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ORIGINAL ARTICLE

Common fetal hemoglobin variants in Lebanese patients bearing the codon 29 beta gene mutation associated with different thalassemia phenotypes

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Abstract

Beta-thalassemia can present with a wide spectrum of phenotypes determined by the coinheritance of α-thalassemia, hereditary persistence of fetal hemoglobin, and polymorphic variants in the *BCL11A*, *HMIP*, and *HBB* clusters. The codon 29 (cd29) mutation in the beta gene has been associated with a broad diversity of thalassemia phenotypes, possibly through genetic modifiers determining the genotype-phenotype relationship. In this study, we evaluated the effect of 10 single nucleotide polymorphisms (SNPs) on β-thalassemia severity in a group of 21 Lebanese patients bearing the cd29 mutation. Hematological parameters and clinical characteristics were evaluated according to transfusion dependence. The proportions and absolute concentrations of HbF were found to be higher in non-transfusion-dependent (NTD) patients than in transfusion-dependent (TD) ones. Iron parameters were found to be higher in TD patients. The SNPs that were evaluated included the XmnI-158 polymorphism in the *HBG* gene and SNPs in the *BCL11A* and *HMIP* loci. It was noted that individuals homozygous or heterozygous for the effect allele in the *BCL11A* and *HMIP* SNPs had higher HbF levels, lower ferritin concentrations, and lower liver iron content and were less likely to be transfusion dependent. Our results showed that HbF production variants may have an important impact on the severity of β-thalassemia, which might provide a severity prediction tool that can help in the anticipation of patients' phenotypes and therefore in future therapeutic decision making.

Keywords Fetal hemoglobin · Single nucleotide polymorphisms · Phenotype · Thalassemia · Transfusion

Introduction

Beta-thalassemia (β-thalassemia) constitutes a group of diseases characterized by decreased or absent β-globin synthesis due to various mutations, resulting in an excess of alpha (α) chains which precipitate in erythroid precursors in the bone marrow, ultimately causing their premature death [1]. According to clinical severity, β-thalassemia is classified into thalassemia major, thalassemia minor, and thalassemia intermedia [2]. Thalassemia major is the most severe phenotypic manifestation of the disease, with children often declaring themselves during the first few years of life due to the seriousness of the involved anemia, almost always imparting transfusion dependence. Thalassemia minor, lying on the other end of the disease spectrum, might often go unnoticed as it rarely causes any symptoms, except for occasional mild anemia. Thalassemia intermedia is phenotypically manifested as too severe to be classified as minor and too sparing to be classified as major. Ineffective hematopoiesis, chronic

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Table 1 Demographic and hematological parameters of the 21 patients included in the study group

Age, mean	27.8 ± 10.5
Age, range	15–57
Males (%)	33
Females (%)	67
Hb level, mean (g/dL)	8 ± 1.3
Hb level, range (g/dL)	5.6–9.8
HbF, mean (%)	25.2 ± 10.8
HbF, range (%)	4.9–66

Hb, hemoglobin

hemolytic anemia, and iron overload define the pathophysiologic basis for all the clinical sequelae that might ensue in this disease entity [3].

The striking phenotypic diversity of the thalassemia syndromes is the end result of a remarkable variability of genotypes, including mild or silent β-globin alleles, α-thalassemia coinheritance, and/or the presence of genetic determinants associated with increased production of gamma (γ) chains and thus the ability to produce fetal hemoglobin (HbF) in adult life [4]. The main mechanism by which these genetic modifiers diversify the phenotypic severity of thalassemia is through reduction of the α/β-globin chain imbalance. Three major loci regulate HbF level: *HBG2- XmnI-158*, which is located in the promoter region of the fetal gamma-globin gene and BCL11A and HBSB1L-cMYB, which are involved in fetal gene silencing in adult life or in cell differentiation and proliferation [5–9].

We have encountered a family from the Beqaa region in Lebanon which presents with a peculiar mutation in the β-globin gene—the codon 29 mutation (cd29, C for T)—where patients manifest clinically either as β-thalassemia major or as β-thalassemia minor. This seems to be congruent with previous reports in the literature in which patients with the cd29 mutation were classified as either thalassemia major or minor [10]. In order to get further insights into the phenotype variability associated to the codon 29 mutation in the Lebanese thalassemia patients, we evaluated the effect of 10 single nucleotide polymorphisms (SNPs) on β-thalassemia severity in a group of 21 Lebanese patients bearing the cd29 β gene mutation.

Materials and methods

Patients

A total of 21 β-thalassemia patients of Lebanese origin followed at the Chronic Care Center in Hazmieh, Lebanon, were recruited. Patients' hematological and clinical data were collected. Among the studied hematological parameters were total hemoglobin (Hb) and fetal hemoglobin (HbF), both in percentage and total quantity. Ferritin levels, liver iron content (LIC), alanine aminotransferase (ALT), and ejection fraction (EF) were also included in the analysis. The presence of complications such as thrombosis, leg ulcers, hypothyroidism, osteoporosis, hypogonadism, and extramedullary hematopoiesis (EMH) and treatment of such comorbidities were considered as well. All patients were either homozygous for the cd29 mutation (c.90T>C) or compound heterozygous for it with concomitant IVSI-1 (c.92+1G>A) or IVSII-1 (c.315+1G>A) mutations.

Patients were divided into two groups according to their transfusion dependence: patients who never or seldom did receive red blood cell transfusions during their lives, or non-transfusion-dependent (NTD) patients, and patients who were dependent on regular transfusions, or transfusion-dependent (TD) patients.

Table 2 Genotyping results for SNPs associated with HbF persistence

Gene	Chr:position	SNP	N	MAF (EA)	N Het (EA)	N Hom (EA)	Global GnomeAD MAF
HBG2-XmnI-158	11:5254939	rs1060499525 C/*T*	21	0.048	2	0	–
HBBP1	11:5242453	rs10128556 C/*T*	21	0.000	0	0	0.248
HIMP	6:135055071	rs28384513 T/*G*	21	0.190	2	3	0.325
	6:135097880	rs9399137 T/*C*	21	0.167	7	0	0.212
	6:135105435	rs4895441 A/*G*	21	0.119	3	1	0.228
	6:135106679	rs9402686 G/*A*	21	0.071	3	0	0.219
BCL11A	2:60490908	rs1427407 G/*T*	21	0.167	5	1	0.809
	2:60492835	rs766432 A/*C*	21	0.167	5	1	0.806
	2:60493111	rs11886868 *C*/T	21	0.357	9	3	0.64
	2:60493816	rs4671393 G/*A*	21	0.143	4	1	0.8

The SNP base associated with the effect is in italics (effect allele). *N*, number of samples; *MAF*, minor allele frequency; *EA*, effect allele; *N Het*, number of heterozygotes for the effect allele; *N Hom*, number of homozygotes for the effect allele. The MAF frequencies reported according to Genome Aggregation Database Genomic are also reported as chromosomal positions in conformity with assembly GRCh38.p7-Annotation 108

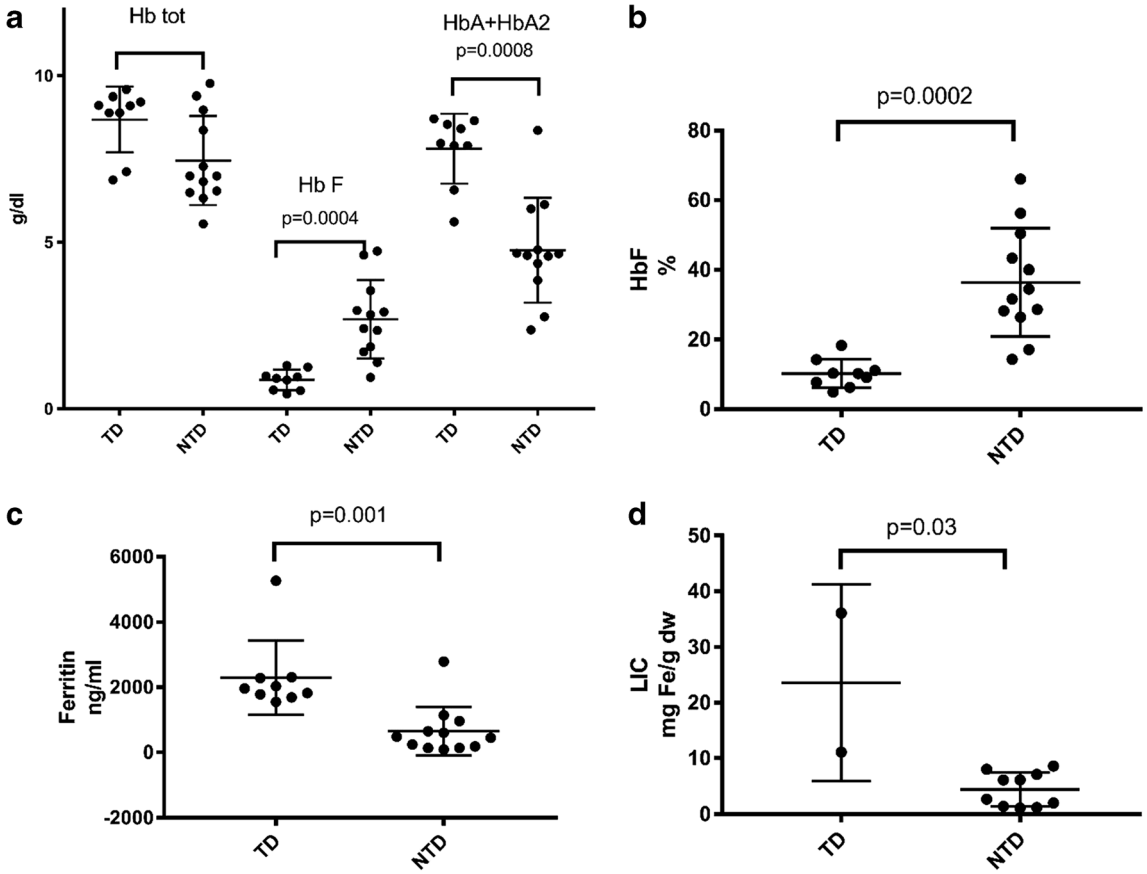

Fig. 1 **a** Association between hemoglobin level and transfusion frequency. **b** HbF percentage as a function of transfusion frequency. **c** Ferritin level in relation to transfusion dependence. **d** Association between LIC and transfusion frequency. Hb, hemoglobin; TD, transfusion-dependent; NTD, non-transfusion-dependent; dw, dry weight

Our study was approved by the Chronic Care Center Institutional Review Board, and all participating patients signed an age-appropriate informed consent form.

Genetic analysis

DNA was extracted from whole-blood samples with Maxwell®16 Blood DNA purification LEV Kit (Promega, USA) in combination with Maxwell®16 Instruments (Promega, USA) following the manufacturer's protocol.

The entire β-globin gene was analyzed as previously described [11]. Briefly, beta gene was amplified with the following primers: A forward primer 5′-CTAAGCCAGTGCCA GAAG-3′; B reverse primer 5′-ACACTGATGCAATC ATTCG-3′; C forward primer 5′-GTGTACACATATTG ACCAAA3′; and D reverse primer 5′-CACT GACCTCCCACAT-TCCC-3′. The amplification was performed with 35 cycles of denaturing at 94 °C (30″), annealing at 56 °C (30″), and extension at 72 °C (45″). PCR products were sequenced using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems by Thermofisher, USA) and analyzed on the ABI Prism 310 Genetic Analyzer (Applied Biosystems by Thermofisher, USA).

The presence of deletions or duplications in the α- and β-globin gene clusters was assessed with the multiplex ligation-dependent probe amplification (MLPA) technique. MLPA was carried out using the SALSA MLPA P140-HBA and P102-HBB Kits (MRC-Holland, the Netherlands) according to the manufacturer's instructions.

Briefly, 150 ng of genomic DNA in 5 μL of low TE buffer was denatured for 5 min at 98 °C and hybridized for 16 h at 60 °C with 1.5 μL of SALSA probe mix and 1.5 μL of SALSA MLPA buffer. Probe ligation was then performed by adding 32 μL of ligation mix and incubating it at 54 °C for 15 min; the reaction was stopped at 98 °C for 5 min. Finally, amplification of ligated probes was carried out by polymerase chain reaction (PCR) with specific SALSA FAM primers in the presence of a specific a deoxynucleotide (dNTP) solution mix and SALSA polymerase. All amplicons were separated by capillary electrophoresis on ABI Prism 310 (Applied Biosystems by Thermofisher, USA) while adjusting the running time to 45 min [12]. Quantitative analysis was carried out by MRC-Coffalyser software v.7 (MRC-Holland). The threshold ratios for deletion and duplication were set at 0.7 and 1.3, respectively.

Table 3 Association of hemoglobin, ferritin, ALT, LIC, and EF values with SNPs in the BCL11A, HMIP, and HBG2 XmnI-158C/T loci

		N (*n* = 21)	Hb (g/dL)	HbF (%)	Hb F (g/dL)	Ferritin (ng/mL)	*N* (*n* = 20)	ALT (U/L)	*N* (*n* = 12)	LIC (mg Fe/g dw)	*N* (*n* = 17)	EF (%)
HBG2-XmnI-158	rs5254939 C/*T*											
	CC	19	8.0	25.0	1.9	1402	18	34.2	11	8.1	15	0.63
	CT	2	8.0	27.7	2.0	963	2	149.2	1	2	2	0.63
	TT	0	–	–	–	–	0	–	0	–	0	–
	p value		0.59	0.72	0.63	0.63		0.10		0.47		0.94
HMIP	rs28384513T/*G*											
	TT	16	7.8	25.2	1.8	1395	15	53.8	9	8.7	13	0.62
	TG	2	7.7	30.5	2	1063	2	16.0	2	6.2	2	0.61
	GG	3	9.2	24.5	2.3	1367	3	24.8	1	1.10	2	0.67
	p value		0.27	0.99	0.82	0.89		0.29		0.27		0.7
	rs9399137T/*C*											
	TT	14	8.2	22.1	1.7	1293	14	52.3	5	6.3	12	0.61
	TC	7	7.5	31.3	2.37	1393	6	30.4	7	9.6	5	0.67
	CC	0	–	–	–	–	0	–	0	–	0	–
	p value		0.31	0.14	0.21	0.18		0.47		0.37		*0.03*
	rs4895441A/*G*											
	AA	17	7.9	23.4	1.7	1441	16	52.9	10	8.9	15	0.62
	AG	3	8.3	26.9	1.9	1309	3	17.7	1	1.2	1	0.62
	GG	1	9.4	50.4	4.7	134	1	14	1	1.1	1	0.74
	p value		0.36	0.41	0.23	0.31		0.22		0.1		0.26
	rs9402686G/*A*											
	GG	18	7.9	22.9	1.8	1488	17	51.0	10	8.9	15	0.62
	GA	3	8.4	28.9	3.1	594	3	15.7	2	1.2	2	0.68
	AA	0	–	–	–	–	0	–	0	–	0	–
	p value		0.55	0.27	0.16	0.07		0.12		*0.03*		0.26
BCL11A	rs1427407G/*T*											
	GG	15	8.1	21.3	1.6	1609	14	35.0	7	10.2	12	0.62
	GT	5	7.5	31.7	2.3	858	5	82.1	4	4.8	4	0.63
	TT	1	9.4	50.4	4.8	134	1	14.0	1	1.1	1	0.74
	p value		0.36	0.12	0.06	0.18		0.5		0.24		0.24
	rs766432A/*C*											
	AA	15	8.1	21.3	1.6	1609	14	35.0	7	10.2	12	0.62
	AC	5	7.5	31.7	2.3	858	5	82.1	4	4.8	4	0.63
	CC	1	9.4	50.4	4.8	134	1	14.0	1	1.1	1	0.74
	p value		0.36	0.12	0.06	0.18		0.5		0.24		0.24

Table 3 (continued)

	N (*n* = 21)	Hb (g/dL)	HbF (%)	Hb F (g/dL)	Ferritin (ng/mL)	*N* (*n* = 20)	ALT (U/L)	*N* (*n* = 12)	LIC (mg Fe/g dw)	*N* (*n* = 17)	EF (%)
rs11886868*C/T*											
TT	9	7.9	18.6	1.4	1829	8	40.7	4	14.1	6	0.64
TC	9	7.9	28.2	2.1	993	9	24.9	6	5.3	9	0.60
CC	3	8.7	36.0	3.0	1055	3	121.5	2	1.5	2	0.70
p value		0.48	0.21	0.10	0.47		0.42		0.16		0.06
rs4671393*G/A*											
GG	16	9.4	22.7	1.7	1523	15	49.8	8	9.16	13	0.62
GA	4	7.6	28.8	2.1	1013	4	38.2	3	5.7	3	0.62
AA	1	8	50.4	4.7	134	1	14.0	1	1.1	1	0.74
p value		0.42	0.24	0.13	0.27		0.56		0.28		0.26

Mean values are displayed. Italicized *p* values denote conventional clinical significance ($p < 0.05$). *Hb*, hemoglobin; *ALT*, alanine aminotransferase; *LIC*, liver iron concentration; *EF*, ejection fraction

Allelic discrimination study was performed by selecting 10 SNPs known for their association with increased HbF levels (*HBG2*, rs1060499525; *HBBP*, rs10128556; *BCL11A*, rs1427407, rs766432, rs11886868, rs4671393; *HIMP* locus, rs28384513, rs9399137, rs4895441, rs9402686). The *HBG2* SNP was genotyped by PCR and direct automated sequencing. One hundred nanograms of genomic DNA was amplified with specific *HBG2* primer pairs and then submitted to automated sequencing with ABI Prism 310 (Applied Biosystems by Thermofisher, USA). SNPs on the *BCL11A* and *HIMP* loci were genotyped by allelic discrimination using Taqman™ MGB specific assays on a 7500 real-time PCR (Applied Biosystems by Thermofisher, USA). In short, for each SNP, 100 ng of DNA was amplified in the presence of Taqman™ specific assay and genotyping master mix and submitted to allelic discrimination PCR. After a pre-read for 30 s at 60 °C, the following cycling protocol was used: 95 °C for 10 min followed by 50 cycles with a denaturation step at 92 °C for 15 s and an annealing step at 60 °C for 1 min. At the end of the PCR, an additional step of fluorescence reading was performed at 60 °C for 30 s. Fluorescence data were then analyzed with 7500 SDS software v.2.3 (Applied Biosystems by Thermofisher, USA).

Statistical analysis

Quantitative and categorical variables were analyzed with the Kruskal-Wallis test and Fisher's exact test, respectively. The software Stata 15 was used for analysis (StataCorp. 2017).

Results and discussion

Fourteen out of the 21 patients (67%) were female. The age range was 15–57 years. Table 1 summarizes the hematological characteristics of the patients.

A total of 14 patients were homozygous for cd29, and seven were compound heterozygotes—three with IVSI-1 and four with IVSII-1. Because phenotypic severity in β-thalassemia patients is partly determined by the degree of β-globin chain deficit and by α-globin chain imbalance [13], MLPA of the α and β genes was performed for all patients to test for the potential effect of *HBB* and *HBA* deletions or duplications on disease severity. None of the patients displayed either deletions or duplications in the β genes, while only two patients co-inherited the $\alpha^{3.7}$ deletion, which was heterozygous in one patient and homozygous in the other.

The minor allele frequencies (MAFs) for the 10 investigated SNPs are shown in Table 2. The rs10128556 *HBBP* gene was excluded from subsequent analysis because all patients had the wild-type allele. The allele frequency of each SNP in our patients was found to be lower than the corresponding reported global GnomeAD MAF. This is probably due to

Table 4 Association of transfusion dependence and splenectomy with SNPs in the BCL11A, HMIP, and HBG2 XmnI-158C/T loci

		Transfusion dependence		Splenectomy	
		N (*n* = 21)	*N* transfused (*N*/total)	*N* (*n* = 18)	*N* splenectomized (*N*/total)
HBG2-XmnI-158	rs5254939 C/*T*				
	CC	19	8/19 = 42%	16	12/16 = 75%
	CT	2	1/2 = 50%	2	2/2 = 100%
	TT	0	–	0	–
	p value		0.7		0.6
HMIP	rs28384513T/*G*				
	TT	16	6/16 = 38%	15	12/15 = 80%
	TG	2	1/2 = 50%	1	1/1 = 100%
	GG	3	2/3 = 67%	2	1/2 = 50%
	p value		0.77		0.55
	rs9399137T/*C*				
	TT	14	7/14 = 50%	13	12/13 = 92%
	TC	7	2/7 = 29%	5	2/5 = 40%
	CC	0	–	0	–
	p value		0.64		*0.04*
	rs4895441A/*G*				
	AA	17	7/17 = 41%	16	13/16 = 81%
	AG	3	2/3 = 67%	1	1/1 = 100%
	GG	1	0/1 = 0%	1	0/1 = 0%
	p value		0.75		0.4
	rs9402686G/*A*				
	GG	18	8/18 = 44%	16	13/16 = 81%
	GA	3	1/3 = 33%	2	1/2 = 50%
	AA	0	–	0	–
	p value		0.61		0.4
BCL11A	rs1427407G/*T*				
	GG	15	9/15 = 60%	12	11/12 = 92%
	GT	5	0/5 = 0%	5	3/5 = 60%
	TT	1	0/1 = 0%	1	0/1 = 0%
	p value		*0.02*		0.08
	rs766432A/*C*				
	AA	15	9/15 = 60%	12	11/12 = 92%
	AC	5	0/5 = 0%	5	3/5 = 60%
	CC	1	0/1 = 0%	1	0/1 = 0%
	p value		*0.02*		0.08
	rs11886868*C*/T				
	TT	9	6/9 = 67%	6	5/6 = 83%
	TC	9	3/9 = 33%	9	8/9 = 89%
	CC	3	0/3 = 0%	3	1/3 = 33%
	p value		0.14		0.17
	rs4671393G/*A*				
	GG	16	9/16 = 56%	13	12/13 = 92%
	GA	4	0/4 = 0%	4	2/4 = 50%
	AA	1	0/1 = 0%	1	0/1 = 0%
	p value		0.06		*0.04*

certain demographic peculiarities unique to the Lebanese population. For example, it was shown in a previous work that the cd29 mutation is almost restricted to the Shiite sect of the Lebanese people, which is mostly related to the scarcity of intercommunity marriages in Lebanon and therefore of genetic exchange, translating to a lower MAF [14].

Hematological parameters and clinical characteristics were evaluated according to transfusion dependence. Hb levels were not significantly different between NTD and TD patients. However, the absolute amount of HbF as well as its percentage was found to be higher in the NTD group ($p = 0.0004$ and $p = 0.0002$, respectively) (Fig. 1, panels a and b), which is expected because an increased HbF level in adults with β-thalassemia is a well-known determinant of less severe disease by compensating the decreased β chain [15]. Iron parameters were found to be higher in TD patients ($p = 0.001$ and $p = 0.03$, respectively, for ferritin and LIC) (Fig. 1, panels c and d), which is attributable to the iron load usually contributed by transfused blood. On the contrary, ALT level and left ventricular ejection fraction did not differ significantly between the two groups (Online Resources 1 – Table 1 and Figure 2).

The association between the polymorphisms involved in HbF persistence and hematological and clinical characteristics was also investigated. The SNPs that were evaluated included the XmnI-158 polymorphism in the *HBG* gene and previously described SNPs in the *BCL11A* and *HMIP* loci (Table 3). Although no conventional statistical significance could be attained, it was noted that individuals homozygous or heterozygous for the effect allele in the *BCL11A* and *HMIP* SNPs had higher HbF percentages and absolute levels and were less likely to be transfusion-dependent (Table 4). This ameliorating effect of the aforementioned SNPs confirms the similar associations elucidated in analogous reports in the literature [4, 16, 17]. The XmnI polymorphism was not frequent in our group (2/21 or 9.5%), and patients carrying the polymorphism did not have higher HbF levels or percentages nor did they demonstrate less transfusion requirement, unlike what was previously described in the literature.

As for measures of iron status, the presence of the effect allele in *BCL11A* and *HMIP* was associated with lower ferritin levels and LIC, which can arguably be explained by the corresponding protective effect of these alleles against transfusion dependence. On the other hand, ALT levels were lower among patients with polymorphisms in the *HMIP* locus only. Notably, rs5254939 was the only XmnI-158 SNP that seemed to influence ALT values. Lower rates of splenectomy were present in patients carrying the *BCL11A* effect alleles than those with variant alleles in the *HMIP* locus (Table 4).

Specific treatment and complication rates were not significantly associated with the investigated SNPs (Online Resources 1 – Table 2), where better outcomes were not observed in patients carrying the effect variants.

Analyzing the single polymorphisms, rs1427407 and rs766432 on *BCL11A* had the highest HbF levels, least transfusion dependence, and least splenectomy rates, yet with no conventional statistical significance. Being only about 2 kb apart, it is conceivable that these polymorphisms are inherited together, thus explaining the similarity in their association with the aforementioned indices. Bauer et al. [18] identified the association of the rs1427407 polymorphism with HbF level as the strongest among other SNPs in *BCL11A*, and they gave it a functional role related to reduce transcription factor binding, decreased BCL11A expression, and elevated HbF.

Our results showed that HbF production variants have an important impact on the severity of β-thalassemia phenotype. Such genetic modifiers can be related to various clinical indices and outcomes, including time to first transfusion, for example, which is a key measure that characterizes the severity of β-thalassemia, as well described by Danjou et al. [4]. This study has a promising predictive value on multiple clinical measures, including transfusion dependence and need for splenectomy, as a function of individual patients' personal genetic background, suggesting the possibility of developing a scoring system based on genetic modifiers to predict the severity of thalassemia. Furthermore, the investigated SNPs can represent attractive therapeutic targets for genome engineering in the realm of the β-hemoglobinopathies, such as disruption of the *BCL11A* enhancer in erythroid precursors with ensuing HbF augmentation. A major limitation to our work, however, remains to be the small sample size, hindering attainment of conventional statistical significance for many of the obtained results. Because distinction between TDT and NTDT is based on clinical criteria and often takes at least 4 years before it can be confirmed, further validation of this prediction tool of severity may help in the anticipation of the type of thalassemia the patient will develop later and may have promising implications for genetic counseling and for therapeutic decision making.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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