Key Determinants of Phenotypic Heterogeneity of Hb E/ β Thalassemia: A Comparative Study from Eastern India

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Received: 19 March 2019 / Accepted: 29 August 2019 / Published online: 6 September 2019
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Abstract HbE Beta thalassemia is phenotypically very diverse disease. We aim to study role of various genetic factors in determining severity of this disease. 243 diagnosed cases of HbE Beta thalassemia were included in this study. Patients were divided in two arms—transfusion dependent and non-transfusion dependent arms. Various factors (percentage of haemoglobin F, hemoglobin E, type of Beta mutation, Xmn1 polymorphism, alpha deletion, HPFH mutation) were evaluated in these patients. Xmn1 polymorphism (homozygous and heterozygous), presence of HPFH mutation and alpha deletion were more prevalent in NTDT arm versus TDT arm (p value < 0.001). Higher prevalence of severe beta mutation IVS 1-5 (G $\rightarrow$ C) mutation {64(61.54%) vs 38(27.34); p value < 0.001 } was found in TDT arm when above factors were excluded from analysis. Higher mean haemoglobin F and mean Hemoglobin E percentage was associated with NTDT arm (p value < 0.001). Various factors (hemoglobin F and E percentage, Xmn1 polymorphism, HPFH mutation, alpha deletion and IVS 1-5 Beta mutation) were identified to affect severity of this cohort.

Keywords NTDT · TDT · HbE Beta thalassemia · Genotype · Phenotype

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12288-019-01176-9>) contains supplementary material, which is available to authorized users.

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Introduction

Hemoglobin E Beta thalassemia is the commonest symptomatic hemoglobinopathy in many Asian countries. The major bulk (around 50%) of symptomatic thalassaemia patients is HbE-Beta thalassemia [1]. Of 2190 thalassemia patients registered at our institute i.e. Institute of hematology and transfusion medicine, Kolkata 1458 (66.57% cases) are of HbE/Beta thalassemia. A multicentric study done by I.C.M.R. “Jai Vigyan S&T Mission Project on Community Control of Thalassemia Syndromes” has shown that prevalence of HbE carrier rate and Beta thalassemia carrier rate is 4.4% and 3.9% respectively [2]. Phenotypic presentation of Haemoglobin E Beta thalassemia is highly variable ranging from those who never require blood transfusion to those who are dependent on it for survival [1]. Its wide diversity and limited understanding of natural history of this disorder makes treatment of this disorder challenging.

In last decade various studies have been published on genetic factors (xmn1 polymorphism, Beta mutation type, alpha gene deletion) affecting severity of this disease. Most of the studies evaluated various factors in isolation; however there are very few large scale studies which have comprehensively evaluated various abovementioned factor [3]. Moreover till date there is no large scale study from India on phenotypic variations of HbE-beta thalassaemia and its determinants. In this study we have documented wide range of phenotypic heterogeneity in these patients and evaluated various factors responsible for such a wide phenotypic heterogeneity in these patients.

Materials and Methods

Study Design Comparative study.

Place of Study Institute of Hematology and Transfusion Medicine (IHTM), Medical College Kolkata.

Study Population Diagnosed cases of HbE beta thalassemia (Transfusion dependent and Non Transfusion Dependent) attending Thalassaemia Clinic and day care of IHTM, Medical College Kolkata, between March 2015 and August 2016. Diagnosis of HbE-beta thalassaemia was done by HPLC using Bio-Rad Variant II machine (beta thalassemia short program was used for analysis). Wherever there was doubt regarding diagnosis, parental screening and mutation studies were done to confirm the diagnosis. All age groups and both the sexes were included in analysis.

Patients were Divided into NTDT and TDT Group Using Standard Guideline

- A. Transfusion Dependent: Those who receive more than 8 units PRBC transfusion per year.
- B. Non Transfusion Dependent: Those who receive less than 8 units PRBC transfusion per year.

Consent Informed consent was obtained from patient if age > 18 years or from the guardian if age of patient is less than 18 years.

Clinical Parameters

A detail history was taken from the patients or their parents and thorough clinical examination was done.

Laboratory Data/Evaluation

- (a) Hematological parameters were analyzed by Sysmex (KX-21) automated cell counter.
- (b) Serum ferritin was done by ELISA based assay.

DNA Isolation and Mutation Analysis

Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) by proteinase-K digestion and a standard high salt extraction method [4]. Ten common known Beta-thalassemia mutations IVS1-5(G → C), IVS1-6, CD15(G → A), CD30(G → C), CD41/42(-CTTT), CD8/9(+G), CD39(C → T), CD5(-CT), CD16(-C), IVS(1-1) were detected by using amplification refractory mutation system polymerase chain reaction (ARMS-PCR) [5]. Hundred ng genomic DNA was amplified with DNA

polymerase (AmpliTaq Gold; Applied Bio systems, Inc.[ABI], Foster City, CA) in two separate PCR reaction mixtures, containing 1 × PCR buffer (Applied Biosystems), 2 mM MgCl₂, 0.25 mM each dNTP, 20 pmol generic antisense primer, and 20 pmol of one of the two allele-specific primers. To assess the success of PCR, an internal control 860-bp nucleotide was amplified in both reactions with specific primers (20 pmol/reaction). The PCR reactions were performed in the following conditions: 94 °C 5 min, 63 °C 60 s, followed by 25 cycles of 72 °C for 90 s, 94 °C for 60 s, 63 °C for 60 s and 72 °C for 3 min for the final extension. The amplified products were separated by electrophoresis on 2% agarose gel stained with 0.5 mg/mL ethidium bromide and visualized and photographed under a UV transilluminator. XmnI polymorphism was identified following primer sets and protocol by Sanjana et al. [6].

Alpha Deletions (3.7 and 4.2) were detected by using the primer set and protocol as described by Liu et al. [7]; with necessary modifications with the concentrations of primer and high GC content stabilizers (DMSO, Betaine) to amplify the respective amplicons using Qiagen Hot Taq polymerase. HPFH mutation was detected protocol using Old et al. [8].

Statistical Analysis

Categorically variables are expressed as number of patients and % of patients and compared across the groups using Pearson's Chi Square test for independence of attributes. Continuous variables are expressed as mean ± standard deviation and compared across the 2 groups using unpaired t test. The statistical software SPSS version 20 has been used for the analysis. An alpha level of 5% has been taken, i.e. if any *p* value is less than 0.05 it has been considered as significant.

Results

Two forty-three HbE-Beta Thalassemia patients were included in the study. Out of them 104 were in transfusion dependent and 139 were in non- transfusion dependent arm. Both the groups were similar in terms of age (Table 1) and sex distribution (Table 1). Various phenotypic parameters were compared in transfusion dependent and non dependent arm (Table 1). Age at presentation was significantly lower in TDT arm (*p* value < 0.001). Average age of presentation in TDT arm was 6.71 years as against 13.38 years in NTDT arm. Forty-nine cases (55%) in TDT arm presented after 4 years of age and 15 cases (14.4%) cases presented after 14 years of age. Whereas, fourteen cases (13.33%) in NTDT arm presented earlier than 4 years of age. TDT arm lagged in terms of linear growth (Fig. 1a,

Table 1 Phenotypic characteristics

	TDT n=104 Mean $\pm$ SD	NTDT n=139 Mean $\pm$ SD	<i>p</i> value
Male	n = 44	n = 54	0.587
Female	n = 60	n = 85	
Age (year)	19.8 $\pm$ 8.92	21.45 $\pm$ 8.84	0.153
Age at diagnosis (year)	6.71 $\pm$ 7.15	13.38 $\pm$ 10.32	< 0.001
Hemoglobin (g/dl)	6.17 $\pm$ 1.2	7.09 $\pm$ 1.34	< 0.001
Hemoglobin F (%)	13.85 $\pm$ 11.16	25.34 $\pm$ 13.81	< 0.001
Hemoglobin E + A2 (%)	40.09 $\pm$ 18.02	59.06 $\pm$ 13.04	< 0.001
Total number of transfusions	114.36 $\pm$ 63.46	9.04 $\pm$ 26.19	< 0.001
Spleen size (cm)	6.46 $\pm$ 1.81	7.1 $\pm$ 1.78	0.013
Height (cm)	139.12 $\pm$ 14.63	141.65 $\pm$ 18.15	0.245
Weight (kg)	35.26 $\pm$ 10.63	38.42 $\pm$ 11.71	0.031
Tanner staging genitals	3.25 $\pm$ 1.41	4.02 $\pm$ 1.24	< 0.001
Ferritin (ng/ml)	1588.57 $\pm$ 1131.7		< 0.001
Tanner pubic hair	3.32 $\pm$ 1.41	4.04 $\pm$ 1.25	< 0.001
Ferritin (ng/ml)	1588.57 $\pm$ 1131.7

Fig. 1 Comparison of height and weight percentile among TDT and NTDT

b) and pubertal growth as compared to NTDT arm (Table 1).

Mean baseline haemoglobin, haemoglobin E (HbE%), haemoglobin F (HbF%) were higher in NTDT arm (Table 1). It was observed that 7.91% (n = 11) cases in NTDT arm had Haemoglobin E less than 40% and 50.36% (n = 70) cases in this arm had greater than 60% of Haemoglobin. As against this, only 16.35% (n = 17) cases in TDT arm had haemoglobin E greater than 60% and 48.08%

(n = 50) cases had haemoglobin E less than 40% in this arm.

This difference in Haemoglobin E distribution was statistically significant (Supplementary Table 1). In further analysis of cases where those with Xmn1 polymorphism, alpha deletion and HPFH mutation were excluded, higher haemoglobin E % was documented in NTDT arm; suggesting high haemoglobin E may be independently responsible for reducing severity (Supplementary Table 2).

For beta mutation status no statistically significant difference was found for frequency of various types of beta mutation (Table 2). When subgroup analysis was done by excluding patients having xmn1 polymorphism, HPFH mutation and alpha deletion, higher prevalence of IVS 1-5 (G $\rightarrow$ C) mutation in the beta globin gene 64 (61.54%) vs 38 (27.34%); *p* value < 0.001 was found in TDT arm compared to NTDT arm (supplementary Table 3).

Xmn1 polymorphism (homozygous and heterozygous), presence of HPFH mutation and alpha deletion were more prevalent in NTDT arm vs TDT arm. *p* value was less than < 0.001 for all three parameters analysed (supplementary Table 3, 4, 5). Higher haemoglobin F % was observed in those with Xmn1 polymorphism (supplementary Table 4, 7).

Discussion and Conclusion

Haemoglobin E Beta thalassemia is a major health burden especially in Eastern India and many developing Asian countries [9]. There is great diversity in presentation of this disorder. Many genetic factors like, XMN1 polymorphisms, alpha deletion are identified as determinants of

Table 2 Beta mutation analysis of patients

	Group		Total	<i>p</i> value
	TDT n (%)	NTDT n (%)		
Beta gene MUTATION				
IVS 1-5 (G → C)	72 (69.23)	100 (71.94)	172 (70.78)	0.17
CD 8/9	3 (2.88)	5 (3.6)	8 (3.29)	
IVS 1-1	3 (2.88)	0 (0)	3 (1.23)	
CD 41/42	2 (1.92)	6 (4.32)	8 (3.29)	
CD 15	10 (9.62)	10 (7.19)	20 (8.23)	
CD 16	1 (0.96)	0 (0)	1 (0.41)	
CD 30	1 (0.96)	2 (1.44)	3 (1.23)	
CD 39	0 (0)	2 (1.44)	2 (0.82)	
CD 5	0 (0)	2 (1.44)	2 (0.82)	
IVS 1-6	0 (0)	3 (2.16)	3 (1.23)	
UNKNOWN	12 (11.54)	9 (6.47)	21 (8.64)	
Total	104 (100)	139 (100)	243 (100)	

severity of this disease. In this study we documented clinical heterogeneity of this disease and evaluated various factors determining severity of this disease.

It was observed that age of presentation was significantly higher in NTDT patients as compared to TDT patients. There is growing understanding that phenotype of a case of E Beta thalassemia may evolve over a period of time. Many non transfusion dependent patients may require regular transfusion and reverse may also be true. What causes this shift in phenotype is poorly understood. There is good evidence to show that transfusion may be stopped successfully in some of these patients without deleterious effect on their health [3]. In this study it was observed that 14.4% cases ($n = 15$) required regular transfusion support after age of 14 years. It was also observed that for 12 of 140 cases (8.57%) regular transfusion could be successfully stopped; similar findings were noted in study from Sri Lanka. These findings suggest that phenotype may evolve over a period of time.

Statistical significance was observed for % of cases below 3rd percentile for weight and height in two arms suggesting significant percentage of patients had growth failure in transfusion dependent arm. Though less than that of TDT arm, 44.6% and 32.37% of cases in NTDT arm had height and weight below 3rd percentile respectively as compared to general population when assessed by IAP (Indian academics of pediatrics) growth chart. Total of 55.14 and % of cases had height and weight less than 3rd percentile respectively, suggesting overall poor growth in these patients. Similar findings suggesting overall poor linear growth was observed in study of 378 Thai HbE/Beta thalassemia patients where 75% subjects had growth retardation [10].

Mean haemoglobin was significantly higher in NTDT arm, but the difference of hemoglobin was just 0.92 g/dl in two arms. This suggests that there is very small difference in average haemoglobin in two arms causing large phenotypic variation. Similar results were noted from Sri Lankan study where average difference was 1 to 2 gm/dl [3].

We also assessed whether the type of beta mutation affects severity of these patients. There have been contrasting results regarding role of beta mutation type in determining severity of these cases. Various studies in last decade suggest that the severity of the β mutation may be an important but do not consistently influence on the clinical diversity of HbE/ β -thalassaemia [11]. In present study there was no statistical difference in frequency of various beta gene mutation in two arms (p value 0.170). IVS 1-5 (G → C) was most common beta mutation detected in both the arms. We did subgroup analysis where patients with *xmn1* polymorphism, alpha deletion and presence of HPFH mutation were excluded. We found that frequency of severe beta mutation IVS 1-5(G → C) was significantly higher in TDT (61.54% vs 27.34%; p value < 0.001) patients suggesting role in pathophysiology.

Mean haemoglobin F was higher in NTDT arm as compared to TDT arm (p value < 0.001). *Xmn1* polymorphism (homozygous or heterozygous) was also more common in NTDT arm (45.32 vs 4.81%; p value < 0.001), correlating with increased hemoglobin F in this group of patients. Average frequency of *Xmn1* polymorphism in West Bengal is 14.295% as assessed by screening of 928 normal subjects during this study. In a study of Thai patients with Hb E/ β - thalassemia, *Xmn1*/+ genotype [12] had higher total hemoglobin (8.5 compared to < 7.0

g/dl) and higher fetal hemoglobin concentrations than those with a XmnI^{−/−} genotype. In this study there was positive correlation between concentrations of fetal hemoglobin and XmnI polymorphism. Similarly in the longitudinal study of HbE/ β -thalassaemia from Sri Lanka, a positive correlation between concentrations of haemoglobin and fetal hemoglobin, and homozygosity for the XmnI polymorphism was reported [3]. Patients with the XmnI^{+/+} genotype were identified only in mildly affected patients; conversely, patients with “severe” phenotypes, including a history of early presentation to medical attention, were more likely to have XmnI^{−/−} [3]. Proportion of patients with no xmnI polymorphism in this study was 95.19% in TDT arm vs 54.68% NTDT arm (p value < 0.001).

Presence of alpha gene deletion has been implicated in milder forms of E Beta thalassemia. In present study alpha deletion was more common in NTDT group (40.29 vs 4.81; p value < 0.001). This was higher than that of tribal population of West Bengal which was reported as 18% [13]. Findings are consistent with studies from Sri Lanka and India where alpha was found to be more common in milder form of disease [14]. Associated presence of hereditary persistence of fetal haemoglobin (HPFH) was also studied during this study. It was found that NTDT group had higher percentage of cases inheriting this mutation (p value 0.0020), suggesting role in decreasing severity of these patients.

It was also noted that average haemoglobin E % is higher in NTDT arm vs TDT arm (59.06 ± 13.04 vs 40.09 ± 18.02 p value < 0.001). We further divided these patients into two groups those with hemoglobin E less than 40% and Hemoglobin E greater than 60%. It was observed that 11% of patients in NTDT arm had hemoglobin E less than 40% and 50.36% cases had hemoglobin E greater than 60%. As against this only 16.35% cases in TDT arm had Haemoglobin E greater than 60% and 50% cases had hemoglobin E less than 40%. To our best of knowledge no study has demonstrated hemoglobin E % modify phenotype in HbE Beta thalassemia. We further did analysis to see correlation between haemoglobin E % and total haemoglobin in NTDT group. We excluded cases with xmnI polymorphism, alpha deletion and HPFH mutation from analysis. We found higher haemoglobin E was associated with lower total haemoglobin (supplementary Table 1 and supplementary Fig. 1). We hypothesised that shift of oxygen dissociation curve to right in presence of high haemoglobin E could be responsible for less severe phenotype.

To conclude, significant number of patients in both the groups had growth failure though it was higher in TDT arm. Serum ferritin was higher in TDT arm. Beta gene mutation has complex interaction with presence of xmnI polymorphisms, alpha deletion and HPFH mutation. When only IVS 1-5 (G $\rightarrow$ C) was analysed in absence of above

polymorphisms it was noted that prevalence of IVS 1-5 (G $\rightarrow$ C) was significantly greater in TDT arm suggesting its role in severity. We have also demonstrated that phenotype is not only modified by haemoglobin F % but also by haemoglobin E %.

Still all the factors responsible for such diverse nature of this disease are not completely understood and further studies are warranted to understand variation in this common disease over range of just 1 gm/dl of total hemoglobin.

Compliance with Ethical Standards

Conflict of interest All the authors declare that there is no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (include name of committee + reference number) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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