

REPORTING TWO RARE MUTATIONS WITH ASSOCIATED CLINICAL FEATURES IN BANGLADESHI Hb E/ β THALASSEMIA PATIENTS



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ABSTRACT

Thalassemia is one of the most common genetic disorders in Bangladesh. We have performed an investigation among 360 patients with Hb E/ β -thalassemia to understand the heterogeneity in thalassemia severity among Bangladeshi patients. The analysis revealed that one of the common mutations CD26 (G>A) is found with two rare mutations HBB: c.-79A>G (-29A>G) and HBB: c.45_46insG (CD14/15 (+G)) in two patients individually which are not prevalent in geographically adjacent populations. Among these two rare mutations, -29A>G is located at the 'TATA' box region of the promoter sequence regulating transcription initiation and CD14/15 (+G) leads to a premature stop codon. We performed transcription factor binding site prediction analysis and found that 'AP-1', 'c-Jun', and 'kr' transcription factors bind at the 'TATA' box region. From this analysis, we can predict that -29A>G mutation can alter the level of HBB transcription whereas the CD14/15 (+G) mutation causes a truncated (and possibly non-functional) HBB protein. Aligned with such assumption, it was observed from patients' medical history that, the patient who had CD14/15 (+G) and CD26 (G>A) mutations was severely blood transfusion-dependent whereas the patient carrying -29A>G and CD26 (G>A) mutations was moderately transfusion dependent. The comprehensive analysis of the mutations offers crucial insights and approaches for enhanced management of thalassemia among the Bangladeshi population.

KEYWORDS: hemoglobin E-beta thalassemia, rare mutation, thalassemia

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Introduction

Thalassemia is a genetic disorder that negatively affects the production of normal hemoglobin, leading to anemia and fatigue. More than 7000 children are estimated to be born with thalassemia every year in Bangladesh (Nur et al., 2022). There are two main types of thalassemia: alpha thalassemia, which involves reduced production of alpha globin chains (HBA), and beta-thalassemia, characterized by insufficient beta globin chain (HBB) production, with varying degrees of severity within each type. When *HBB* contains a mutation (G>A) at codon number 26 (CD26), the resultant variant of the *HBB* is called hemoglobin E (Hb E) where the amino acid at codon 26 changes from glutamic acid to lysine (Nancy et al., 2011). Any additional beta-thalassemia mutation together with CD26 (G>A) mutation of the *HBB* gene results in hemoglobin E-beta-thalassemia (Hb E/ β -thalassemia). Codon 26 mutation triggers the activation of a splicing site at the intersection of exon 1 and intron 1 (Nancy et al., 2011). As a result, it leads to irregular alternative splicing and reduced production of functional mRNA for the HBB globin chain. One having only CD26 (G>A) mutation generally shows symptoms similar to mild thalassemia. However, in any other β -thalassemia mutation with the CD26 (G>A) mutation, the symptoms may be similar

to β -thalassemia intermedia or β -thalassemia major depending on the severity of the β -thalassemia mutation (Suthat and David, 2012). Thus, although CD26 (G>A) mutation alone does not result in notable clinical complications, its co-presence with different mutations of the β globin gene and the resultant Hb E/ β -thalassemia may be manifested with a broad spectrum of clinical syndromes of varying degrees of severity (Suthat and David, 2012), such as infections and pulmonary hypertension tend to occur more frequently in individuals with a severe type of Hb E/ β -thalassemia and homozygous β -thalassemia compared to those with the intermediate type of Hb E/ β -thalassemia and compound heterozygous β -thalassemia (Dustin and Roberto, 2016). The finding of another research revealed that the codon 26 mutation was associated with severe hypochromic microcytic anemia (Hanan et al., 2023). This result was consistent with earlier studies where the CD26 (G>A) mutation was identified among Malaysians, Indonesians, as well as populations from Singapore, and Thailand (Elizabeth 2013; Manit et al., 2014; Yetti et al., 2022). Our study investigated 360 individuals (186 male and 174 female) diagnosed with Hb E/ β -thalassemia. Apart from common pathogenic mutations previously reported in the

Bangladeshi population (Abdul et al., 2020), we found two rare mutations in two different patients, which are not common in the neighboring populations. One of the mutations is HBB: c.-79A>G and the other one is CD14/15 (+G). In both cases, CD26 (G>A) mutation was present making them compound heterozygous Hb E thalassemia that tends to exhibit more prominent symptoms, commonly presenting as thalassemia intermedia and sometimes even displaying a thalassemia major phenotype (Suthat and David, 2012).

Our findings in this study report those two mutations from Bangladesh with associated clinicopathological features.

In an earlier study, CD14/15 (+G) was found in 1 out of 232 samples in prenatal screening which was reported as a rare mutation in our country (Abdul et al., 2020). However, it couldn't exhibit clinical features as the study was carried out with the carrier mother. In this study, our findings showed how these mutations are correlated with clinicopathological features of beta-thalassemia patients in Bangladesh.

Materials and Methods

Sample collection

This study was designed for Hb E/ β -thalassaemia patients who came to a tertiary-level hospital named Bangladesh Shishu (Children) Hospital and Institute (BSHI) from all over Bangladesh. The study was approved by the Institutional Review Board (IRB) of BSHI. Questionnaire data regarding the severity of Hb E/ β -thalassaemia were collected from patients or patient guardians. A written Consent form for this study was taken by participants/guardians. For the study, a 2 mL blood sample was collected from each participant in an EDTA vacutainer and stored at 2°C to 8°C for 2-3 days.

DNA extraction

DNA extraction was done from nucleated blood using PureLink™ Genomic DNA Purification Mini Kit (Invitrogen). The protocol was followed according to the manufacturer's instructions. The concentration and the purity index of the extracted DNA were measured through a Qubit 2.0 Fluorometer (Invitrogen, USA). The extracted DNA was stored at -20°C for later use.

PCR and Sequencing

The detection of the most common mutations in Bangladesh was performed using the amplification refractory mutation

system (ARMS) PCR method. Genomic DNA was amplified using specific primers for exons 1, 2, and 3 through PCR. The sequence of the primers is shown in supplementary data. For the identification of rare or novel mutations, Sanger sequencing was performed. The sequencing procedure covered the whole coding region (exons 1, 2, and 3), a segment of introns 1 and 2, the promoter region, and the splice site of the β -globin gene.

The sequencing was carried out using the BigDye terminator cycle sequencing kit (Thermo Fisher Scientific). The analysis of the sequencing data was performed using automated capillary electrophoresis (CE) in the Applied Biosystems 310 Genetic Analyzer. The results from capillary electrophoresis were aligned with the Reference Sequence of the β -globin gene using the Seqscape sequence alignment version 2.5 software from Applied Biosystems. The HbVar and ClinVar databases which contain information on hemoglobin (Hb) variants and thalassemia mutations were explored to determine the clinical significance and genetic variants (Tânia et al., 2017).

In silico analysis

Bioinformatics analyses were performed to prognosticate the potential impact of the mutation. NCBI Entrez database was visited to retrieve the HBB mRNA sequence. The OMIM database was explored to detect the occurrence of mutations previously reported in different populations (Ada et al., 2005). The promoter sequence was predicted by Eukaryotic Promoter Database (EPD) (René 2015) and putative transcription factor binding sites were identified using the PROMO version 2.0 web tool (Domènec 2003). Transcription factor binding sites were also investigated using the AliBaba2.1 prediction tool (Niels 2002). It predicts transcription factor binding sites by dynamically creating matrices based on TRANSFAC 4.0 sites. Furthermore, the presence of the polymorphism at *XmnI* in the 5' region of the $G\gamma$ site was examined. The presence of a restriction enzyme site was denoted by a "+" sign, while its absence was indicated by a "-" sign.

Result

In our study, two of the 360 patients had two uncommon mutations in the *HBB* gene, which have been presented in Fig.1 along with other common mutations. These rare mutations, namely -29 (A>G) and CD 14/15 (+G) are not prevalent in our neighborhood population. The patients who are carrying these rare mutations also have a common CD 26 (G>A) mutation.

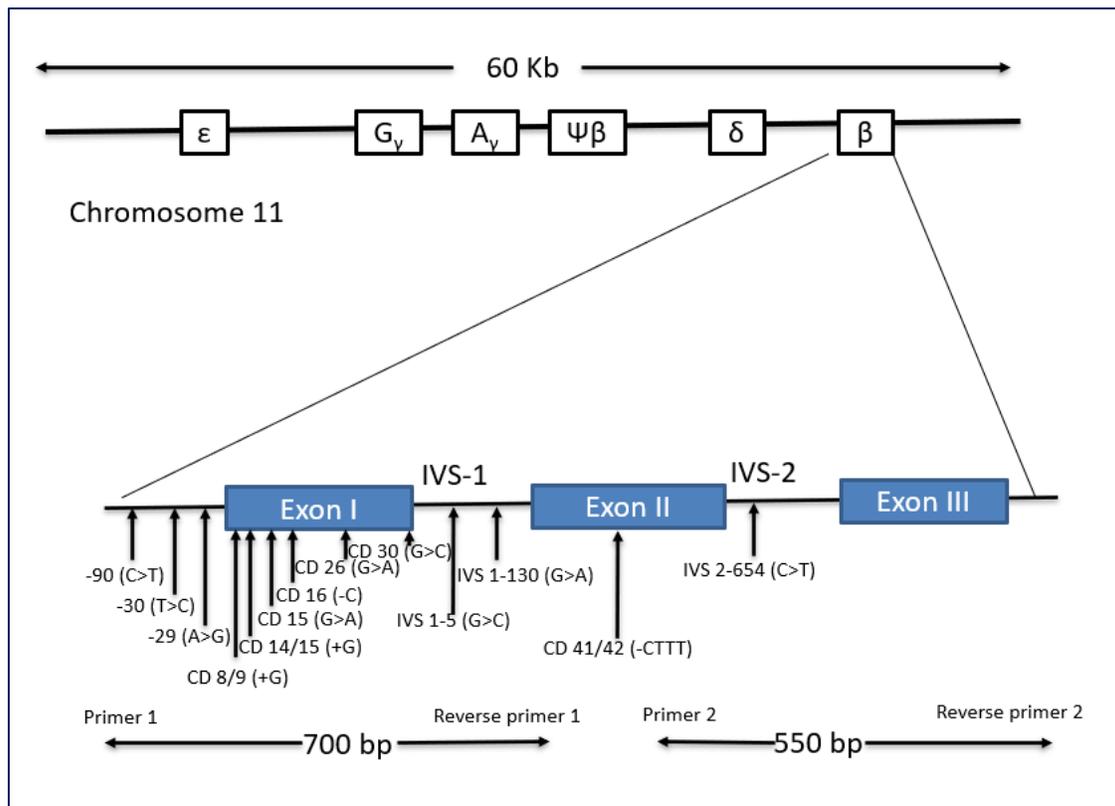


Figure 1. Map of human β -globin (HBB) gene cluster on chromosome 11 and common indicated mutations along with their location. Seven common mutations including IVS 1-5 (G>C), CD 26 (G>A), CD 8/9 (+G), CD 41/42(-CTTT), CD 30 (G>C), CD 15 (G>A), CD 16 (-C) and four uncommon mutations including -90 (C>T), -30 (T>C), IVS 1-130 (G>A) and IVS 2-654 (C>T) and two rare mutations including -29 (A>G) and CD 14/15(+G) found in patients in Bangladesh.

The clinical background of the two patients who carried these rare mutations has been presented in Table 1. Patient 1 had a hemoglobin percentage of 7.3% and was heterozygous for *XmnI* polymorphism. She was moderately severe transfusion-dependent, but the application of HU medicine was responsive and reduced her transfusion dependency. Patient 2 was sixteen

years old, had a hemoglobin percentage of 6.2%, and was heterozygous for *XmnI* polymorphism. She was regular transfusion dependent, but the application of HU medicine had no response. Both patients had comorbidities- patient 1 had non-insulin dependent diabetes, while patient 2 had facial deformity.

Table 1. Medical history of two patients carrying a rare mutation

Sample	Mutation	Age	District Name	Transfusion	Comorbidity	<i>XmnI</i> polymorphism	Hydroxyurea (HU) Medicine
Patient 1	-29 (A>G)	18 year s	Pabna	Moderately severe	Non-insulin-dependent diabetes	Heterozygous (-/+)	Response
Patient 2	CD 14/15 (+G)	16 year s	Bagerhat	Regular transfusion dependent	Facial deformity	Heterozygous (-/+)	No-response

The hematological characteristics of two patients with two rare mutations (-29 (A>G) and CD 14/15 (+G)) are presented in Fig.2. Patient 1, who carried the -29 (A>G) mutation, had a hemoglobin (Hb) concentration of 7.3 g/dL, while patient 2 carrying the CD 14/15 (+G) mutation had a hemoglobin concentration of 6.2 g/dL. Both patients had significantly low hemoglobin levels compared to the normal range of 12.5-16 g/dL. The mean corpuscular volume (MCV) in patient 1 and patient 2 were 59.6 fL and 60.6 fL, respectively, which was

lower than the normal range. The mean corpuscular hemoglobin (MCH) concentration was also lower than the normal range for both patients, with 20.1 pg/cell and 19.1 pg/cell, respectively. The mean corpuscular hemoglobin concentration (MCHC) did not change in them compared to the normal range. The red blood cell distribution width (RDW) was higher in both patients, while the hematocrit cell was lower than the normal range. In both cases, the red blood cell count was lower than the normal range (Fig 2).

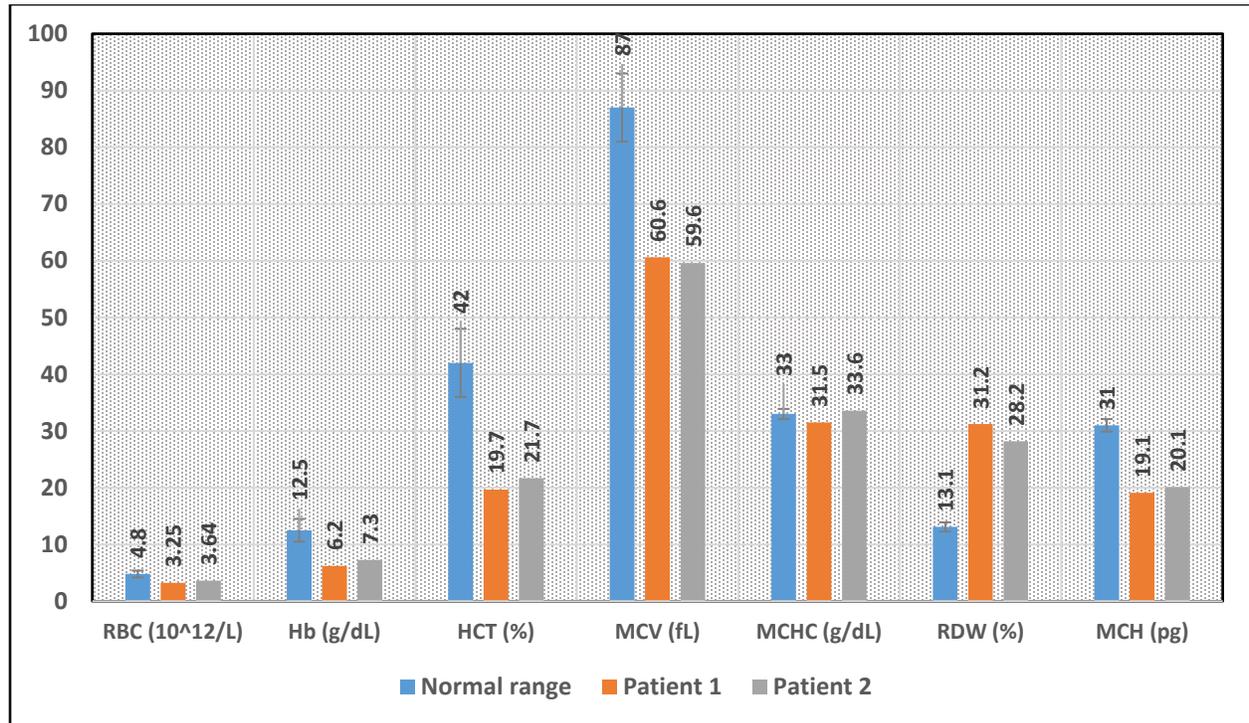


Figure.2. Hematological and clinical data of the thalassemia patients carrying the rare mutation. Here, patient 1 obtained CD 14/15(+G) and CD 26(G>A) mutation whereas patient 2 carried -29(A>G) and CD 26(G>A) mutation.

The sequence analysis of the patients showed that both had compound heterozygous Hb E thalassemia. Patient 1 had -29 (A>G) and CD 26 (G>A) mutation (Figure.3) whereas patient 2 had CD 14/15 (+G) and CD 26 (G>A) mutations (Figure.4)

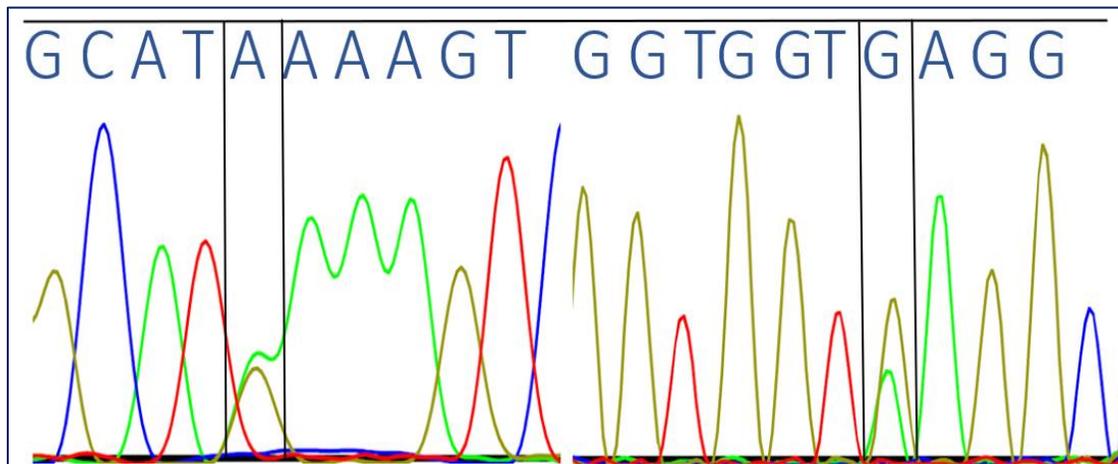


Figure 3. Chromatogram showing compound heterozygous of -29 (A>G) and CD 26 (G>A) mutation in patient 1 DNA

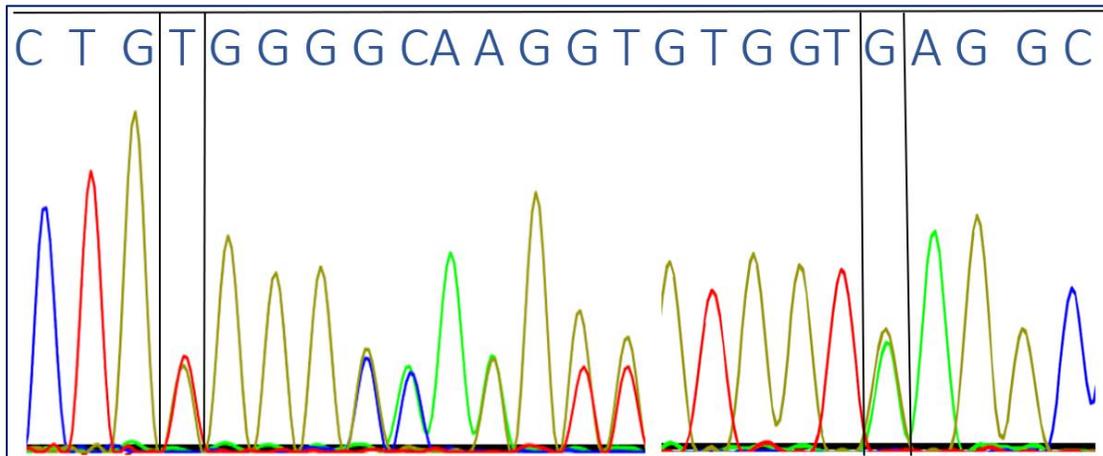


Figure 4. Chromatogram showing compound heterozygous of CD 14/15 (+G) and CD 26 (G>A) mutation in patient 2

The rare mutation HBB:-79A>G (-29A>G) is located upstream of the transcription start site (TSS). Changes in the segment of the 'TATA' box of the promoter, specifically occurring between positions -26 and -30, led to a notable reduction in transcription levels. Our computational analysis showed the presence of a putative binding site for transcription factors 'AP-1' and 'c-Jun' in Fig. 5a. The c-Jun, protein of the activator protein-1 (AP-1) complex, plays a crucial role in cellular processes, including proliferation, survival, tumorigenesis,

apoptosis, and tissue morphogenesis (Qinghang and Ying 2011). Using another prediction tool, we found that the 'TATA' box is the binding site for the 'kr' transcription factor (Fig. 5b). On the other hand, the addition of a nucleotide (+G) between codons 14 and 15 of the β -globin gene leads to a frameshift mutation, which causes premature termination of the protein due to generating a stop codon downstream 20 bases of the coding sequence.

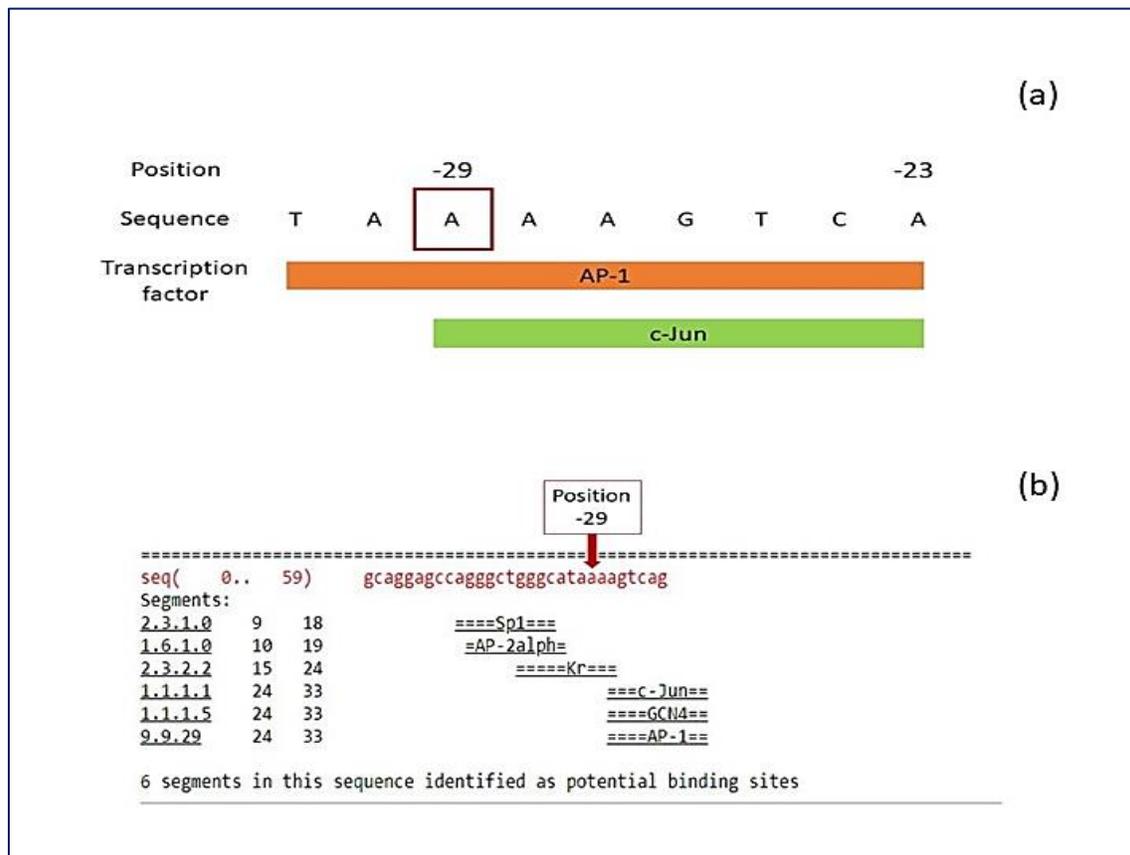


Figure 5. Predicted binding site for transcription factor at position -29 upstream of transcription start site by Promo (a) and Alibaba2 (b) prediction tool

Discussion

Hemoglobin E (Hb E) which results from the mutation CD 26 (G>A) in the *HBB* gene, is a widely prevalent variant of hemoglobin in numerous Asian countries. When combined with beta-thalassemia mutations, Hb E often leads to a moderately severe phenotype due to a reduction in the synthesis of beta globin protein (Edward et al., 1981). Having compound heterozygosity for Hb E beta-thalassemia can lead to a wide range of outcomes, varying from having no symptoms to requiring regular blood transfusions. Here we have reported two rare mutations- *HBB*:c.-79A>G [-29 (A>G)] and *HBB*:c.45_46insG [CD 14/15 (+G)] in two cases that are not prevalent in neighboring populations. The *HBB* c.-79A>G variant was observed in patients either in a homozygous state or in combination with another pathogenic *HBB* variant (Stylianou et al., 1984; Gisele et al., 2017). Functional analysis of this variant reveals a substantial decrease in beta-globin transcription, reducing it to approximately 25 percent of normal levels (Stylianou et al.,

1984). It causes a 13-fold decrease in the rate at which TBP complexes and the TATA box associate, compared to those found in healthy individuals. Additionally, the Kd value, which represents the affinity between TBP and TATA, is reduced by 10-fold. The other reported rare mutation CD 14/15 (+G) leads to an early stop codon in the gene, which is expected to result in a shortened or completely missing protein due to a process called nonsense-mediated decay. CD 14/15 (+G) has been unveiled in various individuals with beta-thalassemia in clinical studies and is commonly referred to as a prevalent mutation among Chinese populations (Vivian et al., 1988; Min et al., 2014). Based on the available information, it can be inferred that this variation is linked to the severe form of thalassemia.

This study has revealed two rare mutations with associated clinicopathological data. Further studies in larger populations are required to establish the actual frequency of these mutations in our country.

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