RESEARCH ARTICLE

Modulatory effect of single nucleotide polymorphism in Xmn1, BCL11A and HBS1L-MYB loci on foetal haemoglobin levels in β-thalassemia major and Intermedia patients

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Abstract

Objective: To evaluate the influence of certain genetic modifiers on foetal haemoglobin levels in thalassemia major and thalassemia intermedia.

Methods: The cohort study was conducted from November 2018 to August 2019, at Department of Haematology, University of Health Sciences, Lahore and comprised beta thalassemia intermedia and thalassemia major patients who were referred by various healthcare facilities across Punjab, Pakistan. Foetal haemoglobin was quantified by high performance liquid chromatography. Primary mutation analysis and single nucleotide polymorphisms were done by amplification refractory mutation system-based polymerase chain reaction. Data was analysed using SPSS 20.

Results: Of the 116 patients, 52(45%) had beta thalassemia intermedia and 64(55%) had thalassemia major. Foetal haemoglobin levels were primarily influenced by alleles of the HBG2 (rs7482144) and BCL11A (rs766432) genes, but single nucleotide polymorphism of HBS1L-MYB (rs9399137) had no significant role ($p>0.05$). The rs7482144 single nucleotide polymorphism explained 8.3% of the variation in the foetal haemoglobin levels, while 5% of trait variation was explained by rs766432.

Conclusion: There was found a clear association between foetal haemoglobin level and single nucleotide polymorphisms in HBG2 (rs7482144) and BCL11A (rs766432) genes. This correlation was additive and was seen both in thalassemia major and thalassemia intermedia cohorts.

Keywords: Beta Thalassemia, Foetal haemoglobin, Polymorphism, Single nucleotide. (JPMA 71: 1394; 2021)

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Introduction

Thalassemias are the most common autosomal recessive disorders of haemoglobin (Hb) worldwide. There is absent or decreased production of alpha (α) globin or beta (β) globin subunits.[1] Southeast Asia is the hub of the most complex thalassaemia genotypes. Mainly, point mutations and occasionally small deletions or insertions in the β-globin gene sequence are the major molecular defects responsible for most β-thalassaemias. Homozygosity of β-thalassaemia results either in severe disease, i.e., thalassemia major, or thalassemia intermedia, which has mild to moderately severe clinical course.[2]

Almost all β-thalassemia patients will have normal foetal gamma (γ) globin genes expressed before birth but switched off during the first year after birth. This gradual change from γ- to β-globin expression is called Hb switching, and 'reversing the switch' can ameliorate the symptoms of β-thalassemia and some related haemoglobinopathies due to availability of γ-globin chains to bind to excess α chains.[3]

The levels of foetal Hb (HbF) in healthy adults is quite variable. This variance is genetically determined and does not originate from a single genetic locus. Instead, variations at multiple genes, possibly from different chromosomes, combine to produce this characteristically high heritability. Genetically, HbF persistence is a quantitative trait (QT), i.e. interplay of multiple cis-acting and transacting elements and factors influencing the β-globin cluster and probably other regulatory loci. Genes together with a small environmental component determine the value measured in any particular individual.[4] The coordinated function of specific transcription factors (TFs) is highlighted in many genome-wide association studies and many quantitative trait loci (QTL) have been outlined.[5] These QTLs are certain genetic elements on various genes that can cause increment in HbF levels, thereby bridging the gap between relative α and β chain imbalance.[5] Strong associations between HbF level and single nucleotide

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polymorphisms (SNPs) have been identified for at least three genomic loci:

Cis-acting factors are related with gene silencing and gene competition.[6] The Xmn1 (C>T) polymorphism is associated with high HbF γ-globin levels. Many pioneering studies conducted on normal individuals and patients with sickle cell disease or β-thalassemia showed correlation between -158C>T (rs7482144) SNP and HbF levels.[7]

BCL11A (B-cell Chronic lymphocytic leukaemia [CLL] / lymphoma 11A) locus is on chromosome 2p16.1, unlinked to β-globin gene cluster. It has emerged as silencer of γ-globin gene.[8] Polymorphism in BCL11A gene, are able to modify the severity of the homozygous beta^0 thalassemia. Among others one of the most frequently BCL11A genetic variants associated with increased Hb-F and β-thalassemia ameliorating effects is rs766432.[9]

Several HBS1L-MYB intergenic (HIMP) variants affect regulatory elements that are occupied by key erythroid TFs within this region. These elements interact with MYB, a critical regulator of erythroid development and Hb-F levels. MYB activates KLF1 and TR2/TR4 in human erythroid cells, thus indirectly activating the BCL11A gene, a robust foetal globin gene repressor. The c-MYB directly activates key γ-globin repressor genes and, thus, fulfils an important role within the established molecular HbF repression mechanism. It is a key player in the emerging TFs network governing γ-globin expression, in which the role of BCL11A and KLF1 is repressive one.[10] The rs9399137 is the SNP in HMIP most significantly associated with HbF among Chinese, Europeans and Africans.[11]

In Pakistan most of the work linking HbF, β-thalassemia phenotype severity and genetic modifiers is limited to -158C>T (rs7482144) SNP.[12] The current study was planned to evaluate the influence of certain genetic modifiers on HbF levels in thalassemia major (TM) and thalassemia intermedia (TI).

Patients and Methods

The cohort study was conducted from November 2018 to August 2019 at Department of Haematology, University of Health Sciences Lahore, and comprised patients referred by various healthcare facilities across Punjab, Pakistan, for diagnostic purposes as part of anaemia workup. The patients were subsequently classified into TM and TI groups on the basis of Hb level, age at presentation and first transfusion, and number of transfusions per year.

Approval for the study was granted by the institutional ethics committee of University Health Sciences Lahore. After obtaining informed consent from parents or patients, 3ml ethylenediaminetetraacetic acid (EDTA) blood samples were drawn. Complete blood count (CBC) analysis was done on Sysmax KX-21 followed by Hb fraction estimation by high performance liquid chromatography (HPLC) (Biorad Variant II; short β-thalassemia programme).

Deoxyribonucleic acid (DNA) was extracted using blood mini kit (Qiagen, GmbH, Hilden, Germany) method. The extracted DNA samples were quantified by nano-drop spectrophotometer. Primary mutation was confirmed by multiplex amplification refractory mutation system-based polymerase chain reaction (ARMS-PCR) method. Primer panel included most common mutations, like Fr 8-9 (+G), IVSI-5(G>C), Fr 41-42(-TTCT), IVS 1-1(G>T), Cd-30(G>C), Del 619, Cd-15(G>A), Cd-5(-CT), Fr 16(-C), and Cap+1(A>C).

Polymorphism of Xmn1 (rs7482144), BCL11A (rs766432) and HBS1L-MYB (rs9399137) was performed by ARMS-PCR. ARMS primers were synthesised and the specific DNA fragment was amplified and run on 2% agarose gel electrophoresis. A modified method was used in for the analysis of Xmn-1 polymorphism.[13] Two separate reactions were prepared for amplification of normal 122bp and mutated 239bp sequences for Xmn-1 polymorphism. The primer sequences of Xmn1 (rs7482144), BCL11A (rs766432) and HBS1L-MYB (rs9399137) were noted (Table-1).

Data was analysed using SPSS 20. Evaluation of the associations, odds ratio (OR), and tests for interaction were performed fitting a logistic regression. OR were calculated with 95% confidence interval (CI). $P<0.05$ was considered statistically significant.

Results

Of the 116 patients, 52(45%) were in the TI group; 24(46%) females and 28(54%) males with an overall age range of 2-32 years. The remaining 64(55%) patients had TM; 33(51.5%) females and 31(48.5%) males with an overall age range of 2-13 years. In both cohorts collectively, 113(97.4%) patients were Punjabis and 3(2.6%) were Balochi-Pathans from southern Punjab. Consanguinity was found in 95(81.9%) cases, while in 21(18.1%) cases the parents were unrelated individuals.

The most common mutations in both TI and TM cohorts were IVSI-5(G>C), Fr8-9(+G), and Cd-30(G>C) (Figure-1). The other less common mutations were Fr41-42(-TTCT),

IVS1-1(G>T), Del619bp, Cd-15(G>A), Cd-5(-CT) and Fr16(-C). A total of 4(3.4%) cases remained uncharacterised on ARMS-PCR. Though the distribution of IVSI-5(G>C) was unremarkable between the groups, Fr8-9(+G) was more frequently present in TM compared to cd-30 which was more prevalent in TI group (p>0.04).

High level of HbF was found in TI group compared to TM (p<0.0001) (Figure-2).

Multivariate analysis for the presence of genotypes showed significant results (Table-2).

Xmn1, rs766432 and rs9399137 were subjected to stepwise multiple regression analysis to predict HbF levels in the patients. The final model comprised rs7482144 (Xmn1) and rs766432. The model showed that the variance by both SNPs to HbF was 13.3% (Table-3).

Table-1: Amplification refractory mutation system (ARMS) primer sequence for Xmn-1 (rs7482144), BCL11A (rs766432) and HBSYL-MYB (rs9399137) genes.

SNP	Wild Type Primer Sequence(5'-3')	Size (bp)	Mutant Primer Sequence (5'-3')	Size (bp)
Xmn-1 (rs7482144)	F- ATGCAAATATCTGTCTGAAACGTTC R- GCCTCACTGGATACTCTAAGAC	122	F- TGGAGCTACAGACAAGAAGG R- TGGGTGGAGTTTAGCCATGA	239
BCL11A (rs766432)	F-TTGTTTCGCTTTAGCTTTATTAAGGTACAA R- GACGTGTTCTGTATCTTGATTTTGGT	135	F- CCAAACAGTTTAAAGGTTACAGACAGACT R- AAAATGAATGACTTTTGTTGTATGTAGAG	116
HBS1L-MYB (rs9399137)	F-GAAATACCATCACTGAGAAAAGCATAAG R- CAGCAGGGTTGCTTGTGAAAAAACTTTA	234	F- AATGTAATTAACTGAACATATGGTTAGTC R-TTTATTGTTACAAGGTTAATTCACTGCC	178

SNP: Single nucleotide polymorphism.

Table-2: Association of genotypes with TI and TM.

Ameliorating alleles	Allele frequency		Multivariate analysis	
	TI (n=52)	TM (n=64)	OR (95% CI)	p-value
Xmn-1 (rs7482144)				
Allele T	0.36	0.21	2.0658 (1.15-3.70)	0. 001
Co-Dominant Model				
CC	15 (28.8%)	37 (44.8%)	1.00	
CT	37 (71.2%)	27 (42.2%)	0.296 (0.14-0.64)	0.002
BCL11A (rs766432)				
Allele C	0.25	0.18	1.5217 (0.81-2.87)	0.19
Co-Dominant Model				
AA	28 (53.8%)	44 (68.8%)	1.00	
AC	22 (42.3%)	17 (26.6%)	0.412 (0.223-1.08)	0.048
CC	2 (3.8%)	3 (4.7%)	0.955 (0.150-6.07)	0.961
HBS1L MYBrs9399137				
Allele C	0.21	0.26	0.7724 (0.42-1.43)	0.40
Co-Dominant Model				
TT	34 (65.4%)	37 (57.8%)	1.00	
TC	14 (26.9%)	21 (32.8%)	1.378(0.60-3.13)	0.44
CC	4 (7.7%)	6 (9.4%)	1.378(0.36-5.30)	0.64

TI: Thalassemia intermedia; TM: Thalassemia major; OR: Odds ratio; CI: Confidence interval.

Table-3: Effect of Xmn1 and BCL11A (rs766432) polymorphism on foetal haemoglobin (HbF) in TI.

Locus	SNP	Allele change	MAF (allele)	Effect size (SE)	P	Variance explained (%)
β-Globin locus	rs7482144 (Xmn-1)	C → T	0.27	1.566 (.414)	<0.001	8.3
BCL11A	rs766432	A → C	0.21	1.078 (.424)	.012	5.0

TI: Thalassemia intermedia; SNP: Single nucleotide polymorphism; MAF: Minor allele frequency; SE: Standard error.

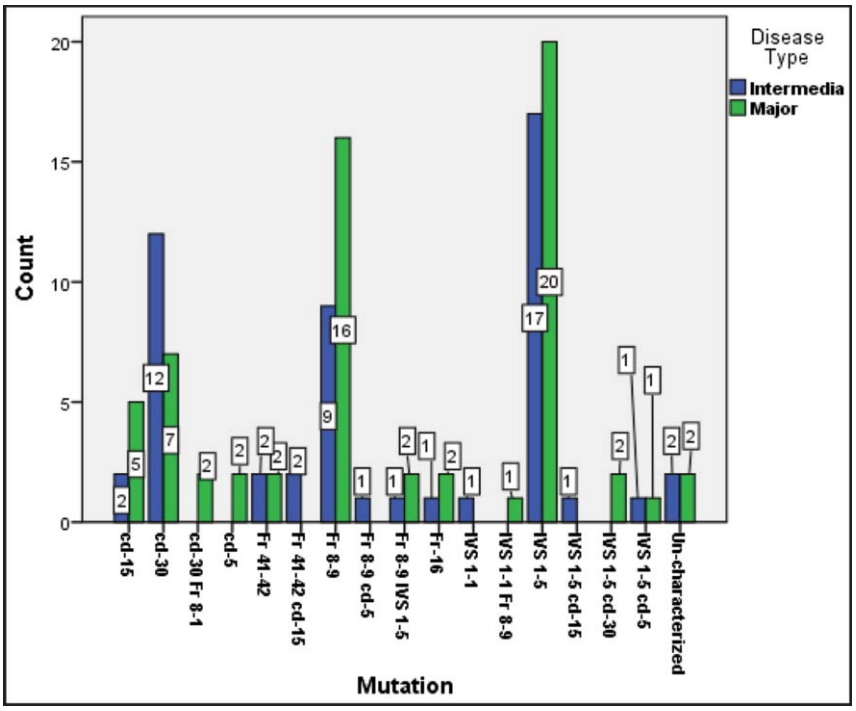

Figure-1: Distribution of beta thalassemia mutations in thalassemia intermedia (TI) and thalassemia major (TM). The figure represents spectrum of mutations in both TI and TM cohorts.

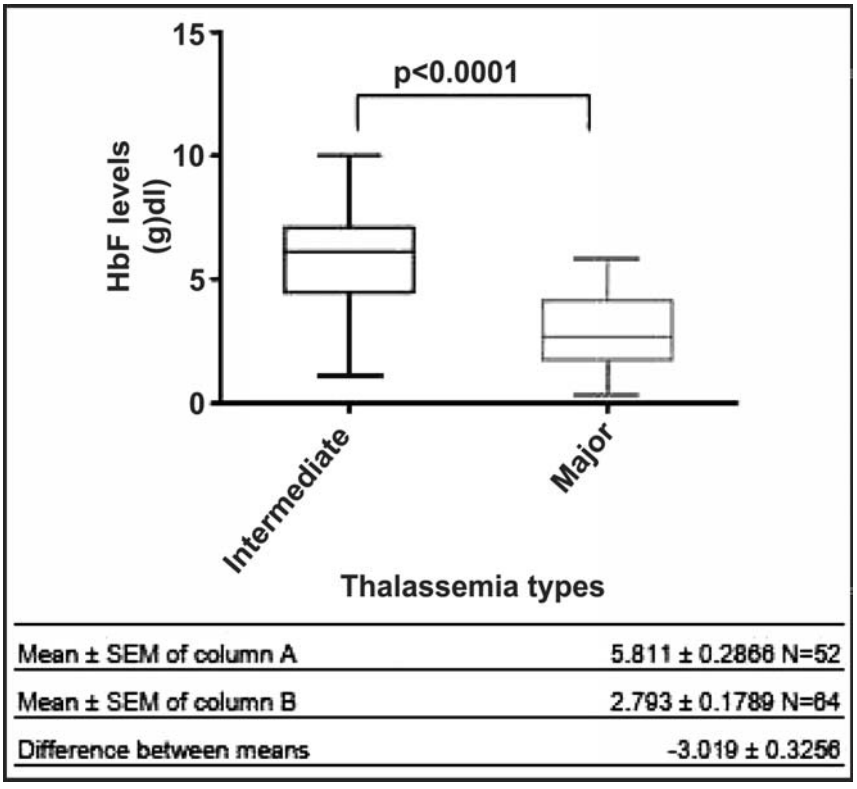

Figure-2: Box plot showing distribution of Hb-F levels in thalassemia intermedia (TI) and thalassemia major ™ cohorts. Bar within each rectangle shows median value for foetal haemoglobin (HbF), whereas each rectangle represents data between the 25th and the 75th quartiles.

Discussion

Influence of the polymorphism in Xmn1 and other QTLs on HbF levels in the current study, the most common primary genetic mutations among TI cohort was were IVSI-5(G>C), Cd-30(G>C) and Fr8-9(+G). This is in accordance with a study conducted in Karachi.[12]

Xmn1 allelic frequency was significantly higher in TI compared to TM in th current study. This higher frequency of C-T polymorphism in TI confirms its pivotal role in reducing the severity of the disease. Similar results were reported earlier.[13]

The current study is the first in Pakistan where prevalence of BCL11A (rs766432) was studied in TI and TM. Similar study was carried out in Indonesia.[14] According to genome-wide association studies for HbF QTL, different ethnic populations across the globe harbour variable sets of genetic variants.[15]

As far as polymorphism of HBS1L MYB (rs9399137) on Pakistani population is concerned, no data is available so far. The current study is the first to analyze the effectiveness of HBS1L MYB (rs9399137) polymorphism on elevation of HbF level in TI. HBS1L MYB (rs9399137) polymorphism was found to have no role in enhancing HbF level in TI in Pakistani population. Similar results were found in Indonesia.[15]

In the current study, HbF level was significantly high in TI than TM. This marked increase in HbF level in TI is caused due to the presence of high frequency of polymorphism in Xmn1 site and BCL11A (rs766432). As discussed, the role of Xmn1 polymorphism is well established in enhancing HbF level and amount of total Hb. However, it is a novel study regarding the role of BCL11A (rs766432) polymorphism in increasing HbF level in Pakistani population.

Xmn1, BCL11A and HBS1L-MYB were subjected to the stepwise multiple regression analysis to predict HbF levels in thalassemic patients. The value of Xmn1, BCL11A SNP in TI was found to be

significant. The level of HbF was 8% higher in TI patients compared to TM due to high incidence of Xmn-1 polymorphism in TI. Similarly, BCL11A (rs766432) polymorphism accounted for 5% variance of HbF level in TI patients. According to data, the effect of Xmn1 polymorphism was more profound than BCL11A (rs766432) polymorphism. In the presence of both SNPs simultaneously, the model collectively predicted 13.3% variance in HbF level in TI patients. According to a previous study, γ-globin production can be increased 11 times due to presence of Xmn1 polymorphism in comparison with control.[16]

The molecular events controlling HbF switch has been well understood and the role of secondary modifiers, especially transacting genes, including BCL11A and HBS1L-MYB, is clearly known. SNP in these genes is associated with better outcome and, secondly, these QTL-harbouring individuals are more responsive to HbF augmentation therapy, like hydroxyurea (HU). Even though there is published data indicating effective induction of HbF that can ameliorate disease phenotype among TM cohort in Pakistan,[17] the knowledge of these QTLs can actually predict responders and non-responders well before time and help in treatment decisions.[4]

There is enormous clinical application and scope of the current study in Pakistan. First, it can address the dilemma in labelling a person as TM or TI at the time of diagnosis, and that helps in deciding whether or when to transfuse. Secondly, in Pakistan there is unchecked use of HU without ensuring whether a patient is responder or non-responder, and, being a cytotoxic drug, it does have potential side effects. Prior knowledge of these genetic modifiers can help in stratification of patient cohorts among thalassemics.[18]

Conclusion

HbF levels were significantly increased among TI patients compared to TM patients, indicating ameliorating impact of increased HbF on disease phenotype. Also, there was significant association between HbF level and SNPs in HBG2 (rs7482144) and BCL11A (rs766432) gene. This correlation was additive and was seen both in TM and TI cohorts; more so in the latter.

Disclaimer: The text is based on a Ph.D. thesis.

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