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HAPLOTYPE ANALYSIS OF BANGLADESHI β-THALASSAEMIA PATIENTS: A PILOT STUDY

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Abstract

β-thalassaemia is one of the major genetic disorders in Bangladeshi population. Nevertheless, systematic study on the genetic basis of this disease in Bangladeshi population is very limited. The major aim of this study was to identify and characterize the β-globin gene cluster haplotype in Bangladeshi β-thalassaemia patients. For this, β-thalassaemia patients diagnosed on haematological observations were tested at the genetic level for different β-globin cluster haplotypes. Twenty-eight β-globin gene clusters of fourteen confirmed β-thalassaemia patients were analyzed using PCR amplification and Restriction Fragment Length Polymorphism (RFLP). Type VII haplotype was found to be the most common β-globin gene cluster haplotype in the studied population. Two of the patients had Type VII haplotype in homozygous form. Only one patient had atypical haplotype in one locus. The study reports β-globin cluster haplotype of fourteen Bangladeshi patients for the first time. However, it should be noted that small size precludes the possibility of determining the detailed distribution of different haplotypes and their association with different β-thalassaemia mutations.

Introduction

Bangladesh has a high β-thalassaemia prevalence rate. According to a study of the World Health Organization (WHO), ~3.0% of the population are carriers of β-thalassaemia(1). Effective management of this disease requires proper diagnosis and appropriate course of treatment. However, lack of information on the genetic background of β-thalassaemia patients in Bangladesh is one of the major impediments in achieving this goal.

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A haplotype is defined as a combination of alleles along a specific chromosome[4]. In a previous study carried out by Orkin and co-workers[5], it was found that β- globin cluster haplotypes showed strong linkage disequilibrium with β-thalassaemia mutations. Thus, β- globin cluster haplotypes can be used for the identification of β-thalassaemia[6] chromosome and would subsequently aid both prenatal and postnatal diagnosis of β-thalassaemia[7] and study of population affinities.

For the purpose of the study, samples from 14 patients were taken for polymerase chain reaction (PCR) amplification followed by restriction fragment length polymorphism (RFLP) analysis. It is to be noted that in a previous study Ayub and co-workers had analysed the β-globin gene mutations for these patients[2]. Seven regions within and around the β-gene cluster were amplified using seven sets of primers as described in previous studies. These regions were chosen because of the presence of polymorphic restriction sites within them. For DNA from each patient, the PCR products were treated with five different enzymes. The polymorphic restriction sites studied were: (1) *Hind*II 5′ of ε; (2) *Hind*III 5′ of $^{G}\gamma$; (3) *Hind*III in the IVSII 5′ of $^{A}\gamma$; (4) *Hinc*II in the ψβ; (5) *Hind*II 3′ of ψβ; (6) *Ava*II in β and (7) *Hinf*I *in* β. For classification of the haplotypes the method of Orkin and co-workers [5] was followed (Table 1).

Table 1. Patterns of Haplotypes (*The β haplotypes have been constructed from polymorphic sites as *Hinc*II/ε, *Hind*III Gγ, *Hind*III Aγ, *Hind* II/3′ψβ, *Hinc*II/5′ψβ, *Ava*II/ β, and *Hinf*I/ β).

Haplotype	Pattern*
I	(+ - - - - + +)
II	(- + + - + + +)
III	(- + - + + + -)
IV	(- + - + + - +)
V	(+ - - - - + -)
VI	(- + + - - - +)
VII	(+ - - - - - +)
VIII	(- + - + - + -)
IX	(- + - + + + +)

To determine the heterozygous haplotypes, it was assumed that the heterozygous patient possessed one common haplotype and a rare haplotype rather than two different rare haplotypes, as assumed by Steinberg *et al*.[11].

Materials and Methods

Sample collection: Samples were collected from the patients of a children's hospital ranging from 1 to 15 years. It is to be noted that these samples were the same as the ones used by Ayub *et al.* in their study [2]. 2 ml blood samples were collected from each patient

using a sterilized disposable hypodermic needle with minimum possible trauma. The samples were transferred to 2.5 ml EDTA coated test tubes and stored at – 20°C.

Isolation of genomic DNA: Lysis buffer was used to remove the non-nucleated cells. Genomic DNA was isolated from nucleated blood cell using modified DNAzol (Invitrogen) method. The concentration of the isolated DNA was measured by a NanoDrop™. The quality of genomic DNA was also tested by gel electrophoresis. The isolated genomic DNA was then stored at –20°C until further use.

PCR amplification: Seven sets of RFLP primers were used in this experiment (Table 2). The primers were dissolved in TE buffer at first, according to the manufacturer's (IDT™) instruction and then diluted 10 times before using for the PCR. The primers were stored at –20°C. Samples from 14 patients were PCR amplified for 40 cycles following the conditions as- hot-start 95°C for 5 min, denaturing at 94°C for 30 sec, annealing for 40 sec according to primers, Tm (Table 2), extension at 72°C for 30 sec, and final extension for 5 min at 72°C. After amplification, the bands were observed by gel electrophoresis in 1.5% agarose gel.

Table 2. PCR primers with their respective Tm values.

Primers		Sequence	Tm (°C)	References
Primer 1	Forward	5′ TCTCTGTTTGATGACAAATTC 3′	50.7	(Rahimi *et al.* 2003)
	Reverse	5′ AGTCATTGGTCAAGGCTGACC 3′	58.5	
Primer 2	Forward	5′AGTGCTGCAAGAAGAACAACTACC 3′	58.8	(Rahimi *et al.* 2003)
	Reverse	5′ CTCTGCATCATGGGCAGTGAGCTC 3′	63.9	
Primer 3	Forward	5′ ATGCTGCTAATGCTTCATTAC 3′	52.6	(Rahimi *et al.* 2003)
	Reverse	5′ TCATGTGTGATCTCTCAGCAG 3′	56.5	
Primer 4	Forward	5′ GAACAGAAGTTGAGATAGAGA 3′	52.6	(Falchi *et al.* 2005)
	Reverse	5′ ACTCAGTGGTCTTGTGGGCT 3′	58.4	
Primer 5	Forward	5′ GTACTCATACTTTAAGTCCTAACT 3′	53.6	(Rahimi *et al.* 2003)
	Reverse	5′ TAAGCAAGATTATTTCTGGTCTCT 3′	53.6	
Primer 6	Forward	5′ GTGGTCTACCCTTGGACCCAGAGG 3′	65.6	(Rahimi *et al.* 2003)
	Reverse	5′ TTCGTCTGTTTCCCATTCTAAACT 3′	55.3	
Primer 7	Forward	5′ AGTTAGAGGCTTGATTTGGAGG 3′	56.7	(Falchi *et al.* 2005)
	Reverse	5′ GTTAAGGTGGTTGATGGTAAC 3′	54.6	

Restriction digestion: Concentrations of the DNA which were confirmed by PCR using specific primers for β-globin gene, were measured with a NanoDrop™. The amount of the required restriction enzyme was calculated from the estimated DNA concentration. 10 µl of PCR product was taken into an Eppendorf tube. 2.5 µl of buffer (supplied with

the enzymes by TAKARA™) was added to it and kept at 4°C for 5 min. Next 0.6 µl of enzymes were added to each tube and the final volume was made 25 µl by adding PCR water. Enzymes were selected based on the studies by Falichi *el al.*[9] and Rahimi *et al.*[10]. The tubes were incubated overnight at 37°C. The digested samples were loaded on a 1.5% agarose gel using DNA loading buffer. The banding pattern was observed under an UV-transilluminator.

Results and Discussion

Twenty-eight β-globin gene clusters from 14 patients were analysed for their haplotypes with specific RFLP primers (Table 3).

Table 3. Patients and their respective haplotypes and *Xmn*I patterns. Patient IDs correspond to the patient number reported in Ayub *et al*[2]. The presence or absence of the *Xmn*I restriction sites have been indicated by the – or + sign on the 5th column of the table.

Patient No	Patient ID	Haplotypes	Digestion Pattern	*Xmn*I Polymorphism
1	Patient 2	+ - - - - + +/ + - - - - - +	Type I/ VII	-
2	Patient 3	+ - - - - - +	Type VII	-
3	Patient 4	+ - - - - - +	Type VII	-
4	Patient 5	+ - - - - + +/ - + - + + - + or + - - - - - +/ - + - + + + +	Type I/ IV or Type VII/ IX	+/ -
5	Patient 6	+ - - - - + +/ + - - - - - +	Type I/ VII	+/ -
6	Patient 8	+ - - - - + +/ + - - - - - +	Type I/ VII	-
7	Patient 9	+ - - - - - +/ + - - - - - -	Type VII/ atypical	-
8	Patient 10	+ - - - - + +/ - + - + + - + or + - - - - - +/ - + - + + + +	Type I/ IV or Type VII/ IX	+/ -
9	Patient 11	+ - - - - + +/ + - - - - - +	Type I/ VII	-
10	Patient 12	+ - - - - + +/ - + - + + - + or + - - - - - +/ - + - + + + +	Type I/ IV or Type VII/ IX	+/ -
11	Patient 13	+ - - - - + +/ - + - + + + +	Type I/ IX	+/ -
12	Patient 14	+ - - - - + +/ - + - + + + +	Type I/ IX	+/ -
13	Patient 15	+ - - - - + +/ + - - - - - +	Type I/ VII	+/ -
14	Patient 16	+ - - - - + +/ + - - - - - +	Type I/ VII	-

Definitive haplotype of 11 patients were determined by following the classification method of Orkin and co-workers[5] and the criteria of Steinberg *et al*.[11]. The remaining three patients (patients 4, 8 and 10) had Type I/IV haplotype or Type VII/IX haplotype. Among the 11 definitive haplotypes, the major observed pattern was Type VII which was found in 11 loci. Of them 7 were heterozygous with other haplotypes (*viz*. Type I (for patient 1, 5, 6, 9, 13, 14) and atypical (for patient 7)) and 4 were homozygous where two loci contributed by each of patients 2, 3. The second most prevalent pattern was type I

which was found to be in heterozygous form in 8 loci. Restriction patterns, haplotypes and *Xmn*I polymorphism of different patients along with their β-thalassaemia mutations[2] are shown in Table 3.

β Thalassaemia has a high frequency in the Mediterranean region, Africa, South-East Asia and the Indian subcontinent. The treatment for β thalassaemia is expensive and imposes a significant burden on the available resources for healthcare [14]. Thus, for a country like Bangladesh, carrier detection, genetic counselling and prenatal diagnosis programmes can play an important role in the management of the disease. Each population group in which the disease is prevalent has a different spectrum of β globin gene mutations and haplotypes[15-17]. This study of 28 β-thalassaemia alleles in patients from different regions of the country shows that there is considerable difference in haplotypes in Bangladeshi population. Thus, results of the current study have implications for disease diagnosis, population genetics and phenotype–genotype correlation of the disease.

Three definite (Type I, Type VII and Type IX) and one possible haplotype pattern (Type IV) of the β-globin gene cluster were found in the Bangladeshi patients. Additionally, one atypical pattern (+ - - - - - -) was also observed. Of the established patterns, the most frequently occurring one was type VII (+ - - - - - +). Eleven chromosomes (out of 28) had this haplotype. Additionally, it is possible that the three patients whose haplotype could not be determined unambiguously, namely patients 4, 8 and 10, had this haplotype in 3 additional chromosomes (Table 3). Previous studies have revealed that a number of populations from diverse ancestries have this haplotype. Examples include populations of Portuguese[18], Mediterranean[9], South-East Asian, Sri Lankan[21], Japanese[22] ancestry and so on. This type VII is the only haplotype which was found to be homozygous in two patients (patients 2 and 3). The remaining haplotypes were in the heterozygous form (Table 3).

The second most prevalent haplotype is type I, which was found in 8 chromosomes (out of 28). The pattern of this type is (+ - - - - + +) which means +ve for *Hinc* II/ε, -ve for *Hind*III Gγ, –ve for *Hind*III Aγ, -ve for *Hind*II/3′ψβ, –ve for *Hinc*II/5′ψβ, +ve for AvaII/ β, and +ve for *Hinf*I/ β. The third major haplotype pattern found in this study is that of type IX. This type consists of a -ve site for *Hinc* II/ε, +ve for *Hind*III Gγ, -ve for *Hind*III Aγ, +ve for *Hind* II/3′ψβ, +ve for *Hinc*II/5′ψβ, +ve for AvaII/ β, and +ve for *Hinf*I/ β, which comprises the pattern (- + - + + + +). This pattern was observed in two chromosomes.

Among others, one chromosome showed atypical haplotype (+ - - - - - -). Six chromosomes of three patients (Patients. 4, 8 and 10) showed ambiguous haplotype pattern, which can be classified as Type 1/ IV or Type VII/ IX. Interestingly, all the three patients carried HbE and IVS-I-5 (G>C) mutations in heterozygous form (Table 3) and Ayub *et al.*[2]. Additionally, they were heterozygous for *Xmn*I polymorphism (Table 3).

Of the fourteen patients, nine had Type VII haplotype pattern in at least one chromosome. Interestingly, six of them (66.66 %) had IVS-I-5 (G>C) mutation in at least one of their chromosomes and among them, four had mutations in both the chromosomes. This, suggests close association of Type VII haplotype with the IVS-I-5 (G>C) mutation. It is to be noted that all the three patients whose haplotypes could not be resolved unambiguously had IVS-I-5 (G>C) mutation. Thus, it is likely that these patients had Type VII haplotype pattern as well, suggesting Type I/VII haplotype pattern considering both chromosomes.

Five patients (Patients 1, 2, 3, 13 and 14) of the present study were homozygous mutants of *HBB* gene as revealed by Ayub *et al.*[2]. However, three of them (Patients 2, 13 and 14) were heterozygous for β-globin gene cluster haplotype. They had Type I/VII haplotype pattern. Additionally, patient 15 was heterozygous for *Xmn*I site. This can be explained as a consequence of either acquisition of new mutations or recombination within the region of β-globin cluster analyzed.

Half of the analyzed patients were heterozygous for *Xmn*I polymorphism, rest were negative for *Xmn*I. Previous studies reported a positive correlation between *Xmn*I polymorphism and HbF level[25]. Nevertheless, detailed study by Neishabury and co-workers[26] failed to identify association between *Xmn*I polymorphism and the incidence of the mild form of β-thalassaemia, thalassaemia intermedia. We aim to extend this study on a larger cohort of patient samples to find more evidence.

The present study attempted at identifying the different haplotypes present in Bangladeshi population. However, the small sample size precludes drawing a conclusion regarding the distribution of different β-globin gene cluster haplotypes. Further study is required to draw definitive association between β-thalassaemia causing mutations in *HBB* gene and different haplotypes in the population, which can be utilized in clinical diagnosis of the disease.

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