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Research Article

HBG2 and HBG1 Nucleotide Substitutions and Hbf Production in Thalassemia Patients

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Abstract

HbF represents the main hemoglobin fraction during fetal life. After birth, the synthesis of HbF decreases gradually and is replaced by HbA so that HbF levels are less than 2% in adults. During adulthood, HbF may be slightly or significantly elevated because of pathological or nonpathological causes such as HPFH. This status is caused either by fetal globin gene promoter variants or large deletions affecting the human fetal globin genes. In thalassemia, an increase in HbF is linked to β-thalassemia major, δβ-thalassemia or thalassemia intermedia and improves the clinical picture as γ globin chains compensate for the lack of functional β- globin chains. The degree of HbF persistence varies greatly among adults and is largely genetically controlled. Research of causes that induce the expression of γ globin gene can impact the definition of clinical condition, as well as provide potential targets for treatment of hemoglobinopathy, as an increase in HbF production ameliorates β-thalassemia severity. In this report we present full DNA analysis of both HBG2 and HBG1 genes in a cohort of 96 β- and/or α-thalassemia subjects with high HbF levels and 30 healthy individuals to update the list of HPFH polymorphisms.

Keywords: Fetal hemoglobin (HbF); Hereditary persistence of fetal hemoglobin (HPFH); γ Globin genes; HPFH polymorphisms; Thalassemia; Hemoglobinopathy

Abbreviations: HbF: Fetal Hemoglobin; HbA: Adult Hemoglobin; HPFH: Hereditary Persistence of Fetal Hemoglobin; BCL11A: B cell CLL/lymphoma 11A; ZBTB7A: Zinc-Finger and BTB-domain-containing 7A; KLF1: Kruppel Like Factor 1; MLPA: Multiplex Ligation Probe Amplification; SNPs: Single Nucleotide Polymorphisms

Introduction

HbF is a high oxygen affinity tetramer consisting of two α and two γ globin chains. Its role is to transfer oxygen from maternal to fetal circulation and represents the main hemoglobin fraction during fetal life. HbF production starts from the sixth week of gestation and replaces the embryonic hemoglobins Gower I, Gower II and Portland produced in the first weeks after conception and at birth it constitutes about 80% of total hemoglobin. After birth, the synthesis of HbF decreases gradually due to silencing of the expression of γ globin genes and reciprocal increase in β-globin gene expression and is replaced by HbA. In normal conditions only trace amounts of HbF (less than 2%) are present in postnatal life after 1 year of age [1]. However, HbF may be slightly or significantly elevated during adulthood. A high level of HbF is mainly due to pathological conditions such as β-thalassemia major, δβ-thalassemia, (as γ globin chains compensate for the lack of functional β-globin chains) erythropoietic stress and bone marrow malignancies, or nonpathological conditions, known as HPFH, such as large deletions within the β-globin gene cluster (deletional HPFH), promoter variants of γ globin genes (non deletional HPFH) or pregnancy [2-4]. While persistence of increased HbF production has no clinical consequences in healthy individuals, HbF is one of the most common and major modifiers of disease severity in individuals with β-thalassemia [5-8].

The degree of HbF persistence varies greatly among adults and is largely genetically controlled [9]. Gene expression is modulated by a large number of polymorphisms on cis-regulatory elements located between nucleotide -202 and -110 relative to the Cap site on promoter region of the HBG1 and HBG2 genes which disrupt transcription factor binding motif (BCL11A, ZBTB7A, MYB, KLF1) [10-12]. The involvement of some variants has a clear association with increased HbF levels, although others are still ambiguous. Gene and genetic variant identification contributing to HbF variation and the molecular mechanisms through which they operate play an important role in diagnostic and prognostic medicine, as well as in research for novel therapeutic markers. In fact, the reactivation of HbF synthesis by several approaches, such as pharmacological

and gene transfer, modulate the phenotype of patients with β-thalassemia [13]. The research of polymorphisms in γ globin genes and direct or indirect transcriptional repressors of γ globin gene expression has recently gained attention by aiding in the development of new therapeutic strategies and pharmacological agents that increase the levels of this hemoglobin [14]. In this report we present full DNA analysis of both HBG2 and HBG1 genes to update the list of HPFH polymorphisms.

Materials and Methods

We screened a cohort of 96 β- and/or α-thalassemia subjects with high HbF levels who were referred to our diagnostic laboratory for ascertainment of possible hemoglobinopathy and 30 healthy individuals with HbF level <2% recruited at Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico in Milan. All subjects signed the informed consent form before blood sampling.

Patients were divided into 4 groups:

1. 8 α-thalassemia carriers (HbF level: 2.7% - 32.6%)
2. 41 β-thalassemia carriers (HbF level: 3% - 50.3%)
3. 4 β-thalassemia intermedia/major patients (HbF level: 67% - 94.6%)
4. 43 γ-thalassemia patients (HbF level: 2.2% - 16.5%)

The hematological parameters were determined on Sysmex XN-9000, and hemoglobins were analyzed routinely by High Performance Liquid Chromatography- HPLC- Biorad D100, Biorad Laboratories, Hercules, CA, USA. Genomic DNA was extracted from peripheral blood samples. The research of polymorphisms in γ globin genes was performed by Sanger sequencing (BigDye Terminator Cycle Sequencing Ready Reaction Kit v.1.1) on the ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The research of deletional HPFH was performed by MLPA method according to the manufacturer's instructions (SALSA® MLPA® Probemix P102-D1 HBB; MRC-Holland, Amsterdam, The Netherlands).

Results and Discussion

Table 1 & 2 show the frequencies in patient and reference groups of HBG2 and HBG1 polymorphisms, respectively. HBB MLPA resulted negative for all analysed subjects (data not shown). In the β- and α-thalassemia carriers and β-thalassemia intermedia/major, an increased HbF level is due to their pathologic condition. In the β-thalassemia carrier group we found the c.227T>C HbF-Sardinia (AγT) variant together with β0 c.118C>T (Codon 39 (C->T)) and an HbF increase of 10% of the total, as expected [15]. Two β0 c.92+1G>A (IVS-I-1 (G->A)) patients present the c.-170G>A (rs35378915) mutation, called Greek HPFH, that affects the binding of three nuclear factors to the CCAAT region of the HBG1 gene (HbF: 10.3-50.3) [16]. In γ- thalassemia patients, the high HbF level is not correlated to β- and α-thalassemia, even if we cannot exclude other malignancies. We identified the following SNPs exclusively in this group: rs73402643, rs531285196, rs904054277, rs757734616 and rs1231067410 in HBG2; rs35993903, rs34844625, rs3020750, rs2860456, rs1234411915, rs537552941, rs567305547, rs112286603 and c.*179G>A in HBG1. To note, c.*179G>A does not have an assigned reference SNP ID number.

Table 1: Allele Frequencies of HBG2 polymorphisms in patients and reference groups.

HBG2 POLYMORPHISMS (NM_000184.3)	α-thalassemia carriers	β-thalassemia carriers	β-thalassemia intermedia/major patients	γ- thalassemia patients	Reference group
c.-558C>G (rs73402643) (c.-437-121C>G) 475 bp before transcription start site				1/86 (f=0.011)	
c.-450_-445delCTTTAA (rs112075505) 392-397 bp before transcription start site	1/16 (f=0.062)				
c.-450C>T (rs531285196) 397 bp before transcription start site				1/86 (f=0.011)	
c.-422C>G (rs112215533) 369 bp before transcription start site		2/82 (f=0.024)		3/86 (f=0.034)	
c.-362A>G (rs112479156) 309 bp before transcription start site	2/16 (f=0.125)	2/82 (f=0.024)		5/86 (f=0.058)	
c.-324T>C (rs113622787) 271 bp before transcription start site	1/16 (f=0.062)				
c.-321G>C (rs904054277) 268 bp before transcription start site				1/86 (f=0.011)	
c.-309 A>G (rs1045222350) 256 bp before transcription start site	1/16 (f=0.062)			2/86 (f=0.023)	
c.-211C>T (rs7482144) 158 bp before transcription start site	6/16 (f=0.375)	19/82 (f=0.231)	4/8 (f=0.500)	29/86 (f=0.337)	21/60 (f=0.350)
c.-69C>G (rs551623060) 16 bp before transcription start site		1/82 (f=0.012)			
c.93-58C>T (rs1894398) intron 1 of 2 position 65 of 122 (intronic)	1/16 (f=0.062)	22/82 (f=0.268)		9/86 (f=0.104)	

c.315+24A>C (rs33993529) intron 2 of 2 position 24 of 886 (intronic)	1/16 (f=0.062)				
c.*18C>T (rs757734616) exon 3 of 3 (3'UTR) position 147 of 218				1/86 (f=0.011)	
c.*54_*55ins A (rs34879481) exon 3 of 3 (3'UTR) before position 184 of 218	6/16 (f=0.375)	18/82 (f=0.219)	1/8 (f=0.125)	23/86 (f=0.267)	21/60 (f=0.350)
c.*160G>T (rs1231067410) 71 bp after transcription end site				1/86 (f=0.011)	

Table 2: Allele Frequencies of HBG1 polymorphisms in patients and reference groups.

HBG1 POLYMORPHISMS (NM_000559.2)	α-thalassemia carriers	β-thalassemia carriers	β-thalassemia intermedia/major patients	γ- thalassemia patients	Reference group
c.-637G>A (rs2855039)		4/82 (f=0.048)		5/86 (f=0.058)	21/60 (f=0.350)
c.-548T>A (rs57966301) 495 bp before transcription start site		1/82 (f=0.012)		2/86 (f=0.023)	
c.-447G>A (rs35993903) 394 bp before transcription start site				1/86 (f=0.011)	
c.-418G>C (rs2855040) 365 bp before transcription start site		1/82 (f=0.012)		2/86 (f=0.023)	
c-405A>G (rs34844625) 352 bp before transcription start site				1/86 (f=0.011)	
c.-373C>T (rs3020750) 320 bp before transcription start site				1/86 (f=0.011)	
c.-366A>G (rs2860456) 313 bp before transcription start site				1/86 (f=0.011)	
c.-320C>T (rs12290216) 267 bp before transcription start site	1/16 (f=0.062)			1/86 (f=0.011)	
c.-275_-274 in sAGCA (rs561507744) 222 bp before transcription start site	10/16 (f=0.625)	57/82 (f=0.695)	4/8 (f=0.500)	50/86 (f=0.581)	51/60 (f=0.850)
c.-214G>A (rs1234411915) 161 bp before transcription start site				1/86 (f=0.011)	
c.-170G>A (rs35378915) 117 bp before transcription start site		2/82 (f=0.024)			
c.-69C>G (rs558015287) 16 bp before transcription start site		1/82 (f=0.012)			
c.-29G>A (rs368698783) exon 1 of 3 (5'UTR) position 25 of 145	3/16 (f=0.187)	12/82 (f=0.146)	2/8 (f=0.250)	17/86 (f=0.197)	21/60 (f=0.350)
c.92+40C>A (rs537552941) intron 1 of 2 position 40 of 122 (intronic)				1/86 (f=0.011)	
c.92+41C>A (rs567305547) intron 1 of 2 position 41 of 122 (intronic)				1/86 (f=0.011)	
c.93-58C>T (rs202216517) intron 1 of 2 position 65 of 122 (intronic)	2/16 (f=0.125)	26/82 (f=0.317)		12/86 (f=0.139)	
c.227T>C p.Ile76Thr (rs1061234) Hb F-Sardinia (AγT)	2/16 (f=0.125)	8/82 (f=0.097)		4/86 (f=0.046)	
c.*3_*6 delTCAC insCTCT (rs386750130) exon 3 of 3 (3'UTR) position 132-135 of 216	11/16 (f=0.687)	50/82 (f=0.609)	4/8 (f=0.500)	49/86 (f=0.569)	43/60 (f=0.716)
c.*15A>C (rs112286603) exon 3 of 3 (3'UTR) position 144 of 216				1/86 (f=0.011)	
c.*55delA (rs3841756) exon 3 of 3 (3'UTR) position 184 of 216	1/16 (f=0.062)	15/82 (f=0.182)		10/86 (f=0.116)	13/60 (f=0.216)
c.*124T>A (rs2402330) 37 bp after transcription end site		2/82 (f=0.024)		1/86 (f=0.011)	
c.*179G>A				1/86 (f=0.011)	

c.*246A>T (rs916111) 159 bp after transcription end site	6/16 (f=0.375)	36/82 (f=0.439)		22/86 (f=0.255)	30/60 (f=0.500)
c.*259A>T (rs1143541) 172 bp after transcription end site	2/16 (f=0.125)	2/82 (f=0.024)		6/86 (f=0.069)	
c.*344G>A (rs147256314) 257 bp after transcription end site				1/86 (f=0.011)	

In comparing patient and reference groups, nucleotide variations identified only in promoter regions of patient groups include rs73402643, rs112075505, rs531285196, rs112215533, rs112479156, rs113622787, rs904054277, rs1045222350 and rs551623060 in HBG2 and rs57966301, rs35993903, rs2855040, rs34844625, rs3020750, rs2860456, rs12290216, rs1234411915, rs35378915 and rs558015287 in HBG1. They are located between c.-558C>G (475bp before transcription start site) and c.-309A>G (256bp before transcription start site) in the HBG2 promoter region, and between c.-548T>A (495bp before transcription start site) and c.-366A>G (313bp before transcription start site) in the HBG1 promoter region.

Considering their position, frequency, absence in reference group and effect on HbF level (range from 2.5-5%), these variations may modify transcription binding sites, resulting in up-regulation of γ globin gene or influence the HBG2 and HBG1 ratio expression. Always considering patients versus reference group, we identified in the HBG2 intron: rs33993529 and the HBG1 intron: rs537552941, rs567305547 and rs1061234. The presence of c.93-58C>T (rs1894398) in HBG2 and c.93-58C>T (rs202216517) in HBG1, are observed only inpatient groups, even if population frequencies, obtained from exomes and whole genomes studies, are ƒ = 0.169(rs202216517) and ƒ = 0.425 (rs1894398) [17]. Analysis of 3'UTR showed the presence of rs757734616 and rs1231067410 in HBG2 and rs112286603, rs2402330, c.*179G>A, rs1143541 and rs147256314 in HBG1. We identified the following SNPs in the promoter region in both patient and reference groups, with differing HbF levels (patient >4% and reference <2%): HBG2: rs7482144 and rs34879481; HBG1: rs2855039, rs561507744, rs386750130, rs3841756 and rs916111.

Given their frequency in our cohort and the population frequency reported, we can assume they are polymorphisms. We cannot exclude that those SNPs induce HBG expression genes under erythropoietic stress conditions. Inducing erythropoietic stress in the reference group followed by HbF analysis could help define their role. We observed in all groups that a high frequency of HBG2 c.-211C>T (rs7482144) expressed concomitant with HBG1 c.-29G>A (rs368698783) is associated with a greater variability in HbF level (range from 0.4-15.1%) [18]. Analysis of 3'UTR showed the presence of polymorphisms with a high frequency in both patient and reference groups, such as c.*54_*55insA (rs34879481) in HBG2 and c.*3_*6delTCACinsCTCT (rs386750130) in HBG1. Polymorphisms on 3'UTR may influence microRNAs binding, whose expression is enhanced/inhibited during erythroid differentiation and induction of HbF production [19]. Recent studies have revealed that some microRNAs influence the expression of the γ-globin gene, showing that high HbF levels in adulthood may result from a range of genetic factors which may explain the observed variations in HbF of healthy subjects and patients with hemoglobinopathies [20,21].

Conclusion

HPFH is a harmless condition characterized by a lack of changes in the synthesis of β-globin chains, resulting in an increase of γ chains and consequently high HbF level [22]. However, slightly or severely increased HbF values in adults may hide a more complex clinical condition, such as β-thalassemia major, δβ-thalassemia, or bone marrow malignancies. As known in literature, HBG2 and HBG1 promoter regions have a regulatory role in gene expression because they contain enhancer and silencer elements [23]. Our study proposes to list SNPs found in HBG2 and HBG1 promoter regions and in 3'UTR in a cohort of patients with hemoglobinopathies and subjects with slightly or severely increased HbF values and in a reference group, to underlie the importance of a correct ascertainment and interpretation of an elevated HbF level.

Our results show several SNPs found either in patient and reference groups, whose correlation to HbF expression isn't clear, although they might be involved in stimulation of HBG2 and HBG1 expression during stress erythropoiesis. Exclusively inpatient groups we have also identified nucleotide variations, which may play a role in transcription binding site modification and regulation of γ globin genes expression. More data is necessary to define a genotype/phenotype correlation. Additional studies are necessary to clarify the possible role of identified SNPs in our cohort to better understand their impact on HbF production in adults and alleviation in thalassemia disease. The level of HbF is an important index in thalassemia because it can hide a complex genotype that could otherwise be misdiagnosed causing diagnostic errors and incorrect reproductive risk.

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Conflict of Interest

The authors declare no conflicts of interest. The necessary consent for publication was obtained from the patients' parents.

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