

## RESEARCH PAPER

# Identification and Integrative Bioinformatics Analysis of Common and Rare Mutations in the Patients with Transfusion Dependent HbE/ $\beta$ and $\beta$ -thalassemia in Chittagong, Bangladesh

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## Abstract

**Background:** Thalassemia is an inherited blood disorder that affects hemoglobin's structure and functions. Among several forms of this life-threatening disorder, HbE/ $\beta$  and  $\beta$ -thalassemia are most common in Bangladesh and worldwide as well. But the molecular and clinical data are not adequate regarding the underlying cause of this genetic disorder in Bangladesh. So, we aimed to identify the genetic mutations within  $\beta$ -globin gene (*HBB*) and to investigate the correlation of the mutations with HBB mRNA structure, gene transcription and hematological status among the patients with blood transfusion dependent HbE/ $\beta$  and  $\beta$ -thalassemia in Bangladesh.

**Methods:** A total of 40 blood samples were collected from the patients with blood transfusion dependent HbE/ $\beta$  and  $\beta$ -thalassemia prior to taking their consent. Detection of mutations within *HBB* gene was carried out by polymerase chain reaction followed by Sanger DNA sequencing method. Identification and characterization of mutations along with their effects on *HBB* gene were analyzed by various bioinformatics approaches. In addition, complete blood count (CBC), and hemoglobin electrophoresis were done for hematological analysis.

**Results:** c.92+5G>C, c.79G>A and c.9T>C genetic mutations were identified within the *HBB* gene, where c.92+5G>C was the most common mutation among the study patients. Mutations along with hematological status and putative transcription factor binding sites revealed that the severity of the disease depends upon the mutation type and its location in the *HBB* gene sequence. In addition, mRNA structure analysis showed that the identified mutations contribute to its structural diversity by altering folding mechanism that ultimately affects the stability and function of the HBB protein among the patients with blood transfusion dependent HbE/ $\beta$  and  $\beta$ -thalassemia.

**Conclusions:** The study showed the underlying cause of HbE/ $\beta$  and  $\beta$ -thalassemia in genetic level by identifying rare and common mutations within *HBB* gene and their effects on with *HBB* gene transcription and mRNA structure. We hope study will contribute in designing effective molecular medicine and other therapeutics for the patients with HbE/ $\beta$  and  $\beta$ -thalassemia to improve their health condition.

**Keywords:** HbE/ $\beta$ -thalassemia,  $\beta$ -thalassemia, Mutations, Transcription factors, mRNA structure.

## Introduction

Thalassemia is an inherited blood disorder caused by abnormal synthesis of hemoglobin. This disorder results in excessive destruction of red blood cells

which in turn leads to moderate to severe anaemia<sup>1</sup>. Mainly thalassemia is two types-are  $\alpha$  and  $\beta$ -thalassemia.  $\alpha$ -thalassaemia is caused by reduced or absent synthesis of alpha globin chains, where  $\beta$ -thalassaemia is caused by reduced or absent synthesis of beta globin chains.<sup>2</sup> When  $\beta$ -thalassemia is inherited together with a hemoglobin E (HbE) allele, this results in HbE/ $\beta$ -thalassemia, is sometimes characterized by a severe, transfusion-dependent thalassemia major.<sup>3</sup> Hemoglobin E (HbE) is an

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abnormal structural variant of hemoglobin, resulting from a substitution mutation G>A in codon 26 (Glu>Lys) of the  $\beta$ -globin gene, mostly prevalent in South-East Asian populations.<sup>4,5</sup>

HbE/ $\beta$  and  $\beta$ -thalassemia are inherited blood disorders caused by the mutations in  $\beta$ -globin (*HBB*) gene on chromosome 11.<sup>6</sup> The mutations down-regulates the  $\hat{\alpha}$ -globin gene that decreases hemoglobin synthesis and red cell survival.<sup>7</sup> More than 200 mutations have been characterized within *HBB* gene including point mutations, small deletion or insertion.<sup>8</sup> These mutations affect *HBB* gene transcription, messenger RNA processing, translation, or hemoglobin structure.<sup>9</sup> Single nucleotide polymorphisms (SNPs) in coding and non-coding region of the gene can interfere in the processing of primary mRNA transcript that has prominent impact on translation process.<sup>10</sup> Altering the native structure of mRNA via SNPs can affect both the stability and the speeds along with its fidelity of translation.<sup>11</sup> Mutations in coding region can affect the structure and/or function of the hemoglobin protein that ultimately affect the function of red blood cell. Mutations in exon-intron splice junction can also affect the splicing site that ablate regular splicing and inducing  $\beta$ -thalassemia.<sup>12</sup>  $\beta$ -thalassemia becomes a common blood genetic disorder in Bangladesh due to geographical position and global population movement.<sup>13</sup> Though several reports on mutation spectrum in Indian subcontinent and Middle East are available, the complete mutational spectrum of  $\beta$ -thalassemia mutations in Bangladeshi patients is not well established. Recent studies showed that the carrier frequency of  $\beta$ -thalassemia is 3.0% of total population.<sup>14,15</sup> These carriers of  $\beta$ -thalassemia phenotypically normal, although they can usually be detected by screening red cell indices and an elevated hemoglobin A<sub>2</sub> level.<sup>16</sup>

Worldwide, the patients with Hb E/ $\beta$ -thalassaemia represent approximately 50% of those affected with severe  $\hat{\alpha}$ -thalassaemia.<sup>17</sup> The highest frequencies of Hb E/ $\beta$ -thalassaemia are found in India, Bangladesh and throughout Southeast Asia, particularly in Thailand, Laos and Cambodia.<sup>18</sup> It is estimated that approximately 60,000–70,000 patients are suffering from  $\beta$ -thalassaemia major and HbE/ $\beta$ -thalassaemia with different levels of severity and nearly 2500 children with thalassaemia major are added every year in Bangladesh.<sup>19</sup> That's why the characterization of disease-causing mutations is essential for prenatal

diagnosis of fetus at risk that regulates and expresses  $\beta$ -globin gene. We aimed to identify the genetic mutations within *HBB* gene and to investigate the correlation of mutations with clinical manifestations among the blood transfusion dependent HbE/ $\beta$  and  $\beta$ -thalassemia patients in Bangladesh.

## Materials and Methods

**Collection of study samples:** 3 ml of venous blood samples were collected in EDTA (anticoagulant) containing sterile tube from 40 patients who were diagnosed with HbE/ $\beta$  and  $\beta$ -thalassemia at the Thalassemia Welfare Centre of Chittagong, Bangladesh. During sample collection, purpose of the study was informed to the patients and their respective guardians. The collected blood samples were transported to the laboratory for analysis maintaining proper guideline.

**Hematological analysis:** Hemoglobin electrophoresis and complete blood count (CBC) were done for analysis of hematological parameters of the study patients. After hematological examination, the blood samples were aliquoted and stored at -20°C for molecular analysis.

**Genomic DNA extraction and PCR:** Genomic DNA was extracted from the collected blood samples using Favor Prep™ blood cell genomic DNA extraction mini kit as per manufacturer's instruction. Polymerase chain reaction (PCR) was done targeting a 587 bp mutational hotspot region of the  $\beta$ -globin gene using one set of primers (forward primer: 5'-gctgtcatcacttagacctca-3' and reverse primer: 5'-cacagtcgagctcactcag-3').<sup>20-22</sup> 1.5% agarose gel electrophoresis was carried out for the confirmation of PCR products.

**Sequencing and Bioinformatics analysis:** The purification of the PCR product was carried out through Wizard® SV Gel and PCR Clean-Up System and sequenced by Sangers Di-deoxy sequencing method. The Nucleotide sequences corresponding to the current NCBI Ref Seq for human *HBB* gene (NG\_000007.3) were analyzed using BLASTseq for pairwise sequence alignment accounting for the mismatch or gap or deletion or substitution of the sequences. The HbVar database was used for the identification and characterization of mutations presented in the sequences. Mutations absent in the database were considered as new mutations.<sup>23</sup> Alibaba 2.1 gene regulation program was used to identify transcription factor binding sites on the mutational region. The

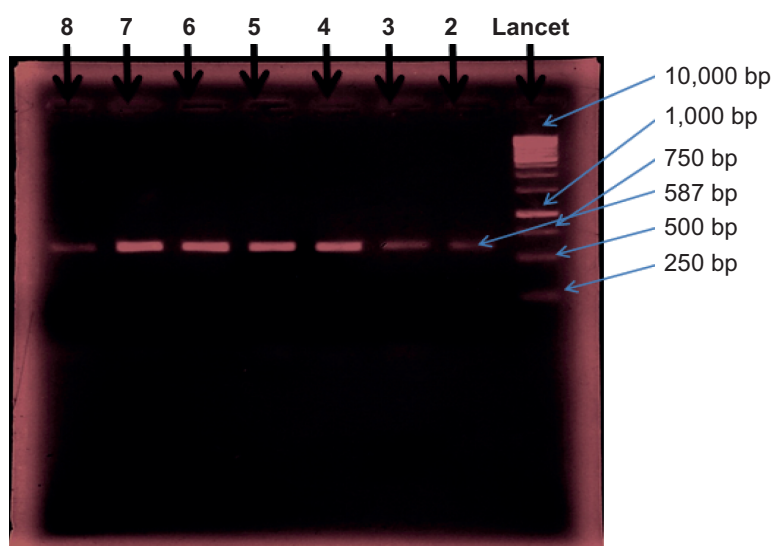
mutational effect on HBB mRNA structure was predicted by using Nucleic Acid Converter (<https://skaminsky115.github.io/nac/>), ViennaRNA Web Services and 3dRNA v2.0 tools sequentially.<sup>24</sup>

## Results

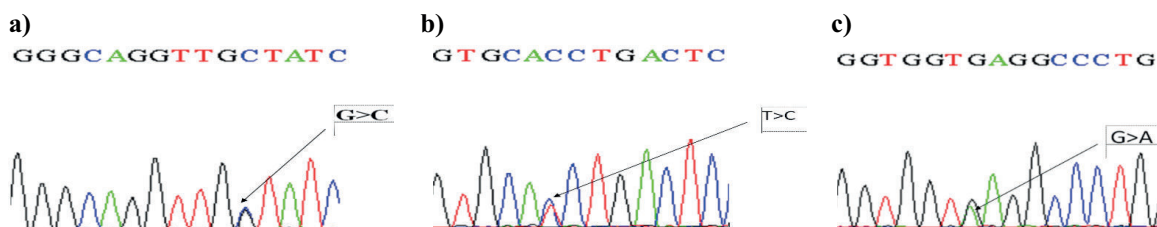
**DNA extraction, PCR and sequencing:** Extraction of genomic DNA was analyzed by 1.5% agarose gel electrophoresis under UV transilluminator of ethidium bromide-stained DNA. It is estimated that there are 30,000-50,000 protein-coding genes in the human genome.<sup>25</sup> From this larger genome, we amplified the specified  $\beta$ -globin gene via PCR technology. For identification of mutation, a hot spot region of 587bp of  $\alpha$ -globin gene was amplified. To ensure successful PCR, the PCR products were analyzed on 1.5% agarose gel electrophoresis under UV transilluminator (Figure 1).

The purified PCR products were sequenced based on Sanger method and the sequence chromatograms with respective SNPs in  $\beta$ -globin gene are shown in figure 2.

**Identification of mutation and bioinformatics analysis:** Based on the chromatogram, sequences were retrieved and aligned with the current NCBI reference sequence for human HBB gene (NG\_000007.3) by BLASTseq. The identified mismatches were analyzed in HbVar database and three characterized mutations as follows: IVS-I-5; G>C/ HBB: c.92+5G>C' (HbVar ID -824), HBB: c.9T>C (HbVar ID- 3042), and HBB:c.79G>A (HbVar ID -277). The intronic mutation "IVS-I-5; G>C" was situated in NG\_000007.3:g.70691 no position and present in all the sequenced samples. The other two mutations HBB: c.9T>C and HBB:c.79G>A were found in the coding region with the nucleotide



**Figure 1:** Results of PCR products of amplified 587bp of HBB gene on 1.5% agarose gel. Here, lane 1 showed the ladder DNA and lane 2, 3, 4, 5, 6, 7 & 8 showed PCR products.



**Figure 2:** Sequence chromatogram showing (a) c.92+5G>C (b) c.9T>C and (c) c.79G>A substitution mutation in HBB gene.

position no, NG\_000007.3:g.70603 and NG\_000007.3:g.70673 respectively.<sup>26</sup>

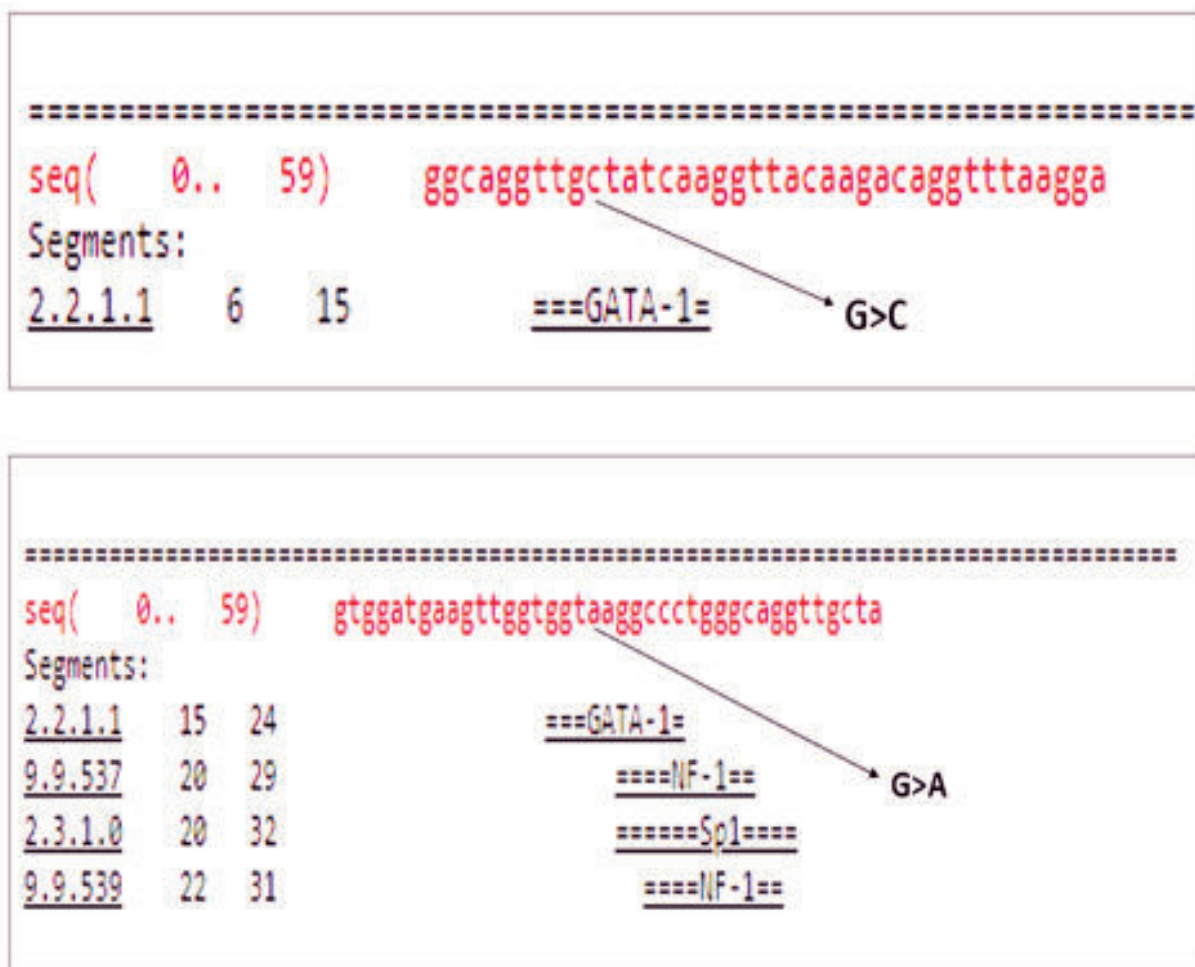
From the analysis of the Alibaba 2.1 program, the important putative transcription factor GATA-1 binding site was identified in the mutational region of the IVS-I-5 (G>C) and HBB: c.79G>A (Figure 3).

The secondary structure of both wild type and mutated *HBB* mRNA was obtained from RNAfold system showed that these mutations drastically affect the mRNA structure of *HBB* gene. Figure 4 showed the centroid-based pair probability where alternation of structural backbone is marked in highlighted boxes.

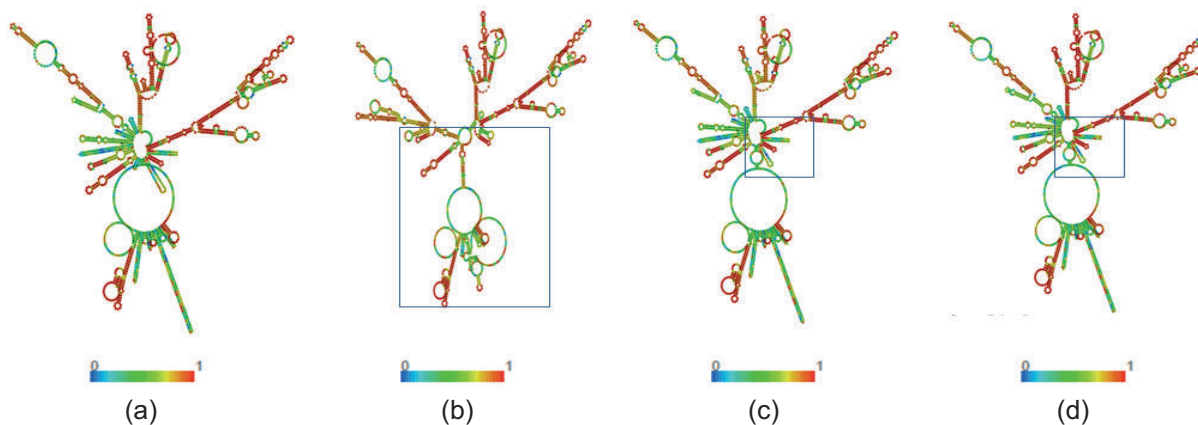
The mountain plots of the thermodynamic characteristics (Minimal free energy calculation, centroid-based pair probability and partition function analysis.) of the *HBB* mRNA are shown in figure 5.

Genetic mutations in *HBB* contribute to the free energy changes in mRNA were shown in table 1.

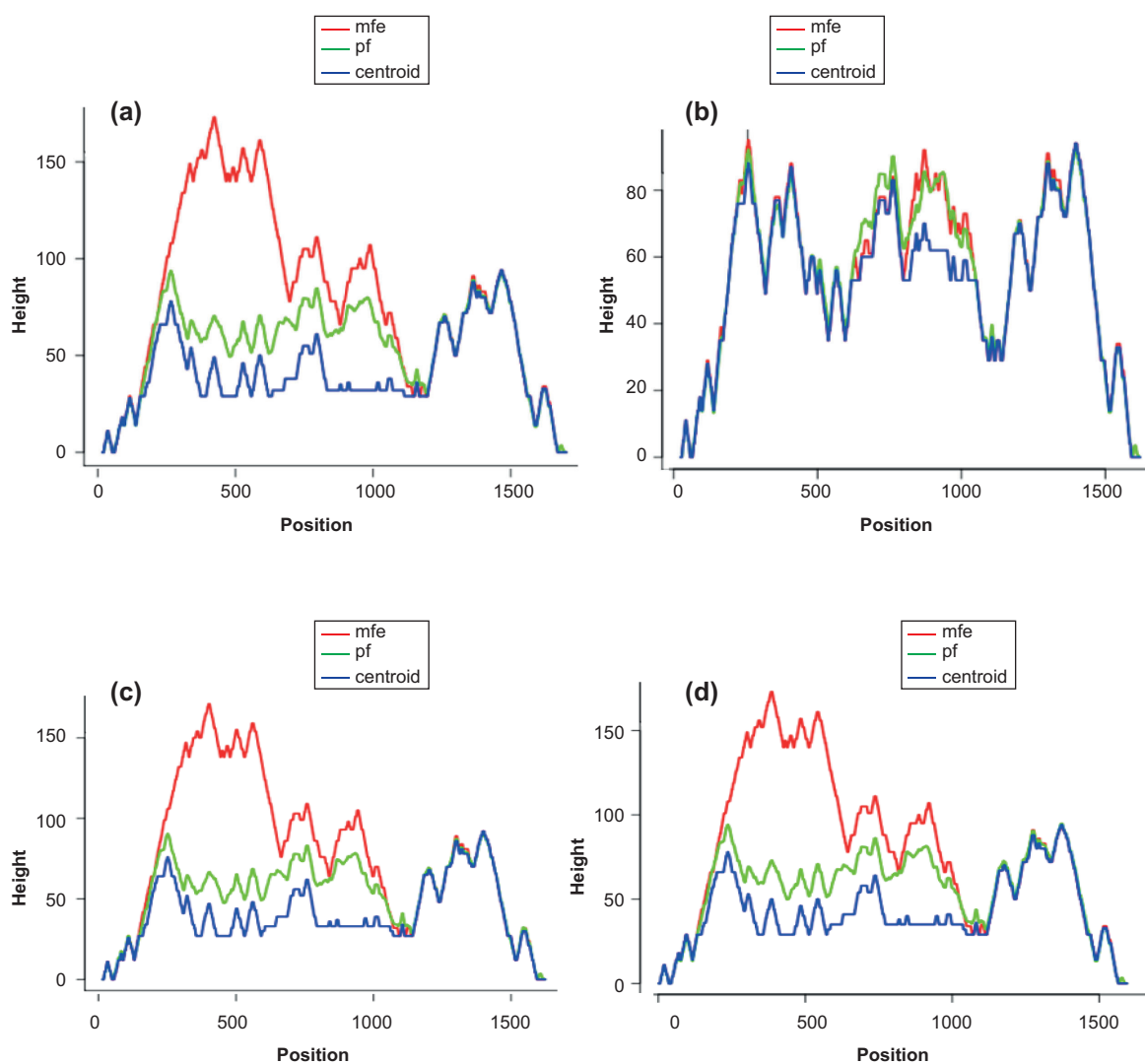
**Hematological studies analysis:** Hemoglobin (Hb), Erythrocyte Sedimentation rate (ESR), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) were analyzed statistically in order to evaluate the hematological status of the patients. The hematological analysis showed remarkable lower Hb, RBC, MCV and HCH level than the normal range among the both blood transfusion-dependent HbE/ $\beta$  and  $\beta$ -thalassemia patients. Hemoglobin electrophoresis showed reduced level of HbA and increased level of HbA<sub>2</sub> and HbF in patients with  $\beta$ -thalassemia than the normal level. In addition, the patients with HbE/ $\beta$ -thalassemia showed reduced level of HbA, and increased level of HbA<sub>2</sub>, HbE and HbF than the normal level.



**Figure 3:** GATA-1 Transcription Factor binding site (a) HBB:c.92+5G>C in the intron-1 and (b) HBB:c.79G>A in the exon-1 of *HBB* gene sequence.



**Figure 4:** Centroid-based pair probability of the human  $\beta$ -globin gene. (a) wild type, (b) HBB: c.92+5G>C, (c) HBB: c.9T>C and, (d) HBB: c.79G>A. Structural changes of RNA are highlighted by blue boxes.

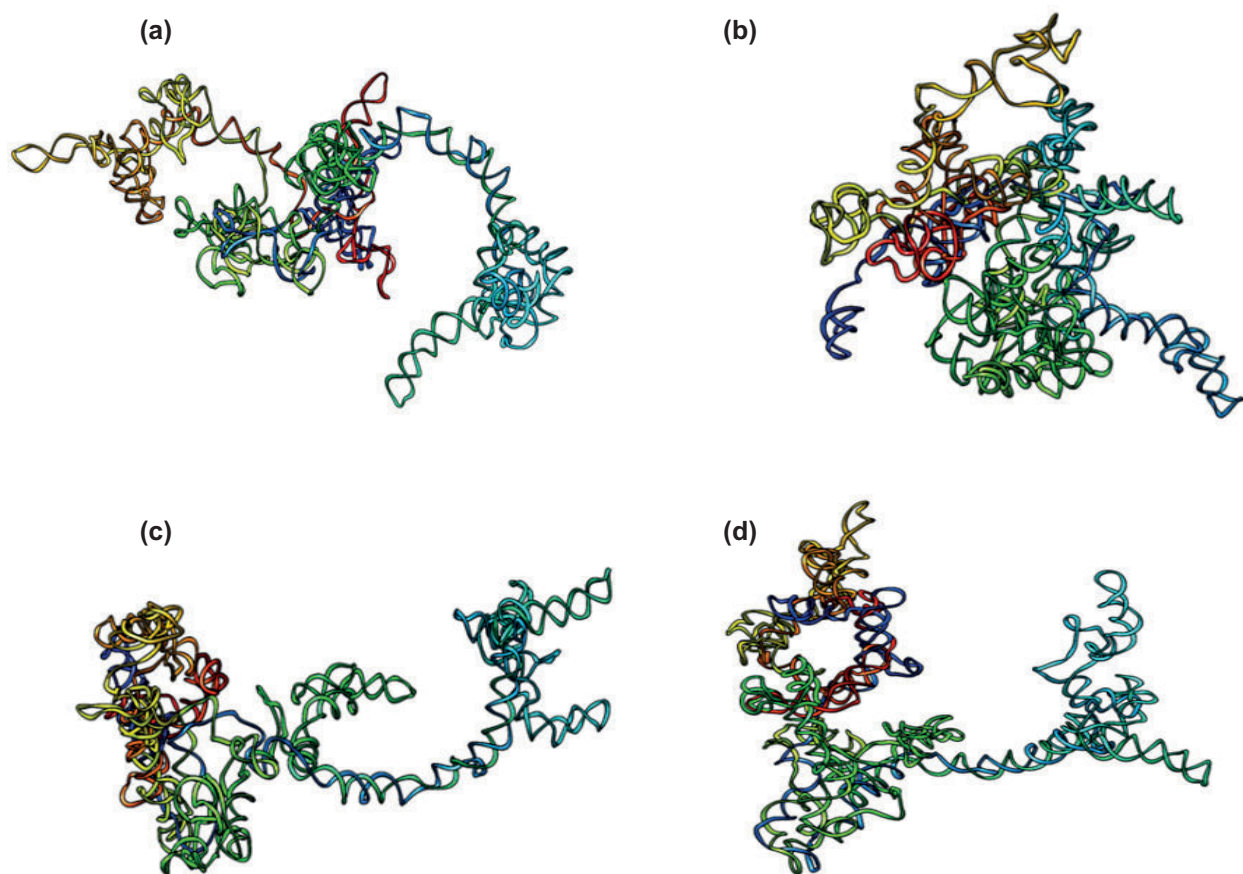


**Figure 5:** Mountain plots of the thermodynamic characteristics of the predicted mRNA of human  $\alpha$ -globin gene (a) Wild type, (b) HBB: c.92+5G>C, (c) HBB: c.9T>C and, (d) HBB:c.79G>A

**Table I:** Thermodynamic information of the secondary structure of mRNAs of wild type *HBB* and mutated *HBB* gene

<i>HBB</i> SNP genotypes	MFE of optimal secondary structure (kcal/mol)	MFE of centroid secondary structure (kcal/mol)	Free energy of the thermodynamic ensemble (kcal/mol)	Ensemble Diversity of RNA
Wild type <i>HBB</i>	-441.60	-321.61	-471.29	398.86
IVS-I-5 G>C	-440.90	-384.21	-470.59	314.75
HBB: c.9T>C	-441.20	-324.01	-470.93	413.87
HBB:c.79G>A	-442.50	-331.51	-472.03	387.25

The 3D structure obtained from 3dRNA v2.0 was visualized with UCSF chimera and shown in figure 6.

**Figure 6:** Tertiary structure of (a) wild type , (b) mutated HBB: c.92+5G>C, (c) mutated HBB: c.9T>C and, (d) mutated HBB: c.79G>A HBB protein.

### Discussion

HbE/ $\beta$  and  $\beta$ -thalassemia are the two most common forms of thalassemia among Asian people including Bangladesh. Point mutations affecting the  $\beta$ -globin

expression belong to three different categories: mutations in promoter and 5' UTR region; mutation in non-coding region that affects mRNA processing; and mutations resulting in abnormal mRNA

translation.<sup>27</sup> After completion of transcription process the mRNA structure is formed. Both the structure and stability of mRNA play an important role in translation of protein.<sup>28</sup> The altered mRNA folding can affect its stability and has an impact on efficiency of translation.<sup>29-31</sup> According to the sequencing result, the mutation c.92+5G>C occurred in the intervening sequence where GATA-1 transcription factor (TF) binding sites was identified in all sequenced samples (Figure 2a). This result corresponded to the previous studies that also found the c.92+5G>C mutation is the most common mutation (60%) in the Bangladesh.<sup>21</sup> This mutation is also common in the Indian Subcontinent, specifically in Srilanka (56%), Pakistan (43.5%) and India (Punjab, 30.51%).<sup>32-34</sup> In thalassemia major patients, c.92+5G>C mutation alone can be formed pathogenic condition in homozygous genotype or it can be formed disease combined with other mutation in both homozygous and heterozygous genotype.<sup>15</sup> The effects of this intronic mutation on mRNA structure affect tremendously. The figure 4(b) showed the centroid-based pair probability where conformational differences are marked in blue box. The marked region indicated the enlargement of loops that makes unstable of the mutated mRNA structure. The mutation leads to a large free energy change that also revealed the decreased stability. The 3D structure obtained from 3dRNA v2.0 were visualized with UCSF chimera and found that this mutation affects the folding of 3D model tremendously (Figure 6b). In this study, we also identified rare mutation HBB: c.9T>C first time in Bangladeshi patient, which was co-inherited with a common substitution mutation c.92+5G>C. This type of SNP present in about 20% of the population of Odisha state, India.<sup>35</sup> HBB: c.9T>C (CAT>CAC, His>His) mutation was identified in 2 no codon of exon-1 sequence of *HBB* gene. Due to codon degeneracy, this mutation has no role in changing amino acid and known as synonymous mutation. This synonymous mutation altered the mRNA folding and affecting its stability that is shown in figure 4(c). Alternation of mRNA folding can change the biological activity and stability changes can lead to abnormal protein production which has strong implications for the manifestation of the disease.<sup>36,37</sup> In the same exon sequence (exon-1) on 26 number codon, mutation named HBB:c.79G>A was identified where glutamine is substituted to lysine (GAG>AAG, Glu> Lys). This mutation results in reduced HbE synthesis due to the formation of functional beta E-mRNA is decreased for abnormal alternative splicing of precursor  $\beta$ -mRNA at a site 5' to the intron 1 (IVS-1).<sup>38</sup> GATA-1 putative transcription factor (TF) binding site was revealed on

this mutational position. This mutation also causes the structure change of  $\beta$ -mRNA.

The hematological data analysis showed that those were the blood transfusion-dependent thalassemia patients. The present study also demonstrated that  $\hat{a}$ -globin gene mutations, responsible for HbE/ $\hat{a}$  and  $\hat{a}$ -thalassemia condition, are highly heterogeneous in Bangladesh. One rare mutation (HBB: c.9T>C) was identified in Bangladeshi patients which was co-inherited with the other two common mutations (c.92+5G>C and c.79G>A). All the identified mutations showed significant impact on both structure and stability of mRNA that tremendously affects the tertiary structure of the beta globin chain. A large amount of data can give more information about the mutational pattern within *HBB* gene as the underlying cause of HbE/ $\beta$  and  $\beta$ -thalassemia in Bangladeshi patients. In future, this study will help to identify the incidence of  $\beta$ -thalassemia trait, HbE/ $\beta$  and  $\beta$ -thalassemia, and their coinheritance, mutations and its impact on mRNA structure as well as to support the patients who are already diagnosed with HbE/ $\hat{a}$  and  $\beta$ -thalassemia in Bangladeshi population for improving treatment strategies.

### Conclusion

Our study demonstrated the underlying cause of HbE/ $\beta$  and  $\beta$ -thalassemia in genetic level by identifying rare and common mutations within *HBB* gene and their effects on with *HBB* gene transcription and mRNA structure. We hope this study will contribute in designing effective molecular drug and other therapeutic approaches for the patients with HbE/ $\hat{a}$  and  $\hat{a}$ -thalassemia.

### Limitation of the study

This study conducted in greater Chattogram only with a small number of samples. More study with larger sample size will give true picture of mutational spectrum within *HBB* gene which will be helpful for the development and implementation of better treatment strategies for the patients with HbE/ $\hat{a}$  and  $\beta$ -thalassemia in Bangladesh.

### Ethical clearance

The research proposal was reviewed and approved by the 'Ethical Committee of Institute of Child Health, Chattogram Maa-O-Shishu Hospital (ICH, CMOSH).

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**Conflict of Interest:** There was no conflict of interest.

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**Ethical approval:** Ethical Committee of Chattogram Maa-O-Shishu Hospital, Institute of Child Health (CMOSH, ICH).

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