ORIGINAL ARTICLE

Coinheritance of Haemoglobin E Trait With Alpha Thalassaemia Among Malay Students in National Thalassaemia Screening Programme in Hospital Sultanah Nur Zahirah Kuala Terengganu

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ABSTRACT

Introduction: Coinheritance of Hb E with alpha thalassaemia may occur. Individuals are asymptomatic for the condition. Further investigations for alpha thalassaemia coinherit with Hb E are dependent upon establishment in lower limit of RBC parameters together with haemoglobin analysis findings. Objectives of the study were to determine coinheritance pattern of Hb E and alpha thalassaemia, significance of RBC parameters and possible cut off level for Hb E for further alpha thalassaemia investigations. **Methods:** This was a cross sectional study using retrospective data among National Thalassaemia Screening Programme (NTSP) Form Four students. Individuals were classified into three groups (Hb E trait individuals with unknown alpha thalassaemia status, without alpha thalassaemia and with alpha thalassaemia). Descriptive data on gender, mean values of the RBC parameters, Hb A2 and Hb E levels were determined, followed by comparative analysis and ROC curve plotting to determine cut-off value to predict coinheritance of alpha thalassaemia in Hb E traits. **Results:** The male and female groups are almost equally distributed. Variability in the Hb A2, Hb E levels and RBC parameters values in between groups were noted and some values show significant differences. The cut-off Hb E level to predict the possibility of alpha thalassaemia in Hb E trait is 23.25 %. **Conclusion:** The proportion of Hb E trait in NTSP of Terengganu state was 17.7 %. Mean RBC parameters between different groups in Hb E trait show variability in values. Hb E ≤23 % can be safely used as a cut-off value to predict coinheritance alpha thalassaemia in Hb E trait.

Keywords: Coinheritance, Hb E trait, Alpha thalassaemia, RBC parameters, Cut off

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INTRODUCTION

Thalassaemia is an autosomal recessive disorder and causes reduced or absent synthesis of one or more of globin chains. On the other hand, haemoglobinopathy is an inherited disorder caused by structural alteration of globin chain. These disorders are public health problem in Malaysia as a total of 7984 thalassaemia patients were reported in 2018 Malaysian Thalassaemia Registry Report (1). Terengganu showed 344 (4.31 %) registered thalassaemia cases with Hb E-beta thalassaemia recorded as 52.33 % from total cases. Hb H disease is 17.73% of all thalassaemia cases reported in Terengganu (1).

Prevalence of Hb E trait and Hb E disease is higher in the northern and eastern states of Malaysia due to close geographical connection to southern Thailand (2). On the other hand, alpha thalassaemia is the most common inherited disorder in Malaysia. It is estimated 6.8 % of the population are carriers for β- or α- thalassaemia trait (3). One study showed prevalence of deletional type alpha thalassaemia carrier which was 9.25 % among our Malaysian population (4). Hence, it is not uncommon for individuals to carry both beta and alpha thalassaemia and it can be easily missed during haemoglobin analysis. In Malaysia, the National Thalassaemia Screening Programme (NTSP) targeting the Form 4 students was established in 2016 to reduce the number of transfusion dependent thalassaemia cases hence reducing the financial burden incurred by this disorder. Many studies encompass on coinheritance of deletional alpha thalassaemia in Hb E trait and had established a cut-off value of Hb E level less than 25 % in Hb E trait via high-performance liquid chromatography (HPLC) method to screen for coinheritance of deletional alpha thalassaemia (5,6). Limited literatures available on the cut off value of Hb E using capillary zone electrophoresis (CZE) method to predict presence of alpha thalassaemia in Hb E trait individuals.

It is important to determine the differences in RBC

parameters and Hb E level between the Hb E trait without known alpha thalassaemia status and Hb E trait with coinheritance alpha thalassaemia (deletional and non-deletional) to ensure that the alpha thalassaemia cases are not missed in Hb E trait individuals. Missed diagnosis of alpha thalassaemia can bring about deleterious effect like foetal death due to hydrops fetalis. For Hb analysis screening, laboratory is required to have at least two methods (7) before further confirmatory investigations; and in Hospital Sultanah Nur Zahirah (HSNZ) the two screening methods employed were CZE and gel electrophoreses.

This study aimed to determine the differences in RBC parameters and haemoglobin analysis findings via CZE method between Hb E trait with unknown alpha thalassaemia status, Hb E trait with alpha thalassaemia (both deletional and non-deletional) and Hb E trait without alpha thalassaemia. In HSNZ a cut-off value of Hb E ≤ 23 % is taken as a criterion for further DNA analysis to detect alpha thalassaemia in Hb E trait individuals when utilising CZE method. However, this value has never been objectively evaluated in our local setting. This cut-off value of less or equivalent to 23 % was based on observations by the haematologists in HSNZ. This study aimed to evaluate this cut-off value of Hb E level when using the CZE method to predict coinheritance of alpha-thalassaemia in Hb E trait individuals.

MATERIALS AND METHODS

The study was conducted in Pathology department, HSNZ Kuala Terengganu. HSNZ is the largest government hospital in Terengganu, and it is the main referral centre for government district hospitals, private hospitals, and health clinics. Around 95 % of Terengganu state population are Malays, hence the study population consist of Malay population only.

This was a cross sectional study using retrospective data of Form Four students from NTSP in the state of Terengganu. Data from July 2016 to July 2018 was obtained from HSNZ Laboratory Information System (LIS). The study was approved by Medical Research Ethics Committee (MREC) NMRR-18-3825-45081. Inclusion criteria for the study were:

1. Haemoglobin analysis done among male students with Hb ≥ 13 and MCH ≤ 27 pg, and female students with Hb ≥ 12 with MCH ≤ 27 pg.
2. Students diagnosed as Hb E trait with the CZE and gel electrophoresis methods from the sample received in HSNZ from July 2016 to July 2018 with a complete demographic and clinical information.

The proportion of Hb E trait individuals from the NTSP haemoglobin analysis screening performed in the year 2017 was calculated using OpenEpi website calculator (www.openepi.com) (8). Subsequently, these samples were divided into three groups based on data at hand. The number of Hb E trait in NTSP from July 2016 to July 2018 was 1217. The data from these 1217 individuals grouped as Hb E trait with unknown alpha thalassaemia status even though 58 DNA analysis samples available to compare data between this group and the other 2 groups with confirmed alpha and without alpha thalassaemia by molecular analysis.

Data of haemoglobin analysis screening including the Form 4 NTSP data was taken from HSNZ LIS. The RBC parameters and haemoglobin analysis results were retrieved according to the sample ID number. DNA analysis results retrieved to identify the coinheritance of alpha thalassaemia among Hb E trait individuals. All data were recorded in proforma for further analysis.

Principle of CZE, gel electrophoresis and DNA analysis

The Sebia CZE and Sebia HYDRAGEL HEMOGLOBIN(E) assays were utilized to screen for Hb E trait cases. Blood samples were sent in EDTA tubes and stored at 4 degree Celcius for up to 1 week. Sebia CZE uses the CAPILLARYS 2 FLEX-PIERCING instrument which gave a quantitative reading of haemoglobin fractions like Hb A, Hb A2, Hb F and other major haemoglobin variants like Hb S, C, E and D. Whole blood samples in EDTA tubes were directly analysed. The assay was performed on the hemolysate of these whole blood samples. The charged molecules of haemoglobin are separated by their electrophoretic mobility in an alkaline buffer (pH 9.4) at a high voltage. The results were displayed as electropherograms. Hb E was detected in zone 4 and relative percentage was less than 35 % and some samples showed presence of small peaks at zone 12, zone 15, zone 2 and Hb E ≤23% prompting suggestion of DNA analysis to exclude alpha thalassaemia.

Sebia HYDRAGEL HEMOGLOBIN(E) assay used as a second method to further screen for the presence of Hb E in Hb E trait individuals. The HYDRAGEL HEMOGLOBIN(E) assay was performed on HYDRASYS instrument where the haemoglobin is separated by electrophoresis on alkaline agarose gels. The visualization of bands on the gels gave a qualitative analysis. The Hb E is detected as a band in at C/E/O-Arab/A2 region on gel electrophoresis at alkaline pH.

Multiplex GAP PCR method was used to detect single or two gene deletion alpha-thalassaemia like $\alpha\alpha/-\alpha^{3.7}$, $-\alpha^{3.7}$ / $-\alpha^{3.7}$, $\alpha\alpha/-\alpha^{4.2}$, $\alpha\alpha$ / $--^{SEA}$. Multiplex ARMS PCR method is used to detect non-deletional alpha-thalassaemia mutations like $\alpha\alpha$ / $\alpha\alpha^{CS}$ and $\alpha\alpha$ / $\alpha\alpha^{CD59}$. Different primers utilised to detect deletion or mutations in alpha gene region. The molecular analysis was conducted in Institute of Medical Research (IMR) Kuala Lumpur. The primers used in IMR for multiplex GAP PCR method can detect $-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{SEA}$, --THAI, --MED, --FIL and –(α)20.5 deletions. Meanwhile, the primers used in multiplex ARMS PCR method can detect mutations in codon 30,

codon 35, codon 59 (Hb Adana), codon 125 (Hb Quong Sze) and Hb Constant Spring.

Data processing and statistical analysis

All results were analysed by standard statistical software package, statistics for Windows, SPSS version 25. Normality test using skewness, kurtosis, histogram, Kolmogorov-Smirnov, and Shapiro Wilk were used.

The proportion of Hb E trait in NTSP in a year was determined using the 2017 samples data. The other descriptive and comparative data are determined using the July 2016 to December 2018 samples result. The distribution of demographic characteristics (gender) was determined by using the frequency distribution and percentage. Descriptive statistics were used for the Hb A2, Hb E levels and RBC parameters in three groups which were Hb E trait with unknown alpha thalassaemia status representing overall Hb E trait individuals in Form 4 NSTP, Hb E trait with alpha thalassaemia (confirmed by DNA analysis) and Hb E trait without alpha thalassaemia (confirmed by DNA analysis) Independent t test was used to determine whether means of Hb A2, Hb E and RBC parameters in between these three groups differ significantly. The determination of appropriate cut-off value of Hb E in Hb E trait to exclude alpha thalassaemia was analysed with receiver operating characteristic (ROC) curve. The sensitivity value was plotted against 1-specificity value and the best cut-off value is determined by taking the value closest to 1 (sensitivity).

RESULTS

Two sets of samples were analysed for this study. The proportion of Hb E trait in NTSP was calculated based on samples of Form 4 students in 2017 which was 3731 total samples. Another set of samples of Form 4 students with Hb E trait from July 2016 to December 2018 were analysed to determine the differences in RBC parameters and haemoglobin analysis values. Out of this 1217 individuals, 58 individuals had DNA analysis results to detect coinheritance alpha thalassaemia.

As shown in Table I, in the year 2017, the proportion of Hb E trait individuals in NTSP was 17.7%. Out of the 1217 Hb E trait individuals, 140 individuals were suggested for DNA analysis to exclude alpha thalassaemia but only 58 individuals took the DNA analysis. Out of 58 DNA analysis performed, it was observed that 35 individuals amongst 1217 Hb E trait individuals have coinheritance alpha thalassaemia. As for the gender distribution, female and male individuals were almost equally distributed, where the female individuals were 633 (52 %) and the male individuals were 584 (48 %).

There were 58 DNA analysis sent to exclude the coinheritance of alpha thalassaemia amongst 1217 individuals with Hb E. Out of the 58 samples, 35 individuals showed coinheritance alpha thalassaemia as shown in Table II, 16 (45.7 %) of them were heterozygous for alpha plus thalassaemia 3.7 deletion ($\alpha\alpha$ /-$\alpha^{3.7}$) with equal distribution of gender. Nine individuals (25.7 %) had concurrent Hb Constant Spring (CS) ($\alpha\alpha$ / $\alpha\alpha^{CS}$) where there were five females and four males, respectively. Two male individuals had concurrent Hb Adana ($\alpha\alpha$ / $\alpha\alpha^{CD59}$), and one female individual was heterozygous for alpha plus thalassaemia 4.2 deletion ($\alpha\alpha$ /-$\alpha^{4.2}$). Four individuals were heterozygous for alpha zero thalassaemia South East Asian deletion ($\alpha\alpha$ / --^{SEA}) where three of them were females. Three individuals (1 female and 2 male) were homozygous for alpha plus thalassaemia $^{3.7}$ deletion (-$\alpha^{3.7}$ / -$\alpha^{3.7}$).

Table III showed, the mean (± 2SD) in between 3 groups (Hb E trait with unknown thalassaemia status, Hb E trait

Table I: The total individuals in Hb analysis screening in Terengganu for the year 2017 and Hb E trait in Form 4 individuals during the study period

Duration	July – Dec 2016	Jan – Dec 2017	Jan – July 2018	Total
Total individuals in Hb analysis screening		4418		
Form 4 individuals from NTSP		3731		
Hb E trait in Form 4 individuals	131	663	426	1217
DNA analysis performed.	11	28	19	58
• With alpha thalassaemia	4	16	15	35
• Without alpha thalassaemia	7	12	4	23

Table II: The demographic details (gender) of Hb E trait individuals with coinheritance alpha thalassaemia

Characteristic	n (%) Female	n (%) Male	Total
Hb E trait with alpha thalassaemia			35
1. Heterozygous for alpha plus thalassaemia 3.7 deletion	8 (22.9)	8 (22.9)	16 (45.8)
2. Heterozygous for alpha plus thalassaemia 4.2 deletion	1 (2.9)	0	1 (2.9)
3. Heterozygous for alpha zero thalassaemia South East Asian deletion	3 (8.6)	1 (2.9)	4 (11.5)
4. Homozygous for alpha plus thalassaemia 3.7 deletion	1 (2.9)	2 (5.7)	3 (8.6)
5. Termination codon (TAA->CAA) mutation (Hb Constant Spring)	5 (14.2)	4 (11.4)	9 (25.6)
6. Codon 59 (GGC->GAC) mutation Hb Adana	0	2 (5.7)	2 (5.7)

Table III: The Hb A2, Hb E levels and RBC parameters in Hb E trait with different alpha thalassaemia status

	Unknown alpha thalassaemia status	No α -thalas-saemia	With alpha thalassaemia					
			$\alpha\alpha/-\alpha^{3.7}$	$\alpha\alpha/-\alpha^{4.2}$	$\alpha\alpha$ / $--^{SEA}$	$-\alpha^{3.7}$ / $-\alpha^{3.7}$	$\alpha\alpha$ / $\alpha\alpha^{CS}$	$\alpha\alpha$ / $\alpha\alpha^{CD59}$
N	1217	23	16	1	4	3	9	2
Hb A2 (%)	3.72 ± 0.64	3.85 ± 0.80	3.71 ± 0.70	3.10	3.93 ± 1.92	4.03 ± 0.70	3.37 ± 0.74	3.85 ± 0.14
Hb E (%)	25.10 ± 2.98	24.4 ± 3.08	22.47 ± 2.22	17.90	16.55 ± 0.66	18.20 ± 1.74	22.88± 1.46	22.75± 0.42
Hb (g/L)	135.73 ± 21.12	135.39 ± 16.76	139.44 ± 24.24	122.00	132.25± 21.42	139.67± 13.32	134.22± 20.20	146.00± 31.12
RBC count ($x10^{12}$/L)	5.57 ± 0.96	5.67 ± 0.90	5.67 ± 1.14	5.41	6.18 ± 0.88	6.06 ± 0.46	5.46 ± 0.68	6.09 ± 0.66
MCV (fL)	73.45 ± 6.46	72.40 ± 6.34	74.25 ± 4.10	70.60	66.10 ± 2.08	70.97 ± 9.76	74.53± 6.12	70.90± 6.78
MCH (pg)	24.43± 2.42	23.95± 2.56	24.66± 1.98	22.60	21.40± 0.92	23.10± 3.82	24.57± 2.22	23.95± 2.40

Data reported as mean ± 2SD

without alpha thalassaemia and Hb E trait with alpha thalassaemia). The mean Hb E level in Hb E trait with alpha thalassaemia group is lower than Hb E trait without alpha thalassaemia group and Hb E trait with unknown alpha thalassaemia status group. The mean Hb E level is less than 20 % in Hb E trait with $\alpha\alpha$ / $--^{SEA}$ and $-\alpha^{3.7}$ / $-\alpha^{3.7}$ and $\alpha\alpha$ /$-\alpha^{4.2}$, while the mean Hb E level is more than 20 % in Hb E trait individuals with $\alpha\alpha/-\alpha^{3.7}$, $\alpha\alpha$ / $\alpha\alpha^{CS}$ and $\alpha\alpha$ / $\alpha\alpha^{CD59}$. Extreme range of Hb E was noticed where the level range in between 15.80 to 28.40 % in Hb E trait with unknown alpha thalassaemia status. In this study, the range of Hb E level for coinheritance $\alpha\alpha$/ $--^{SEA}$ was 16.10 to 16.80 %. The mean Hb A2 in all three groups were above the normal Hb A2 level except in Hb E trait with $\alpha\alpha/-\alpha^{4.2}$.

The Hb level in all three groups were normal ranging between 120 g/L to 176 g/L because only Form 4 individuals with normal haemoglobin level were screened for thalassaemia. The mean RBC count are on the higher side in all three groups except in coinheritance $\alpha\alpha$ /$-\alpha^{4.2}$. The mean value of RBC count in Hb E trait individuals with deletional alpha thalassaemia ($\alpha\alpha/-\alpha^{3.7}$, $\alpha\alpha/-\alpha^{4.2}$, $\alpha\alpha$ / $--^{SEA}$ and $-\alpha^{3.7}$ / $-\alpha^{3.7}$) were in between 5.41 to 6.18 X 1012/L.

The mean value of MCV in Hb E trait individuals with different alpha thalassaemia were lower compared to Hb E trait individuals with unknown status of thalassaemia and Hb E trait individuals without alpha thalassaemia ranging from 66.10 to 74.53 fL except in $\alpha\alpha/-\alpha^{3.7}$ and $\alpha\alpha$/ $\alpha\alpha^{CS}$. The mean level of MCH in all three groups was less than 25 pg.

Table IV showed comparison of the Hb A2, Hb E levels and RBC parameters between Hb E individuals with unknown alpha thalassaemia status and Hb E individuals with alpha thalassaemia. There was significant difference in the mean value of Hb E amongst Hb E trait individuals with unknown alpha thalassaemia status and Hb E trait individuals with alpha thalassaemia ($\alpha\alpha/-\alpha^{3.7}$, $\alpha\alpha$ / $--^{SEA}$, $-\alpha^{3.7}$ / $-\alpha^{3.7}$, $\alpha\alpha$ / $\alpha\alpha^{CS}$). There was also significant difference in the mean value of MCH in between Hb E trait individuals with unknown alpha thalassaemia status and Hb E trait individuals with $\alpha\alpha$ / $--^{SEA}$.

As shown in Table V, there were significant differences seen in RBC parameters (RBC count, MCV and MCH), Hb A2 and Hb E levels when Hb E trait without alpha thalassaemia group compared to Hb E trait with $\alpha\alpha$ / $--^{SEA}$ group. There was also significant difference observed, in the Hb A2 level in between Hb E trait without alpha thalassaemia group compared to Hb E trait with $\alpha\alpha$ / $\alpha\alpha^{CS}$ group. Similarly, significant difference was also observed in the MCV level in between Hb E trait without alpha thalassaemia group compared to Hb E trait with $\alpha\alpha/-\alpha^{3.7}$ group.

The fifty-eight samples with DNA analysis were analysed to determine sensitivity and specificity of the different cut-off value of the Hb E level to predict the coinheritance of alpha thalassaemia in Hb E trait individuals. In this study, the best cut-off value of Hb E level to predict coinheritance of alpha thalassaemia using the ROC curve was less than 23.25 % using CZE method with sensitivity of 82.6 % and specificity of 85.7 %. The AUC value for the ROC curve plotted is 0.891 as shown in Figure 1.

Other findings observed were not all Hb E trait individuals with coinheritance Hb CS had eluted in zone 2 of CZE. Out of nine individuals with coinheritance Hb

Table IV: The comparison of Hb A2, Hb E levels and RBC parameters in Hb E trait with unknown alpha thalassaemia status and Hb E trait with alpha thalassaemia

Variables	Hb E trait with unknown alpha thalassaemia status	Hb E trait with alpha thalassaemia	t statistic (df)	p value
Hb A2	3.72 (0.64)	αα/-$\alpha^{3.7}$ 3.71(0.70)	0.04(15)	0.97
		αα/-$\alpha^{4.2}$ 3.10	-	-
		αα / --^{SEA} 3.93(1.92)	-1.92(3)	0.15
		-$\alpha^{3.7}$ / -$\alpha^{3.7}$ 4.03(0.70)	-0.77(2)	0.52
		αα / αα^{CS} 3.37(0.74)	0.90(8)	0.40
		αα / αα^{CD59} 3.85(0.14)	-1.50(1)	0.37
Hb E	25.10(2.98)	αα/-$\alpha^{3.7}$ 22.47(2.22)	2.30(15)	**0.04***
		αα/-$\alpha^{4.2}$ 17.90	-	-
		αα / --^{SEA} 16.55(0.66)	23.03(3)	**0.00***
		-$\alpha^{3.7}$ / -$\alpha^{3.7}$ 18.20(1.74)	6.90(8)	**0.02***
		αα / αα^{CS} 22.88(1.46)	2.87(8)	**0.02***
		αα / αα^{CD59} 22.75(0.42)	9.04(1)	0.07
Hb	135.73(21.12)	αα/-$\alpha^{3.7}$ 139.44(24.24)	-0.30(15)	0.77
		αα/-$\alpha^{4.2}$ 122.00	-	-
		αα / --^{SEA} 132.25(21.44)	0.29(3)	0.79
		-$\alpha^{3.7}$ / -$\alpha^{3.7}$ 139.67(13.32)	-0.51(2)	0.66
		αα / αα^{CS} 134.22(20.20)	0.14(8)	0.89
		αα / αα^{CD59} 146.00(31.12)	-0.54(1)	0.69
RBC count	5.57(0.96)	αα/-$\alpha^{3.7}$ 5.67(1.14)	-0.17(15)	0.86
		αα/-$\alpha^{4.2}$ 5.41	-	-
		αα / --^{SEA} 6.18(0.88)	-1.24(3)	0.30
		-$\alpha^{3.7}$ / -$\alpha^{3.7}$ 6.06(0.46)	-1.82(2)	0.21
		αα / αα^{CS} 5.46(0.68)	0.29(8)	0.78
		αα / αα^{CD59} 6.09(0.66)	-1.27(1)	0.43
MCV	73.45(6.46)	αα/-$\alpha^{3.7}$ 74.25(4.10)	-0.38(15)	0.71
		αα/-$\alpha^{4.2}$ 70.60	-	-
		αα / --^{SEA} 66.10(2.08)	6.31(3)	0.08
		-$\alpha^{3.7}$ / -$\alpha^{3.7}$ 70.97(9.76)	0.44(2)	0.70
		αα / αα^{CS} 74.53(6.12)	-0.34(8)	0.75
		αα / αα^{CD59} 70.90(6.78)	0.61(1)	0.65
MCH	24.43(2.42)	αα/-$\alpha^{3.7}$ 24.66(1.98)	-0.22(15)	0.83
		αα/-$\alpha^{4.2}$ 22.60	-	-
		αα / --^{SEA} 21.40(0.92)	5.87(3)	**0.01***
		-$\alpha^{3.7}$ / -$\alpha^{3.7}$ 23.10(3.82)	0.61(2)	0.61
		αα / αα^{CS} 24.57(2.22)	-0.12(8)	0.91
		αα / αα^{CD59} 23.95(2.40)	0.33(1)	0.80

Data are presented as mean (2SD)
* indicates p value < 0.05 which is statistically significant

Table V: The comparison of Hb A2, Hb E levels and RBC parameters in Hb E trait without alpha thalassaemia and Hb E trait with alpha thalassaemia

Variables	Hb E trait without alpha thalassaemia	Hb E trait with alpha thalassaemia	t statistic (df)	p value
Hb A2	3.85(0.80)	αα/-$\alpha^{3.7}$ 3.71(0.70)	-1.15(37)	0.26
		αα/-$\alpha^{4.2}$ 3.10	1.84(22)	0.08
		αα / --^{SEA} 3.93(1.92)	-0.38(25)	0.71
		-$\alpha^{3.7}$ / -$\alpha^{3.7}$ 4.03(0.70)	-0.77(24)	0.45
		αα / αα^{CS} 3.37(0.74)	3.13(30)	**0.00***
		αα / αα^{CD59} 3.85(1.42)	-0.01(23)	0.99
Hb E	24.47(3.08)	αα/-$\alpha^{3.7}$ 22.47(2.22)	-4.44(37)	**0.00***
		αα/-$\alpha^{4.2}$ 17.90	4.18(22)	**0.00***
		αα / --^{SEA} 16.55(0.66)	10.09(25)	**0.00***
		-$\alpha^{3.7}$ / -$\alpha^{3.7}$ 18.20(1.74)	6.83(24)	**0.00***
		αα / αα^{CS} 22.88(1.46)	2.94(30)	**0.01***
		αα / αα^{CD59} 22.75(0.42)	-1.55(23)	0.14
Hb	135.39(16.76)	αα/-$\alpha^{3.7}$ 139.44(24.24)	1.24(37)	0.23
		αα/-$\alpha^{4.2}$ 122.00	1.56(22)	0.13
		αα / --^{SEA} 132.25(21.44)	0.67(25)	0.51
		-$\alpha^{3.7}$ / -$\alpha^{3.7}$ 139.67(13.32)	-0.84(24)	0.41
		αα / αα^{CS} 134.22(20.20)	0.34(30)	0.74
		αα / αα^{CD59} 146.00(31.20)	1.63(23)	0.12
RBC count	5.67(0.90)	αα/-$\alpha^{3.7}$ 5.67(1.14)	0.03(37)	0.98
		αα/-$\alpha^{4.2}$ 5.41	0.56(22)	0.58
		αα / --^{SEA} 6.18(0.88)	-2.09(25)	**0.04***
		-$\alpha^{3.7}$ / -$\alpha^{3.7}$ 6.06(0.46)	-1.44(24)	0.16
		αα / αα^{CS} 5.46(0.68)	1.22(30)	0.23
		αα / αα^{CD59} 6.09(0.66)	-1.26(23)	0.22
MCV	72.40(6.34)	αα/-$\alpha^{3.7}$ 74.25(4.10)	2.05(37)	**0.04***
		αα/-$\alpha^{4.2}$ 70.60	0.56(22)	0.58
		αα / --^{SEA} 66.10(2.08)	3.89(25)	**0.00***
		-$\alpha^{3.7}$ / -$\alpha^{3.7}$ 70.97(9.76)	0.70(24)	0.49
		αα / αα^{CS} 74.53(6.12)	-1.73(30)	0.10
		αα / αα^{CD59} 70.90(6.78)	-0.64(23)	0.53
MCH	23.95(2.56)	αα/-$\alpha^{3.7}$ 24.66(1.98)	1.85(37)	0.07
		αα/-$\alpha^{4.2}$ 22.60	1.04(22)	0.31
		αα / --^{SEA} 21.40(0.92)	3.89(25)	**0.00***
		-$\alpha^{3.7}$ / -$\alpha^{3.7}$ 23.10(3.82)	1.04(24)	0.31
		αα / αα^{CS} 24.57(2.22)	-1.26(30)	0.22
		αα / αα^{CD59} 23.95(2.40)	-0.00(23)	1.00

Data are presented as mean (2SD)
* indicates p value < 0.05 which is statistically significant

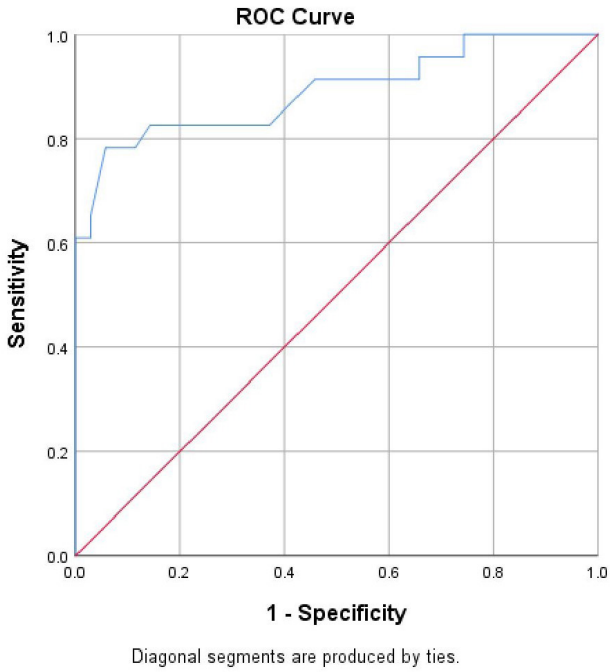

Figure 1: The ROC curve to determine best cut-off of HB E level to predict coinheritance of alpha thalasaemia in Hb E trait individuals. Area under the curve (AUC)=0.891

CS, five individuals showed Hb peak in zone 2 ranging from 0.2 to 0.3 %. In other four Hb E trait individuals with coinheritance Hb CS, no Hb was eluted in zone 2 in CZE. These individuals had Hb E level ranging from 21.9-24.3 % and Hb A2 ranging from 2.8 -3.9 %. This study showed that only 55.6 % cases of Hb E trait with coinheritance Hb CS cases showed Hb peak in zone 2 of CZE. On the other hand, seven individuals had eluted Hb peak ranging from 0.1-0.8 % in zone 2 of CZE, but DNA analysis did not show any presence of Hb CS mutation.

DISCUSSION

In this study, the proportion of Hb E trait in Form 4 individuals in NTSP was 17.7%. Previously, a study in secondary school in Ampang showed proportion of Hb E trait of 12.5 % (2) which was similar as the finding of this study. It was noted that the proportion of Hb E trait individuals during Hb analysis screening in NTSP was higher than the overall prevalence of Hb E trait of 2.6 to 4 % in Malaysia (2,5,9). This is because in this study proportion determination focused only on individuals with normal haemoglobin level and MCH < 27 pg and this directed haemoglobin analysis gave a higher proportion of Hb E trait. Geographical factor also likely to give a higher percentage of Hb E cases in Terengganu. The proportion of Hb E trait with coinheritance of alpha thalassaemia could not be determined due to limitation in this study. The proportion of coinheritance of alpha thalassaemia in Hb E trait in this study is not representative of actual value because not all Hb E trait individuals were subjected to DNA analysis to exclude alpha thalassaemia which is one of the limitations of this study. A study in Malaysia, stated that 11. 1 % individuals with Hb E trait had concomitant deletional alpha thalassaemia (5). A larger comprehensive study needed to determine true proportion of both deletional and non-deletional alpha thalassaemia in Hb E trait.

Six types of alpha thalassaemia were detected in Hb E trait individuals in this study. Other studies had mentioned detection of other alpha thalassaemia types beside αα /-α3.7, -α3.7 / -α3.7, αα/-α4.2, αα / --^{SEA}, αα / αα^{CS} and αα / αα^{CD59}. A study in Thailand had mentioned presence of αα / αPSα, -α3.7 / αCSα, --^{SEA} /-α3.7, --^{SEA} /-α4.2 (11). In this study, the study population were from Malay race and all the individuals were 16 years old. Around 45.7 % of 35 individuals with alpha thalassaemia were heterozygous for alpha plus thalassaemia 3.7 deletion which is expected as this deletion is common in Malays compared to Chinese in Malaysia. Data from IMR revealed that αα/-α3.7 has global distribution among all ethnic groups in Malaysia (12).

About 25.7 % Hb E trait individuals had concomitant Hb CS. The incidence of Hb CS in Malays was 5.6 % (12). Incidence of Hb Adana was around 1.4 % (12) in Malay race and 5.7 % Hb E trait individuals in this study had concurrent Hb Adana. This comparative data needed to be interpreted cautiously as this study focused on proportion of coinheritance of alpha thalassaemia in Hb E trait rather than solely on alpha thalassaemia or Hb E trait.

The gender distribution in this study was almost equal just like other studies on thalassaemia demographic distribution (2,10) as thalassaemia/hemoglobinopathy is an autosomal recessive disorder.

As shown in Table III, our study demonstrated that interaction of Hb E trait with alpha thalassaemia may or may not influences the Hb A2, Hb E level and RBC parameters. The mean value of Hb A2 in Hb E trait individuals was higher in all groups than normal due to mild thalassaemic effect and compensatory increase in δ globin (13). The mean value of Hb E level in this study among Hb E trait individuals with unknown thalassaemia status was 25.09 % which is almost comparable with other studies which mentioned mean Hb E level of 24.28 % to 24.9 % when CZE method used (13,14). In this study, we observed the Hb E level is lower in Hb E trait individuals with coinheritance alpha thalassaemia compared to Hb E trait individuals without alpha thalassaemia. Similar findings were found in multiple studies in Malaysia and Thailand (5,9,11,15). The presence of alpha thalassaemia in individual with Hb E trait can result in decrease in abnormal haemoglobin amount (11).

The Hb E level is lower in Hb E trait with alpha thalassaemia-1 and Hb E trait with homozygous alpha

thalassaemia-2 (-α3.7/-α3.7) compared to Hb E trait with alpha thalassaemia-2 (αα/-α3.7) and Hb E trait with non-deletional alpha thalassaemia (αα/αα^{CS}, αα/αα ^{CD59}). With these findings, the Hb E level can be used to establish a cut-off value to differentiate Hb E trait individuals with alpha thalassaemia and Hb E trait individuals without alpha thalassaemia.

The mean Hb level in Hb E trait individuals in this study was above 120 g/L, due to the study inclusion criteria. Some individuals may show mild anaemia attributed to concomitant iron deficiency or α-1 thalassaemia (11). Individuals were only subjected to Hb analysis in condition where the hypochromia was persistent with a normal iron study.

It was observed that the hypochromia and microcytosis was much greater in Hb E trait individuals with alpha thalassaemia-1 and homozygous alpha thalassaemia-2. In 2007, a study in Thailand revealed that combination of Hb E <26 % with either MCV <74fL or MCH < 24 pg was recommended for screening of α0 thalassaemia (– –^{SEA} and – –THAI deletions) in Hb E trait individuals (15). All the combination mentioned above was fulfilled in the Hb E trait cases with α0 thalassaemia (--/αα^{SEA}) in this study. In this study, Hb E trait individuals with coinheritance --/αα^{SEA} showed significant differences in RBC parameters like RBC count, MCV and MCH and Hb E levels when compared to Hb E trait individuals without alpha thalassaemia. However, a cut-off value with all combination of Hb E, MCV, MCH and RBC count was not established due to inadequate sample for analysis. None the less, based on observation, data from the 4 individuals with Hb E trait and coinheritance (--/αα^{SEA}), the MCV level is less than 70 fL and Hb E level is less than 17 %.

The RBC count was variable in the Hb E trait individuals and the count tend to be higher in Hb E trait individuals with alpha thalassaemia especially alpha thalassaemia-1 and homozygous alpha thalassaemia-2 compared to Hb E trait without alpha thalassaemia. RBC count increment is a compensatory mechanism relative to the low MCV and MCH count in thalassaemia carriers. The RBC count in this study was within the means values reported in other studies in Klang and Thailand (9,11).

A study in Thailand found statistically significant differences when MCV, MCH, Hb E values were compared between groups of Hb E trait with and without alpha thalassaemia (10). In this study, noticeable reduction was seen in Hb E level mounting to statistical significance in Hb E trait individuals with alpha thalassaemia except in Hb E trait with Hb Adana when compared to Hb E trait individuals without alpha thalassaemia. Greater hypochromia and microcytosis were observed in Hb E trait individuals with alpha thalassaemia-1 and homozygous alpha thalassaemia-2 compared to Hb E trait individuals without alpha thalassaemia. Similar findings were observed in multiple studies regarding Hb E trait with alpha thalassaemia (5,9,11,15).

Our study provided some information on the Hb E trait with non-deletional alpha thalassaemia. Recognition of Hb Adana in Hb E trait individuals was not straight forward. Hb Adana can be easily missed as there was no significant differences in all RBC parameters and Hb E level when compared to Hb E trait individuals without alpha thalassaemia or Hb E trait individuals with unknown alpha thalassaemia status. Only two cases of Hb Adana recognized in this study by taking the cut-off value of Hb E ≤ 23 % in CZE to exclude alpha thalassaemia. A different study with higher number of samples is needed for further evaluation of different parameters to recognize Hb Adana in Hb E trait individuals. Detection of Hb CS in Hb E trait individuals was more straight forward with presence of Hb CS peak in the HPLC or CZE method. From this study, in the absence of Hb CS peak in CZE, by taking the Hb E ≤ 23% with CZE method as proposed in HSNZ to exclude alpha thalassaemia, this study detected 3 cases of coinheritance Hb CS in Hb E trait individuals.

This study focused on evaluation of Hb E less or equivalent to 23 % as a cut-off value to suggest DNA analysis to exclude alpha thalassaemia in Hb E trait individuals in settings where HPLC method is not utilised as first or second method. Many studies focused on establishment of a cut-off Hb E level to suspect alpha thalassaemia in Hb E trait using HPLC method (5,6,16). Limited literature on the establishment of cut-off value of Hb E to predict alpha thalassaemia (deletional and non-deletional) using CZE method is available, hence comparison of data is not possible. Based on observation, the mean Hb E level in concurrent α-thalassaemia 2 and α-thalassaemia 1 was lower (16.37 to 21.42 %) compared to Hb E trait individuals without alpha thalassaemia (14). This value of Hb E is much lower than the cut-off of Hb E ≤23 % utilised by HSNZ to suspect coinheritance alpha thalassaemia. In this study, the ROC curve indicated that Hb E value less than 23.25 % with CZE method can be used to predict alpha thalassaemia with sensitivity of 82.6 % and specificity of 85.7%. As previously mentioned, HSNZ utilised the cut-off value of Hb E ≤ 23 % with CZE method to predict coinheritance alpha thalassaemia based on observation. A study in Kelantan stated that Hb E level using CZE method in Hb E trait with deletional alpha thalassaemia was lower, but no cut-off value of Hb E was mentioned (15).

This study showed that only 55.6 % cases of Hb E trait with coinheritance Hb CS cases had shown peak in zone 2 using CZE method. This finding is not consistent with finding of a study in Thailand where the CZE method detected 100 % Hb CS cases among Hb E trait individuals (17,18). By taking cut off value of Hb E less or equivalent to 23 % to predict coinheritance of alpha

thalassaemia, the rate of coinheritance Hb CS detection is raised to 75 % in Hb E trait individuals without Hb CS peak using CZE in this study. Detection of Hb CS in haemoglobin analysis is dependent on initial Hb CS level and storage of blood specimen as Hb CS is easily degradable (19).

However, the presence of Hb Adana in Hb E trait individuals can go undetected unless the Hb E value was less or equivalent to 23 % is taken to predict non-deletional alpha thalassaemia like Hb Adana as Hb Adana cannot be detected with CZE or HPLC. Unlike Hb CS which can present as a peak in HPLC and CZE method, Hb Adana cannot be visualised as a peak in both HPLC and CZE method. Detection of coinheritance Hb CS or Hb Adana in Hb trait is equally important as detection of deletional alpha thalassaemia to prevent occurrence of birth with Hb H-CS or Hb H-Adana. The data obtained in this study, should provide useful information in detecting alpha thalassaemia especially alpha thalassaemia-2 and non-deletional alpha thalassaemia in Hb E trait individuals for the prevention of alpha thalassaemia with clinically significant presentation.

There were few limitations to this study. The correct percentage of coinheritance of alpha thalassaemia could not be determined as not all Hb E trait individuals were subjected to DNA analysis to diagnose coinheritance of alpha thalassaemia. The sample size was not sufficient to compare the three different groups in this study. The RBC parameters and haemoglobin analysis values in this study group need to be interpreted cautiously as the samples were subjected to some degree of storage changes. The DNA analysis performed in IMR was limited to common deletion and mutation based on epidemiology in Malaysia. Uncommon or novel mutations that are diagnosed with MLPA and DNA sequencing may be missed.

CONCLUSION

In conclusion, the proportion of Hb E trait individuals in NTSP of Terengganu state was higher compared to proportion in other studies. The established mean and ± 2SD value, in between different concomitant alpha thalassaemia groups in Hb E trait individuals showed variability in values. Significant differences in the mean value of Hb A2, Hb E levels and RBC parameters was seen when Hb E trait without alpha thalassaemia group compared to Hb E trait with αα/--^{SEA} group. Hb E level less or equivalent to 23 % with CZE method can be utilised to predict the coinheritance of alpha thalassaemia in unavailability of concurrent HPLC method to diagnose thalassaemia. A comparative analysis of different values of Hb E and Hb A2 levels using CZE and HPLC methods need to be performed to predict the coinheritance of alpha thalassaemia in Hb E trait individuals.

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