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Assessment of molecular and clinical characteristics in blood transfusion dependent HbE/β-thalassemia patients

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Abstract

Hb E/β-thalassemia is one of the most common forms of hemoglobinopathies worldwide. This life-threatening genetic disorder is not completely curable. This study aims to investigate the correlation of mutations with clinical manifestations among the transfusion dependent Hb E/β-thalassemia patients using molecular, hematological and biochemical methods. A total of 60 blood samples were collected from regular blood transfusion-dependent HbE/β-thalassemia patients. Mutations within *HBB* gene were analysed by Sanger sequencing, BLAST 2.0, HbVar database and Alibaba 2.1. Clinical data were analysed based on CBC, Hemoglobin Electrophoresis and Serum Ferritin assay. Results demonstrate that a total of three mutations-deletion of A (+23bp in *HBB* promoter region), c.9 T>C and c.79 G>A were detected among the study population, where deletion of A (+23bp in *HBB* promoter region) is a novel mutation. A total of three (USF, AP-1 and GATA-1) important putative binding sites were identified within the mutated region of *HBB* gene. Hematological analysis also showed remarkable correlation between the mutations and clinical manifestations in the patients. Reduced levels of RBC, Hb, MCV and MCH were found among the patients than the normal. Highly increased level of serum ferritin was also found among the patients due to the frequent destruction of RBCs. In conclusion, the findings of this study will be helpful to understand the mutational and hematological status of HbE/β-thalassemia patients. And this study will also be beneficial for effective molecular drug designing, drug response and other therapeutic approach for HbE/β-thalassemia patients.

Keywords: Hb E/β-thalassemia, HbVar database, Mutations, Transcription factor.

INTRODUCTION

Thalassemia (also known as Mediterranean anemia, Cooley's anemia, Beta-thalassemia or Alpha thalassemia) is an inherited blood disorder affected by an abnormal form of hemoglobin. This specific type of blood disease results in excessive destruction of red blood cells which in turn leads to anaemia (Weatherall *et al.,* 1996). The two main types of thalassemia are α and β-thalassemia. Clinically, thalassemia presents as beta-

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thalassemia trait or minor (β+ or β^0), intermedia (β+/β+; β+/β^0) or major (β^0/β^0) (George, 1994). When β-thalassemia is inherited together with a hemoglobin E (HbE) allele, the resulting condition, HbE-βthalassemia (HbE-βThal), is sometimes characterized by a severe, transfusion-dependent thalassemia major (Weatherall, 2000).

The Hb E/β-thalassemia is one of the most common forms of hemoglobinopathies worldwide (Weatherall *et al.*, 1996). Hemoglobin E (β26; Glu>Lys) is the most common hemoglobinopathy in Southeast Asia and the second prevalent worldwide (Vichinsk, 2005; Rees *et al.*, 1998). The HbE is a splice variant of normal β-globin protein. This cryptic splice site (related to HbE) is not normally used for mRNA processing. This new splice site competes with the normal splice site and thus produces a protein with a Lys instead of a Glu at position 26 (Weatherall, 2000). This variant (HbE) is thus the protein, produced by the said mutation. HbE can be present in both heterozygous and homozygous state. The primary clinical importance of HbE trait arises when the β^E allele interacts with other β-thalassaemia mutations leading to moderate-to-severe anaemia known as HbE/β-thalassaemia (Orikin *et al*., 1982).

Worldwide, patients with hemoglobin E-beta-thalassaemia (Hb E/β-thalassaemia) represent approximately 50 per cent of those affected with severe beta thalassaemia (Ong-Ajyooth *et al*., 1987). The highest frequencies are observed in India, Bangladesh and throughout Southeast Asia, particularly in Thailand, Laos and Cambodia, where it is common for individuals to inherit alleles for both hemoglobin E (HbE) and beta-thalassaemia (Modell & Darlinson, 2008). The number of patients suffering from thalassemias (beta major and HbE beta) with different levels of severity is estimated to be approximately 60,000–70,000 in Bangladesh. With the birth rate of 21.6/1000, it could be estimated that nearly 2500 thalassemia major cases are added every year in Bangladesh (Weatherall *et al.*, 2001).

Since, the mutational and clinical data of the thalassemia patients are not adequate in Bangladesh, the present study aimed to investigate the correlation of genetic polymorphisms in *HBB* gene with clinical manifestations among the blood transfusion dependent HbE/β-thalassaemia patients in Bangladesh.

MATERIALS AND METHODS

Study patients: This study was approved by the institutional review board of the department of Genetic Engineering and Biotechnology, University of Chittagong and Thalassemia Welfare Centre, Chittagong, Bangladesh. A total of 60 blood transfusion dependent HbE/β-thalassemia patients were enrolled in the study. The purpose of blood collection regarding this research work was informed to the patients. A detailed history was taken from the patients and their parents including sex, age, age at diagnosis, frequent blood transfusion time and clinical symptoms at presentation. Blood samples were collected from the patients after one month of blood transfusion.

DNA isolation: Blood genomic DNA isolation from the collected samples was carried out using ™ Blood Cell Genomic DNA Extraction Mini Kit, FABGK 001, followed by isolated DNA quantification using (Nano Drop 2000 Spectrophotometer, Thermo Fisher Scientific, US) technology. Then the extracted DNA was checked by 1.5% agarose gel electrophoresis on UV transilluminator.

PCR amplification: A 168 bp of mutational hot spot region for HbE/β-thalassaemia was targeted for amplification from *HBB* gene using two primers (Forward: 5'-GGCAGAGCCATCTATTGCTTAC-3' and Reverse: 5'-AGCAACCTGGCCAGGGCCTT -3'). The PCR program was followed by: Initial denaturation at 95°C for 15 min, denaturation at 95°C for 15sec, annealing at 62°Cfor 30 sec, extension at 72°Cfor 20 sec and final extension at 72°C for 5 min. Total no. of cycle was 35. The PCR products were then checked in 1.5% agarose gel on UV trans-illuminator.

PCR product purification and Sequencing: PCR products were purified using QIA quick PCR Purification Kit (Qiagen, Hilden, Germany) and sequencingfrom the purified PCR products was carried out commercially at Gene Bio, South Korea by Sanger method.

Mutational data analysis: The characterization of mutation was carried out by the following steps: Firstly, the reference sequence of Hemoglobin (Accession NG_000007.3) was obtained from NCBI Ref Seq database. Secondly, BLAST tool(https://blast.ncbi.nlm.nih.gov/Blast.cgi)wascarried out to compare the obtained sequence with the Reference sequence. Thirdly, HbVar (http://globin.cse.psu.edu/hbvar/menu.html) was carried out to detect the mutations present in *Hbβ-globin (HBB)* gene. These mutations were searched in HbVar Database to match with already reported β-globin mutations. Finally, AliBaba2.1 (http://gene-regulation.com/pub/programs/alibaba2/index.html) program was used to predict the transcription factor binding site of the mutational region of the sequences.

Clinical examinations: Hematological examination, hemoglobin electrophoresis andserum ferritin were performed to investigate the clinical manifestations due to the changes in genetic level of the regular transfusion-dependent Hb E/β-thalassemia patients. Hematological examinations were carried out (Automated Hematology Analyzer Sysmex XP-300TM, U.S.A), hemoglobin electrophoresis was conducted using the Sebia Fully Automated Capillary 2 FP System, France and serum ferritin test was carried out usingBeckman Coulter AU clinical chemistry analyzer, USA. All the clinical examinations were performed at Chevron Clinical Laboratory (PTE) Limited, Chittagong.

RESULTS AND DISCUSSION

A fragment of 168 bp from the *HBB* gene was amplified using two specific primers and the amplified products were detected as mutational hot spot region in *HBB* gene on 1.5% agarose gel (Fig. 1).

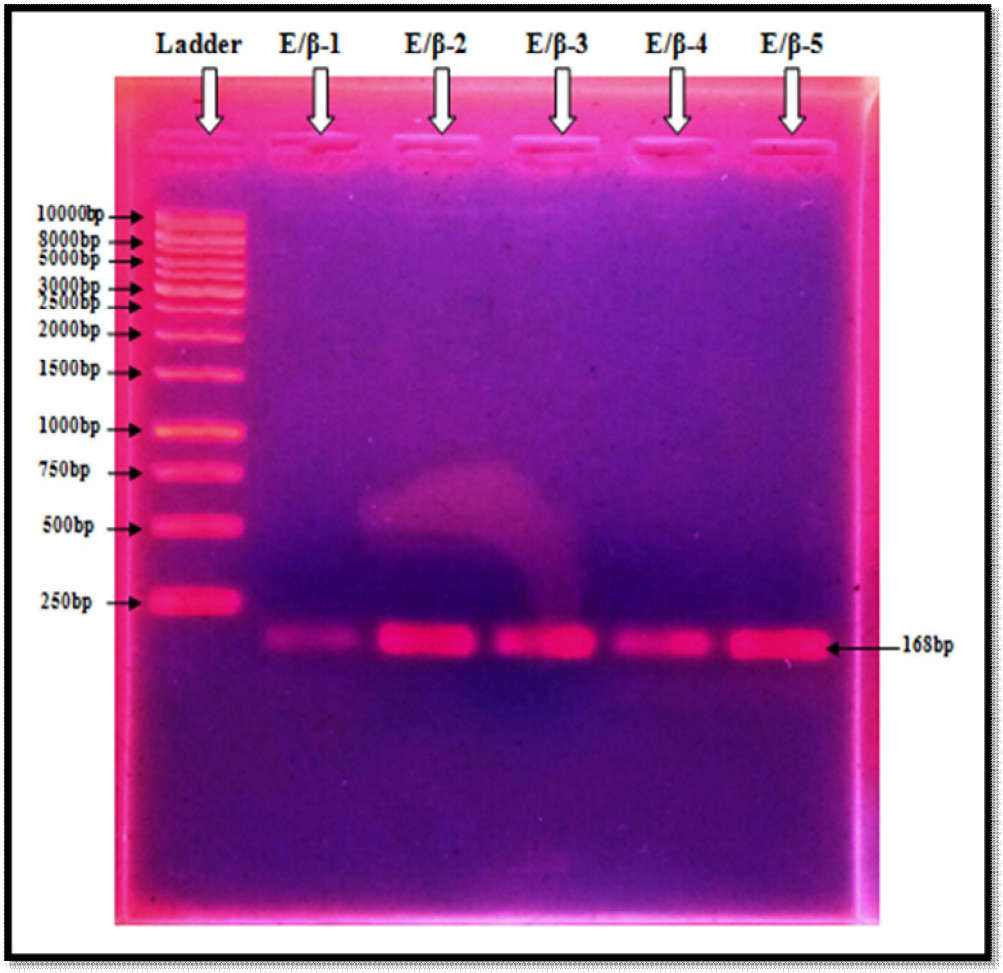

Fig. 1. Visualization of PCR amplified a 168 bp fragment correspond to the mutational hot spot region of *β-globin* gene on 1.5% agarose gel

Sequencing results: Based on the sequence chromatogram, retrieved sequences were analysed for mutation, applying BLAST and HbVar Database. Summarized result of the mutation analysis is given bellow in Table1.

Table 1. Summarized result of mutations in *HBB* gene among the transfusion dependent Hb E/β-thalassemia patients

Characteristics	Mutations in *HBB* gene		
	c.79 G>A	**c.9 T>C**	**Deletion of A**
HbVar ID	277	3042	Uncharacterized
Category	Both variant and thalassemia	Rare variant	Uncharacterized
Mutation location	Codon 26, Exon 1 hg38: chr11 5,226,943	Codon 2, Exon 1 hg38: chr11 5,227,013	+23bp in *HBB* promoter region (70572) ntd
HGVS name	HBB:c.79G>A	HBB:c.9T>C	Uncharacterized
Amino acid change	**G**AG>**A**AG(Glu>Lys)	CA**T**>CA**C** (His>His)	-
Clinical Significance	Pathogenic mutation which results in the production of hemoglobin E and can be associated with mild anemia when found in the homozygous state17	Non-pathogenic mutation16	Uncharacterized
Frequency	100%	100%	20%

During the analysis of the retrieved sequences, a total of three mutations among the patients. HBB: c.9 T>C and HBB: c.79 G>A are characterized on HbVar database, and deletion of A at+23bp in *HBB* promoter region (70572 nts) in *HBB* gene is still uncharacterized in HbVar database. So, this is a novel mutation (Fig. 2a, 2b & 2c).

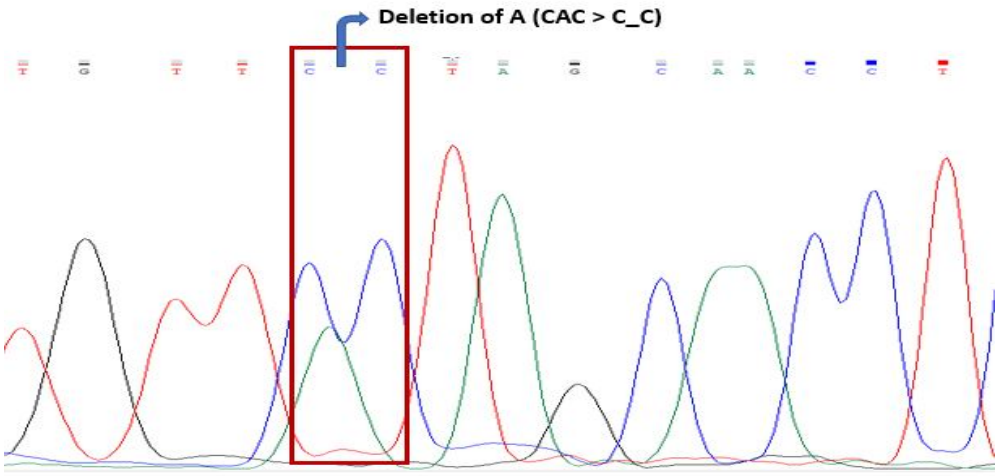

Fig. 2a. Identification of Deletion of A in +23bp promoter region from sequence chromatogram of *HBB* gene

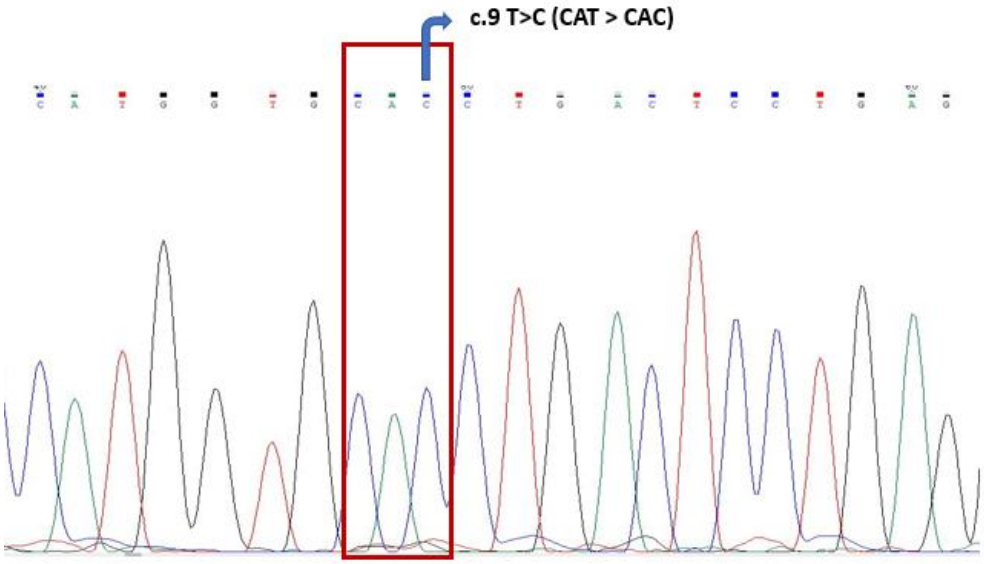

Fig. 2b. Identification of c.9 T>C (CAT>CAC) mutation from sequence chromatogram of *HBB* gene

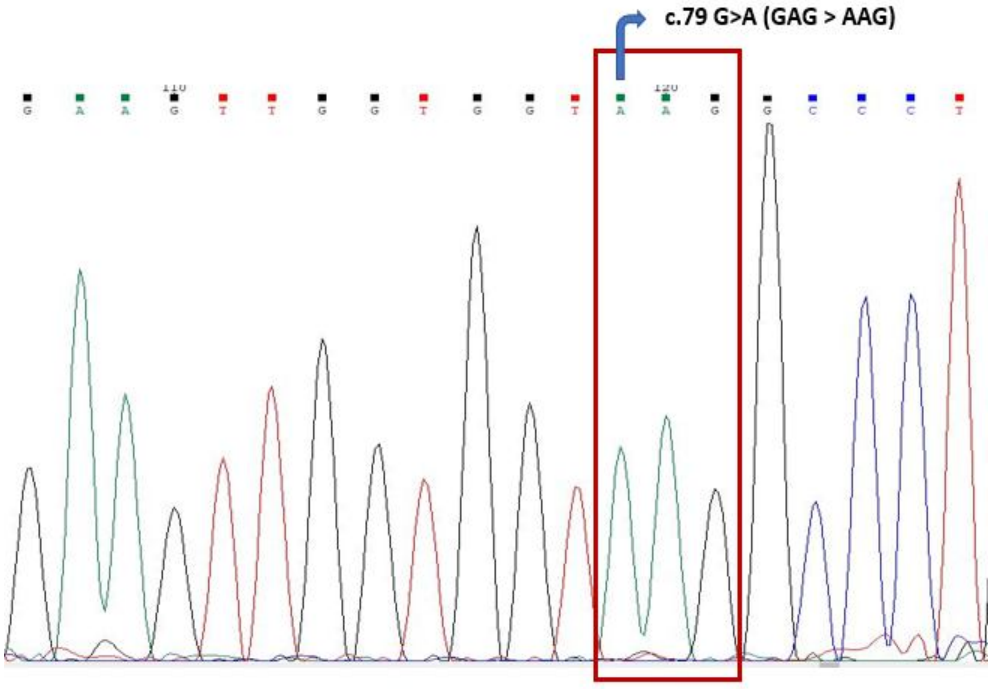

Fig. 2c. Identification of c.79 G>A (GAG>AAG) mutation from sequence chromatogram of *HBB* gene

Transcription factor binding sites of the retrieved sequences were also analysed. And a total of three important putative binding sites for transcription factor GATA-1, USF and AP-1 were revealed in the analysed sequenced (Fig. 3)

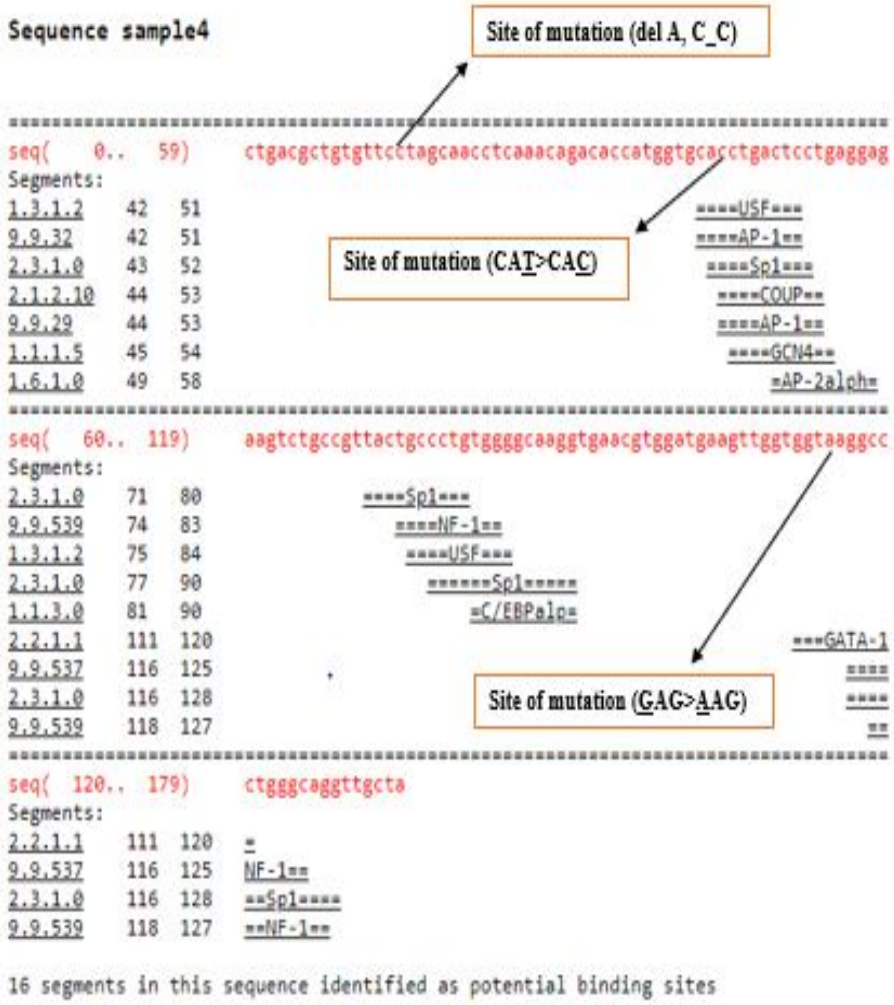

Fig. 3. Putative USF, AP-1 and GATA-1 binding site present at the mutation site. Transcription factor binding site predicted by Ali Baba 2.1 program (Grabe, 2002), using the TRANSFAC database

Hematological analysis: The result of hematological examination including some important parameters given in Table 2.

Table 2. Comparative analysis of haematological parameters between Healthy and the patients with HbE/β-thalassemia

Characteristics	Normal/Healthy	HbE/β-thalassemia
Hb (g/dl)	12-18	8.13 ± 1.4
RBC (million/µl)	4.2-6.2	3.78 ± 0.75
MCV (fL)	76-96	69.53 ± 8.90
MCH (pg)	26.6-33.5	21.98 ± 3.98

SD=Standard Deviation; Hb=Hemoglobin; RBC=Red Blood Cell; MCV=Mean Corpuscular Volume; MCH=Mean Corpuscular Hemoglobin.

The hematological analysis showed remarkable significance among the regular blood transfusion-dependent Hb E/β-thalassemia patients with lower Hb and RBC level than the normal participants. Because the defective hemoglobin molecule and RBCs are structurally abnormaland they can't survive in blood as normal. That's why MCV and MCH volume are also lower than normal.

Hemoglobin Electrophoresis: Hemoglobin electrophoresis from fresh blood samples of the study patients showed significantly reduced level of HbA and presence of HbA2, HbF and HbE as increased in level than the normal level.

Serum ferritin test: It was found from serum ferritin test that the average level of serum-ferritin in all patients was significantly higher as 2318 ng/ml on average where the normal level is 18-370 ng/ml in male and 9-120 ng/ml in female.

Frequency of Age, Sex and Blood group among the patients: Among the study patients the average age was 13 years, ranging from 2 -30 years. We found that the highest frequency of age group was 0-10. The age group of above 30 represented the lowest frequency.

The distribution of sex was analysed to understand the frequency of HbE/β-thalassemia in male & female and the analysis showed the frequency of female patients was higher than the male patients (Fig. 4)

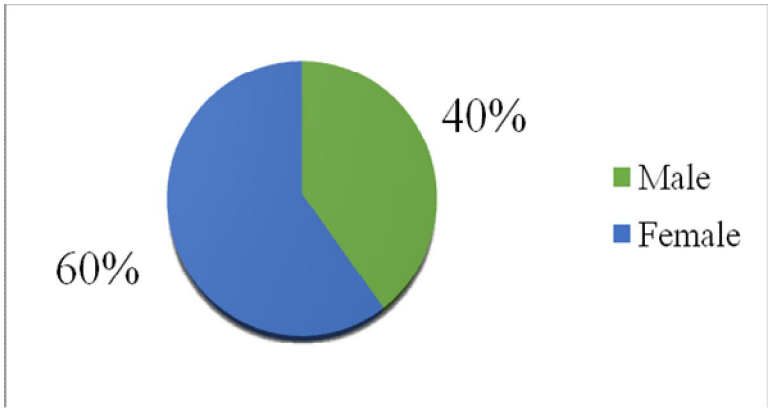

Fig. 4. Frequency of Sex among the transfusion-dependent HbE/β-thalassemia patients

The distribution of blood group among the blood transfusion-dependent HbE/β-thalassemia patients was also analysed to understand the frequency of Hb E/β-thalassemia prone blood group. It was found that the O+ve blood group was more frequent (40%) and AB+ve was less frequent (20%) of all analysed blood groups (Fig. 5).

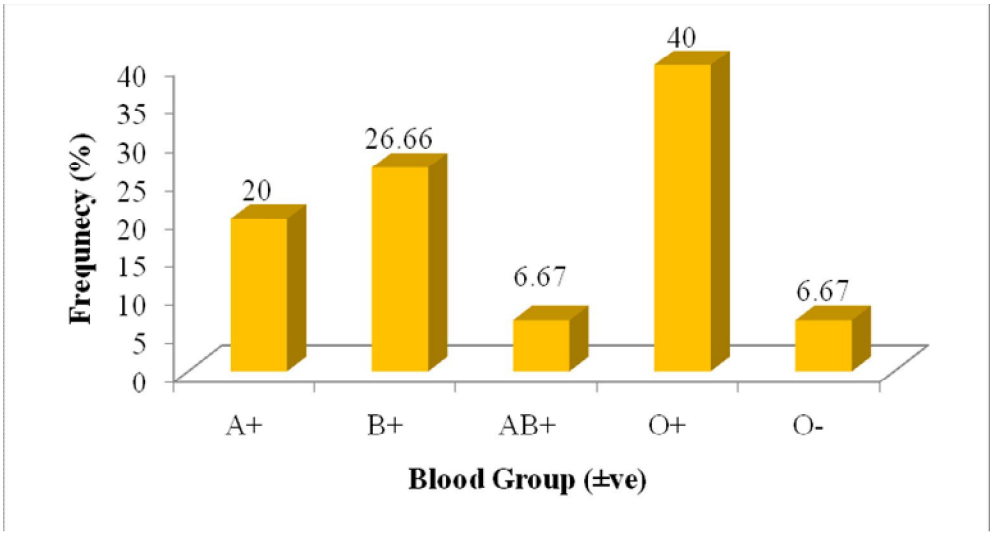

Fig. 5. Frequency of Blood Group among the transfusion-dependent HbE/β-thalassemia patients

Thalassemia, a group of genetic disorder occur mainly due to defective formation of globin chain of the hemoglobin moiety of the RBC. This specific type of blood disease results in excessive destruction of red blood cells which in turn leads to anaemia. In this disease RBC breakdown occur at an early stage due to abnormal globin chain unable to protect RBC in oxidative stress. Resulting destruction of RBC leads to produce bilirubin production which ultimately metabolized in liver for excretion. In thalassemia the rate of destruction of RBC is so rapid that it exceeds the liver capacity to metabolize the excess bilirubin (Mandall *et al*., 2014).

HbE/β-thalassemia, an inherited disorder of hemoglobin is characterized by chronic hemolytic anaemia, typically requires life-long blood transfusion (BT) therapy for patients' survival (Palit *et al*., 2012). Pathophysiology is complex which involves ineffective erythropoiesis, apoptosis, Oxidative damage and shortened red cell survival (Borgna *et al*., 2014).

Discussion on mutational analysis: A total of three mutations were identified among the transfusion dependent HbE/β-thalassemia patients. Deletion of A at 70752nt position (+23bp in HBB promoter region) of *HBB* gene was detected as uncharacterized in HbVar database. The T>C substitution at 70603nt position in *HBB* gene which is characterized as HBB: c.9 T>C. This T>C substitution results in CAT>CAC (His>His) change in codon 2 of exon-1 in *HBB* gene. Mutation HBB: c. 9T > C is a silent mutation. This

polymorphism was also found in Mediterranean populations (Atweh *et al.,* 1986). The G>A substitution at 70673 nt position in *HBB* gene is characterized as HBB: c.79 G>A in HbVardatabase.GAG>AAG (Glu>Lys) change occurs in codon 26 ofexon-1 of *HBB* gene. This mutation is pathogenic and responsible for the production of structurally abnormal hemoglobin E. The interaction of HbE and beta-thalassemia results in thalassemia phenotypes ranging from a condition indistinguishable from thalassemia major to a mild form of thalassemia intermedia (Galanello & Origa, 2010). Along with this mutation, c.9 T>C and deletion of A were responsible for HbE/β-thalassemia. Since HBB: c.9 T>C (His>His) is rare but not pathogenic, deletion of A at +23nt position of promoter region of *HBB* gene along with c.79G>A mutation could be effective enough to create HbE/β-thalassemia as moderate to severe. That's why the patients were transfusion-dependent. And deletion of A at +23nt position of promoter region of *HBB* gene was detected as a new mutation in Bangladesh as well as worldwide. The frequency of c.79G>A, c.9 T>C and deletion of A in +23bp in HBB promoter region was 100%, 100% and 20% respectively among the studied participants (Table 1).

In this study, putative binding sites for transcription factors in the *HBB* gene were detected. A total of three transcription factor as GATA-1, USF and AP-1 binding sites were identified in the mutated region of the *HBB* gene (Fig. 3). The zinc finger transcription factor GATA-1 is expressed in erythroid, megakaryocytic, eosinophilic, and mast cells and plays multiple roles in hematopoiesis (Orkin, 1998). For instance, GATA-1 is essential for the differentiation of erythroid and megakaryocytic precursors, as demonstrated by gene targeting in the mouse (Fujiwara *et al.,* 1996; Shivdasani *et al.,* 1997; Vyas *et al.,* 1999). GATA1 was first described as a transcription factor that activates the hemoglobin B gene in the red blood cell precursors of chickens (Evans *et al.,* 1988). Subsequent studies in mice and isolated human cells found that GATA1 stimulates the expression of genes that promote the maturation of precursor cells (e.g. erythroblasts) to red blood cells while silencing genes that cause these precursors to proliferate and thereby to self-renew (Welch *et al.,* 2004; Cheng *et al.,* 2009). GATA1 stimulates this maturation by, for example, inducing the expression of genes in erythroid cells that contribute to the formation of their cytoskeleton and that make enzymes necessary for the biosynthesis of hemoglobins and heme, the oxygen-carrying components of red blood cells. GATA1-inactivating mutations may thereby result in a failure to produce sufficient numbers of and/or fully functional red blood cells (Katsumura *et al.,* 2013). USF is a family of transcription factor characterized by a highly conserved basic-helix-loop-helixleucine zipper (bHLH-zip) DNA-binding domain (Luo & Sawadogo, 1996). USF binds DNA as a dimer at specific sites that are characterized by a central CACGTG motif (Sawadogo & Roeder, 1985). Activator protein 1 (AP-1) is a transcription factor that regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, stress, and bacterial and viral infections (Hess *et al.,* 2004). AP-1 controls a number of cellular processes including differentiation, proliferation, and apoptosis (Ameyar *et al.,* 2003). Putative GATA-1, USF and AP-1 binding sites presented at the mutation sitesare given in result section.

Discussion on Clinical Analysis: Hematological analysis showed that the average hemoglobin (Hb) level of the transfusion dependent HbE/β-thalassemia patients were 8.1 g/dl which was much lower than the normal level. In male patients, the normal level of Hb is 14-18 g/dl where the male patients had 8.9 g/dl on average. In addition, the normal level of hemoglobin is 12-16 g/dl in female where the female patients had 7.5 g/dl on average. A 25years aged female patient was suffering from severe form of HbE/β-thalassemia because of extreme lower level of hemolglobin (5.9 g/dl). Patients with severe forms of thalassemia suffer with hemoglobin level ranging from2 to 8 g/dl. In a recent study by Tahura (2017) showed that HbE/β-thalassemia& Hbβ-thalassemia major patients possessed Hb level as 6.1g/dl & 4.9g/dl respectively. So, it can be said that the Hb level is lower in β-thalassemia patients than HbE/β-thalassemia patients. The summarized result of hematological parameters is given in Table 2.

The RBC count is usually decreased in anemia and thalassemia due to the destruction of RBCs just after synthesis because c.79 G>A mutation in *HBB* gene produces abnormal and unstable hemoglobin. So, thalassemia patients contain abnormal RBCs with abnormal hemoglobin. As a result, RBC's normal life span (120 days) is decreased remarkably. That's why the thalassemia patients need blood transfusion after 1-2 months and thalassemia major patients need twice or more times blood transfusion in a month. In this study it has been found that HbE/β-thalassemia patients showed decreased level of total RBCs than the normal range. In both male and female patients showed the RBCs level as 3.77 million/µl on average where the normal level is 4.2-6.2 million/µl in both male & female (Table 2).

The MCV count is generally decreased in thalassemia patients. Normal level of MCV is 76-96 fL in both male & female. All the subjects of this study possessed the MCV count as slightly decreased, 69.5 fL on average. But in female patients the MCV level was found as lower than male patients (Table 2).

TheMCH count is generally decreased as MCV count in thalassemia patients. Normal level of MCH is 26.6-33.5 pg in both male & female. MCH count was also found as decreased form, 21.98pg on average in the study patients. In female patients the average MCH level was found as lower than male patients (Table 2). A 10-year-old female patient showed the lowest MCH count, 15.7pg.

Hemoglobin electrophoresis studies of the Hb E/β-thalassemia patients showed remarkable changes in hemoglobin concentration and variants. It was found that HbA was relatively lower and the HbE was higher as >40%, because of the mutated hemoglobin. HbA2 level was found as higher than the normal range due to the imbalance of α-β globin chain.

In serum-ferritin studies, we found that the average level of serum-ferritin in all the Hb E/β-thalassemia patients was significantly higher as 2318 ng/ml on average where the normal level is 18-370 ng/mL in male and 9-120 ng/mL in female. Due to the breakdown

of mutated RBCs and accumulation of access iron, ferritin level was found as extremely high in the transfusion dependent Hb E/β-thalassemia patients.

Hb E/β-thalassemia is more common hemoglobin disorder in not only in Bangladesh but also worldwide. This hereditary genetic disorder is life threatening. The findings of this research will be beneficial for effective molecular drug designing, drug response and other therapeutic approach for HbE/β-thalassemia patients.

Authors' contributions: Nazneen Naher Islam designed the work. Nazneen Naher Islam and Mohabbat Hossainwrote the manuscript. Mohabbat Hossain conducted the lab work. Mohabbat Hossain, Shuvo Chandra Das and Sabrina Alam Seuti collected the blood samples. Mahmood Ahmed Chowdhury helped to manage the blood samples and clinical examinations. All authors read and approved the final manuscript.

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Ethics approval: Ethical permission for the study was taken from the Chittagong Maa-o-Shishu Hospital and Department of Genetic Engineering and Biotechnology, University of Chittagong, Bangladesh.

Competing interests: The authors declare that they have no competing interests.

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