ORIGINAL REPORT

Molecular analyses of *GYPB* in African Brazilians

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The molecular background of variant forms of *GYPB* is not well studied in Brazilians of African descent. The present study was carried out to determine the molecular bases of the S–s– phenotype and the frequency of *GYPB*S* silent gene for the S–s+ phenotype in a blood donor population of African Brazilians. In this study, 165 blood samples from African Brazilians (Northeastern Brazil) who phenotyped as S–s– (n = 17) and S–s+ (n = 148) by hemagglutination were selected. Allele-specific (AS)-PCR and PCR-restriction fragment length polymorphism (RFLP) were used to identify the variant forms of *GYPB.* In 13 of 17 S–s– samples (76.5%), both *GYPB* were deleted. In 137 of the 148 S–s+ samples (92.6%), the AS-PCR was consistent with the S–s+ phenotype. In 4 of the S–s– samples (23.5%) and 11 of the S–s+ samples (7.4%), the AS-PCR showed the presence of a *GYPB*S* allele associated with silencing of S. In the 4 donors with the S–s– phenotype, there was homozygosity (or hemizygosity) for the *GYP(P2)* allele (n = 2), homozygosity (or hemizygosity) for the *GYP(NY)* allele (n = 1), and heterozygosity for the *GYP(P2)* and *GYP(NY)* alleles (n = 1). In the 11 donors with the S–s+ phenotype, there was heterozygosity for *GYP(P2)* allele (n = 8) and heterozygosity for *GYP(NY)* allele (n = 3). This study reports for the first time the molecular mechanisms responsible for the S–s– phenotype in a population of African Brazilians and provides new information about the frequency and molecular bases of the *GYPB*S* silent gene (7.4%) in this population. ***Immunohematology* 2008;24:148-153.**

Key Words: African Brazilians, S–s– phenotype, *GYPB(P2), GYPB(NY),* MNS blood group system, *GYPB*S* silent gene, blood group genotyping.

The MNS blood group system is a highly complex system that consists of more than 40 distinct antigens.[1] These antigens are carried on glycophorin A (GPA), glycophorin B (GPB), or hybrid proteins that arise from unequal crossover, nucleotide substitution, or gene conversion events between the glycophorin genes.[2]

The genes encoding GPA *(GYPA)* and GPB *(GYPB)* are located on chromosome 4 together with a third gene of this glycophorin family, *GYPE.* In addition to sequence homology and proximity between the glycophorin genes, recombination hot spots have been identified and demonstrated to generate many different hybrid *GYP* gene products at the RBC surface, as a result of reciprocal and nonreciprocal exchange of nucleic acids.[3] These hybrid molecules often carry one or more novel antigens of the MNS blood group system. Furthermore, the expression of more common MNS system antigens, such as S or s, may be affected if the encoding sequence is close to the crossover site, manifested by unexpected results with some antisera.[4] In addition, Storry et al.,[5] studying the molecular bases of the weakened expression of S or s associated with the low-incidence antigens M^v, s^D, and Mit, showed that Arg35 is important for full expression of S and Pro39 and Thr3 were also important for full expression of s.

The absence of GPB on the RBCs as a result of a *GYPB* gene deletion is characterized by the S–s– phenotype,[6] which is found in persons of African descent. RBCs of approximately 1 percent of African Americans, and up to 37 percent of West Africans, type as S–s–. The S–s– phenotype is associated either with the absence of the high-prevalence antigen, U, or with weakened expression of U (U^{+var}). U is commonly found in all populations. The S–s–U– phenotype is found among Black ethnic groups and appears to correlate with the geographic prevalence of malaria infection as a result of the deletion of *GYPB,* whereas the S–s–U^{+var} phenotype has been associated with two variant GPB proteins.[7–9]

Storry et al.[4] reported in 2003 a large cohort study of S–s– donors to determine the molecular alterations of the S–s– phenotype and their distribution in the African American population. In this study, it was confirmed that DNA from donors whose RBCs failed to react with a potent anti-U/GPB was not amplified by allele-specific or gene-specific primers designed to detect *GYPB.* In contrast, donors whose RBCs were reactive with anti-U/GPB demonstrated the presence of variant forms of *GYP.He* (in the majority of samples) or of *GYPB* by allele-specific assays.

The *GYP.He* variant is an altered form of *GPB* (B-A-B hybrid gene) as a result of a gene recombinational event. The ultimate result of this event was the generation of a composite sequence defining the Henshaw (He) epitope with a concomitant abolition of the GPB-associated "N" antigen in some Africans.[7] All these samples demonstrated the presence of the silenced *GYPB*S* allele. The absence of S at the RBC surface was demonstrated to be caused by a change at nucleotide 208 (G>T) and 230 (C>T) of exon 5 (named variant *GYPB[NY],* or *GYPHe[NY]*), or a g>t change at +5 of intron 5 (named variant *GYPB[P2],* or *GYPHe[P2]*), which led to partial or complete exon skipping. Sequencing and PCR-restriction fragment length polymorphism (RFLP) analyses showed that the *GYP(P2)* allele was the most common mechanism in donors and patients with the S–s–U^{+var} phenotype. The *N*-terminus of glycophorins carrying either GP(P2) or GP(NY) can express "N" or He antigens.[4]

In this study, DNA-based assays were used to identify variant forms of *GYPB* in Brazilian blood donors of African descent with the S–s– and S–s+ phenotypes. *GYP(P2)* and *GYP(NY)* alleles have been identified as the mechanisms for the S–s–U^{+var} and have also explained the presence of the *GYPB*S* allele with silencing of S in S–s+ phenotypes in this donor population.

Materials and Methods

Blood Samples

EDTA blood samples were obtained from healthy volunteer blood donors, who self-identified their ethnicity as African Brazilians, at the Sao Rafael Hospital Blood Bank. Institutional Review Board–approved informed consent was obtained from each blood donor.

Serologic Analysis

The S/s/U status of donors' RBCs was determined by IAT-hemagglutination in gel cards (DiaMed AG, Morat, Switzerland) using commercially available polyclonal anti-S and anti-s (Immucor, Norcross, GA) and single-source plasma or serum samples from donors or patients (human polyclonal anti-U). After the determination of the antigen profile, aliquots of 200 µL of 17 S–s–U– and 148 S–s+ samples were subjected to DNA analysis.

Genomic DNA Extraction

Genomic DNA was isolated by a whole-blood DNA extraction kit (Easy DNA, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The DNA solutions were analyzed for quality by agarose gel electrophoresis. Quantification tests were not performed after extraction by this procedure; however, the expected yield was 3 to 12 µg of DNA.

Allele-Specific PCR

Allele-specific PCR (AS-PCR) for the S/s alleles were performed in all 17 S–s–U– and 148 S–s+ samples to characterize the alleles present. The sequences of primer combinations and control primers that amplified an unrelated gene (human growth hormone gene) were previously published.[4] AS-PCR was carried out under the following conditions: 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP mix, 100 ng of sense and antisense primers, 100 ng of control primers, and 2.5 U Taq DNA polymerase. Two microliters of genomic DNA was used per 50-µL assay. Amplification was performed using a standard 35-cycle protocol, with an annealing temperature of 62°C.

GYPB *Exon 5 Combination AS/PCR-RFLP Assay*

Genomic DNA samples from the 4 nondeletion type S–s–U^{+var} samples and 11 S–s+ samples were amplified with the GPB4/5, GPBIVS5, and GPB5T primers, using a combination AS/PCR-RFLP assay[4] to determine whether *GYPB* is present or absent and to distinguish the variant *GYPB* gene products in nondeletion type S–s– and S–s+ (*GYPB*S* silent gene) donors. The PCR products were digested with *Eco*RI (MBI, Fermentas, Amherst, NY) during an overnight incubation at 37°C. The uncut and digested products were analyzed on a 10% polyacrylamide gel.

Results

Serologic Analysis

RBCs from 165 blood samples were initially tested with polyclonal anti-S and anti-s by IAT-hemagglutination in gel test cards to determine the S/s profile. Of these, 17 samples were phenotyped as S–s– and 148 were S–s+. None of the S–s– samples reacted with our human polyclonal anti-U (n = 17) and were serologically classified as S–s–U–.

Allele-Specific PCR

Genomic DNA from the 165 samples was analyzed by AS-PCR assay for *GYPB**S/s. The *GYPB*S*-specific primer pair amplified products in 4 of the 17 S–s– and 11 of the 148 S–s+ samples (Table 1). No amplification

Table 1. Results of AS-PCR for *GYPB*S/s*

Serologic results	Number	*GYPB*S* amplified product	No *GYPB*S* amplified product
S–s–U–	17	4	13*
S–s+	148	11	137

*No *GYPB*S* or *GYPB*s* amplified products.

Table 2. Results of testing 17 S–s– DNA samples by AS/PCR-RFLP assay

Number	Variant gene	Type of nucleotide change
2	*GYP(P2)*	+5 intron 5(g>t)
1	*GYP(NY)*	nt208 (G>T) and nt230 (C>T)
1	*GYP(NY)/GYP(P2)*	nt208 (G>T) and nt230 (C>T)/+5 intron 5 (g>t)
13	*GYPB* deletion	–

Table 3. Results of testing 11 S–s+ DNA samples by AS/PCR-RFLP assay

Number	Variant gene	Type of nucleotide change
8	*GYP(P2)*	+5 intron 5(g>t)
3	*GYP(NY)*	nt208 (G>T) and nt230 (C>T)

was obtained in the other 13 S–s– samples, indicating that *GYPB* was deleted in these samples. The remaining 137 S–s+ samples only gave amplified products with the *GYPB*s*-specific primer, suggesting homozygosity or hemizygosity for *GYPB* (Fig. 1).

GYPB *Exon 5 Combination AS/PCR-RFLP Assay*

Genomic DNA from the 4 S–s– and 11 S–s+ samples that gave amplified products for *GYPB*S*-specific primer were analyzed by the *GYPB* exon 5 combination AS/PCR-RFLP assay, and the results are summarized in Tables 2 and 3, respectively. This combination assay was validated by Storry et al.[4] to determine whether *GYPB* is present or absent and, if *GYPB* is amplified, whether it is the GP(P2) or the GP(NY) form (Fig. 2).

The digested products from the four S–s– samples showed the following patterns: two were homozygous (or hemizygous) for the nucleotide change at +5 (g>t) of intron 5, consistent with variant *GYP(P2),* one was homozygous (or hemizygous) for nucleotide changes at nt208 (G>T) and nt230 (C>T), consistent with variant *GYP(NY),* and one was a heterozygous variant form for both *GYP(NY)/GYP(P2)* alleles.

In the 11 samples that phenotyped as S–s+, we found the following electrophoresis profile in the digested products: 8 had a nucleotide change at +5 (g>t) of intron 5, consistent with variant *GYP(P2),* and 3 presented with changes at nt208 and nt230, consistent with variant *GYP(NY).* The African Brazilians' samples phenotyped as S–s+ were included in this study to extend the molecular characterization of variant *GYPB.* They provided evidence of a high prevalence (7.4%) of *GYPB*S* in this population. In samples that were apparently homozygous for the *GYPB*s* allele, it is not possible to determine whether the partner chromosome has a deleted *GYPB.*

Discussion

In the present study, the molecular background of variant forms of *GYPB* in Brazilian blood donors of African descent was determined for the first time through analyses of S–s– and S–s+ phenotypes. This is particularly important because the ability to detect these variant forms within a population of patients with sickle cell disease (SCD) or other hemoglobinopathies and of Black donors ensures the best match and transfusion safety. Moreover, the *GYPB* exon 5 combination assay is useful to readily identify *GYPB*-deleted donors for those patients whose antibody is compatible only with *GYPB*-deleted RBCs and also permits the identification of the silent S allele to resolve the discrepancies between hemagglutination and DNA-based assays. Clinical issues must be addressed for S–s–U– patients or for those who are S–s–U^{+var}; as shown by Storry et al.,[4] they can produce anti-U when exposed to antigen-positive RBCs. This premise is consistent with other reports of clinically significant transfusion reactions in SCD patients[10] and several cases of hemolytic disease of the fetus and newborn[11–13] caused by anti-U. Transfusion of alloimmunized patients requiring S–s–U– RBC components is a challenge for any transfusion service because of the lack of well-characterized serologic screening reagents. PEG-IAT and MTS-gel with broadly reactive anti-U/GPB are the methods of choice to detect S–s–U^{+var}.[14] However, current knowledge of the molecular bases associated with expression of variant antigens makes DNA testing an important tool for both screening donors and typing patients. Therefore, it is now feasible to genotype donors to identify S–s–U– phenotypes.

In 1987, Huang et al.[6] reported two unrelated individuals who exhibited the S–s–U– phenotype lacking GPB and observed that the absence of this protein correlated with deletion of *GYPB.* Gene deletion is a common mechanism for some human genetic disorders, most notably thalassemias.[15]

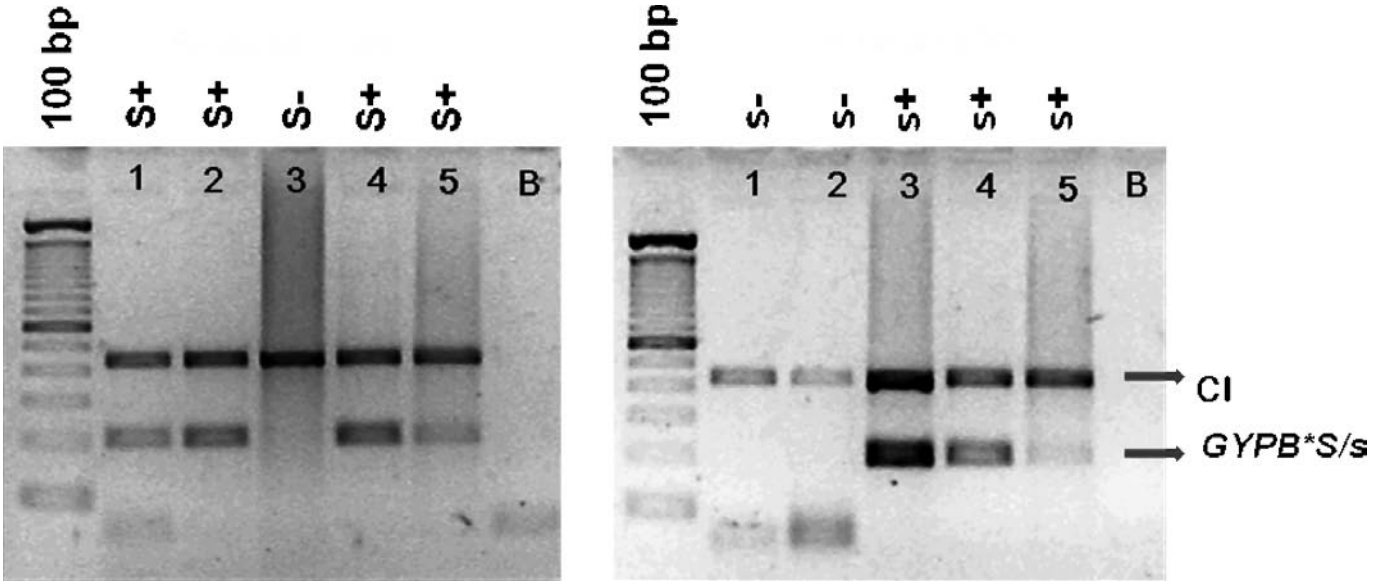

Fig. 1. AS-PCR for *GYPB*S/s*. Electrophoresis profile in agarose gel. B = water blank control. CI = internal control.

Certain thalassemias occur in geographically defined groups of individuals, for which it has been presumed that the absence of the α or β globin gene may have represented a selective advantage. It is therefore of interest that the S–s–U– phenotype is prevalent among selected populations, in particular among Blacks from certain regions in Africa where the incidence of this phenotype ranges between 1 percent and 35 percent[16] as compared with an incidence of less than 0.001 percent in the White population. However, several studies[7–9] have associated the S–s– phenotype not only with the absence of the high-prevalence antigen, U, but also with weakened expression of U (the so-called U variant [U^{+var}] phenotype). The S–s–U– phenotype is attributable to the deletion of *GYPB,* whereas the S–s–U^{+var} phenotype is associated with a variant GPB protein that often expresses He.[4]

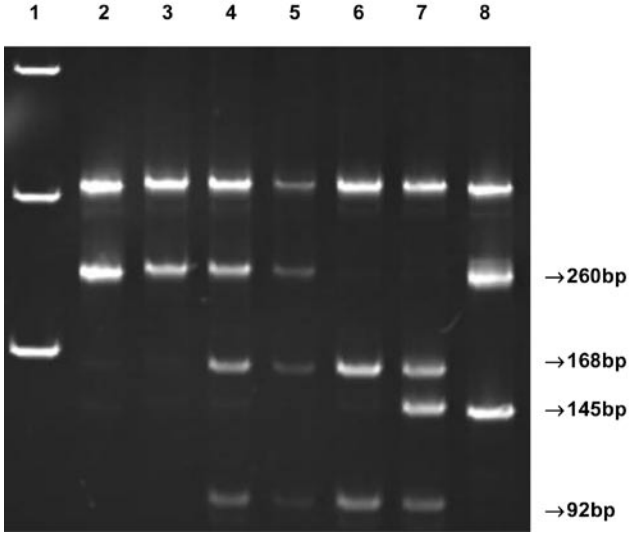

Fig. 2. Analysis of *GYPB* exon 5 combination AS/PCR-RFLP assay after digestion with *Eco*RI. A 10% polyacrylamide gel electrophoresis demonstrating the band patterns for interpretation of variant forms of *GYPB* in African Brazilians phenotyped as S–s– or S–s+; (M = mutant; W = wild). Lane 1 is the 100-bp ladder; Lane 2 is the uncut PCR product; Lane 3 is digestion pattern of an M/M (no *Eco*RI site present), a *GYP(P2);* Lanes 4 and 5 are digestion patterns of an M/W; Lane 6 is the digestion pattern of a W/W, *GYPB;* Lane 7 is the digestion pattern with the allele-specific band *GYP(NY);* Lane 8 is the uncut PCR product of the *GYP(NY)* showing the allele-specific band.

Our findings were slightly different from previously reported data when comparing the molecular events implicated in the nature of *GYPB* variant forms found in our African Brazilian population. We report that 23.5 percent of African Brazilians phenotyped as S–s– presented a variant *GYPB* and that the +5 nucleotide change in intron 5 was the most common (50% of samples) mechanism for generating the S–s–U^{+var} phenotype, followed by a change at nucleotide 208 and 230 (25% of samples with a variant *GYPB*) of exon 5 and a heterozygous change at nt208 and nt230 of exon 5 and +5 of intron 5 (25% of samples with a variant *GYPB*). However, in this study, we have not been able to define the zygosity status of the variant *GYPB* in the donors with S–s–U^{+var} phenotype owing to the apparent inability of the AS/RFLP-PCR assay to detect the deleted *GYPB* gene when *GYP(P2)* or *GYP(NY)* is present as an apparent homozygote.

All samples from S–s– donors failed to react with human polyclonal anti-U. The absence of reactivity of our S–s–U^{+var} samples with human polyclonal anti-U used in our laboratory raised questions about the nature of U on RBCs of normal and variant phenotypes. The molecular basis of U has not been identified so far; however, there is evidence to suggest a possible interaction of GPB with Rh-associated glycoprotein, RhAG,[17,18] which could lead to conformational changes of variant GPB on the RBC surface. This is supported by the observation that some anti-U are compatible with all S–s– RBCs, even S–s–U^{+var}. Apparently rare in an electronic literature search, the inclusion of African Brazilian blood samples phenotyped as S–s+ in this study, to complement and confirm the molecular mechanisms responsible for the S–s– phenotype, provided the knowledge of a high prevalence of *GYPB*S* in this population (7.4% of S–s+ samples), not described in the literature, showing heterozygous variant forms of *GYP(P2)* (73% of samples) followed by *GYP(NY)* (27% of samples). The implication of this molecular characteristic for blood transfusion practice is unknown and remains a challenge for future identification studies of variant forms; however, it is important that molecular methods incorporate the analysis of known *GYPB* variants, especially in a specific population.

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References

1. Daniels GL, Fletcher A, Garratty G, et al. Blood group terminology 2004: from the International Society of Blood Transfusion committee on terminology for red cell surface antigens. Vox Sang 2004;87:304–16.
2. Reid ME, Lomas-Francis C. Blood group antigen factsbook. 2nd ed. San Diego: Academic Press, 2004.
3. Blumenfeld OO, Huang C-H. Molecular genetics of the glycophorin gene family. The antigens for MNSs blood groups; multiple gene rearrangements and modulation of splice site usage result in extensive diversification. Hum Mutat 1995; 6:199–209.
4. Storry JR, Reid ME, Fetics S, Huang C-H. Mutations in *GYPB* exon 5 drive the S–s–U^{+var} phenotype in persons of African descent: implications for transfusion. Transfusion 2003;43:1738–47.
5. Storry JR, Reid ME, MacLennan S, Lubenko A, Nortman P. The low-incidence MNS antigens M^{v}, s^{D}, and Mit arise from single amino acid substitutions on GPB. Transfusion 2001;41:269–75.
6. Huang C-H, Johe K, Moulds JJ, Siebert PD, Fukuda M, Blumenfeld OO. ∂-Glycophorin (Glycophorin B) gene deletion in two individuals homozygous for the S–s–U– blood group phenotype. Blood 1987;70:1830–5.
7. Huang C-H, Reid ME, Blumenfeld OO. Remodeling of the transmembrane segment in human glycophorin by aberrant RNA splicing. J Biol Chem 1994;269:10804–12.
8. Huang C-H, Blumenfeld OO, Reid ME, et al. Alternative splicing of a novel glycophorin allele *GPHe(GL)* generates two protein isoforms in the human erythrocyte membrane. Blood 1997; 90:391–7.
9. Reid ME, Storry JR, Ralph H, et al. Expression and quantitative variation of the low incidence blood group antigen He on some S–s– RBCs. Transfusion 1996;36:719–24.
10. Rosse WF, Gallagher D, Kinney TR, et al. Transfusion and alloimmunization in sickle cell disease. The cooperative study of sickle cell disease. Blood 1990;76:1431–7.
11. Dhandsa N, Williams M, Joss V, Patten J, James D, Sinclair L. Haemolytic disease of the newborn caused by anti-U. Lancet 1981;2:1232.
12. Gottschall JL. Hemolytic disease of the newborn with anti-U. Transfusion 1981;21:230–2.
13. Smith G, Knott P, Rissik J, Win N. Anti-U and haemolytic disease of the fetus and newborn. Br J Obstet Gynaecol 1998;105:1318–21.
14. Reid ME, Storry JR, Maurer J, Nance ST. Practical method for determination of the U status of S–s– erythrocytes. Immunohematology 1997;13:111–14.

15. Kan YW. The William Allan Memorial Award Address: Thalassemia: molecular mechanism and detection. Am J Hum Genet 1986;38:4–12.
16. Lowe RF, Moores P. S–s–U– red cell factor in Africans of Rhodesia, Malawi, Mozambique and Natal. Hum Hered 1972;22:344–50.
17. Habibi B, Fouillade MT, Duedari N, et al. The antigen Duclos. A new high frequency red cell antigen to Rh and U. Vox Sang 1978;34:302–9.
18. Dahr W, Moulds JJ. High-frequency antigens of human erythrocyte membrane sialoglycoproteins, IV. Molecular properties of the U antigen. Biol Chem Hoppe-Seyler 1987;368:659–67.

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