Association of polymorphisms in the HBG1-HBD intergenic region with HbF levels

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Abstract

Background: Increased levels of fetal hemoglobin (HbF) can improve the clinical course of the patients with sickle cell anemia (SCA) or β-thalassemia. The HBG1-HBD intergenic region plays an important role in this process. However, very few studies investigated whether the variations in this region have an effect on HbF expression.

Methods: We retrieved all the SNP data in the HBG1-HBD intergenic region and defined the haplotype blocks, then performed cluster analysis and selected a tagSNP. A total of 500 normal individuals and 300 β-thalassemia carriers were enrolled. After routine blood and hemoglobin capillary electrophoresis testing, β-thalassemia mutations were detected using PCR-reverse dot blot. The genotypes of the rs4910736 (A > C) and rs10128556 (C > T) were determined using Sanger sequencing; the relationship between the two SNPs and the levels of HbF was analyzed.

Results: Two haplotype blocks were constructed. Block 1 included seven haplotypes divided into two groups M and N by 11 tagSNPs, among which rs4910736 was selected as a tagSNP, while block 2 included three haplotypes. We found that the haplotypes of block 1 were statistically associated with HbF levels, but the non-tagSNP rs10128556 was shown to be more strongly associated with HbF levels than rs4910736.

Conclusion: This work proved that the haplotypes in the HBG1-HBD intergenic region and SNP rs10128556 are both statistically associated with HbF levels, revealing the association of polymorphisms in the HBG1-HBD intergenic region with HbF levels.

Keywords
fetal hemoglobin, haplotype, HBG1-HBD intergenic region, single nucleotide polymorphism
1 | INTRODUCTION

Elevated fetal hemoglobin (HbF; αβγδ) plays an important role in improving the clinical course and general well-being of patients with sickle cell anemia (SCA) or β-thalassemia.1-4 Earlier studies revealed that even patients with the same genotype could have large heterogeneity in the degree of anemia.5 A primary modifier for clinical heterogeneity among patients with SCA or β-thalassemia may be the individual differences in the HbF levels. Patients with an increased HbF often tend to have a relatively mild clinical phenotype.6,7 Therefore, the discovery of genetic expression markers of HbF has been a main focus for decades to find therapeutic targets for SCA and β-thalassemia.

HbF is the major hemoglobin during gestation and constitutes approximately 60%-80% of the total hemoglobin in full-term newborns.8 It is then almost completely replaced by adult hemoglobin (hemoglobin A, HbA; α2β2) by the approximate age of 6-12 months, and it amounts to less than 1% of the total hemoglobin in adults. The variations of HbF expression in adult life is a quantitative trait and it amounts to less than 1% of the total hemoglobin in adults. The variations in the levels of HbF, indicating that additional loci are involved.6,7

Many studies have shown that the HBG1-HBD intergenic region plays an important regulatory role in the process of γ to β-globin gene switch.14,15 However, the potential effect of variation in this region on the levels of HbF is not well studied. Galanneau et al16 found that rs10128556 in the HBG1-HBD intergenic region had a stronger effect on HbF expression than the Xmn1 site which is located in upstream of HBG2. However, there were no other reports on the association of HbF expression with the rs10128556 polymorphism locus. In this study, we constructed haplotypes of the HBG1-HBD intergenic region and then selected a tagSNP and rs10128556 to assess the correlation of polymorphisms in the HBG1-HBD intergenic region with the levels of HbF.

2 | MATERIALS AND METHODS

2.1 | Haplotypes construction and tagSNP selection

All the SNP data of the Chinese Han population of Beijing in the HBG1-HBD intergenic region were retrieved from the HapMap database (http://www.hapmap.org). The haplotype blocks were defined using the Haplovew 4.0 software following the approach suggested by Gabriel et al17 Within a region of DNA sequence, if the upper limit of the 95% confidence interval of D’ values between more than 95% SNPs is >0.98 and the lower limit is >0.70, there is almost no genetic recombination within this region and these SNPs constitute a haplotype block. The SNPs for haplotype construction were selected from the haplotype block on the condition that the linkage disequilibrium (LD) measure r2 ≥ .8 and the logarithm of odds (LOD) > 3; the haplotypes were then generated using the Haplovew 4.0 software. The SNPs with a high LD (r2 ≥ .9) between each other were selected as a tagSNP for the haplotype block.18

2.2 | Haplotype cluster analysis

The haplotypes with frequencies greater than 1% in the haplotype block were selected for cluster analysis using the MEGAS.10 software. The maximum likelihood method was used to screen out the phylogenetic tree with the maximum probability according to the theory of phylogenetic reconstruction which may reflect the real situation. Common haplotypes were divided into different groups according to the genetic distance in the cluster analysis.

2.3 | Subjects

In the years 2017 and 2018, 800 individuals visited Guizhou Provincial People’s hospital for β-thalassemia genetic testing or healthy examination. These were enrolled in the study and included 500 normal individuals aged between 6 and 87 years who did not have β-thalassemia or other disorders with a high level of HbF and 300 individuals who were β-thalassemia carriers aged more than 3 years with hypochromic microcytic anemia (MCV < 82 fl, MCH < 27 pg). All the β-thalassemia carriers were confirmed by genetic testing to be heterozygotes of β/β or β/β, and the uncombined α-triplet or α-quadruplet. This study was approved by the Ethics Review Committee of the Guizhou Provincial People’s Hospital. Written informed consent was obtained from all subjects prior to their participation.

2.4 | Sample collection and processing

We collected 2 mL peripheral blood from the participants by venipuncture into EDTA-Na2 anticoagulant tubes. After routine blood testing within 30 minutes, hemoglobin capillary electrophoresis was performed on a CAPILLARYS 2 instrument (Sebia) within 12 hours after blood samples were collected. Using the PCR-reverse dot blot (PCR-RDB) technique, 17 common β-thalassemia mutations in the Chinese population were detected, including codons 41/42 (−CTTT), IVS-II-654 (C > T), codon 17 (A > T), −28 (A > G), codons 71/72 (+A), β6 (codon 26 G > A), codon 43 (G > T), −29 (A > G), codons 27/28 (+C), IVS-1-1 (G > T), IVS-1-5 (G > C), codon 31 (−C), −30 (T → C), −32 (C > A), codons 14/15(+G), initiation codon (ATG → AGG), and Cap
The PCR reaction mixture was prepared in a total volume of 25 μL of the EDTA-anticoagulated blood using the Tiangen Blood Genomic DNA Kit following to the manufacturer's recommendations. DNA segments in which rs4910736 (A > C) and rs10128556 (C > T) are located were separately amplified using the ABI Veriti 96-Well Thermal Cycler. The PCR primers sequences were forward: 5′-TATATGCTCTGACTGGGGACT-3′ and reverse: 5′-ACCTTATCTCCTACCTGCTCTG-3′ for rs4910736 (A > C), and forward: 5′-AGCCTGTATCTGTAGGGTAA-3′ and reverse: 5′-TGTATGTGATGACTGGGGACT-3′ for rs10128556 (C > T). The PCR reaction mixture was prepared in a total volume of 25 μL including 100 ng genomic DNA, 12.5 μL Tap PCR MasterMix, 1 μL of forward and reverse primers. The thermocycling conditions for PCR included one cycle of denaturation at 94°C for 5 minutes, followed by 35 cycles at 94°C for 60 seconds, 55°C for 30 seconds, 72°C for 90 seconds, and the last extension at 72°C for 5 minutes. The genotypes of rs4910736 (A > C) and rs10128556 (C > T) were determined by DNA sequence analysis of the PCR amplified products using the ABI 3130 Genetic Analyzer.

2.5 Statistical analysis

Hardy-Weinberg equilibrium was performed to test the deviations of the observed genotype frequencies from those expected using the chi-square test. The HbF level data were converted to normalize the quantitative trait distribution via logarithmic transformation, and the differences between the groups were then compared using the non-parametric Mann-Whitney test. The positive rate of HbF was compared with the chi-square test. All the data were analyzed using the SPSS 20.0 software (SPSS Inc.). A P-value <.05 was considered to be statistically significant.

3 RESULTS

The demographic characteristics and hematological parameters of the investigated population are shown in Table 1. From the 500 normal individuals (normal group 1), 300 were randomly selected (normal group 2) for analysis of the relationship between rs10128556 and HbF levels.

3.1 Haplotype construction

The data of 52 SNPs of the Chinese Han population of Beijing in the HBG1-HBD intergenic region were acquired from the HapMap database (HapMap Data Rel 24/phasell Nov 08, on NCBI B36 assembly, dnSNP b126). Twenty-seven SNPs with a minor allele frequency (MAF) > 0.05 were selected for haplotype construction. Block 1 and block 2 within the HBG1-HBD intergenic region were identified (Figure 1). The total length of these haplotype blocks accounted for 76.5% of the length of the HBG1-HBD intergenic region, and the SNPs in the haplotype blocks accounted for 81% of the total selected SNPs. There were seven haplotypes made up of 19 SNPs in block 1, which were represented by B1H1-B1H9, shown in Table 2 in which all the underlined bases represent 11 highly correlated SNPs (r^2 ≥ .9) which are considered as a tagSNP for each other in block 1. There were only three haplotypes made up of three SNPs in block 2 that were represented by B2H1-B2H3; thus, the tagSNPs were not analyzed.

3.2 Cluster analysis

Seven kinds of haplotypes were divided into two distinct evolutionarily distant groups by the 11 highly correlated SNPs in block 1 using cluster analysis, the designated group M included B1H2, B1H4, B1H5, and B1H9 and the second group N included B1H1, B1H3 and B1H6 (Figure 2). The smaller the genetic distance between the groups, the higher the similarity was. Rs4910736 was randomly selected as a tagSNP from the 11 highly associated SNPs to explore the association between different haplotype combinations of the HBG1-HBD intergenic region and the HbF levels. Alleles A and C of rs4910736 represented the group N and M haplotype, respectively.

3.3 Relationship between haplotypes and HbF level

Of the 500, 497 normal individuals were genotyped, and the remaining three samples had no signal. The allele numbers of the N and M groups were 798 and 196, accounting for 80.28% and 19.72%, respectively. The cases of the haplotype combinations NN, MN and MM were 315, 168 and 14, accounting for 63.38%, 33.8% and 2.82%, respectively. Since the HbF level is rather low in normal population, the correlation between the haplotype combinations and HbF level was qualitatively analyzed. Samples with HbF can be detected by capillary electrophoresis are designated HbF positive. The HbF-positive rates for the haplotype combinations of NN, MN and MM were 9.84%, 23.81% and 42.86%, respectively. The difference in the HbF-positive rate among these three groups was statistically significant (Table 3).

In the β-thalassemia carriers' group, 285 cases were genotyped, including 216 cases that were NN, 67 cases that were MN, and two cases that were MM. Therefore, the MN and MM combinations were combined into one group for statistical analysis. The medians and interquartile ranges of HbF in the NN group and the MN+MM group were 0.60 (1.50) and 0.80 (2.25), respectively. There was no statistically significant difference between the two groups in the distributions of HbF levels using non-parametric test (log-transformed, P = .100) (Figure 3A). However, the rate of HbF ≥ 2% was...
The expression levels of HbF significantly vary in the population, presenting quantitative traits that are related to multiple genes. The three loci of Xmn1-HBG2 (rs7482144) on chromosome 11p, the HBS1L-MYB intergenic region (HMIP) on chromosome 6q23, and BCL11A on chromosome 2p16 are considered to be QTLs for HbF levels. In addition, earlier studies found that the HBG1-HBD intergenic region plays an important regulatory role in the γ- to β-globin gene switching process.\footnote{14,15} Importantly, there exists BCL11A, GATA-1 and HDAC1 binding sites in a 3.5 kb region near the 5′ portion of HBD which may be related to HbF silencing.\footnote{19,20} Since an SNP at a regulatory DNA binding site may alter the affinity of the regulatory protein, it is worth investigating whether SNP sites or haplotypes in this region are correlated with the levels of HbF.

In this study, the LD analysis of all SNPs in the HBG1-HBD intergenic region in the Chinese Han population in Beijing was carried out using the HapMap database and the Haploview 4.0 software, and two haplotype blocks were constructed: block 1 including seven haplotypes and block 2 containing three haplotypes, with all frequencies greater than 1%. Using cluster analysis, similar haplotypes can be combined into one group. The haplotypes in block 1 were divided after the cluster analysis into the groups M and N whose haplotypes were distinguished by 11 highly linkage disequilibrium SNPs. These 11 SNPs could represent each other and be selected as tagSNPs reducing the number of SNPs for block 1 haplotype-based association analysis from 19 to 1, which not only reduced the workload, but also provided a new understanding of the haplotype of the whole intergenic region.

Rs4910736 was selected as a tagSNP. The results of the HbF qualitative analysis showed that the positive rate of HbF in individuals with the NN (9.84%) genotype was significantly lower than that of individuals with the MN (23.81%) and MM genotypes.
(42.86%) in the normal group. Similarly, in the \( \beta \)-thalassemia carriers’ group, the proportion of HbF \( \geq 2\% \) in individuals with the NN genotype (16.20%) was significantly lower than that of individuals with the MN+MM genotypes (28.99%). It is suggested that the haplotype of the \( HBG1-HBD \) intergenic region has a certain effect on the levels of HbF. However, there was no significant difference between the two subgroups in the \( \beta \)-thalassemia carriers’ group, although the median HbF of individuals with the NN genotype (0.60) was lower than that of individuals with the MN+MM genotypes (0.80); this indicates that the haplotype of the \( HBG1-HBD \) intergenic region may not be the main factor affecting the levels of HbF. This effect needs to be further verified in different races and populations.

Galarneau\textsuperscript{16} found that rs10128556 was more strongly associated with HbF levels than the Xmn1 site upstream of \( HBG2 \). Rs10128556 is not a tagSNP, with a T allele in the haplotype \( B_1H_2 \).
and B₁H₅ and a C allele in other five haplotypes. In this work, we found that there was a significant correlation between rs10128556 and HbF level. The positive rate of HbF in individuals with the CC genotype in the normal group (14.68%) was significantly lower than that of individuals with the CT+TT genotypes (28.38%). In the β-thalassemia carriers’ group, the proportion of HbF ≥ 2% in individuals with the CC genotype (17.51%) was significantly lower than that of individuals with the CT genotype (34.21%). Moreover, the median HbF of individuals with the CC genotype (0.60) in the β-thalassemia carriers’ group was significantly lower than that of individuals with

TABLE 3  The relationship between different haplotype combinations of HBG1-HBD intergenic region as well as genotypes of rs10128556 and HbF level in normal group

| SNP       | Genotype combination | HbF Positive rate (%) | \( \chi^2 \) | P       |
|-----------|----------------------|------------------------|-------------|---------|
| rs4910736 | NN                   | 9.84                   | 24.567      | .000    |
|           | MN                   | 23.81                  |             |         |
|           | MM                   | 42.86                  |             |         |
|           | Total                | 15.49                  |             |         |
| rs10128556| CC                   | 14.68                  | 6.088       | .014    |
|           | CT+TT                | 28.38                  |             |         |
|           | Total                | 18.15                  |             |         |

Note: For rs4910736, allele A and C represent group N and M haplotype, respectively.
TABLE 4 The relationship between haplotype combinations of HBG1-HBD intergenic region and genotypes of rs10128556 with HbF level in β-thalassemia carrier group

| SNP          | Genotype combination | Cases (N) | HbF ≥ 2% (n) | Rate of HbF ≥ 2% (%) | χ²       | P        |
|--------------|----------------------|-----------|--------------|----------------------|----------|----------|
| rs4910736    | NN                   | 216       | 35           | 16.20                |          |         |
|              | MN+MM                | 69        | 20           | 28.99                | 5.486    | .023     |
|              | Total                | 285       | 55           | 19.30                |          |         |
| rs10128556   | CC                   | 257       | 45           | 17.51                |          |         |
|              | CT                   | 38        | 13           | 34.21                | 5.846    | .019     |
|              | Total                | 295       | 58           | 19.66                |          |         |

Note: For rs4910736, allele A and C represent group N and M haplotype, respectively.

the CT genotype (1.05). This suggests that although rs10128556 is not a tagSNP, it is more strongly associated with HbF levels than the tagSNP rs4910736.

As the HBG1-HBD intergenic region belongs to a non-coding sequence, we speculate that the effect of these SNPs on the HbF expression may result from changing the binding sites of the transcription factors (TFs) that are related to the process of γ- to β-globin gene switching. Interestingly, Liu et al.21 also screened out the correlation between rs4910736 and HbF levels using gene chip assays in patients with SCA. Predicted by the Tfsitescan software, the G allele created a growth factor independence 1 (GFI1) DNA motif. GFI1 and its paralog GFI1B are critical transcriptional regulators for the proliferation and maturation of hematopoietic stem cells. We also used the Tfsitescan software to predict the TFs binding sites of the other ten tagSNPs. The TFs that are related to the regulation of the globin gene expression include C/EBP (rs4320977 G allele), Myb (rs4402323 T allele), NF-E2 (rs968857 G allele), FOX family (rs2105819 G allele, rs968857 A allele), TFIID (rs10837643 A allele, rs4320977 A allele, rs2105819 C allele), YY1 (rs3813727 T allele), and the ones that are related to the proliferation and differentiation of the erythroid stem cells include FOXO (rs4320977 A allele) and TCF/LEF (rs2105819 G allele, rs968857 A allele). Although it is unclear whether these binding sites are related to the repression of the γ-globin transcription, our analysis provides an evidence for a potential regulatory sequence in the HbF silencing region. However, there was no difference in the binding sites predicted for rs10128556 with either the C or T allele, suggesting that a variant in the linkage disequilibrium with rs10128556 might be the functional one.

In conclusion, we constructed haplotypes of the HBG1-HBD intergenic region and analyzed their association with HbF levels. The haplotypes in this region were related to HbF levels both in the normal and β-thalassemia carriers’ groups. The SNP rs10128556 is more strongly associated with HbF than the tagSNPs of the HBG1-HBD intergenic region but may not be a functional variant regulating the γ-globin gene expression.

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