Genotype - Phenotype Correlation Among Haemoglobin E β -thalassaemia Patients

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Abstract

Background and objectives: Haemoglobin E β -thalassaemia has a variable clinical presentation. This study describes the clinical spectrum of these patients in two thalassaemia centers in Malaysia in addition to determining the prevalence of selected primary and secondary genetic modifiers which may influence its phenotype.

Methods: A total of 99 patients were recruited in this cross-sectional study. Clinical parameters and severity scoring were determined. Molecular analysis was performed: Sanger sequencing and MLPA for β globin mutations; multiplex PCR for α -globin gene deletions and RFLP-PCR for *XmnI* polymorphism.

Results: Patients with mild HbE β -thalassaemia were diagnosed at a later age as compared to the severe group (mean 3.14 and 1.6 years, $p = 0.03$). Haemoglobin level at diagnosis was higher for the mild group as compared to severe group ($7.9 \text{ g/dL} \pm 1.97$ and $6.0 \text{ g/dL} \pm 1.00$, $p = 0.02$). The commonest β mutation in Malays were IVS1-5 (51.69%) and CD41/42 (20.2%) whereas in the Chinese, IVS2-654(44.4%) and CD41/42(33.4%). Single α -gene deletion ($-\alpha 3.7/\alpha\alpha$) was found in 4% of patients and none were homozygous for *XmnI* (+/+) polymorphism.

Conclusion: Age at presentation and haemoglobin level at initial diagnosis is useful as clinical predictors of disease severity. The majority of our patients had β° gene mutation i.e. IVS1-5 and CD41/42, which accounted for the moderate to severe phenotype based on clinical severity scoring.

Introduction

Haemoglobin E (HbE) β -thalassaemia is an important variant of thalassaemia. HbE is common in many Asian countries. With the relatively high prevalence of β thalassaemia alleles in these populations, the co-inheritance of HbE and β thalassaemia frequently occurs. [1,2] In Malaysia, approximately 2140 Malay patients have HbE β -thalassaemia. HbE is common amongst Malays with a carrier rate of 5%; the ratio is ten Malays with Hb E to one Malaysian Chinese ethnicity. [3]

The decision to initiate regular blood transfusions is often a dilemma. This is because, in addition to having a wide spectrum of clinical phenotype, HbE β -thalassaemia patients are also able to adapt to lower haemoglobin levels. [4] An accurate diagnosis facilitates an early start to transfusion programme in thalassaemia major phenotype and prevents unnecessary transfusion in thalassaemia intermedia.

Genetic modifiers are responsible for the wide spectrum of clinical phenotype in HbE β -thalassaemia patients. The primary modifier is due to heterogeneity of β -thalassaemia alleles, [5] where more than 200 mutations have been characterized. Majority of these mutations involve single nucleotide substitutions which affect the function of β -globin gene. [3]

Secondary modifiers relate to reduced α -globin production or increased gamma-globin synthesis in adulthood. [6] Co-inheritances of α -thalassaemia ameliorates whereas triplication of the α -globin genes

worsens β -thalassaemia severity. Several genetic modifiers increase HbF synthesis thus reduces the disease severity in HbE β -thalassaemia. Examples of these are the inheritance of *Xmn1* polymorphism [CD158 (C→T)] (polymorphism of HBG2 at position -158) and hereditary persistence of fetal Hb (HPFH) synthesis. HbF level is increased only in the homozygous state of *Xmn1* polymorphism (+/+) which ameliorates the clinical severity. [7]

The objective of our study was to describe the clinical spectrum of patients with HbE β -thalassaemia in two thalassaemia centres. We also determined the primary and secondary genetic modifiers which may influence the variable phenotype of this group of patients. These included the type of β gene mutation, co-inheritance of α thalassaemia and presence of *Xmn1* polymorphism.

Materials And Methods

Study Population

This cross-sectional study was conducted from 1st March 2016 until 31st October 2017. Patients diagnosed with HbE β -thalassaemia treated at Institut Pediatrik Hospital Kuala Lumpur (IPHKL) and Universiti Kebangsaan Malaysia Medical Centre (UKMMC) were identified from the thalassaemia registry. A universal sampling of all-HbE β -thalassaemia patients was done. From a total of 143 patients, 99 patients were recruited into the study. The remaining 44 patients were missed in the sampling period due to time constraint, infrequent appointment and patient refusal for consent. Ethical approval was obtained from Universiti Kebangsaan Malaysia ethical committee. All experiments were performed in accordance with good clinical practice guidelines and regulations and all persons or caregiver gave their informed consent before their inclusion in the study.

Data Collection

Clinical parameters of the patients were extracted from their medical records. Haemoglobin level (Hb) at steady state is defined as the mean of haemoglobin level pre-transfusion for the previous one year or the mean haemoglobin level for the past one year for patients who have never been transfused. History of severe infection is defined as patients with a clinically documented infection that require admission and intravenous antibiotic therapy. [8] Transfusion frequency is defined as (i) regular - requiring blood transfusion every three weeks to three months; (ii) occasional - every 4 months to once a year; and (iii) none or rare - patients who have never been transfused, or once in several years. [9]

Definition of endocrinopathies is based on the Thalassaemia International Federation (3rd edition) clinical guideline. [10] Diagnosis of cardiomyopathy is based on clinical evaluation including echocardiography by a paediatric cardiologist. Presence of gallstones is confirmed by an abdominal ultrasound. Patients' severity score was determined based on the Sripichai scoring. Patients with a total score ranging from 0 to 3.5 were categorized as mild phenotype; 4 to 7 as moderate while 7.5 to 10 was severe. [9]

Laboratory Methods

DNA was extracted from leukocytes in the peripheral blood samples of patients using the Macherey-Nagel DNA isolation kit. The quality of the DNA was assessed using Nanodrop spectrophotometer (Thermoscientific), Qubit dsDNA HS assay kit and gel electrophoresis. β globin mutations were identified by Sanger sequencing; large deletions and duplications were tested via Multiplex Ligation-dependent Probe Amplification (MLPA). α globin gene deletions (- α 3.7 / $\alpha\alpha$ deletion and - α 4.2 / $\alpha\alpha$ deletion) were identified by multiplex PCR. Restriction Fragment Length Polymorphism (RFLP) PCR was used to identify the *Xmn-1* polymorphism.

Statistical Analysis

All statistical data were analyzed using SPSS version 23. Chi-square test was used for categorical data, ANOVA for normative continuous variables, Kruskal- Wallis test for non-normative continuous variables. Significance level was set at a p -value of less than 0.05. For calculation of p -value for categorical data, the authors combined the mild and moderate categories into the non-severe group as opposed to the severe group.

Results

Patients characteristic

In this study, 99 patients with HbE β -thalassaemia were enrolled. The median age at the time of enrolment was 13 years (range from 1 to 38 years). Eighty-one patients were transfusion dependant while 18 were non-transfusion dependant. A total of seven patients were in the mild category, 78 in the moderate category while 14 patients were from the severe category (Table 1).

The age of diagnosis amongst the three groups was statistically significant [$p = 0.03$ (Table 1)]. Inspection of the mean rank suggests that patients from the severe group were diagnosed at an earlier age (mean age of 1.6 years) as compared to the mild group who were diagnosed at a later age (mean age of 3.14 years). All patients from the severe group had their first blood transfusion at less than 5 years of age, as compared to the non-severe group with 62.2% (51 patients) who received a blood transfusion at less than 5 years of age [$p = 0.005$ (Table 2)]. The mean Hb at presentation were significant among the three groups with a p -value of 0.02. Post-Hoc comparison using the Tukey HSD test indicated that the mean haemoglobin for the mild group (7.9 g/dL $\pm$ 1.97) was significantly different from the severe group (6.0 g/dL $\pm$ 1.00). There was no statistical difference in pre-transfusion Hb between all groups in our study (p -value 0.10) with Hb difference of 1 to 2g/dL. Only 87 (87.8%) patients had a documented HbF level at diagnosis; the data was incomplete for 12 patients. There was no difference in HbF level (%) at diagnosis amongst the three groups [p value 0.297, Table 1].

A total of 27 patients had a major infection. The documented infections were: pneumonia, bacteraemia (*Bacillus sp* and *Klebsiella pneumoniae*), meningitis, superficial skin abscess, liver abscess, pancreatic abscess, ascending cholangitis, lymphadenitis, infective endocarditis, *E.Coli* urinary tract infection, infected skin ulcer and acute otitis media.

In our study, the risk of infection was not associated with splenectomy [$p=0.17$ (Table 3)]. A total of five patients had osteoporosis while eight patients had osteopenia. Nine patients had cholelithiasis; of these seven underwent cholecystectomy. Ten patients had endocrinopathies which comprised: 1 hypothyroidism, 2 diabetes mellitus and 7 hypogonadisms. Forty-eight patients had short stature (Table 4).

Genotypic data

Excluding HbE mutation, a total of 14 β -gene mutation was found. The most common mutation in our population were IVS 1-5(G>C) found in 47 patients followed by CD 41/42 (-TTCT) found in 21 patients, IVS-II-654 (C>T) and CD17(A>T) with six patients each (Table 5). Other β gene mutations in our study population were: CD 19 (A>G) or Hb Malay and IVS-I-1(G>T) with four patients each, β -Filipino (three patients), nt -28 (A>G) (two patients), and one each of the following mutations: CD 15(G-A), CD 30, CD 123/124/125, IVS 1-2(T > C), CD35/36 and β -Lepore. The commonest β mutation in Malays were IVS 1-5(G>C) (58.8%) and CD 41/42 (-TTCT) (23.8%) whereas in the Chinese, IVS-II-654 (C>T) (44.4%) and CD 41/42 (-TTCT) (33.3%).

Of the 98 genotyped patients, only four patients had a single α gene deletion ($\alpha 3.7/ \alpha\alpha$); two were mildly affected and the other two moderately affected. None of our patients was homozygous for *XmnI* (+/+) polymorphism. Six patients within the mild group, 59 patients from the moderate group and six from the severe group were heterozygous for *XmnI* (+/-) polymorphism.

Genotypic phenotypic correlation

For the six common β -gene genotypes, haemoglobin level at diagnosis was low for most of the mutations (Table 5). The pattern of fetal haemoglobin was similar for each mutation. Majority patients with IVS 1-5(G>C), CD 41/42 (-TTCT), IVS-II-654 (C>T) and CD17(A>T) mutation were in the moderate and severe group.

However, patients with CD 19 (A>G) mutation were mostly presented with mild to moderate phenotype. The haemoglobin level for this mutation also was higher at diagnosis with mean of 7.4 g/dL. One patient with CD 41/42 (-TTCT) had a mild phenotype that is explained by the presence of one α gene deletion. His HbF level also was low with 17.6% and he was heterozygous for *XmnI* (+/-) polymorphism.

Discussion

Clinical profile of Malaysian HbE β -thalassaemia

HbE β -thalassaemia is a clinically diverse disease. In our study population, 78.79% had a moderate clinical severity based on the Sripichai scoring system (Table 1). Our percentage of transfusion-dependent patients were also higher i.e. 81.8% as compared to the study by Premawardhena et al, which ranged from 30% to 57% and the cohort of HbE β -thalassaemia patients in Singapore i.e. 49%. [11,12] Two possible explanation for this difference is the small number of mildly affected patients in our cohort in addition to those who were missed during the sampling period because of the infrequent clinic appointment.

In our study cohort, HbE β -thalassaemia patients with a severe phenotype presented at a significantly younger age of 1.6 years old (p -value 0.03). As described by Taher et al, transfusion-dependent or severe HbE β -thalassaemia patients present at the age of less than two years old, while those with thalassaemia intermedia present after two years of age. [13]

The mean Hb at presentation was significantly lowest for the severe group which was 6.07g/dL (p -value 0.02). This finding is similar to that of Taher et al where patients with a major phenotype present with a Hb of between 6 to 7 g/dL whereas those with an intermedia phenotype had a higher Hb level range of 7 to 10 g/dL. [13]

Our results did not show any statistical difference in initial HbF level among the three severity groups. In contrast to the study by Nuinoon et al in Thailand, where among their 618 HbE β -thalassaemia patients (of which 235 were phenotypically mild and 383 patients were severe), the HbF level was higher (42.5%) in the mild group in comparison to the severe group (31.9%). [14] A possible explanation is that our cohort had only a small number of patients within the mild and severe group.

Our study showed that 29 from a total of 99 patients (29.2%) required splenectomy. This is comparable to other case series which reported that the rate of splenectomy ranged from 17% to 50%. [11,12] The incidence of severe infections caused by encapsulated organisms was higher among splenectomised thalassaemia patients with the incidence of sepsis being 11.6% and the mortality rate of 7.3%. [15] Our results, however, showed that the risk of infection was not associated with splenectomy (p -value 0.13). Interestingly, a prospective control study by Jetsrisuparb et al among HbE β -thalassaemia paediatric patients revealed that infection rates did not differ between all severity groups of HbE β -thalassaemia regardless of splenectomy status in comparison to the normal population. Antibiotic prophylaxis and awareness of infection has helped to decrease the occurrence of severe infections among splenectomised patients. [16]

Only nine of our patients had cholelithiasis. Chronic hyperbilirubinemia and cholelithiasis may significantly worsen the phenotype of HbE β -thalassaemia patients. This is due to the homozygous inheritance of the 7/7 genotype of the promoter of glucuronyltransferase 1 gene (UGTA1A promoter) which results in increased bilirubin level hence the formation of gall stones. [4] This genotypic variant was however not addressed in our patients.

Genotype-phenotype correlation

This study revealed the distribution of β globin gene mutations among Malaysian HbE β -thalassaemia with genotype-phenotype correlation. The two most common β^0 gene mutations in our population are IVS 1-5(G>C) and CD 41/42 (-TTCT). This finding is similar to Thailand and Indonesia. [17,18] Thailand have CD 41/42 as the common mutation with 48.6 % in their HbE β -thalassaemia population while Indonesia have IVS 1-5(G>C) as the commonest. [17,18] The type of β globin gene mutations would explain the diversity of clinical phenotype of HbE β -thalassaemia patients. Patient with β^0 allele and severe β^+ allele have more severe clinical phenotype as compare to mild β^+ allele who presented with a milder phenotype. [17,19] Our result shows a similar finding where most of our patient with β^0 allele and severe β^+ allele presented with moderate to severe phenotype. These includes IVS 1-5(G>C) , CD 41/42 (-TTCT), IVS-II-654 (C>T) and CD17(A>T).

The second factor that affecting the clinical phenotype in HbE β -thalassaemia is the presence of α globin gene deletion. [17] One of our patient with β^0 allele; CD 41/42 (-TTCT) have mild phenotype as he is also co-inherit $-\alpha 3.7/\alpha\alpha$ deletion. Those who co-inherit α globin gene deletion would have lesser alpha-globin chain production, which results in a more balanced globin synthesis, thus a milder phenotype. [20] In a study comprising over 900 Thai HbE β -thalassaemia patients, 8.8% of patients in this group were shown to have α -thalassaemia, of which the most common α globin gene deletion was $-\alpha 3.7 / \alpha\alpha$ deletion (n=51), and $-\alpha 4.2 / \alpha\alpha$ deletion (n=8). [21]

The third factor is the presence of other genetic modifiers which would help to compensate for the reduced beta-globin chains. [17] For instance, the presence of homozygous *XmnI* (+/+) polymorphism allele help to reduce the disease severity by increased synthesis of foetal haemoglobin and higher Hb level. [20] Eventually, none of our patients was homozygous (+/+) for *XmnI* polymorphism. Our study revealed 59 (75.6%) patients with heterozygous *XmnI* (+/-) polymorphism had moderate severity. This is similar to Wong et al with heterozygous *XmnI* (+/-) polymorphism being the most common finding among 58 Chinese and 49 Malays with β -thalassaemia. [22]

Our study is limited by the relatively small number of patients from both the mild and severe phenotypic group.

Conclusion

The age of presentation and haemoglobin level at initial diagnosis are useful as clinical predictors of disease severity. Our study did show genotype phenotypic correlation of β gene mutation of the most prevalent beta-thalassemia types in Malaysia children; thus it may play a role in influencing the disease severity. This will help the clinician in their decision making for early intervention and genetic counselling. However, it is not always possible to consistently predict the phenotype based on beta genotype alone as there are other genetic modifiers like α gene deletion and *XmnI* polymorphism. Infections in HbE β -

thalassemia is an important complication that should be promptly treated regardless of the splenectomy status.

Declarations

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Conflicts of interest/Competing interests:

The authors have no relevant financial or non-financial interests to disclose.

Code availability:

not applicable

Authors' contributions:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by F.M, S.A.A.R and Z.A.L. The first draft of the manuscript was written by F.M and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

F.M, Z.A.L: data curation, investigation, writing—original draft, writing -editing and review, supervision. S.A.A.R, Z.M: data curation, investigation, writing—original draft and review. N.A.A.M, D.L.S.C, C.K.L, R.Z.A.R.S, N.A.A, H.I: writing—editing and review.

Ethics approval:

Approval was obtained from the ethics committee of Universiti Kebangsaan Malaysia. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Consent to participate:

Informed consent was obtained from all individual participants / legal guardians (for patients less than 18 years old) included in the study.

Consent for publication:

Not applicable

Availability of data and material:

All the data generated in the experiments are presented in manuscript

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Tables

Table 1

Demographic and haematological profile of patients with HbE β -thalassaemia

Characteristic	Mild (n=7)	Moderate (n=78)	Severe (n=14)	p-value
Gender, n				
Male	3 4	38	7	-
Female		40	7	
Ethnicity, n				
Malay	7	71	12	
Chinese	0	7	2	-
Age, years (mean $\pm$ sd)	11.3 $\pm$ 4.79	14.27 $\pm$ 8.11	21.79 $\pm$ 10.14	0.01*
Age at diagnosis (mean)	3.14 $\pm$ 1.86	2.96 $\pm$ 1.84	1.6 $\pm$ 0.9	0.03**
Age at receiving first transfusion				
< 5 years, n				
5-10 years, n	2	49	14	
>10 years , n	3	27	0	
	0	1	0	-
Haemoglobin level at diagnosis ,g/dL (mean $\pm$ sd)	7.9 $\pm$ 1.97	6.76 $\pm$ 1.49	6.07 $\pm$ 1.00	0.02**
Haemoglobin level at steady state, g/dL (mean $\pm$ sd)	9.36 $\pm$ 1.5	8.69 $\pm$ 1.23	8.21 $\pm$ 1.52	0.10*
Haemoglobin F level ^a , (%)	34.1 $\pm$ 20.9	45.05 $\pm$ 14.57	40.18 $\pm$ 18	0.297**
Haemoglobin F level ^b (g/dL, mean)	2.74 $\pm$ 1.86	3.05 $\pm$ 1.31	2.87 $\pm$ 0.95	0.732*
Transfusion interval				
Regular, n	1	66	14	
Occasional, n	1	7	0	-
None/rare , n	5	5	0	
^a HbF (%) is the value calculated from the quantitative measurement of HPLC with the relative value to total hemoglobin types.				

^b Absolute HbF is the absolute value calculated from the Hb level and percentage of HbF.

* *p* values were calculated by Kruskal Wallis test.

** *p* values were calculated by ANOVA.

Table 2

Age of first blood transfusion among severe vs non-severe group (mild and moderate group)

Age of first blood transfusion	Non severe	Severe	<i>p</i> -value
< 5 years old	51	14	0.005
> 5 years old	31	0	

Table 3

History of infection and splenectomy status

History of infection	Splenectomised (n=29)	Not splenectomised (n= 70)	<i>p</i> -value
Had infection	11	16	0.13
No infection	18	54	

Table 4

Spectrum of complications in HbE β -thalassaemia patients

Characteristic/complication	Mild (n=7)	Moderate (n=78)	Severe (n=14)
History of major infection, n	2	21	4
Spleen size (cm) (mean $\pm$ sd)	0.7 $\pm$ 1.25	3.23 $\pm$ 3.48	3.00 $\pm$ 4.24
Splenectomy n	0	17	12
Gall stone, n	1	5	3
Endocrinopathy, n	0	7	3
Cardiomyopathy, n	0	2	1
Height (percentile)			
<3 rd centile, n	3	38	7
3 rd -25 th centile, n	3	36	7
>25 th centile , n	1	4	0
Ferritin level ng/ml, (mean $\pm$ sd)	618 $\pm$ 268.9	1877 $\pm$ 1473	4909 $\pm$ 5539

Table 5

Common genotype and haematological data of HbE β -thalassaemia patients

B gene mutation Parameter	IVS1-5 (G>C) (n = 47)	CD41/42 (-TTCT) (n=21)	IVS-II-654 (C>T) (n=6)	CD 17 (A>T) (n=6)	CD 19 (Hb Malay) (n=4)	IVS-I-1 (G>T) (n=4)
Type of β allele	β°	β°	severe β^+ allele	β°	Mild β^+	β°
Clinical Severity						
Mild	3	1	0	0	2	1
Moderate	36	17	5	5	1	3
Severe	8	3	1	1	1	0
Ethnicity						
Malay	47	19	2	4	4	4
Chinese	0	3	4	2	0	0
Age at diagnosis (years)	2.8 $\pm$ 1.9	2.4 $\pm$ 1.7	2.7 $\pm$ 0.6	1.9 $\pm$ 1.8	2.4 $\pm$ 1.5	3.3 $\pm$ 2.1
Age receiving first transfusion (years)	2.8 $\pm$ 1.9	2.4 $\pm$ 1.7	2.7 $\pm$ 0.9	1.9 $\pm$ 1.8	2.4 $\pm$ 1.5	3.3 $\pm$ 2.1
Splenectomy (number)	12	9	2	1	1	0
Hb level at diagnosis (g/dL)	6.6 $\pm$ 1.4	7.0 $\pm$ 1.6	6.5 $\pm$ 1.7	6.3 $\pm$ 1.1	7.4 $\pm$ 3.4	6.5 $\pm$ 1.6
Hb level at steady state (g/dL)	8.8 $\pm$ 1.3	8.5 $\pm$ 1.2	7.8 $\pm$ 1.1	8.6 $\pm$ 0.9	9.4 $\pm$ 2.1	9.3 $\pm$ 0.5
Hb F level (%)	42.5 $\pm$ 10.5	58.1 $\pm$ 16.9	58.1 $\pm$ 16.9	44.7 $\pm$ 24.9	32.9 $\pm$ 23	48.9 $\pm$ 30.9
Hb F level (g/dL)	2.9 $\pm$ 1.0	3.1 $\pm$ 1.2	3.5 $\pm$ 1.9	3.1 $\pm$ 1.9	1.8 $\pm$ 1.1	3.3 $\pm$ 2.4
Note : Data shown as mean $\pm$ sd or number						