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Concurrent Inheritance of Deletional α-thalassaemia in Malays with HbE Trait

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ABSTRACT

Introduction: HbE is the commonest beta haemoglobin (Hb) variant in Southeast Asia. It causes a reduction in synthesis of the beta-globin E (β^E) chain. Studies indicate HbE coinherited with α-thalassaemia leads to a milder clinical phenotype. This study investigates the concomitant inheritance of α-thalassaemia in Malays with HbE. **Methods:** Four hundred and fourteen (414) blood samples were screened for haemoglobinopathy using primarily the first 3 steps of the BHES [(B) blood counts, blood film; (H), HPLC; (E), electrophoresis; (S), stability)] protocol. Complete blood counts were generated on an automated blood cell analyser, Hb typing with cation exchange high-performance liquid chromatography (HPLC) and Hb electrophoresis at an alkaline pH (pH 8.5). Forty-five (10.9%) were identified as HbE trait and DNA analysis was done for deletional α-thalassaemia using a single-tube multiplex-PCR assay. **Results:** Among the 45 subjects with HbE trait, 4 (8.9%) were found to have alpha-thalassaemia-2 (α^+) (α -$^{3.7}$ kb deletion) and 1 (2.2%) the alpha-thalassaemia-1 (α^0) (—^{SEA} 20.5 kb deletion) defects respectively. **Discussion:** These findings show that 11.1% of Malays with HbE inherit alpha-thalassaemia concurrently. The most prevalent interaction found was a double heterozygote for HbE/α-thalassaemia 2, followed by HbE/α-thalassaemia 1. **Conclusion:** Molecular screening of deletional α-thalassemia identified its concurrent inheritance in 11.1% of Malays who were HbE carriers. This information will guide genetic counseling and the planning of treatment modalities in patients with HbE alpha-thalassaemia.

Keywords: α-thalassaemia, concurrent inheritance, HbE trait, Malays

INTRODUCTION

Genetic defects of haemoglobin synthesis (haemoglobinopathies) are the most common genetic disorders worldwide. It occurs mainly in tropical and subtropical areas.[1] HbE is the commonest beta-globin chain variant in Southeast Asia. The frequency of HbE approaches 60% in many regions of Thailand, Laos and Cambodia.[2,3,4,5] It is also found in Sri Lanka, North Eastern India, Bangladesh, Pakistan, Nepal and Vietnam. In Malaysia, micromapping studies indicate 4% of Malays are carriers of HbE.[6]

In HbE, an alternate splicing site found within exon 1 of the beta-globin gene in the primary mRNA transcript causes reduced production of the β^E-globin chain.[2,5]

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Heterozygous HbE (haemoglobin E trait or HbAE) has one normal β-globin gene and one variant haemoglobin E β gene.[5,7,8] Heterozygous HbE individuals have approximately 25-30% of HbE and are asymptomatic with no clinical relevance except for the risk of transmitting the gene to their offspring.[2,5,8] Most individuals with HbE trait have reduced mean corpuscular volume (MCV) and mean cell haemoglobin (MCH), with or without mild anaemia.[2,5,7,8]

α-thalassaemia is a common autosomal recessive disorder worldwide. α-thalassaemia can be caused by mutations within the α-globin gene complex or by deletion of either one, two, three or four globin genes, which can form various thalassaemia genotypes: carriers of α-thalassaemia (-α/αα, --/αα or –α/-α), haemoglobin H (HbH) disease (--/-α), or haemoglobin Bart's hydrops foetalis (--/--).[7,8,9]

Studies show that HbE can occur concurrently with either β- or α- thalassaemia. Variable clinical manifestations are presented depending on the type of thalassaemia gene involved.[4,9,11] HbE trait individuals with concurrent inheritance of α-thalassaemia have variable severity conditions in accordance to the number of non-functional α-globin genes involved.[12,13,14] HbE trait subjects with concomitant inheritance of α-thalassaemia often show a lower percentage of HbE and a milder clinical phenotype while co-inheritance of α-globin gene triplication will show more severe thalassaemia.[4,12,13] Accurate DNA testing must be carried out to identify the mutations involved for proper diagnosis and clinical management.[13]

In Malaysia, there has been no reported study to identify deletion α-thalassaemia in HbE even though the frequency of HbE and α-thalassaemia is high. The aim of this study was to identify concurrent inheritance of α-thalassaemia among Malays with HbE trait.

METHODS

Subjects and Haematological Analysis

A total of 414 blood samples, collected in ethylenediamine tetraacetic acid (EDTA) tubes and sent to the Institute of Medical Research (IMR) Kuala Lumpur, Malaysia for thalassaemia diagnosis, formed the study group. The first three steps of the BHES [(B) blood counts, blood film; (H), HPLC; (E), electrophoresis; (S), stability)] protocol was applied for presumptive identification of haemoglobinopathy. Complete blood counts (CBC) were determined using an automated blood cell analyser (CellDyn® 1800, Abbott Laboratories, USA). Haemoglobin subtypes and quantitation were performed by cation exchange high-performance liquid chromatography (HPLC) Hb analyser using variant β-thalassaemia short program (Bio-Rad Laboratories, Hercules, United States) which provides an accurate quantitation of haemoglobins A, A_2, F and other abnormal variants.[9] Alkaline electrophoresis was done at pH 8.5 to identify haemoglobin types (Sebia Hydrays, Sebia Inc., Georgia, USA).[9] Statistical comparison of data was done with non parametric Kruskal-Wallis test using the Statistical Package for the Social Sciences statistical software (SPSS, Chicago, IL, USA). *P*-value <0.05 was considered statistically significant.

DNA Analysis

Genomic DNA was extracted from leucocytes in peripheral whole blood samples using QIAamp DNA mini kit (Qiagen Ltd., West Sussex, UK). Quality of DNA was determined by

electrophoresis using 0.8% agarose gel (genomic D-1, LE) in 1 x tris-borate-EDTA (TBE) buffer at 10 volts/cm for 30 minutes, stained with ethidium bromide. DNA was quantitated using a spectrophotometer (Brand Biowave II, Company Biochem Ltd, England).

A previously published single-tube multiplex-PCR assay which is capable of detecting 7 common deletional determinants ($-\alpha^{3.7}$; $-\alpha^{4.2}$; $--^{SEA}$; $--^{THAI}$; $--^{MED}$; $--^{FIL}$; $-\alpha^{20.5}$) of α-thalassaemia was used in this study.[14,15] Different sets of primers were used to amplify the junction fragments of the α-thalassaemia determinants (Table 1). *LIS1 gene* 3'UTR fragment served as an amplification control.

PCR amplification was carried out in 50μl reaction containing 200μM of each dNTP, 1.5 mM $MgCl_2$, 1x Q-solution (Qiagen GmbH, Hilden, Germany), 2.5 U HotStart Taq DNA polymerase (Qiagen GmbH, Hilden, Germany), 100-200ng of genomic DNA, and 16 different primer pairs at various concentrations shown in Table 1. Cycling conditions were done using a thermalcycler (Mastercycler ep S, Eppendorf, Hamburg,Germany), with an initial denaturation for 15 minutes at 96°C, followed by 30 cycles of 98°C denaturation for 45 seconds, 60°C annealing for 1 minute 30 seconds, 72°C extension for 2 minutes 15 seconds and a final extension for 5 minutes at 72°C. Fourteen (14) μl of each amplified product was analysed using 1% gel electrophoresis in 1 x TBE buffer at 10 volts/ cm for an hour, stained in ethidium bromide, and visualised on an ultraviolet transilluminator.

RESULTS

Four hundred and fourteen (414) Malays were screened for HbE. This group included a patient who was Malay-Thai. Forty-five (10.9%) were identified as HbE carriers. On the BioRad Variant Hb analyser, HbE is presumptively identified as HbE when the HbA_2 level is greater than 10%. HbA_2 and HbE have the same retention time (RT) value in the chromatogram generated by HPLC. The normal HbA_2 is less than 4% in healthy adults. In this study, HbE subjects showed mean 'HbA_2' of 30.4% and HbF of 2.3% respectively. In HbE carriers, the mean corpuscular volume (MCV) was 72.9fL and mean corpuscular haemoglobin (MCH) was 24.4pg. The presence of HbE was further identified by Hb electrophoresis.

DNA was extracted from blood and tested for concurrent occurrence of α-thalassaemia using single-tube multiplex-PCR assay to detect 7 common deletional determinants ($-\alpha^{3.7}$; $-\alpha^{4.2}$; $--^{SEA}$; $--^{THAI}$; $--^{MED}$; $--^{FIL}$; $-\alpha^{20.5}$) (Figure 2). Amplification of deletional determinant with normal α_2-globin gene sequence showed heterozygous state while absence of normal α_2-globin gene amplification showed homozygous state. Appearance of one of the deletional determinants of α-thalassaemia involved in this study disturbs normal amplification of α_2 gene; therefore α_2-globin gene was used as a marker in this study to identify normal amplification of α-globin gene. (Figure 1)

Heterozygosity for the $-\alpha^{3.7}$ rightward single α-globin gene deletion ($-\alpha^{3.7}/\alpha\alpha$) was confirmed in 4 (8.9%) HbE carriers. Heterozygosity for Southeast Asian type of α^0- thal ($--^{SEA}/\alpha\alpha$) was found in the Malay-Thai who had microcytosis and hypochromia (MCV 63.8fL; MCH 21.2pg). There were 40 (88.9%) pure HbE carriers. Deletional determinants $-\alpha^{4.2}$; $--^{THAI}$; $--^{MED}$; $--^{FIL}$; $-\alpha^{20.5}$ were not observed.

Haematological phenotypes of heterozygous HbE with various forms of α-thalassaemia were listed and compared with those of pure HbE traits in Table 2. There were no significant

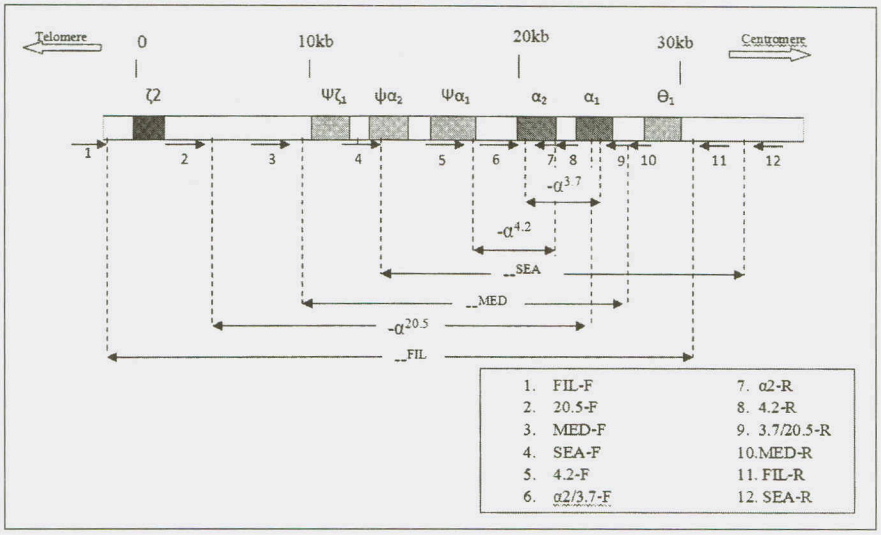

Figure1. Schematic diagram representing part of the α-globin gene clusters with the locations and orientations of amplification primers used in the single tube multiplex-PCR assay for detection of 7-deletional forms of alpha determinants

Table 1. Primer sequences for α-thalassemia multiplex PCR and expected amplicon sizes [15,16]

Primer	5'- 3' seq	Region	Amplicon size (bp)
LIS-F	ATACCATGGTTACCCCATTGAGC	LIS 3' UTR	2350bp
LIS-R	AGGGCTCATTACATGTGGACCC		
$\alpha_{2/3.7}$-F	CCCCTCGCCAAGTCCACCC	- $\alpha^{3.7}$	2022bp
3.7/20.5-R	AAAGCACTCTAGGGTCCAGCG		
$\alpha_{2/3.7}$-F	As above	α^2	1800bp
α_2-R	AGACCAGGAAGGGCCGGTG		
4.2 - F	GGTTTACCCATGTGGTGCCTC	- $\alpha^{4.2}$	1628bp
4.2 - R	CCCGTTGGATCTTCTCATTTCCC		
SEA - F	CGATCTGGGCTCTGTGTTCTC	--SEA	1349bp
SEA -R	AGCCCACGTTGTTGTTCATGGC		
THAI - F	GACCATTCCTCAGCGTGGGTG	--THAI	1153bp
THAI - R	CAAGTGGGCTGAGCCCTTGAG		
20.5 - F	GCCCAACATCCGGAGTACATG	- $\alpha^{20.5}$	1007bp
3.7/20.5-R	As above		
MED – F	TACCCTTTGCAAGCACACGTAC	-- MED	807bp
MED – R	TCAATCTCCGACAGCTCCGAC		
FIL – F	TTTAAATGGGCAAAACAGGCCAGG	--FIL	546bp
FIL – R	ATAACCTTTATCTGCCACATGTAGC		

Table 2. Haematological phenotypes of Hb E carriers with deletional alpha-thal

	1Hb E trait No α-thal	2Hb E trait α-thal 2	3Hb E trait α-thal 1
n	40	4	1
ethnicity	Malay (n=40);	Malay	Malay-Thai
age	5M-60 yr old	9M-34 yr old	20 yr old
α-genotype	αα/αα	αα/-$\alpha^{3.7}$	αα/--^{sea}
RBC (x10^{12} M/μl)	4.72[a]±.7279(2.49-6.53)	4.82±1.3047(3.17-6.14)	5.86
Hb (g/dL)	12.17[a]±4.5089(6.4-37.4)	12.20±3.7112(9.7-17.7)	12.4
MCV (fL)	73.65[a]±6.6700(50.7-84.9)	74.63±15.3691(53.7-89.5)	63.8
MCH (pg)	24.53[a]±2.2978(16.3-28.8)	25.80±5.2580(18.7-30.6)	21.2
MCHC (g/dL)	33.31[a]±1.2793(29.5-35.8)	34.58±0.5909(34.0-35.3)	33.2
RDW (%)	17.15[a]±3.6520(13.5-31.8)	17.43±3.2191(15.2-22.1)	17.7
Hb A_2 (%)	30.03[a]±14.1805(18.6-82.1)	23.88±4.3208(17.5-26.9)	20.1
Hb F (%)	2.45[a]±5.7861(0.00-35.10)	2.15±2.1810(0.30-4.90)	0.5

Data are presented as mean±SD and range.

($p< 0.05$) differences in Kruskal-Wallis test for all haematological parameters between group 1, 2 and 3.

DISCUSSION

HbE ($\alpha_2\beta_2^{26\ Glu->lys}$) is a haemoglobin variant commonly found in Southeast Asians. It is a β-globin chain defect which results from a single amino substitution of glutamic acid to lysine due to a point mutation (GAG to AAG) in codon 26 of the β-globin gene. The β^E chain is synthesised at a reduced rate compared with β^A due to activation of a cryptic mRNA splice site which interferes mRNA processing.[4,5,8,9,11] The β^E haemoglobin is mildly unstable, which contributes microcytosis (MCV less than 75fl) and hypochromia.[9]

The prevalence rate of α-thalassaemia genes in the Southeast Asian populations is estimated to be as high as 30%.[10] Alpha-thalassaemia shows a defect in the α-globin gene loci at 16p13.3 on chromosome 16. In alpha-thalassaemia, there is a quantitative defect of the α-globin chain synthesis. The alpha globin gene complex consists of four α-globin genes per individual.[2,7] The reduced or total absence in synthesis of the α-globin chain is caused by the deletion of one (-) or both (--/) cis-linked α-globin genes on chromosome 16 and less frequently due to non-deletional mutations ($\alpha^T\alpha$ or $\alpha\alpha^T$). Individuals with three functional α-globin genes (-α/αα) are asymptomatic and subjects with either homozygous α-thal 2(α^+)(-α/-α) or heterozygous α-thal 1(α^0) present with hypochromic microcytic indices with normal Hb levels or mild anaemia.[2,9] HPLC to quantify Hb subtypes and Hb electrophoresis are unable to detect α-thalassaemia and thus α-chain synthesis studies or DNA analysis are needed to be certain of the diagnosis.[9]

Investigations on co-inheritance of α-thalasaemia in HbE carriers carried out in Thailand showed occurrence of co-inheritance α-thalasaemia in HbE carriers was high.[11,12,13] 25.2%

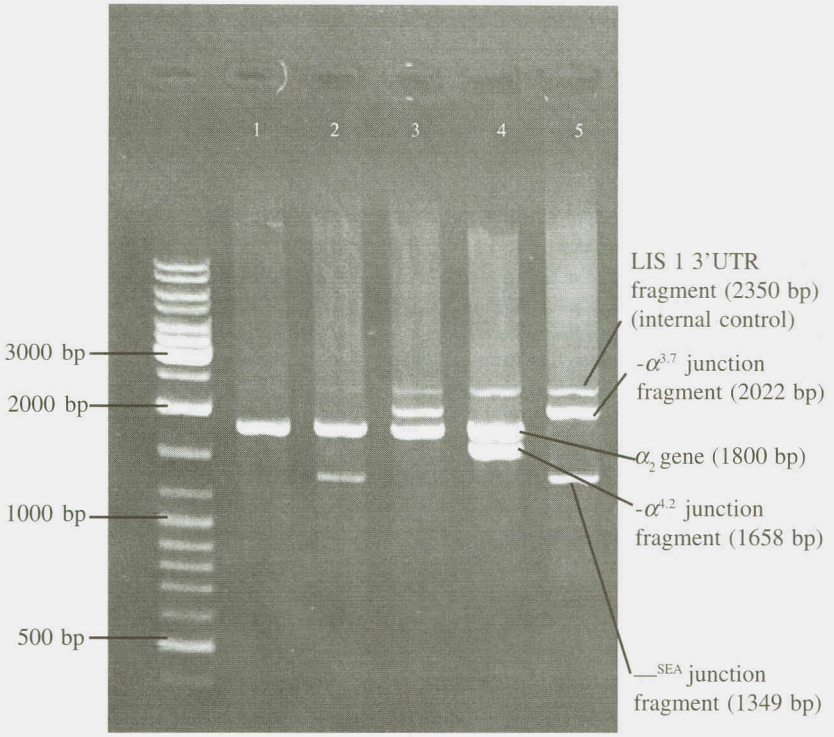

Figure 2. Electrophoresis on 1.0% agarose gel of the amplified DNA for detecting 7 common deletional forms of α-thalassaemia. lane 1: normal α_2 gene ($\alpha\alpha/\alpha\alpha$); lane 2: genotype $\alpha\alpha/—^{SEA}$; lane 3: genotype $\alpha\alpha/-\alpha^{3.7}$; lane 4: genotype $\alpha\alpha/-\alpha^{4.2}$; lane 5: Hb H disease, genotype $—^{SEA}/-\alpha^{3.7}$.

of HbE carriers inherited α-thal 2 trait while 10.4% had α-thal 1.[13] Sripichai *et al.* showed that 6.4% of 925 Thai β^{o}-thalassaemia/HbE patients co-inherited α-thal 2 trait while 0.22% with α-thal 1 trait.[13] In Malaysia, Rosline *et al.* showed 38.5% of the thalassaemia subjects had positive results in H-inclusion test and were suspected as HbE/α-thalassaemia subjects.[16] The H-inclusion test is used in the presumptive screening for α-thal 1(α^{0}) and a negative result does not rule it out. Only DNA studies identify α-thal 1(α^{0}) accurately.

In this study, we have examined HbE carriers in Malays for the presence of deletional α-thalassaemia. α-thalassaemia and HbE were co-inherited in 5(11.1%). The most prevalent type of deletional α-thalassaemia, α-thalassaemia 2 ($-\alpha^{3.7}/\alpha\alpha$) was found in 4(8.9%) and deletional α-thalassaemia 1 ($--^{SEA}/\alpha\alpha$) was found in one Malay-Thai HbE carrier. The SEA deletion was most likely inherited from the Thai parent. These results indicate a higher prevalence of HbE/$-\alpha^{3.7}$ compared to HbE/$--^{SEA}$, consistent with the study done by Sanchaisutriya *et al.* [12]

A study by Wee *et al.* showed that double alpha-globin gene deletion, α-thal 1 ($–^{SEA}$ deletion) was significantly higher in the Chinese compared to the Malays while $-\alpha^{3.7}$ deletion was distributed equally in the three races in Malaysia.[17] We found that $-\alpha^{3.7}$ was found in

Malays while --^{SEA} was in a Malay-Thai individual. Studies show that the Hb E in pure HbE carriers usually comprises about 30% of the Hb subtypes seen. Individuals with less than 25% of HbE on quantifying of Hb subtypes by HPLC analysis were more prone to have co-existing α-thalassemia trait.[18] In our study, the HbE/A_2 levels were lower in HbE carriers with alpha-thalassaemia. (Table 2). Co-inheritance of alpha-thalassaemia with HbE results in a lesser amount of unmatched α-globin chains, which lead to the more balanced α-/non α-globin ratio and a milder form of β-thalassaemia phenotype.[19] In conclusion, co-inheritance of α-thalassaemia and HbE carrier was identified in 11.1% of Malays. This result provides new information on the frequency of co-inheritance of HbE with deletional α-thalassaemia in Malays. The determination of accurate genotypes requires DNA analysis for both α- and β-globin genes in all cases of HbE trait. The presence of concurrent alpha-thalassaemia is suspect when the HbE level is reduced in comparison to pure HbE carriers. Ethnic background and family studies will also assist in diagnosis of α-thalassaemia in HbE carriers. Information on gene-gene interactions will guide genetic counseling and the planning of treatment modalities of patients with HbE α-thalassaemia.

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