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Fetal hemoglobin altering effects of *KLF1*, *BCL11A* rs11886868 and *Xmn*I-HBG2 on transfusion dependent beta thalassemia patients: Preeliminary study

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Abstract

Aim: High fetal hemoglobin value is one of the quantitative trait in beta thalassemia and may effect transfusion dependency status of beta thalassemia cases. There are population-based differences about known genetic modifiers of different fetal hemoglobin values. We aimed to find if high fetal hemoglobin value are caused by *Xmn*I-HBG2 polymorphism, rs11886868 of *BCL11A* or *KLF1* whole gene mutations.

Materials and Methods: Genotyping procedure of thirty well re-defined and characterized transfusion dependent beta thalassemia patients was conducted via either sanger sequencing or and PCR-RFLP. Statistical analysis of groups and multiple logistic regression analysis of related genotypes were performed.

Results: We found strong correlations between transfusion dependency and fetal hemoglobin levels ($p<0.05$). IVS.I.110 (G>A) homozygous mutation was found to be predominant in *HBB* gene. Lower fetal hemoglobin levels were seen in IVS.I.110 (G>A) homozygous group ($p<0.05$). Total count of variations among the three modifier genes *BCL11A* polymorphism was leading first. We did not observe any statistically significant relationship in patients with beta thalassemia major patients who have high fetal hemoglobin values between three modifiers group ($p>0.05$).

Conclusion: This is the first research report from Turkey in terms of 3 different modifiers were analyzed and evaluated. Since some cases have more than one variations in these three modifiers, involving higher sample size may overcome this challenge. Other genomic alterations rather than *Xmn*I-HBG2, variations of *BCL11A* rs11886868 and mutation profile of *KLF1* gene, which could decrease or abolish the effect of gamma globin repressors, may have more direct role with high fetal hemoglobin levels in patients with transfusion dependent beta thalassemia in Turkey.

Introduction

Beta thalassemia major (BTM, #613985) is mainly caused by mutations in beta globin gene (*HBB*-141900). More than 250 mutations nominated on http://globin.bx.psu.edu/cgi- bin/hbvar/query_vars3 web site [1]. Low hemoglobin, MCH, MCV and high RDW values and low beta globin amounts on electrophoresis results are indicative in beta thalassemia [2]. There are distinct modifying genetic factors have been related in Genome Wide Association Studies (GWAS) which alters phenotypic features as in fetal hemoglobin (HBF) values [3,4].

One of the mostly studied HBF quantitative trait loci, *Xmn*I -HBG2-polymorphism (rs7482144), has been shown to clarify around 10% of HBF variance in healthy Europeans [3]. In hematologically normal Iranians, *Xmn*I-HBG2-polymorphism but not B-Cell CLL Lymphoma 11A (*BCL11A*) polymorphisms were found to have HBF modifying effect [5]. In one more study showed that *Xmn*I-HBG2 polymorphism has more ameliorating effect contrary to *BCL11A* variations in thalassemia cases [6].

SNPs (rs11886868, rs4671393) in *BCL11A* have been related to different HBF values in distinct populations [4,5,7–9]. *KLF1* (Kruppel Like Factor 1); in 2010, although *KLF1* wasn't been pointed on QTLs for HBF variation, it was showed that *KLF1* regulates fetal to

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adult-globin switching by affecting *BCL11A* expression [10]. After detailed genetic analysis of a Maltese family with hereditary- persistence of fetal-hemoglobin (HPFH), relationship between high HBF and haploinsufficiency of *KLF1*, opened a new research area on HBF studies [11]. More than 65 *KLF1* mutations have been introduced within six years after its' first discovery [12–15].

Since there are inter-population differences between different HBF modifiers, we aimed to find whether there are relationships between *KLF1* mutations, *BCL11A*-rs11886868, *Xmn*I polymorphism (rs7482144) and HBF levels in adult patients with BTM. We also tried to explain whether clinical severity of the disease based on their transfusion dependency status is affected in an independent or a dependent manner on genotypes, regarding relatively higher HBF levels.

In order to explain fetal hemoglobin altering contributions of three different modifiers we used sequence based methods to *Xmn*I-HBG2, *KLF*1 whole gene and *BCL11A-rs* 11886868 polymorphism.

Materials and Methods

Authors confirms that the study design was approved by local ethics committee (Akdeniz University, Faculty of Medicine, Clinical Research Ethics Committee with the number of 08.01.2013/22) and signed informed consents were obtained from all of subjects.

Study design, subjects and primers

We employed probable sampling methods and invited patients to ask whether they want to participate current study. Thirty well re-defined Turkish transfusion dependent beta thalassemia (TDBT) patients' whole blood counting and hemoglobin electrophoresis results (Biorad Variant™ Hemoglobin-Testing- System II) were obtained just before routine blood transfusion day of patients (Table1). After genomic DNA isolation of peripheral blood samples of participants genetic analysis pertaining to *KLF1* gene, *BCL11A*-rs11886868 and *Xmn*I polymorphism (rs7482144) were conducted via Sequencing for the first two QTLs following PCR reactions. PCR-RFLP was applied with *Xmn*I enzyme (New England Biolabs, Inc). *Xmn*I polymorphism were confirmed by sequencing (Applied Biosystems, ABI-3130- XL-DNA-Sequencer-Genetic-Analyser).

Primer sequences for rs7482144, forward 5'-GAACTTAAGAGATAATGGCCTAA-3', reverse 5'-ATGACCCATGGCGTCTGGACTAG-3', for BCL11A, forward 5'-CATGGATGAATCCCAGAATC- 3', reverse 5'-CGTCCACCAGTCTAGAAAG-3'. To be able to amplify *KLF*1 gene, we used 5 different primer couples. Primer sequences are;

forward 1: 5'-TTTGACTTGGCTTTGGACAC-3' reverse 1: 5'-GACCCCAAGATCTGTGACTG-3',

forward 2: 5'-CAAAGCCTCTGCGTCAGAG-3' reverse 2: 5'-GGGGTACCCGGACAGTAG-3',

forward 3: 5'-TCCTCGGGTGGCTACTTC-3' reverse 3: 5'-GTCTCGGCTATCACACCTG-3',

forward 4: 5'-GGGAGGAAGAGGACGATGA-3' reverse 4: 5'-GGACAAGGAAGCCATAAGC-3',

forward 5: 5'-GGACATGACTGGGCAGAC-3' reverse 5: 5'- GGTTTACAGCCTCCTGCC-3'.

PCR conditions are available upon request.

Statistical analysis

Statistical analysis of genomic mutations and variations were calculated by performing IBM SPSS Statistics for Windows- MacOS, Version 20.0 (Chicago, IL).

Firstly, we explored if groups are distributed normally or not. Measurable parameters were transfusion units, transfusion dependency, transfusion scores and hemoglobin values (Table 1). Descriptive data were presented as n and percentages (%) in categorical input. Allele frequencies of three modifier genes were also presented in this current study.

Continues data were depicted as mean±standard deviation or median (minimum-maximum). Non parametric Mann-Whitney-U-Test was employed to compare paired-groups. We hypothesized whether higher HBF levels were caused by mutation/polymorphism profile of different modifiers and *HBB* genotypes. We accepted HBF level>2.0 as high value and compared them to different genotypes by one one, firstly. We tried to find any statistically significant genotype(s) by together in any genotype combinations.

We also achieved multiple regression analysis in case combinations of these SNPs may in aggregate have a better predictive value of HBF and phenotype rather than individual SNPs. Multiple logistic regressions where the dependent variable were HBF (being critical cut of value of fifteen) was conducted.

Results

HBB gene mutation profile and demographic features

HBB mutation profiles of patients were diverse while the most frequent mutation was IVS.I.110 (G>A)/IVS.I.110

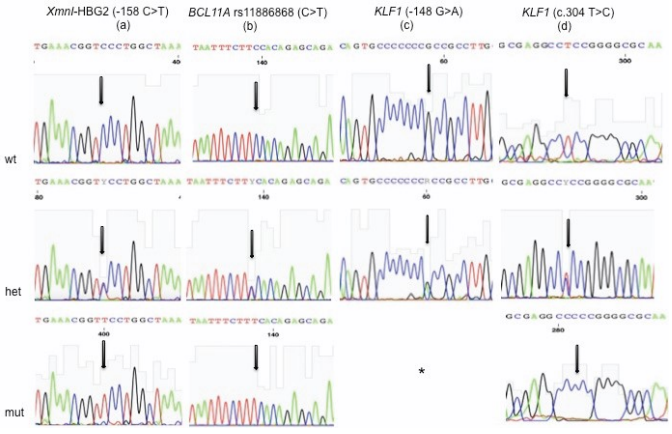

Figure 1. Sanger sequencing images of *Xmn*I-HBG2, *BCL11A* rs11886868, *KLF1* gene in this study. * In our study group there is no AA homozygous mutation allele in *KLF1* gene (-148 G>A).

Table 1. Demographic, genotypic and hematological features of thirty cases with TDBT.

Case	Age-Sex	Beta Globin Gene (HBB)	XmnI	BCL11A	-148G>A	c.304T>C	SP	HB	MCV	MCH	MCHC	RDW
1	27,6-M I	VS.I.110 (G>A)/IVS.I.110 (G>A)	CC	CT	GG	TC	YES	8.3	84.2	26.9	31.9	15.9
2	25,9-M	IVS.II.1 (G>A)/IVS.II.745 (C>G)	CC	CT	GG	TT	NO	7.8	77.3	26	33.6	16.5
3	26,6-M	(-30T>A/IVS.II.745 (C>G))	CC	TT	GG	TC	YES	8.6	82	27.2	33.2	16.1
4	22,5-F	IVS.II.1 (G>A)/IVS.II.1 (G>A)	TT	TT	GG	TC	NO	8.5	82.4	27.5	33.4	16.7
5	20,5-F	IVS.I.110 (G>A)/IVS.II.1 (G>A)	CT	TT	GA	TC	NO	8.8	83	27.5	33.2	14.6
6	24,7-M	IVS.I.110 (G>A)/IVS.I.6 (T>C)	CC	CC	GG	TT	YES	8.7	85.2	25.2	29.5	19.7
7	29-F	IVS.I.110 (G>A)/IVS.I.110 (G>A)	CC	CT	GG	TC	YES	8.6	83.7	27	32.2	18.5
8	22,4-M	IVS.I.1 (G>A)/IVS.I.110 (G>A)	CC	CC	GG	TT	NO	8.9	78.3	25.6	32.7	19.4
9	27,5-M	(-30T>A/IVS.I.110 (G>A))	CC	CT	GG	TT	YES	9.8	81.1	24.9	30.7	25.6
10	29,1-M	IVS.I.110 (G>A)/IVS.I.6 (T>C)	CC	CC	GG	TT	YES	7.8	81.3	26.8	32.9	15.6
11	39,7-F	IVS.I.110 (G>A)/IVS.I.6 (T>C)	CC	TT	GG	TT	YES	8.1	86.9	27	31	16.7
12	40,4-F	HBKNOSSOS/IVS.II.1 (G>A)	CT	CT	GG	TT	YES	8.5	77.1	24.6	31.9	29.9
13	36,5-F	IVS.I.110 (G>A)/IVS.II.1 (G>A)	CC	CC	GG	CC	YES	7.8	78.8	25.9	32.9	16.5
14	62,5-F	(-30T>A/-30T>A)	CC	TT	GG	TT	YES	8.4	76.7	22.7	29.5	28.6
15	22,3-F	IVS.I.110 (G>A)/IVS.I.110 (G>A)	CC	CT	GG	TT	YES	10	85.2	28.3	33.2	17.3
16	26,5-F	IVS.I.110 (G>A)/IVS.I.110 (G>A)	CC	CC	GG	TT	YES	10.1	86.6	28.2	32.5	14.7
17	37,3-F	IVS.I.110 (G>A)/IVS.I.110 (G>A)	CC	CT	GG	TT	YES	10.1	87.1	29.2	33.5	15.4
18	25,2-M	IVS.I.110 (G>A)/IVS.I.110 (G>A)	CC	CT	GG	TT	YES	9.3	87.8	28.4	32.3	14.4
19	27,2-M	IVS.I.110 (G>A)/IVS.I.110 (G>A)	CC	TT	GG	TT	YES	8.8	71.8	22	30.6	36
20	24,4-F	IVS.I.110 (G>A)/IVS.I.110 (G>A)	CC	CT	GA	TT	YES	8	74.7	22	29.5	31
21	20,9-M	IVS.I.110 (G>A)/IVS.I.110 (G>A)	CC	TT	GG	TT	NO	8.5	75	25.7	34.2	27.4
22	19,7-M	IVS.I.110 (G>A)/IVS.I.110 (G>A)	CC	TT	GG	TT	NO	8.5	79.8	27.1	34	14.8
23	29-F	IVS.I.110 (G>A)/IVS.I.110 (G>A)	CC	TT	GG	TT	NO	9.6	83	28.4	34.2	14.2
24	20,6-M	FSC 44(-C)/FSC 44(-C)	CC	CT	GG	TT	NO	8.1	78.8	26.2	33.2	15.3
25	29,5-F	(-30T>A/IVS.II.1 (G>A)	CT	CC	GG	TT	NO	8.5	77.3	25.4	32.9	19.8
26	37,7-F	IVS.I.6 (T>C)/IVS.I.6 (T>C)	CC	CT	GG	TC	YES	9. 2	78.6	24.3	30.9	27.2
27	39,4-M	IVS.I.110 (G>A)/IVS.I.6 (T>C)	CC	CT	GG	TC	YES	8.4	81.7	25.5	31.3	21.2
28	23,3-F	IVS.I.110 (G>A)/IVS.I.110 (G>A)	CC	CT	GG	TC	YES	7.4	84.4	27.4	32.5	17.5
29	27-M	IVS.I.110 (G>A)/IVS.I.110 (G>A)	CC	CT	GG	TC	YES	8.1	84.8	27.1	31.9	14.8
30	24,3-F	IVS.I.110 (G>A)/IVS.I.6 (T>C)	CC	CT	GG	TC	YES	8.6	89.3	26.8	30.1	17.1

SP:splenectomy status, HB:hemoglobin (g/dL). MCV:Mean Corpuscular Volume (fL), MCH:Mean Corpuscular Hemoglobin (pg/cell), MCHC:MCH Concentration (g/dL), RDW:Red cell Distribution Width (per).

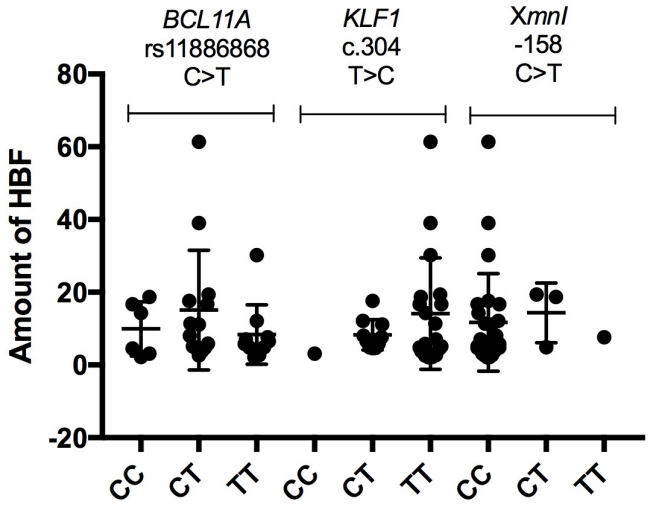

Figure 2. HBF values of *BCL11A*, *KLF1* and *Xmn*I groups*

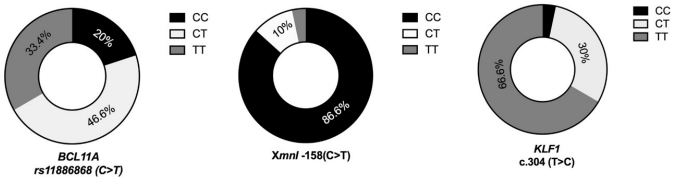

Figure 3. Percent distribution of groups#.

Frequencies smaller than 5% were not stated.

(G>A). Mean blood transfusion scores, the amount of transfusion-unit, transfusion dependency-day were 104.5±30.2 times, 1.7±0.3 units and 21.1±13.0 days, respectively. Mean amount of adult hemoglobin was 8.6±0.7 g/dL, mean HBF percentage was 11.8 (min 2.1, max 61.4). There was a history of splenectomy in 70% of our cases with 12.3 mean value of HBF (n = 13).

Among all mutation profile *HBB* gene, in patients with IVS.I.110 (G>A) homozygous state, HBF levels were the lowest (p=0.038, Mann-Whitney U) with the mean value of 10.8.

XmnI polymorphism-rs7482144

rs7482144 (Figure 1a) was found to be in heterozygous state in 3 patients and homozygous in one patient. Mean HBF were 14.3% and 11.9 % on CT and CC group, re-

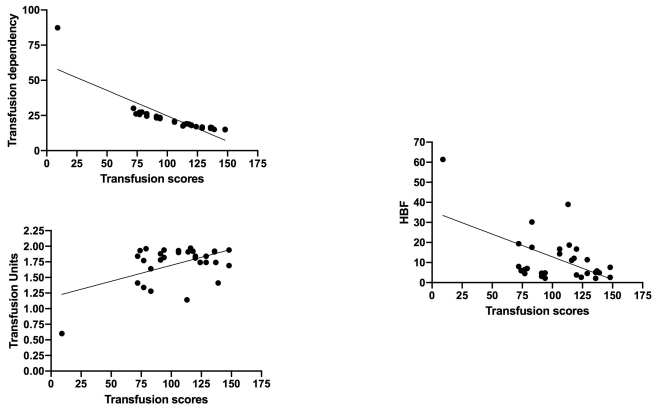

Figure 4. Correlations among non-genomic* groups.
*Measurable groups such as transfusion units, transfusion dependency, transfusion scores and HBF values were stated as non-genomic groups.

spectively (Figure 2). Since there is only one patient with TT genotype, HBF-increasing effect of T allele were calculated with Mann- Whitney U test (p=0.223). Cytosine allele frequency was 0.92. Two out of three heterozygous cases (case 12 and 25) were found to have CT or CC alleles in terms of rs11886868-*BCL11A*.

BCL11A-rs11886868

Only 6 cases were carrying homozygous wild-type alleles (CC), while 14 patients were heterozygous (CT) and 10 cases were with TT homozygous mutated allele (Figure 1b). Cytosine and thymidine allele frequencies were 0.57 and 0.43, respectively. Mean HBF values (Figure 2) were %9.9, %15.1 and %8.4 (Kruskal Wallis, p=0.5395, SDs were 14.9, 12.7, 14.8, respectively). Case number 5 was carrying heterozygous polymorphic *Xmn*I (CT) and *KLF1* promoter mutation (GA) alleles while case number 4 was only homozygous for *Xmn*I (TT) allele (Table 1).

KLF1 mutation profile

We found -148 (G>A) promoter mutation (Figure 1c) in only two cases (case 5 and 20), heterozygous in *KLF1*. Guanine allele frequency was 0.97. Previously considered as benign variation (NP_006554.1:p.Ser102Pro, c.304T>C) were found in 10 cases (Figure 1d) heterozygous and in 1 case with homozygous state. In this group, mean HBF value was calculated as 8.3±12.7 and this value weren't found significant neither.

We obtained different range of allelic distributions in different groups (Figure 3) while the largest genotypic diversity was found in *BCL11A*-rs11886868 among these three modifiers.

Although the effect of combinations of variant alleles on HBF levels were studied with multiple logistic regression models, no significant relationship could be detected (Supplement Table 1). MCV, MCH and HBA1 levels were found to be higher when HBF level was selected as a value of fifteen and reverse was valid regarding RDW values (Supplement Table 2). We found that there were strong negative correlation between HBF values and transfusion units (Figure 4). It means; higher HBF lower transfusion requirement (r=-0.789, p<0.001).

Discussion

We found a rare Hb Knossos/IVS.II.1 (G>A) compound heterozygosity in one case. Hb Knossos and other β-globin mutations like IVS.II.745 (C>G) [16], -101 (C>T), -30 (T>A), IVS.I.5 (G>A), IVS.I.5 (G>C), IVS.I.110 (G>A) [17], FSC8 (-AA) [18], Cod 39 (C>T) [19] have been known in different studies. *Xmn*I-HBG2-polymorphism and *BCL11A*-rs11886868 genotypes were both heterozygous in our case while HBF value was 19.4 (case 12, table 1). It isn't clear if high enough HBF value caused by this togetherness or not.

rs7482144 (HBG2) may exist more common in BTI patients rather than BTM [20,21]. Although high HBF values with XmnI-HBG2 polymorphism were seen in our study we couldn't find an association. Due to the small number of positive cases (4 cases) with the co-inheritance of the *Xmn*I-HBG2 polymorphism and *BCL11A*-rs11886868, we preferred to refrain from testing whether the *Xmn*I polymorphism has an HBF-enhancing effect in our cases. We also revealed that amount of HBF is not affected by splenectomy status of beta thalassemia patients (p=0.39, Mann- Whitney U Test). An article also stated that even if HBF values were higher in sickle cell anemia patients in TT allele group of *Xmn*I-polymorphism, overall disease severity was not related [22].

BCL11A-rs11886868 variation; although the lowest HBF values were seen in TT risk allele group, we couldn't connect any association. The highest HBF values (61.4) in our patients were belong to case-20, who has heterozygous rs11886868 and *KLF1* promoter-mutation. However, the lowest transfusion dependency in this case (0.6 units and 87.4 days) was apparent, this may be related to high HBF level as well as reduction of α-globin genes.

Screening of *KLF1* whole gene mutations revealed 2 previously known variations. One of them was - 148 (G>A) promoter mutation in two cases, heterozygous. The latter one was p.Ser102Pro benign variation in only one case but homozygous. The first one (-148 (G>A) mutation) has been related to cause high hemoglobin levels by abolishing Sp1 transcription factor binding [23]. In that study, Serbian origin adult women showed elevated HBF levels (11%) while they were 4.9% and 61.4% in our cases. It was also showed in the same study that -148 G>A promoter mutation and p.Ser102Pro benign variations are present at the same time. In our case p.Ser102Pro was only present for the case whose hemoglobin f level is 4.9%. Last but not least, while rs11886868 SNP of *BCL11A* gene was present for our case (HBF, 61.4%) but absent for the case who has 11% of HBF level. One study that accepted the threshold value of 1.5 for HBF showed that p.Ser102Pro caused decreased level of HBF when mutated C allele is observed (C allele frequency was 0.29) in fifty two volunteers (HBF levels: mean:2.0, min:1.5, max:3.8) [24]. In that study, they detected 5' UTR variation (-85G>A) in two out of three carriers of p.Ser102Pro in homozygous mutation state which has no role of changing HBF levels alone. It was not studied whether that decreased level of HBF might be the reason of this togetherness or not.

We took into account that normal HBF level as <2.0% and released the mutational profile of these 3 modifiers at the same time in transfusion dependent beta thalassemia patients with higher hemoglobin levels than 2.0% but not in normal population.

This is the first study which the effects of HBF modifiers; *Xmn*I-HBG2-polymorphism, *BCL11A*- rs11886868 and *KLF1* gene, in which were evaluated on TDBT patients at the same time.

Conclusion

Fetal hemoglobin levels of transfusion dependent beta thalassemia patients may be effected several modifier genes such as *Xmn*I-HBG2-polymorphism, *BCL11A* gene polymorphisms and *KLF1* gene variations. Although we did not find proper determinant among those three modifiers, their cross interactions may decide final HBF levels in different patients. New candidates or mutation profiles of unknown modifiers need to be clarified in our patient cohort.

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Disclosure of interest

The authors declare that they have no competing interest.

Ethical approval

Current prospective study was approved by Ethics Committee of Akdeniz University, Faculty of Medicine (decision number with 08.01.2013/22). This study is also employed in line with the principles of "Helsinki Declaration".

Informed Consent

All of the applicants were informed about the scopes and the results of the study. Subjects also signed informed consent documents as volunteers.

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Data availability

The data in this manuscript are available from the corresponding author upon reasonable request.

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