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Disease-Modifying Effect of HBS1L-MYB in HbE/β-Thalassemia Patients in Bangladeshi Population

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Abstract: Background: Thalassemias are a group of autosomal recessive disorders and the most common inherited disease worldwide. Fetal hemoglobin (HbF) is the main oxygen carrier protein in the human fetus. Elevated HbF level is known to ameliorate the severity of HbE/β and β-thalassemia. This study aimed to investigate whether two commonly known HbF-associated SNPs (rs28384513 and rs4895441) in the HBS1L-MYB region are associated with HbF level and disease severity in Bangladeshi HbE/β-thalassemia patients. **Methods:** Blood samples were collected from 160 participants (120 HbE/β-thalassemia patients and 40 healthy controls). Hematological analysis was performed using complete blood count (CBC) and capillary Hb electrophoresis. After genomic DNA extraction, real-time PCR-based high-resolution melting (HRM) for SNP detection, targeting the HBS1L-MYB intergenic region, was done. **Results:** Patients carrying rs28384513 and rs4895441 SNPs had significantly higher HbF (1.29 ± 1.63 and 1.49 ± 1.7 g/dL, respectively) compared to major allele 'TT' and 'AA' (0.87 ± 1.1 and 1.19 ± 1.65 g/dL, respectively) with a p-value of 0.01 and 0.03, respectively. It has been detected that HbF levels in SNP-carrying patients significantly correlated with the higher transfusion interval (60 days, r = 0.38, $p < 0.0001$) and age of first transfusion (65 months, r = 0.26, $p < 0.0028$) in these patients. Further, non-transfusion-dependent patients had the highest HbF level (2.03 ± 2.05 g/dL) compared to transfusion-dependent moderate (0.58 ± 0.78 g/dL) and severe (0.84 ± 1.27 g/dL) patients generating a significant p-value < 0.0001 in One-Way ANOVA test. The minor allele frequencies of rs28384513 (G) and rs4895441 (G) were found to be 0.43 and 0.11 respectively. **Conclusions:** These findings suggest that SNPs of HBS1L-MYB may have a role in elevated HbF levels and ameliorating disease severity in terms of transfusion in HbE/β-thalassemia patients.

Keywords: HbE/β-thalassemia; HBS1L-MYB; fetal hemoglobin (HbF); hemoglobin (Hb)

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1. Introduction

Thalassemia is an inherited hematological disorder categorized by a decrease or absence of one or more of the globin chain synthesis. β-thalassemia is caused by one or more mutations in the β-globin gene. The absence or reduced amount of β-globin chains causes ineffective erythropoiesis which leads to anemia [1]. WHO reported that approximately 1.5% of the global population are carriers of β-thalassemia and 68,000 children are born each year with various thalassemia syndromes [2]. However, precise and up-to-date data on the frequency of β-thalassemia carriers and patients are missing due to a lack of accurate diagnostic approach, limited access to information, and the absence of national screening programs in many of the thalassemia-inflicted countries. In Bangladesh 11.89% carriers of β-globin gene mutations, 8.68% had HbE trait (ETT) and 2.24% had β-thalassemia trait

(BTT). Among eight divisions, Rangpur had the highest carrier frequency of 27.1% [3]. Hemoglobin E (HbE) is an abnormal form of hemoglobin with a single point mutation in the β chain. There is a change in the amino acid, from glutamic acid to lysine (E26K) at position 26. The mutation affects β-gene expression creating an alternate splicing site in the mRNA at codons 25–27 of the β-globin gene resulting in abnormal form HbE [4]. Despite having similar genetic backgrounds, patients show remarkable disease heterogeneity, ranging from nearly asymptomatic to severe transfusion dependence. A scoring system that reflects the clinical severity of b-thalassemia/ Hb E is proposed. It allows the separation of patients into three distinctive severity groups: mild, moderate, and severe [5]. One of the mechanisms for the different clinical expressions may be primarily from the elevated HbF level in patients. As defective gene function leads to the partial suppression of β-globin protein production, the increased number of γ-chains helps to neutralize the large proportion of unbound α-chains and results in higher HbF levels. Increased HbF levels can ameliorate the severity of these disorders. HbF acts as one of the major ameliorating factors. Elevation of HbF level is associated with different quantitative trait loci (QTLs) like BCL11A, HBG2 promoter, and HBS1L-MYB intergenic region. QTLs, located in the HBS1L-MYB intergenic region and in the BCL11A gene, are either directly involved in HbF gene silencing in adult life or in cell proliferation and differentiation [6–9]. BCL11A (2p16.1), HBS1L-MYB (6q23.3), and HBG2 promoter regions account for approximately 20–50% of HbF variation depending on the population studied, with the remaining variance in HbF level unaccounted for, indicating that additional loci are involved [10,11]. Therefore, the objective of this study was to evaluate whether genetic variability especially rs28384513 and rs4895441 at HBS1L-MYB intergenic region influences HbF levels in HbE/β-thalassemia patients in the Bangladeshi population.

2. Materials and Methods

2.1. Study Population

A total of 120 HbE/β-thalassemia patients were enrolled in the study. Forty unrelated healthy individuals' blood samples were also collected for comparison. The samples were collected from the "Thalassemia Samity Hospital" with the full consent of the hospital authority from September 2018 to April 2019. All the patients or legal guardians provided their ethical compliance by filling up a consent form before contributing to the study. After collection, the hematological, molecular, and genetic laboratory experiments were conducted in Biosafety Level 2 (BSL-2) facilities of the Genetics and Genomics Laboratory at the Institute for Developing Science and Health Initiatives (ideSHi). Bangladesh Medical Research Council (BMRC) had provided the ethical approval.

The study was designed to experiment with 160 participants (120 HbE/β-thalassemia patients and 40 healthy controls) in two parts: (1) Hematological analysis by Complete Blood Count (CBC) and Capillary Hb electrophoresis, (2) Real-Time PCR followed by High-Resolution Melting (HRM) for SNPs detection lying in HBS1L-MYB intergenic region (Figure 1).

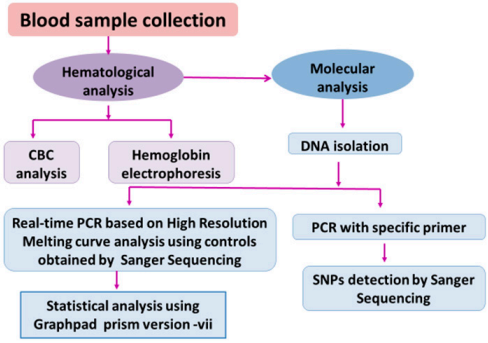


Figure 1. Workflow of the study.

2.2. Study Site and Ethical Approval

All the samples were collected with full consent from the patients and their legal guardians from Thalassemia Samity Hospital as well as all the hematological, molecular, and genetic laboratory experiments were conducted in Biosafety Level 2 (BSL-2) facilities of the Genetics and Genomics Laboratory at Institute for Developing Science and Health Initiatives (ideSHi). Bangladesh Medical Research Council (BMRC) had provided the ethical approval. The samples were collected from the "Thalassemia Samity Hospital" with the full consent of the hospital authority from September 2018 to April 2019. All the patients or legal guardians provided their ethical compliance by filling out a consent form before contributing to the study.

2.3. Hematological and Molecular Test

For complete blood count analysis, blood in an EDTA tube was used in 'Automated hematology analyzed Sysmex kx-21 (Sysmex Corporation, Kobe, Japan)' according to the manufacturer's instructions. MCV, MCH, RDW, and hemoglobin levels were used in this current study. Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH) are the most widely recommended RBC indices for the preliminary screening of thalassemia. In the present study, MCV less than 80 fL and/or MCH less than 27 pg were used as cut-off values to initially suspect the participants to be carriers of thalassemia [12]. Hemoglobin electrophoresis was performed for all the samples irrespective of the values of MCV and MCH as it is the gold standard method for thalassemia carrier detection. HbA2 level > 3.5% was used as a cut-off value for screening for beta-thalassemia [13]. Hemoglobin electrophoresis was performed by 'CAPILLARYS 2 FLEX-PIERCING (Serbia, France)'. CAPILLARYS HEMOGLOBIN (E) kit was used for the analysis. Relative quantification of individual hemoglobin fractions such as HbA, HbF, and HbA2 was performed automatically at the end of the analysis. High-resolution separation of the major hemoglobin variants (HbS, HbC, HbD, and HbE) is allowed by CAPILLARYS instruments and accurate quantification of the HbA2 and HbF [14]. DNA was extracted by using the QIAGEN FlexiGeneR DNA kit (QIAGEN, Hilden, Germany) manual, genomic DNA was extracted from whole blood [15]. DNA concentration was measured with (Nanodrop 2000).

The DNA sequence of the human HBS1L-MYB intergenic region was retrieved from the nucleotide database of the National Center for Biotechnology Information (NCBI) to design the primers for the amplification of the human HBS1L-MYB gene. The primer pair is designed to amplify the 456 bp for rs28384513 and 439 bp for rs4895441 HBS1L_MYB intergenic region. By using the oligo-analyzer tool of IDT (Integrated DNA Technologies, United States of America, the properties of primers e.g., -GC%, Tm, heterodimer, and self-dimer were checked as well as by using the NCBI primer BLAST tool, specificity and product length of the amplified products of primers were checked. Two modified gene variants were studied (HBS1L-MYB, rs28384513, and rs4895441). PCR High-resolution Melting (HRM) method was used for genotyping. The basic information on two SNPs is displayed in Table 1 [16]. The results of the genotype were confirmed by Sanger sequencing.

Table 1. The basic information of two SNPs of our study.

SNPs	Chromosome	Location	Gene	Function	Mutation
rs28384513	6	135,376,209	HBS1L-MYB	Intergenic region	C/A
rs4895441	6	135,426,573	HBS1L-MYB	Intergenic region	G/A

Primer sequences and other information for the reactions of these two SNPs are displayed in Table 2.

Table 2. Primer sequence and other information for real-time PCR HRM and Sanger Sequencing.

Primer Name	Sequence (5′-3′)	Tm	Product Length (bp)
HBS1L-MYB_rs2838p4513_HRM_F	TTGGACTAAATGTTGCAAGCGG	65.93	456
HBS1L-MYB_rs28384513_HRM_R	ACTGAGCGCATAGCTTTCTCAG	62.36	
HBS1L-MYB_rs4895441_HRM_F	ATGGGGGTAAGAAGGAAACCAG	58.17	439
HBS1L-MYB_rs4895441_HRM_R	CTCCCTGTCCCCAGATACTTAC	60.18	
HBS1L-MYB_rs28384513_SEQ_F	CGGCAATGCCTCAGGGTCACTG	65.93	456
HBS1L-MYB_rs28384513_SEQ_R	TATGTTGCTCAGGCTGGTCTCG	62.36	
HBS1L-MYB_rs4895441_SEQ_F	GTGTTGGGATATAGGCCATAGAC	58.17	439
HBS1L-MYB_rs4895441_SEQ_R	GGTCTACAAAGCCCTACAGGATC	60.18	

2.4. Scoring System

The groups of patients were scored. The score from 0 to 2.8 indicated a mild form of HbE/b-Thalassemia, from 3.2 to 5.6 indicated moderate, and from 6 to 8 indicated a severe form of HbE/β-Thalassemia as four clinical criteria (Hb at steady state, age of first blood transfusion, transfusion interval, and splenectomy data) were used in this study [5].

2.5. Statistical Analysis

To find statistical significance, all the results were subjected to statistical analysis. With the data set, a two-tailed *T*-test, one-way ANOVA test, and correlation analysis were performed by using the software 'Graph-Pad Prism version-7′ (Graph-Pad Software, La Jolla, CA, USA). The data were articulated as suitable units depending on the variable mentioned such as number (N), percentage, and Mean ± Standard deviation (SD). The Probability (P) value less than 0.05 and correlation (r) near or above 0.5 were considered significant values when the data were compared.

3. Results

3.1. Demographic Information of the Study Population

A total hundred and twenty (120) HbE/β thalassemia patients were recruited with complete information including hematological data, age of first transfusion, transfusion interval, history of splenomegaly or splenectomy, etc. Demographic data of the study participants are shown in Table 3.

Table 3. Demographic data of the study.

Parameters		Total Number of Patients (n = 120)
Age	Range	3 years to 65 years
	Mean ± SD	19.66 ± 10.22
Pediatric (n = 45, 37.5%)	Male, n (%)	23 (19.2%)
	Female, n (%)	22 (18.3%)
Adult (n = 75, 62.5%)	Male, n (%)	45 (37.5%)
	Female, n (%)	30 (25.0%)

We found significant variance in MCV levels between the pediatric and adult groups with a *p*-value of 0.007. While other parameters total Hb and HbF showed no significant (*p* values 0.37 and 0.11 respectively) variance between these two groups (Table 4).

Table 4. Comparison of hematological parameters between pediatric and adult groups of HbE/β-Thalassemia patients.

Group	MCV (fl/red Cell)	HB (g/dL)	Hemoglobin-F
Pediatric	71.07 ± 6.52	7.51 ± 1.27	1.19 ± 1.87
Adult	67.53 ± 7.97	7.41 ± 1.37	1.25 ± 1.30
p Value	0.007	0.37	0.11

3.2. Comparison of Hematological Parameters and Severity of the Disease Among Three Groups of HbE/β-Thalassemia Patients

The age of the first blood transfusion (months) and transfusion interval (Days) of the three groups are summarized in Table 5. The NTD/mild group has the highest mean ± SD of transfusion interval as well as the first age of transfusion. On the other hand, the Severe group has the lowest first age of transfusion (months) and transfusion interval (Days). This comparison showed significant variation with a *p*-value of <0.0001 (Figure 2I,J).

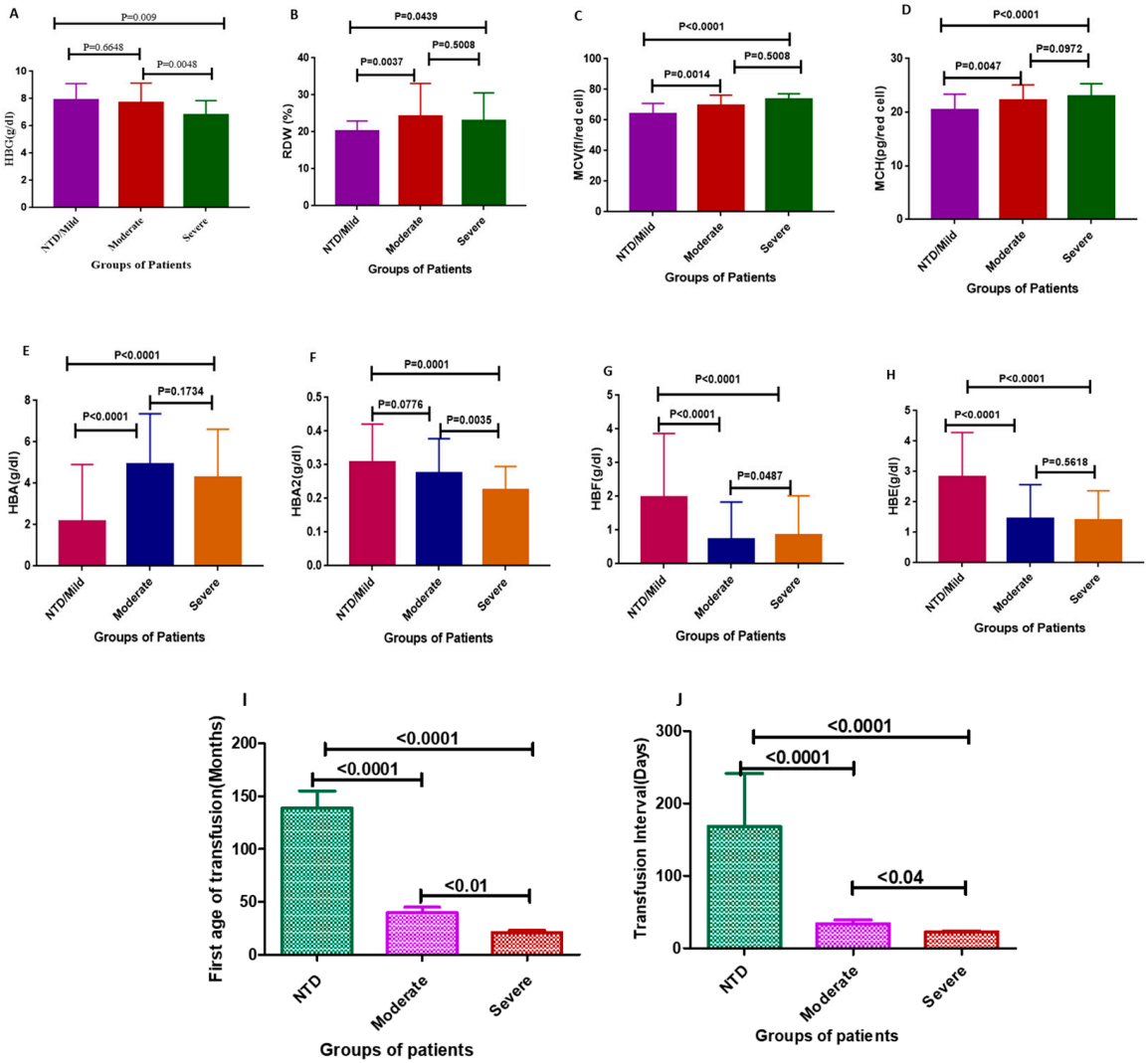


Figure 2. Comparisons of Hematological parameters among three groups by ANOVA and *t*-test.

Table 5. Severity of the disease among the HbE/β-Thalassemia patients enrolled in the Study.

Group	HGB (g/dL)	RDW (%)	MCV (fl/red Cell)	MCH (pg/red Cell)	Hemoglobin-A	Hemoglobin-A2	Hemoglobin-F	Hemoglobin-E	First Age of Transfusion (Months) Mean ± SD	Transfusion Interval (Days) Mean ± SD
NTD	7.88 ± 1.23	27.05 ± 8.5	64.11 ± 7.24	20.21 ± 2.9	2.27 ± 2.81	0.33 ± 0.1	2.03 ± 2.05	2.88 ± 1.47	141.79 ± 131.5	100.88 ± 91.48
Moderate	7.69 ± 1.44	24.24 ± 8.87	69.46 ± 6.85	21.99 ± 3.08	5.33 ± 2.61	0.27 ± 0.1	0.58 ± 0.78	1.36 ± 1.1	33.76 ± 44.3	36.14 ± 41.29
Severe	6.82 ± 1.03	22.97 ± 7.59	73.26 ± 3.82	23.28 ± 2.27	4.39 ± 2.42	0.23 ± 0.07	0.84 ± 1.27	1.35 ± 0.97	17.16 ± 16.11	22.23 ± 9.79
p Value	0.009	0.0439	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	<0.0001	<0.0001	<0.0001

There was no significant difference in HGB and RDW levels (Figure 2A,B). When compared among all three groups, there were statistically significant differences in MCV and MCH levels ($p < 0.0001$, Table 5 and Figure 2C,D).

The hemoglobin variants such as HbA, HbA2, HbF, and HbE were significantly different (p-value < 0.0001, 0.0001, <0.0001, and <0.0001 respectively) among Mild, Moderate, and Severe patients (Figure 2E–H). Table 5 shows that HbA2, HbF, and HbE levels were highest in NTD/mild groups, while the lowest HbA2 and HbE levels were in severe groups, but HbF was higher than in moderate groups. On the other hand, unexpectedly moderate groups and severe groups show the highest HbA level.

3.3. Genotype Distribution and Genotype Frequencies of Two SNPs

3.3.1. Genotype Distributions Among HbE/β-Thalassemia Patients

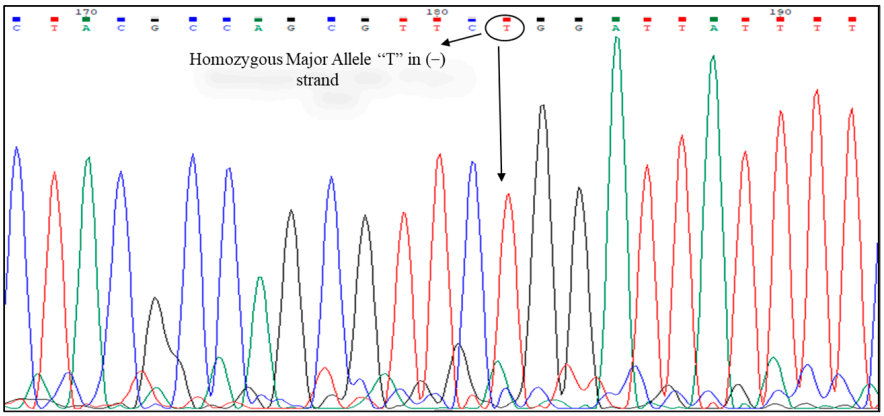
The genotype results of two SNPs using the PCR-HRM method were 100% consistent with direct sequencing. SNP rs28384513 of HBS1L-MYB was detected by Sanger Sequencing. We found, homozygous 'TT', Heterozygous 'TG', and Homozygous 'GG' in the study population. In the case of SNP rs4895441 of HBS1L-MYB, heterozygous AG, and homozygous 'AA' genotypes were found but no homozygous genotype 'GG' was found (Figure 3).

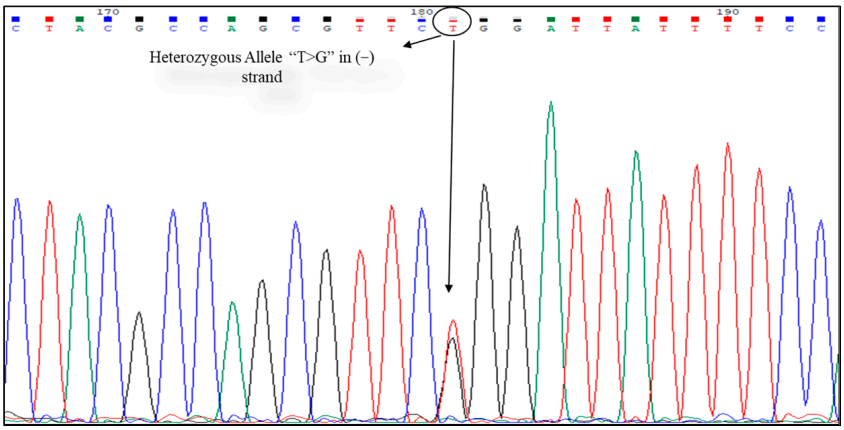
Secondly, the sequenced samples were used as reference samples with known genotypes, and the rest of the samples were tested for the presence of the two SNPs (rs28384513 and rs4895441) by Real-time PCR followed by High-Resolution Melting (HRM) Curve Analysis in the patients of HbE/β-Thalassemia. These temperature-shifted curves showed the differences in melting temperatures in the presence of the polymorphic alleles. Blue curves in Figure 3 showed the homozygous 'TT' genotype, Green showed the homozygous 'GG' genotype and Red showed the heterozygous 'TG' genotype of rs28384513 SNP of HBS1L-MYB gene in HbE/β-thalassemia patients (Figure 3).

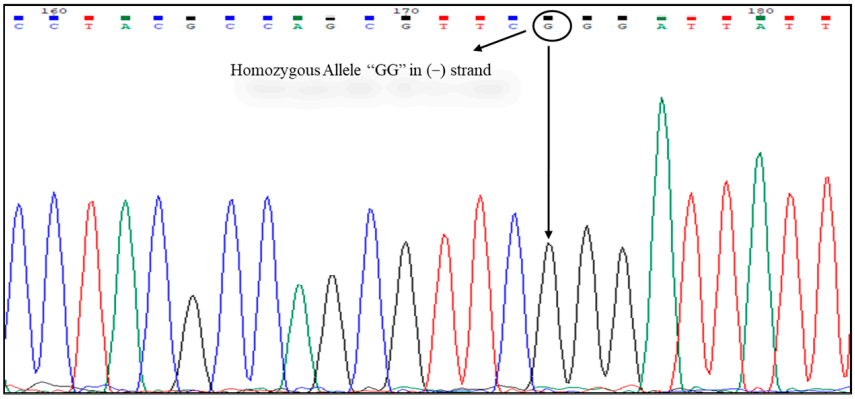
3.3.2. Genotype Frequency Among HbE/β-Thalassemia Patients

Among one hundred and twenty (120) HbE/β-Thalassemia patients, in the case of rs2838513 NTD/mild patients carried the highest (25.86%) TG + GG genotype compared to the other two groups. On the other hand, in the case of rs4895441 Homozygous genotype 'AA' showed a greater percentage (26.67%) compared to the heterozygous 'AG' genotype. That means rs28384513 (T>G) is more frequent in our population (Table 6).

rs28384513 SNP :







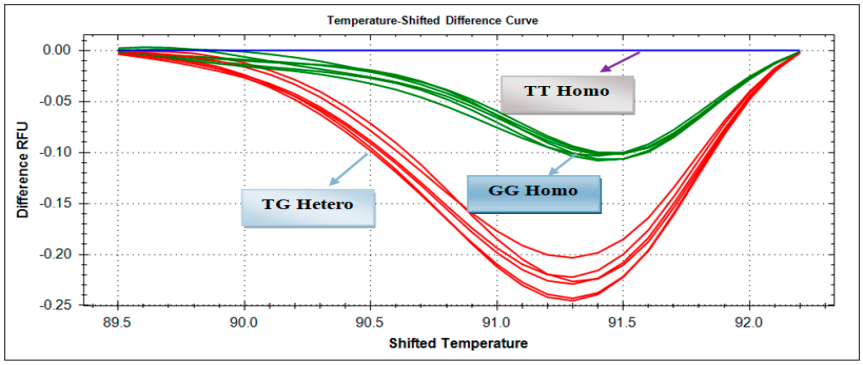


Figure 3. *Cont.*

rs4895441 SNP:

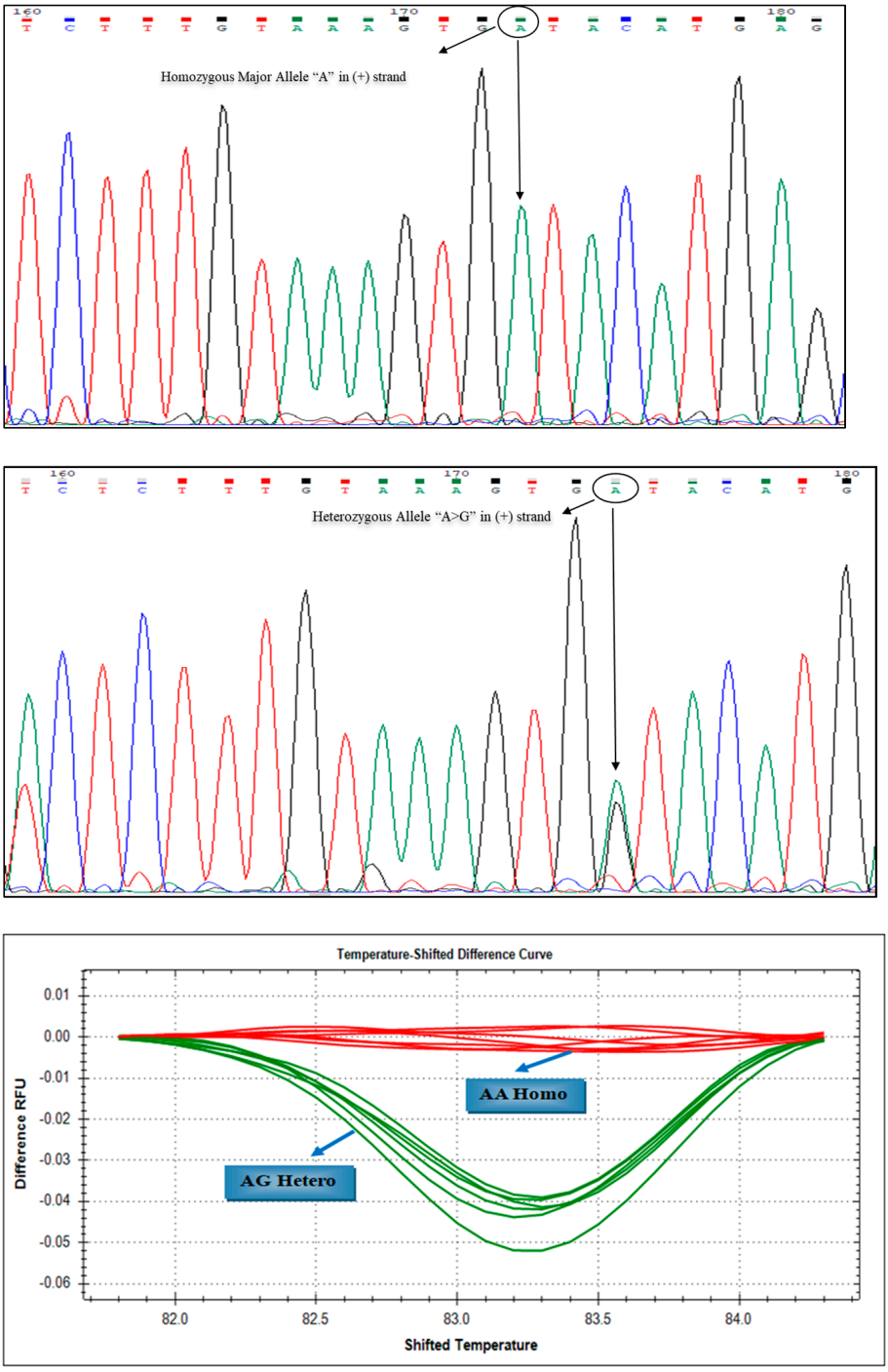


Figure 3. Genotyping by HRM and confirmation by Sanger sequencing of 2 SNPs. In our population, in rs28384513, homozygous 'TT' and 'GG' and heterozygous 'TG' had been found which comprised 30% of 'TT' and 70% of 'TG + GG' genotype. On the other hand, in rs4895441, about 79% AA and 21% AG genotypes were reported in this study among the enrolled patients (Figure 4).

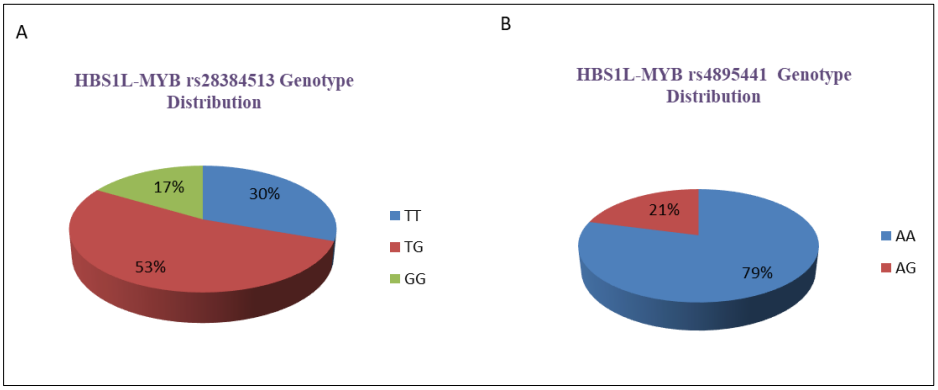


Figure 4. Allele Distributions among HbE/β-Thalassemia patients of rs28384513 (**A**) and rs4895441 HBS1L-MYB (**B**).

Table 6. Allele Frequency of rs28384513 and rs4895441 among three groups.

Locus	Genotype	NTD/Mild	Moderate	Severe
rs**28384513(T>G)**	TT	13 (10.83%)	19 (15.83%)	5 (4.17%)
	TG + GG	31 (25.83%)	30 (25%)	22 (18.33%)
rs**4895441(A>G)**	AA	32 (26.67%)	40 (33.33%)	22 (18.33%)
	AG	12 (10%)	9 (7.5%)	5 (4.17%)

3.4. Association of SNPs in HBS1L-MYB with Disease Severity of HbE/β-Thalassemia Patients

3.4.1. Comparison of HbF Level Between HbE/β-Thalassemia Patient Groups with Different SNPs of HBS1L-MYB

In the case of rs28384513, genotype 'TG + GG' showed the highest (1.29 ± 1.63) HbF values as expected than the 'TT' genotype while significant differences had been found in HbF (p-value = 0.01). Similarly, in rs4895441 both 'AA' and 'AG' genotypes showed expected statistically significant differences of HbF (p = 0.03), shown in (Table 7). The comparison of Mean ± SD of HbF (g/dL) was done by T-test and the results are shown in Figure 5.

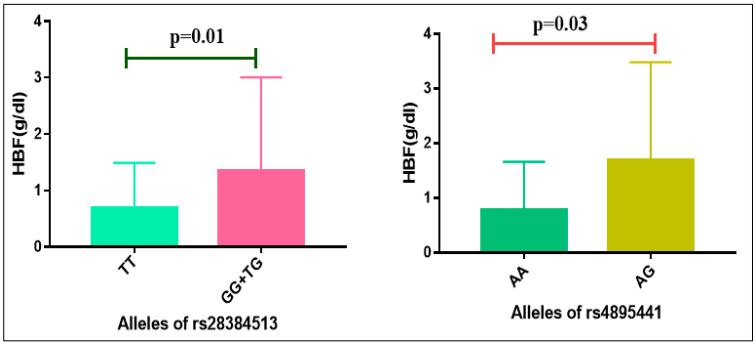


Figure 5. Comparing HbF through t-test results between major and mutant alleles of rs28384513 and rs4895441.

Table 7. Comparison of fetal hemoglobin level among HbE/β-Thalassemia patients with different SNPs of HBS1L-MYB.

Gene (Choromosoe)	Locus	Genotype	Numbers	Mean ± SD (HbF)	t Test (p)
HBS1L-MYB(6q23)	rs28384513 (T>G)	TT	37 (31%)	0.87 ± 1.1	0.01
		TG + GG	83 (69%)	1.29 ± 1.63	

Table 7. *Cont.*

Gene (Choromosoe)	Locus	Genotype	Numbers	Mean ± SD (HbF)	*t* Test (*p*)
HBS1L-MYB(6q23)	rs4895441 (A>G)	AA	95 (79%)	1.19 ± 1.65	0.03
		AG	25 (21%)	1.49 ± 1.7	

3.4.2. Correlation Studies of HBF Concentration and Disease Severity

There is a significant correlation of HBF (g/dL) with transfusion interval (Days) including r = 0.38, R squared = 0.14, and p <0.0001 *, and a negatively significant correlation was found with Clinical Score (r = −0.31, R squared = 0.0.09 and p value 0.0003 *).

However, less correlation was found between HBF (g/dL) and age of first blood transfusion where r = 0.26, R squared = 0.07, and p = 0.0028 *. So, it can be said that there was a significant but less correlation found of HbF (g/dL) with the age of first blood transfusion (Figure 6).

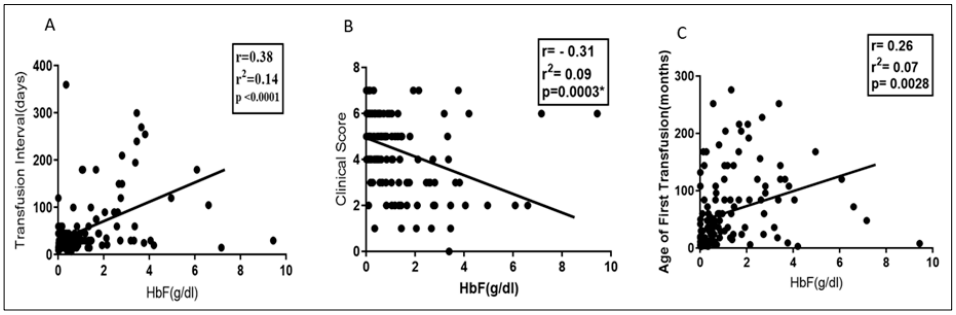


Figure 6. Pearson Correlation test of HBF (g/dL) with Transfusion Interval (Days) (**A**), Age of first blood Transfusion (Months) (**B**), and Clinical Score (**C**).

3.5. Demographic, Hematological Information and Genotype Frequency Among Healthy Individuals

Forty (40) healthy controls including 22 (55%) males and 18 (45%) females were with a mean age of 21.64 ± 3.31 years recruited in this study. All of them had completely normal hematological and hemoglobin parameters. In SNP rs28384513, 31% homozygous genotype TT, 46% heterozygous genotype TG, and 23% homozygous genotype were found. In SNP (rs4895441), 75% homozygous genotype AA and 25% heterozygous genotype AG were found (Figure 7).

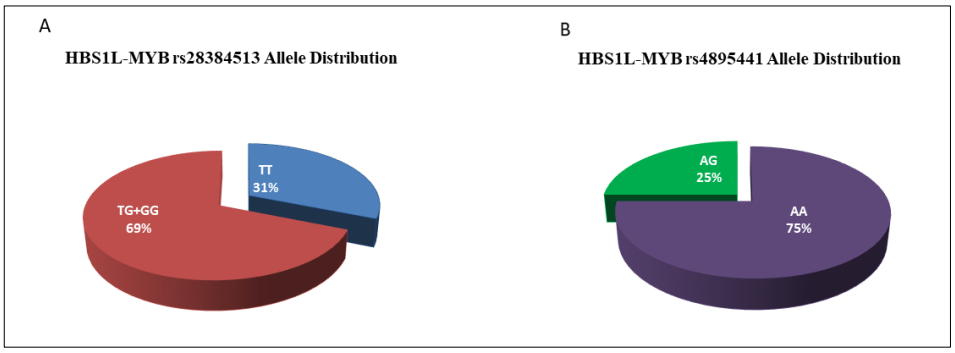


Figure 7. Allele Distributions among Healthy Individuals of rs28384513 (**A**) and rs4895441 (**B**) SNP of HBS1L-MYB.

3.6. Allele Frequency of rs28384513 and rs4895441 SNP of HBS1L-MYB in Total Study Population

For rs2838513, major allele T and minor allele G were detected. In the case of rs4895441 major and minor alleles A and G were detected respectively. The minor allele (G) frequency

of rs28384513 and rs4895441 in the HBS1L-MYB was found as 0.43 and 0.11 respectively in our study population (Table 8).

Table 8. Allele frequency of total study population.

Genotype	Total Sample (160)
Allele Frequency of rs28384513	
T allele	184 (0.58)
G allele	136 (0.43)
Allele Frequency of rs28384513	
A allele	285 (0.89)
G allele	35 (0.11)

4. Discussion

HbF is one of the indicators that play an important role in disease modifiers as it is able to reimburse the deficiency of β-globin chains and HbA. High HbF levels are correlated with reduced morbidity and mortality. Genetic studies have identified common variants within the intergenic region (HBS1L-MYB) between GTP-binding elongation factor HBS1L and myeloblastosis oncogene MYB on chromosome 6q that are associated with elevated HbF levels and alterations of other clinically important human erythroid traits. It is unclear how these noncoding sequence variants affect multiple erythrocyte characteristics [15,16]. The association between HbF levels and DNA polymorphisms in the HBS1L-MYB intergenic region has been demonstrated in different ethnic backgrounds [6,7,9,16,17].

This study aimed to show the effect of SNPs of HBS1L-MYB intergenic region with disease severity and clinical heterogeneity. In this study, we identified an association between rs28384513 and rs4895441 on erythropoiesis and HbF levels in the Bangladeshi population. In different literature reviews from Asian countries, we identified that the HBS1L-MYB gene is strongly associated with HbF fluctuation in thalassemia patients. So, we targeted three SNPs (rs4895441, rs9399137, and rs28384513) from this intergenic region in our study [6,7,16–22]. In adults, the level of HbF is ~1% of total hemoglobin which is genetically controlled and can be a critical modifier of the clinical severity of the major β-hemoglobin disorders. β-globin chain is deficient in β-thalassemia. So, increased γ-globin expression reduces the imbalance of the α- and β-globin chains and affects various clinical heterogeneity. Patients with transfusion-dependent HbE/β-thalassemia disease require lifelong regular blood transfusion for survival, while NTD patients generally have mild anemia and do not require regular blood transfusion for survival [23,24]. Here, in our study, Non-Transfusion Dependent patients showed the highest HbF level as well as the highest transfusion interval and age of first transfusion (100.88 ± 91.48 and 141.79 ± 131.5) than moderate (36.14 ± 41.29 and 33.76 ± 44.3) or severe (22.23 ± 9.79 and 17.16 ± 16.11) patients indicating ameliorated disease severity which unambiguously indicated the heterogeneity of clinical phenotypes (Table 5). One-way ANOVA test among NTD, Moderate, and Severe generates a significant p-value < 0.0001 indicating significantly different HbF values among these three groups (Figure 2G).

In our study, the allelic distribution of rs28384513 in the HBS1L-MYB intergenic region was 30% TT and 70% GG + TG and for rs4895442, there were 79% AA and 21% AG (Figure 4A,B). In our study, both SNPs rs28384513 and rs4895441 showed higher mean ± SD 1.29 ± 1.63 and 1.49 ± 1.7 respectively for HbF compared to wild type as well as significant association with induction of HbF (g/dL) (p = 0.01 and p = 0.03) in HbE/β-thalassemia patients (Table 7). These SNPs were significantly associated with either HbF levels or the degree of clinical patients reported by another study performed on the Indian population [25]. Genotype analysis revealed that the allele of HBS1L-MYB QTL rs4895441 is predominantly associated with β-thalassemia. This is consistent with previous reports from European, Chinese, and African β-thalassemia intermedia and SCD patients. Fine-mapping

uncovered that rs28384513 was independently associated with HbF levels [26]. A study on the Saudi β-thalassemia patients, rs4895441 also showed a significant association for the induction of HbF. They concluded that stimulation of HbF gene expression may provide alternative therapies for the amelioration of the disease severity of β-thalassemia [21]. In a study in France SNPs in the HBS1L-MYB region did not show statistically significant correlations with HbF levels [22]. All these studies clearly showed the role of SNPs in the HBS1L-MYB region on either clinical expression or HbF levels of β-thalassemia patients.

The current study has shown that both SNPs (rs28384513 and rs4895441) of HBS1L-MYB had significant effects on HbF. Besides, these SNPs also have a significant positive correlation of HbF value with transfusion interval (r = 0.38) and age of first transfusion (r = 0.26) but a negative correlation with clinical score (r = −0.31). Both the age of first blood transfusion and transfusion interval are proportional to disease severity. Therefore, disease severity is greatly varied with genetic heterogeneity which is an influencing factor for HbF induction. In HbE/β-thalassemia patients increased level of HbF can be the compensatory factor while HbA decreased in level. A review article concluded that the expression of HbF with a genetic modifier is crucial in determining the severity of anemic diseases, and genetic modification of HbF expression may offer clinical benefits in diagnosis and disease management [7]. Among β-thalassemia patients, a similar study in Indonesia found no correlation was found for any single-nucleotide polymorphisms and clinical appearance [27]. However, in this study, the total Hb level is lowest (6.82 ± 1.03 g/dL) in severe patients among all three groups in patients according to severity score (Table 5). Still, in the case of healthy individuals, 14.08 ± 2.48 g/dL Hb had been observed. A study in Northeast Thailand showed the baseline Hb levels ranging from 7.0 to 9.0 g/dL and did not develop splenomegaly in NTDT patients while in this study 7.88 ± 1.23 g/dL Hb level was found in NTD/Mild HbE/β-thalassemia patients (Table 5). This study also showed that HbF level in NTD/Mild patients is much higher than in severe patients who cannot grow and develop adequately without blood transfusion, thus a study conducted in Sri Lanka on mild and severe patients of β-thalassemia followed the same results as this study [28,29].

All parameters (HbF, HbE, HbA, HbA2, MCV, and MCH) are statistically significant (ANOVA; $p < 0.05$) among three groups but while doing significant tests individually (between NTD/Mild and Moderate) significant values had been found for HbF, HbE, HbA, RDW, MCV and MCH where HbA2 (g/dL) showed non-significant value where opposite aptitude had been found between moderate and severe groups of patients. On the other hand, in comparison with healthy individuals, RDW (15%) showed superior values in all three groups but Hb (8 g/dL), MCV (75 fl), and MCH (25 pg) showed lower values as well. Approximately close trends had been seen for hemoglobin variants where HbF and HbA2 showed significant values even individually but HbE and HbA did not show statistically significant values individually between Moderate and Severe ($p = 0.05$). Therefore, regardless of the SNPs rs28384513 and rs4895441 of HBS1L-MYB, the hemoglobin indices were still lower or different from normal indices of healthy individuals. The minor allele frequency of rs28384513 (G) and rs4895441 (G) in the HBS1L-MYB was found as 0.43 and 0.11 respectively in HbE/β-Thalassemia patients. Among all healthy individuals minor allele 'G' frequency for rs28384513 and rs4895441 46% and 13% respectively had been identified (Table 8).

We have herein reported that SNPs of the HBS1L-MYB intergenic region, rs28384513, and rs4895441 have a relationship with HbF levels and the clinical appearance among HbE/β-thalassemia patients in the Bangladeshi cohort. Cyrus et al., 2017 reported these elevated HbF (g/dL) levels as an amelioration factor for HbE/β-thalassemia. Hanafi et al., 2016 demonstrated that high HbF levels are correlated with reduced morbidity and mortality [21,30].

Apart from these two SNPs, other SNPs from three major QTLs for HbF including the B-cell lymphoma/leukemia (BCL11A) gene, the HBS1L-MYB intergenic region, and Krüppel-like factor 1 (KLF) have been identified by previous studies. A study in India demonstrated the association of HbF levels in thalassemia major patients with the polymorphisms in BCL11A (rs11886868 rs7557939; rs1427407 and rs766432) and HBS1L-MYB (rs9399137)

gene. The results of this study indicated that the presence of polymorphisms on modifier genes is strongly associated with an increase in HbF levels in thalassemia major patients. Interestingly, in contrast with our study, they found no association with fluctuations in HbF levels with rs9376090 and rs28384513 [25]. Another study in Thailand reported no statistically significant differences in allele and genotype frequencies of SNPs in the BCL11A gene (rs1427407, rs10189857, and rs11886868) between the groups of β-thalassemia/HbE patients and homozygous HbE subjects with low and high HbF levels. In contrast, SNPs in the HBS1L-MYB gene (rs4895441, rs9399137, and rs28384513) found a significant effect on HbF production and the variation of hematological parameters in homozygous HbE subjects [31].

The identification of novel trans-acting genetic variants that are associated with the modulation of HbF is a key step toward the development of novel diagnostic and therapeutic applications. Till now, more than 70 different HbF inducers have been identified. Natural compounds, like Resveratrol, Ripamycin, and Bergaptene, with limited cytotoxicity and high efficacy have started capturing the attention of researchers. Hydroxyurea (HU) and histone deacetylase (HDAC) inhibitors are considered as an inducer of HbF production by direct inhibition of repressing epigenetic enzymes. Genome editing (GE) is one of the most efficient and useful molecular approaches to correct the effects of gene mutations in hereditary monogenetic diseases, including β-thalassemia. CRISPR-Cas9 gene editing has been proposed for effective correction of the β-thalassemia mutation, obtaining high-level "de novo" production of adult hemoglobin (HbA). In addition to the correction of the primary gene mutations causing β-thalassemia, several reports demonstrate that gene editing can be employed to increase HbF, obtaining important clinical benefits in treated β-thalassemia patients [32–34]. Furthermore, increasing knowledge and understanding of the genetics of HbF regulation will support the development of innovative therapeutic targets, including the development of novel drug therapies.

Our biggest drawback is we cannot look into all the QTL and its SNPs associated with HbF, due to time and resource limitations. One of the drawbacks of this study was the smaller sample size for three different groups. This underpins the importance of further research on the functional significance of genetic variations in this region. A better understanding of how HbF production is regulated can lead to new strategies to modify the disease course of severe hemoglobin disorders.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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