

The *DAU* Allele and Anti-D Alloimmunization Present With High Frequency in Brazilian Sickle Cell Disease Patients

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Abstract

Background: Antigens *DIIIa*, *DAR* and *DAU* are common in people of African descent and are involved in anti-D alloimmunization. Sickle cell disease (SCD) patients frequently need blood therapy and are vulnerable to alloimmunization.

Methods: The study included SCD patients from the Brazilian state of Bahia, which has the highest incidence of the disease in Brazil; 241 SCD patients and 220 healthy individuals were studied. Alleles were characterized by PCR-RFLP and PCR-SSP techniques.

Results: The *DAU* allele was found in 22.3% (43/193) of the SCD patients. Two (1%) patients had the *DIIIa/D* wild-type genotype, one (0.5%) had the *DIIIa/D-* genotype, 11 (5.7%) had the *DAR/D* wild-type genotype and three (1.6%) had the *DAR/D-* genotype. Two patients were positive for the 667T>G mutation and the 1136C>T mutation, one (0.5%) had the genotype *DIIIa/DAU*, and one (0.5%) had the genotype *DAR/DAU*.

Conclusion: There was statistical significance when the allele frequencies were evaluated among SCD, sickle cell anemia (HbSS) patients and healthy individuals. The frequencies of the *DIIIa*, *DAR* and *DAU* alleles among SCD patients differ from those of healthy individuals from the same population, and a high frequency of the *DAU* variant was associated with anti-D alloimmunization in these patients.

Keywords: Sickle cell disease; Anti-D alloimmunization; *DIIIa*;

DAR; *DAU* alleles

Introduction

The Rh system is polymorphic and is composed of antigens characterized by high immunogenicity, especially the D antigen. The D antigen (ISBT 004001; RH1) is a red blood cell (RBC) membrane antigen associated with a blood group that is of great importance in blood therapy, because Rh-negative individuals easily develop antibodies when exposed to D-positive RBC [1].

The partial D phenotype is characterized by the absence of one or more epitopes of the original D antigen that were replaced by other amino acid sequences. Those changes are promoted mostly by missense point mutations in the *RHD* gene or by gene rearrangements between the *RHD* and *RHCE* genes due to the high homology and the short distance between them, and they qualitatively change the D protein in its extracellular localization [2-4].

Partial D antigens are rare in the Caucasian population, with frequencies of less than 1%, but are frequent among Africans and individuals of African descent [2-4]. The variant *DIIIa* was first described in 1996 as one of the six categories of variants of the Rh-positive antigen among individuals who developed anti-D antibody [5]. The *DAR* variant is characterized by the complete absence of at least nine of the 37 epitopes and is commonly found in Rh-positive individuals. The *DAU* variant is common in people of African descent, but it is rare in Caucasians. In 1989, du Toit et al conducted a study in the region of Cape Town, South Africa, and reported that 11% of pregnant women were anti-D Rh-positive [6].

Sickle cell disease (SCD) is a genetic disease characterized by an autosomal recessive inheritance. It shows a high incidence and prevalence in Brazil, especially in the Bahia state [7]. Sickle cell anemia (HbSS) is the most severe type of SCD, and individuals with this need frequent blood therapy and are vulnerable to alloimmunization, which elevates the risk of blood transfusion reactions that can be severe or even fatal due to overall organ damage [4].

Therefore, the present study aimed to investigate the frequencies of the *DIIIa*, *DAR* and *DAU* alleles associated with

Manuscript submitted January 8, 2017, accepted February 14, 2017

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doi: <https://doi.org/10.14740/jh316w>

Table 1. Synthetic Oligonucleotides Used to Amplify Exons 3, 5, 7 and 8 of the *RHD* and *HBB* Genes

Gene	Sequence (5'→3')	Exon <i>RHD</i> /mutation and <i>HBB</i> gene	Reference
<i>RHD14'</i>	F: TAAGCACTTCACAGAGCGG	Exon 5/667T>G	[8]
<i>EX5R</i>	R: TCTTGCTGATCTTCCCTTGG		
<i>RHDEX3F</i>	F: TATTCGGCTGGCCACCATGA	Exon 3/455A>C	[8]
<i>RHD13R</i>	R: TTCTAATTTTGCTGTAATC		
<i>RHD16F</i>	F: TCATAGTGTGGTCCGTA AAC	Exon 7/1025T>C	[9]
<i>RHDEX7R</i>	R: CAATCATGCCCATG GCC		
<i>DAU 1B</i>	F: TTGGCCATCGTGATAGCTCACAT	Exon 8/1136C>T	[10]
<i>DAUB</i>	R: GGAGATGGGGCACATAGACATC		
<i>HBB108</i>	F: CAAGGACAGGTACGGCTGTGTCATCAC	Promoter region	[11]
<i>HBB109</i>	R: TTTTCCAAGTTTGA ACTAGCTCTTC	IVS-2 region	

F: forward; R: reverse. HBB: internal control beta globin gene.

the partial D antigen among SCD patients from the North-east of Brazil and to estimate the risk of anti-D alloimmunization.

Materials and Methods

Study population

A cross-sectional study was developed during the period of 2003 - 2007. The population studied was composed of 241 SCD patients selected from the Hematology Outpatient Clinic of the Fundacao de Hematologia e Hemoterapia do Estado da Bahia (HEMOBA). All patients were phenotyped as D positive or D weak. Clinical data were collected from the medical records, and demographic data were provided by interviews with the patients or with their parents or guardians when the patient was less than 21 years of age. The eligibility criteria included only SCD patients. All patients were at the steady state of the disease, which was characterized as a period without any acute events, and had not had blood therapy prior to blood sampling. The exclusion criteria included the presence of infectious disease, previous blood therapy (less than 4 months before the study) and inflammatory episodes during the study.

The study also included a group of 220 healthy individuals from the same population as the patient group. This group was included to test the frequency of the studied alleles in the same general population. The healthy group was composed of volunteers selected by the Clinical Analysis Laboratory of the Faculdade de Farmacia of Universidade Federal da Bahia (UFBA).

The study was approved by the Centro de Pesquisa Goncalo Moniz de Fundacao Oswaldo Cruz (CPqGM-FIOCRUZ) Human Board, and all subjects provided written informed consent. For patients younger than 21 years, parents or guardians signed written informed consent followed by the child's agreement. The study was developed in accordance with the Declaration of Helsinki of 1975 as revised in 2000. Clinical information was collected from the patients' charts and their physicians.

Hemoglobin profile and hematological data

The hemoglobin profile was confirmed by high-performance liquid chromatography (HPLC) using the VARIANT I (Bio-Rad, CA, USA) equipment, which uses the principle of ion exchange. Calibrators were evaluated before the samples, according to the manufacturer's recommendations.

Molecular biology analysis

Genomic DNA was extracted from leukocytes in 200 µL of peripheral blood using the DNA Kit (Qiagen, Foster City, CA, USA) according to the manufacturer's recommendations. The DNA concentration was measured spectrophotometrically using the Nanodrop ND-1000 (Isogen Life Science, Amsterdam, The Netherlands).

Molecular biology analysis was performed by polymerase chain reaction (PCR) amplification using specific oligonucleotides for study-specific gene regions.

The restriction fragment length polymorphism (PCR-RFLP) technique was used to identify gene polymorphisms associated with the *DIIIa* and *DAR* alleles (Table 1). All samples containing the *HincII* restriction enzyme site had the 667C>G (exon 5) mutation in the *RHD* gene and were subjected to a second PCR reaction to search for the 455A>C (exon 3) and the 1025T>C (exon 7) mutations. The DNA fragment amplified by PCR was digested with the *BanI* restriction enzyme, to identify the 455A>C mutation specific for the *DIIIa* allele, and by the *HphI* restriction enzyme, to identify the 1025T>C mutation specific for the *DAR* allele [8, 9].

The identification of the *DAU* allele was performed by PCR-SSP using the primers *dau1b* and *daub* to amplify the mutant allele located in exon 8 of the *RHD* gene [10]. The *beta globin* (*HBB*) gene was amplified as an internal control (Table 1) [11].

Statistical analysis

The analysis of the probability distribution of the variables was

Table 2. *RHD* Gene Mutation Frequencies Among the SCD Patients and Healthy Individuals

Genotype	SCD (N = 193) (%)	Healthy group (N = 220) (%)	OR (95% CI); P
667T>G			
T/T	174 (90.1)	211 (95.9)	2.56 (1.07 - 6.29); 0.034 [§]
T/G	15 (7.8)	6 (2.7)	
G/D-	4 (2.1)	3 (1.4)	
455A>C (<i>DIIIa</i>)			
A/A	189 (97.9)	220 (100)	0.047 ^{§§}
A/C	3 (1.5)	0	
C/D-	1 (0.6)	0	
1025T>C (<i>DAR</i>)			
T/T	178 (92.2)	211 (95.9)	1.99 (0.8 - 5.05); 0.16 [§]
T/C	12 (6.2)	6 (2.7)	
C/D-	3 (1.6)	3 (1.4)	
1136C>T (<i>DAU</i>)			
Absence	150 (77.7)	212 (96.4)	7.65 (3.34 - 18.18); < 0.000 [§]
Presence	43 (22.3)	8 (3.6)	
<i>DIIIa/DAR</i> or <i>1136C>T (DAU)</i>			
Absence	114 (78.6)	203 (92.3)	3.25 (1.65 - 6.43); 0.002 [§]
Presence	31 (21.4)	17 (7.7)	

[§]Chi-squared Yates corrected. ^{§§}Fisher's exact test. SCD: sickle cell disease; OR: odds ratio; CI: confidence interval.

performed using the Kolmogorov-Smirnov test together with ANOVA parametric tests or non-parametric Kruskal-Wallis tests. The ANOVA test was used to analyze the distribution of means of quantitative or numerical variables with Gaussian distribution within categories. Moreover, to determine whether there was likely to be a difference between the mean values, we conducted multiple comparisons of means by the Bonferroni test (or *post hoc*). The non-parametric Kruskal-Wallis test was used for non-Gaussian distribution.

Analyses of qualitative or categorical variables with three or more groups were performed by non-parametric Chi-squared tests, properly corrected by Mantel-Haenszel and Yates tests. When values less than 4 were obtained, a Fisher's exact test was used, and 95% confidence intervals (CIs) and prevalence ratios (PRs) were calculated for these variables.

The Mann-Whitney test and the independent *t*-test were used to analyze two quantitative variables by comparing two groups of values within a variable, taking into account their distribution.

The data analysis was performed using EPIInfo 6.04 (CDC, Atlanta, GA), the Statistics Data Analysis (STATA) SE 10 (StataCorp, TX, USA) and GraphPad Prism 5.0. A P value of less than 0.05 was considered statistically significant.

Results

Patient characteristics

We investigated 241 SCD patients with an average \pm standard

deviation (SD) age of 17.3 ± 10.9 years, of whom 134 (49.8%) were female. All of these subjects had hemoglobin patterns compatible with SCD, 171 (71%) with HbSS and 56 (29%) with SC disease (HbSC). A healthy control group was included in the study and was composed of 220 individuals with an average age of 36 ± 9.2 years, of whom 98 (44.2%) were female. All of the individuals in the healthy group had normal hemoglobin patterns.

RHD gene mutation frequencies

Tables 2 and 3 show the 667C>G (exon 5) mutation frequencies among the SCD and HbSS patients. The presence of the D partial phenotype was confirmed by searching for the *DII-Ia* allele associated with the 455A>C mutation (exon 3), the *DAR* allele associated with the 1025T>C mutation (exon 7) and the *DAU* allele associated with the 1136C>T mutation in the *RHD* gene. All results were compared with the frequency of the same allele among healthy individuals from the control group.

Of 241 SCD patients selected, 193 were analyzed for the 667T>G (exon 5) mutation and 90.1% (174/193) had the wild-type allele, while 9.9% (19/193) had the mutant allele. Of these 19 SCD patients with the 667T>G mutant allele, 78.9% (15/19) were heterozygous, and 21.1% (4/19) were hemizygotes with a negative *D* allele (*D*667T>G/*D*-). The results from the group of healthy individuals showed that 95.9% (211/220) had the wild-type allele for the 667T>G mutation and 4.1% (9/220) had the mutant allele. Of these, 66.7% (6/9)

Table 3. Distributions of *DIIIa*, *DAR* and *DAU* Alleles Frequencies in the *RHD* Gene Among Individuals With HbSS and Healthy Individuals

Genotypes	HbSS (N = 137) (%)	Healthy individual group, N (%)	OR (95% CI); P
667T>G			
T/T	122 (89.1)	211 (95.9)	2.88 (1.15 - 7.38); 0.021 [§]
T/G	13 (9.5)	6 (2.7)	
G/D-	2 (1.4)	3 (1.4)	
455A>C (<i>DIIIa</i>)			
A/A	134 (97.8)	220 (100)	0.05 ^{§§}
A/C	2 (1.4)	0	
C/D-	1 (0.8)	0	
1025T>C (<i>DAR</i>)			
T/T	125 (91.2)	211 (95.9)	2.25 (0.86 - 5.95); 0.11 [§]
T/C	11 (8.0)	6 (2.7)	
C/D-	1 (0.8)	3 (1.4)	
1136C>T (<i>DAU</i>)			
Absence	109 (79.6)	212 (96.4)	6.81 (2.84 - 16.89); < 0.000 [§]
Presence	28 (20.4)	8 (3.6)	
<i>DIIIa/DAR</i> or 1136C>T (<i>DAU</i>)			
Absence	71 (77.2)**	203 (92.3)	3.53(1.67 - 7.48); < 0.000 [§]
Presence	21 (22.8)**	17 (7.7)	

**N = 92. [§]Chi-squared Yates corrected. ^{§§}Fisher's exact test. OR: odds ratio; CI: confidence interval; HbSS: sickle cell anemia.

were heterozygous, and 33.3% (3/9) were hemizygotes with a negative *D* allele. The 455 A>C mutation, which characterizes the *DIIIa* allele, was found in 2.1% (4/193) of SCD patients. Of these, 75% (3/4) were heterozygous, and 25% (1/4) were hemizygous (*DIIIa* /D-). Interestingly, the 455A>C mutation was not found among the group of healthy individuals. The 667T>G and 1136C>T (*DAU*) mutations were associated with *DIIIa/DAR* or 1136C>T (*DAU*) alleles that were not in Hardy-Weinberg equilibrium in either the SCD group or the HbSS group.

No difference was observed when the allele frequencies of SCD and HbSS patients groups were compared for 667T>G, 1136C>T (*DAU*), and the associated *DIIIa/DAR* or 1136C>T (*DAU*).

Distribution of *DIIIa*, *DAR* and *DAU* alleles

Table 4 shows the distribution of the *DIIIa*, *DAR* and *DAU* alleles in the *RHD* gene among the SCD, HbSS and HbSC disease patients.

Distribution of partial D genotype frequencies

Table 5 shows the genotype distribution of the *DIIIa*, *DAR* and *DAU* alleles of the *RHD* gene in the SCD, HbSS, and HbSC groups and in the group of healthy individuals. Patients with the D genotype associated with hemizygoty (D partial/D

negative) or homozygosity or compound heterozygosity (D partial/D partial) were subject to alloimmunization. From a total of six patients, one (16.6%) had the genotype *DIIIa*/D-, three (50%) had the genotype *DAR*/D-, one (16.6%) had the genotype *DIIIa*/DAU, and one (16.6%) had the genotype *DAR*/DAU. Three (1.4%) of the healthy individuals were hemizygous and had the genotype *DAR*/D-.

Distribution of the “C”, “c”, “E”, and “e” antigens of the Rh system and the *DIIIa*, *DAR* and *DAU* alleles of the *RHD* gene

Phenotypic data for the antigens “C”, “c”, “E” and “e” of the Rh system were investigated in the medical records of the patients. This information was available for 75 SCD and 60 HbSS patients. The most common phenotype found in the SCD group and the HbSS group was “ccee”, with 37.3% (28/75) and 36.7% (22/60) of patients demonstrating this phenotype, respectively. The second most common phenotype was “Ccee”, with 29.3% (22/75) in the SCD group and 30.0% (18/60) in the HbSS group.

In analysis of the frequencies of the phenotypes of the Rh system antigens in 36 patients with SCD who had a mutation in the *RHD* gene, the phenotype “ccee” was the most frequent at 56.8% (21/37), followed by “Ccee” at 18.9% (7/37) and “ccEe” at 18.9% (7/37) (Table 6).

The distribution of the Rh system phenotypes among the SCD patients showed that the incidence of 667T>G and

Table 4. Distributions of the *DIIIa*, *DAR* and *DAU* Alleles in SCD and Healthy Groups

<i>RHD</i> alleles	SCD patients (n = 193)	Healthy controls (n = 220)	OR (95% CI)	P
667T>G	19 (9.8%)	9 (4.1%)	2.56 (1.07 - 6.29)	0.034*
<i>DIIIa</i>	4 (2.1%)	0	-	0.047**
<i>DAR</i>	15 (7.8%)	9 (4.1%)	1.99 (0.8 - 5.05)	0.16*
1136C>T (<i>DAU</i>)	43 (22.3%)	8 (3.6%)	7.65 (3.34 - 18.2)	< 0.000*
	HbSS (n = 137)			
667T>G	15 (10.9%)	9 (4.1%)	2.88 (1.15 - 7.38)	0.021*
<i>DIIIa</i>	3 (2.2%)	0	-	0.05**
<i>DAR</i>	12 (8.8%)	9 (4.1%)	2.25 (0.86 - 5.95)	0.110*
1136C>T (<i>DAU</i>)	28 (22.2%) (n = 126)	8 (3.6%)	6.81 (2.84 - 16.89)	< 0.000*
	HbSC (n = 56)			
667T>G	4 (7.1%)	9 (4.1%)	-	0.31**
<i>DIIIa</i>	1 (1.8%)	0	-	0.20**
<i>DAR</i>	3 (5.4%)	9 (4.1%)	-	0.71**
1136C>T (<i>DAU</i>)	14 (20.9%) (n = 67)	8 (3.1%)	7.00 (2.58 - 19.38)	< 0.0001*

*Chi-squared Yates corrected. **Fisher's exact test. OR: odds ratio; CI: confidence interval; SCD: sickle cell disease; HbSS: sickle cell anemia; HbSC: hemoglobin SC.

Table 5. Distribution of Partial D Genotype Frequencies in the SCD, HbSS and Healthy Groups

<i>RHD</i> gene alleles	SCD (N = 193) (%)	HbSS (N = 137) (%)	Healthy individual group (N = 220) (%)
<i>DIIIa/D</i> normal	2 (1.0)	1 (0.7)	-
<i>DAR/D</i> normal	11 (5.7)	11 (8.0)	6 (2.7)
<i>DIIIa/D-</i>	1 (0.5)	0	-
<i>DAR/D-</i>	3 (1.6)	1 (0.7)	3 (1.4)
<i>DIIIa/DAU</i>	1 (0.5)	1 (0.7)	-
<i>DAR/DAU</i>	1 (0.5)	0	-
<i>DAU/D-</i> , <i>D</i> normal or <i>DAU/DAU</i>	40 (20.7)	27 (19.7)	8 (3.6)

SCD: sickle cell disease; HbSS: sickle cell anemia.

1136C>T mutations with the R1R phenotype was more frequent among carriers of the wild-type allele, representing 36.4% (8/22) of the patients, followed by R0r with 22.7% (5/22). Regarding the presence of the 667T>G mutation and the 1136C>T mutation, the phenotype R0r was the most fre-

quent, corresponding to 56.8% (21/37) of the patients, followed by the phenotype R1R with 18.9% (7/37).

The mutations 667T>G and 1025T>C, which are related to the *DAR* allele, were associated with a high risk of hospitalization (Fig. 1).

Table 6. Distribution of the "C", "c", "E", and "e" Antigens of the Rh System and the *DIIIa*, *DAR* and *DAU* Alleles of the Gene *RHD* Among Sickle Cell Disease Patients

<i>RHD</i> alleles	ccee	Ccee	ccEe	CcEe	Not conclusive
<i>DIIIa/D</i> normal	-	1	-	-	-
<i>DAR/D</i> normal	5	2	2	-	-
<i>DIIIa/D-</i>	-	-	-	-	1
<i>DIIIa/DAU</i>	1	-	-	-	-
<i>DAR/DAU</i>	1	-	-	-	-
<i>DAU/D-</i> , <i>D</i> normal or <i>DAU/DAU</i>	14	4	5	1	-
Total (%)	21 (56.8)	7 (18.9)	7 (18.9)	1 (2.7)	1 (2.7)

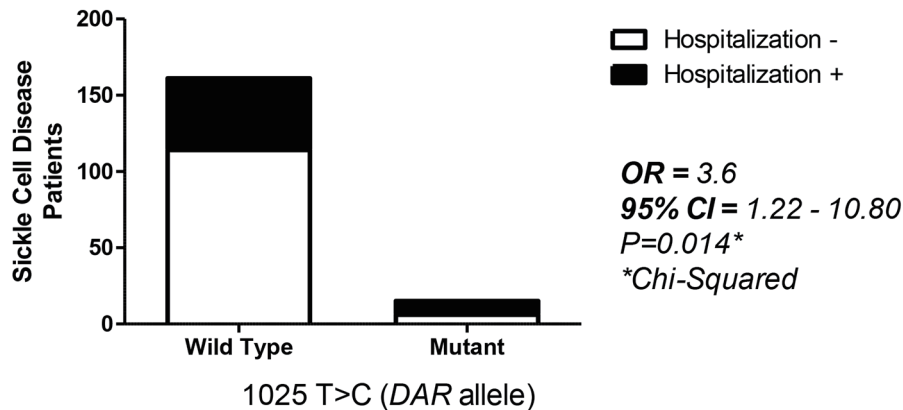


Figure 1. Distribution of 1025T>C, which is related to the *DAR* allele, and its association with a high risk of hospitalization.

Discussion

The Rh system is extremely polymorphic and has important features related to ancestry, as changes described in this system are not homogeneously distributed among different ethnic groups [12]. Partial D antigens are rare in the Caucasian population but have high frequencies among individuals from Africa or of African descent [2-4].

Partial D antigens are involved in anti-D alloimmunization because they are recognized as weakly D positive by the monoclonal and polyclonal antisera typically used in serology. Individuals with these antigens usually have been transfused with Rh-positive RBC [13].

In this study, we investigated the distributions of the *DIIIa*, *DAR* and *DAU* alleles among SCD individuals from the Brazilian state of Bahia after identifying the 667T>G, 455A>C, 1025T>C, and 1136 C>T mutations in the *RHD* gene. Individuals with SCD that participated in this study had the highest frequencies of the *DIIIa*, *DAR* and *DAU* alleles. Of 241 SCD patients, 25.7% were positive for at least one of the *RHD* gene mutations investigated. Our data corroborate previous reports that showed high frequencies of the partial D alleles among African people [3, 4, 14].

The 667C>G mutation is associated with both the *DIIIa* and *DAR* alleles and was therefore used as an indicator of either of these alleles. The 667C>G mutation was frequent among SCD patients, with a 2.5-fold higher probability for the presence of the *DAR* or *DIIIa* allele in SCD patients compared with healthy individuals. When the HbSS patient group was considered, the 667C>G mutation was found at a higher frequency, with a 2.9-fold higher probability of being found among patients compared with individuals from the healthy group. However, no differences were found between the allele frequencies in HbSC patients and HbSS patients, indicating that the alloimmunization risk is high even among HbSS patients. The observation of a higher allele frequency among SCD patients could represent a selection of patients with severe disease that attend the outpatient clinic and have a high risk of developing RhD antibodies, increasing the risk of alloimmunization.

In our work, 2.1% of SCD patients had the 667C>G and the 455A>C mutations, indicating the presence of the *DIIIa* allele. Only one patient was hemizygous for this allele. The *DAU* and *DAR* alleles were found in 22.3% and 7.8% of the patients, respectively. Castilho et al in 2005 reported the *DIIIa* allele frequency among Brazilian SCD patients from the city of Campinas, located in the South of Brazil, a region with a predominance of Caucasian individuals, where 9.2% of patients had the 667C>G mutation, comprising 3.1% of the genotype *DIIIa/D* wild type, and 6.1% had the genotype *DIIIa/D-*. While Castilho et al in 2005 reported that hemizygous individuals (*DIIIa/D-*) predominated, the most common genotype in our study was *DIIIa/D* normal [9]. However, the finding of a greater number of hemizygous individuals (*DIIIa/D-*) may suggest an increased risk of alloimmunization among SCD patients from Campinas.

The *DIIIa* allele was not found among individuals from the control group, and analysis of this allele among SCD, HbSS and HbSC patients and the healthy group did not show significant differences. Three of four patients with the 667C>G mutation were from the HbSS group, providing an estimate of the incidence of a partial D genotype *DIIIa*, of 2.2% of these patients. These findings corroborate the observation that a high frequency of this mutation is common in people of African origin and uncommon among individuals with a Caucasian genetic heritage [8].

We found the *DAR* allele in 7.8% of SCD patients and in 8.8% of HbSS patients, which is higher than reported in other studies. Hemker et al studied 326 individuals from South Africa and reported a frequency of 4.9% for the *DAR* allele, representing 1.5% of all homozygotes or hemizygotes [15]. In populations of Caucasian origin, however, the frequency of the *DAR* allele is lower than in populations of African origin, with reported frequencies lower than 0.1% [10]. The frequency of the *DAR* allele reported by Avent in 2009 was 0.0002%, while the frequency of the *DAU* allele was 0.0001% among 33,864 healthy individuals from a German population. In our study, the frequency of the *DAR* variant was 7.8% of the mutant alleles in SCD patients [16]. Castilho et al in 2005 described a frequency of 6.9% for this allele in SCD patients from Campinas [9].

A study conducted in the region of Cape Town, South Africa, demonstrated that approximately 11% of pregnant women had Rh-positive anti-D. Molecular biology analysis revealed that all of these women were positive for a D partial allele [6]. This variant is very common in African populations according to previous reports [17]. In 2009, Hustinx et al studied people of African descendant and found a frequency of 24.5% for the *DAU* allele identified by PCR-SSP [18]. In Brazil, the *DAU* allele has not been reported previously, and the high frequency of this allele among our SCD patients demonstrates the relevance of this work. In our study, the variant *DAU* was identified by a single codon mutation, 1136C>T [10]. Thus, the *DAU* allele, compared with *DIIIa* and *DAR*, was the most frequent in the SCD patients studied. These data reinforce the report that the *DAU* allele, not the *DIIIa* allele, is the most frequent *RHD* allele in the Brazilian population of Bahia, followed by the *DAR* allele. Castilho et al, in 2005, found 9/130 (6.9%) HbSS patients with the *DAR* allele, but only 5/130 (3.9%) with the *DIIIa* allele [9]. On the basis of the results obtained here, the *DAU* allele may be associated with a heterogeneous distribution of hemoglobin S (HbS) in Brazil, but this allele was not investigated in this study. In Northeast Brazil, mainly in Bahia, the population is a tri-racial mixture of Europeans (mostly Portuguese), Africans and indigenous people. The frequency of HbS in the state of Bahia is the highest in Brazil, varying from 4.5% to 14.7% in several population groups studied [7].

The distribution of the “C”, “c”, “E” and “e” antigens of the Rh system, according to positivity for the 667T>G and 1136C>T mutations, indicated that the phenotype R1R was the most common at 36.4% (8/22) of patients, followed by R0r with 22.7% (5/22). Among patients who were positive for any of the mutations, the most common phenotype was R0r at 56.8% (21/37), followed by R1R at 18.9% (7/37). Another study detected a *DAR* variant allele correlated with the “ce” phenotype in an Afro-Caribbean woman [19]. A group of European Caucasians individuals with the 1136C>T mutation exhibited the R0R2 phenotype more frequently than the Brazilian population [18].

For a similar Bahia population, D-typing strategies may be appropriate because of the variety of alleles presented by African clusters that are difficult to identify. In the case of SCD patients, this picture is even worse. In patients with anemia, the disease may be aggravated due to hemolysis caused by a transfusion reaction. This may decrease the effectiveness of transfusion therapy, generating free hemoglobin with the potential to damage kidneys and lungs, and increasing the level of reactive oxygen species, which can aggravate the disease pathogenesis [20]. These effects may partly account for the 3.6-fold increase in the risk for hospitalization in the SCD patients with the *DAR* allele in our study and indicate the need for improving transfusion therapy, for example, by genotyping patients for *DAR*, *DIIIa* and *DAU*, especially those who are candidates for chronic transfusion, to prevent alloimmunization [21].

Conclusions

Distributions of the variants of *DIIIa*, *DAR*, and *DAU* among

SCD patients from the Brazilian state of Bahia differ from the frequencies observed in healthy individuals from the same population. The data presented here show that the *DIIIa*, *DAR* and *DAU* alleles are common in SCD and HbSS patients when analyzed separately, with a high frequency of the *DAU* allele; this is the first description of these high incidences in Brazil. The variant *DAU* was associated with the occurrence of anti-D alloimmunization among the SCD patients investigated. However, further studies should be performed to confirm the alloimmunization occurrence in two double hemizygous *DAR/D-* and *DIIIa/D-* patients who were submitted to several transfusion events, but had negative indirect Coombs.

Acknowledgments

This work was supported by grants from the Brazilian National Council of Research (CNPq) (3065427/2007-5 and 484457/2007-1) (M.S.G.); the Foundation of Research and Extension of Bahia (FAPESB) (1431040053063; 9073/2007 and 6234/2010) (M.S.G.); and MCD/CNPq/MS-SCTIE-DECIT (409800/2006-6) (M.S.G.). The sponsors of this study are public or nonprofit organizations that support science in general. They had no role in gathering, analyzing or interpreting the data.

Competing Interests

The authors declare no competing financial interests.

References

1. Avent ND, Reid ME. The Rh blood group system: a review. *Blood*. 2000;95(2):375-387.
2. Wagner FF, Flegel WA. *RHD* gene deletion occurred in the Rhesus box. *Blood*. 2000;95(12):3662-3668.
3. Wagner FF, Flegel WA. Review: the molecular basis of the Rh blood group phenotypes. *Immunohematology*. 2004;20(1):23-36.
4. Flegel WA, Wagner FF. Molecular biology of partial D and weak D: implications for blood bank practice. *Clin Lab*. 2002;48(1-2):53-59.
5. Tippett P, Lomas-Francis C, Wallace M. The Rh antigen D: partial D antigens and associated low incidence antigens. *Vox Sang*. 1996;70(3):123-131.
6. du Toit ED, Martell RW, Botha I, Kriel CJ. Anti-D antibodies in Rh-positive mothers. *S Afr Med J*. 1989;75(9):452.
7. Azevedo ES, Alves AF, Da Silva MC, Souza MG, Muniz Dias Lima AM, Azevedo WC. Distribution of abnormal hemoglobins and glucose-6-phosphate dehydrogenase variants in 1200 school children of Bahia, Brazil. *Am J Phys Anthropol*. 1980;53(4):509-512.
8. Westhoff CM, Vege S, Halter-Hipsky C, Whorley T, Hue-Roye K, Lomas-Francis C, Reid ME. *DIIIa* and *DIII* Type 5 are encoded by the same allele and are associated with altered *RHCE*ce* alleles: clinical implications. *Transfu-*

- sion. 2010;50(6):1303-1311.
9. Castilho L, Rios M, Rodrigues A, Pellegrino J, Jr., Saad ST, Costa FF. High frequency of partial DIIIa and DAR alleles found in sickle cell disease patients suggests increased risk of alloimmunization to RhD. *Transfus Med.* 2005;15(1):49-55.
 10. Wagner FF, Ladewig B, Angert KS, Heymann GA, Eicher NI, Flegel WA. The DAU allele cluster of the RHD gene. *Blood.* 2002;100(1):306-311.
 11. Goncalves MS, Nechtman JF, Figueiredo MS, Kerbauy J, Arruda VR, Sonati MF, Saad SO, et al. Sickle cell disease in a Brazilian population from Sao Paulo: a study of the beta s haplotypes. *Hum Hered.* 1994;44(6):322-327.
 12. Cartron JP. RH blood group system and molecular basis of Rh-deficiency. *Baillieres Best Pract Res Clin Haematol.* 1999;12(4):655-689.
 13. Daniels G. *Human Blood Groups*. 2nd ed. Blackwell, Oxford, 2002.
 14. Wagner FF, Eicher NI, Jorgensen JR, Lonicer CB, Flegel WA. DNB: a partial D with anti-D frequent in Central Europe. *Blood.* 2002;100(6):2253-2256.
 15. Hemker MB, Ligthart PC, Berger L, van Rhenen DJ, van der Schoot CE, Wijk PA. DAR, a new RhD variant involving exons 4, 5, and 7, often in linkage with ceAR, a new Rhce variant frequently found in African blacks. *Blood.* 1999;94(12):4337-4342.
 16. Avent ND. Large-scale blood group genotyping: clinical implications. *Br J Haematol.* 2009;144(1):3-13.
 17. Wagner FF, Moulds JM, Tounkara A, Kouriba B, Flegel WA. RHD allele distribution in Africans of Mali. *BMC Genet.* 2003;4:14.
 18. Hustinx H, Poole J, Bugert P, Gowland P, Still F, Fontana S, Scharberg EA, et al. Molecular basis of the Rh antigen RH48 (JAL). *Vox Sang.* 2009;96(3):234-239.
 19. Silvy M, Chapel-Fernandes S, Callebaut I, Beley S, Dourousseau C, Simon S, Lauroua P, et al. Characterization of novel RHD alleles: relationship between phenotype, genotype, and trimeric architecture. *Transfusion.* 2012;52(9):2020-2029.
 20. Noizat-Pirenne F. Relevance of alloimmunization in haemolytic transfusion reaction in sickle cell disease. *Transfus Clin Biol.* 2012;19(3):132-138.
 21. Miller ST, Kim HY, Weiner DL, Wager CG, Gallagher D, Styles LA, Dampier CD, et al. Red blood cell alloimmunization in sickle cell disease: prevalence in 2010. *Transfusion.* 2013;53(4):704-709.