DNA analysis offers a definite diagnosis for α-thalassaemia. Common conventional DNA analysis techniques such Gap-polymerase chain reaction (PCR), allele-specific PCR, reverse dot blot (RDB) analysis, real-time PCR with melting curve analysis, multiplex ligation-dependent probe amplification (MLPA), Sanger sequencing and NGS can be use to look for alpha gene abnormalities thus giving the definitive diagnoses but all with some limitations (Jiang et al., 2022). Multiplex GAP-PCR method with melting curve analysis developed for α-thalassaemia genotyping which the primes are able to specifically amplify two deletion fragments, -^{SEA} and -^{THAI} deletions and two normal other normal fragments Ψζ- and α2-globin gene. This method allows differentiation α-thalassaemia 1 heterozygotes, α-thalassaemia 2 homozygotes, Hb H disease, and α-thalassaemia 1 homozygote (Hb Bart's hydrops fetalis) (Munkongdee et al., 2010). Multiplex ligation-dependent probe amplification (MLPA) is frequently used to detect rare deletions or duplications in α- or β- globin genes although accurate information of positions for unknown deletions or duplication cannot be provided and certain unknown mutations is unable to be detected. The advantage is, it is easy to use and only requires a thermocycler and CE equipment (Lei et al., 2019). The determination of thalassaemia genotype also become difficult whenever deletions and duplications coexists (Chen et al., 2020; Xu et al., 2020). The next-generation sequencing (NGS) is one of recent technique used although it is sometimes inaccurate in repeat regions due to high homology sequence between HBA1 and HBA2, short read length and PCR amplification during library preparation and actual sequencing reaction (Guan & Sung, 2016). The latest advancement of next-generation sequencing is the third-generation sequencing, the single-molecule real-time (SMRT) (Ardui et al., 2018; Xu et al., 2020). However, more loci such as genetic modifiers that have significant effects on clinical manifestation should be included in the NGS screening making it more precise in diagnosing thalassaemia thus allowing appropriately right treatment. Other limitations are expensive cost and limited technical expertise. Therefore, more studies should be conducted to verify this technique.

In this study, we evaluated alpha thalassaemia genotypes in cases referred for molecular analysis in Hospital Kuala Lumpur and compare with the alpha thalassaemia genotypes in Southeast Asia, focusing on the

molecular analysis by Multiplex Gap PCR and Multiplex ligation-dependent probe amplification (MLPA) methods.

Material And Methods

A total of 5100 cases were referred to Haematology unit, Pathology Department, Hospital Kuala Lumpur from all over countries of Malaysia within 5 months duration in year 2015 for the alpha thalassemia diagnosis. All haematological data such as red cell indices, Hb quantification and frequency of different types of alpha gene abnormalities were traced from Laboratory Information System (LIS). The haemoglobin and red cell indices was determined using automated blood cell counter (Sysmex XN1500). The quantitation of Hb A, Hb A2 and Hb F were performed using Sebia Capillary Electrophoresis and Bio-Rad Variant Beta II Short Program HPLC. All cases were subjected for molecular diagnosis using Multiplex GAP and ARMS PCR to detect deletional alpha (α20.5, α3.7, SEA, THAI, FIL, MED, α4.2) and non-deletional alpha (initiation Cd ATG>A-G, Codon 35, Codon 30, Adana, Quang Sze, Constant Spring). A total of 160 negative samples with ARMS and GAP analysis and MCH < 27 pg were selected and analysed with MLPA.

The alpha thalassaemia genotypes in Southeast Asia search was conducted using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (Page et al., 2021) Statement guidelines of quantitative and qualitative studies. Three electronic databases, Scopus, ScienceDirect, and PubMed were searched for the relevant studies from January 2010 to March 2021. The search terms such as 'alpha thalassaemia', 'Malaysia', 'Southeast Asia', 'molecular genetics', 'multiplex gap PCR', and 'MLPA' used were according to the Medical Subject Headings (MeSH) terminology and related to the molecular genetic abnormalities of alpha thalassaemia in Southeast Asia combined with search functions and Boolean operators in the electronic databases. Research questions were formulated based on the Population, Intervention, Comparison, Outcome (PICO) criteria, a tool that assists in the development of relevant research questions for the review. Based on these concepts, four main aspects were included in the review, namely Southeast Asian (Population), DNA analysis of alpha thalassaemia using GAP PCR, ARMS PCR and MLPA (Intervention), different regions of Southeast Asia (Comparison) and alpha genotyping abnormalities which were distinctively according to the Southeast Asia regions (Outcome) which then guide the authors to formulate its main research question – What is the molecular spectrum of alpha thalassaemia in the Southeast Asian population? All studies that met the PICO criteria and were published between January 2010 to March 2021 were included for review, while articles that were not original research such as (e.g., drafts, case studies, conference posters or abstracts) were excluded from the synthesis. This review was restricted to articles published in the English language. Figure I illustrates the identification process of studies collected and screened.

Result

For a period of 5 months in 2015, molecular analysis confirmed the diagnosis of alpha thalassemia in 2586 cases from 5100 cases received by Hospital Kuala Lumpur. Most of the positive cases were Malays (80.24%) followed by Chinese (11.3%), Sabah & Sarawak Ethnics (3.4%) and Indians (2%). While small positivity was detected among others ethnicity (2%) and Orang Asli (1.1%). The percentage of distribution of alpha thalassaemia genotype are majority of αα/-α3.7 (41%), αα/-SEA (26%), αα/ααCS (13.1%), -α3.7/-α3.7 (4.6%), -α3.7/-SEA (3.3%) and αα/-α4.2 (3%). While percentage of other uncommon genotype were αα/ααCD59 (2.2%), -α3.7/ααCS (1.3%) and others (each less than 1%) which were, αα/-THAI, αα/-FIL, αα/ααCD125, αα/ααCD30, -α3.7/-α4.2, -α4.2/-α4.2, -α3.7/ααCD59, -α3.7/ααCD125, -α3.7/-FIL, -α3.7/THAI, -α4.2/SEA, -α4.2/ααCS, -α4.2/ααCD59, ααCD125/-SEA, ααCS/-THAI, αα/-SEA, ααCS/αα125, ααCS/ααCD59, and ααCS/ααCS) **(Table I)**. Remaining 160 cases with abnormal red cell indices (MCH < 27 pg) but showed normal findings by multiplex PCR were subjected for MLPA analysis. Analysis by MLPA detected positivity for alpha genotype abnormalities in 4 out of 160 cases. They were 1 case each of single alpha gene deletion -3.7, double alpha gene deletion FIL, double alpha gene deletion SEA and HBZ. While 139 cases were reported as normal finding and 17 cases were inconclusive. Haematological parameters were also analysed in relation to the alpha genotypic abnormalities **(Table II)**. Cases which were known to have underlying iron deficiency, chronic medical illness, or concomitant with other thalassemia and haemoglobinophaties were exclude from this analysis.

The alpha thalassaemia genotypes article review in Southeast Asia yielded in total, 114 articles from electronic databases. We removed 64 articles, including non-published articles, those published before January 2010, published with no full assess available, non-english, and did not meet the criteria by browsing the titles and abstracts. This includes another 10 duplicates, which then came to 40 full-text articles eligible for review. Further 35 articles exclusion was made on those which do not focus on the six main keywords used for searching the database. Finally, 5 full-test studies were included and analysed in this review. A data extraction done based on the research question with no further validation process. Findings revealed that the highest alpha thalassemia prevalence was observed in Vietnam (51.5%) followed by Cambodia (39.5%), Laos (26.8%), Thailand (20.1%), and Malaysia (17.3%) (Goh et al. 2020). All the five articles reported that α+-thalassaemia

(predominantly -α3.7) was the most prevalent form in Southeast Asia. It showed high allele frequencies of 26% in Cambodia, 16.8% in Sabah (Malaysian Borneo) and up to 14.4% in Vietnam. The highest allele frequencies of α^0-thalassaemia was detected in Thailand, predominantly SEA deletion (--SEA), with a prevalence of 4.46% (Hockham et al., 2019). The non-deletional α-thalassaemia, predominantly Hb Constant Spring, is the commonest molecular abnormality in central Peninsular Malaysia with a frequency of 16.25%. It was also detected in high frequencies in Cambodia and Vietnam with a percentage of 8% and 14.3% respectively. The -α3.7 and -α4.2 were found at similar frequencies in Java, Indonesia (Husna & Handayani, 2021). These results are summarized in the **(Table III)**.

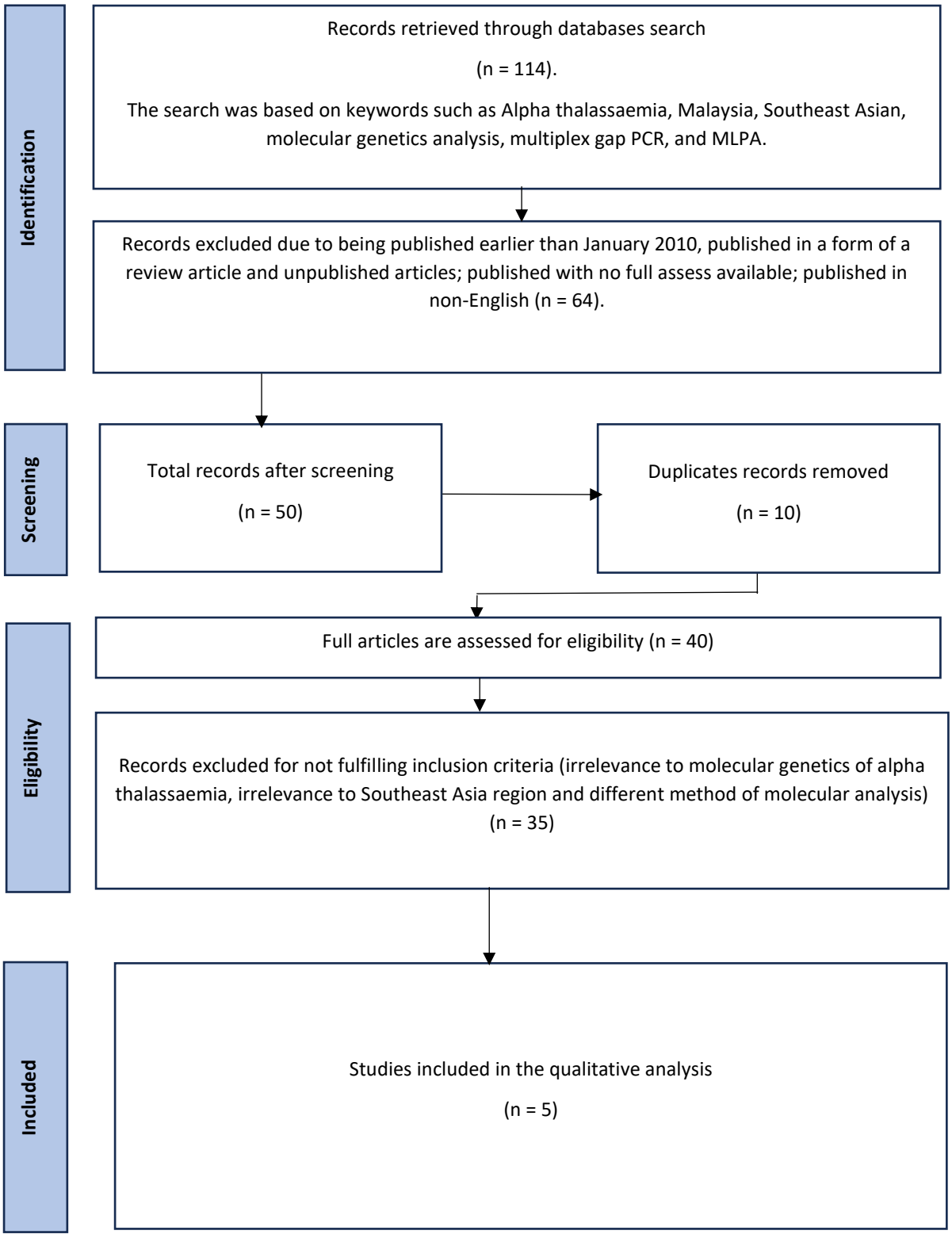

Figure I. Flow diagram of the relevant article search for qualitative synthesis.

Table I. Distribution of incidence of alpha thalassaemia in different ethnic groups

Genotype	Malay	Chinese	Indian	Sabah & Sarawak Ethnics	Orang Asli	Others	Total
αα/-$\alpha^{3.7}$	921 (86.9%)	30 (2.8%)	37 (3.5%)	43 (4.1%)	7 (0.7%)	21 (2%)	1059 (41.0%)
αα/-$\alpha^{4.2}$	67 (85.9%)	5 (6.4%)	3 (3.8%)			3 (3.9%)	78 (3.0%)
αα/--^{SEA}	448 (64.5%)	213 (3.1%)		10 (1.4%)	7 (1.0%)	10 (1.4%)	695 (26.9%)
αα/--^{THAI}	14 (93.3%)	1 (6.7%)					15 (0.6%)
αα/--^{FIL}	8 (40.0%)	1 (5.0%)	1 (5.0%)	9 (45.0%)		1 (5.0%)	20 (0.8%)
αα/αα^{CS}	319 (94.1%)	7 (2.1%)		3 (0.9%)	3 (0.9%)	7 (2.1%)	339 (13.1%)
αα/αα^{CD125}	5 (45.5%)	6 (54.5%)					11 (0.4%)
αα/αα^{CD30}	1 (100.0%)						1(0.0%)
αα/αα^{CD59}	51 (91.1%)				5 (8.9%)		56 (2.2%)
-$\alpha^{3.7}$/-$\alpha^{3.7}$	83 (69.7%)	1 (0.8 %)	9 (7.6%)	21 (17.6%)	1 (0.8%)	4 (3.4%)	119 (4.6%)
-$\alpha^{3.7}$/-$\alpha^{4.2}$	12 (92.3%)		1 (7.7%)				1 (0.5%)
-$\alpha^{4.2}$/-$\alpha^{4.2}$	1 (100%)						1 (0.0%)
-$\alpha^{3.7}$/αα^{CD59}	11 (100%)						11 (0.4%)
-$\alpha^{3.7}$/αα^{CD125}	2 (66.7%)	1 (33.3%)					3 (0.1%)
-$\alpha^{3.7}$/αα^{CS}	34 (100%)						34 (1.3%)
-$\alpha^{3.7}$/--^{FIL}	3 (75.0%)					1 (25.0%)	4 (0.2%)
-$\alpha^{3.7}$/--^{SEA}	64 (75.3%)	15 (17.6%)	1 (1.2%)		2 (2.4%)	3 (3.6%)	85 (3.3%)
-$\alpha^{3.7}$/--^{THAI}	2 (100.0%)						2 (0.1%)
-$\alpha^{4.2}$/--^{SEA}	5 (62.5%)	3 (37.5%)					8 (0.3%)
-$\alpha^{4.2}$/αα^{CS}	4 (100.0%)						4 (0.2%)
-$\alpha^{4.2}$/αα^{CD59}	2 (100.0%)						2 (0.1%)
αα^{CD125}/--^{SEA}		2(100.0%)					2 (0.1%)
αα^{CD59}/--^{SEA}		1 (100.0%)					1 (0.0%)
αα^{CS}/--^{THAI}	1 (100.0%)						1 (0.0%)
αα^{CS}/--^{SEA}	15 (83.3%)	2(11.1%)				1 (5.6%)	18 (0.7%)
αα^{CS}/αα^{CD125}	1 (100.0%)						1 (0.0%)
αα^{CS}/αα^{CD59}	2 (100.0%)						2 (0.1%)
αα^{CS}/αα^{CS}	1 (100.0%)						1 (0.0%)
Total	2074 (80.2%)	291 (11.3%)	51 (2.0%)	87 (3.4%)	27 (1.1)	53 (2.0%)	2586

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Table II: HB and Red cell indices in alpha thalassaemia cases (Mean +/- SD)

	No of Patients	RBC (10^{12}/L)	Hb (g/dL)	HCT	MCV (fl)	MCH (pg)	MCHC
Slient Alpha Thalasemia							
αα/-α$^{3.7}$	1007	4.89 ± 0.62	11.91 ± 1.69	37.11 ± 5.78	76.74 ± 7.44	24.64 ± 3.29	31.78 ± 2.63
αα/-α$^{4.2}$	73	4.88 ± 0.62	11.95 ± 1.71	36.59 ± 5.78	76.82 ± 5.48	24.47 ± 2.13	31.74 ± 1.80
αα/αα^{CS}	310	4.74 ± 0.68	11.72 ± 3.81	36.65 ± 18.23	75.52 ± 7.65	24.32 ± 2.99	31.69 ± 2.91
αα/αα^{CD125}	11	5.47 ± 0.79	12.69 ± 1.93	36.33 ± 12.42	75.38 ± 5.36	24.30 ± 2.79	28.81 ± 851
αα/αα^{CD30}	1	4.52	11.00	32.00	72.60	24.30	33.50
αα/αα^{CD59}	48	4.99 ± 0.77	11.82 ± 1.53	35.90 ± 4.64	73.67 ± 6.51	23.64 ± 2.05	32.11 ± 1.29
Alpha Thalasemia trait							
αα/--^{SEA}	659	5.50 ± 0.79	11.32 ± 1.51	35.90 ± 5.09	65.99 ± 5.63	20.82 ± 2. 73	31.51 ± 11.31
αα/--^{THAI}	15	5.31 ± 0.81	10.54 ± 1.14	34.16 ± 3.91	65.13 ± 6.97	20.03 ± 1.68	31.76 ± 2.58
αα/--^{FIL}	20	5.50 ± 0.82	11.24 ± 1.43	35.20 ± 4.36	64.37 ± 5.51	20.52 ± 1.46	31.95 ± 1.64
-α$^{3.7}$/-α$^{3.7}$	107	5.18 ± 0.85	11.71 ± 5.72	35.58 ± 4.88	70.44 ± 5.24	22.13 ± 2.63	31.19 ± 2.17
-α$^{3.7}$/-α$^{4.2}$	13	4.84 ± 0.61	10.41 ± 2.06	34.18 ± 2.80	70.47 ± 4.83	22.43 ± 1.83	31.50 ± 4.88
-α$^{4.2}$/-α	1	4.40	10.00	32.60	73.60	23.50	31.90
-α$^{3.7}$/αα^{CD59}	11	4.01 ± 0.75	8.72 ± 1.11	28.42 ± 3.51	71.84 ± 6.74	22.03 ± 1.89	30.68 ± 0.81
-α$^{3.7}$/αα^{CD12}	3	4.92 ± 0.47	10.43 ± 0.60	31.90 ± 0.42	65.97 ± 4.90	20.73 ± 1.85	22.43 ± 16.24
-α$^{3.7}$/αα^{CS}	29	4.96 ± 0.87	10.59 ± 1.89	32.88 ± 5.71	70.79 ± 6.07	21.98 ± 1.87	30.50 ± 2.40
-α$^{4.2}$/αα^{CS}	4	4.87 ± 0.75	10.20 ± 0.73	33.48 ± 2.81	69.25 ± 5.22	21.18 ± 1.94	30.55 ± 0.87
-α$^{4.2}$/αα^{CD59}	2	4.37 ± 0.04	9.45 ± 0.77	31.30 ± 3.11	71.40 ± 8.06	21.50 ± 1.97	30.15 ± 0.63
αα^{CS}/αα^{CD125}	1	4.86	9.6	32	66	20	29.9
αα^{CS}/αα^{CD59}	2	3.67 ± 1.27	8.00 ± 1.56	28.30 ± 2.69	80.70 ± 20.64	22.40 ± 3.53	28.10 ± 2.82
αα^{CS}/αα^{CS}	1	4.85	10.30	30.10	62.10	21.20	34.20
Hb H Disease							
-α$^{3.7}$/--^{FIL}	4	5.54 ± 0.01	9.15 ± 0.07	30.55 ± 1.6	55.10 ± 3.11	16.50 ± 0.14	30.00 ± 1.41
-α$^{3.7}$/--^{SEA}	77	5.28 ± 0.83	9.98 ± 8.11	30.84 ± 4.14	58.9 ± 4.15	17.24 ± 1.923	30.13 ± 5.90
-α3.7/--^{THAI}	2	5.54 ± 0.014	9.15 ± 0.07	30.55 ± 1.62	55.10 ± 3.11	16.50 ± 0.14	30.00 ± 1.41
-α$^{4.2}$/--^{SEA}	6	5.71 ± 0.68	9.68 ± 0.90	33.05 ± 4.39	58.28 ± 7.65	20.68 ± 8.02	29.52 ± 2.51
αα^{CD125}/--^{SEA}	2	3.56 ± 2.21	7.40 ± 4.24	26.05 ± 15.62	73.15 ± 1.20	21.15 ± 1.20	29.00 ± 1.27
αα^{CD59}/--^{SEA}	1	2.90	6.00	23.70	71.70	20.70	25.30
αα^{CS}/--^{THAI}	1	3.84	8.60	27.50	71.60	22.40	31.30
αα^{CS}/--^{SEA}	16	4.32 ± 0.72	8.51 ± 1.14	31.28 ± 4.79	73.17 ± 7.84	20.14 ± 2.01	27.65 ± 3.24

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Table III. Summary of studies included in the review of alpha thalassaemia genotypes in Southeast Asia

	Author	Journal and year of publication	Title	Sample size	Study design	Finding summary
1.	Ahmad et al.	International Journal of Molecular Sciences (2013)	Distribution of Alpha Thalassaemia Gene Variants in Diverse Ethnic Populations in Malaysia: Data from the Institute for Medical Research	5016 patients referred from various hospitals to the Institute for Medical Research for α thalassaemia screening from 2007 to 2010	Retrospective study	High incidence of α–3.7 deletion observed in Malays, Indians, Sabahans, Sarawakians and Orang Asli people. The ––SEA deletion was the most common cause of alpha thalassaemia in Chinese. Statistical analysis showed a significant difference in the distribution of α thalassaemia determinants amongst the various ethnic groups.
2.	Azma et al.	Malaysian Journal of Pathology (2014)	Molecular characteristic of alpha thalassaemia among patients diagnosed in UKM Medical Centre	1623 cases referred to laboratory in Universiti Kebangsaan Malaysia Medical Centre (UKMMC) for the diagnosis of α-thalassaemia from October 2001 to December 2012	Retrospective study	The most common gene abnormality was αα/--SEA (64.0%) followed by αα/-α3.7 (19.8%), -α3.7 /--SEA (6.9%), αα/ααCS (3.0%), --SEA/--SEA (1.2%), -α3.7/-α3.7 (1.1%), This data indicates that the molecular abnormalities of α-thalassaemia in the Malaysian population is heterogenous.
3.	Hockham et al.	eLIFE Research Communication (2019)	Estimating the burden of a-thalassaemia in Thailand using a comprehensive prevalence database for Southeast Asia	Compilation a geodatabase of a-thalassaemia prevalence and genetic diversity surveys. Using geostatistical modelling methods to generate the continuous maps of a-thalassaemia mutations in Thailand and sub-national estimates of the number of newborns with severe forms in 2020	Retrospective and prospective study	Estimated 3595 (95% credible interval 1,717–6,199) newborns will be born with severe a-thalassaemia in Thailand in 2020, which is considerably higher than previous estimates.
4.	Goh et al.	International Journal of Environmental Research and Public Health (2020)	Prevalence of Alpha (α)-Thalassemia in Southeast Asia (2010–2020): A Meta-Analysis Involving 83,674 Subjects	Twenty-nine studies with 83.674 subjects to provide an update from year 2010 to 2020 on the prevalence of-thalassemia in Southeast Asia	Meta-analysis study	Highest-thalassemia prevalence was observed in Vietnam (51.5%) followed by Cambodia (39.5%), Laos (26.8%), Thailand (20.1%), and Malaysia (17.3%).
5.	Husna et al.	Reports of Biochemistry & Molecular Biology (2021)	Molecular and Haematological Characteristics of alpha-thalassemia carrier screening	Blood samples from 173 healthy volunteers from	Prospective study	17 (9.8%) of the volunteers were confirmed to have alpha thalassemia trait with four genotypes were identified namely –

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	Thalassemia Deletions in Yogyakarta Special Region, Indonesia	in Yogyakarta Special Region were used for alpha thalassaemia analysis.	α3.7/αα (58.8%), –α4.2/αα (5.9%), –α3.7/–α4.2 (5.9%) and ––SEA/αα (29.4%).

Discussion

Alpha thalassaemia is an important public health problem in Malaysia. The prevalence of alpha thalassaemia is 17.3 % in the general population of Malaysia (Goh et al., 2020). It is an important cause of ineffective erythropoiesis with some sorts of chronic haemolytic anaemia leading to extramedullary hemopoiesis, foetal hydrops and transfusion dependent anaemia which caused high disease burden to the community and the country. The National Thalassaemia Screening Programme in Malaysia has started in year 2004 by premarital, antenatal and familial thalassaemia screening. However, these type of thalassaemia screening are not effective since Malaysia has high incidence of thalassaemia carriers (Azma et al., 2018; Ezalia, Irmi Elfina et al., 2014; Mohd Ibrahim et al., 2020). Thus, the programme have evolved rapidly, with the current guideline of the form four secondary school thalassaemia screening introduced in year 2016. This program was able to cover larger population and younger patients diagnosed early, allowing counselling given in more effective way in the prevention of the thalassaemia (National Thal Registry 2019). HKL is one of the tertiary hospital in Malaysia that received referral cases from all over Malaysia for the molecular diagnosis of alpha thalassaemia. Most of the samples received were anaemia cases with abnormal red cells parameters, family history or cascade screenings for thalassaemia and prenatal diagnosis of hydrops foetalis for couples with underlying alpha globin gene abnormalities. Requests for molecular analysis for alpha thalassaemia gene abnormalities were made after the Hb analysis showed no abnormalities for beta thalassaemia or cases that needed confirmation by molecular analysis after being diagnosed alpha thalassaemia by Hb analysis such as Hb Constant Spring (Azma et al., 2014).

Initial screening work up by looking at the red blood cells indices with hallmark features of hypochromic microcytic subsequently by haemoglobin analysis is quiet effective in detecting major α-globin gene mutation. Unfortunately, silent carrier of alpha thalassaemia with nearly normal haemoglobin level and red blood cells indices can be missed. In this analysis **(Table II)**, the carriers presented mild anaemia (Mean Hb < 12 g/dl), except for αα/ααCD125 (Hb 12.69 g/dl), with mildly low MCV (≤ 76 fl) and MCV (≤ 25 pg). Alpha thalassemia trait patients are typically asymptomatic, with mild to moderate microcytic hypochromic anaemia. Previous study have presented that α+ thalassemia carrier are generally normal both clinical and haematological especially α3.7 and non-deletion affecting the α1 gene. Such individuals may be normocytic or borderline hypochromic without anaemia (Harteveld & Higgs, 2010). In this study shows α0 heterozygous had a lower Hb (< 11.4 g/dl), MCV (< 67 fl) and (MCH < 21pg). While α+ heterozygous shows lower of Hb (< 11.7 g/dl), MCV (71 fl) and MCH (< 23) pg. MCH value spotted mild in compound Quang Sze and homozygous Constant Spring less than 66 fl and 62 fl respectively. Hb H disease is characterized by having one functional alpha gene lead to moderately severe microcytic hypochromic anaemia. Patients with non-deletional type of Hb H disease known to be severe affected than those with the deletion types (Harteveld & Higgs, 2010; Laosombat et al., 2009). For deletion type, Hb value approximately 9 g/dl, MCV and MCH markedly reduced 55-58 fl and 16-20 pg. Non-deletional type Hb value varies between 6.0-8.51 g/dl, MCV less than 73 fl, and MCH less than 21 pg.

This study conveyed more than half (n=2586, 50.71%) cases were diagnosed as alpha thalassemia, exactly 2074 Malays, 291 Chinese, 51 Indians, 87 Sabah & Sarawak Ethnics, 27 Orang Asli, and 53 others (including mixed marriage and immigrant). **Table I** showed the molecular characteristic of alpha genotypic abnormalities in cases with alpha thalassaemia. A total of 2051 cases (40.23%) was reported as αα/αα and 463 cases (9.06%) still remain undiagnosed due to limited panel. Malays showed the highest incidence of the alpha gene abnormalities, most predominant for αα/-α3.7 (89.6%), α/-α4.2 (85.9%), αα/αCSα (94.1%), αα/αCD59α (91.0%) and αα/--SEA (64.5%). αα/-α3.7 was known to have a global distribution among all ethnic groups, up to 30% of the population (Chow et al., 2013). It is known to be prevalent in most of the tropical and subtropical countries. Various studies showed a high incidence of αα/-α3.7 in Melanesia (70%), Cambodia (23.73%), Vietnam (9.64%), Indonesia (2.0%), and Filipino (1.6%) (Bui Thi Kim et al., 2016; Fucharoen et al., 2011; Setianingsih et al., 2003). While αα/-α4.2 was reported less frequently and mainly in Southeast Asia, specifically 2.4% in Vietnam and 0.8% in Cambodia (Jomoui et al., 2020; Munkongdee et al., 2016). In this study, αα/-α3.7 was observed in all ethnic group but αα/-α4.2 was only observed in major ethnic groups. Hb Constant Spring (CS) is the most common alpha globin structural variant in Southeast Asian countries, up to 8% were carriers (Vichinsky, 2009). It was reported about 47% incidence in Singapore, 26.2% in Vietnam and 5.03% in Cambodia (Bui Thi Kim et al., 2016; Lam et al., 2014; Munkongdee et al., 2016). Hb CS was also observed occur at low incidence in others ethnic group except Indian, no gene detected concordance with previous studies (Ahmad et al., 2013; Azma et al., 2014; Rahimah et al., 2012). Hb Adana was only observed in Malay, Orang Asli, and Chinese which is common in Mediterranean but in Southeast Asia, it was reported in Malaysia, Philippine, Singapore and Indonesia (Alauddin et al., 2014; Lam et al., 2014; Setianingsih et al., 2003). There were two highly rare variant was observed in this study which were Hb Quong Sze (QS) and Codon 30 (-GAG) mutation. Hb Quong Sze (QS) was reported in the Chinese, Thailand and Singapore populations (Huang et al., 2015; Lam et al., 2014; Laosombat et al., 2003; Yang et al., 2013; Yu et al., 2015). Codon 30 (-GAG) mutation was only described in Chinese population (Ma et al., 2001; Yang et al., 2013).

Majority of alpha thalassaemia trait of --SEA deletion will be followed with –α3.7/-α3.7, --FIL deletion and --THAI deletion. --SEA deletion was a common two gene deletion in Southeast Asia and often describe in Chinese (Azma et al., 2014; Huang et al., 2015; Ma et al., 2001; Rahimah et al., 2012; Yang et al., 2013; Yu et al., 2015) with prevalence of 87.35% in Vietnam (Bui Thi Kim et al., 2016), 8% in Indonesia (Setianingsih et al., 2003), 14% in Thailand (Chui et al., 2005), 1.53% in Cambodia (Munkongdee et al., 2016) and 3% in Filipino (Harteveld & Higgs, 2010). Previous study pertaining to –THAI deletion was found mainly in Thailand, minority in Malaysia and Vietnam (Ahmad et al., 2013; Ardui et al., 2018; Rahimah et al., 2012). While --FIL deletion more commonly seen in Filipinos (0.2 %) (Bui Thi Kim et al., 2016). It was also reported in Thailand, Vietnam and Malaysia (Ahmad et al., 2013; Ardui et al., 2018). Due to multi ethnicity country and mixed marriage, Codon 30 mutation was observed in Malay, Hb Adana in Chinese and -- SEA deletion in Indian (Guan et al., 2021). This factors contributed to the gene diversity and complexity in alpha thalassemia genotyping of Malaysian population.

The current gold standard technique of detecting alpha thalassaemia is by molecular analysis. Two common conventional molecular techniques are DNA analysis by Multiplex Gap PCR and Multiplex ligation-dependent probe amplification (MLPA) methods. Multiplex Gap PCR is currently used in Molecular Genetic Laboratory Unit, Hospital Kuala Lumpur for detecting deletions and point mutation type of α-thalassaemia. For deletion types, it consist of screening panel for -α3.7, -α4.2, - α20.5, --SEA, --MED, --Thai, and –Fil while for point mutation, it consist of screening panel for Constant spring, QuangSze, Adana, Codon 30 and Codon 35. Multiplex PCR amplification (MLPA) also used concurrently and able to show the presence of mutation in cases of missed detection of some uncommon deletional cases by Multiplex Gap PCR method. Thus, it is widely utilised as a secondary or further genotyping method for α-thalassemia when the common panel yields negative results (Phylipsen et al., 2010).

The complex compound types of α-globin chain mutation are expected, in view of various multi-ethnicity in Malaysia. Currently the data on molecular characteristics beyond the common alpha thalassaemia mutation is limitedly available and lacks of study on the type of genetic modifiers has been done in Malaysia. Therefore, our problem is a substantial proportion of clinically diagnosed thalassaemia syndrome cannot be diagnosed definitely in the laboratory because of absence of data on the complete gene abnormalities. Definitive diagnosis of inherited diseases is crucial for appropriate optimum management, genetic counselling and early preventive measures.

Conclusion

The multi ethnicity of the Malaysian population representing the Southeast Asian population, resulted in diverse alpha thalassaemia molecular genetics, which causing greater difficulty in the diagnosis of alpha thalassaemia in the future. Therefore, there is a need to regularly update the molecular genetics data as numerous interactions of alpha genotypes with a wide range of clinical phenotypes may be encountered. Anticipating the difficulty in the diagnosis of α-thalassaemia in the future, there is an urgency to develop simple and cost-effective strategies to improve the panel of detection for the diagnosis.

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Statement conflict of interest

The author declared that there was no conflict of interest.

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